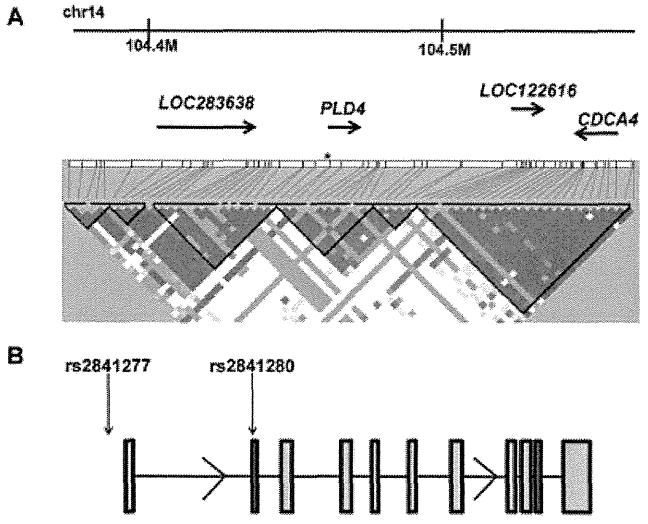

Figure 1. Linkage disequilibrium (LD) block around the *PLD4* region and the *PLD4* structure. **A,** LD block and genes around *PLD4*. The LD block is based on HapMap phase 3 data. **Asterisk** indicates rs2841277. **B,** Schematic view of *PLD4* structure. Rectangles represent exons of *PLD4*.

further stringent analysis excluding patients with other autoimmune diseases demonstrated significant associations of the 3 genes (see Supplementary Table 2). When we compared SSc patients with and those without other autoimmune diseases for the associated alleles, no differences were observed (data not shown).

Subanalysis of types of SSc. Previous studies have revealed that the genetic background of SSc varies between different types of SSc (11,18). Thus, subanalyses of the 5 regions examined in the combined study were performed, in which the allele frequencies of the control subjects were compared with those of the patients with lcSSc or dcSSc. The control subjects were the same as those used in the first study or the combined study. Although *PLD4* and *TNFAIP3* did not display a preference for either SSc phenotype, *IRF8* and *ARID5* showed suggestive preferences for lcSSc, and *CD83* showed a suggestive preference for dcSSc (Table 3).

We also investigated whether the susceptibility loci affect autoantibody status and severe complications. The association studies revealed an association of *TNFAIP3* with SSc patients who possess anticentromere antibodies (ACAs) (see Supplementary Table 3, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37777/abstract), but intracase analyses did not demonstrate clear significance ($P = 0.043$). We did not observe other associations between the susceptibility loci and clinical phenotypes of SSc, in either case–control analyses or intracase analyses.

Efficacy of the current study. In the current study, a candidate gene analysis was performed based on a meta-analysis of RA GWAS, because many susceptibility genes for autoimmune disease have been reported

Table 3. Associations of the 2 SSc subtypes*

					Limited cutaneous SSc (n = 408)			Diffuse cutaneous SSc (n = 318)		
SNP	Chr	Gene	Allele 1/2	Controls, allele 1 frequency	Allele 1 frequency	*P*	OR (95% CI)	Allele 1 frequency	*P*	OR (95% CI)
rs766449	1	*PADI4*	T/C	0.40	0.39	0.52	0.94 (0.77–1.14)	0.36	0.11	0.85 (0.69–1.04)
rs11900673	2	*B3GNT2*	T/C	0.29	0.25	0.096	0.82 (0.66–1.03)	0.31	0.32	1.11 (0.9–1.38)
rs2867461	4	*ANXA3*	A/G	0.44	0.42	0.40	0.92 (0.75–1.12)	0.44	0.97	1.00 (0.82–1.22)
rs657075	5	*IL3-CSF2*	A/G	0.36	0.34	0.54	0.94 (0.76–1.15)	0.33	0.23	0.88 (0.72–1.08)
rs12529514	6	*CD83*	C/T	0.14	0.15	0.79	1.03 (0.85–1.25)	0.18	0.0075	1.32 (1.08–1.62)
rs1571878	6	*CCR6*	C/T	0.49	0.48	0.81	0.98 (0.80–1.19)	0.46	0.20	0.88 (0.72–1.07)
rs6932056	6	*TNFAIP3*	C/T	0.069	0.093	0.0062	1.40 (1.1–1.78)	0.10	0.00063	1.57 (1.21–2.04)
rs2233434	6	*NFKBIE*	G/A	0.21	0.20	0.60	0.94 (0.73–1.20)	0.22	0.70	1.05 (0.83–1.33)
rs10821944	10	*ARID5B*	G/T	0.36	0.40	0.0085	1.22 (1.05–1.41)	0.38	0.30	1.09 (0.93–1.29)
rs3781913	11	*PDE2A-CENTD2*	T/G	0.69	0.69	0.98	1.00 (0.81–1.24)	0.69	0.90	1.01 (0.82–1.25)
rs2841277	14	*PLD4*	T/C	0.69	0.73	0.0067	1.24 (1.06–1.45)	0.74	0.0049	1.29 (1.08–1.55)
rs2841280	14	*PLD4*	C/G	0.64	0.69	0.0011	1.30 (1.11–1.52)	0.69	0.0086	1.27 (1.06–1.51)
rs2847297	18	*PTPN2*	G/A	0.34	0.33	0.67	0.96 (0.78–1.18)	0.34	0.87	1.02 (0.83–1.25)
rs4937362	11	*ETS1-FLI1*	T/C	0.68	0.68	0.75	0.97 (0.78–1.19)	0.69	0.92	1.01 (0.82–1.25)
rs3783637	14	*GCH1*	C/T	0.74	0.73	0.69	0.96 (0.77–1.19)	0.73	0.65	0.95 (0.76–1.18)
rs1957895	14	*PRKCH*	G/T	0.39	0.40	0.84	1.02 (0.84–1.25)	0.42	0.16	1.15 (0.95–1.41)
rs6496667	15	*ZNF774*	A/C	0.35	0.39	0.088	1.19 (0.97–1.45)	0.34	0.75	0.97 (0.79–1.19)
rs7404928	16	*PRKCB1*	T/C	0.62	0.61	0.60	0.95 (0.78–1.16)	0.66	0.15	1.17 (0.95–1.44)
rs2280381	16	*IRF8*	T/C	0.84	0.88	0.0038	1.36 (1.11–1.68)	0.86	0.21	1.16 (0.92–1.45)

* SSc = systemic sclerosis; SNP = single-nucleotide polymorphism; Chr = chromosome; OR = odds ratio; 95% CI = 95% confidence interval.

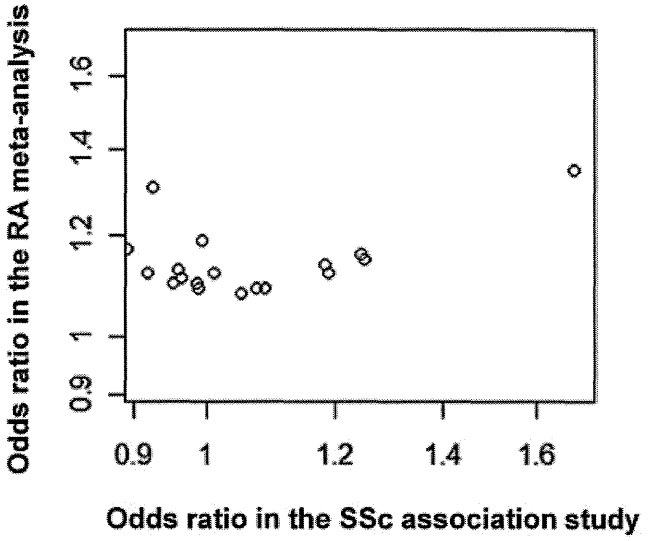

Figure 2. Comparison of associations for systemic sclerosis (SSc) and rheumatoid arthritis (RA). The odds ratios obtained for 18 genes in association studies of SSc and RA are plotted.

to be shared by a wide range of diseases. As a result, 3 susceptibility genes for SSc in Japanese were identified. Thus, we analyzed whether the candidate gene approach taken in the current study for detecting novel susceptibility genes for SSc was effective. When the likelihood of finding 3 susceptibility genes among 18 genes by chance was calculated, the likelihood was determined to be 2.5×10^{-8}. These results indicated that our approach to identifying novel susceptibility genes for systemic diseases is effective. It would be interesting to compare the risk direction of the genotyped markers between RA and SSc. Although the 3 susceptibility loci for SSc shared risk direction with RA, no correspondence of the risk directions of the markers between the 2 diseases was detected (Figure 2). This indicated that a large proportion of the 18 RA markers are not shared by SSc, and that the lack of association between the 13 markers and SSc was not attributable to the low power produced by the relatively small number of SSc patients included in this study.

DISCUSSION

Because SSc can lead to severe complications, poor quality of life, and shortened survival, clarifying the characteristics of SSc is important. Clarification of the disease would aid the search for novel therapeutic targets and the development of new therapeutic strategies. Detecting susceptibility genes using GWAS or a candidate gene approach would also help to uncover the pathophysiology underlying SSc.

Previous studies have revealed that more than 15 markers and loci are associated with SSc. However, the markers detected so far cannot fully explain the genetics of SSc, indicating that many susceptibility genes are yet to be identified. Because a relatively large proportion of RA susceptibility genes are shared by other autoimmune diseases (24), a candidate gene approach using novel markers observed in GWAS of RA is a fascinating way of identifying new SSc markers. In fact, some of the novel susceptibility markers for RA identified in the meta-analysis were shown to be susceptibility markers for systemic lupus erythematosus (SLE) and Graves' disease (31).

In the current study, we successfully identified 3 susceptibility genes for SSc in Japanese. No studies have identified *PLD4* as an SSc-associated locus. The current study is also the first to detect *TNFAIP3* and *IRF8* as susceptibility genes for SSc in a Japanese population. The best-fit models for each association are shown in Supplementary Table 4, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.002/art.37777/abstract.

