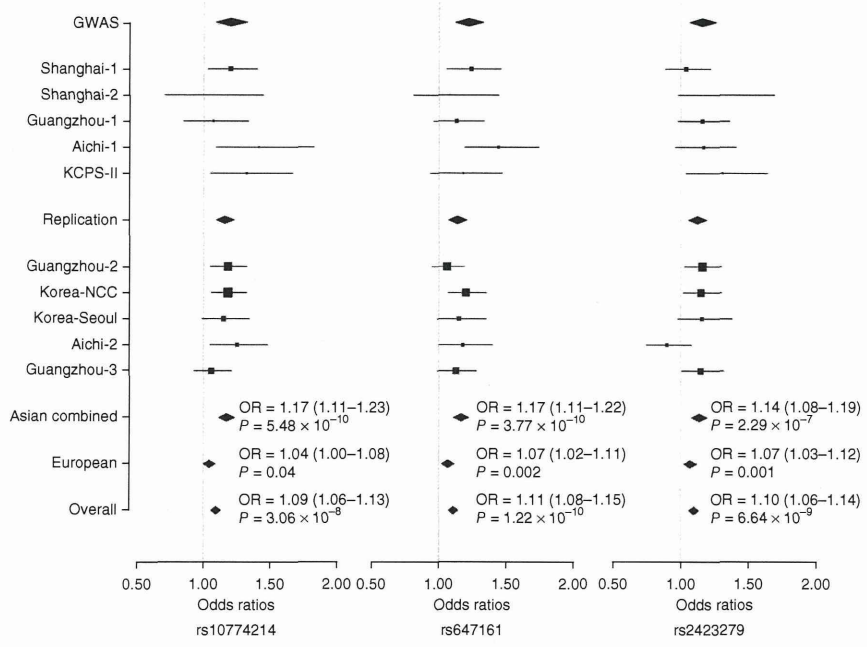

Figure 1 Forest plots for the three SNPs showing evidence of an association with CRC risk. Per-allele ORs are presented, with the area of each box proportional to the inverse variance weight of the estimate. Horizontal lines represent 95% confidence intervals.

Asians and Europeans (**Supplementary Fig. 4**). It is possible that causal variants in these regions are tagged by different SNPs in these two populations or that there is allelic heterogeneity, in which different underlying causal variants exist in populations of Asian and European ancestry. The difference in LD structure between Asian and European descendants and possible allelic heterogeneity in these two populations might explain, in part, why these loci were not discovered in previous studies conducted in individuals of European ancestry. The fourth SNP evaluated in the GECCO and CCFR sample, rs1665650, however, was not replicated in individuals with European ancestry (OR = 0.96, $P = 0.05$).

Stratification analyses showed that the association of CRC risk with each of the three replicated SNPs was generally consistent in Chinese, Korean and Japanese individuals ($P_{het} > 0.05$), although the association with rs2423279 was not statistically significant in Japanese, perhaps owing to a small sample size (**Supplementary Table 5**). Associations of these three SNPs with CRC risk were similar for men and women ($P_{het} > 0.05$) (**Supplementary Table 6**).

The rs10774214 SNP is located just 15 kb upstream of *CCND2*, the gene encoding cyclin D2 (**Fig. 2a**), a member of the D-type cyclin family, which also includes cyclins D1 and D3. These cyclins have a critical role in cell cycle control (from G1 to S phase) through activation of cyclin-dependent kinases (CDKs), primarily CDK4 and CDK6 (ref. 22). *CCND2* is closely related to *CCND1*, a well-established human oncogene[22,23]. Although *CCND2* has been less well studied than *CCND1*, several studies, including The Cancer Genome Atlas (TCGA), have shown that *CCND2* is overexpressed in a substantial proportion of human colorectal tumors[22–25]. Overexpression of this cyclin may be an independent predictor of survival in individuals with CRC[24]. Several other genes, including *PARP11*, *FGF23*, *FGF6*, *C12orf5* and *RAD51AP1*, are also in close proximity to the SNP identified in our study, of which both *C12orf5* (also known as *TIGAR*, encoding TP53-induced glycolysis and apoptosis regulator) and *RAD51AP1* were found to be overexpressed in CRC tissue included in TCGA[25]. rs10774214 is in strong LD with several SNPs that are located in potential transcription factor–binding sites, as determined using the TRANSFAC database[26]. Additional research may be warranted regarding possible mechanisms by which this SNP is related to CRC risk.

The rs647161 SNP is located on chromosome 5q31.1, where a cluster of SNPs were associated with CRC risk (**Fig. 2b**). Of the genes in this region (including *PITX1*, *CATSPER3*, *PCBD2*, *MIR4461* and *H2AFY*), *PITX1* is the closest to rs647161 (approximately 129 kb upstream). The *PITX1* gene (encoding paired-like homeodomain 1) has been described as a tumor suppressor gene and may be involved in the tumorigenesis of multiple human cancers[27–31], including CRC[27,32]. *PITX1* has been reported to suppress tumorigenicity by downregulating the RAS pathway, which is frequently altered in colorectal tumors[27]. Inhibition of *PITX1* induces the RAS pathway and tumorigenicity, and restoring *PITX1* in colon cancer cells inhibits tumorigenicity[27]. It also has been reported that *PITX1* may activate *TP53* (ref. 33) and regulate telomerase activity[34]. Consistent with its possible function as a tumor suppressor gene, *PITX1* has been found to be downregulated in human cancer tissue samples and cell lines[27–30,32]. CRC tissue expressing wild-type KRAS showed significantly lower expression of *PITX1* than tissue with mutant KRAS[32]. Most recently, low *PITX1* expression was found to be associated with poor survival in individuals with CRC[35]. In addition, rs6596201, which is in moderate LD with rs647161 ($r^2 = 0.25$), is an expression quantitative trait locus (eQTL) ($P = 2.42 \times 10^{-28}$) for the *PITX1* gene[36]. Several other genes at this locus, including *C5orf24*, *H2AFY* and *NEUROG1*, were also found to be highly expressed in colorectal tumors included in TCGA ($P < 0.001$)[25]. Additional studies are warranted to explore a possible role for these genes in the etiology of CRC.

Table 2 Association of CRC risk with the top three risk variants in European descendants and east Asian and European descendants combined

		MAF[b]		European-ancestry populations[c]				East Asian and European-ancestry populations combined[c]			
SNP	Alleles[a]	Cases	Controls	Cases	Controls	OR (95% CI)	P_{meta}	Cases	Controls	OR (95% CI)	P_{meta}
rs10774214	T/C	0.385	0.379	11,870	14,190	1.04 (1.00–1.09)	0.040	19,165	25,736	1.09 (1.06–1.13)	3.06×10^{-8}
rs647161	A/C	0.680	0.667	11,870	14,190	1.07 (1.02–1.11)	0.002	19,185	25,754	1.11 (1.08–1.15)	1.22×10^{-10}
rs2423279	C/T	0.263	0.252	11,870	14,190	1.07 (1.03–1.12)	0.001	19,195	25,750	1.10 (1.06–1.14)	6.64×10^{-9}

[a]Alelles (minor/major) for east Asians. [b]MAF in European-ancestry populations. [c]Summary statistics were generated using inverse variance–weighted fixed-effects meta-analysis.

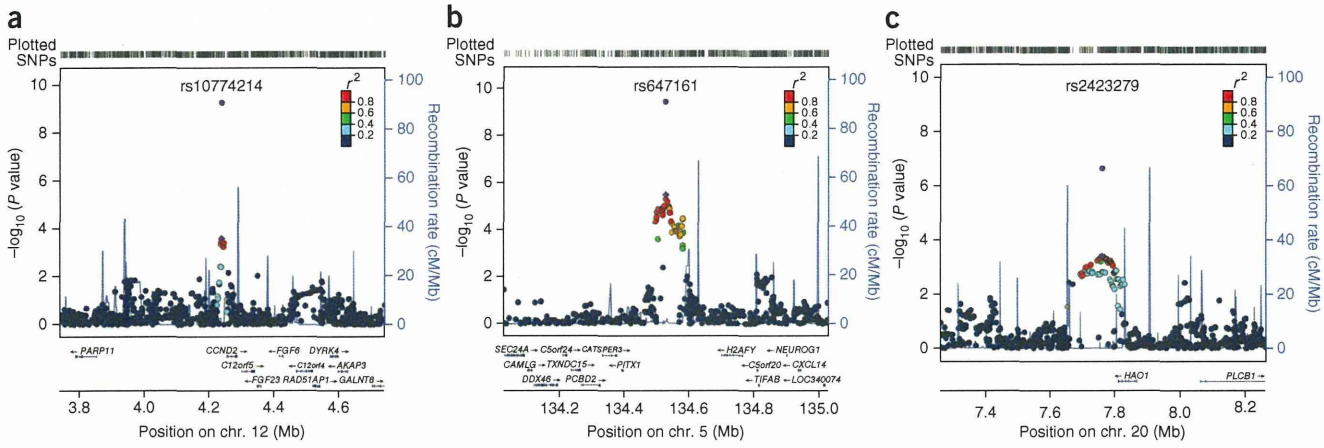

Figure 2 Regional plots of association results and recombination rates for the three SNPs showing evidence of association with CRC risk. Genotyped and imputed data from GWAS samples are plotted on the basis of their chromosomal position in NCBI Human Genome Build 36.3. For each region, the SNP selected for stage 2 replication is denoted with a diamond, and the *P* value from the combined analysis of stage 1 and 2 data is provided. (**a**–**c**) Data are shown for rs10774214 (**a**), rs647161 (**b**) and rs2423279 (**c**).

