showed strong evidence of heterogeneity across collections (Cochran's Q $P<0.001$).

We first analyzed all samples together (n = 2,706), regardless of drug type. We found no clear evidence of association with treatment response measured by ΔDAS (Figure 1A). Similar results were obtained using the binary phenotype of EULAR responder versus EULAR non-responder status (Figures S1 and S2).

We next separately analyzed patients treated with either etanercept (n = 733), infliximab (n = 894) or adalimumab (n = 1,071) (Figure 1B–1D), under the hypothesis that different genetic loci affect response to the different drugs based on their mechanism of action or other biochemical properties. GWAS results are publicly available for all SNPs tested at the Plenge laboratory and RICOPILI Web sites (see URLs). GWAS results for all SNPs achieving $P<10^{-6}$ from any analysis are detailed in the Table S3.

For etanercept-treated RA patients, a locus on chromosome *1q23* achieved near-genome-wide significance (rs6427528, $P_{META} = 8\times10^{-8}$) (Figure 1B, Figure 2A, and Figure 3), but not in the infliximab or adalimumab subsets ($P>0.05$) (Figure S3). SNPs in linkage disequilibrium (LD) showed consistent association results (rs1503860, $P = 1\times10^{-7}$, $r^2 = 1$ with rs6427528 in HapMap; three perfect-LD clusters of SNPs exemplified by rs3737792, rs10908787 and rs11265432 respectively; $P<5\times10^{-6}$; $r^2 = 0.83$, 0.63 and 0.59 with rs6427528, respectively). No single collection was responsible for the signal of association, as the effect size was consistent across all collections (Figure S4). The top SNP rs6427528 was genotyped in the ReAct dataset (Illumina Omni Express genotyping chip), and was well imputed across all other datasets (imputation quality score INFO ≥0.94, which is an estimate of genotype accuracy; the range of INFO scores is 0–1, where 1 indicates high confidence). All of these SNPs had minor

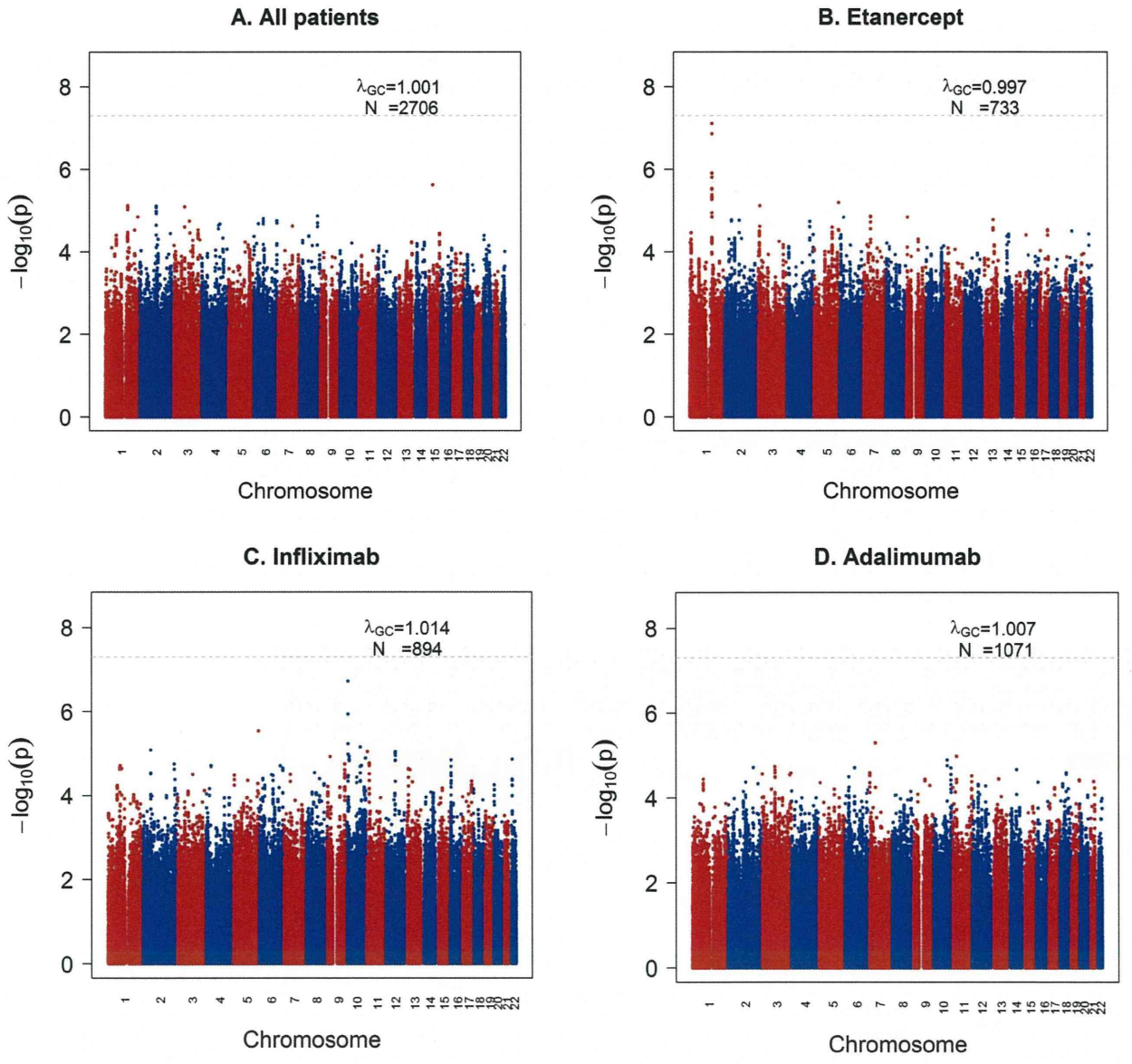
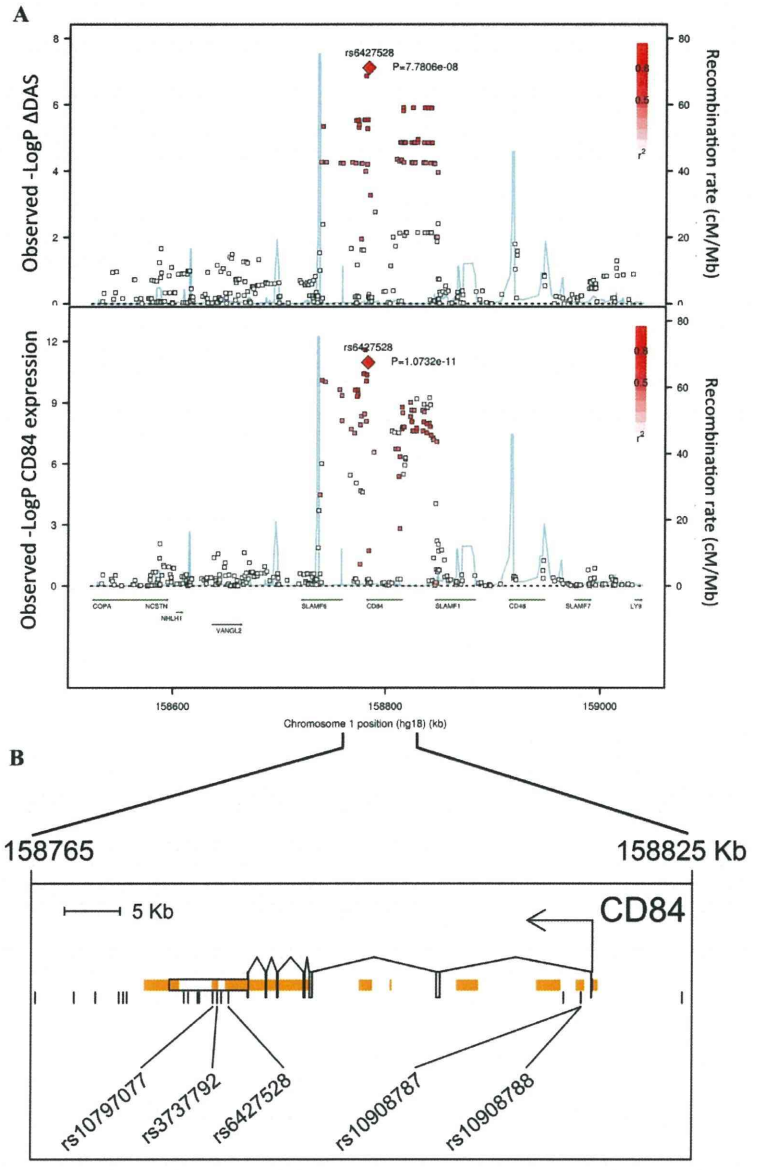


Figure 1. GWAS results for the ΔDAS phenotype. Shown are strengths of association (−Log10 P-value) for each SNP versus position along chromosomes 1 to 22. A) All samples (n = 2,706). B) Etanercept-treated patients (n = 733). C) Infliximab-treated patients (n = 894). D) Adalimumab-treated patients (n = 1,071).
doi:10.1371/journal.pgen.1003394.g001



C.