It is conceivable that these 3 associations might have been obtained due to the overlap of RA and SSc. Even after excluding the patients with both RA and SSc based on physicians' reports, the significant associations for the 3 loci were still observed (Table 3). Information regarding rheumatoid factor (RF) and anti–citrullinated protein antibody (ACPA) was available for 371 SSc patients without RA and 65 SSc patients without RA, respectively, of whom 21.6% and 10.8% were positive for RF and ACPA, respectively. These prevalences are compatible with those previously observed in SSc patients without RA (35,36). Moreover, we showed that the effect sizes and risk direction of the markers tested in this study were dissociated between SSc and RA. In addition, further stringent analysis comprising SSc patients without any autoimmune disease also showed the associations of the 3 loci. These results indicate that the associations of the 3 loci are not attributable to overlapping of RA or other diseases.

Although the associations of the *ARID5B* and *CD83* loci with SSc did not reach a stringently significant level in the combined study, the tendencies toward an association with SSc displayed by rs10821944 in the *ARID5B* locus and rs12529514 in the *CD83* region in the first study were maintained in the replication study. This indicates that these loci are potential susceptibility regions for SSc. Further replication studies are needed to

address the associations of these 2 loci with SSc in a Japanese population.

Because *TNFAIP3* was reported to be strongly associated with SSc in a European population (18), the significant associations detected in the combined study indicate that *TNFAIP3* displays general associations with SSc that go beyond ethnic boundaries. In addition, rs6932056, which displayed a strong association with SSc in a European population (18), is in strong LD with rs5029939 ($r^2 = 0.85$) in the Japanese population. SNP rs6932056 also displays strong LD with rs2230926, a missense mutation of *TNFAIP3* ($r^2 = 0.85$), in Japanese. The rs2230926 missense mutation leads to an amino acid alteration in the OTU (ovarian tumor) domain of the A20 protein, which is considered to result in decreased NF-κB signaling. Because we did not observe strong associations between rs6932056 and SSc in the replication study, it will be necessary to reexamine the association between *TNFAIP3* and SSc using independent sample sets of Japanese patients with SSc, in spite of the significant associations detected in this study.

PLD4 is a recently reported member of the phospholipase family without phospholipase D activity. *PLD4* is expressed in the spleen and early postnatal microglia in the white matter of mice (37). The phenotypes of *Pld4*-deficient mice have not been reported. In addition, little is known about the expression or distribution of *PLD4* in humans. Although the functions of *PLD4* are also poorly understood, it is known to be involved in the phagocytosis of microglia (38). The expression of *PLD4* around the marginal zone in the spleen might support the functional involvement of *PLD4* in immunologic systems. It is interesting that rs2841280, which alters an amino acid of PLD-4, is associated with SSc. Minor allele G of rs2841280 is associated in a protective manner. The impact of an amino acid alteration brought by rs2841280 on the effect of PLD-4 protein is not known.

When we analyzed the impact of the amino acid alteration using in silico analysis (SIFT software; http://sift.jcvi.org/), it was shown to result in a small effect. However, the association raises the possibility that this polymorphism leads functional modulation of PLD-4, and it is feasible to analyze the functional change of PLD-4 protein with rs2841280, using animal models of SSc. When we performed an in silico analysis of the effect of rs2841277 and rs2841280 on *PLD4* expression, we did not detect any clear associations between the 2 genotypes and *PLD4* transcription ($P > 0.05$) (39). Therefore, in spite of the association of these 2 mutations, it has not been confirmed whether one of these 2 polymorphisms is the causative mutation.

Although the detection of a P value less than 5×10^{-8} in a GWAS is stringent evidence of an association between a marker and a particular disease, the detection of suggestive associations between the *PLD4* region and SSc in European GWAS would indicate that associations exist between *PLD4* and SSc in other populations. However, when we examined the associations between the *PLD4* locus or nearby loci and SSc in GWAS involving a European population, we did not detect any strong associations ($P < 10^{-4}$) (8,9). According to the HapMap database, the European population displays a higher risk allele frequency for rs2841277 than the Japanese population. In addition, the HapMap database also indicates that the LD block spanning *PLD4*, which includes rs2841277, is similar in Europeans and Japanese. Nevertheless, a European population did not show a strong association between *PLD4* and SSc, suggesting that *PLD4* has a stronger effect on autoimmune diseases in Japanese than in Europeans. There is also a possibility that these 2 polymorphisms are only markers, and that a rare variant in LD with the 2 markers affects disease onset. A rare causative variant might explain a different association of *PLD4* with SSc between populations.

IRF8 was shown to be associated with SLE in a European population (40). Interferon regulatory factor 8 (IRF-8) protein is a transcription factor involved in the interferon pathway. The interferon pathway has been shown to be involved with a broad range of autoimmune diseases, including SSc (41). Thus, it is interesting that *IRF5* and *IRF8*, both of which belong to the IRF family, displayed associations with SSc. Although a European GWAS of SSc patients revealed suggestive associations between the *IRF4* locus and SSc, the results were not successfully replicated (8), indicating that the different functional roles of each IRF family molecule might influence the development of SSc. *IRF8* promotes B cell differentiation; however, the roles and importance of B cells in skin fibrosis in SSc patients have not been established (42–44). *IRF8* and its mutant variants are also known to be involved in the development of dendritic cells (45). Thus, the association between *IRF8* and SSc might indicate the involvement of B cells and dendritic cells in the development of SSc.

When the patients with SSc were classified as having either lcSSc or dcSSc and subanalyses were performed, *ARID5B*, *IRF8*, and *CD83* displayed stronger associations with one of the 2 phenotypes. However, the associations of these 3 markers with the phenotypes

were not strong enough to provide convincing evidence of a clear distinction between the genetic backgrounds of the 2 SSc phenotypes. When the associations of the SSc subtypes with the other 13 markers in the first set were analyzed, no strong association was detected ($P > 0.05$). Other subanalyses of the susceptibility loci in the combined set did not show significant results between disease phenotypes, due to lack of power. Because classification according to disease phenotypes resulted in limited numbers of subjects in each subset, we conducted this subanalysis only in the combined set. The association between *TNFAIP3* and ACAs should be confirmed in a large-scale association study.

Although GWAS are an extremely powerful way to detect novel susceptibility genes for diseases, GWAS of patients with SSc have been performed only in European populations. Our study detected strong evidence for the sharing of susceptibility genes between RA and SSc in a Japanese population. In addition, the current study indicated that a candidate gene approach based on the results of GWAS of other diseases that display pathologic signaling pathways or mechanisms similar to those associated with the disease being examined is an effective approach to identifying novel susceptibility genes.

It will be interesting to perform GWAS of Japanese patients with SSc and analyze the similarities and differences in the detected associations not only between Japanese and Europeans but also between Japanese patients with SSc and Japanese patients with RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Terao had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Terao, Ohmura, Kawaguchi, Nishimoto, Kawasaki, Takehara, Furukawa, Kochi, Ota, Ikari, Sato, Tohma, Yamada, Yamamoto, Kubo, Yamanaka, Kuwana, Tsuchiya, Matsuda, Mimori.

Acquisition of data. Terao, Ohmura, Kawaguchi, Nishimoto, Kawasaki, Takehara, Furukawa, Kochi, Ota, Ikari, Sato, Tohma, Yamada, Yamamoto, Kubo, Yamanaka, Kuwana, Tsuchiya, Matsuda, Mimori.

Analysis and interpretation of data. Terao, Ohmura.

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Inverse Association between Air Pressure and Rheumatoid Arthritis Synovitis

Chikashi Terao[1*9], Motomu Hashimoto[2 9], Moritoshi Furu[2], Shuichiro Nakabo[3], Koichiro Ohmura[3], Ran Nakashima[3], Yoshitaka Imura[3], Naoichiro Yukawa[3], Hajime Yoshifuji[3], Fumihiko Matsuda[1], Hiromu Ito[2], Takao Fujii[2], Tsuneyo Mimori[3]

1 Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan, **2** Department of the Control for Rheumatic Diseases, Kyoto University Graduate School of Medicine, Kyoto, Japan, **3** Department of Rheumatology and Clinical Immunology, Kyoto University Graduate School of Medicine, Kyoto, Japan

Abstract

Rheumatoid arthritis (RA) is a bone destructive autoimmune disease. Many patients with RA recognize fluctuations of their joint synovitis according to changes of air pressure, but the correlations between them have never been addressed in large-scale association studies. To address this point we recruited large-scale assessments of RA activity in a Japanese population, and performed an association analysis. Here, a total of 23,064 assessments of RA activity from 2,131 patients were obtained from the KURAMA (Kyoto University Rheumatoid Arthritis Management Alliance) database. Detailed correlations between air pressure and joint swelling or tenderness were analyzed separately for each of the 326 patients with more than 20 assessments to regulate intra-patient correlations. Association studies were also performed for seven consecutive days to identify the strongest correlations. Standardized multiple linear regression analysis was performed to evaluate independent influences from other meteorological factors. As a result, components of composite measures for RA disease activity revealed suggestive negative associations with air pressure. The 326 patients displayed significant negative mean correlations between air pressure and swellings or the sum of swellings and tenderness ($p = 0.00068$ and 0.00011, respectively). Among the seven consecutive days, the most significant mean negative correlations were observed for air pressure three days before evaluations of RA synovitis ($p = 1.7 \times 10^{-7}$, 0.00027, and 8.3×10^{-8}, for swellings, tenderness and the sum of them, respectively). Standardized multiple linear regression analysis revealed these associations were independent from humidity and temperature. Our findings suggest that air pressure is inversely associated with synovitis in patients with RA.

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* E-mail: a0001101@kuhp.kyoto-u.ac.jp

9 These authors contributed equally to this work.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by joint synovitis and resultant joint destruction. Patients with RA present with swellings and tenderness of their joints, especially small joints such as metacarpophalangeal joints and proximal interphalangeal joints. Joint swellings and tenderness are closely related with future joint destruction [1,2] and this is why joint swellings and tenderness are included in the items for composite measures used for evaluation of RA activity [3–6]. Disease activity index (DAS) 28 is the most common composite measure in RA used for evaluation of daily RA activity and is composed of erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) as inflammatory parameters, swollen joint count (SJC) and tender joint count (TJC) for the 28 joints with or without visual analogue scale (VAS).