The rs2423279 SNP is located on chromosome 20p12.3, close to the *HAO1* and *PLCB1* genes (**Fig. 2c**). *HAO1* encodes hydroxyacid oxidase, which oxidizes 2-hydroxyacid. *PLCB1* encodes phospholipase C-β1, which has an important role in the intracellular transduction of many extracellular signals. Overexpression of the *PLCB1* gene has been observed in CRC tissue[25]. Possible mechanisms by which these genes are involved in CRC carcinogenesis are unknown. The rs2423279 SNP is 1,408,069 bp downstream of rs961253, a SNP previously identified in a European GWAS as being associated with CRC risk[10]. However, these two SNPs are not correlated in east Asians ($r^2 = 0$) or in Europeans ($r^2 = 0$). Adjustment for rs961253 did not change the results for rs2423279 (data not shown).

To our knowledge, this is the largest GWAS performed for CRC in east Asians, a population that differs from populations of European ancestry in CRC risk and certain aspects of genetic architecture. Results from our study, along with data from a large study conducted in a population of European ancestry, provide convincing evidence of associations with CRC risk for three new independent susceptibility loci at 5q31.1, 12p13.32 and 20p12.3. Results from this study provide new insights into the genetics and biology of CRC.

URLs. Cancer Genetic Markers of Susceptibility (CGEMS), http://cgems.cancer.gov/; Database of Genotypes and Phenotypes (dbGaP), http://www.ncbi.nlm.nih.gov/gap; EIGENSTRAT, http://genepath.med.harvard.edu/~reich/EIGENSTRAT.htm; eqtl.uchicago.edu, http://eqtl.uchicago.edu/Home.html; GTEx eQTL Browser, http://www.ncbi.nlm.nih.gov/gtex/GTEX2/gtex.cgi; Haploview, http://www.broad.mit.edu/mpg/haploview/; HapMap Project, http://hapmap.ncbi.nlm.nih.gov/; IntOGen, http://www.intogen.org/home; LocusZoom, http://csg.sph.umich.edu/locuszoom/; MaCH 1.0, http://www.sph.umich.edu/csg/abecasis/MACH/; mach2dat, http://genome.sph.umich.edu/wiki/Mach2dat:_Association_with_MACH_output; METAL, http://www.sph.umich.edu/csg/abecasis/Metal/; PLINK version 1.07, http://pngu.mgh.harvard.edu/~purcell/plink/; R version 2.13.0, http://www.r-project.org/; SAS version 9.2, http://www.sas.com/; SNAP, http://www.broadinstitute.org/mpg/snap/; TRANSFAC, http://www.gene-regulation.com/pub/databases.html; UCSC Genome Browser, http://genome.ucsc.edu/; WHI investigators, https://cleo.whi.org/researchers/SitePages/Write%20a%20Paper.aspx.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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W.Z. conceived and directed ACCC as well as the Shanghai-Vanderbilt Colorectal Cancer Genetics Project. W.-H.J., Y.-X.Z., K.M., A.S., Y.-B.X., S.H.J., D.-H.K., U.P.

and G.C. directed CRC projects at Guangzhou, Aichi, Korea-NCC, Shanghai, KCPS-II, Korea-Seoul, GECCO and CCFR, respectively. B.Z., Q.C. and W.W. coordinated the project. Q.C. directed laboratory operations. J.S. performed genotyping experiments. B.Z., J.L. and W.W. performed statistical analyses. W.Z. wrote the manuscript with substantial contributions from B.Z., Q.C., J.L., X.-O.S. and R.J.D. Z.R., G.Y., B.-T.J., Z.-Z.P., F.M., Y.-T.G., J.H.O., Y.-O.A., E.J.P., H.-L.L., J.W.P., J.J., J.-Y.J. and S.H. contributed to data and biological sample collection in the original studies included in ACCC and contributed to manuscript revision. Members of GECCO and CCFR contributed to data and biological sample collection in studies included in these consortia.

