Association of *STAT4* with Rheumatoid Arthritis in the Korean Population

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A recent study in the North American White population has documented the association of a common STAT4 haplotype (tagged by rs7574865) with risk for rheumatoid arthritis (RA) and systemic lupus erythematosus. To replicate this finding in the Korean population, we performed a case-control association study. We genotyped 67 single nucleotide polymorphisms (SNPs) within the STAT1 and STAT4 regions in 1123 Korean patients with RA and 1008 ethnicity-matched controls. The most significant four risk SNPs (rs11889341, rs7574865, rs8179673, and rs10181656 located within the third intron of STAT4) among 67 SNPs are identical with those in the North American study. All four SNPs have modest risk for RA susceptibility (odds ratio 1.21–1.27). A common haplotype defined by these markers (TTCG) carries significant risk for RA in Koreans (34 percent versus 28 percent, $P = 0.0027$, OR (95 percent CI) = 1.33 (1.10–1.60)). By logistic regression analysis, this haplotype is an independent risk factor in addition to the classical shared epitope alleles at the HLA-DRB1 locus. There were no significant associations with age of disease onset, radiographic progression, or serologic status using either allelic or haplotypic analysis. Unlike several other risk genes for RA such as PTPN22, PADI4, and FCRL3, a haplotype of the STAT4 gene shows consistent association with RA susceptibility across Whites and Asians, suggesting that this risk haplotype predates the divergence of the major racial groups.

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune arthritis characterized by progressive joint destruction and autoantibody formation such as anti-cyclic citrullinated peptide (anti-CCP) and rheumatoid factor (RF). Both genetic and environmental factors, and their interaction, play a role in the development of RA (1–3). A common set of alleles at the HLA-DRB1 locus (the "shared epitope" alleles) has been associated with RA in populations of White European and Asian ancestry (4,5). However, commonality of other risk loci in these population groups has been difficult to demonstrate.

Over the last several years, additional risk genes for RA have been identified in both White and Asian populations. PTPN22 was discovered as a risk factor for RA by genome-wide association scanning of functional SNPs (6) and has been replicated in many White RA cohorts. In addition, PTPN22 confers risk for several other autoimmune diseases such as Type1 diabetes and systemic lupus erythematosus (SLE), providing evidence for common pathways of pathogenesis in these disorders (7). However, the PTPN22 risk allele (R620W) is extremely rare in the Asian population and, so far, there is no evidence of association of PTPN22 with RA in non-White populations (8,9). In contrast, PADI4, SLC22A4, and FCRL3 have been associated with RA in the Japanese population and replicated in the other Asian groups (10–12), but have given weak or negative results in populations of European ancestry (13,14). These divergent results suggest genetic heterogeneity of RA across the major racial groups (15).

Recently, a study in the North American White population has documented the association of a common STAT4 haplotype with both RA and SLE using a combined positional mapping and candidate-gene approach catalyzed by finding a linkage peak on chromosome 2q (16). In this report we demonstrate that a common STAT4 haplotype confers a similar degree of

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risk for RA in both Asian and White populations.

MATERIALS AND METHODS

Study Population

We included 1128 Korean RA patients who were enrolled consecutively from the outpatient clinic of The Hospital for Rheumatic Diseases, Hanyang University, Seoul, South Korea and 1022 ethnically matched controls. All RA patients met the American College of Rheumatology 1987 classification criteria for RA (17). Written informed consent was obtained from all participants. HLA-DRB1 typing was performed in cases (N = 1120) and controls (N = 1002) by a polymerase chain reaction sequence based typing (PCR-SBT) method using the reference protocol of the Twelfth International Histocompatibility Workshop (18).

Clinical and Laboratory Data of the Studied Subjects

Clinical data such as sex and age at disease onset from medical records and from interviews conducted at the time of enrollment were used in the analysis.