A large number of studies have tried to elucidate basic mechanisms of joint synovitis in RA and revealed the importance of inflammatory cytokines such as TNF-alpha and IL-6 to which biological agents were developed to target [7]. On the contrary, environmental factors which influence joint synovitis are scarcely known. Several studies with a relatively large number of subjects have reported seasonal variations of RA symptoms or joint destruction [8,9], but the relationship is still inconclusive [10–12]. Detailed meteorological differences among various seasons which cause changes in RA symptoms have not been clarified.

Through our daily medical care for patients with RA, we noticed that many patients with RA told us about the fluctuations in their joint symptoms according to changes in air pressure. In particular, many of them recognized worsening of their symptoms when the air pressure decreased such as during a typhoon in the summer. While many rheumatologists have heard of this, there have been no large-scale association studies to date addressing the relationship between air pressure and joint synovitis in RA. Previous studies addressing correlations between RA synovitis and meteorological changes included less than 100 patients with RA and the results were not consistent [10,13–18]. Previously, it was reported that a consistent microenvironment would ease joint symptoms in patients with RA [13]. While one study showed that temperature and humidity were associated with joint pain in 88 patients with RA [15], another study did not find statistically

significant associations between meteorological changes and arthritic symptoms in 70 patients [16]. Another study where researchers observed 18 patients with RA for more than one year did not find significant associations and the authors concluded that this subjective belief in association by RA patients is simply an assumption that people have believed in for thousands of years [19]. However, none of the studies analyzed a large-number of joint assessments and the association is still inconclusive.

Recently, Kyoto University developed a large-scale database named "KURAMA (Kyoto University Rheumatoid Arthritis Management Alliance)" to accumulate detailed clinical information and specimen of patients with RA to uncover the basics of RA [20]. Here, we obtained 23,064 joint assessments for patients with RA from the KURAMA database and analyzed correlations between air pressure and joint synovitis in RA.

Results

Firstly, whether air pressure was correlated with daily disease activity in RA was addressed. DAS28 was selected as evaluation of RA activity for the association study. Table 1 shows the basic characteristics of the DAS28 scores and its components. The mean DAS28 score in the current patient group was 3.28, indicating low to moderate disease activity [21]. 14,999 DAS28 scores with three variables including SJC, TJC and ESR did not show a significant association with air pressure ($p = 0.18$, Table S1). Because DAS is a composite measure for assessing disease activity in RA, other RA composite measures could be associated with air pressure. Correlation analyses between air pressure and SJC, TJC, ESR, patients' VAS (pVAS) and Dr's VAS (dVAS) showed that all elements except for ESR showed suggestive or significant inverse associations with air pressure (Table S1). We also found that the sum of SJC and TJC showed a significant association with air pressure (Table S1). These results suggest that air pressure is associated with RA synovitis across different evaluations and different patients with RA. However, these analyses might be influenced by intra-patient correlations. Considering that significant associations were observed for SJC and the sum of SJC and TJC with air pressure, and that the largest number of data was available for SJC and TJC among the components of composite measures, we adopted SJC, TJC, and the sum of the two counts as best candidates showing associations with meteorological factors to obtain the maximum power for further analyses.

Secondly, correlation between air pressure and RA synovitis in each patient was analyzed to control intra-patient correlations. As the distribution of number of evaluations varied in the patients with RA, we extracted patients with more than 20 evaluations to confirm the correlations between air pressure and joint synovitis across different patients. In total, 12,061 evaluations from 326 patients were used for this analysis. The means of SJC and TJC were comparable with those in the 2,131 patients (Table 1). The overall fluctuations between SJC, TJC, or the combination of the two and air pressure are illustrated in Figure S1. Because we could not find the regular strong tendency of association between air pressure and joint synovitis through the figure, correlation coefficients between air pressure and signs of joint synovitis were calculated for each of the 326 patients. The correlation coefficients in items of synovitis demonstrated normal distributions in each item ($p \geq 0.78$, Shapiro-Wilk test, data not shown), justifying the application of t-test. The mean correlation coefficients across the 326 patients revealed significant negative correlations of air pressure with SJC and the combination of SJC with TJC (mean rho $= -0.0410$ and -0.0455, $p = 0.00068$ and 0.00011, respectively, Table 2). TJC showed a suggestive negative correlation (mean rho $= -0.0306$ and $p = 0.010$, Table 2). Figure 1 illustrates the smallest, largest and median correlation coefficients between air pressure and SJC, TJC or combination of the two among the 326 patients with RA, suggesting that negative mean correlation coefficients of RA synovitis were not brought about by patients demonstrating extreme negative correlation coefficients.

Table 1. Basic characteristics of the subjects in the current study.

Items	Overall	326 patients
Evaluation	23,064	12,061
Patient	2,131	326
Age (mean±SD)	60.7±15.0	62.4±13.6
Female ratio (%)	80.9	82.5
Stage* (mean±SD)	2.63±1.18	2.86±1.16
Class* (mean±SD)	1.92±0.70	1.94±0.70
Disease duration (year, mean±SD)	14.0±11.9	16.1±11.7
Smoking** (%)	33.6	30.8
Biologics*** (%)	19.0	46.6

*Steinbrocker's Stage and Class.
**Current smoker and ex-smoker.
***Patients who have been treated by biological agents between 2005 and 2012.
SD:standard deviation.
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Thirdly, we analyzed which day showed the most significant associations between air pressure and items of joint synovitis, because it was likely that air pressure affected RA joint synovitis by an indirect mechanism taking several days. The air pressure data of six consecutive days before the date of evaluation was obtained and the same analyses using the same data set of the 326 patients were performed to evaluate associations with items of joint synovitis. As a result, the mean correlation coefficients of SJC, TJC, and the sum of SJC and TJC showed a "U-pattern" in the consecutive days (Figure 2A). The strongest associations were found three days before the joint evaluations for the three items ($p = 1.7\times10^{-7}$, 0.00027, and 8.3×10^{-8}, for SJC, TJC, and the sum of SJC and TJC, respectively, Figure 2A and 2B). These results strengthened correlations between air pressure and joint synovitis in RA.

Finally, whether these correlations were mainly brought about by other meteorological factors or not was addressed. Data of daily mean temperatures and humidity were obtained from the same period as air pressure. Multiple standardized linear regression analyses were performed to assess independent correlations between joint synovitis and air pressure. As a result, air pressure showed significant associations with joint synovitis in the 21,940 evaluations which were independent from temperature and humidity ($beta \leq -0.0765$ and $p \leq 0.0031$, Table 3). The analyses suggested that humidity also negatively influenced RA joint synovitis (Table 3). When multiple standardized linear regression analyses were performed for air pressure three days before the evaluations in each of the 326 patients with more than 20 evaluations of the 28 joints, mean coefficients of air pressure showed significant negative associations with joint synovitis ($p = 0.00023$, 0.036 and 0.0015, for SJC, TJC and the sum of SJC and TJC, respectively, Table 4). The inverse association between humidity and air pressure was also observed in this analysis ($p = 0.0019$, 0.016 and 0.0023, for SJC, TJC and the sum of SJC and TJC, respectively, data not shown).

Table 2. Mean correlation coefficients between joint synovitis and air pressure in the 326 patients.

Synovitis	Mean±SD(median)	Mean ρ±SD	P*
SJC	2.07±1.99 (1)	−0.0410±0.210	0.00068
TJC	2.08±2.19 (1)	−0.0306±0.212	0.010
SJC+TJC	4.15±3.70 (2)	−0.0455±0.207	0.00011

*p-values for Student's t-test. SD:standard deviation.
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Discussion

Because environmental effects on RA synovitis are not well established, analysis with convincing results would be beneficial to manage patients with RA properly. This is the first study to address the correlations between air pressure and joint synovitis in RA with a large number of RA patients, and the first to detect significant associations between them. Our results indicate that low air pressure is associated with worsening of joint synovitis. This matches the complaints from RA patients, that they feel worsening of RA synovitis when typhoons come in the summer. Since SJC is an objective element and TJC is a subjective element of patients with RA, significant associations of air pressure with the two cannot be explained by subjective feelings of patients. Previous studies addressing correlations between meteorological effects and joint synovitis did not give consistent results [10,13–19]. The limited number of subjects (not more than 88 patients with RA) is assumed to have caused this inconsistency. In addition, although we found significant associations, the small mean correlation coefficients (ρ: −0.04∼) made the previous studies difficult to detect these small effects. Because RA patients at Kyoto University Hospital frequently told us of the fluctuations in their symptoms in accordance with air pressure, among all the various meteorological