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1. Jemal, A. *et al.* Global cancer statistics. *CA Cancer J. Clin.* **61**, 69–90 (2011).
2. de la Chapelle, A. Genetic predisposition to colorectal cancer. *Nat. Rev. Cancer* **4**, 769–780 (2004).
3. Dong, L.M. *et al.* Genetic susceptibility to cancer: the role of polymorphisms in candidate genes. *J. Am. Med. Assoc.* **299**, 2423–2436 (2008).
4. Zanke, B.W. *et al.* Genome-wide association scan identifies a colorectal cancer susceptibility locus on chromosome 8q24. *Nat. Genet.* **39**, 989–994 (2007).
5. Tomlinson, I. *et al.* A genome-wide association scan of tag SNPs identifies a susceptibility variant for colorectal cancer at 8q24.21. *Nat. Genet.* **39**, 984–988 (2007).
6. Broderick, P. *et al.* A genome-wide association study shows that common alleles of *SMAD7* influence colorectal cancer risk. *Nat. Genet.* **39**, 1315–1317 (2007).
7. Jaeger, E. *et al.* Common genetic variants at the *CRAC1* (*HMPS*) locus on chromosome 15q13.3 influence colorectal cancer risk. *Nat. Genet.* **40**, 26–28 (2008).
8. Tenesa, A. *et al.* Genome-wide association scan identifies a colorectal cancer susceptibility locus on 11q23 and replicates risk loci at 8q24 and 18q21. *Nat. Genet.* **40**, 631–637 (2008).
9. Tomlinson, I.P. *et al.* A genome-wide association study identifies colorectal cancer susceptibility loci on chromosomes 10p14 and 8q23.3. *Nat. Genet.* **40**, 623–630 (2008).
10. Houlston, R.S. *et al.* Meta-analysis of genome-wide association data identifies four new susceptibility loci for colorectal cancer. *Nat. Genet.* **40**, 1426–1435 (2008).
11. Houlston, R.S. *et al.* Meta-analysis of three genome-wide association studies identifies susceptibility loci for colorectal cancer at 1q41, 3q26.2, 12q13.13 and 20q13.33. *Nat. Genet.* **42**, 973–977 (2010).
12. Cui, R. *et al.* Common variant in 6q26-q27 is associated with distal colon cancer in an Asian population. *Gut* **60**, 799–805 (2011).
13. He, J. *et al.* Generalizability and epidemiologic characterization of eleven colorectal cancer GWAS hits in multiple populations. *Cancer Epidemiol. Biomarkers Prev.* **20**, 70–81 (2011).
14. Zheng, W. *et al.* Genome-wide association study identifies a new breast cancer susceptibility locus at 6q25.1. *Nat. Genet.* **41**, 324–328 (2009).
15. Bei, J.X. *et al.* A genome-wide association study of nasopharyngeal carcinoma identifies three new susceptibility loci. *Nat. Genet.* **42**, 599–603 (2010).
16. Jee, S.H. *et al.* Adiponectin concentrations: a genome-wide association study. *Am. J. Hum. Genet.* **87**, 545–552 (2010).
17. Nakata, I. *et al.* Association between the *SERPING1* gene and age-related macular degeneration and polypoidal choroidal vasculopathy in Japanese. *PLoS ONE* **6**, e19108 (2011).
18. Li, Y., Willer, C.J., Ding, J., Scheet, P. & Abecasis, G.R. MaCH: using sequence and genotype data to estimate haplotypes and unobserved genotypes. *Genet. Epidemiol.* **34**, 816–834 (2010).
19. Willer, C.J., Li, Y. & Abecasis, G.R. METAL: fast and efficient meta-analysis of genomewide association scans. *Bioinformatics* **26**, 2190–2191 (2010).
20. Peters, U. *et al.* Meta-analysis of new genome-wide association studies of colorectal cancer risk. *Hum. Genet.* **131**, 217–234 (2012).
21. Figueiredo, J.C. *et al.* Genotype-environment interactions in microsatellite stable/microsatellite instability–low colorectal cancer: results from a genome-wide association study. *Cancer Epidemiol. Biomarkers Prev.* **20**, 758–766 (2011).
22. Musgrove, E.A., Caldon, C.E., Barraclough, J., Stone, A. & Sutherland, R.L. Cyclin D as a therapeutic target in cancer. *Nat. Rev. Cancer* **11**, 558–572 (2011).
23. Mermelshtein, A. *et al.* Expression of D-type cyclins in colon cancer and in cell lines from colon carcinomas. *Br. J. Cancer* **93**, 338–345 (2005).
24. Sarkar, R. *et al.* Expression of cyclin D2 is an independent predictor of the development of hepatic metastasis in colorectal cancer. *Colorectal Dis.* **12**, 316–323 (2010).
25. Gundem, G. *et al.* IntOGen: integration and data mining of multidimensional oncogenomic data. *Nat. Methods* **7**, 92–93 (2010).
26. Matys, V. *et al.* TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. *Nucleic Acids Res.* **34**, D108–D110 (2006).
27. Kolfschoten, I.G. *et al.* A genetic screen identifies PITX1 as a suppressor of RAS activity and tumorigenicity. *Cell* **121**, 849–858 (2005).
28. Chen, Y. *et al.* Decreased *PITX1* homeobox gene expression in human lung cancer. *Lung Cancer* **55**, 287–294 (2007).
29. Chen, Y.N., Chen, H., Xu, Y., Zhang, X. & Luo, Y. Expression of pituitary homeobox 1 gene in human gastric carcinogenesis and its clinicopathological significance. *World J. Gastroenterol.* **14**, 292–297 (2008).
30. Lord, R.V. *et al.* Increased *CDX2* and decreased *PITX1* homeobox gene expression in Barrett's esophagus and Barrett's-associated adenocarcinoma. *Surgery* **138**, 924–931 (2005).
31. Nagel, S. *et al.* Activation of paired-homeobox gene *PITX1* by del(5)(q31) in T-cell acute lymphoblastic leukemia. *Leuk. Lymphoma* **52**, 1348–1359 (2011).
32. Watanabe, T. *et al.* Differential gene expression signatures between colorectal cancers with and without *KRAS* mutations: crosstalk between the KRAS pathway and other signalling pathways. *Eur. J. Cancer* **47**, 1946–1954 (2011).
33. Liu, D.X. & Lobie, P.E. Transcriptional activation of p53 by Pitx1. *Cell Death Differ.* **14**, 1893–1907 (2007).
34. Qi, D.L. *et al.* Identification of *PITX1* as a *TERT* suppressor gene located on human chromosome 5. *Mol. Cell Biol.* **31**, 1624–1636 (2011).
35. Knösel, T. *et al.* Loss of desmocollin 1-3 and homeobox genes *PITX1* and *CDX2* are associated with tumor progression and survival in colorectal carcinoma. *Int. J. Colorectal Dis.* **27**, 1391–1399 (2012).
36. Zeller, T. *et al.* Genetics and beyond--the transcriptome of human monocytes and disease susceptibility. *PLoS ONE* **5**, e10693 (2010).

ONLINE METHODS

Study populations. After quality control filtering, 7,456 cases and 11,671 controls from 10 studies were included in the consortium (**Supplementary Table 2**). Detailed descriptions of participating studies and demographic characteristics of study participants are provided in the **Supplementary Note**. Briefly, the consortium included 10,730 Chinese participants, 5,544 Korean participants and 2,853 Japanese participants. Chinese participants were from five studies: the Shanghai Study 1 (Shanghai-1, $n = 3,102$), the Shanghai Study 2 (Shanghai-2, $n = 485$), the Guangzhou Study 1 (Guangzhou-1, $n = 1,613$), the Guangzhou Study 2 (Guangzhou-2, $n = 2,892$) and the Guangzhou Study 3 (Guangzhou-3, $n = 2,638$). Korean participants were from three studies: the Korean Cancer Prevention Study-II (KCPS-II, $n = 1,301$), the Seoul Study ($n = 1,522$) and the Korea–National Cancer Center (Korea-NCC) Study ($n = 2,721$). Japanese participants were from two studies: the Aichi Study 1 (Aichi-1, $n = 1,346$) and the Aichi Study 2 (Aichi-2, $n = 1,507$). We also evaluated associations for the top 4 SNPs using data from 11,870 CRC cases and 14,190 controls of European ancestry included in GECCO and CCFR, which included 14 studies from the United States, Europe, Canada and Australia[4,20,21]. Approval was granted from the relevant institutional review boards at all study sites, and all included participants gave informed consent.

Genotyping and quality control procedures. Detailed descriptions of genotyping and quality control procedures as well as design of plates and control samples are given in the **Supplementary Note**. Briefly, in stage 1, 481 cases and 2,632 controls from Shanghai-1 were genotyped using the Affymetrix Genome-Wide Human SNP Array 6.0 as described previously[14]. The average concordance percentage of quality control samples was 99.7%, with a median value of 100% in Shanghai-1 (refs. 14,37,38). Stage 1 genotyping for 296 cases and 257 controls in Shanghai-2 was performed using Illumina HumanOmniExpress BeadChips. The same method was used to genotype cases from the Guangzhou-1 ($n = 694$) and Aichi-1 ($n = 497$) studies in stage 1. The positive quality control samples in these studies had an average concordance percentage of 99.41% and a median value of 99.97%. Cases and controls in KCPS-II were genotyped using the Affymetrix Genome-Wide Human SNP Array 5.0 (ref. 16). Controls for the Guangzhou-1 and Aichi-1 studies were genotyped previously using the Illumina Human610-Quad BeadChip[15] and Illumina Infinium HumanHap610 BeadChip[17] platforms, respectively. Details of quality control procedures for these samples have been described previously[15–17]. We excluded from the analysis samples that were genetically identical or duplicated, had a genotype-determined sex that was inconsistent with self-reported data, had unclear population structure, had close relatives with a PI-HAT estimate greater than 0.25 or had a call rate of <95%. Within each study, SNPs were excluded if (i) MAF was <5%, (ii) the call rate was <95%; (iii) the genotyping concordance percentage was <95% in quality control samples; (iv) the P value for Hardy-Weinberg equilibrium was $<1.0 \times 10^{-5}$ in controls; or (v) SNPs were not on the 22 autosomes. The final numbers of cases, controls and SNPs remaining for analysis in each participating study are presented in **Supplementary Table 1**.

Genotyping for stage 2 was completed using the iPLEX Sequenom MassARRAY platform as described previously[14,39]. With the exception of samples from the Guangzhou-3 study, which were genotyped at Fudan University (Shanghai), all other samples were genotyped at the Vanderbilt Molecular Epidemiology Laboratory. The average concordance percentage of the genotyping data for positive control samples was >99% with a median value of 100% for each of the five studies. SNPs were excluded from the analysis if (i) the call rate was <95%, (ii) the genotyping concordance percentage was <95% in control samples, (iii) there was an unclear genotype call or (iv) the P value for Hardy-Weinberg equilibrium was $<7.8 \times 10^{-4}$. The numbers of SNPs remaining for analysis in each participating study in stage 2 are presented in the **Supplementary Note**.

Genotyping for samples included in the GECCO and CCFR GWAS was conducted using Illumina BeadChip arrays, with the exception of the Ontario Familial Colorectal Cancer Registry study, for which Affymetrix arrays were used[4,20,21]. Details of the quality control procedures for these samples are presented in the **Supplementary Note**.

SNP selection for replication. SNPs were selected for stage 2 replication if (i) data were available in each of the five stage 1 studies; (ii) MAF was >5% in each stage 1 study; (iii) no heterogeneity was detected across the five studies included in stage 1 ($P_{het} > 0.05$ and $I^2 < 25\%$); (iv) there was no LD ($r^2 < 0.2$) with any known risk variant reported from previous GWAS; (v) there was no LD ($r^2 < 0.2$) with the other SNPs identified in this study; (vi) there was high imputation quality in each of the five studies (RSQ > 0.5); and (vii) $P < 0.01$ in combined analysis of all stage 1 studies.