The staging system proposed by Steinbrocker et al was used as a marker of the radiographic severity of RA (19). Subjects were initially classified into stages I–IV and were then dichotomized into two groups: stage I and stages II–IV. Serum levels of rheumatoid factor (RF) were measured nephelometrically. Serum anti-CCP levels were quantitatively measured in duplicate from all RA patients by enzyme-linked immunosorbent assay using the DIASTAT Anti-CCP kit FCCP 200 (Axis-Shield Plc, Scotland, UK) according to the manufacturer's instructions. The upper limit of the reference range of anti-CCP is five units and anti-CCP levels were not titered out beyond 100 units.

Genotyping

We first confirmed that the most significant risk SNP (rs7574865) of the STAT4 gene in the North American RA population was also polymorphic in Asian population (MAF 0.28 and 0.33 in

Table 1. The Distribution of 52 SNPs of STAT1/STAT4 Region in Cases and Controls

SNP Name	Assoc Allele	Location (Build 35)	MAF in cases	MAF in controls	*P* value	SNP call rate(%)
rs3088307	C	191537657	0.92	0.91	0.4452	89.5
rs16824035	C	191545879	0.92	0.91	0.4246	98.2
rs6718902	T	191546449	0.52	0.52	0.7731	97.8
rs13395505	G	191546759	0.39	0.39	0.8549	99.4
rs1547550	C	191553970	0.92	0.91	0.1849	89.3
rs2280234	A	191558344	0.83	0.82	0.721	90.4
rs2280233	C	191558811	0.12	0.12	0.8711	98
rs2280232	G	191559011	0.17	0.17	0.7421	90.8
rs11887698	A	191563119	0.74	0.73	0.4329	90.8
rs10199181	A	191581798	0.28	0.28	0.8878	99.3
rs13029532	C	191584146	0.13	0.13	0.9841	99.4
rs10208033	C	191587662	0.74	0.73	0.4587	84.4
rs1467199	G	191588747	0.51	0.49	0.2539	98.7
rs16833177	C	191595877	0.45	0.43	0.1504	99.4
rs4853456	A	191600008	0.81	0.8	0.4204	91.2
rs3024904	A	191603447	0.81	0.8	0.5432	98.3
rs3024936	C	191603621	0.94	0.93	0.2285	91.5
rs925847	T	191605785	0.54	0.51	0.1183	99.5
rs6749371	A	191610429	0.88	0.87	0.5736	99.5
rs6715106	A	191621279	0.88	0.87	0.5935	99.2
rs16833215	G	191622044	0.48	0.45	0.0406	98
rs3024866	C	191631086	0.52	0.48	0.0229	97.8
rs1517352	A	191639709	0.51	0.47	0.0148	98
rs13017460	A	191640801	0.51	0.48	0.0124	98.9
rs7601754	A	191648696	0.84	0.82	0.0715	86
rs11889341[a]	T	191651987	0.35	0.3	0.0003	99.4
rs6434435	G	191662109	0.86	0.85	0.1702	98
rs7574865[a]	T	191672878	0.39	0.33	0.0004	91.1
rs8179673[a]	C	191677586	0.39	0.34	0.002	99.2
rs10181656[a]	G	191678124	0.39	0.34	0.0029	99.4
rs13401064	C	191678575	0.89	0.89	0.9341	97.4
rs16833260	G	191679810	0.54	0.49	0.0051	90.5
rs6752770	G	191681808	0.22	0.2	0.1662	97.1
rs4341966	G	191690450	0.78	0.77	0.2879	90.5
rs2356350	G	191710783	0.47	0.46	0.5645	99.3
rs11685878	C	191717700	0.59	0.58	0.2676	97.9
rs4853546	G	191717897	0.7	0.7	0.9996	98
rs1031509	A	191718434	0.31	0.3	0.9336	90.3
rs12327969	C	191719016	0.31	0.3	0.66	90.8
rs10497711	G	191722166	0.13	0.12	0.4322	98.9
rs7572482	A	191723317	0.46	0.46	0.9282	99.2
rs2278940	A	191724173	0.13	0.12	0.3094	98.2
rs897200	A	191726016	0.46	0.46	0.8733	90.4
rs16833437	T	191727617	0.46	0.46	0.8786	90
rs1031507	T	191728863	0.46	0.46	0.785	91
rs13001658	G	191733149	0.67	0.66	0.6955	89.2
rs1869624	A	191738636	0.13	0.12	0.2238	98.8
rs4853550	T	191739472	0.52	0.52	0.7674	98
rs4853551	T	191739508	0.12	0.11	0.7171	99.4
rs12467660	A	191739895	0.28	0.27	0.442	98
rs2054090	A	191741945	0.33	0.33	0.8721	99.1
rs7595886	T	191746983	0.28	0.26	0.307	91

[a]Indicates the most significant SNPs with less than *P* value 0.005.

