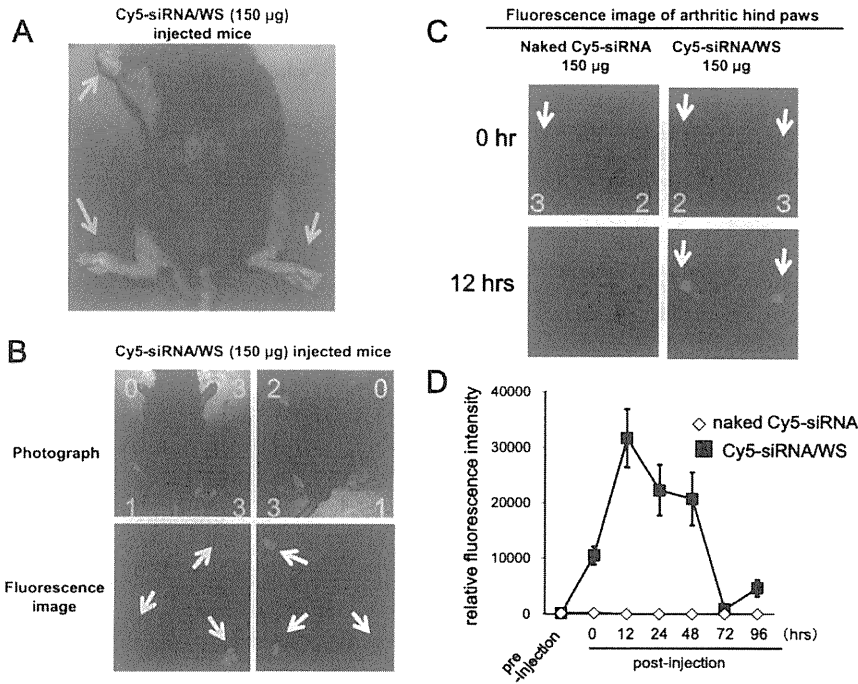

Fig. 1. Accumulation of systemically administered siRNA/WS in arthritic sites. At day 7 after induction of CIA, mice with arthritis (scored more than 2 at least in a paw) were given injections of saline, naked Cy5-siRNA, or Cy5-siRNA/WS. A, mice were examined 12 h later for accumulation of the Cy5-fluorescence with a stereoscopic microscope. The arrows indicate the sites where the fluorescence was observed. B, arabic numerals on each paw indicate arthritis score (top), and arrows show the accumulation of the fluorescence observed in the corresponding paws (bottom). C, fluorescence observed in arthritic hind paws of mice injected either with naked Cy5-siRNA (150 μg/body) or Cy5-siRNA/WS (150 μg/body) at 0 min (immediately after the injection) and 12 h. D, time course of the fluorescence intensity in arthritic hind paws (arthritis score = 3) of mice injected with naked Cy5-siRNA (150 μg/body) or Cy5-siRNA/WS (150 μg/body). Data are the mean ± S.E.M.; n = 3. A–C, representative photographs of eight mice are shown.

synovium of naked Cy5-siRNA-injected CIA mice (Fig. 2A). Moreover, the Cy5 intensity was significantly enhanced in synovial cells from mice injected with Cy5-siRNA/WS compared with that in those injected with naked Cy5-siRNA, with the ratio of mean fluorescence intensity (MFI) (Cy5-siRNA/WS versus naked Cy5-siRNA) of 4.4 (Fig. 2B). The Cy5 intensity in bone marrow cells and peripheral blood leukocytes of naked Cy5-siRNA-injected CIA mice was also higher; however, the Cy5 intensity in mice injected with Cy5-siRNA/WS was not enhanced (MFI ratios 0.97 and 0.57, respectively). Of note, the MFI of Cy5 was much higher in synovial cells from Cy5-siRNA/WS-injected mice compared with bone marrow cells and peripheral blood leukocytes (Fig. 2B). On the other hand, the fluorescence intensity was not significantly altered in splenocytes.

Next, we determined the cells that incorporate the Cy5-siRNA/WS in the synovium. At 24 h after injection, the synovial cells of mice treated with naked Cy5-siRNA or Cy5-siRNA/WS were analyzed by flow cytometry after staining with anti-CD11b, -F4/80, -CD3 or -CD19 mAbs. In the Cy5-siRNA/WS-injected mice, 81% of the synovial cells were Cy5-positive, and most of the Cy5-positive cells (>75%) were

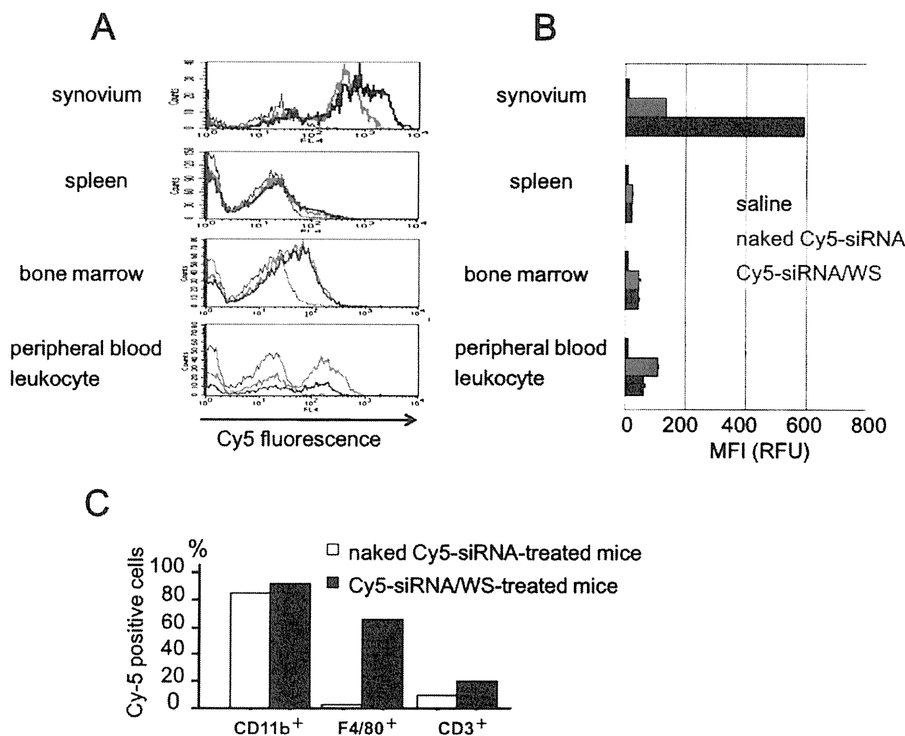

Fig. 2. Tissue distributions of systemically administered siRNA/WS. At day 7 after induction of CIA, mice with arthritis (scored more than 2 at least in a paw) were given injections saline, naked Cy5-siRNA, or Cy5-siRNA/WS. A, twelve hours after the injection, the levels of Cy5 fluorescence in cells extracted from the synovium, spleen, bone marrow, and peripheral blood leukocytes were analyzed by flow cytometry. B, MFI of Cy5 in the tissues. RFU, relative fluorescence unit. C, synovial cells from the mice injected with naked Cy5-siRNA or Cy5-siRNA/WS were stained with anti-CD11b, -F4/80, or -CD3 mAb, and Cy5 expression in $CD11b^+$, $F4/80^+$, or $CD3^+$ cells was analyzed by flow cytometry. The percentage of Cy5-positive cells was determined for each cell type. n = 2 to 3 for each condition (A–C). Synovial cells from the three mice were analyzed altogether (A–C).