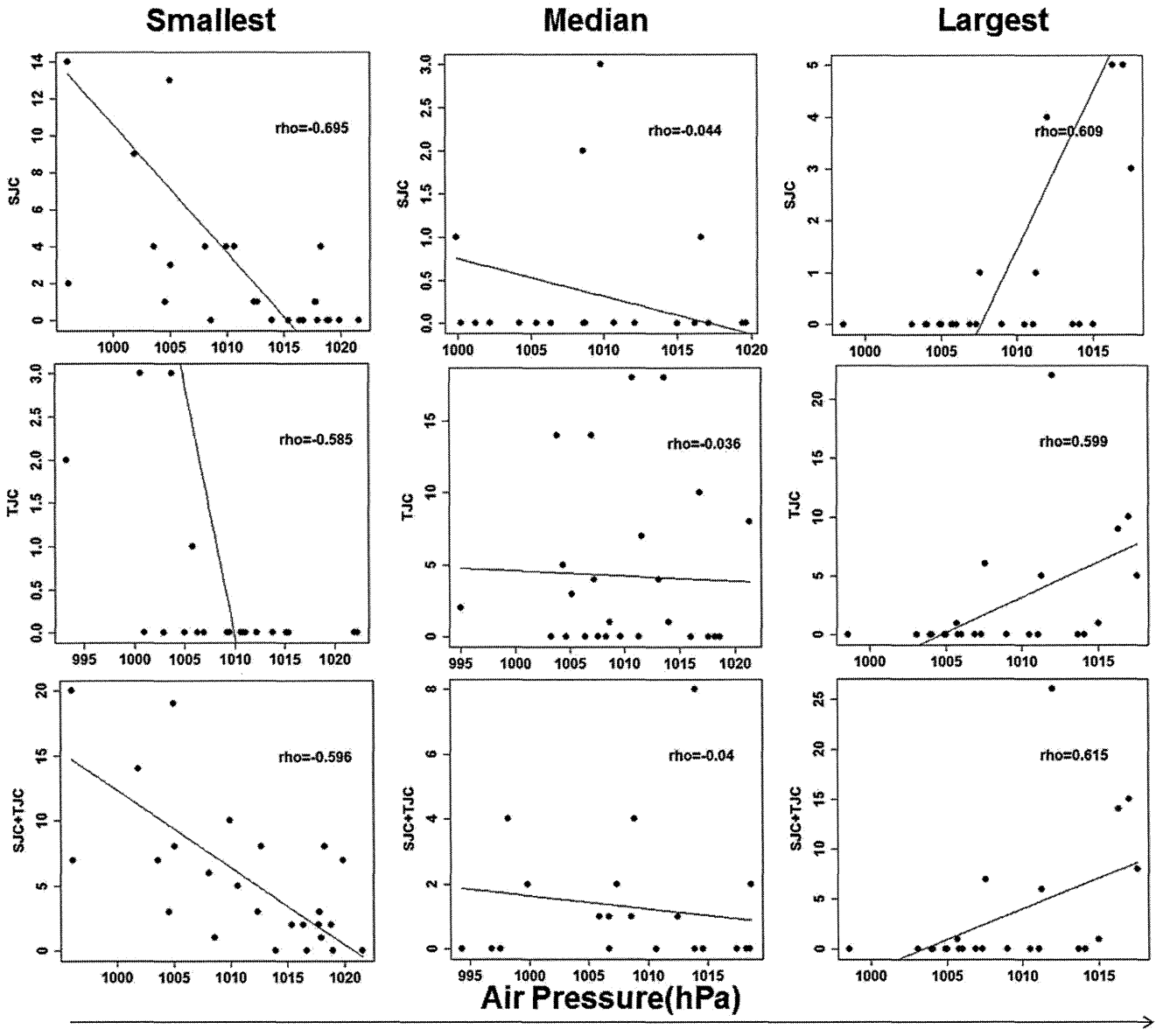

Figure 1. Correlations between joint synovitis and air pressure in RA patients. Correlation plots in patients demonstrating the smallest, median and largest correlation coefficients between joint synovitis and air pressure are illustrated in the left, middle and right panels, respectively.
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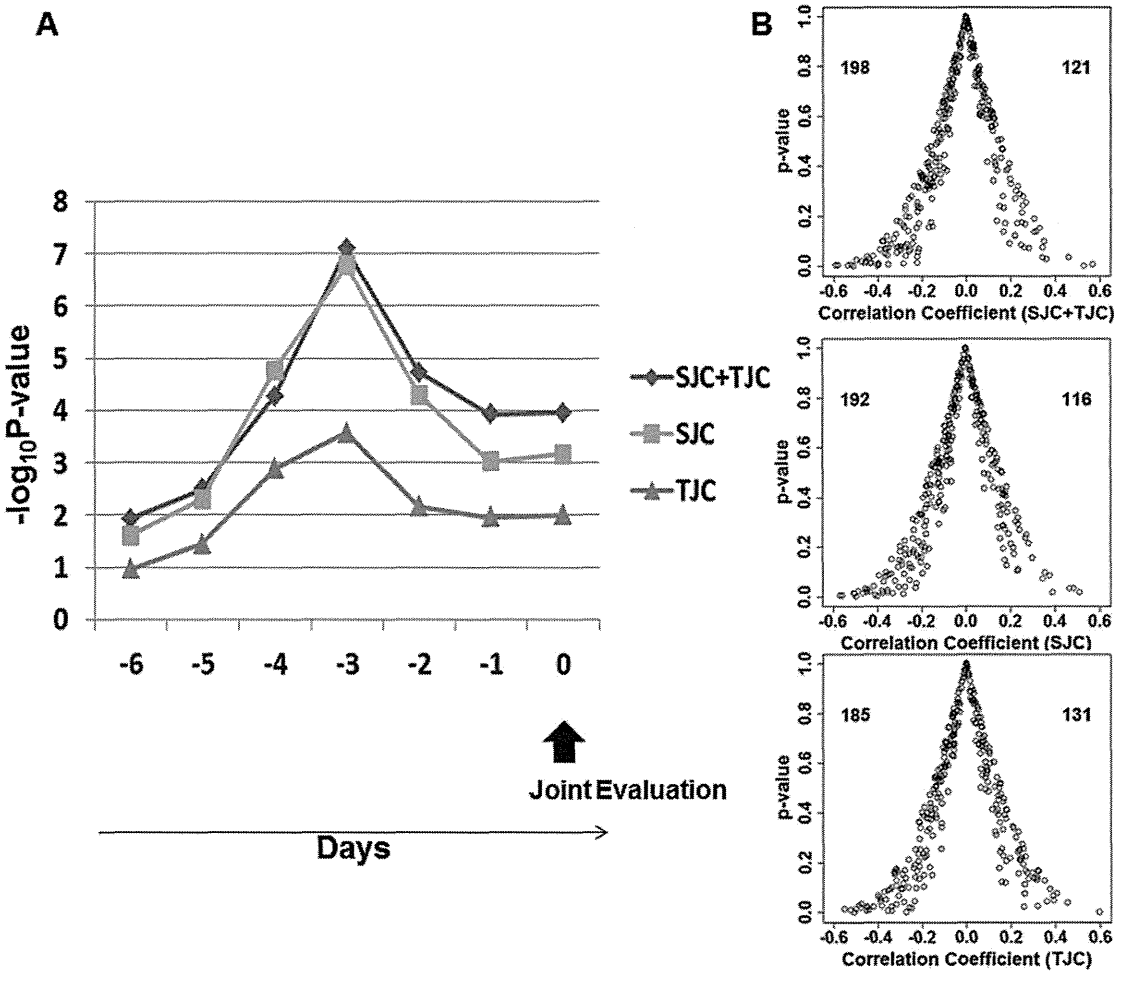

Figure 2. The strongest associations between joint synovitis and air pressure three days before joint evaluations. The 326 patients with more than 20 evaluations were analyzed. A)Associations between air pressure and joint synovitis for the seven consecutive days. Y axis indicates $-\log_{10}$P-value of Student's t-test. X axis indicates days before joint evaluations. B)Volcano plots for distribution of RA patients demonstrating positive or negative associations between joint synovitis and air pressure three days before joint evaluations. X and Y axes indicate Spearman's correlation coefficient and P-value, respectively. Numbers in the panels indicate RA patients showing positive or negative correlation coefficients. Because a part of the patients showed consistent TJC or SJC across all evaluations, the sum of the two numbers in each panel does not make 326.
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factors we focused on air pressure. Another reason we only focused on air pressure was to avoid type I statistical error by performing multiple association studies.

Our data set revealed low to moderate disease activity of RA on average, which reflects the appropriateness of the treatment the patients were receiving at Kyoto Unvesity Hospital. Considering large variation of the synovitis data and large number of joint evaluations, the current data should be enough to detect correlations.

The first analysis addressing correlation between air pressure and DAS28 did not result in a significant association. However, it should be noted that all of the elements of composite measures showed the same negative direction of association with air pressure. These results may suggest common associations between

Table 3. Results of multiple standardized linear regression analysis between air pressure and joint synvoitis.

Synovitis	-	Air pressure (hPa)		Temperature (°C)		Humidity (%)	
	Mean±SD(median)	1009.34±6.42 (1009.25)		16.10±8.77 (16.45)		63.83±9.75 (64)	
	-	Beta	P	Beta	P	Beta	P
SJC	1.96±3.06 (1)	−0.0765	0.0031	−0.0419	0.097	−0.0996	2.8×10^{-6}
TJC	2.12±3.70 (1)	−0.105	0.00082	−0.0535	0.079	−0.0976	0.00015
SJC+TJC	4.08±5.87 (2)	−0.181	0.00027	−0.0954	0.049	−0.197	1.4×10^{-6}

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Table 4. Standardized multiple linear regression analysis for air pressure 3 days before evaluations and synovitis among the 326 patients.

Synovitis	Mean±SD(median)	mean Beta±SD	P*
SJC	2.07±1.99 (1)	−0.126±0.0425	0.00023
TJC	2.08±2.19 (1)	−0.0893±0.0339	0.036
SJC+TJC	4.15±3.70 (2)	−0.215±0.0672	0.0015

*p-values for Student's t-test. SD:standard deviation.
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air pressure and RA joint synovitis instead of limited association between air pressure and a specific element of RA joint synovitis. At the same time, these results may suggest that composite measures composed of multiple elements showing weak association with air pressure are not sensitive enough to detect the influence of air pressure. In addition, because many medical doctors evaluate synovitis of many patients with RA, variations among doctors in each element would be amplified in the composite measure and result in difficulty to detect significant correlations. Considering the strong association between air pressure and sum of SJC and TJC, the fine direction of association between different elements and air pressure should partly overlap but partly differ. PVAS showed the lowest mean correlation coefficient among items of composite measures. This matches our observation that many of the RA patients feel correlation between their symptoms and air pressure. Although the number of data for pVAS is limited, 81 patients with more than 15 data for pVAS revealed a suggestive inverse correlation with air pressure (mean rho = −0.0469, data not shown). Accumulation of more data for each element would detect a significant association between each element and air pressure and a fine direction of association with air pressure in each element.

Because the number of joint assessments differed among patients and it was likely that analyses using all assessments would be influenced by the specific patients with large number of joint assessments, we performed the analyses focusing on the 326 patients with more than 20 assessments to avoid intra-patient correlations. We hypothesized that correlations between air pressure and joint synovitis should be largely different among patients but a large number of patients would result in a significant deviation. We calculated a correlation coefficient in each patient separately. The results supported our hypothesis and showed a significant deviation of the mean correlation coefficient from the null hypothesis. Figure 1 presenting patients with median correlations along with Table S2 indicated that overall distributions of correlations between air pressure and joint synovitis shifted to negative correlations. Considering the size of correlation coefficients, although RA patients show a negative correlation in average between air pressure and joint synovitis, the correlation greatly vary among patients and the correlation should not be generalized.

We found the strongest associations between air pressure and signs of synovitis three days before evaluations. This may indicate that slow mechanisms underlie the correlations or that joint synovitis is prone to unknown factors which reflect past air pressure.