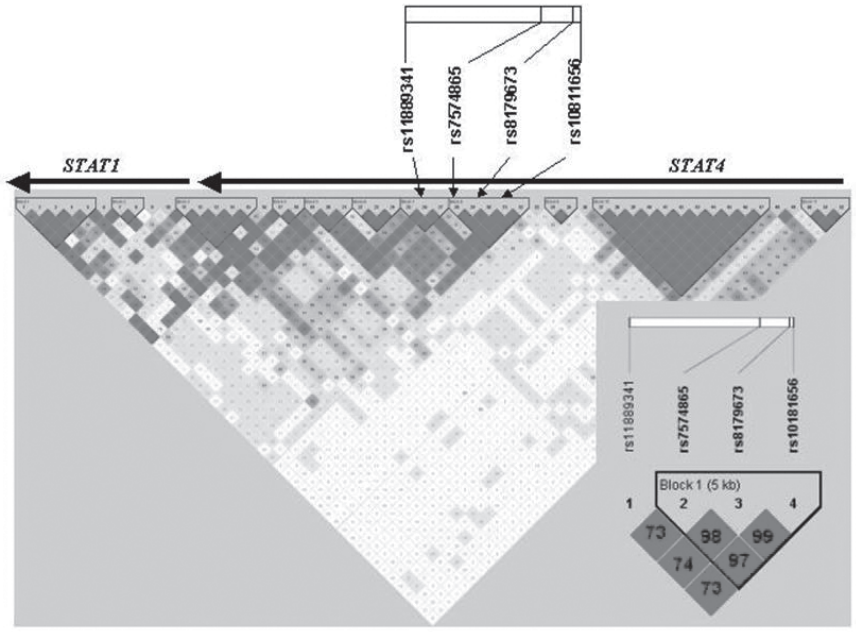

Figure 1. LD of 52 SNPs in STAT1/STAT4 region in 1128 Korean RA cases and 1022 controls. The location of the most significant four risk SNPs are indicated. The LD pattern of these risk four SNPs with r^2 values are shown in the bottom right. This figure was obtained from Haploview version 3.32.

Japanese and Chinese population, respectively) using HapMap Phase II data (http://www.hapmap.org). We therefore genotyped the same panel of 67 SNPs as in our previous report (16). These were selected from Hapmap Phase II data and included all non-synonymous coding SNPs reported in dbSNP, and covered both the STAT1 and STAT4 genes on chromosome 2q.

The DNAs were genotyped using a multiplexed primer extension method (Sequenom, Inc., San Diego, CA). In brief, multiplex PCR was used to amplify DNA products containing up to 28 SNPs in one reaction from 5 ng genomic DNA. Synthetic oligonucleotides that bind adjacent to the SNP site were then hybridized and extended with nucleotides complementary to the template SNP site using modified nucleotides that terminate the extension reaction at the interrogated SNP, thus generating alternate products of sufficiently different masses to be separated by mass spectrometry. The extended products were separated by MALDI-TOF mass spectrometry and the genotypes determined with SpectroTyper software (Sequenom). Calls were evaluated and edited by cluster analysis performed with the SpectroTyper software.

Data Analysis

We excluded all the case or control samples with missing genotypes on more than 50 percent of the 67 studied SNPs. We also excluded SNPs with significant deviation from Hardy Weinberg equilibrium (P values less than 0.005) in the control group, and we did not consider SNPs with minor allele frequency (MAF) less than 0.01. Association tests for each SNP in cases and controls were determined using Haploview version 3.32.

Alleles/genotypes or haplotypes of STAT4 were analyzed for association with clinical or laboratory variables using chi-square test, Mann-Whitney U test, or logistic regression test, using SAS software, version 9.1 (SAS Institute, Cary, NC, USA). $P < 0.05$ is considered significant.