$CD11b^+$. In addition, 92% of $CD11b^+$ and 67% of $F4/80^+$ cells were Cy5-positive, whereas, only 20% of $CD3^+$ cells were Cy5-positive. Although the majority of $CD11b^+$ cells in the synovium of naked Cy5-siRNA-treated mice were also Cy5-positive (82%), fewer $F4/80^+$ and $CD3^+$ cells were Cy5-positive (1 and 10%, respectively) compared with Cy5-siRNA/WS-treated mice (Fig. 2C). $CD11b^+$ synovial cells in CIA mainly consisted of macrophages (70%) and secondary dominant neutrophils (30%) (data not shown). Of the synovial cells, very few were $CD19^+$ (less than 0.1%).

These results suggest that both selective delivery and retention of Cy5-siRNA to inflamed synovium could be achieved using the WS as a carrier of the siRNA and that systemically administered siRNA/WS would efficiently target macrophages and neutrophils in inflamed joints.

Efficacy of siRNA Targeting TNF-α/WS for CIA. Experimental evidence indicates that functional blockade of TNF-α effectively inhibits CIA (Kim et al., 2002). Thus, we analyzed the effect of systemic administration of siRNA/WS targeting TNF-α on CIA. Mice treated with TNF-α siRNA/WS at 1 and 10 μg showed substantially decreased and delayed incidence of arthritis over those treated with control siRNA/WS ($P < 0.05$, TNF-α siRNA/WS 10 μg versus control 10 μg, at day 7) (Fig. 3A). Treatment with 10 μg of siRNA/WS targeting TNF-α significantly lowered the disease severity expressed as clinical arthritis score and paw thickness, compared with control siRNA/WS (Fig. 3, B and C). On the other hand, naked TNF-α siRNA had no effect on CIA (data not shown). In addition, TNF-α mRNA levels in the paws of TNF-α siRNA/WS (10 μg)-treated mice were significantly lower than those in mice treated with control siRNA/WS (Fig. 3D). No signs of toxicity were observed in either control siRNA/WS or TNF-α siRNA/WS-treated mice. These results suggest that down-regulation of TNF-α expression in the synovial tissue by systemically administered TNF-α-targeting siRNA/WS inhibited CIA.

Discussion

In this study, we showed that systemically administered siRNA/WS complex accumulated in arthritic joints in murine CIA, where the complex was preferably incorporated into $CD11b^+$ macrophages and neutrophils. Moreover, treatment with siRNA/WS targeting TNF-α ameliorated CIA.

RA is characterized by chronic synovitis affecting multiple joints, and synovial macrophages play central roles in the pathogenesis by producing various proinflammatory cytokines such as TNF-α, interleukin (IL)-1, and IL-6. Treatment with anti-TNF-α mAb was effective for murine CIA (Williams et al., 1992). Moreover, TNF-α blockade was efficacious in the treatment of RA (Smolen et al., 2007). However, systemic administration of TNF-α inhibitor could impair the immune system, which increases the development of infectious diseases (Komano et al., 2011), including reactivation of latent infections such as tuberculosis (Keane et al., 2001). Therefore, selective delivery of siRNA that targets macrophages in the affected synovia at multiple anatomical sites is an ideal strategy. Our results demonstrated that this goal can be achieved using the siRNA/WS formation, and such treatment might be potentially useful for RA in humans by inhibition of cytokine production by synovial macrophages. Our method could also target other proinflammatory cytokines or intracellular molecules in the synovial macrophages that are known to play some roles in chronic joint inflammation (Mosser and Edwards, 2008).

In arthritic synovium, incorporation of siRNA/WS was observed in a majority of the $CD11b^+$ cells and in more than half of the $F4/80^+$ cells, but only in a few in the $CD3^+$ cells. $CD11b^+$ synovial cells in our CIA model mainly consisted of macrophages and neutrophils. It was reported that $F4/80^+$ synovial cells were mature macrophages (Morris et al., 1991). The mechanism of the selective incorporation of siRNA/WS in macrophages and neutrophils in the inflamed synovium is not fully understood. It is unlikely that the selective siRNA delivery by the siRNA/WS complex depends simply on the innate phagocytic capability of the cells. Investigators in our group reported that the complex was able to transduce therapeutic siRNA selectively into cancer cells in mice in vivo (Yagi et al., 2009). It is possible that the siRNA/WS is preferentially delivered and enters the affected synovium as a result of increased angiogenesis and vascular permeability in such inflammatory tissues. Indeed, the size of the siRNA/WS

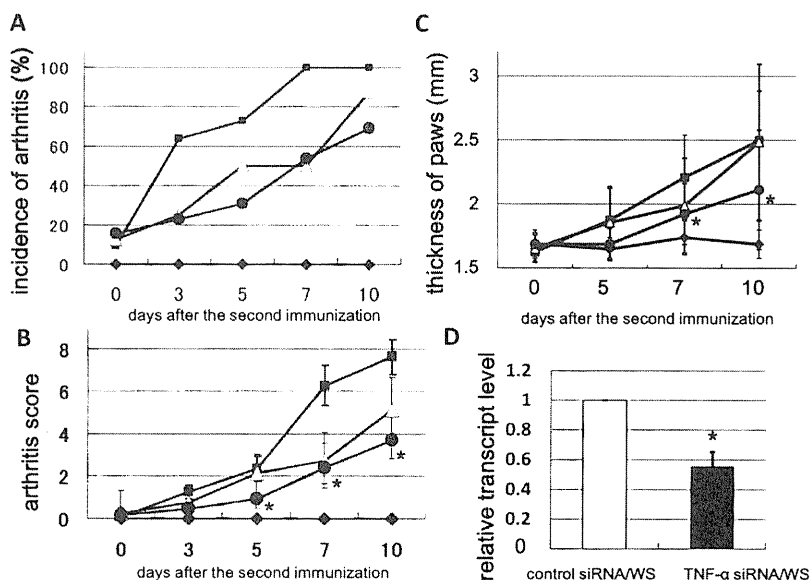

Fig. 3. Effects of systemic administration of siRNA targeting TNF-α/WS on CIA. A–C, TNF-α siRNA/WS (1 or 10 μg) or control siRNA/WS (10 μg) was injected intravenously in CIA mice three times per week starting from the day of the second immunization (day 0) to day 10. The incidence of arthritis (A), arthritis score (B), and paw thickness (C) were evaluated continuously until day 10. D, at day 12, TNF-α mRNA expression in the paw was analyzed by real-time RT-PCR. Data are the mean ± S.E.M. of 5 to 13 mice in each group. *, $P < 0.05$ mice treated with TNF-α siRNA/WS 10 μg versus control siRNA/WS. ■, control siRNA/WS, 10 μg/body; △, TNF-α siRNA/WS, 1 μg/body; ●, TNF-α siRNA/WS, 10 μg/body; ◆, normal.

is designed to maximize enhanced permeability and retention effect (Hall et al., 2007). In addition, we recently found that systemically injected siRNA/WS was accumulated in 12-*O*-tetradecanoylphorbol-13-acetate-treated skin area in a mouse model of skin inflammation (N. Yagi, unpublished observations). These data also indicate that the siRNA/WS has the nature of accumulating in the inflamed tissue, probably due to an increase in vascular permeability. As an alternative, activated macrophages and neutrophils in the inflamed synovium may have enhanced siRNA/WS uptake activity. Further studies are needed to examine these possibilities and to determine the mechanism of action of siRNA/WS. The siRNA/WS system could potentially be applicable to other systemic diseases in which macrophages play a central pathogenic role. Understanding the molecular mechanism of inflammatory tissue-selective siRNA/WS delivery should help us further improve the therapeutic effect of WS-mediated siRNA delivery.