Evaluation of population structure. We evaluated population structure in each of the five participating studies included in stage 1 by using principal-components analysis (PCA). Genotyping data for uncorrelated genome-wide SNPs were pooled with data from HapMap to generate the first ten principal components using EIGENSTRAT software[40] (see URLs). The first two principal components for each sample were plotted using R (see URLs). We identified and excluded one participant of KCPS-II who was more than 6 s.d. away from the means of principal components 1 and 2 (**Supplementary Fig. 1**). The remaining 7,847 samples showed clear east Asian origin, and these samples were included in the final genome-wide association analysis. Cases and controls in each of the five studies were in the same cluster as HapMap Asian samples. The estimated inflation factor λ ranged from 1.02 to 1.04 in these studies after adjusting for age, sex and the first ten principal components, with a λ of 1.01 for combined stage 1 data (**Supplementary Fig. 2** and **Supplementary Table 1**).

Imputation. We used the MaCH 1.0 program[18] (see URLs) to impute genotypes for autosomal SNPs that were present in HapMap Phase 2 release 22 separately for each of the five studies included in stage 1. Genotype data from the 90 Asian subjects from HapMap were used as the reference. For Guangzhou-1 and Aichi-1, cases and controls were genotyped using different platforms. To improve imputation quality[41], we identified SNPs for which data were available in both cases and controls (250,612 SNPs in Guangzhou-1 and 232,426 SNPs in Aichi-1) and used them to impute genotyping data. A total of 1,636,380 genotyped SNPs or imputed SNPs with high imputation quality (RSQ > 0.50) in all five studies were tested for association with CRC. To directly evaluate the imputation quality for the top four SNPs identified in our study, we genotyped them in approximately 2,500 samples included in stage 1. The agreement of genotype calls derived from direct genotyping and imputation was very high, with mean concordance rates of 98.05%, 95.61%, 99.84% and 97.90% for rs647161, rs10774214, rs2423279 and rs1665650, respectively (**Supplementary Table 7**).

Statistical analyses. Dosage data for genotyped and imputed SNPs for participants in each stage 1 study were analyzed using the program mach2dat[18] (see URLs). We coded 0, 1 or 2 copies of the effect allele as the dosage for genotyped SNPs, and, for imputed SNPs, we used the expected number of copies of the effect allele as the dosage score. This approach has been shown to give unbiased estimates in meta-analyses[42]. Associations between SNPs and CRC risk were assessed using ORs and 95% CIs derived from logistic regression models. ORs were estimated on the basis of the log-additive model and adjusted for age, sex and the first ten principal components. PLINK version 1.07 (see URLs) also was used to analyze genotype data[43] and yielded results virtually identical to those derived from dosage data using mach2dat[18]. Meta-analyses were performed using the inverse-variance method, assuming a fixed-effects model, and calculations were implemented in the METAL package[19] (see URLs).

Similar to stage 1, we used logistic regression models to derive ORs and 95% CIs for the 64 selected SNPs in stage 2, assuming a log-additive model with adjustment for age and sex. We performed joint analyses to generate summary results for combined samples from all studies, with additional adjustment for study site. We also conducted stratification analysis for the top four SNPs by population ancestry (Chinese, Korean or Japanese) and by sex. We used Cochran's Q statistic to test for heterogeneity[44] and the I^2 statistic to quantify heterogeneity[45] across studies as described elsewhere in detail[46]. Analyses for stage 2, as well as combined stage 1 and 2 data, were conducted using SAS, version 9.2 (see URLs), with the use of two-tailed tests. P values of $<5\times10^{-8}$ in the combined analysis was considered statistically significant.

We used Haploview version 4.2 (see URLs; ref. 47) to generate a genome-wide Manhattan plot for results from the stage 1 meta-analysis. Forest plots

and quantile-quantile plots were drawn using R. We drew regional association plots using the website-based tool LocusZoom, version 1.1 (see URLs; ref. 48). LD plots were generated using Haploview[47] and the UCSC Genome Browser (see URLs).

37. Long, J. *et al.* Genome-wide association study in east Asians identifies novel susceptibility loci for breast cancer. *PLoS Genet.* **8**, e1002532 (2012).
38. Shu, X.O. *et al.* Identification of new genetic risk variants for type 2 diabetes. *PLoS Genet.* **6**, pii: e1001127 (2010).
39. Zheng, W. *et al.* Genetic and clinical predictors for breast cancer risk assessment and stratification among Chinese women. *J. Natl. Cancer Inst.* **102**, 972–981 (2010).
40. Price, A.L. *et al.* Principal components analysis corrects for stratification in genome-wide association studies. *Nat. Genet.* **38**, 904–909 (2006).
41. Sinnott, J.A. & Kraft, P. Artifact due to differential error when cases and controls are imputed from different platforms. *Hum. Genet.* **131**, 111–119 (2012).
42. Jiao, S., Hsu, L., Hutter, C.M. & Peters, U. The use of imputed values in the meta-analysis of genome-wide association studies. *Genet. Epidemiol.* **35**, 597–605 (2011).
43. Purcell, S. *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81**, 559–575 (2007).
44. Lau, J., Ioannidis, J.P. & Schmid, C.H. Quantitative synthesis in systematic reviews. *Ann. Intern. Med.* **127**, 820–826 (1997).
45. Higgins, J.P. & Thompson, S.G. Quantifying heterogeneity in a meta-analysis. *Stat. Med.* **21**, 1539–1558 (2002).
46. Zhang, B., Beeghly-Fadiel, A., Long, J. & Zheng, W. Genetic variants associated with breast-cancer risk: comprehensive research synopsis, meta-analysis, and epidemiological evidence. *Lancet Oncol.* **12**, 477–488 (2011).
47. Barrett, J.C., Fry, B., Maller, J. & Daly, M.J. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* **21**, 263–265 (2005).
48. Pruim, R.J. *et al.* LocusZoom: regional visualization of genome-wide association scan results. *Bioinformatics* **26**, 2336–2337 (2010).

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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

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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

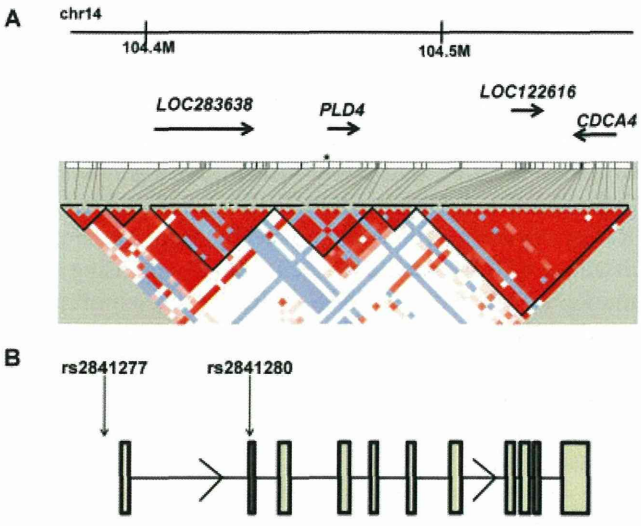

Figure 1. Linkage disequilibrium (LD) block around the *PLD4* region and the *PLD4* structure. **A,** LD block and genes around *PLD4*. The LD block is based on HapMap phase 3 data. **Asterisk** indicates rs2841277. **B,** Schematic view of *PLD4* structure. Rectangles represent exons of *PLD4*.

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.

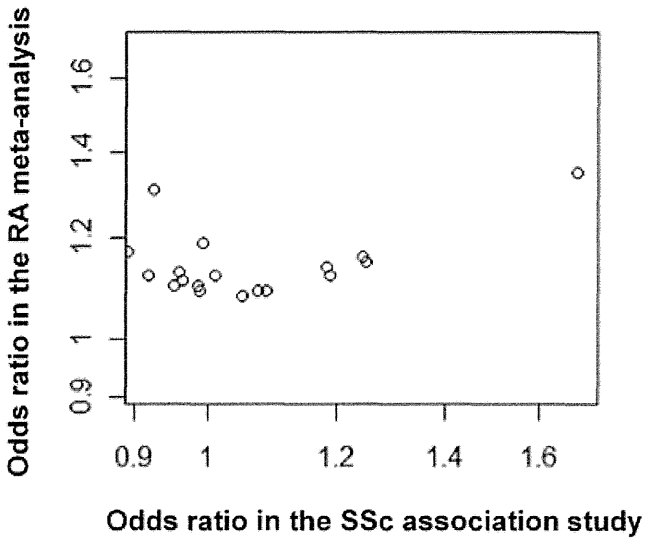

Figure 2. Comparison of associations for systemic sclerosis (SSc) and rheumatoid arthritis (RA). The odds ratios obtained for 18 genes in association studies of SSc and RA are plotted.