RESULTS

Association of a STAT4 Haplotype with Rheumatoid Arthritis

We analyzed 2131 samples (1123 cases and 1008 controls) out of an initial 2150 samples (1128 cases and 1022 controls) after excluding 19 samples (five cases and 14 controls) with more than 50 percent missing genotypes. In the remaining subjects, the average call rate in the 67 SNPs was 95.6 percent (84.3–99.7 percent). After filtering all SNPs with minor allele frequency (MAF) less than 0.01 (n = 12) or SNPs with significant deviation from Hardy Weinberg equilibrium, i.e. P values less than 0.005 in the control group (n = 3), we analyzed the distribution of allele frequency of 52 SNPs in the RA cases and controls. We removed 15 SNPs out of initial 67 studied SNPs based on Hardy Weinberg Equilibrium less than P value 0.005 (rs3024839, rs10931481, and rs7596818) or MAF less than 0.01 (rs17749316, rs13005843, rs3024933, rs16833220, rs932169, rs2459611, rs13010752, rs12998748, rs13011805, rs7599504, rs10194402, rs17769459). Call rates for each of the 52 SNPs is shown. (Table 1). The most significant four SNPs ($P <$ 0.005) in the present study are identical with those in the North American study.

The LD pattern of the 52 SNPs within the region of STAT1/STAT4 is shown in Figure 1. The four most significant disease-associated SNPs (rs11889341, rs7574865, rs8179673, and rs10181656) are in strong linkage disequilibrium ($D' > 0.93$; $r^2 > 0.73$). A haplotype association analysis of these four risk SNPs revealed that the same haplotype (TTCG) as the one in the North American study showed significant risk for RA [0.34 versus 0.28, $P =$ 0.0027, OR (95 percent CI) = 1.33 (1.10–1.60)] (Table 2).

Stratification Analysis by Clinical or Laboratory Variables

We examined patient subgroups for association with STAT4 using logistic regression. There was no significant

Table 2. Association of the Genotypes and Haplotypes of the Most Significant Four SNPs Within the Large Third Intron of STAT4 with RA Susceptibility

				Dominant[a]		Allele[b]
SNPs	Genotype	RA	Control	*P* value	OR (95% CI)	OR (95% CI)
rs11889341	TT	149	91	0.0039	1.29 (1.08–1.53)	1.27 (1.12–1.45)
	CT	485	414			
	CC	484	496			
rs7574865	TT	157	95	0.0065	1.29 (1.07–1.54)	1.27 (1.11–1.45)
	GT	481	411			
	GG	394	402			
rs8179673	CC	172	113	0.0181	1.23 (1.04–1.47)	1.22 (1.07–1.38)
	CT	520	456			
	TT	424	430			
rs10181656	GG	172	114	0.0234	1.22 (1.03–1.46)	1.21 (1.07–1.37)
	CG	521	458			
	CC	424	428			

	Frequency		*P*	ORs
Haplotypes	RA	Control	value	(95% CI)
C,G,T,C	0.60	0.64	0.003	0.83(0.73–0.94)
T,T,C,G	0.34	0.28	0.0027	1.33(1.10–1.60)
C,T,C,G	0.05	0.05	0.45	0.90(0.68–1.19)
T,G,T,C	0.0122	0.0126	0.80	0.93(0.53–1.64)
C,G,C,G	0.0015	0.0026	0.48[c]	0.73(0.30–1.77)[c]
T,G,C,G	0.0014	0.0019		
C,G,T,G	0.0009	0.0015		
C,G,C,C	0.0004	0		

[a]Homozygote of risk allele and heterozygote versus homozygote of non-risk allele.
[b]*P* values are the same as ones in Table S1 (0.0003, 0.0004, 0.002, and 0.0029 in order).
[c]Four minor haplotypes (CGCG, TGCG, CGTG, and CGCC) are combined to calculate *P* value and ORs (95% CI).