There are only a few studies that reported the success of siRNA-based therapy for RA in animal models (Inoue et al., 2005; Schiffelers et al., 2005; Khoury et al., 2006, 2008). Khoury et al. (2006, 2008) reported that systemic administration of a cationic liposome carrying siRNA directed against TNF-α or a cocktail of siRNAs directed against proinflammatory cytokines, including IL-1, -6, and -18, was effective against CIA. However, the cationic liposome used in the above studies could rapidly be distributed to reticuloendothelial systems because of the micron-sized diameter and positive surface property. In contrast, siRNA/WS was designed to be nanosized (100 nm in diameter) and neutrally charged, resulting in its efficient distribution into peripheral inflamed tissue (Yagi et al., 2009). Indeed, we confirmed remarkable accumulation and cellular incorporation of the siRNA-encapsulated WS in inflamed synovium; thus, the relative contribution of macrophages in the affected joints to the therapeutic effect of siRNA might be higher in the WS than in the previously reported cationic liposome.

Treatment with TNF-α siRNA/WS from day 0 (when clinical arthritis just started developing) showed a therapeutic effect in CIA. However, when we started the treatment from day 7 (when the arthritis were established), the CIA was not suppressed (data not shown). Combining siRNA/WS with a specific targeting approach (e.g., surface modification of liposome) may improve the therapeutic efficacy of this system (Koning et al., 2006).

In conclusion, our results showed that the siRNA/WS system enabled efficient delivery of siRNA to arthritic joints. The siRNA/WS was incorporated into CD11b^{+} macrophages and neutrophils in the inflamed synovium, suggesting that it could be a therapeutic tool to silence the expression of various molecules associated with the pathogenesis of RA produced by these cells.

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Authorship Contributions

Participated in research design: Komano, Miyasaka, and Nanki.
Conducted experiments: Komano, Yagi, Onoue, and Kaneko.
Contributed new reagents or analytic tools: Yagi.
Performed data analysis: Komano.
Wrote or contributed to the writing of the manuscript: Komano, Miyasaka, and Nanki.

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Association of *EMCN* with Susceptibility to Rheumatoid Arthritis in a Japanese Population

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ABSTRACT. ***Objective.*** Endomucin, an endothelial-specific sialomucin, is thought to facilitate "lymphocyte homing" to synovial tissues, resulting in the major histopathologies of rheumatoid arthritis (RA). We examined the association between RA susceptibility and the gene coding endomucin, *EMCN*.

Methods. Association studies were conducted with 2 DNA sample sets (initial set of 1504 patients, 752 controls; and validation set, 1113 patients, 940 controls) using 6 tag single-nucleotide polymorphisms (SNP) from the Japanese HapMap database. Immunohistochemistry for the expression of endomucin was conducted with synovial tissues from 4 patients with RA during total knee arthroplasty. Electromobility shift assays were performed for the functional study of identified polymorphisms.

Results. Within the initial sample set, the strongest evidence of an association with RA susceptibility was SNP rs3775369 (OR 1.20, p = 0.0075). While the subsequent replication study did not initially confirm the observed significant association (OR 1.13, p = 0.062), an in-depth stratified analysis revealed significant association in patients testing positive to anti-cyclic citrullinated peptide (anti-CCP) antibody in the replication data set (OR 1.15, p = 0.044). Investigating 2 sample sets, significant associations were detected in overall and stratified samples with anti-CCP antibody status (OR 1.17, p = 0.0015). Positive staining for endomucin was detected in all patients. The allele associated with RA susceptibility had a higher binding affinity for HEK298-derived nuclear factors compared to the nonsusceptible allelic variant of rs3775369.

Conclusion. A significant association between *EMCN* and RA susceptibility was detected in our Japanese study population. The *EMCN* allele conferring RA susceptibility may also contribute to the pathogenesis of RA. (First Release Dec 15 2010; J Rheumatol 2011;38:221–8; doi:10.3899/jrheum.100263)

Key Indexing Terms:
RHEUMATOID ARTHRITIS GENE SUSCEPTIBILITY ENDOMUCIN

The contribution of genetic factors to the etiology of rheumatoid arthritis (RA) has been well established through extensive genealogical and twin-based studies[1,2]. HLA-DRB1 has been primarily associated with RA susceptibility among most major ethnic groups, including those of European and Asian descent[3,4,5]. Many other genes are also believed to contribute to the pathogenesis of RA, presumably operating at small to modest risk levels.

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Over the past decade, significant efforts have been made to identify putative RA susceptibility genes residing outside the HLA region. Often, these rely on a candidate gene or systematic approach, including genome-wide linkage scans. Such efforts have implicated *PTPN22* and *PADI4* as causative genes for RA susceptibility in European and Asian populations, respectively[6,7,8]. Additionally, *STAT4* has been shown to contribute a similar level of genetic risk for development of RA in European and Asian populations[9,10,11,12]. As well, *TNFAIP3*, *CD40*, *PRKCQ*, *KIF5A*, and *CCL21* were identified as RA-associated loci, by means of chip-based, genome-wide association studies and comprehensive replication studies among people of European origin[13,14,15,16,17,18].

However, more than half the genetic contribution to pathogenesis of RA is purported to arise from as-yet unknown genetic variance[18]. Considering that multiple molecular pathways are implicated in the onset and progression of the disease, it is likely that undetected genes of small to modest risk ultimately contribute to the pathogenesis of RA. Detection of such unknown genes outside the well studied HLA region is essential in understanding the genetic basis for RA susceptibility.

RA is characterized by chronic inflammation of the synovial tissues in joints and tendons. Entry of mononuclear cells, particularly T lymphocytes, into the synovium and the subsequent formation of aggregates into secondary ectopic lymphoid structures contributes to the systematic inflammation associated with RA via production of proinflammatory chemokines or pathogenic autoantibodies[19,20,21,22]. Recruitment of lymphocytes to synovial tissues is one of the key steps in the pathogenesis of RA. The homing of lymphocytes to secondary lymphoid structures is initiated by the interaction between sialomucin, a protein secreted on the endothelial cell surface, and cell adhesion molecules on lymphocytes, such as L-selectin. This group of several endothelial adhesion molecules that collectively bind L-selectin are commonly termed peripheral node addressins (PNAd). The secretion of PNAd in the vasculature of RA synovial tissues can be detected by immunostaining with the antiadhesive molecule MECA-79[20,23]. Thus, the PNAd are thought to play an important role in RA pathogenesis by facilitating lymphocyte homing to synovial tissues.

Endomucin, a PNAd, is known to be expressed in vascular endothelial cells of a variety of tissues including the heart, skin, and kidney[24,25]. While the expression of endomucin in synovial tissue has not been identified to date, it facilitates lymphocyte homing by interacting with L-selectin on respective lymphocytes like other PNAd[26]. Additionally, endomucin is overexpressed as a result of stimulation of tumor necrosis factor-α, a pleiotropic inflammatory cytokine central to a range of autoimmune diseases[27]. The implication of endomucin's involvement in key molecular processes suggests that *EMCN*, the endomucin coding gene, is a candidate susceptibility gene associated with RA. Our objective was to assess the association between *EMCN* and RA susceptibility within a Japanese population, and to investigate which, if any, associated polymorphisms have any directly identifiable biological effect on disease susceptibility.

MATERIALS AND METHODS

Study subjects. The study was approved by the relevant local ethics committee of each participating organization. Written informed consent was obtained from all participants prior to enrollment. All patients with RA had been diagnosed according to the revised criteria devised by the American College of Rheumatology[28].

Initial set. For initial analysis, case subjects were recruited from the Institute of Rheumatology Rheumatoid Arthritis (IORRA) associated with Tokyo Women's Medical University[29]. The IORRA series, including a case-control study for each haplotype tag single-nucleotide polymorphism (SNP) and haplotype analysis, consisted of 1504 cases and 752 controls. Control subjects were obtained from the DNA collection of the Pharma SNP Consortium, Tokyo, Japan, currently entrusted to the Health Science Research Resources Bank, Osaka, Japan.

Validation set. In addition, a RIKEN/BioBank series consisting of 1113 cases and 940 controls was prepared for a validation study of the SNP that had the best evidence of association with the initial set from IORRA. Case subjects were derived from the BioBank Japan Project DNA collection and controls were recruited from the Rotary Club of Osaka-Midosuji District 2600 Rotary International in Japan[29].