SNP (Major/ minor alleles)	Conserv-ation score[1]	DNase[2]	Transcription factor motifs altered		
			TF Motif	LOD(min) - LOD(maj)	Position weight matrix logo[3]
rs10797077 (G/A)	2.1	T-47D	AIRE_2	>6.6	
rs3737792 (G/A)	-1.3	-	-		
rs6427528 (A/G)	-2.8	-	KROX	>3	
			SREBP_4	-2.2	
rs10908787 (A/G)	-3.4	GM12878, Jurkat	-		
rs10908788 (A/G)	-7.8	GM12878	-		

Figure 2. Association results and SNP annotations in the *1q23 CD84* locus. A) Regional association plots with ΔDAS (top panel) and with *CD84* expression (bottom panel), showing strengths of association (−Log10 P-value) versus position (Kb) along chromosome 1. B) Schematic of *CD84* gene structure (RefSeq gene model, box exons connected by diagonal lines, arrow indicates direction of transcription) with strong enhancer chromatin states (orange rectangles) and SNPs in high LD (r2>0.8) with rs6427528 (vertical ticks). SNPs in enhancers are labeled below. C) Annotations of strong-enhancer rs6427528 proxy SNPs; listed are SNP rs-ID (major and minor alleles), conservation score, cell line with DNAse footprint if present, and transcription factor binding sites altered. 1- Genomic evolutionary rate profiling (GERP) conservation score, where a score >2 indicates conservation across mammals. 2- DNase footprint data are compiled from publicly available experiments by HaploReg. 3- Position weight matrix logos show transcription factor consensus binding sites with nucleotide bases proportional to binding importance. SNP position is boxed. Note that the rs10797077 AIRE_2 and the rs6427528 SREBP_4 motifs are on the minus strand (base complements correspond to SNP alleles), with the SREBP motif shown upside down to align with the rs6427528 KROX motif on the positive strand. Data are from HaploReg.
doi:10.1371/journal.pgen.1003394.g002

allele frequencies ranging from 7–10%. The SNP explains 2.6% variance in response to etanercept treatment.

For patients treated with infliximab, we observed a suggestive result on chromosome *10p14* (rs12570744, $P = 2\times10^{-7}$). No highly significant or suggestive results were observed for the ΔDAS phenotype in patients treated with adalimumab ($P_{META} > 10^{-5}$).

Qualitatively similar results were attained in the analysis of our secondary phenotype, EULAR good responder vs non-responder status (Figures S1 and S2). For SNPs at the *1q23* locus, the pattern of association with responder/non-responder status (etanercept-treated patients) was consistent with the results for ΔDAS ($P = 6\times10^{-3}$ for rs6427528 and rs1503860). We also identified potential novel associations, with suggestive results for infliximab (rs4336372, chromosome *5q35*, $P = 8\times10^{-7}$) and adalimumab (rs940928, chromosome *2q12*, $P = 2\times10^{-6}$).

eQTL and sequence analysis of the *CD84* gene

For each SNP with $P<10^{-6}$ identified by our GWAS (n = 6 independent SNPs), we searched for biological evidence to support a true positive association. We used genome-wide sequence data from the 1000 Genomes Project to search for putative functional variants in LD with the index SNP (defined as SNPs predicted to change protein-sequence or mRNA splicing). We also used genome-wide expression data to search for an expression quantitative trait locus (eQTL) in public databases and in peripheral blood mononuclear cells (PBMCs) in 228 non-RA patients and in 132 RA patients.

While we did not identify any variants disrupting protein-coding sequences or mRNA splicing, we did find that the *1q23* SNP associated with response to etanercept therapy was a strong eQTL in PBMCs (Figure 2A and Figure 3). In an analysis of 679 SNPs for cis-regulated expression of five genes in the region of LD (*SLAMF6*, *CD84*, *SLAMF1*, *CD48*, and *SLAMF7*), we found that rs6427528-*CD84* (and SNPs in LD with it) was the top eQTL of all results (n = 228 subjects; Figure 2A). This SNP was specifically associated with *CD84* expression, and was not an eQTL for other genes in the region (P>0.36 for the other genes).

We replicated our eQTL finding in 132 RA patients with both GWAS data and genome-wide expression data. PBMC expression data were available from RA patients in the Brigham RA Sequential Study. (BRASS) and Autoimmune Biomarkers Collaborative Network (ABCoN) collections. We observed a significant association between rs6427528 genotype and *CD84* expression (linear regression adjusted for cohort P = 0.004, rank correlation P = 0.018). The direction of effect was the same as in the PBMC samples from 228 non-RA patients. A combined analysis of RA patients and the non-RA patient eQTL data (described above) yielded rank correlation $P = 3\times10^{-10}$ (n = 360 total individuals).

We searched sequence data to determine if rs6427528, or any of the SNPs in LD with it, were located within conserved, non-coding motifs that might explain the eQTL data. We used HaploReg [16] to examine the chromatin context of rs6427528 and 26 SNPs in LD with it (at $r^2>0.50$). We found that 5 SNPs occur in strong enhancers inferred from chromatin marks (Figure 2B) [17]. Two of these 5 SNPs, rs10797077 and rs6427528 ($r^2 = 0.74$ to each other), are predicted to disrupt transcription factor binding sites, and rs10797077 occurs at a site that shows conservation across mammalian genomes [18]. Figure 2C shows the DNA sequence position weight matrices of the transcription factor binding sites changed by rs10797077 (the minor allele creates a stronger binding site for the AIRE transcription factor) and rs6427528 (the minor allele creates a binding site for KROX and SREBP).

Expression of *CD84* as a biomarker of disease activity and treatment response

Because the genetic data demonstrates that the allele associated with better response is associated with higher *CD84* expression, this suggests that *CD84* expression itself may serve as a useful biomarker of disease activity or treatment response. We tested both hypotheses using PBMC expression data from the BRASS and ABCoN collections. First, we tested if *CD84* expression is associated with cross-sectional DAS, adjusting for age, gender and cohort (Figure 4). We observed a significant inverse association between *CD84* expression and cross-sectional DAS in 210 RA patients (beta = −0.3, P = 0.02, $r^2 = 0.02$). That is, higher *CD84* expression was associated with lower DAS, regardless of treatment.

Second, we tested *CD84* for association with our primary treatment response phenotype, ΔDAS. The sample size for this analysis was smaller than for the cross-sectional analysis, as we required that patients be on anti-TNF therapy and have pre- and post-treatment DAS. We found that *CD84* expression levels showed a non-significant trend towards an association with ΔDAS in 31 etanercept-treated patients (beta = 0.2, $r^2 = 0.002$, P = 0.46) and in all 78 anti-TNF-treated patients (beta = 0.14, $r^2 = 0.004$, P = 0.4). The effect is in the same direction one would predict based on the genetic association at rs6427528: the allele associated with better response is also associated with higher *CD84* expression (Figure 3), and in 31 RA patients, higher *CD84* expression (regardless of genotype) is associated with a larger ΔDAS (i.e., better response; Figure 4).

Replication of genetic data in a small, multi-ethnic cohort

Since most of the samples available to us as part of our international collaboration were included in our GWAS, few additional samples were available for replication. In addition, the remaining samples available to us were from different ethnic backgrounds. Nonetheless, we sought to replicate the associations of rs6427528 with ΔDAS in these additional samples. We genotyped 139 etanercept-treated patients from a rheumatoid arthritis registry in Portugal (Reuma.pt) and 151 etanercept-treated patients from two Japanese collections (IORRA, n = 88 patients on etanercept and Kyoto University, n = 63 on etanercept). Replication sample sizes, clinical data and results for these

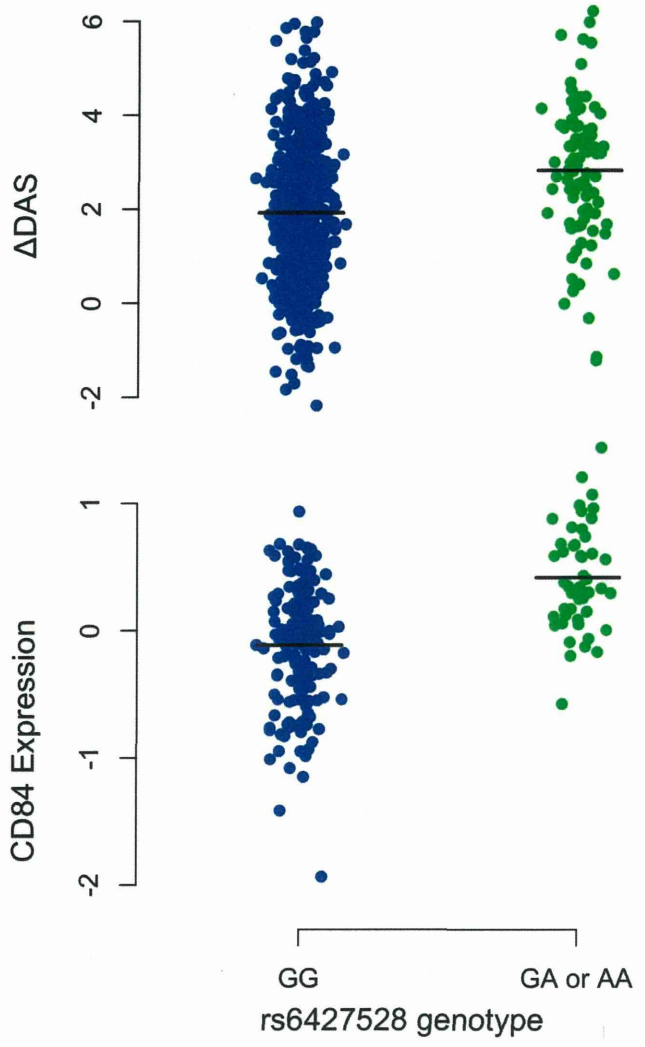


Figure 3. *1q23/CD84* genotype association plots for ΔDAS and *CD84* gene expression. Shown are ΔDAS in our GWAS in etanercept-treated patients (top panel, n = 733; n = 634 with the GG genotype and n = 99 with the GA or AA genotype) and *CD84* expression in our eQTL results (bottom panel, n = 228 non-RA patients; n = 178 with the GG genotype and n = 50 with the GA or AA genotype). The rare-allele homozygous genotype AA was observed four times in our ΔDAS GWAS and was pooled with the heterozygous GA genotype for this figure; AA homozygotes were not observed in the *CD84* eQTL data. Association analyses reported in the text regressed phenotype (ΔDAS, $P = 8 \times 10^{-8}$; *CD84* expression, $P = 1 \times 10^{-11}$) on minor-allele dosage (range 0–2).
doi:10.1371/journal.pgen.1003394.g003