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.

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AUTHOR CONTRIBUTIONS

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

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

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

Analysis and interpretation of data. Terao, Ohmura.

REFERENCES

1. Chifflot H, Fautrel B, Sordet C, Chatelus E, Sibilia J. Incidence and prevalence of systemic sclerosis: a systematic literature review. Semin Arthritis Rheum 2008;37:223–35.
2. Kawut SM, Taichman DB, Archer-Chicko CL, Palevsky HI, Kimmel SE. Hemodynamics and survival in patients with pulmonary arterial hypertension related to systemic sclerosis. Chest 2003;123:344–50.
3. Ioannidis JP, Vlachoyiannopoulos PG, Haidich AB, Medsger TA Jr, Lucas M, Michet CJ, et al. Mortality in systemic sclerosis: an international meta-analysis of individual patient data. Am J Med 2005;118:2–10.
4. Bhattacharyya S, Wei J, Varga J. Understanding fibrosis in systemic sclerosis: shifting paradigms, emerging opportunities. Nat Rev Rheumatol 2012;8:42–54.
5. Romano E, Manetti M, Guiducci S, Ceccarelli C, Allanore Y, Matucci-Cerinic M. The genetics of systemic sclerosis: an update. Clin Exp Rheumatol 2011;29:S75–86.
6. Arnett FC, Cho M, Chatterjee S, Aguilar MB, Reveille JD, Mayes MD. Familial occurrence frequencies and relative risks for systemic sclerosis (scleroderma) in three United States cohorts. Arthritis Rheum 2001;44:1359–62.
7. Terao C, Ohmura K, Katayama M, Takahashi M, Kokubo M, Diop G, et al. Myelin basic protein as a novel genetic risk factor in rheumatoid arthritis: a genome-wide study combined with immunological analyses. PLoS One 2011;6:e20457.
8. Radstake TR, Gorlova O, Rueda B, Martin JE, Alizadeh BZ, Palomino-Morales R, et al. Genome-wide association study of systemic sclerosis identifies CD247 as a new susceptibility locus. Nat Genet 2010;42:426–9.
9. Allanore Y, Saad M, Dieude P, Avouac J, Distler JH, Amouyel P, et al. Genome-wide scan identifies TNIP1, PSORS1C1, and RHOB as novel risk loci for systemic sclerosis. PLoS Genet 2011;7:e1002091.
10. Gorlova O, Martin JE, Rueda B, Koeleman BP, Ying J, Teruel M, et al. Identification of novel genetic markers associated with clinical phenotypes of systemic sclerosis through a genome-wide association strategy. PLoS Genet 2011;7:e1002178.
11. Rueda B, Broen J, Simeon C, Hesselstrand R, Diaz B, Suarez H, et al. The STAT4 gene influences the genetic predisposition to systemic sclerosis phenotype. Hum Mol Genet 2009;18:2071–7.
12. Dieude P, Guedj M, Wipff J, Avouac J, Fajardy I, Diot E, et al. Association between the IRF5 rs2004640 functional polymorphism and systemic sclerosis: a new perspective for pulmonary fibrosis. Arthritis Rheum 2009;60:225–33.
13. Gourh P, Agarwal SK, Divecha D, Assassi S, Paz G, Arora-Singh RK, et al. Polymorphisms in TBX21 and STAT4 increase the risk of systemic sclerosis: evidence of possible gene–gene interaction and alterations in Th1/Th2 cytokines. Arthritis Rheum 2009;60:3794–806.
14. Dieude P, Guedj M, Wipff J, Ruiz B, Riemekasten G, Airo P, et al. NLRP1 influences the systemic sclerosis phenotype: a new clue for the contribution of innate immunity in systemic sclerosis-related fibrosing alveolitis pathogenesis. Ann Rheum Dis 2011;70:668–74.
15. Bossini-Castillo L, Broen JC, Simeon CP, Beretta L, Vonk MC, Ortego-Centeno N, et al. A replication study confirms the association of TNFSF4 (OX40L) polymorphisms with systemic sclerosis in a large European cohort. Ann Rheum Dis 2011;70:638–41.
16. Dieude P, Guedj M, Truchetet ME, Wipff J, Revillod L, Riemekasten G, et al. Association of the CD226 Ser^{307} variant with systemic sclerosis: evidence of a contribution of costimulation pathways in systemic sclerosis pathogenesis. Arthritis Rheum 2011;63:1097–105.
17. Gourh P, Agarwal SK, Martin E, Divecha D, Rueda B, Bunting H, et al. Association of the C8orf13-BLK region with systemic sclerosis in North-American and European populations. J Autoimmun 2010;34:155–62.
18. Dieude P, Guedj M, Wipff J, Ruiz B, Riemekasten G, Matucci-Cerinic M, et al. Association of the TNFAIP3 rs5029939 variant

with systemic sclerosis in the European Caucasian population. Ann Rheum Dis 2010;69:1958–64.
19. Tsuchiya N, Kawasaki A, Hasegawa M, Fujimoto M, Takehara K, Kawaguchi Y, et al. Association of STAT4 polymorphism with systemic sclerosis in a Japanese population. Ann Rheum Dis 2009;68:1375–6.
20. Ito I, Kawaguchi Y, Kawasaki A, Hasegawa M, Ohashi J, Hikami K, et al. Association of a functional polymorphism in the IRF5 region with systemic sclerosis in a Japanese population. Arthritis Rheum 2009;60:1845–50.
21. Ito I, Kawaguchi Y, Kawasaki A, Hasegawa M, Ohashi J, Kawamoto M, et al. Association of the FAM167A–BLK region with systemic sclerosis. Arthritis Rheum 2010;62:890–5.
22. Hasebe N, Kawasaki A, Ito I, Kawamoto M, Hasegawa M, Fujimoto M, et al. Association of UBE2L3 polymorphisms with diffuse cutaneous systemic sclerosis in a Japanese population. Ann Rheum Dis 2012;71:1259–60.
23. Zhou X, Lee JE, Arnett FC, Xiong M, Park MY, Yoo YK, et al. HLA–DPB1 and DPB2 are genetic loci for systemic sclerosis: a genome-wide association study in Koreans with replication in North Americans. Arthritis Rheum 2009;60:3807–14.
24. Suzuki A, Kochi Y, Okada Y, Yamamoto K. Insight from genome-wide association studies in rheumatoid arthritis and multiple sclerosis. FEBS Lett 2011;585:3627–32.
25. Plenge RM, Seielstad M, Padyukov L, Lee AT, Remmers EF, Ding B, et al. TRAF1-C5 as a risk locus for rheumatoid arthritis: a genomewide study. N Engl J Med 2007;357:1199–209.
26. Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G, Behrens TW, et al. STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. N Engl J Med 2007;357:977–86.
27. Plenge RM, Cotsapas C, Davies L, Price AL, de Bakker PI, Maller J, et al. Two independent alleles at 6q23 associated with risk of rheumatoid arthritis. Nat Genet 2007;39:1477–82.
28. Thomson W, Barton A, Ke X, Eyre S, Hinks A, Bowes J, et al. Rheumatoid arthritis association at 6q23. Nat Genet 2007;39: 1431–3.
29. Begovich AB, Carlton VE, Honigberg LA, Schrodi SJ, Chokkalingam AP, Alexander HC, et al. A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. Am J Hum Genet 2004;75:330–7.
30. Diaz-Gallo LM, Gourh P, Broen J, Simeon C, Fonollosa V, Ortego-Centeno N, et al. Analysis of the influence of PTPN22 gene polymorphisms in systemic sclerosis. Ann Rheum Dis 2011; 70:454–62.
31. Okada Y, Terao C, Ikari K, Kochi Y, Ohmura K, Suzuki A, et al. Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population. Nat Genet 2012;44:511–6.
32. Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 1980;23:581–90.
33. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA Jr, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988;15:202–5.
34. 1000 Genomes Project Consortium. A map of human genome variation from population-scale sequencing. Nature 2010;467: 1061–73.
35. Mimura Y, Ihn H, Jinnin M, Asano Y, Yamane K, Yazawa N, et al. Rheumatoid factor isotypes and anti-agalactosyl IgG antibodies in systemic sclerosis. Br J Dermatol 2004;151:803–8.
36. Santiago M, Baron M, Miyachi K, Fritzler MJ, Abu-Hakima M, Leclercq S, et al. A comparison of the frequency of antibodies to cyclic citrullinated peptides using a third generation anti-CCP assay (CCP3) in systemic sclerosis, primary biliary cirrhosis and rheumatoid arthritis. Clin Rheumatol 2008;27:77–83.
37. Yoshikawa F, Banno Y, Otani Y, Yamaguchi Y, Nagakura-Takagi Y, Morita N, et al. Phospholipase D family member 4, a transmembrane glycoprotein with no phospholipase D activity, expression in spleen and early postnatal microglia. PLoS One 2010;5: e13932.
38. Otani Y, Yamaguchi Y, Sato Y, Furuichi T, Ikenaka K, Kitani H, et al. PLD4 is involved in phagocytosis of microglia: expression and localization changes of PLD4 are correlated with activation state of microglia. PLoS One 2011;6:e27544.
39. Stranger BE, Forrest MS, Dunning M, Ingle CE, Beazley C, Thorne N, et al. Relative impact of nucleotide and copy number variation on gene expression phenotypes. Science 2007;315: 848–53.
40. Cunninghame Graham DS, Morris DL, Bhangale TR, Criswell LA, Syvanen AC, Ronnblom L, et al. Association of NCF2, IKZF1, IRF8, IFIH1, and TYK2 with systemic lupus erythematosus. PLoS Genet 2011;7:e1002341.
41. Higgs BW, Liu Z, White B, Zhu W, White WI, Morehouse C, et al. Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. Ann Rheum Dis 2011;70:2029–36.
42. Whitfield ML, Finlay DR, Murray JI, Troyanskaya OG, Chi JT, Pergamenschikov A, et al. Systemic and cell type-specific gene expression patterns in scleroderma skin. Proc Natl Acad Sci U S A 2003;100:12319–24.
43. Lafyatis R, Kissin E, York M, Farina G, Viger K, Fritzler MJ, et al. B cell depletion with rituximab in patients with diffuse cutaneous systemic sclerosis. Arthritis Rheum 2009;60:578–83.
44. Smith V, Van Praet JT, Vandooren B, Van der Cruyssen B, Naeyaert JM, Decuman S, et al. Rituximab in diffuse cutaneous systemic sclerosis: an open-label clinical and histopathological study. Ann Rheum Dis 2010;69:193–7.
45. Hambleton S, Salem S, Bustamante J, Bigley V, Boisson-Dupuis S, Azevedo J, et al. IRF8 mutations and human dendritic-cell immunodeficiency. N Engl J Med 2011;365:127–38.