When we classified patients into subgroups based on positivity of disease duration, smoking, Stage, Class and usage of biological agents during the observation period, we did not find significant difference among RA subsets (data not shown). The analysis of confounding factors revealed that the association of air pressure with joint synovitis was not derived from humidity and temperature, which were selected since air pressure, humidity and temperature are representatives of meteorological factors. Although both humidity and temperature showed correlations with air pressure (rho:0.19 and 0.58, between air pressure and humidity or temperature, respectively), their correlations could not explain the association between air pressure and RA synovitis. The analysis also revealed that humidity showed a negative association with joint synovitis that is independent from temperature and air pressure. A previous study reported that a combination of increase in humidity and decrease in air pressure were associated with worsening of joint pain [14]. Their findings matched our results for air pressure, but the association of humidity was opposite to ours. Thus, the association between humidity and joint synovitis is inconclusive and further studies are required. It is notable that mean temperature was not associated with joint synovitis in a multiple standardized linear regression analysis. As increase or decrease of blood flow due to temperature is supposed to influence signs of synovitis in RA, the lack of the association may be explained by rapid influence of finely conditioned temperature in hospitals.

It is difficult to assume the basic mechanisms underlying the correlation between joint synovitis and air pressure. One possible explanation is that air pressure directly presses joint structures in patients with RA. Low air pressure results in reduced outside pressure of joints which allow joints to be swollen more easily. Enlarged space of joints would allow more inflammatory cells to enter joint space and produce inflammatory cytokines. Another explanation is involvement of autonomic nerves to regulate threshold of pain. A Japanese group reported that both decreased air pressure and temperature led to worsening of joint pain in an animal model [22,23]. The group also showed that these correlations in the animal model were mediated by sympathetic nerve, whose excitement and increase of circulating noradrenaline were brought about by decrease of air pressure [24,25]. As the current study did not reveal a strong association between joint tenderness and air pressure, involvement of sympathetic nerve with pain cannot fully explain the current results. Variations of B-cell activity due to meteorological changes could be another possibility. Our previous study reported seasonal variation of IgG in rheumatic diseases [26]. Analysis incorporating altitude of residence for each patient, whose information is not available in the current study, would give a clue for the mechanism underlying the association.

We could not conclude whether air pressure directly influences RA synovitis or if it is just a confounding factor of yet-to-be-determined factors with direct effects on RA synovitis. However, our analysis supports the patients' subjective feelings of relationship between air pressure and joint synovitis. Another study addressing correlations between air pressure and joint synvoitis estimated by imaging including ultrasound seems necessary. Further experiments and analyses between air pressure and joint symptoms in humans would clarify the detailed mechanisms. It will be interesting to determine the characteristics of patients who are susceptible to change of air pressure.

Materials and Methods

Ethical statements

The analyses in the current study were performed under policy of data analysis of the KURAMA database approved by Kyoto University Hospital Ethical Committee [20]. Written informed consent to enroll in the database described below was obtained

from most of the patients, but for some patients the information regarding the construction of this database was disclosed instead of obtaining written informed consent. Participants who were informed regarding the construction of the database (instead of obtaining written informed consent) were allowed to withdraw from the study if desired. All data were de-identified and analyzed anonymously. This study was designed in accordance with the Helsinki Declaration.

Data of joint synovotis in patients with RA

A total of 23,064 evaluations of disease activity from 2,131 patients with RA were obtained with the corresponding dates of evaluations from the KURAMA database. The evaluation data contained some or all of the SJC, TJC in the 28 joints, ESR, pVAS and dVAS as well as DAS 28 as a composite measure for RA disease activity. The sum of SJC and TJC was also calculated for each evaluation. 326 patients with more than 20 evaluations of disease activity were extracted for further analysis.

Data of air pressure and other meteorological factors

Data of daily mean air pressure in Kyoto from 2005 to 2012 was obtained from the homepage of Japan Meteological Agency (http://www.jma.go.jp/jma/index.html). Data of daily mean temperature and humidity in Kyoto were also obtained in the same manner.

Statistical analysis

Correlations between mean air pressure on the day of joint evaluation and DAS28, SJC, TJC, ESR, pVAS, dVAS or sum of SJC and TJC were estimated by Spearman's correlation coefficients, using 23,064 evaluations of joint synovitis or evaluations in each of the 326 patients. The mean Spearman's correlation coefficients among the 326 patients with RA were estimated by Student's t-test under the null hypothesis that the mean was zero. Normality of distribution of correlation coefficients in the 326 patients was analyzed by Shapiro-Wilk test. A daily mean air pressure of the six days before the day of joint evaluation was also analyzed for correlations with signs of joint synovitis across the 326 patients with RA. To analyze independent effects of air pressure on joint synovitis from humidity and temperature, multiple standardized linear regression analysis was performed for 23,064 evaluations and each of the 326 patients with more than 20 evaluations. Mean beta values in the multiple standardized linear regression analysis among the 326 patients were assessed by Student's t-test under the null hypothsis that the mean beta values were zero. P-values less than 0.0071 were regarded as significant based on Bonferroni's correction. Data analysis was performed by R software (http://www.r-project.org/) or SPSS (ver 18).

Supporting Information

Figure S1 Fluctuations of air pressure and joint synovitis in the 326 patients. Fluctuations of each item are illustrated between 2005 and 2012. The three figures of SJC, TJC and combination of SJC and TJC are composed of 326 lines presenting fluctuations in the 326 patients.
(TIF)

Table S1 Correlation coefficients of RA joint synovitis in association with air pressure across different evaluations.
(DOC)

Table S2 Detailed information of the 326 RA patients.
(DOC)

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Author Contributions

Conceived and designed the experiments: CT MH FM HI TF TM. Analyzed the data: CT. Contributed reagents/materials/analysis tools: CT MH MF SN KO RN YI NY HY HI TF TM. Wrote the paper: CT MH.

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Two Susceptibility Loci to Takayasu Arteritis Reveal a Synergistic Role of the *IL12B* and *HLA-B* Regions in a Japanese Population

Chikashi Terao,[1,2,*] Hajime Yoshifuji,[2] Akinori Kimura,[3] Takayoshi Matsumura,[4] Koichiro Ohmura,[2] Meiko Takahashi,[1] Masakazu Shimizu,[1] Takahisa Kawaguchi,[1] Zhiyong Chen,[3] Taeko K. Naruse,[3] Aiko Sato-Otsubo,[5] Yusuke Ebana,[6] Yasuhiro Maejima,[7] Hideyuki Kinoshita,[8] Kosaku Murakami,[9] Daisuke Kawabata,[2] Yoko Wada,[10] Ichiei Narita,[10] Junichi Tazaki,[11] Yasushi Kawaguchi,[12] Hisashi Yamanaka,[12] Kimiko Yurugi,[13] Yasuo Miura,[13] Taira Maekawa,[13] Seishi Ogawa,[5] Issei Komuro,[4] Ryozo Nagai,[14] Ryo Yamada,[1] Yasuharu Tabara,[1] Mitsuaki Isobe,[7] Tsuneyo Mimori,[2] and Fumihiko Matsuda[1]

Takayasu arteritis (TAK) is an autoimmune systemic vasculitis of unknown etiology. Although previous studies have revealed that HLA-B*52:01 has an effect on TAK susceptibility, no other genetic determinants have been established so far. Here, we performed genome scanning of 167 TAK cases and 663 healthy controls via Illumina Infinium Human Exome BeadChip arrays, followed by a replication study consisting of 212 TAK cases and 1,322 controls. As a result, we found that the *IL12B* region on chromosome 5 (rs6871626, overall $p = 1.7 \times 10^{-13}$, OR = 1.75, 95% CI 1.42–2.16) and the *MLX* region on chromosome 17 (rs665268, overall $p = 5.2 \times 10^{-7}$, OR = 1.50, 95% CI 1.28–1.76) as well as the *HLA-B* region (rs9263739, a proxy of HLA-B*52:01, overall $p = 2.8 \times 10^{-21}$, OR = 2.44, 95% CI 2.03–2.93) exhibited significant associations. A significant synergistic effect of rs6871626 and rs9263739 was found with a relative excess risk of 3.45, attributable proportion of 0.58, and synergy index of 3.24 ($p \leq 0.00028$) in addition to a suggestive synergistic effect between rs665268 and rs926379 ($p \leq 0.027$). We also found that rs6871626 showed a significant association with clinical manifestations of TAK, including increased risk and severity of aortic regurgitation, a representative severe complication of TAK. Detection of these susceptibility loci will provide new insights to the basic mechanisms of TAK pathogenesis. Our findings indicate that *IL12B* plays a fundamental role on the pathophysiology of TAK in combination with HLA-B*52:01 and that common autoimmune mechanisms underlie the pathology of TAK and other autoimmune disorders such as psoriasis and inflammatory bowel diseases in which *IL12B* is involved as a genetic predisposing factor.

Introduction

Takayasu arteritis (TAK [MIM 207600]) is an autoimmune systemic vasculitis that was first reported from Japan.[1] It is estimated that TAK affects around 0.004% of the population in Japan, especially young women aged between 15 and 35. Although TAK was originally thought to affect individuals of mainly Asian origin, individuals with TAK have been identified worldwide, though with lower prevalence compared to Asia.[2] TAK is characterized by the involvement of large arteries, especially the aorta and its large branches, and is grouped into "vasculitis affecting large vessels" according to the Chapel Hill classification.[3] Individuals with TAK develop a wide range of symptoms such as fatigue, syncope, and lowering of vision in addition to its characteristic complications including aortic regurgitation (AR), pulselessness, and difference of blood pressure between right and left upper limbs. Previous studies have revealed that genetic components are involved in the pathogenesis of TAK, and HLA-B*52:01 is so far the only established genetic factor across the world.[4–7] Other genetic components especially outside of the HLA locus have not been confirmed to date. Establishment of association with non-HLA regions would lead to a deeper understanding of the basics of TAK pathology and the development of a novel therapy for this vasculitis. Here, we performed a genome-scanning study of TAK to identify the genetic predisposing factors for TAK.