genotypic or haplotypic association with sex, radiographic severity, or age of disease onset (data not shown). When we stratified the RA cases into anti-CCP positive and negative groups, we observed significant associations of all four risk SNPs and the risk haplotype of STAT4 with anti-CCP positive RA (Table 3), but not with anti-CCP negative RA compared with controls. However, because we observed the similar trend of distribution between both groups, this lack of significance in anti-CCP negative RA probably resulted from the smaller sample size (anti-CCP negative RA, N = 111). This finding suggests that the STAT4 contribution to disease risk may not be restricted to the anti-CCP + RA subset. Using a logistic regression analysis, the STAT4 risk haplotype is an independent risk factor ($P = 0.049$) in addition to SE ($P < 0.0001$) for RA.

To address the relationship of the disease-associated STAT4 variants with anti-CCP and RF titer, we divided the cases with anti-CCP into high (≥ 100) and low titer groups. This was necessary because CCP titers were truncated at the high end. This analysis did not show an effect of the STAT4 risk alleles or haplotype on the frequency of high anti-CCP group (data not shown). There was a suggestion of an effect on RF titer, but this was not significant, as shown in Table 4.

DISCUSSION

The North American Rheumatoid Arthritis Consortium (NARAC) recently reported on two new RA linkage regions at chromosomes 2q33 and 11p12 with logarithm of odds (LOD) scores of 3.52 and 3.09, respectively (20). Dense SNP mapping of the 2q RA linkage peak led to identification of a new susceptibility gene, STAT4, for RA (16). This association was replicated in several independent White RA case and control populations (16). In the current study, we have now confirmed that STAT4 is associated with RA in a large Korean population dataset, with the same common haplotype, which is more common in Koreans than in North American populations, but nevertheless confers a similar degree of risk.

A significant source of variability in the RA genetics literature has been the inability to replicate genetic findings across the major racial groups, particularly Whites and Asians. An interesting example of this is the association of the intracellular phosphatase, PTPN22, with RA and other autoimmune diseases. These disease associations have been widely replicated in White populations(7), but the PTPN22 risk allele (R620W) is exceedingly rare in Asian populations(15). Furthermore, attempts to identify other risk variants of PTPN22 that might be associated with RA in Asians have been unrevealing (9,H-S Lee unpublished). This has raised the possibility that there is true locus heterogeneity for RA among these major racial groups.

Several other examples have arisen in which associations are observed in Asian populations, but not in Whites. The most robust of these examples is the association of PADI4 with rheumatoid arthritis. PADI4 is a compelling candidate gene, because it encodes one of the enzymes responsible for citrullination of endogenous proteins, and an antibody response to citrullinated peptides is highly specific for RA(21). Numerous studies in Asian

Table 3. The Frequency (%) of Risk Alleles and Haplotype in Both Anti-CCP Positive and Negative RA Groups

Risk[a] SNP/ haplotype	AntiCCP+ (n = 612)	AntiCCP– (n = 111)	Control	*P* value CCP + vs. CCP-[b]	*P* value CCP + vs. Control[c]	*P* value CCP- vs. Control[d]
rs11889341	35.2	34.5	29.7	0.84	0.001	0.14
rs7574865	38.8	38.6	32.9	0.96	0.001	0.11
rs8179673	38.9	40.0	34.0	0.77	0.005	0.08
rs10181656	38.9	40.0	34.2	0.76	0.007	0.09
Haplotype, TTCG	34.2	32.4	28.5	0.62	0.0009	0.23

[a]Only 723 cases with anti-CCP data were compared with a total of 1008 controls.
[b]All alleles and haplotype had statistically significant risk for anti-CCP positive RA compared with control (ORs (95% CI) 1.29 (1.11–1.50), 1.28 (1.10–1.51), 1.24 (1.07–1.43), 1.23 (1.06–1.42), and 1.29 (1.11–1.51) in order)
[c]The distribution of risk alleles and haplotype between antiCCP positive and negative groups were not significantly different.
[d]*P* values of the risk alleles and haplotype in the anti-CCP negative group were not statistically significant level compared with control group.