SNP selection and genotyping. Six SNP were selected that provided an adequate description of the haplotypes detected in the Japanese-derived genotype data obtained from HapMap (http://www.hapmap.org) using HaploView 4.1[30]. SNP genotyping data were extracted from *EMCN* in the region 3 kb upstream from the start of the first identified exon and 1 kb downstream from the end of the last exon, as described[31,32]. SNP of minor allele frequency (> 0.1) were included, while SNP deviating from Hardy-Weinberg equilibrium ($p < 0.05$) were excluded. A total of 33 SNP were identified and 6 tag SNP genotyped in this study revealed 32 SNP (96%). Genotyping was performed using the TaqMan SNP genotyping assay, following the manufacturer's specifications (Applied Biosystems, Tokyo, Japan)[7]. Duplicate samples and negative controls were included to ensure the accuracy of the genotyping protocol.

Stratified analyses on anti-CCP antibody status. Owing to the heterogeneity of RA, and the occurrence of distinct subsets characterized by the presence or absence of anticitrullinated peptide antibody (anti-CCP)[33], a stratified analysis was conducted for both the IORRA and RIKEN/BioBank-derived sample sets. The serum level of anti-CCP antibody was measured by a standard ELISA.

Statistical analysis. The differences in allele and genotype frequencies of each SNP between cases and controls were examined by the chi-squared test and the Cochran-Armitage test for trend, respectively. Chi-squared distribution was additionally applied to the stratified analyses on the level of anti-CCP antibody in RA patients, as well as in the statistical assessment of Hardy-Weinberg equilibrium. In subsequent combined analyses of the 2 case-controlled studies, a Breslow-Day test was used to assess significant heterogeneity and the Mantel-Haenszel procedure was performed to provide a common odds ratio when heterogeneity was denied. Statistical power for chi-squared test was calculated with alpha set as 0.05. These analyses were performed using the R software package (http://www.r-project.org/). Haplotypes were constructed using HaploView 4.1[30]. Multiple comparisons were corrected using the Bonferroni method. Biological interaction between the identified SNP and anti-CCP antibody status was evaluated with departure from additivity of effects. To quantify the amount of interaction, the attributable proportion due to interaction (AP) was calcu-

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lated based on relative risks from logistic models using SPSS for Windows (version 17.0; SPSS Inc., Chicago, IL, USA). If there is no interaction, AP is equal to 0. A detailed description regarding definition and calculation of AP is described elsewhere[34,35].

Immunohistochemistry. Synovial tissues were obtained from 4 RA patients during total knee arthroplasty. Each specimen was frozen in liquid nitrogen within optimal cutting-temperature compound and cut on a cryostat. The expression of endomucin in derived synovial tissues was evaluated by immunohistochemical assays using the HISTAR detection system (STAR3000A; AbD Serotec, Kidlington, UK) in accord with manufacturer's specifications. Rat anti-human-endomucin antibody (ReproCell Inc., Yokohama, Japan) was used as a primary antibody and rat IgG was used as a negative control.

Electromobility shift assays (EMSA). HEK293 cell lines were cultured in Dulbecco's modified Eagle's medium containing fetal bovine serum albumin (10%), penicillin (1%), and streptomycin (1%). Nuclear extracts were prepared using an NE-PER Nuclear Extraction Reagent Kit (Pierce Biotechnology, Cramlington, UK) in accord with specifications. Nuclear extracts were stored in aliquots at –80°C until required. Biotin-labeled and unlabeled single-strand oligonucleotide probes (20 bp) were purchased (Invitrogen), corresponding to the haplotype-tagging SNP rs3775369 sequence of allele A (minus strand: 5'-GGT TAC ACC CAT GCG TAG CTG-3'; plus strand: 5'-CAG CTA CGC ATG GGT GTA ACC-3') and allele G (minus strand: 5'-GGT TAC ACC CGT GCG TAG CTG-3'; plus strand: 5'-CAG CTA CGC ACG GGT GTA ACC-3').

EMSA was performed using Lightshift chemiluminescent EMSA kit (Pierce). HEK293 nuclear extracts (2 μg) were incubated with the relevant biotin-labeled oligonucleotide probe for 20 min at 20°C in 10 mM Tris buffer (pH 7.5) containing 100 mM KCl, 1 mM DTT, 0.6 μg Poly(dI-dC), 0.05% NP-40, and 5 mM $MgCl_2$. For competition experiments, 25-, 50-, and 100-fold molar excesses of each unlabeled oligonucleotide probe were incubated 20 min with the nuclear extracts before addition of the labeled oligonucleotide probes. Each reaction mixture was then separated by electrophoresis on 6% polyacrylamide gel (Novex DNA Retardation Gel; Invitrogen) using 0.5 × TBE buffer. After electroblotting onto nylon membrane (Roche), the band shift was visualized according to the specifications of the Lightshift kit.

EMCN expression in relation to genotypes. EMCN expression data were extracted from the Genevar database (http://www.sanger.ac.uk/humgen/genevar/) for the HapMap samples as described[36]. The association between EMCN genotypes and expression was accessed using linear regression methods.

RESULTS

Association of SNP within EMCN locus with RA and haplotype analysis. All 6 of the identified SNP were in Hardy-Weinberg equilibrium within the identified disease cases and the controls. The genotyping success rate was over 99% for all SNP. Importantly, significant differences were observed in allele frequency between cases and controls in 4 of the 6 characterized SNP (Table 1). An SNP rs3775369 located within intron 1 showed the most significant association with disease susceptibility (OR 1.21, allele p = 0.0075, genotype p = 0.0070; Figure 1). After applying Bonferroni correction for multiple testing, only rs3775369 was still significant (allele p = 0.045, genotype p = 0.0042). The minor allele A in rs3775369 was associated with increased risk of RA.

Statistically, all 6 characterized SNP were in linkage disequilibrium. Four haplotypes were estimated to account for 90% of all haplotypes (Table 2). Significant differences between the identified disease cases and the controls were detected in the second and third common haplotype, haplotype 2 and haplotype 3, respectively. Haplotype 2 was more prevalent in RA patients (OR 1.19, p = 0.013); conversely, haplotype 3 was observed more frequently within the control group (OR 0.82, p = 0.036). However, these differences were no longer significant after Bonferroni correction (p = 0.09 and 0.29, respectively).

Validation study and combined analysis. To validate the association detected in the initial set, a replication study was conducted using an independent case-control study, derived from the RIKEN/BioBank sample set. The SNP rs3775369 was genotyped for the validation study as it demonstrated the strongest association. Although a recognizable trend of association was observed in the validation set, significant differences between the RA cases and controls were not detected (OR 1.13, allele p = 0.062, genotype p = 0.0058). Because no heterogeneity of allelic distribution was observed within the case-control sample set (IORRA and RIKEN/BioBank) (p = 0.54), all data were combined to provide common OR with significant statistical difference (Table 3; OR 1.17, allele p = 0.0015, genotype p = 0.0051).

Publicly available genome-wide association data with RA samples, all in Caucasian populations, were also analyzed to determine whether the association of *EMCN* with RA was supported[37]. We found no evidence of positive association between RA and the SNP located within the linkage disequilibrium block including *EMCN* in the database (data not shown).

Table 1. Case-control data on HapMap tag single-nucleotide polymorphisms of *EMCN* locus in the IORRA series.