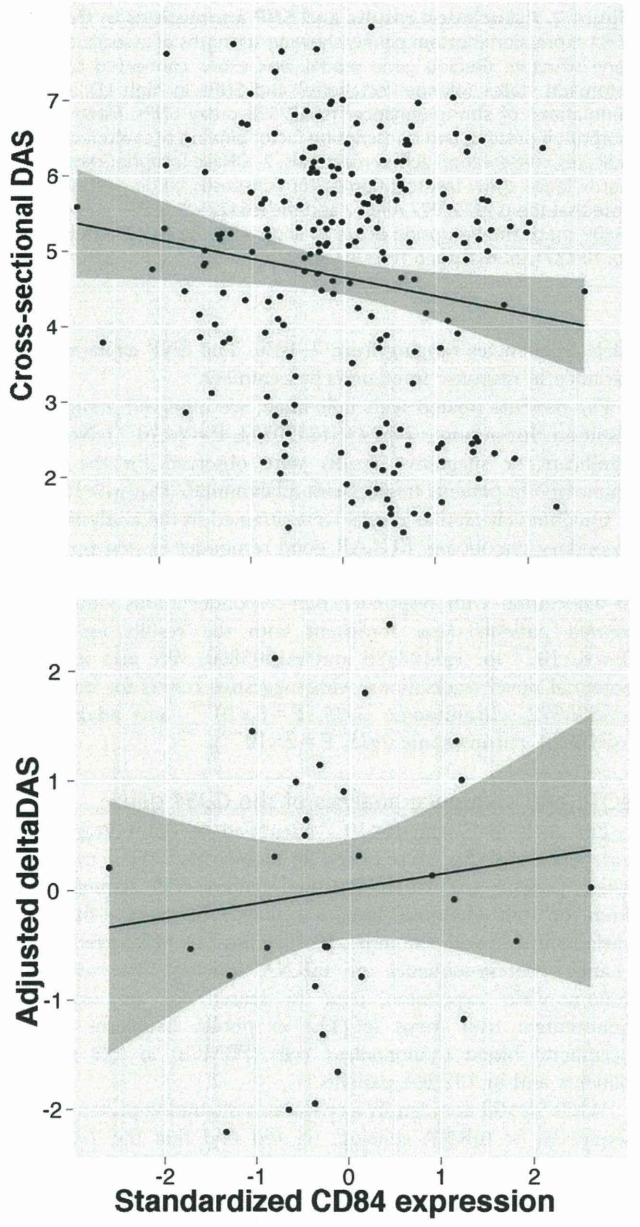


Figure 4. *CD84* expression level and clinical features. Analyses are shown in RA patients from the BRASS and ABCoN registries, for baseline DAS (top panel, n = 210; $R^2 = 0.02$, p = 0.02) and ΔDAS (bottom panel, n = 31; $R^2 = 0.001$, p = 0.46). Best-fit linear regression lines are shown in black, with shaded regions showing linear regression model (slope and intercept) 95% confidence intervals. *CD84* expression levels were quantile normalized, and ΔDAS values were adjusted for age, gender and baseline DAS.
doi:10.1371/journal.pgen.1003394.g004

two SNPs are shown in Table S4. Based on the observed effect size in the GWAS and observed allele frequency in the replication samples, we had 32% power to replicate this finding in the Portuguese samples and 17% power to replicate this finding in the Asian samples at $P<0.05$. The same association analysis as for GWAS was carried out: linear regression assuming an additive genetic model and using ΔDAS as phenotype, adjusted for baseline DAS. Replication results are shown in Figure 5.

While the SNPs fail to replicate in these patient collections at $P<0.05$, the direction of effect is the same in the Portuguese and Kyoto replication samples as in our GWAS. In a combined analysis limited to subjects of European-ancestry (GWAS data and Portuguese replication samples), rs6427528 remained highly suggestive ($P = 2 \times 10^{-6}$). Including the Japanese subjects, the overall GWAS+replication combined meta-analysis P-value remained suggestive ($P = 5 \times 10^{-4}$).

Discussion

Here we present the largest GWAS to date on anti-TNF therapy response in 2,706 RA patients. We find a significant association at the *1q23/CD84* locus in 733 etanercept treated patients ($P = 8 \times 10^{-8}$), but not in RA patients treated with drugs that act as a monoclonal antibody to neutralize TNF (infliximab or

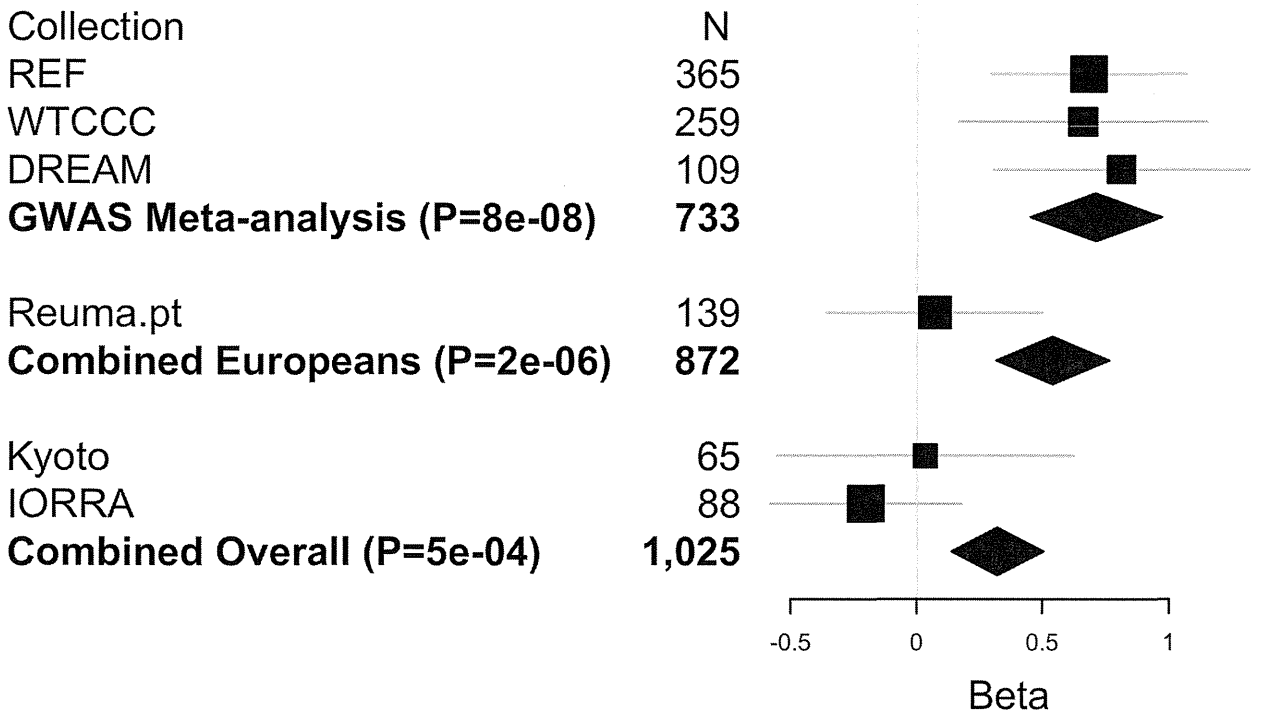


Figure 5. Replication and overall results for the *CD84* SNP rs6427528. Forest plot shows each cohort, sample size and linear regression beta coefficient estimates with symbol size proportional to cohort sample size and thin horizontal lines showing beta 95% CIs. Inverse variance weighted meta-analysis results are shown in bold for GWAS, GWAS+European (Portuguese) replication samples, and for GWAS+European+Asian (Japanese) replication samples, with diamond widths indicating beta 95% CIs.
doi:10.1371/journal.pgen.1003394.g005

adalimumab). The allele associated with a larger ΔDAS (i.e., better response) was associated with higher *CD84* expression in PBMCs from non-RA patients ($P = 1 \times 10^{-11}$) and in RA patients ($P = 0.004$).

We first conducted a GWAS of both categories of anti-TNF drugs (the soluble receptor drug, etanercept, and two monoclonal antibody drugs, infliximab and adalimumab). However, this analysis revealed no strongly associated SNPs. When we subset our GWAS by each of the three individual drugs, several SNPs in the *1q23* locus were highly significant in etanercept-treated patients, and SNPs in three other loci (*10p15*, *5q35* and *2q12*) were associated in infliximab or adalimumab subset analyses. Furthermore, the top SNPs for each analysis (Table S3) showed little correlation across the three anti-TNF drugs. This simple observation suggests that genetic control of treatment response may be different for different drugs. This finding is consistent with the clinical observation that RA patients who fail one anti-TNF drug may still respond to a different anti-TNF drug, albeit at lower rates of response [19]. If confirmed in larger samples and more comprehensive analyses, then this could have major implications for how physicians prescribe these drugs.

The most significant finding from our GWAS was a set of equivalent SNPs in LD with each other from the *1q23* locus in etanercept-treated RA patients (Figure 1 and Figure 2A). While the top SNP did not reach genome-wide significance in predicting treatment response, it did reach genome-wide significance as an eQTL in PBMCs ($P = 1 \times 10^{-11}$; Figure 2A). This finding indicates that the SNP (or another variant in LD with it) is indeed biologically functional in a human tissue that is important in the immune response. Two SNPs, rs10797077 and rs6427528, disrupt transcription factor binding sites, and represent excellent candidates for the causative allele to explain the effect on *CD84* expression (Figure 2C).