Subjects and Methods

Study Subjects

A total of 379 TAK cases and 1,985 controls were enrolled in this study. All the cases were diagnosed based on the criteria of

[1]Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan; [2]Department of Rheumatology and Clinical Immunology, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan; [3]Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-8510, Japan; [4]Department of Cardiovascular Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan; [5]Cancer Genomics Project, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan; [6]Department of Bioinformational Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-8510, Japan; [7]Department of Cardiovascular Medicine, Tokyo Medical and Dental University, Tokyo 113-8510, Japan; [8]Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan; [9]Osaka Red Cross Hospital, Osaka 543-8555, Japan; [10]Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8510, Japan; [11]Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan; [12]Institute of Rheumatology, Tokyo Women's Medical University, Tokyo 162-0054, Japan; [13]Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, Kyoto 606-8507, Japan; [14]Jichi Medical University, Tochigi 329-0498, Japan
*Correspondence: a0001101@kuhp.kyoto-u.ac.jp
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Table 1. Summary of Study Subjects

	Case	Control
Genome Scanning		
Number	167	663
Age[a]	45.7 ± 15.2	53.5 ± 13.5
Female ratio	0.92	0.74
Age at onset[a]	30.5 ± 14.5	NA
Genotyping	Illumina Infinium Human-Exome BeadChip	Illumina Infinium Human-Exome BeadChip
Subjects with clinical information	AR:87; CRP:89	NA
Institutions	Kyoto University; Tokyo Women's Medical University	Kyoto University
Replication Study		
Number	212	1,322
Age[a]	46.6 ± 17.6	53.3 ± 13.4
Female ratio	0.94	0.62
Age at onset[a]	27.0 ± 11.8	NA
Genotyping	Taqman assay	Illumina Infinium Human Omni 2.5-4 BeadChip, Illumina Infinium Human Omni 2.5-8 BeadChip
Subjects with clinical information	AR:102; CRP:None	NA
Institutions	Tokyo Medical and Dental University; Kyoto University; Niigata University	Kyoto University

Abbreviations are as follows: NA, not applicable; AR, aortic regurgitation; CRP, C-reactive protein.
[a]Mean ± standard deviation (SD).

American College of Rheumatology[8] or guideline provided by Japanese Circulation Society.[9] The control subjects were collected as a part of the Nagahama Prospective Genome Cohort for Comprehensive Human Bioscience (The Nagahama Study), a community-based prospective multiomics cohort study conducted by Kyoto University.[10] This study was approved by the local ethical committees at each institution, and written informed consent was obtained from each subject involved in the study.

Genome Scanning

Illumina Infinium Human Exome BeadChip arrays (Illumina) were used for genome scanning of the cases and the controls. The genome scanning was conducted in Center for Genomic Medicine, Kyoto University Graduate School of Medicine.

Quality Control of Genome Scanning

Polymorphisms showing success rates less than 0.95 in either cases or controls, departure from Hardy-Weinberg equilibrium (HWE) ($p < 1.0 \times 10^{-5}$), or minor allele frequencies less than 0.05 in both cases and controls were excluded from the analysis. Subjects who showed success rates less than 0.95 or evidence of relatedness with other subjects were also excluded. Kinship between study subjects were estimated by PLINK.[11] Quantile-quantile plot (QQ plot) was used to assess the population stratification of the study. Because 1,827 markers over 24,487 were located in the HLA locus in which polymorphisms are very closely linked with each other, the 22,660 markers in the non-HLA regions were used for QQ plot.

Replication Study

The SNPs with p values less than 1.0×10^{-5} in the genome scanning were selected for the replication study. Because the association found in the *HLA-B* region (MIM 142830) was largely attributable to HLA-B*52:01, rs9263739, a proxy of HLA-B*52:01, was selected as a representative of the *HLA* locus. In the replication study, case samples were genotyped by Taqman Assay (Applied Biosystems) and control genotypes were extracted from array data (Table 1).

Combined Study and Association Study for Genotypes

Association studies of genotypes were performed by chi-square test based on 2 × 2 contingency tables. Combined study of the two studies was performed by inverse-variance method, assuming a fixed-effects model from the effect size (logarithm of odds ratio [OR]) in each study. A significant level for detecting susceptibility genes was set as 2.0×10^{-6}, which was obtained by Bonferroni's correction. A stringent cut-off level of 5.0×10^{-8} was also applied to assess overall significance.

Imputation of Genotypes

Mach dat2 software[12] was used for imputation of the whole genomes based on the results of genome scans with the use of the East Asian panel of HapMap phase II data as reference. SNPs with low imputation scores (Rsq < 0.3) were excluded from the analysis.

Calculation of Linkage Disequilibrium

LD between SNPs in the Illumina Infinium Human Exome BeadChip was assessed based on the genome-scanning data. HapMap project phase II data was used when SNPs were not contained in the array. LD between HLA-B*52:01 and SNPs was calculated by combining our previous HLA-genotyping data of the 173 TAK cases (C.T., unpublished data) by WAKFlow system (Wakunaga Pharmaceutical) with the genome-scanning data.

Estimation of Interaction

We used the method for evaluation of interaction proposed by Andersson et al.[13] Gene-gene interaction was defined as departure from additivity of two loci and measured by three indices based on calculation of relative risk (RR); relative excess risk due to interaction (RERI), attributable proportion (AP), and synergy index (SI). We considered an interaction as significant only when both RERI and AP were different from 0 and additionally SI was more than 1. The very low prevalence of TAK justifies to approximate OR by RR. For instance, when we assessed the interaction between rs9263739 and rs6871626 through these three indices, the subjects were classified into four groups: negative for both rs9263739 T allele and rs6871626 A allele, positive for rs9263739 T allele and negative for rs6871626 A allele, negative for rs9263739 T allele and positive for rs6871626 A allele, and positive for both rs9263739 T allele and rs6871626 A allele. Logistic models were used to calculate the indices.

In Silico Analysis of Association between the Gene Expression and rs6871626

We used two methods to assess the effect of rs6871626 on the *IL12B* (MIM 161561) expression. Gene expression data for *IL12B*

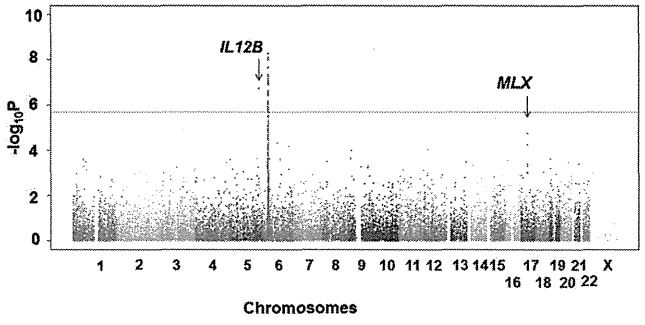

Figure 1. Manhattan Plot of Genome Scanning
The horizontal line indicates the significant level based on Bonferroni's correction. The HLA locus on chromosome 6 and the *IL12B* region on chromosome 5 reached the significant level.

in lymphoblastoid cells were obtained from GEO database (accession number GSE6536)[14] and analyzed for association with genotypes of rs6871626 obtained from HapMap project. Genevar software was used for analyzing the *IL12B* expression in adipose and skin in association with the rs6871626 genotypes.[15] Associations between genotypes and gene expression were evaluated by a linear regression analysis.

Associations between Genotypes and Clinical Phenotypes of TAK

Data of age at onset were analyzed for the association with the susceptibility alleles. AR, ischemic heart disease, and pulmonary infarction were selected for the association with genotypes as representative complications of TAK because cardiovascular event was the major cause of death in TAK individuals[16] and it was previously demonstrated that these phenotypes were associated with HLA-B*52:01,[17] suggesting that genetic backgrounds were at least partly responsible for these clinical manifestations. Data of the clinical manifestations were collected in Kyoto University Hospital or Tokyo Medical and Dental University by medical doctors who were blinded to genotype data reviewing clinical charts. Although AR evaluated by transthoracic echocardiography or angiography was positive for 44% of cases, other complications were found in less than 16%. Only AR was analyzed because of lack of power for other manifestations. Data for severity of AR assessed by the three categories[18] (mild, moderate, and severe) were also collected. C-reactive protein (CRP) was focused on as a biomarker reflecting disease activity. We calculated time-averaged CRP and dosage of prednisolone. Individuals who had visited hospitals for less than 500 days were excluded from the analysis of CRP. The associations between genotypes and clinical phenotypes were assessed by logistic regression analysis for existence of AR or linear regression analysis for severity of AR, time-averaged CRP, and age at onset. Time-averaged CRP was analyzed in condition with time-averaged dosage of prednisolone alone or in combination with rs3093059 genotypes in the *CRP* (MIM 123260) region. Associations between genotypes and clinical manifestations with p values less than 0.05 were regarded as significant.

Statistical Analysis

Statistical analyses were performed by PLINK v.1.07, R statistical software, or SPSS v.18.0.

Results

A summary of basic information of the subjects in our study is shown in Table 1. DNA samples from 167 cases and 663 healthy controls were genome scanned with the use of Illumina Human-Exome arrays containing 247,730 SNPs. One sample of the TAK cases and six samples in controls with success rates of less than 0.95 or with evidence of relatedness with other subjects (PI_HAT > 0.2 calculated by PLINK, see Subjects and Methods) were excluded from further analysis. The genotyping revealed that more than 80% of the markers in the array were monomorphic and 9% of the markers showed low minor allele frequency (<0.05) in the Japanese population, respectively. A total of 24,487 markers remained after filtering of SNPs that showed success rates of less than 0.95, deviation from HWE ($p < 1 \times 10^{-5}$) in either cases or controls, or minor allele frequencies of less than 0.05 in both cases and controls. The mean success rate of individuals was 0.999 after filtering.