			Allele	Genotypes of Patients					Genotypes of Controls						p	
SNP	Location	Position*	1/2	1/1	1/2	2/2	Total	MAF	1/1	1/2	2/2	Total	MAF	OR (95% CI)	Allele	Genotype
rs3816056	Intron 1	25986294	G/T	1072	383	39	1494	0.154	495	236	20	751	0.184	0.81 (0.69–0.96)	0.012	0.012
rs3775369	Intron 1	25980974	G/A	681	669	145	1495	0.321	384	301	59	744	0.282	1.21 (1.05–1.38)	0.0075	0.0070
rs1501096	Intron 1	25980569	A/C	1037	405	46	1488	0.167	480	247	24	751	0.196	0.82 (0.70–0.97)	0.015	0.015
rs3796655	Intron 1	25980365	A/T	1043	407	46	1496	0.167	480	247	24	751	0.196	0.82 (0.70–0.96)	0.015	0.014
rs12501083	Intron 1	25970515	T/C	1218	265	14	1497	0.098	617	127	7	751	0.094	1.05 (0.84–1.30)	0.67	0.67
rs736517	Intron 2	25946759	G/C	1057	396	43	1496	0.161	511	217	24	752	0.176	0.90 (0.76–1.06)	0.20	0.20

* Positions are according to genomic contig NT_016354.18. MAF: minor allele frequency. IORRA: Institute of Rheumatology Rheumatoid Arthritis.

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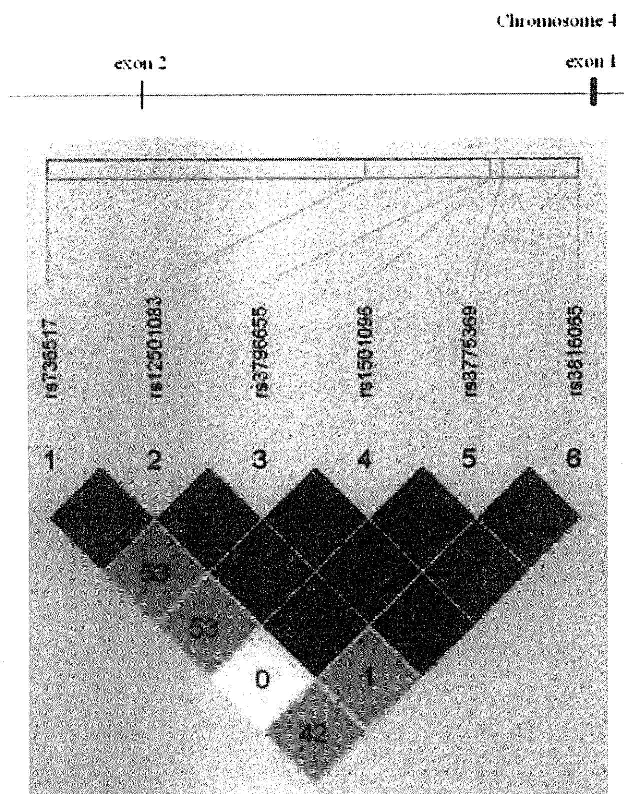

Figure 1. Linkage disequilibrium (LD) structure of 6 tagging SNP genotyped in all case and control subjects in the IORRA series. The key SNP rs3775369 is located within intron 1 of *EMCN*. Connected pairs of SNP show the standard color D'/LOD scheme using Haploview 4.1 (bright red: D' = 1, LOD score ≥ 2; shades of pink/red: D' < 1, LOD score ≥ 2; and white: D' < 1, LOD score < 2). Value in each diamond represents r^2.

Stratified analysis. Anti-CCP antibody measurements were available for the majority of patients in the IORRA data set (96%) and all patients (100%) in the RIKEN/BioBank data set. The frequency of the risk-inducing allele in patients returning positive results for the anti-CCP antibody was significantly higher than that in the controls, in both the IORRA and RIKEN/BioBank series (p = 0.012 and p = 0.044, respectively). Conversely, the incidence of patients with RA returning a negative result to the anti-CCP antibody was not significantly different from that in the control group in either of the sample series (Table 4). Combined analysis of both sample series also revealed a significant difference in the allelic distribution between RA patients who returned a positive result to the anti-CCP antibody and the control group (OR 1.17, p = 0.0015).

Interaction effects between rs3775369 and anti-CCP antibody status were estimated with a departure-from-additivity model by calculating the attributable proportion due to interaction in the IORRA data set. However, we found no evidence of a significant interaction (AP = 0).

Immunohistochemistry. Although endomucin is reportedly expressed in vascular endothelial cells of a variety of tissues, including the heart, skin, and kidney, expression in synovial tissues has not been elucidated. The expression of endomucin was thus examined by immunohistochemical staining in human synovial tissues from patients with confirmed RA following total knee arthroplasty. Within this sample set, positive staining confirmed the presence of endomucin in all patients, with expression specific to the endothelial cells of blood vessels (Figure 2). No staining was observed within the negative control (i.e., samples incubated without a primary antibody; data not shown).

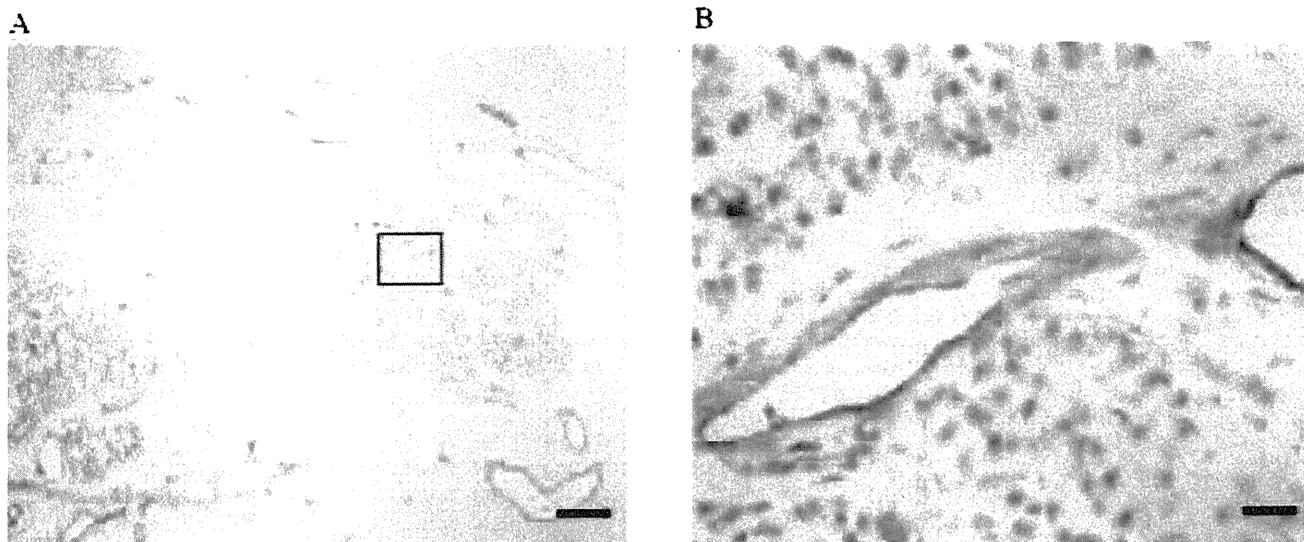

Figure 2. Immunohistochemical staining for expression of human endomucin in endothelial cells of vessels of RA synovial tissues. Brown stain indicates endomucin expression and sections are counterstained with hematoxylin (blue). A. Low-power image with inset shown in (B) at high-power magnification. Bars represent 200 μm (A) and 100 μm (B).

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Table 2. *EMCN* haplotypes and association with RA.

Haplotype	Sequence a	b	c	d	e	f	Frequency Patients	Controls	OR (95% CI)	p	p_{corr}*
1	G	G	A	T	T	G	0.41	0.43	0.95 (0.84–1.08)	0.45	1.00
2	G	A	A	T	T	G	0.29	0.25	1.19 (1.03–1.38)	0.013	0.09
3	T	G	C	A	T	C	0.11	0.13	0.82 (0.67–0.99)	0.036	0.25
4	G	G	A	T	C	G	0.09	0.09	1.06 (0.85–1.32)	0.63	1.00
5	T	G	C	A	T	G	0.04	0.05	0.83 (0.61–1.14)	0.21	1.00
6	G	A	A	T	T	C	0.03	0.03	1.11 (0.77–1.63)	0.59	1.00
7	G	G	C	A	T	C	0.02	0.02	1.10 (0.68–1.84)	0.71	1.00

* After Bonferroni correction. a: rs3816056, b: rs3775369, c: rs1501096, d: rs3796655, e: rs12501083, f: rs736517.