Our findings suggest that *CD84* genotype and/or expression could be a biomarker for etanercept treatment response among individuals of European ancestry. The genetic and expression data predict that *CD84* expression should be positively associated with treatment response (i.e., higher expression is associated with better response; Figure 3). While we did not observe a significant association between *CD84* levels and ΔDAS, we did observe a trend consistent with this prediction (Figure 4). Importantly, we note that power was extremely limited with the small sample sizes for which we had *CD84* expression as well as drug response data ($n = 31$ RA patients treated with etanercept).

The *CD84* gene is a compelling candidate for immune response, belonging to the CD2 subset of the immunoglobulin superfamily. It has been implicated in T-cell activation and maturation [20]. CD84 localizes to the surface of CD4+ and CD8+ T cells, and acts as a costimulatory molecule for IFN-gamma secretion [21]. *CD84* is also expressed in B-cells, monocytes and platelets. *CD84* has not been previously implicated in genetic studies of RA risk, disease activity, disease severity, or treatment response.

A limitation of our study is the small sample size available for replication ($n = 290$ etanercept-treated patients), and the lack of replication observed for the top *CD84* SNP (rs6427528) among patients of Portuguese and Japanese ancestry. The simplest explanation is that our original observation in the GWAS data represents a false positive association. However, the eQTL and gene expression data argue against this possibility. Explanations for a false negative finding in our replication collections include: (1) lack of power, especially if the effect size observed in the GWAS represents an over-estimate of the true effect size (the Winner's Curse) – we estimate that we had 32% and 17% power (at $P = 0.05$) to detect an association in the Portuguese and Japanese sample collections, respectively; (2) clinical heterogeneity, which is

always a possibility in pharmacogenetic studies, especially those conducted in different countries; and (3) ethnic differences, including different patterns of LD between the underlying causative allele (which is as yet unknown) and marker SNPs tested in our study. We did observe subtle differences in local patterns of LD between Asians and Europeans using genetic data from the 1000 Genomes Project (Figure S5). We note that the rs6427528 minor allele A has a frequency of ~5–10% in European and East Asian populations, and ~50% in the African YRI population (HapMap2 and 1000 Genomes); therefore, it may be of interest to test African American samples in replication.

What are the options for increasing sample size in pharmacogenetic studies, thereby providing an opportunity to replicate our *CD84* genetic and expression findings? While it might seem trivial to collect more samples through traditional registries, this is extremely challenging for phenotypes pertaining to treatment efficacy. To underscore this point, we highlight our study design, where we organized samples and clinical data from 16 different collections across 7 different countries in order to obtain the samples for the current study. Going forward, non-traditional strategies to collect biospecimens linked with clinical data (e.g., online registries, electronic medical records) may be required to achieve clinical collections of sufficient size to discover pharmacogenomic predictors of efficacy.

In conclusion, we conducted the largest GWAS to date for response to anti-TNF therapy in RA patients. Our genetic and expression data suggest that *CD84* genetic variants and/or expression levels could be developed as predictive biomarkers for etanercept treatment response in RA patients of European ancestry.

Methods

Samples and clinical data

All patients met 1987 ACR criteria for RA, or were diagnosed by a board-certified rheumatologist. In addition, patients were required to have at least moderate disease activity at baseline (DAS>3.2). All patients gave their informed consent and all institutional review boards approved of this study. A total of 13 collections from across 5 countries were included in GWAS [11,12,13,22]: Autoimmune Biomarkers Collaborative Network (ABCoN) from the U.S. (N = 79); the Genetics Network Rheumatology Amsterdam (GENRA, N = 53); the Dutch Behandelstrategieen voor Rheumatoide Arthritis (BeSt, N = 85); the U.K. Biological in Rheumatoid arthritis Genetics and Genomics Study Syndicate (BRAGGSS, N = 140); the U.S. Brigham Rheumatoid Arthritis Sequential Study (BRASS, N = 55); the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA, N = 298); the Immunex Early Rheumatoid Arthritis study (eRA N = 57); the Swedish Karolinska Institutet study (KI, N = 77); the Netherlands collection from Leiden University Medical Center (LUMC, N = 43); and the U.S. Treatment of Early Aggressive RA (TEAR, N = 109). We refer to these collections as the American College of Rheumatology Research and Education Foundation (REF) collection, as funding for GWAS genotyping was provided by the "*Within Our Reach*" project. We included additional samples from BRAGGSS (N = 595) [12]; the Dutch Rheumatoid Arthritis Monitoring registry (DREAM) in the Netherlands, and the ApotheekZorg (AZ) database (which facilitates the Dutch distribution of adalimumab; N = 880) [23,24], together referred to as DREAM; and the French Research in Active Rheumatoid Arthritis (ReAct, N = 272) [25].

Additional samples were collected for replication of SNPs in the *1q23* locus. These included the Rheumatic Diseases Portuguese Register (Reuma.pt, N = 378) from the Portuguese Society of Rheumatology (SPR), which captures more than 90% of patients treated with biological therapies and managed in rheumatology departments across Portugal [26]. Additional replication samples (N = 374) of East Asian ancestry were included from the IORRA and Kyoto University Hospital registries, part of the Japanese Genetics and Allied research in Rheumatic diseases Networking consortium (GARNET) [27].

Clinical data were collected in each cohort, including disease activity scores at baseline and at least one time point after treatment, gender, age, methotrexate use, as well as autoantibody status (RF or CCP). The composite disease activity scores for 28 joints (DAS28) included laboratory values for erythrocyte sedimentation rate (ESR) for most samples and C-reactive protein (CRP) for 191 samples in the REF collection (ABCoN, BRASS and eRA cohorts). DAS28 values were available at baseline and at 3–12 months after initiating anti-TNF therapy. Our primary phenotype was defined as ΔDAS = baseline DAS - end DAS, and responder status was also determined according to EULAR criteria for start and end DAS [15]. Clinical variables were assessed for association with phenotype in multivariate linear or logistic regression models for both the ΔDAS and EULAR responder-status phenotypes. Clinical variables that were significant in these analyses were retained as covariates in genetic association tests, except for methotrexate co-therapy. Including a covariate for methotrexate co-therapy reduced sample size substantially due to missing clinical data, so results were compared for our primary analysis and a secondary analysis with the covariates (and with reduced sample size) and the results were verified not to be impacted (not shown).

Genotyping and data processing

A total of eleven genotyping batches were processed separately. (1) BRASS samples were genotyped using Affymetrix 6.0 chip [28]; (2) WTCCC samples were genotyped on Affymetrix 500K chip [12]. All other cohorts were genotyped using Illumina platform arrays (see Table 1). Our American College of Rheumatology Research Education Fund (REF) collection was made up of smaller cohorts from throughout North America and Europe, including BRASS samples. Also included in REF: (3) ABCoN [13] and (4) EIRA [29] were separately genotyped on the Illumina 317K genotyping array; (5) eRA on the Illumina 550K chip; and (6) GENRA, BeSt, BRAGGSS (a subset of N = 53 samples), KI and LUMC were genotyped in one batch, and (7) BRAGGSS (N = 87) and TEAR were genotyped in a second batch, both using Illumina 660k chips, at the Broad Institute (8–10). DREAM and AZ samples were genotyped in three batches, one on 550K chip and two on 660K chips (manuscript in preparation), and (11) ReAct samples were genotyped on Illumina OmniExpress chips. Quality control (QC) filtering was done in each genotyping batch, including filtering individuals with >5% missing data, and filtering SNPs with >1% missing data, minor allele frequency (MAF) <1% and Chi-squared test of Hardy Weinberg equilibrium $P_{HWE}<10^{-5}$. We then used individual-pairwise identity-by-state estimates to remove occasional related and potentially contaminated samples. Data processing and QC were performed in PLINK [30]. Principal Components Analysis (PCA) was performed using EIGENSTRAT [31] (default settings) on the combined dataset using 20,411 SNPs genotyped across all datasets. Ethnicity outliers including all individuals of non-European decent were identified and removed, and the first three eigenvectors were used as covariates in GWAS.

Imputation was conducted on each of eleven datasets separately, using the IMPUTE v1 software [32] and haplotype-phased

HapMap Phase 2 (release 22) European CEU founders as a reference panel. Imputation of BRASS and EIRA was previously reported [28,33], and we followed the same imputation procedures for the remaining datasets. Imputation yielded posterior genotype probabilities as well as imputation quality scores at SNPs not genotyped with a minor allele frequency ≥1% in HapMap CEU. We removed imputed SNPs with imputation 'info' scores <0.5 or MAF <1% in any of the datasets.

Expression profile and eQTL data

Gene expression levels were quantified using mRNA derived from peripheral blood mononuclear cells (PBMCs) using Affymetrix Human Genome U133 Plus 2.0, for 255 multiple sclerosis patients in the Comprehensive Longitudinal Investigation of MS at the Brigham and Women's Hospital [34], either untreated (N = 83) or treated with interferon-beta (N = 105) or glatiramer acetate (N = 67). The raw intensity values were subject to quality control based on the recommended pipeline available in the simpleaffy and affyPLM R Bioconductor packages, and were then normalized using GCRMA (N = 228). The data are available on the Gene Expression Omnibus website (GSE16214). Expression levels for 17,390 probes mapping to 9,665 Ensembl transcripts were adjusted for confounding factors including age, gender, drug and batch using principle components and Bayesian factor analysis [35], and used in eQTL association analyses. Genotype data were collected on the Affymetrix 550K GeneChip 6.0 platform as a part of a previously published study [36]. Allelic dosages from imputed data (HapMap Phase II CEU samples; >2 million SNPs, MACH imputation quality >0.1 and MAF>=0.05) were used for association analysis. *Cis*-eQTLs were identified +/−1 Mb of transcription start sites (TSS) in the *1q23* locus region. Significance was evaluated by 10,000 permutations per gene, and false discovery rates were calculated based on cis-eQTL analyses in the total of 9,665 genes [37].