Association of Genetic Variants on 8p21 and 4q12 with Age-Related Macular Degeneration in Asian Populations

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Purpose. To evaluate the association of genetic variants at chromosomes 8p21 and 4q12 with the risk of developing AMD and its two main subtypes, choroidal neovascular membrane (CNV) and polypoidal choroidal vasculopathy (PCV), in Asian populations.

Methods. The study population comprised 2360 patients with neovascular AMD (1013 typical AMD-CNV and 1282 PCV), and 3598 controls from four independent cohorts, two of Japanese (n = 4859) and two of Chinese (n = 1099) ethnicity. We performed a meta-analysis in case-control studies of two reported single nucleotide polymorphisms (SNPs) (rs13278062 at *TNFRSF10A-LOC389641* on 8p21 and rs1713985 at *REST-C4orf14-POLR2B-IGFBP7* on 4q12) by using logistic regression analysis adjusted for age and sex. Subgroup analysis by CNV and PCV subtypes were performed to evaluate the significance of these two variants.

Results. The reported association between rs13278062 at 8p21 and neovascular AMD was replicated in this population ($P = 1.12 \times 10^{-4}$, odds ratio [OR] = 0.79, 95% confidence interval [CI] = 0.70–0.89). However, there was no association of rs1713985 at 4q12 with neovascular AMD, or its two subtypes, typical AMD-CNV and PCV (all $P > 0.05$). The study sample size had a statistical power of greater than 99% to detect an association of a risk allele with AMD with an OR of 1.30, as reported in the original study of rs1713985 and AMD.

Conclusions. The present results did not replicate the reported association between rs1713985 at 4q12 and neovascular AMD. However, we confirmed the association between rs13278062 at 8p21 and neovascular AMD in Asian populations. (*Invest Ophthalmol Vis Sci.* 2012;53:6576-6581) DOI:10.1167/iovs.12-10219

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AMD is the leading cause of visual impairment in the elderly and the most common cause of blindness in developed countries.[1] Several genes have been reported to be associated with this disease,[2-10] and these genetic studies have helped to reveal the mechanisms underlying the development of AMD, specifically suggesting an inflammation-based model of AMD pathogenesis.[11-13]

There is increasing recognition that the prevalence of AMD in Asian populations is as high as in Caucasian populations.[14] However, it has been suggested that the frequency of AMD subtypes may differ between the Asians and Caucasians.[15,16] In Asian populations, neovascular AMD ("wet" AMD) is the major type of late AMD, and is associated with choroidal neovascular membrane (CNV), collectively referred to as AMD-CNV in this study. However, recent studies revealed that almost all reported genetic risk factors for developing AMD in Asians are identical to those for developing Caucasian AMD.[17-20]

Polypoidal choroidal vasculopathy (PCV) is the other subtype of neovascular AMD, and it is usually diagnosed clinically by indocyanine green angiography.[21] The prevalence of PCV in Asian populations with neovascular AMD has been reported to be higher than in Caucasians.[22-24] Previous studies

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have revealed several genes that are linked to the development of PCV.[17–20,25] However, because PCV has many similarities with AMD, including genetic factors, demography, pathology, and clinical manifestation, it remains controversial whether PCV is a subtype of AMD or a distinct clinical entity.[16,22,24,26]

Recently, Arakawa et al.[27] suggested that two new loci (rs13278062 at *TNFRSF10A-LOC389641* on chromosome 8p21 and rs1713985 at *REST-C4orf14-POLR2B-IGFBP7* on chromosome 4q12) conferred risk for the development of AMD-CNV in case-control studies involving a Japanese population. They noted that *TNFRSF10A* on 8p21 and all four genes on 4q12 were expressed in human adult retinal pigment epithelium (RPE) and rod photoreceptors in mice, which suggests that these loci have functional roles in the development of AMD. To accurately evaluate the gene–disease association, it is important to replicate the positive association reported in previous studies using the same or different ethnic cohorts. In addition, it is important to evaluate the significance of these two variants for typical AMD-CNV and PCV in a larger number of cases, because the original study by Arakawa et al.[27] involved subgroup analysis of AMD with relatively small numbers of patients (298 typical AMD-CNV and 480 PCV).

The aim of this study was to investigate whether the two suggested loci play a significant role in the development of AMD in Asians and its subtypes, typical AMD-CNV and PCV, by assessing 6000 participants from Japanese and Chinese populations.

Methods

We conducted a case-control study with meta-analysis for 2360 patients with neovascular AMD (1013 typical AMD-CNV and 1282 PCV) and 3598 controls from four cohorts.

All procedures in this study adhered to the tenets of the Declaration of Helsinki. This study was approved by the ethics committee of each institute involved (Kyoto University Graduate School and Faculty of Medicine, ethics committee, the ethical committee of Fukushima Medical University, the ethical committee of Kobe City Medical Center General Hospital, the ethical committee at Aichi Cancer Center, the Ethics and Gene Analysis Committee in the Faculty of Medicine, University of Yamanashi, the Singapore Eye Research Institute (SERI) institutional review board, and the Ethics Committee on Human Research, the Chinese University of Hong Kong). All the patients were fully informed about the purpose and procedures of this study, and written consent was obtained from each.

Patients and Controls

Kyoto Cohort. The Kyoto cohort consisted of 1364 neovascular AMD cases and 3057 general healthy population controls. Neovascular AMD patients were recruited from the Department of Ophthalmology at Kyoto University Hospital, Fukushima Medical University Hospital, and Kobe City Medical Center General Hospital. The diagnoses of AMD were based on the International Classification System for age-related maculopathy.[28] Of these patients, 720 were diagnosed as PCV. As proposed by the Japanese Study Group of PCV,[29] the diagnoses were based on indocyanine green angiography, which showed a branching vascular network terminating in polypoidal swelling. Typical AMD-CNV showed classic CNV, occult CNV, or mixed CNV with clear images of vascular CNV networks or diffuse staining of the CNV membrane without polypoidal lesions in indocyanine green angiography.