Table 3. Validation study of association between rs3775369 and RA susceptibility in RIKEN/BioBank series and combined analysis.

Series	Genotypes of Patients GG	GA	AA	Total	MAF	Genotypes of Controls GG	GA	AA	Total	MAF	OR (95% CI)	p Allele	Genotype
RIKEN/BioBank	493	497	119	1109	0.331	442	416	76	934	0.304	1.13 (0.99–1.30)	0.062	0.058
IORRA	681	669	145	1495	0.321	384	301	59	744	0.282	1.21 (1.05–1.38)	0.0075	0.0070
Combined	1174	1166	264	2604	0.325	826	717	135	1678	0.294	1.17 (1.06–1.28)	0.0015	0.0051

MAF: minor allele frequency.

Table 4. Analyses of sr3775369 stratified on anti-CCP status in patients with RA.

Series	Status	No. Alleles G	A	Total	Frequency of Risk Allele (A)	Patients vs Controls OR (95% CI)	p
IORRA	Controls	1069	419	1488	0.28		
	Patients Anti-CCP (+)	1723	809	2532	0.32	1.20 (1.04–1.38)	0.012
	Anti-CCP (–)	248	120	368	0.33	1.23 (0.96–1.59)	0.092
RIKEN/BioBank	Controls	1300	568	1868	0.30		
	Patients Anti-CCP (+)	1225	617	1842	0.34	1.15 (1.00–1.33)	0.044
	Anti-CCP (–)	258	118	376	0.31	1.05 (0.82–1.34)	0.71
Combined	Patients Anti-CCP (+)	2948	1426	4374	0.33	1.17 (1.06–1.30)	0.0015
	Anti-CCP (–)	506	238	744	0.32	1.13 (0.96–1.35)	0.16

Anti-CCP: anti-cyclic citrullinated peptide antibody.

Electromobility shift assay (EMSA) results. Based on the finding of a significant association with susceptibility to RA, rs3775369 and other SNP identified in associated linkage disequilibrium may be the most likely candidates for causative SNP. However, a high level of linkage disequilibrium between rs3775369 and other SNP was not identified, according to the r^2 or LOD scores ($r^2 < 0.2$, LOD score < 2) derived from Haploview 4.1 in conjunction with the HapMap JPT database. The rs3775369 SNP was thus independently considered a likely candidate as a causative polymorphism. Owing to the location of rs3775369 within intron 1, allelic differences within this region may in turn alter the affinity for various nuclear factors, and hence the level of resultant transcriptions. To determine whether polymorphisms in rs3775369 affect the DNA binding abilities by various nuclear factors, an EMSA was conducted using nuclear extracts from human HEK293 cell lines and oligonucleotide probes corresponding to the sequence surrounding the SNP. Although labeled probes containing each alternative allele each produced retarded bands, significant differences in band intensity were not detected between the 2 recognized variants, the A and G alleles (Figure 3). Extending this technique to an additional competition analysis, excess non-labeled probe corresponding to the G allele did not significantly inhibit the ability of HEK293-derived nuclear extracts to bind to the labeled A allele. However, excess non-labeled probe corresponding to the A allele did in fact inhibit binding of nuclear extracts in a concentrate-dependent manner. This suggests a degree of difference in the binding ability of the 2 allelic variants, with allele A demonstrating higher binding affinity.

EMCN expression in relation to genotypes. Gene expression of *EMCN* was accessed with JPT HapMap subjects to evaluate whether there was any genotypic effect on *EMCN*

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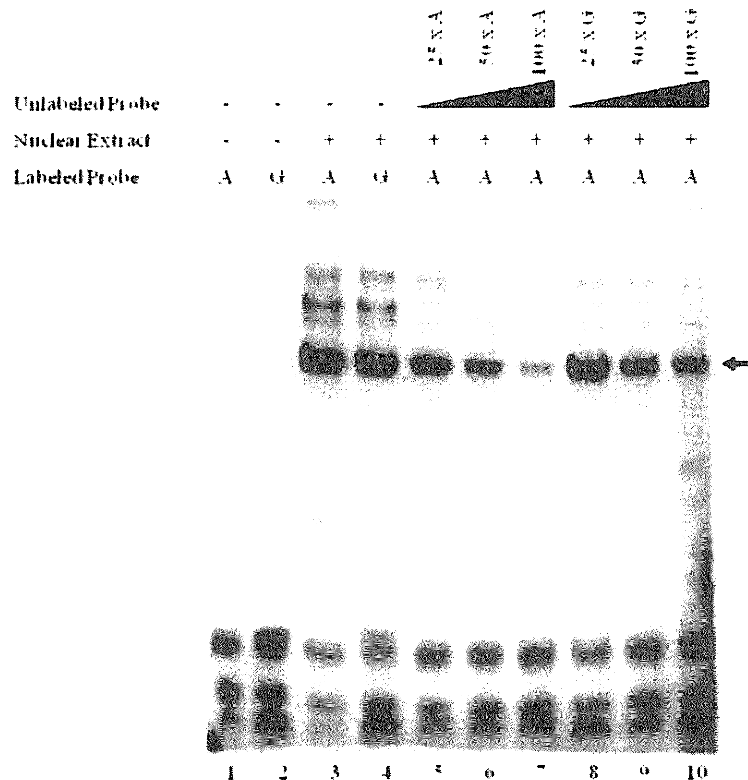

Figure 3. Electromobility shift assays with oligonucleotide probes corresponding to SNP rs3775369. Labeled probe alone containing allele A (lane 1) and allele G (lane 2); incubation with nuclear extracts from the HEK293 cell line, allele A (lane 3) and allele G (lane 4); competition experiments with increasing amounts of nonlabeled probe A (lanes 5–7) and nonlabeled allele G (lanes 8–10). Arrow indicates the band that was shifted with the addition of nuclear extracts.

expression. No association was observed between *EMCN* expression and rs3775369 genotypes (data not shown).

DISCUSSION

Our study identified a significant association between polymorphisms in *EMCN* and susceptibility to RA, using 2 independent sample sets within the Japanese population (OR 1.17, allele p = 0.0015, genotype p = 0.0051). The effect that *EMCN* itself has on disease susceptibility is moderate, based on the odds ratio obtained from this study. This finding is in accord with data for other RA susceptibility genes identified outside the HLA region; these also present a moderate risk. This is not surprising as it is generally accepted that the overall risk contribution of each genetic factor is small in common diseases such as RA. The complex etiology observed in RA likely results from multiple genetic and environmental factors, both known and unknown. To account for even a small level of associated risk, a large sample size was used to obtain sufficient statistical power. In our study, statistical power was calculated at 91% in the combined data set analysis.

The identified association between *EMCN* and RA suggests that variation within *EMCN* may contribute to the development and subsequent pathogenesis of RA. Endomucin, as encoded by *EMCN*, is thought to regulate L-selectin-mediated lymphocyte homing on the surface of vascular endothelial cells[26]. We established that endomucin is indeed abundantly expressed in the vascular endothelial cells of RA synovial tissues. Endomucin could therefore serve to enhance inflammation of synovial tissues through the process of lymphocyte homing in RA patients. The genetic association between *EMCN* polymorphisms and RA identified in this study suggests that *EMCN* may contribute to the pathogenesis of the disease. Recently, a genetic association with CCL21, a chemokine also involved in lymphocyte homing, was identified in a powerful metaanalysis of 2 genome-wide association studies in Caucasians[18]. The recognition of additional genetic components that contribute to lymphocyte homing is critical for developing an understanding of the genetic basis of RA, and ultimately for identifying key targets for effective treatment of RA[38].