Additional expression profile data were available for subsets of samples that were part of two cohorts in our GWAS. Expression data from patients enrolled in the BRASS registry have been previously published [38]. Expression data were collected on Affymetrix Gene Chip U133 Plus 2 microarrays. BRASS patients had either cross-sectional expression data (n = 132, assayed at the time the patient was enrolled in BRASS) or pre- and post-treatment expression data (n = 17 samples, 8 treated with etanercept). Of these, n = 87 patients had expression and GWAS data. For patients with pre- and post-treatment data, we used the "baseline" pre-treatment expression data for cross-sectional analysis. In ABCoN, 65 RA patients (n = 23 treated with etanercept) had both pre- and post-treatment expression data, as well as ΔDAS clinical data [39], and n = 45 patients had expression and GWAS data. As with BRASS, we use the "baseline" pre-treatment expression data for cross-sectional analysis. For ABCoN expression profile data were collected on Illumina Human WG6v3 microarrays and were quantile normalized according to Illumina recommended protocols. Within both BRASS and ABCoN, expression data were normalized to the mean and standard deviation within each collection. For prospective analyses of expression data and ΔDAS, we combined BRASS and ABCoN to include 31 etanercept-treated patients and 78 anti-TNF-treated patients.

Statistical analyses

In our primary GWAS analysis, we tested each SNP for association with ΔDAS using linear regression adjusted for baseline DAS and the first 3 PCA eigenvectors in each collection. In our secondary GWAS analysis, we modeled SNPs predicting EULAR good response *versus* EULAR non-response using logistic regression, again adjusting for start-DAS value and the first three eigenvectors. Association analysis was done using SNPTEST [32] assuming an additive genetic model. Genomic control λ_{GC} values [40] for genotyped SNPs only and all SNPs were calculated, and no inflation or deflation was observed in the distributions of association test results. We then conducted inverse variance-weighted meta-analysis to combine results across the four datasets, and conducted Cochran's Q tests for heterogeneity using the β coefficients [41]. We further divided samples into 3 subsets according to drug (etanercept, infliximab or adalimumab). GWAS analysis for each group followed the same analysis procedure. Meta-analysis and heterogeneity tests were conducted using SAS. Expression analyses utilized linear regression or Spearman rank correlation, also using SAS. We tested for effects of cohort, age, gender and concurrent methotrexate, and results are shown using significant covariates as indicated.

Supporting Information

Figure S1 Quantile–quantile (QQ) plots for ΔDAS and response analysis, with genomic control λ_{GC} values.
(TIF)

Figure S2 GWAS results for the good response versus non-response phenotype. Shown are strengths of association (−Log10 P-value) for each SNP versus position along chromosomes 1 to 22. A) All samples (n = 1,708). B) Etanercept-treated patients (n = 472). C) Infliximab-treated patients (n = 599). D) Adalimumab-treated patients (n = 636).
(TIF)

Figure S3 Forest plot of replication results for the CD84 SNP rs6427528, in patients treated with anti-TNF drugs other than etanercept (infliximab & adalimumab).
(TIF)

Figure S4 Forest plot of CD84 result in patients treated with etanercept, subset by all collections.
(TIF)

Figure S5 Patterns of linkage disequilibrium (LD) at the CD84 locus in HapMap. Shown patterns of LD for CEU (top panel) and CHBJPT (bottom panel).
(TIF)

Table S1 Sample information for each of thirteen clinical batches.
(DOC)

Table S2 Clinical multivariate model for the ΔDAS phenotype.
(DOC)

Table S3 GWAS results for all SNPs achieving $P<10^{-6}$ from any analysis.
(XLS)

Table S4 Sample and clinical data summary for replication samples.
(DOC)

Acknowledgments

We thank Lars Klareskog and Lars Alfredsson for their contribution to the EIRA cohort, and the Swedish Rheumatology Register and Biologics Register (ARTIS) for providing follow-up data for the patients in the EIRA and KI study groups. We thank participants in the Biologics in Rheumatoid Arthritis Genetics and Genomics Study Syndicate (BRAGGSS).

Author Contributions

Conceived and designed the experiments: JC EAS RMP. Performed the experiments: JC EAS RMP. Analyzed the data: JC EAS RMP. Contributed reagents/materials/analysis tools: SS CM DD GT TR MUM HC KI CT YO SW JA HY SM AT KO FM TM NG MK AWM JDI AGW KLH MH MED P-PT JBAC IEvdH-B GJW PLCMvR MvdL H-JG NAS CFA TWJH REMT RPK SLB LAC LWM JEF NdV BES PLDJ SR MEW PKG XM AB LP MJHC EWK. Wrote the paper: JC EAS RMP. All authors reviewed and approved the manuscript.

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ARTHRITIS & RHEUMATISM
Vol. 65, No. 2, February 2013, pp 472–480
DOI 10.1002/art.37777

PLD4 as a Novel Susceptibility Gene for Systemic Sclerosis in a Japanese Population

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Objective. **Systemic sclerosis (SSc) is an autoimmune disease for which multiple susceptibility genes have been reported. Genome-wide association studies have shown that large numbers of susceptibility genes are shared among autoimmune diseases. Recently, our group identified 9 novel susceptibility genes associated with rheumatoid arthritis (RA) in a Japanese population. The aim of this study was to elucidate whether the 18 genes that displayed associations or suggestive associations for RA in our previous study are associated with SSc in Japanese.**

Methods. **We performed an association study that included 415 patients with SSc and 16,891 control subjects, followed by a replication study that included 315 patients and 21,054 control subjects. The 18 markers reported to display association with RA were analyzed for their associations with SSc in the first study, and 5 markers were further analyzed in the replication study. The inverse variance method was used to evaluate the associations of these markers with SSc in a combined study.**

Results. **In the phospholipase D4 gene (*PLD4*), rs2841277 displayed a significant association with SSc in Japanese patients ($P = 0.00017$). We observed that rs2841280 in exon 2 of *PLD4* was in strong linkage disequilibrium with rs2841277 and introduced an amino acid alteration. We also observed associations between SSc and rs6932056 in *TNFAIP3* and rs2280381 in *IRF8* ($P = 0.0000095$ and $P = 0.0030$, respectively), both of which displayed associations with SSc in a European population.**

Conclusion. **We determined that *PLD4* is a novel susceptibility gene for SSc in Japanese, thus confirming the involvement of *PLD4* in autoimmunity. Associations between SSc and *TNFAIP3* or *IRF8* were also detected in our Japanese population. SSc and RA appear to share relatively large proportions of their genetic backgrounds.**

Systemic sclerosis (SSc) is a connective tissue disease that affects 7–489 individuals per million worldwide and is characterized by the excess production of extracellular matrix molecules and fibrosis (1). Patients with SSc display skin sclerosis, obliterative microvasculopathy such as Raynaud's phenomenon, and multiorgan involvement. Severe complications of SSc sometimes develop, including interstitial lung disease, pulmonary hypertension, and renal crisis. These severe symptoms

Supported by grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, by research grants from the Japan Rheumatism Foundation, the Waksman Foundation, and the Mitsubishi Pharma Research Foundation, and by the Genetics and Allied Research in Rheumatic Diseases Networking consortium.

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Submitted for publication June 4, 2012; accepted in revised form October 23, 2012.

and complications of SSc result in a poor prognosis and a shortened lifespan (2,3). No effective method for preventing or curing SSc has been established (4).

It is well known that SSc has genetic components (5); for example, a US study revealed that the incidence of SSc was much higher among the families of patients with SSc compared with the general population (6). Recent technologic developments enabled the use of genome-wide association studies (GWAS) to identify novel susceptibility loci for autoimmune diseases (7). GWAS of European patients with SSc revealed that *CD247* (8), HLA (8), *TNIP1*, *PSORS1C1*, and *RHOB* (9) are susceptibility loci for SSc. In addition, another GWAS identified associations between *IRF8*, *GRB10*, and *SOX5* and limited cutaneous SSc (lcSSc) in a European population (10). Furthermore, studies adopting a candidate gene approach based on subjecting genes to functional inference analysis led to the identification of *STAT4* (11), *IRF5* (12), *TBX21* (13), *NLRP1* (14), *TNFSF4* (15), *CD226* (16), *BLK* (17), and *TNFAIP3* (18) as novel susceptibility genes for SSc in Europeans. SSc association studies in Japanese populations confirmed that *STAT4* (19), *IRF5* (20), and *BLK* (21) are associated with SSc and identified *UBE2L3* as a susceptibility gene for diffuse cutaneous SSc (dcSSc) (22). An association between HLA and SSc was also detected in Asians (23). These findings suggest a clear overlap in the genetic background of SSc between different populations.

It is well known that susceptibility genes are shared by various autoimmune diseases (24). In fact, HLA (25), *STAT4* (26), and *TNFAIP3* (27,28), which are susceptibility genes for SSc, have also been reported to be associated with rheumatoid arthritis (RA). In addition, *PTPN22*, which was shown to be strongly associated with RA in a European population (29), showed a suggestive association with SSc in Europeans (30). The sharing of these susceptibility genes between RA and SSc raises the possibility that newly identified susceptibility genes for RA could also be susceptibility genes for SSc. Recently, a large Japanese consortium, the Genetic and Allied research in Rheumatic diseases Networking consortium, identified 9 novel susceptibility genes and 6 candidate susceptibility genes for RA using a meta-analysis of GWAS and replication studies (31). Four other genes, namely, HLA, *PADI4*, *CCR6*, and *TNFAIP3*, were also confirmed to display associations with RA. Here, we performed a 2-stage association study of Japanese patients with SSc, in which we genotyped these genes as candidate susceptibility loci.