Patients displaying any of the following characteristics were excluded from the study: (1) high myopia (spherical equivalent < -6.00 diopters [D]), (2) geographic atrophy or drusen only, (3) patients with one eye affected by typical choroidal neovascularization and the other with polypoidal lesions, or (4) an old lesion without a clear diagnosis. The healthy Japanese individuals were obtained from the three studies, which have been reported elsewhere: the Pharma SNP Consortium (PSC),[30] the Aichi Cancer Center Research Institute (with patients confirmed not to have cancer according to the cancer registry, medical record, and self-reporting)[31] and the Japanese Single Nucleotide polymorphism (JSNP) database.[32] We recruited them without ophthalmic data, and they served as general population controls. All subjects in this cohort were unrelated and of Japanese ethnicity.

Yamanashi Cohort. The Yamanashi cohort consisted of 323 neovascular AMD cases and 115 controls. All participants were recruited from the Macular Clinic, Department of Ophthalmology, University of Yamanashi Hospital. Of the patients, 211 patients were diagnosed with PCV. All diagnoses were made as described for the Kyoto cohort. All control subjects were confirmed not to have any signs of AMD by funduscopic examination. All subjects in this cohort were unrelated and of Japanese ethnicity.

Singapore Cohort. The Singapore cohort consisted of 240 neovascular AMD cases and 151 controls. The AMD cases were recruited from a tertiary eye hospital, the Singapore National Eye Center, between September 2007 and April 2008.[33] Controls comprised participants from the Singapore Chinese Eye Study[15] without any sign of AMD.[34] Of the AMD patients, 118 were diagnosed with PCV. All diagnoses were based on criteria as described for the Kyoto cohort. All subjects in this cohort were unrelated and of Chinese ethnicity.

Hong Kong Cohort. The Hong Kong cohort consisted of 433 neovascular AMD cases and 275 controls. All participants were recruited from two tertiary ophthalmic centers in Hong Kong, the Hong Kong Eye Hospital and the Prince of Wales Hospital. Of these patients, 233 were diagnosed with PCV. All diagnoses were made as described for the Kyoto cohort. All control subjects were confirmed not to have any signs of AMD by funduscopic examination. All subjects in this cohort were unrelated and of Chinese ethnicity.

Genotyping

We targeted rs13278062 at *TNFRSF10A-LOC389641* on chromosome 8p21 and rs1713985 at *REST-C4orf14-POLR2B-IGFBP7* on chromosome 4q12, which have been described to have a positive association with development of AMD in a prior study.[27] In the Kyoto cohort, all

Table 1. Characteristics of the Study Population

	Japanese				Chinese			
	Kyoto		Yamanashi		Singapore		Hong Kong	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
n	1364	3057	323	115	240	151	433	275
Age, y	74.3	45.3	74.0	72.5	70.8	65.7	71.6	74.3
SD	8.7	15.4	8.5	9.4	8.7	5.0	9.1	7.6
Range	42 to 96	20 to 79	47 to 93	45 to 91	44 to 93	60 to 84	43 to 94	60 to 94
Male, %	71.6	50.2	74.1	59.8	65.0	53.6	62.8	44.0
PCV, %	52.8	-	65.3	-	49.2	-	53.8	-

TABLE 2. Distribution of Genotypes and the Results of the Association Tests for TNFRSF10-LOC389641 rs13278062

		Genotype								Association Analysis			
		Cases				Controls				Nominal		Adjusted*	
	n	GG	GT	TT	MAF	GG	GT	TT	MAF	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)
Japanese													
Kyoto	4421	506	642	205	0.39	1327	1354	348	0.34	5.22×10^{-6}	0.80 (0.73-0.88)	0.040	0.84 (0.71-0.99)
Yamanashi	438	114	163	46	0.39	60	41	14	0.30	0.011	0.66 (0.48-0.91)	0.012	0.65 (0.47-0.91)
Chinese													
Singapore	391	111	103	26	0.32	85	56	10	0.25	0.033	0.71 (0.51-0.97)	0.051	0.72 (0.51-1.00)
Hong Kong	708	206	186	41	0.31	143	113	19	0.27	0.161	0.84 (0.67-1.07)	0.110	0.82 (0.64-1.04)
Meta-analysis													
Total	5958									1.98×10^{-8}	0.79 (0.73-0.86)	1.12×10^{-4}	0.79 (0.70-0.89)

* Adjusted for age and sex.

case samples and PSC samples were genotyped using TaqMan SNP assays with an ABI PRISM 7700 system (Applied Biosystems, Foster City, CA). Controls from the Aichi Cancer Center Research Institute were genotyped using Illumina Human610-Quad BeadChips (Illumina Inc., San Diego, CA). In the Yamanashi cohort, all samples were genotyped using TaqMan genotyping assays with a 7300/7500 Real-Time PCR System (Applied Biosystems). In the Singapore cohort, all samples were genotyped using Illumina Human610-Quad BeadChips (Illumina Inc.). In the Hong Kong cohort, the SNPs were genotyped using TaqMan genotyping assays on an ABI Prism 7000 Sequence Detection System, according to the manufacturer's instructions (Applied Biosystems).

Statistical Analyses

Deviations in genotype distributions from the Hardy-Weinberg equilibrium (HWE) were assessed with the HWE exact test. Statistical analyses for differences in the observed genotypic distributions were performed by the χ^2 test for trend; logistic regression analyses were performed for age and sex adjustments. Meta-analyses were conducted using a weighted-inverse variance approach in METAL (http://www.sph.umich.edu/csg/abecasis/metal/; provided in the public domain by Center for Statistical Genetics, The University of Michigan, Ann Arbor, MI).[35] The *P* values of heterogeneity across the population were determined using a Cochran's Q test. The coordinates presented were from NCBI Built 36.1 (http://www.ncbi.nlm.nih.gov/; provided in the public domain by National Center for Biotechnology Information, Bethesda, MD). The statistical power calculation was performed using QUANTO version 1.2.[36] *P* value correction was not performed because the SNPs were analyzed independently. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Demographics of the four Japanese and Chinese cohorts are shown in Table 1. Overall, genotype and allele frequencies of the two reported SNPs (rs13278062 at *TNFRSF10A-LOC389641* and rs1713985 at *REST-C4orf14-POLR2B-IGFBP7*) were analyzed in the 2360 patients with neovascular AMD, and compared with those of the 3598 controls. The genotyping of evaluated SNPs was more than 99.3% successful, and the distributions of the genotypes for all study groups were in HWE ($P > 0.05$). Details of allele frequencies and summary statistics for rs13278062 are shown in Table 2. The minor allele frequency (MAF) of rs13278062 was lower in Chinese populations (0.25-0.32) than in Japanese populations (0.30-0.39). After age and sex adjustments, meta-analyses of the four cohorts revealed a significant association of rs13278062 with developing neovascular AMD ($P = 1.12 \times 10^{-4}$, odds ratio [OR] = 0.79, 95% confidence interval [CI] = 0.70-0.89).

Table 3 shows details of allele frequencies and summary statistics for rs1713985. The MAF of rs1713985 was similar between Japanese and Chinese populations. We found no significant association between rs1713985 and neovascular AMD ($P = 0.785$, OR = 1.02, 95% CI = 0.90-1.15). Had there been a true association between rs1713985 and development of AMD at the level reported in the original study (OR = 1.30),[27] our sample size had more than 99.9% power to detect it (unmatched case-control design, log-additive genotype model, 0.67% for the prevalence of exposure in general population,[37] and 0.22-0.31 for allele frequency). We found no evidence of heterogeneity in these meta-analyses for rs13278062 and rs1713985 (heterogeneity $P > 0.05$).

TABLE 3. Distribution of Genotypes and the Results of the Association Tests for REST-C4orf14-POLR2B-IGFBP7 rs1713985

		Genotype								Association Analysis			
		Cases				Controls				Nominal		Adjusted*	
	n	GG	GT	TT	MAF	GG	GT	TT	MAF	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)
Japanese													
Kyoto	4421	109	557	689	0.29	274	1204	1547	0.29	0.730	0.98 (0.89-1.09)	0.359	0.92 (0.77-1.10)
Yamanashi	438	22	147	154	0.30	7	39	69	0.23	0.058	1.40 (0.99-1.99)	0.058	1.39 (0.99-2.05)
Chinese													
Singapore	391	20	102	118	0.30	4	59	88	0.22	0.023	1.47 (1.05-2.06)	0.131	1.32 (0.92-1.89)
Hong Kong	708	39	183	210	0.30	25	121	129	0.31	0.725	0.96 (0.76-1.21)	0.530	0.93 (0.73-1.18)
Meta-analysis													
Total	5958									0.250	1.12 (0.92-1.37)	0.785	1.02 (0.90-1.15)

* Adjusted for age and sex.