In stratified analyses derived from anti-CCP antibody assays, the association between *EMCN* and RA in both data sets was predominantly through the subgroup of RA patients

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that tested positive to the autoantibody. However, this predominance might not be specific to this subgroup, since no interaction between *EMCN* and anti-CCP antibody status was found. Nevertheless, this correlation is perhaps understandable, as many studies have indicated that autoantibody-positive and negative RA may confer different levels of associated genetic risk[33]. Interestingly, ectopic lymphoid structures present in RA synovial tissues were reported to be involved in autoantibody production, including the anti-CCP antibody[19]. *EMCN* may indeed contribute to RA pathogenesis by facilitating lymphocyte homing to secondary lymphoid organs, subsequently causing the production of anti-CCP antibody.

While a positive association between *EMCN* and RA was detected in this study, there is still a possibility of false positives. False-positive associations may result from population stratification, or errors in sampling, phenotyping, genotyping, or multiple testing, as well as other potential biases[39,40]. Unaccounted or unidentified population stratification has the potential to produce spurious genetic associations. Although the Japanese population is known to have limited genetic diversity, 2 main population clusters have been identified, the Hondo and the Ryukyu[41]. To account for this, both case-control data series in our study were recruited from the Hondo area. Nevertheless, as the 2 data series were not tested for population stratification, this may remain a limitation and potential source of error.

Age and sex of cases and controls are not matched in the IORRA and RIKEN/BioBank series (IORRA cases, mean age 59.3 yrs, 83.8% female; controls, mean age 36.4 yrs, 50% female. RIKEN/BioBank cases, mean age 60.4 yrs, 82.0% female; controls, mean age 52.6 yrs, 25% female). This mismatch might also be a limitation of our study. With regard to errors derived from phenotyping, autoantibody testing reduced the possibility of phenotyping error by providing positive correlations with RA diagnosis. However, there remained some limited potential for phenotyping errors. Genotyping error was unlikely, owing to an overall genotyping success rate of 99% and a genotype concordance rate of 100%, assessed by duplicate samples. Although multiple testing does not eliminate false positives, the identified associations remained significant even after Bonferroni correction in the IORRA series (allele $p = 0.045$, genotype $p = 0.0042$).

The SNP allele frequencies of control subjects in this study had some deviations from JPT HapMap data, although the minor alleles of the SNP in this study were identical to those in the JPT HapMap data. This deviation might be a result of the relatively small numbers of samples used in the HapMap database. However, the possibility remains of false-positive results derived from the discrepancy.

Because the SNP rs3775369 is located within intron 1, it is suggested that alternative alleles may influence the binding ability of nuclear factors and thus their resultant transcription activity. Our EMSA revealed that the sequence containing the RA susceptibility allele A had a higher binding affinity to nuclear extracts than the alternative non-susceptible allele G. The Transcription Element Search System (TESS; http://www.cbil.upenn.edu/cgi-bin/tess/tess) predicted that the transcription factor AP-2α has the potential to bind to the sequence surrounding the SNP rs3775369 when the allelic variant is the RA susceptibility allele A. Variations in the *EMCN* allele may alter the binding affinity of AP-2α and hence regulate subsequent transcription of the gene. However, the genotypic effect on *EMCN* gene expression was not confirmed using the Genevar database with HapMap samples. Additional experiments are needed to fully deduce the changes of these genetic variation effects in the function of *EMCN*. Moreover, as the HapMap database does not fully account for the breadth of genetic variability, it is necessary to find other novel SNP that may also influence or determine the risk haplotype. Additional examination of the functional variants of *EMCN* is essential to establish a solid genetic association.

Our study clearly identified an association between *EMCN* and RA susceptibility within the Japanese population. The association was chiefly with RA patients who tested positive to anti-CCP antibody. This key finding suggests that variation within a gene engaged in lymphocyte homing may be critical in the pathogenesis of RA. The identified variation within the intronic SNP was significantly associated with RA and may alter transcription activity; further investigation is required to elucidate the mechanism of action. Identification of the RA disease-causing variant of *EMCN*, in conjunction with replicate studies in other populations, is central to reaching a definitive conclusion.

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PADI4 polymorphism predisposes male smokers to rheumatoid arthritis

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ABSTRACT

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

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

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

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

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

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

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

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

METHODS

Subjects

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

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subjects entered into this study provided informed consent prior to participation in the study, and all study protocols were preapproved by the ethics committees of each institute.

Smoking status

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

SNP genotyping

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

Statistical analysis

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

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

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

*rs112033673 (T/C, T is the minor allele) and rs174803 (T/C, T is the minor allele) were genotyped for the test. Both case and control subjects were stratified with smoking status in the first set, whereas only case subjects were stratified with smoking status in the second set.

MAF, minor allele frequency; *PADI4*, peptidyl arginine deiminase 4; RA, rheumatoid arthritis.

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allele frequencies for patients and controls were used to calculate the OR and the 95% CI using the method of Woolf.[15] Gene–environmental interactions were assessed by both 'case-only' analysis and logistic regression analysis.[16] All statistical analysis were performed using Plink software.[17]

RESULTS AND DISCUSSION

A significant association between the *PADI4* polymorphism and RA susceptibility was observed in the whole set of case–control subjects in the first Japanese set (rs11203367; per allele OR (OR_{allele}) 1.14; 95% CI 1.00 to 1.29; p value for a trend test (p_{trend})=0.045; table 1). In a stratified analysis with sex, the *PADI4* polymorphism was significantly associated only in men (OR_{allele} 1.44; 95% CI 1.14 to 1.81; p_{trend}=0.0022), but not in women (OR_{allele} 1.02; 95% CI 0.82 to 1.25; p_{trend}=0.84). Similarly, when subjects in both cases and controls were stratified for smoking status, the *PADI4* polymorphism had a greater effect in ever-smokers (OR_{allele} 1.35; 95% CI 1.11 to 1.65; p_{trend}=0.0024) compared with never-smokers (OR_{allele} 1.03; 95% CI 0.86 to 1.23; p_{trend}=0.71). Further stratification analysis with sex and smoking status revealed that the *PADI4* polymorphism had the highest risk in the subpopulation of male ever-smokers (OR_{allele} 1.61; 95% CI 1.24 to 2.09; p_{trend}=0.00031). Similar findings were also observed, when only ACPA-positive patients were analysed (supplementary table 3, available online only).

To support these observations, we also analysed other case–control sets in the Japanese population and Dutch population (unstratified controls for smoking status were used in both sets as no information was available). In the second Japanese set, the highest risk in the subpopulation of male ever-smokers was replicated in rs1748033 (OR_{allele} 1.34; 95% CI 1.02 to 1.75; p_{trend}=0.039; table 1). In the Dutch set, the association of the *PADI4* polymorphism (rs1748033) was statistically significant in a dominant model (OR_{dom} 1.32; 95% CI 1.02 to 1.72; p_{dom}=0.03; table 2), but not in a trend test, when evaluated in total (p_{trend}=0.14). When patients were stratified by sex or/and smoking status and compared with control subjects, OR in the dominant model was higher for men (OR 1.36; 95% CI 0.90 to 2.06; p=0.13) than for women and was higher for ever-smokers (OR 1.56; 95% CI 1.06 to 2.31; p=0.02) than for never-smokers. Furthermore, it was highest in male ever-smokers (OR 1.79; 95% CI 0.98 to 3.27; p=0.043).