Association studies were performed by chi-square test to compare allele frequencies between cases and controls. Population stratification was evaluated by QQ plot. The results indicated a lambda value of 1.05 in the QQ plot, indicating no excess population stratification in our study. Manhattan plot revealed that a region on chromosome 5 as well as the HLA locus showed significant associations that satisfied the genome-wide significant threshold obtained by Bonferroni's correction ($p = 2.0 \times 10^{-6}$; Figure 1). The associations were also confirmed by the imputed results (Figure S1 available online). rs4947248 in the *HLA-B* region, which is a known susceptibility gene to TAK, showed the strongest association ($p = 5.1 \times 10^{-9}$, OR = 2.17, 95% CI 1.67–2.82). rs9263739, a proxy of HLA-B*52:01 ($r^2 = 0.94$), similarly showed a significant association ($p = 8.0 \times 10^{-9}$, OR = 2.30, 95% CI 1.72–3.07; Table 2) and in moderate LD with rs4947248 ($D' = 0.95$, $r^2 = 0.58$). Because rs4947248 did not show evidence of an independent association from rs9263739 in logistic regression analysis ($p = 0.04$), we assumed that the top association in the HLA locus was attributable to HLA-B* 52:01. rs6871626 in the *IL12B* region on chromosome 5 also showed a significant association ($p = 1.8 \times 10^{-7}$, OR = 1.90, 95% CI 1.49–2.42; Table 2 and Figure 2A). Four other loci showed suggestive associations in our study ($p < 5.0 \times 10^{-5}$; Table 2). No departure from HWE was observed for these six SNPs ($p \geq 0.041$).

A replication study was performed with the use of DNA samples from 212 cases and 1,322 controls. The six SNPs with p values less than 5.0×10^{-5} in the genome scanning were genotyped in the replication study. rs9263739 was selected as a representative of the associations in the HLA locus. As a result, the significant associations of TAK with rs6871626 and rs665268 in the *MLX* (MAX dimerization protein [MIM 602976]) region on chromosome 17 as well as rs9263739 were replicated ($p = 1.1 \times 10^{-7}$, 0.0032, and 6.0×10^{-15}, respectively; Table 2, Figures 2A

Table 2. Results of Association Studies for TAK Susceptibility

						Genome Scan			Replication			Meta-analysis	
SNP	Chr	Position	Gene	Ref(A1)	Var(A2)[a]	Case A2freq	Cont A2freq	p	Case A2freq	Cont A2freq	p	p	OR (95% CI)
rs10934853	3	129521063	*EEFSEC*	A	C	0.59	0.45	1.3×10^{-5}	0.52	0.47	0.066	2.6×10^{-5}	1.40 (1.20–1.64)
rs6871626	5	158759370	*IL12B*	C	A	0.53	0.37	1.8×10^{-7}	0.53	0.39	1.1×10^{-7}	1.7×10^{-13}	1.75 (1.42–2.16)
rs9263739	6	31219335	*CCHCR1*	C	T	0.27	0.14	8.0×10^{-9}	0.30	0.14	6.0×10^{-15}	2.8×10^{-21}	2.44 (2.03–2.93)
rs1570843	6	84577239	*RIPPLY2*	C	T	0.62	0.50	4.6×10^{-5}	0.54	0.51	0.19	3.1×10^{-4}	1.34 (1.14–1.57)
rs12102203	15	49578851	*DMXL2*	G	A	0.64	0.49	3.8×10^{-6}	0.53	0.54	0.71	0.0081	1.24 (1.06–1.46)
rs665268	17	37975555	*MLX*	A	G	0.58	0.44	1.7×10^{-5}	0.49	0.42	0.0032	5.2×10^{-7}	1.50 (1.28–1.76)

Abbreviations are as follows: chr, chromosome; ref, reference allele; var, variant allele; CaseA2freq, variant allele frequency in cases; ContA2freq, variant allele frequency in controls; OR, odds ratio; CI, confidence interval. Positions are according to National Center for Biotechnology Information (NCBI) build 36.
[a]Risk alleles for TAK based on the results of the genome scanning are set as variant alleles.

and 2B). The suggestive association on chromosome 15 (Figures S1 and 1) was not replicated. Again, no departure from HWE was observed ($p \geq 0.11$).

A combined study in which the associations in the two studies were integrated by inverse-variance method demonstrated that rs6871626, rs665268, and rs9263739 showed significant associations ($p = 1.7 \times 10^{-13}$, 5.2×10^{-7}, and 2.8×10^{-21}; OR = 1.75, 1.50, and 2.44; 95% CI 1.42–2.16, 1.28–1.76, and 2.03–2.93, respectively; Table 2) satisfying the significance obtained by Bonferroni's correction. rs6871626 and rs9263739 satisfied the more stringent, widely accepted genome-wide significance ($p = 5.0 \times 10^{-8}$).

Because it was suggested that genetic components had influence on the manifestations of the disease,[17] we analyzed whether the variant of the *IL12B* region had clinical effects on the disease course or severity. Age at onset was not associated with rs6871626 ($p = 0.36$), whereas a significant association between rs6871626 and development of AR was observed in a recessive model ($p = 0.0046$; Figure 3A). Focusing on the cases with AR, a significant association between rs6871626 and severity of AR was observed in the recessive model ($p = 0.0018$; Figure 3B). Risk allele of rs6871626 (A allele) also demonstrated a significant association with increased level of time-averaged CRP, which was a representative marker of the disease activity ($p = 0.021$; Figure 3C). The association between rs6871626 and CRP levels was independent from rs3093059 in the *CRP* region ($p = 0.029$), which showed the strongest association with circulating CRP levels in Japanese.[19] These associations between rs6871626 and clinical manifestations were independent from rs9263739 (conditioned p value of rs6871626 ≤ 0.020). Although rs665268 also demonstrated a significant association with development of AR in a dominant model ($p = 0.0089$; Figure S2A), the association was not significant in condition with rs9263739 ($p = 0.080$). No significant associations were observed between rs665268 and other clinical phenotypes (Figures S2B and S2C).

Next, we investigated the interaction between the *IL12B* and *HLA-B* loci to TAK susceptibility. The risk of TAK in the population positive for both rs6871626 A allele and rs9263739 T allele surpassed the product and sum of the risk in those who were positive for either rs6871626 A allele or rs9263739 T allele alone (Figure 4). The analysis revealed that those who were positive for both had OR of 6.00 (95% CI 4.22–8.55), whereas those who were positive for either rs9263739 T allele or rs6871626 A allele showed OR of 1.80 (95% CI 1.11–2.93) or 1.74 (95% CI 1.23–2.47), respectively. Interaction measures revealed RER of 3.46 ($p = 1.4 \times 10^{-5}$, 95% CI 1.90–5.01), AP of 0.58 ($p = 1.0 \times 10^{-12}$, 95% CI 0.42–0.73), and SI of 3.24 ($p = 0.00028$, 95% CI 1.72–6.11). This significant interaction between *IL12B* and *HLA-B* on TAK susceptibility could be observed in both studies (Table 3). The synergistic interaction effects between rs6871626 and rs9263739 were not evident in the clinical manifestations associated with rs6871626 (Figure S3). When we analyzed the interaction between the *MLX* and *HLA-B* regions, we observed suggestive interaction with RER of 1.73, AP of 0.43, and SI of 2.29 ($p \leq 0.027$; Figure S4 and Table S1). The associations between the interaction and clinical manifestations were not significant (Figure S5).

IL12B encodes a common subunit of the IL12 and IL23 protein, known as p40. Because previous studies showed that the *IL23R/IL12RB2* (MIM 607562/601642) region was associated with Behçet disease[20] (MIM 109650), another connective tissue disease where vasculitis is involved in its pathology, we investigated this region for the possible associations in the current study. As a result, no suggestive association was found, either in our study or in the imputed results (Figure S6).

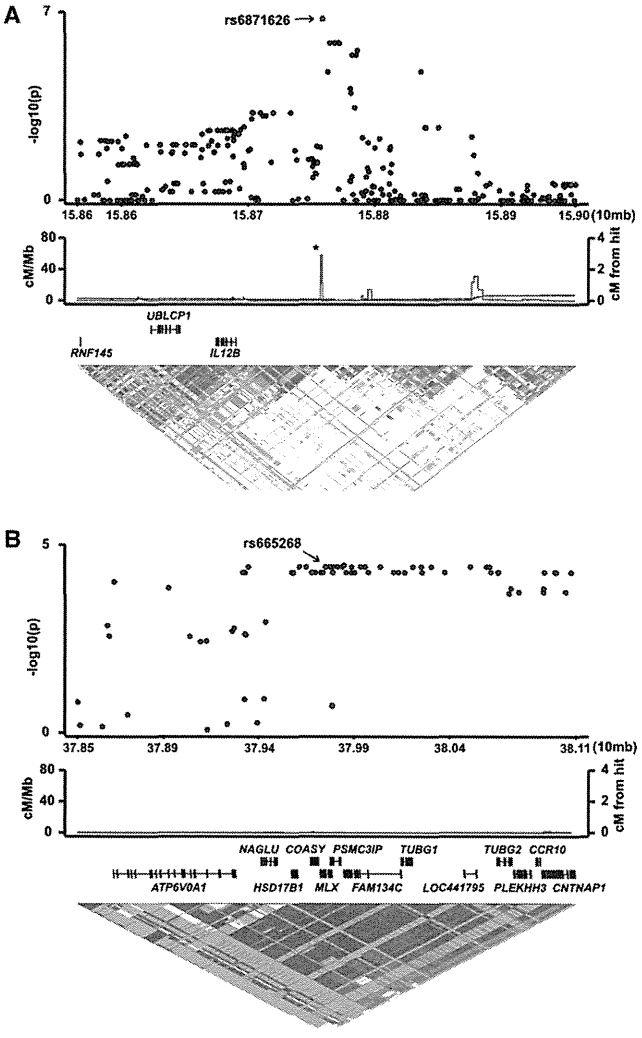

Figure 2. Associations of the *IL12B* and *MLX* Regions with the Susceptibility to TAK

Associations of SNPs in the (A) *IL12B* and (B) *MLX* regions in the genome scanning are plotted according to the position of the markers. Red circles indicate results of the current genome scanning. Blue circles indicate results of the imputation analysis based on the current results. The middle panel indicates recombination rates. The lower panel indicates LD of markers. Asterisk indicates a recombination hot spot in the *IL12B* region.