Table 4. The Relationship of Risk Alleles and Haplotype of STAT4 with High CCP and RF Titer

	Haplotype TTCG	Haplotype Others	*P* value
High antiCCP[a]	0.50	0.48	0.46
High RF[a]	0.42	0.39	0.33
RF titer[b]			
Mean	219.9	181.9	0.29
Median	79.4	73.5	
25 percentile	36.2	36.2	
75 percentile	215.0	183.5	

[a]We divided the cases into high or low anti-CCP/RF group based on the titer ≥ 100 units; Values indicate the frequency of cases with high anti-CCP or high RF out of given subjects; All risk alleles (data not shown) and haplotype do not affect the high anti-CCP or high RF titer frequency using chi square test (ORs (95% CI) 1.09 (0.86–1.38) and 1.11 (0.90–1.38) in order).
[b]RF titer was not significantly related to risk haplotype using nonparametric statistics despite a trend toward higher titer in the risk groups.

populations have demonstrated the association of PADI4 with RA (10,22–25), but these associations are either absent or very weak in populations of European ancestry (13,14). It is possible that this difference reflects an interaction of PADI4 genetic susceptibility with environmental factors, because citrullination may also be related to smoking or other environmental exposures (1,2,26).

The current report is the first clear demonstration of non-MHC related susceptibility gene for RA that confers a similar degree of risk among both White and Asian populations. Furthermore, it appears that the risk haplotype is likely to be identical in the two racial groups, suggesting that the responsible functional variant is ancient in origin. Indeed, the same haploytpe also is found in African populations (TTCG, 0.14 in the Yoruba people of Ibadan, Nigeria, www.hapmap.org) and it will be of great interest to see if the STAT4 associations with RA and lupus are also present in this population group . The associated haplotype is located primarily in the third intron of the STAT4 gene, and the actual functional allele(s) remain to be identified. A full resequencing of the STAT4 gene is in progress, and this will help to direct future studies of splice variation and/or expression differences that may explain the disease associated haplotype.

STAT4 encodes a transcription factor that lies in the signaling pathway of several important cytokines, including IL-12 and type I interferons, as well as IL-23 (27). STAT4 is present in the cytosol and upon cytokine signaling it becomes phosphoryated and translocates to the nucleus. The target genes for STAT4 include γIFN and therefore it plays a key role in the IL12 induced differentiation of T cells into the Th1 pathway. In addition, STAT4 may also be involved in the production of IL17 by Th17 cells, in response to IL23 (28). At the same time, γIFN production tends to inhibit differentiation toward the Th17 pathway(29). Thus, while genetic differences in STAT4 dependent signaling may be involved in regulating the balance of Th1 vs. Th17 responses, the expected effects of increased vs. decreased STAT4 activity are not obvious. Furthermore, compared with the mouse, the production of IL17 is not as clearly restricted to an easily definable Th17 subset in humans (30,31), and recent work suggests that some subsets of CD4 cells can produce both IL17 and γIFN (29). Therefore, it will be important to try to relate the major risk haplotype of STAT4 to phenotypic differences in these various T cell subsets.

Given the current evidence of Th17 involvement in chronic inflammation in RA (29), it would be expected that the STAT4 risk alleles would generally enhance a Th17 response. Several studies in animal models strongly suggest a key regulatory role of STAT4 in experimental arthritis (32). There is evidence that STAT4 may play a role both during initiation of disease as well as in the maintenance of the inflammatory process, leading to the idea that STAT4 may be a useful therapeutic target as has been demonstrated in murine collagen induced arthritis (32). In this context, it may be relevant that STAT4 also is involved in signaling responses to type 1 interferons in activated monocytes, macrophages, and mature dendritic cells. The dependence of type 1 interferon signaling on STAT4 is of particular interest in view of our recent observation that in addition to RA, STAT4 has a strong association with systemic lupus (16), a disease in which dysregulation of interferon pathways is prominent.

The finding of a common risk haplotype for RA among Asian and White

populations shows that replication studies across racial boundaries may be useful for confirming at least some risk loci. In view of the large number of potential risk alleles that are coming out of whole genome scans (33), our data suggest that comparison across racial groups can be a reasonable approach to gene identification, and we hope to be able to carry out a genome-wide study of the Korean RA population in the near future.

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