Table 2 Association of *PADI4* polymorphism and RA stratified with sex and smoking status in a Dutch population*

	Sum		MAF		Genotype frequency test (dominant model)	
	Case	Contol	Case	Contol	OR	p Value
rs11203367						
All	646	385	0.44	0.42	1.19 (0.90 to 1.56)	0.2
Men	218	180	0.47	0.40	1.49 (0.96 to 2.33)	0.063
Women	398	188	0.42	0.43	1.06 (0.73 to 1.56)	0.7
Ever-smoker	174	385	0.43	0.42	1.14 (0.76 to 1.70)	0.5
Never-smoker	178	385	0.45	0.42	1.06 (0.72 to 1.57)	0.7
Male ever-smoker	76	180	0.45	0.40	1.46 (0.78 to 2.71)	0.2
Male never-smoker	40	180	0.53	0.40	1.38 (0.62 to 3.10)	0.4
Female ever-smoker	98	188	0.41	0.43	0.99 (0.58 to 1.72)	0.9
Female never-smoker	138	188	0.43	0.43	0.99 (0.61 to 1.61)	0.9
rs1748033						
All	635	391	0.34	0.30	1.32 (1.02 to 1.72)	0.03
Men	215	183	0.35	0.28	1.36 (0.90 to 2.06)	0.13
Women	389	191	0.32	0.31	1.33 (0.93 to 1.91)	0.11
Ever-smoker	158	391	0.36	0.30	1.56 (1.06 to 2.31)	0.02
Never-smoker	178	391	0.31	0.30	1.06 (0.73 to 1.53)	0.7
Male ever-smoker	70	183	0.38	0.28	1.79 (0.98 to 3.27)	0.043
Male never-smoker	41	183	0.35	0.28	1.35 (0.65 to 2.82)	0.4
Female ever-smoker	88	191	0.34	0.31	1.48 (0.86 to 2.55)	0.13
Female never-smoker	137	191	0.30	0.31	1.03 (0.65 to 1.64)	0.9

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

These stratified analyses suggested gene–environmental interactions between *PADI4* and sex, and/or between *PADI4* and smoking status. We performed case-only analysis to test these interactions statistically, by comparing the allele frequency of the *PADI4* polymorphism in the stratified subpopulation of patients (the first and second Japanese sets were combined). Allele frequency was significantly higher in men than in women (rs11203367; 0.48 vs 0.42; p_{trend}=0.0016) and in ever-smokers than in never-smokers (rs11203367; 0.47 vs 0.41; p_{trend}=0.00077), suggesting the presence of gene–environmental interactions for *PADI4*. Similar results were obtained for rs1748033. In addition to stratified analyses using the contingency tables, we analysed these gene–environmental interactions using logistic regression models. The first Japanese set was used for analysis because of the availability of smoking status. The *PADI4* polymorphism was associated with RA susceptibility in an additive model, adjusted by sex and smoking status (rs11203367; OR_{add} 1.18; 95% CI 1.01 to 1.38; p_{add}=0.035). When an interaction term between SNP genotype and sex (a product term of genotype×sex) was introduced into the regression model, the logistic coefficient for the term was significant (p=0.029). Similarly, when an interaction term between SNP genotype and smoking status (a product term of genotype×smoking status) was introduced into the model, the coefficient for the term was again significant (p=0.034). We also added the age of subjects into the model, because it could be a confounding factor considering that smoking prevalence has been decreasing in recent decades, especially in Japanese men (OECD Health Data, 2009).[18] The interaction term for SNP and smoking remained significant (p=0.038), whereas the significance level of the interaction term for SNP and sex became marginal (p=0.075).

Finally, we examined the association between the *PADI4* polymorphism and ACPA status in the patients of Japanese sets. The allele frequency of *PADI4* showed a higher trend in ACPA-positive patients compared with ACPA-negative patients (rs11203367; 0.43 vs 0.41; p_{trend}=0.54). When the genotype frequency was compared in a recessive model, the *PADI4* polymorphism was significantly associated with the ACPA status in ever-smokers (rs11203367; OR_{rec} 2.33; 95% CI 1.23 to 4.39; p_{rec}=0.0072; table 3), suggesting that the *PADI4* polymorphism may be involved in the appearance of ACPA in smokers.

Gene–environmental interactions in RA susceptibility have been well described between polymorphisms in *HLA-DRB1* and *PTPN22* genes and smoking habit in populations of European descent.[19 20] Our observations here indicate that the *PADI4* polymorphism is another genetic risk that would interact with smoking in RA susceptibility, although why this interaction is prominent in men remains to be solved. The status of sex hormones may influence the role of *PADI4*, as it is profoundly involved in the onset of RA.[21] Another possible explanation could be gender differences in smoking behaviour, which has also been argued in other smoking-related diseases.[22] Quantitative analysis of smoking history, such as pack-years smoked, may be needed to investigate further for the gender difference.

Smoking prevalence rates differed highly among the populations, and the attribution of smoking to the onset of RA may thus differ among populations. A recent epidemiological survey

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Table 3 Association of *PADI4* polymorphism and ACPA status in a Japanese population*

	Sum		MAF		Genotype frequency test (recessive model)	
	ACPA+	ACPA−	ACPA+	ACPA−	OR	p Value
rs11203367						
All	1614	401	0.43	0.41	1.25 (0.93 to 1.68)	0.14
Men	295	80	0.46	0.45	1.22 (0.65 to 2.28)	0.52
Women	1319	321	0.42	0.40	1.27 (0.90 to 1.78)	0.17
Ever-smoker	523	116	0.46	0.41	2.33 (1.23 to 4.39)	0.0072
Never-smoker	1091	285	0.41	0.41	0.99 (0.70 to 1.39)	0.96
Male ever-smoker	245	55	0.49	0.44	1.90 (0.85 to 4.25)	0.11
Male never-smoker	50	25	0.34	0.48	0.28 (0.08 to 1.01)	0.045
Female ever-smoker	278	61	0.43	0.39	3.20 (1.11 to 9.22)	0.024
Female never-smoker	1041	260	0.41	0.41	1.09 (0.75 to 1.56)	1
rs1748033						
All	1614	400	0.37	0.37	1.39 (0.98 to 1.98)	0.063
Men	295	80	0.41	0.42	1.40 (0.69 to 2.83)	0.34
Women	1319	320	0.36	0.36	1.41 (0.93 to 2.12)	0.10
Ever-smoker	523	115	0.40	0.38	2.15 (1.08 to 4.28)	0.026
Never-smoker	1091	285	0.35	0.37	1.13 (0.75 to 1.72)	0.54
Male ever-smoker	245	55	0.43	0.41	2.09 (0.84 to 5.16)	0.10
Male never-smoker	50	25	0.30	0.44	0.34 (0.08 to 1.43)	0.13
Female ever-smoker	278	60	0.37	0.36	2.28 (0.78 to 6.65)	0.12
Female never-smoker	1041	260	0.35	0.36	1.27 (0.81 to 1.98)	0.29

Anti-citrullinated protein antibody (ACPA)+ and ACPA−, ACPA-positive and ACPA-negative rheumatoid arthritis (RA) patients, respectively.
*rs112033673 (T/C, T is the minor allele) and rs174803 (T/C, T is the minor allele) were genotyped for the test. Case subjects of Japanese sets (first and second) were combined for analysis.
MAF, minor allele frequency; *PADI4*, peptidyl arginine deiminase 4.

has shown that smoking prevalences are generally higher in men from Asian countries than in western European countries: Japan, 45.8%; Korea, 46.6%; UK, 25.0%; The Netherlands, 35.0%; Sweden, 13.9%; and USA, 19.1% in 2005.[18] Considering our observation that the *PADI4* polymorphism has the highest risk in male ever-smokers, the attribution of the *PADI4* polymorphism may be relatively high in populations with high smoking prevalences among men, such as Japan and Korea, corresponding to the positive results in association studies for *PADI4* polymorphisms in these countries.[2 4 5]

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

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Competing interests None.

Patient consent Obtained.

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