Discussion

This study provides a convincing evidence of associations between non-HLA genes and TAK susceptibility along with a synergistic role of susceptibility genes to TAK. The lack of evidence for associations of non-HLA genes with TAK so far is attributable to the lack of GWASs of TAK performed to date. Low prevalence of this disease had made it difficult to collect DNA samples to obtain sufficient power to detect susceptibility genes and perform a GWAS. Previous studies have revealed that the *IL12B* region was associated with a wide variety of autoimmune disorders and infectious diseases, including psoriasis[21–23] (MIM 177900), ankylosing spondylitis[24] (MIM 106300), Crohn disease[25] (CD [MIM 266600]), ulcerative colitis[26] (UC [MIM 191390]), and leprosy[27] (MIM 609888). rs6871626 showed a significant association with UC and leprosy over the genome-wide significance. Notably, rs6871626 A allele is susceptible to UC but protective against leprosy. A previous study from Turkey reported a suggestive association of TAK with rs3212227 in the 3′ UTR of the *IL12B* region.[28] rs3212227 is not in strong LD with rs6871626 in the Japanese population ($r^2 = 0.11$) and in Europeans ($r^2 = 0.06$) because of a recombination hot spot adjacent to rs6871626 (Figure 2A). In fact, an imputed association of rs3212227 with TAK in the current study resulted in only a suggestive association ($p = 0.0027$). There is a possibility that rs6871626 was responsible for the suggestive association between rs3212227 and TAK reported in the Turkish population. The association between gene expression and SNPs in the *IL12B* region appears to be complicated and inconsistent across different studies. rs3212227 in the 3′ UTR and rs17860508, an ins/del polymorphism in the promoter region of *IL12B*, were shown to have potential effects on the gene expression.[29,30] However, the previous studies showed that the association patterns varied according to the cell type and the protocol used for stimulation.[31–33] No previous report analyzed the effects of rs6871626 on the gene expression of *IL12B*. Although our in silico analysis failed to show the effects of rs6871626 on *IL12B* expression (data not shown, see Subjects and Methods), specific cell types or stimulus could lead to a significant association. Because a recent study showed that a haplotype of SNPs in the *IL12B* region could influence the gene and protein expression of *IL12B*,[22] a combination of rs6871626 and other SNPs in the *IL12B* region might lead to consistent results.

The associations between rs6871626 and clinical manifestations of TAK suggest the fundamental effects of IL-12p40 protein on TAK progression as well as TAK onset. We found that HLA-B*52:01 was associated with AR as reported previously ($p = 0.00014$). This finding supported the accuracy of our data. Although the risk allele of rs6871626 was associated with a significant dose-dependent increase in risk and severity of AR and in circulating CRP levels ($p = 0.013$, 0.030, and 0.023, respectively), these associations were more evident in a recessive manner. This raises a possibility that those who are homozygous for rs6871626 have strong disease activity that exceeds the additive disease activity of cases with single risk alleles, leading to severe destruction of aortic valve. Genetic variations in *IL12B* are known to influence the risk of psoriasis[21–23] and CD.[25] Because ustekinumab, a monoclonal antibody against IL-12p40, is an effective treatment for both diseases,[34,35] our findings would raise a possibility of its therapeutic use for TAK by targeting the IL-12/23 pathway. A previous study reported that the level of IL-12 protein was increased in TAK cases compared to healthy populations,[36] whereas there have been no reports addressing the circulating levels of IL-23 in TAK cases. IL-12 directly leads to type 1 helper T cell proliferation[37] and IL-23 upregulates IL-17 production and

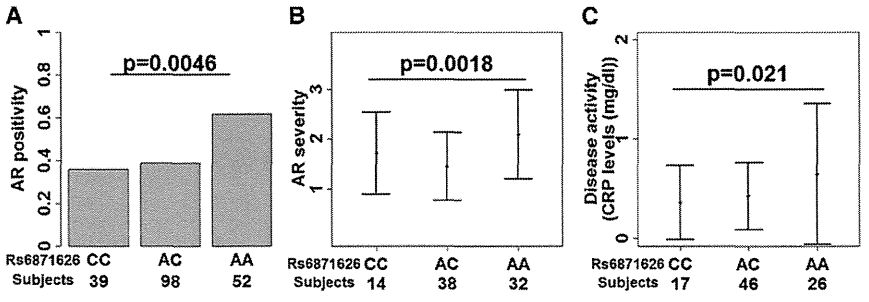

Figure 3. Associations between rs6871626 Genotypes and Clinical Manifestations of TAK

An association between rs6871626 genotypes and (A) development of AR, (B) severity of AR, and (C) time-averaged CRP levels in TAK cases. The p value was calculated by (A) logistic regression analysis, (B) linear regression analysis, and (C) linear regression analysis with time-averaged dosage of prednisolone as covariate. The recessive model is applied to all calculations. Severity of 1 to 3 in AR corresponds to mild, moderate, and severe, respectively. Mean ± SD are indicated for (B) and (C).

supports survival of activated Th17 cells.[38] Further analyses addressing circulating T cells in individuals with TAK or cell types infiltrating the artery specimen obtained from cases would provide clues to specify a critical pathway in TAK pathology.

The synergistic effect between rs6871626 and HLA-B*52:01 was notable. Those carrying both risk alleles had OR of 6.00 in comparison with those not carrying any risk alleles. Combination of rs6871626 and HLA-B*52:01 showed tendency of severe clinical phenotypes. Thus, we assume that increase of subjects and extraction of subjects who are homozygous for rs6871626 and positive for HLA-B*52:01 would provide evidence for significant effects of the combination on the disease phenotypes. The synergistic effect of these two loci raises a possibility that immune-related cells that recognize a yet-to-be-determined antigen through HLA-B*52:01 can be overactivated by IL-12/23 whose expression or function is modulated by rs6871626. In vitro analysis of immune-related cells from cases with TAK or healthy individuals would provide functional evidence of this synergistic role in the TAK pathogenesis.

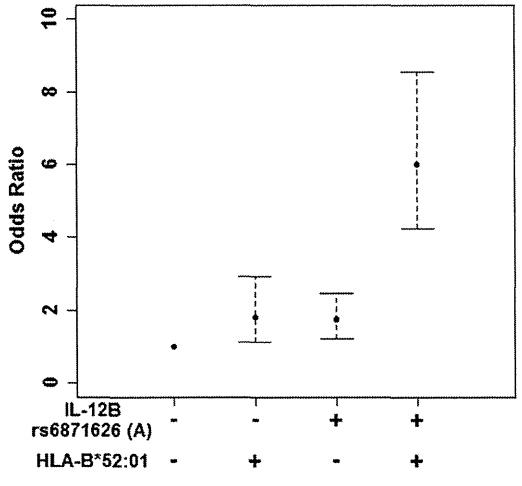

Figure 4. A Synergistic Effect between *IL12B* and HLA-B*52:01 on TAK Susceptibility

ORs are shown for the four strata of subjects according to combination of rs6871626 and rs9263739 genotypes. Those who are negative for rs9263739 T allele, a proxy of HLA-B*52:01, and rs6871626 A allele are used as reference. ORs and 95% CI are indicated.

rs665268 is a missense mutation of *MLX* that alters the 139th glutamine to arginine (Gln139Arg). MLX is a member of the basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factor family and regulates gene expression by forming heterodimers with Mad protein.[39] The 17q21 region, whose associations with other autoimmune diseases including psoriasis[40] and CD[41] were shown, contains a number of genes including immune-related genes and polymorphisms that are in strong LD with each other (Figure 2B), so the corresponding gene to TAK susceptibility was inconclusive. Because risk allele frequency of rs665268 is comparable to that of rs6871626, the lack of associations between rs665268 and clinical manifestations and the weaker interaction between rs665268 and HLA-B*52:01 compared to rs6871626 might be a reflection of the milder effect of rs665268 on TAK progression. No interaction was observed between rs665268 and rs6871626 (data not shown).

We set the relatively low cut-off value of imputation score (Rsq $\geq$ 0.3) in the imputation analysis to increase sensitivity at the expense of specificity, but we failed to find other candidates of susceptibility loci. Another imputation analysis based on the data from the 1000 Genomes Project[42] revealed the same signals as the current study (data not shown). However, because the array used in the current study focused on SNPs in exons or nearby genes, it did not fully cover the whole genome with dense markers even in imputation analysis. There is a possibility that other SNPs not tagged by the markers on the array are associated with TAK. When the associations in the HLA locus were conditioned by rs9263739 or rs4947248, the most significantly associated SNPs, suggestive association signals in this locus could still be observed (the smallest p value = 5.5×10^{-5}, data not shown). The use of arrays with denser markers especially in intergene regions and using an increased number of cases could lead to the discovery of other susceptibility regions or independent associations in the HLA locus. Considering that both of the non-HLA susceptibility loci to TAK found in the current study are also associated with psoriasis and inflammatory bowel diseases, further analysis of TAK susceptibility genes would reveal other overlapping loci and common autoimmune mechanisms between TAK and other autoimmune diseases. It is feasible to

Table 3. Synergistic Effects between *IL12B* and HLA-B*52:01 in Each Study

	RERI		AP		SI	
Study	**(95% CI)**	**p**	**(95% CI)**	**p**	**(95% CI)**	**p**
Genome scanning	2.90 (0.60–5.20)	0.014	0.50 (0.23–0.78)	0.00034	2.57 (1.08–6.09)	0.032
Replication study	3.87 (1.70–6.05)	0.00049	0.62 (0.42–0.81)	4.7×10^{-10}	3.76 (1.51–9.32)	0.0043
Combined study	3.46 (1.90–5.02)	1.4×10^{-5}	0.58 (0.42–0.73)	1.0×10^{-12}	3.24 (1.72–6.11)	0.00028

Abbreviations are as follows: RERI, relative excess risk; AP, attributable proportion; SI, synergy index; CI, confidence interval.

analyze whether these two loci are associated with TAK and whether the interactions are observed in other populations.

Taken together, the current study identified two susceptibility genes to TAK and provided evidence of a common immunological pathway exerted by the *IL12B* region that is involved in the etiology of TAK and other autoimmune disorders and of its synergistic role with HLA in the susceptibility to TAK.

Supplemental Data

Supplemental Data include six figures and one table and can be found with this article online at http://www.cell.com/AJHG/.

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Web Resources

The URLs for data presented herein are as follows:

Gene Expression Omnibus (GEO), http://www.ncbi.nlm.nih.gov/geo/
Genevar (Gene Expression Variation), http://www.sanger.ac.uk/resources/software/genevar/
International HapMap Project, http://hapmap.ncbi.nlm.nih.gov/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/
R statistical software, http://www.r-project.org/

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