PATIENTS AND METHODS

Study subjects. DNA samples were obtained from 415 patients with SSc at Kyoto University Hospital and Tokyo Women's Medical University; these samples comprised the first set. Independent DNA samples were obtained from 315 patients with SSc at Keio University Hospital, Sagamihara National Hospital, and Kanazawa University Hospital; these samples were used as the replication set. All patients were Japanese, all had a diagnosis of SSc as determined by a rheumatologist, and all fulfilled the 1980 American College of Rheumatology classification criteria for SSc (32). The patients with SSc for whom clinical information was available were classified as having lcSSc or dcSSc, according to the definitions developed by LeRoy et al (33). The control samples were described in detail in our previous study (31). The current study was approved by the local ethics committees at each institution, and written informed consent was obtained from all subjects. The basic characteristics of the study subjects are shown in Table 1.

Genotyping. The 9 novel susceptibility markers, 6 potentially associated markers, and 4 confirmed markers of RA that were identified in our previous study in a Japanese population (31) were chosen as candidate susceptibility markers for SSc in Japanese. Eighteen of the 19 markers (HLA was excluded; see Results), none of which had previously been reported to be associated with SSc in Japanese individuals, were genotyped in the current study. The 5 candidate markers in the first set that showed associations with P values less than 0.1 were further genotyped in the replication study. Single-nucleotide polymorphisms (SNPs) rs2841280 and rs894037 were chosen as candidate causative variants in the phospholipase D4 gene (*PLD4*) region. Because rs894037 was shown to be monomorphic in Japanese, rs2841280 was genotyped in 334 control subjects, in addition to all patients, for imputation reference. The patients in the first and replication studies were genotyped at Kyoto University or Tokyo Women's Medical University and at Keio University or University of Tsukuba, respectively, using TaqMan assays (Applied Biosystems). The genotyping methods in control subjects were described in detail in our previous study (31).

Briefly, control genotypes in the first set were imputed based on the genome-scanning data, using mach2dat software with HapMap Phase II East Asian Populations as reference. The control genotypes for the replication study were extracted from genome-scanning data for the markers included on Illumina HumanHap610 Quad BeadChips. The genotypes for rs6932056 (which is not included in the array) were imputed based on the genome-scanning data, using mach2dat software with HapMap Phase II East Asian Populations as reference, and were used as control data for the replication set. The genotypes for rs2841280 (which is not included in the HapMap data or the array) were also imputed in control subjects, based on the genome-scanning data, using mach2dat software. Genotyping data for the 334 control subjects as determined by TaqMan assay in combination with genome-scanning data were used as reference.

Statistical analysis. The associations between the genotyped markers and SSc were analyzed using a Cochran-Armitage trend test in both the first and replication studies. Subanalyses were performed by comparing the genotypes of

Table 1. Characteristics of the study population*

	Patients	Controls
First set		
Institutions	Kyoto University, Tokyo Women's Medical University	Kyoto University, Tokyo Women's Medical University, BioBank Japan
Typing	TaqMan assay	Illumina HumanHap610 Quad BeadChip, Illumina HumanHap550 BeadChip, Affymetrix Genome-Wide Human SNP Array 6.0
Limited SSc/diffuse SSc, %	49.6/50.4	Not applicable
Anti–topo I/ACA, %	30.6/31.1	Not applicable
Interstitial lung disease, %	48.9	Not applicable
Age, mean ± SD years	50.9 ± 14.7	60.9 ± 12.5
Female, %	91.3	44.9
Replication set		
Institutions	Keio University, Sagamihara National Hospital, Kanazawa University	Kyoto University, BioBank Japan
Typing	TaqMan assay	Illumina HumanHap550 BeadChip, Illumina HumanHap610 Quad BeadChip
Limited SSc/diffuse SSc, %	63.8/34.6	Not applicable
Anti–topo I/ACA, %	29.5/35.2	Not applicable
Interstitial lung disease, %	43.2	Not applicable
Age, mean ± SD years	51.4 ± 14.1	59.3 ± 14.2
Female, %	87.3	48.4

* The first set included 415 patients with systemic sclerosis (SSc) and 16,891 control subjects. The replication set included 315 patients with SSc and 21,054 control subjects. Anti–topo I = anti–topoisomerase I; ACA = anticentromere antibody.

the control subjects with those of patients in the SSc subgroups based on the disease phenotypes. The subanalyses used the same control subjects as were used in the association studies. Intracase analyses based on phenotypes were also performed.

Odds ratios (ORs) and 95% confidence intervals were also calculated. The associations detected in the first and replication studies were then meta-analyzed using the inverse variance method. The resultant *P* values were corrected using the Benjamini-Hochberg false discovery rate (FDR) criterion, and corrected *P* values less than 0.05 were regarded as significant in both the combined study and the subanalyses. The efficiency of the current study was estimated by calculating the likelihood of detecting 3 significant markers (after correcting the *P* values using the FDR method) among 18 randomly selected markers. After the statistically significant markers were identified, the best-fit model for each association was analyzed using dominant, recessive, trend, and allelic chi-square tests or models. Statistical analyses were performed using R or SPSS (version 18) software.

RESULTS

Analyses of candidate genes for SSc in a Japanese population. The 415 patients with SSc and 16,891 control subjects in the first set were genotyped for the 18 markers that were shown to have associations or suspected associations with RA in our previous study. The HLA region was excluded from the genotyped markers, because this region has already been shown to be associated with SSc in Asians. The allele frequencies of the patients were compared with those of the control subjects, using a Cochran-Armitage trend test.

As a result, 3 markers that demonstrated associations with *P* values less than 0.01 in the first set (Table 2) were identified, namely, rs6932056 in the *TNFAIP3* region ($P = 0.0000038$, OR 1.69), rs10821944 in the *ARID5B* region ($P = 0.0025$, OR 1.25), and rs2841277 in the *PLD4* region ($P = 0.0054$, OR 1.25). Two loci that showed suggestive associations with *P* values less than 0.1 (Table 2) were also identified, namely, rs12529514 in the *CD83* region ($P = 0.083$, OR 1.18) and rs2280381 in the *IRF8* region ($P = 0.095$, OR 1.19). The *TNFAIP3* and *IRF8* regions were previously reported to display associations with SSc and lcSSc, respectively, in European populations (10,18). These 5 markers were selected as candidate susceptibility markers for SSc in Japanese and were subjected to validation.

Next, a replication study consisting of 315 patients with SSc and 21,054 control subjects was performed to validate the associations of the 5 markers with SSc. The patients were genotyped for the 5 markers. The genotypes of the control subjects for the 5 markers, except rs6932056, were extracted from the Illumina Infinium HumanHap610 Quad array, as reported previously (31). The genotypes for rs6932056 were imputed based on genome-scanning data using mach2dat soft-

Table 2. Association studies of Japanese patients with SSc*

				Allele 1 frequency								
				First set			Replication set			Combined study		
SNP	Chr	Gene	Allele 1/2	Controls	Patients	*P*	Controls†	Patients	*P*	*P*, patients vs. controls	OR (95% CI)	*P*, patients without overlapping RA vs. controls
rs766449	1	*PADI4*	T/C	0.40	0.37	0.12	–	–	–	–	–	–
rs11900673	2	*B3GNT2*	T/C	0.29	0.28	0.65	–	–	–	–	–	–
rs2867461	4	*ANXA3*	A/G	0.44	0.43	0.57	–	–	–	–	–	–
rs657075	5	*IL3-CSF2*	A/G	0.36	0.34	0.25	–	–	–	–	–	–
rs12529514	6	*CD83*	C/T	0.14	0.16	0.083	0.15	0.16	0.31	0.046	1.15 (1.00–1.33)	0.040
rs1571878	6	*CCR6*	C/T	0.49	0.47	0.28	–	–	–	–	–	–
rs6932056	6	*TNFAIP3*	C/T	0.069	0.11	3.8×10^{-6}	0.067	0.079	0.23	9.5×10^{-6}	1.50 (1.25–1.80)	5.4×10^{-6}
rs2233434	6	*NFKBIE*	G/A	0.21	0.21	0.93	–	–	–	–	–	–
rs10821944	10	*ARID5B*	G/T	0.36	0.41	0.0025	0.36	0.37	0.64	0.0073	1.16 (1.04–1.29)	0.010
rs3781913	11	*PDE2A-CENTD2*	T/G	0.69	0.69	0.91	–	–	–	–	–	–
rs4937362	11	*ETS1-FLI1*	T/C	0.68	0.68	0.88	–	–	–	–	–	–
rs2841277	14	*PLD4*	T/C	0.69	0.74	0.0054	0.69	0.73	0.012	0.00017	1.25 (1.11–1.41)	0.00052
rs3783637	14	*GCH1*	C/T	0.74	0.73	0.54	–	–	–	–	–	–
rs1957895	14	*PRKCH*	G/T	0.39	0.41	0.26	–	–	–	–	–	–
rs6496667	15	*ZNF774*	A/C	0.35	0.37	0.33	–	–	–	–	–	–
rs7404928	16	*PRKCB1*	T/C	0.62	0.63	0.51	–	–	–	–	–	–
rs2280381	16	*IRF8*	T/C	0.84	0.86	0.095	0.83	0.87	0.0099	0.0030	1.26 (1.08–1.47)	0.0021
rs2847297	18	*PTPN2*	G/A	0.34	0.34	0.85	–	–	–	–	–	–

* SSc = systemic sclerosis; SNP = single-nucleotide polymorphism; Chr = chromosome; OR = odds ratio; 95% CI = 95% confidence interval; RA = rheumatoid arthritis.
† The control rs6932056 genotypes used in the replication study were imputed using genome-scanning data obtained for 3,765 subjects.

ware, because rs6932056 was not included in the array. As a result, rs2841277 in the *PLD4* region and rs2280381 in the *IRF8* region showed relatively strong associations with SSc ($P = 0.012$, OR 1.25 and $P = 0.0099$, OR 1.37, respectively) (Table 2). Interestingly, we observed that all 5 of the markers that displayed associations in the first study also demonstrated the same association directions in the replication study.

The inverse variance method was used to combine the data for the first and replication studies. SNPs rs2841277 in the *PLD4* region, rs6932056 in the *TNFAIP3* region, and rs2280381 in the *IRF8* region showed significant associations with SSc even after correcting the associated *P* values using the FDR method for multiple testing (Table 2). Importantly, all 3 of these loci shared risk alleles with RA. Although rs6932056 in the *TNFAIP3* region did not show a strong association with SSc in the replication study, its association was significant in the combined study. The *PLD4* region was shown to be a novel susceptibility gene for SSc, and, for the first time, the *TNFAIP3* and *IRF8* regions were confirmed to be associated with SSc in Japanese.

The association between rs2841277 and SSc was then investigated in detail. When the 200-kbp region around rs2841277 was evaluated, 2 hypothetical genes and cell division cycle associated 4 gene (*CDCA4*) were located at the region, in addition to *PLD4*. *PLD4* was the only gene whose region showed moderate to strong linkage disequilibrium (LD) with rs2841277, indicating *PLD4* as a susceptibility gene (Figure 1A). We vigorously searched candidate markers in exons of *PLD4* that showed strong LD with rs2841277 and selected 2 markers registered in the 1000 Genomes Project (34) that displayed >5% frequency in genotyped subjects, namely, rs2841280 (Figure 1B) and rs894037 in exon 2. Genotyping of these polymorphisms revealed strong LD between rs2841280 (E27Q) and rs2841277 ($D' = 0.98$, $r^2 = 0.75$) and monomorphism of rs894037 in Japanese. An association study of rs2841280 using control genotypes obtained by imputation supported association of *PLD4* with SSc ($P = 6.3 \times 10^{-5}$) (see Supplementary Tables 1 and 2, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.002/art.37777/abstract).

Because the 3 loci were associated with RA in a Japanese population, we analyzed whether the associations with SSc in the current study were contributed by patients with both RA and SSc. When 22 patients who had RA as well as SSc were excluded, significant associations for the 3 loci were still observed (Table 2). A

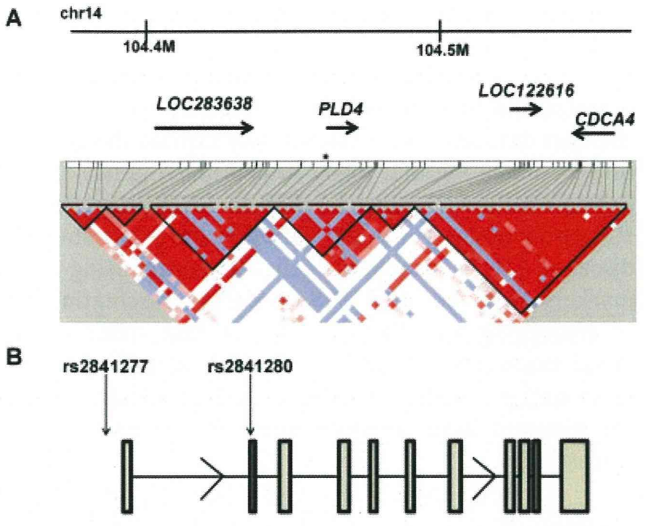

Figure 1. Linkage disequilibrium (LD) block around the *PLD4* region and the *PLD4* structure. **A,** LD block and genes around *PLD4*. The LD block is based on HapMap phase 3 data. **Asterisk** indicates rs2841277. **B,** Schematic view of *PLD4* structure. Rectangles represent exons of *PLD4*.

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*

SNP	Chr	Gene	Allele 1/2	Controls, allele 1 frequency	Limited cutaneous SSc (n = 408)			Diffuse cutaneous SSc (n = 318)		
					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.

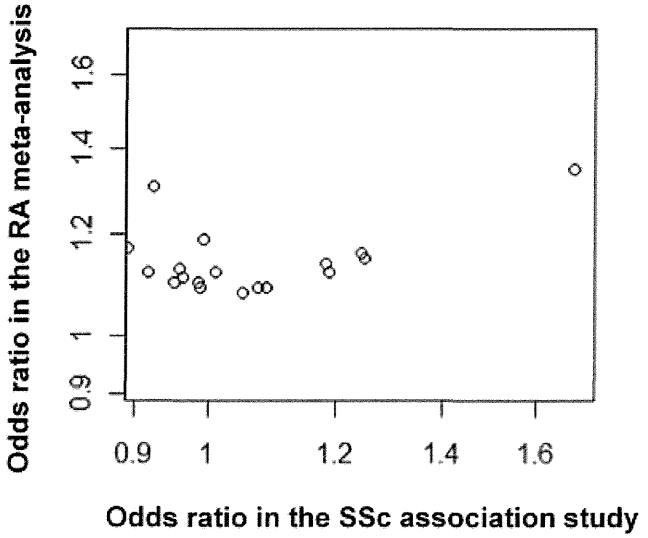

Figure 2. Comparison of associations for systemic sclerosis (SSc) and rheumatoid arthritis (RA). The odds ratios obtained for 18 genes in association studies of SSc and RA are plotted.

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.

ACKNOWLEDGMENT

We thank the staff of the BioBank Japan Project for collecting DNA samples from control subjects.

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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Diabetol Int (2013) 4:66–70
DOI 10.1007/s13340-012-0082-y

CASE REPORT

A patient who developed symptomatic reactive hypoglycemia 14 years after total gastrectomy and was successfully treated with miglitol

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Received: 21 July 2011 / Accepted: 14 June 2012 / Published online: 19 July 2012

Abstract We report the case of an 85-year-old man who had undergone total gastrectomy 14 years prior to admission at our hospital. He lost consciousness and was urgently hospitalized. His blood samples showed severe hypoglycemia (plasma glucose level 8 mg/dl) with hyperinsulinemia (immunoreactive insulin level 174.4 μU/ml) on admission. Laboratory data and imaging studies showed there was no insulinoma. He took garenoxacin mesilate hydrate for flu several days before admission. Such severe hypoglycemia did not recur after cessation of garenoxacin, and it was reported that hypoglycemia was induced by garenoxacin, which promoted insulin secretion, which indicated that garenoxacin had the possibility of inducing severe hypoglycemia. After admission, reactive hypoglycemia by dumping syndrome was diagnosed by mixed-meal tolerance test. The patient began developing symptoms after medication with irbesartan (angiotensin receptor blocker) for essential hypertension 1 month before admission. This preceded irbesartan, which is known to increase insulin sensitivity and may cause symptomatic dumping syndrome. Laboratory data also showed the presence of insulin autoimmune syndrome with a high titer of anti-insulin autoantibody. Although the antibody showed a low affinity and a high bound capacity, blood glucose profile during admission only showed the pattern of reactive hypoglycemia. Therefore, insulin autoimmune syndrome was unlikely the cause of hypoglycemic events in this case. The meal tolerance test showed better efficacy of miglitol to inhibit postmeal hyperglycemia and subsequent hyperinsulinemia than did acarbose and voglibose. We speculate that the angiotensin receptor blocker caused symptomatic dumping syndrome, and only miglitol among α-glucosidase inhibitors suppressed the symptom.

Keywords Hypoglycemia · Hyperinsulinemia · Dumping syndrome · Angiotensin receptor blocker · Insulin autoimmune syndrome · α-Glucosidase inhibitor

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Case report

An 85-year-old man was followed up monthly due to essential hypertension and post-total gastrectomy due to gastric cancer. He had not experienced dumping syndrome, including hypoglycemic events, after gastrectomy. Angiotensin receptor blocker (Irbesartan) was started on 28 February 2009. Several days later, symptoms suggesting hypoglycemia (cold sweats, palpitations) began manifesting several hours after meals. He also had the flu and received antibiotics (garenoxacin mesilate hydrate) on 11 March 2009. The patient lost consciousness while bathing at around 1700 hours on 17 March 2009 and was rushed to a nearby clinic. No abnormality was observed during tests, which included computed tomography (CT) of the head. Abnormal hypoglycemia [blood glucose level 8 mg/dl, immunoreactive insulin (IRI) 174.7 μU/ml] was observed

Table 1 Clinical data on admission

Patient characteristics			
Urinalysis		Cl	104 mEq/l
Protein	–	CRP	0.43 mg/dl
Glucose	–	Glucose	95 mg/dl
Occult blood	–	HbA1c	6.4 %
Ketone	–	Endocrinology	
Complete blood count		IRI (fasting)	19 μIU/ml
WBC	7600/μl	CPR (fasting)	2.8 ng/ml
RBC	397 × 10^6/μl	Free T3	2.1 pg/ml
Hb	11.1 g/dl	Free T4	1.3 μU/ml
Plt	24.1 × 10^4/μl	TSH	4.14 ng/dl
Blood chemistry		Glucagon	115 pg/ml
TP	6.7 g/dl	GH	0.33 ng/ml
Alb	3.6 g/dl	Cortisol	15.2 μg/dl
T-bil	0.7 mg/dl	ACTH	4.17 pg/ml
AST	40 IU/l	Tumor markers	
ALT	42 IU/l	CEA	5.9 ng/ml
LDH	169 IU/l	CA19-9	1.0 U/ml
ALP	283 IU/l	Immunology	
γ-GTP	50 IU/l	Anti-insulin auto-antibody	90 %
AMY	152 IU/l		
CPK	87 IU/l		
BUN	13.9 mg/dl	Scatchard plot analysis	
Cre	0.8 mg/dl	K1	2.06 × 10^{-1} (1/10^{-8}M)
T-CHOL	135 mg/dl	K2	2.01 × 10^{-3} (1/10^{-8}M)
TG	73 mg/dl	R1	1.16 (1/10^{-8}M)
Na	142 mEq/l	R2	12.8 (1/10^{-8}M)
K	4.9 mEq/l	HLA-DRB1	0406

Hb hemoglobin, *IRI* immunoreactive insulin, *WBC* white blood cells, *RBC* red blood cells, *Pil* platelets, *T-bil* total billirubin, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactate dehydrogenase, *γ-GTP* gamma guanosine triphosphate, *AMY* amylase, *CPK* creatine phosphokinase, *BUN* blood urea nitrogen, *Cre* creatinine, *T-chol* total cholesterol, *TG* triglycerides, *Na* sodium, *K* potassium, *Cl* chloride, *CRP* C-reactive protein, *TSH* thyroid-stimulating hormone, *ACTH* adrenocorticotropic hormone, *CEA* carcinoembryonic antigen, *CA 19-9* cancer antigen 19-9, *HLA* human leukocyte antigen

following blood collection. Although consciousness improved after glucose administration, he was admitted to our hospital the next day to elucidate the cause of hypoglycemia. Irbesartan was canceled after admission. His history showed he had a total gastrectomy and partial pancreatectomy due to gastric cancer in 1995 and was diagnosed with essential hypertension in 1998. He had no history of medication with insulin or drugs containing thiol. He had no family history of diabetes or another diseases, and he neither consumed alcohol nor smoked.

The patient was 156 cm tall, weighted 46 kg, and his body mass index (BMI) was 18.2 kg/m^2. His blood pressure was 113/59 mmHg, with 82 bpm regular pulse rate and 36.4 °C body temperature. He was lucid, with no yellowing in the bulbar conjunctiva, anemia in the palpebral conjunctiva, thyromegaly, or abnormal lymph node. There was an abrasion in the occipital region (which occurred when he lost consciousness). Heart sounds were regular without murmur, vesicular pulmonary sounds were normal, and his abdomen was soft and flat. No palpation was conducted on the liver/spleen/kidney, and there was a surgical scar on the abdomen. No edema was evident on the lower legs and back, and neurological findings and reflexes were normal. According to the results of the tests conducted upon hospitalization (see Table 1), hemoglobin $A1_c$ (HbA1c) was 6.8 % (International Reference Value; Japan Diabetes Society value 6.4 %).

After starting continuous glucose administration, hypoglycemia did not recur, and blood glucose level and consciousness were stable. Therefore, we stopped glucose infusion 3 days after admission, and again, hypoglycemia did not recur. Because insulinoma was first suspected due to the high level of IRI at the onset of hypoglycemia, we performed the fasting test; no hypoglycemia was observed. The contrast-enhanced CT of the abdomen showed no abnormal findings in the residual pancreas. The another endocrine tests and tumor markers (see Table 1) were all within normal limits. After initiating diet therapy at 1,600 kcal (35 kcal/kg), mild hypoglycemia (55–70 mg/dl) was observed 3–5 h after each meal without fasting hypoglycemia. Interestingly, although the serum level of C-peptide [C-reactive protein (CPR)] on hypoglycemic

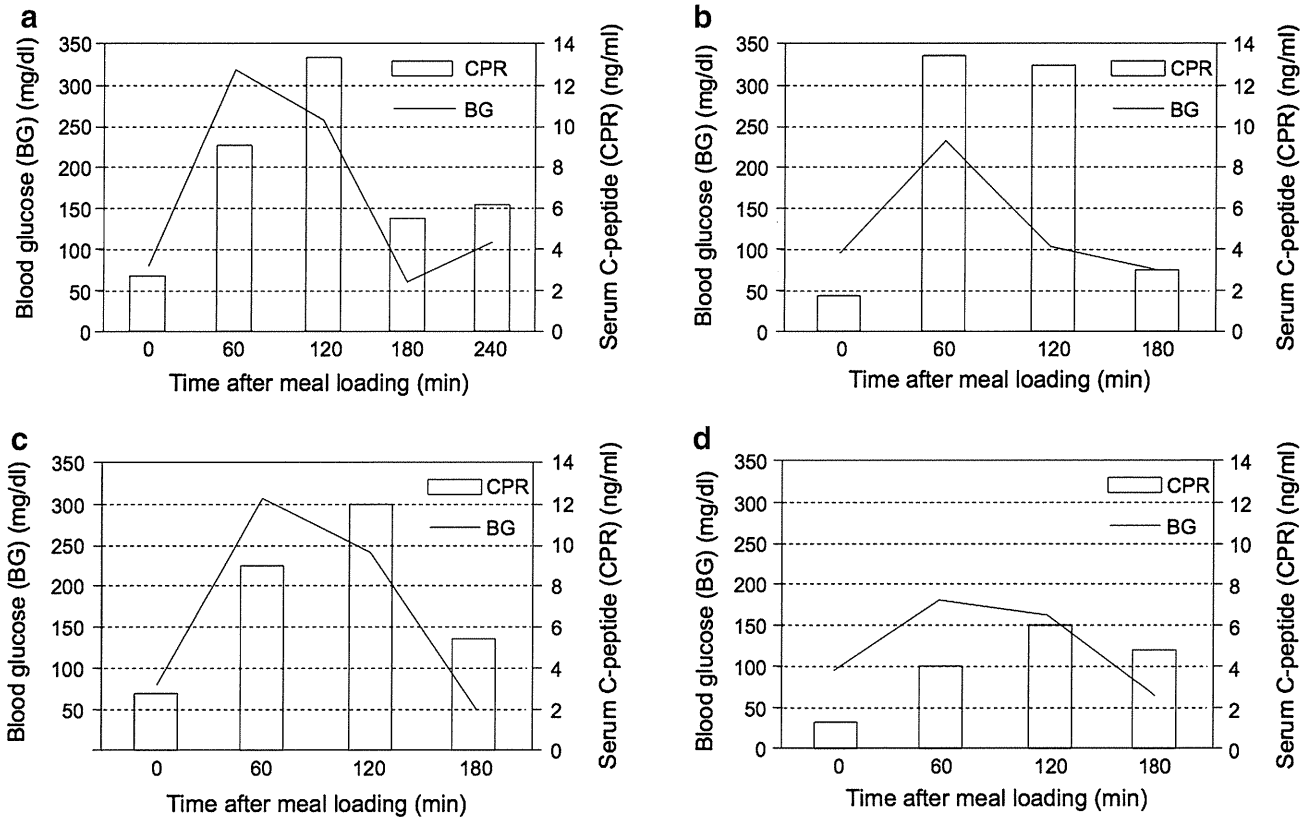

Fig. 1 Results of mixed-meal tolerance tests (MMT). *White bar* indicates serum c-peptide level (ng/ml). *Solid line* indicated blood glucose level (mg/dl). **a** Results of MMT performed 14 days after admission (irbesartan had been canceled after admission). Results of MMT under medication of the α-glucosidase inhibitor, **b** acarbose, **c** voglibose, and **d** miglitol

events after meals were suppressed (4.5 ng/ml), those of IRI were relatively high, at approximately 50 μIU/ml. In order to confirm whether dumping syndrome was associated with these hypoglycemic events, mixed-meal tolerance test (MMT) using the test meal (Kewpie, ingredients: energy 460 kcal; protein 18 g; fat 18 g; carbohydrates 56.5 g) was performed (see Fig. 1). Hyperglycemia (320 mg/dl) was observed 60 min after meal loading. Subsequently, CPR levels peaked 120 min after meal loading, and hypoglycemia was developed reactively. Hypoglycemic symptom was not observed after admission. Furthermore, the presence of anti-insulin autoantibody was tested because IRI and CPR levels were dissociated. Results showed a high titer of anti-insulin autoantibody (>90 %),despite the patient having no history of diabetes or insulin usage. Based on these findings, the patient was diagnosed with dumping syndrome accompanied by insulin autoimmune syndrome. We analyzed the effects of α-glucosidase inhibitors (αGI), because reactive hypoglycemia continued. First, voglibose (Basen®) 0.9 mg before every meal with a 1,600 kcal diet were given, but there was no improvement in the reactive hypoglycemia. Therefore, meal time was divided into six times a day, which resulted in reduced hypoglycemia. For further improvement, we tested and evaluated the effects of another α-glucosidases available in Japan, acarbose and miglitol (the drug was changed just after the meal tolerance tests, and the patient was observed for at least 1 week). As shown in Fig. 1b–d, while the profile of blood glucose and CPR after taking acarbose was similar to those after taking voglibose, oxy-hyperglycemia and reactive hyperinsulinemia were clearly suppressed by miglitol during MTT, resulting in complete suppression of the subsequent hypoglycemia. Finally, miglitol medication was continued thereafter, and the patient was discharged without any hypoglycemic attack or adverse effects on 20 April 2009. He visits our hospital as an outpatient, takes miglitol three times a day before meals, and self-monitors his blood glucose. Neither hypoglycemia nor side effects, such as abdominal symptoms, have been observed.

Discussion

In this case, two types of hypoglycemia were recognized. One was severe hypoglycemia due to hyperinsulinemia, which caused loss of consciousness prior to admission. The other was reactive hypoglycemia that occurred 3–4 h after