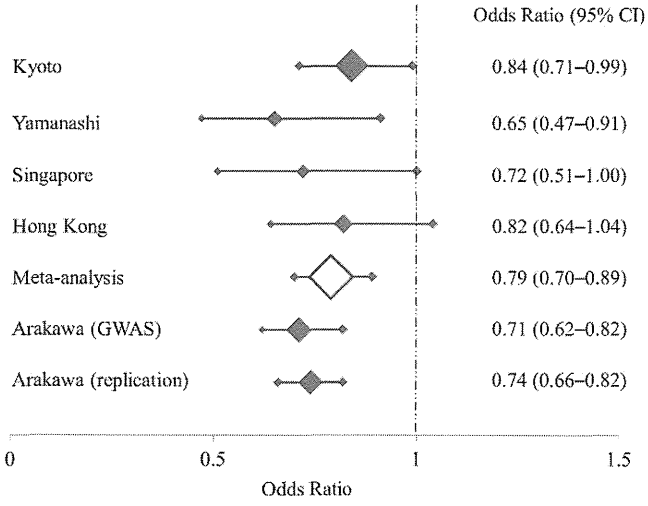

Figure 1. Forest plot of the combined analysis of six case-control cohorts for rs13278062 at 8p21. *Horizontal lines* represent the study-specific odds ratio and 95% CI, and various-sized squares correspond to the sample size of cases. Data from all studies suggested that the T allele of rs13278062 conferred risk for AMD. We found no evidence of heterogeneity in this evaluation ($P = 0.53$).

Next, we evaluated whether these two SNPs were associated with developing typical AMD-CNV or PCV. In this subgroup analysis (Table 4), 1013 typical AMD-CNV and 1282 PCV patients were evaluated. In the meta-analysis, *TNFRSF10A-LOC389641* rs13278062 was found to be significantly associated with development of both typical AMD-CNV ($P = 8.21 \times 10^{-3}$, OR = 0.81, 95% CI = 0.70–0.95) and PCV ($P = 3.79 \times 10^{-5}$, OR = 0.74, 95% CI = 0.65–0.86) after age and sex adjustments, although rs1713985 did not show significant association with either typical AMD-CNV or PCV ($P > 0.05$). In the evaluation of rs1713985 in the development of PCV, we found significant evidence of heterogeneity ($P = 0.02$).

Discussion

A recent article by Arakawa et al.[27] suggested that two loci (rs13278062 at *TNFRSF10A-LOC389641* on chromosome 8p21 and rs1713985 at *REST-C4orf14-POLR2B-IGFBP7* on chromosome 4q12) conferred risk for the development of neovascular AMD in case-control studies of a Japanese population. In the present study, we evaluated these variants by using a larger number of cases ($n = 2360$) than in the previous study ($n = 1536$). However, our current study, including four studies of several Asian cohorts analyzed both independently and collectively, shows no significant association between rs1713985 at *REST-C4orf14-POLR2B-IGFBP7* and neovascular AMD ($P = 0.785$, OR = 1.02, 95% CI = 0.90–1.15). Although one of the cohorts (Yamanashi) suggested marginal significance ($P = 0.058$), this result is likely due to the limited sample size of the control group in the Yamanashi cohort, and, thus, is theoretically more prone to sampling error. In fact, the MAF of rs1713985 in the pooled controls (Japanese: 0.287, Chinese: 0.279) were almost the same as in the previous study that used 18,894 individuals with various diseases as controls (0.282–0.286).[27] Considering similarity in the MAF of the controls, the discrepancy of the association for rs1713985 may be due to the population substructure of the cases in the previous study.

On the other hand, the current study confirmed the significance of rs13278062 at *TNFRSF10A-LOC389641* on chromosome 8p21 for development of AMD in Asians ($P =$

Table 4. Subgroup Analysis of the Two SNPs for Neovascular AMD

	No. of Cases	rs1713985					rs13278062				
			Nominal		Adjusted*			Nominal		Adjusted*	
		MAF	*P*	OR (95% CI)	*P*	OR (95% CI)	MAF	*P*	OR (95% CI)	*P*	OR (95% CI)
tAMD											
Kyoto	579	0.29	0.844	0.99 (0.86–1.13)	0.687	0.95 (0.76–1.20)	0.39	2.89×10^{-4}	0.78 (0.69–0.89)	0.196	0.87 (0.70–1.08)
Yamanashi	112	0.27	0.303	1.25 (0.82–1.91)	0.340	1.24 (0.81–1.93)	0.42	0.010	0.60 (0.41–0.89)	0.016	0.61 (0.41–0.91)
Singapore	122	0.31	0.024	1.56 (1.06–2.29)	0.104	1.46 (0.92–2.32)	0.30	0.179	0.77 (0.53–1.13)	0.337	0.80 (0.52–1.25)
Hong Kong	200	0.30	0.634	0.93 (0.71–1.24)	0.860	0.97 (0.73–1.30)	0.30	0.347	0.87 (0.66–1.16)	0.330	0.86 (0.65–1.16)
Meta-analysis	1013		0.378	1.09 (0.90–1.34)	0.598	1.04 (0.89–1.22)		7.22×10^{-6}	0.78 (0.70–0.87)	8.21×10^{-3}	0.81 (0.70–0.95)
PCV											
Kyoto	720	0.29	0.882	0.99 (0.87–1.12)	0.242	0.89 (0.73–1.08)	0.39	1.69×10^{-4}	0.79 (0.71–0.90)	0.044	0.82 (0.68–0.99)
Yamanashi	211	0.31	0.035	1.49 (1.03–2.15)	0.040	1.95 (1.19–3.19)	0.38	0.032	0.69 (0.49–0.97)	0.029	0.67 (0.48–0.96)
Singapore	118	0.28	0.099	1.39 (0.94–2.06)	0.256	1.28 (0.84–1.96)	0.34	0.020	0.64 (0.44–0.94)	0.017	0.63 (0.43–0.92)
Hong Kong	233	0.31	0.890	0.98 (0.75–1.28)	0.830	0.97 (0.72–1.30)	0.32	0.154	0.82 (0.63–1.08)	0.030	0.71 (0.53–0.97)
Meta-analysis	1282		0.245	1.12 (0.92–1.36)	0.820	1.02 (0.88–1.18)		8.41×10^{-7}	0.78 (0.70–0.86)	3.79×10^{-5}	0.74 (0.65–0.86)

tAMD, typical age-related macular degeneration with choroidal neovascular membrane.
* Adjusted for age and sex.

1.12×10^{-4}, OR = 0.79, 95% CI = 0.70–0.89). Although the calculated risk for developing neovascular AMD was smaller than that reported in the previous study (OR = 0.73),[27] data from all four studies analyzed here suggested that the T allele of rs13278062 conferred risk for AMD (Fig. 1).

In the subgroup analysis targeting the development of typical AMD-CNV and PCV, an association similar to that of all neovascular AMDs was obtained; rs1713985 did not show a significant association for either typical AMD-CNV or PCV, while rs13278062 showed a significant association for both typical AMD-CNV and PCV. In particular, rs13278062 showed almost the same susceptibility for developing typical AMD-CNV and PCV (OR = 0.82 and 0.75, respectively), which suggests that there is no difference between typical AMD and PCV with regard to the role of rs13278062 in disease development. These results are consistent with previous studies that have investigated the significance of genetic backgrounds for both typical AMD-CNV and PCV.[17–20]

Currently, several genes have been reported to be associated with developing AMD in Asians, including the following major AMD-associated loci in Caucasians: (1) age-related maculopathy susceptibility 2 and high-temperature requirement factor A1 genes (*ARMS2/HTRA1*) locus,[2,3] (2) complement factor H gene (*CFH*),[4–7] and (3) complement component 2 and factor B genes (*C2/CFB*) locus.[8] Considering the reported OR of each major locus was approximately 2.0,[2–8,17–20] the susceptibility of rs13278062 at *TNFRSF10A-LOC389641* for developing AMD would be smaller than those of the other major loci. In fact, the population attributable risk (PAR)[38] for rs13278062 was 5.9% in our cohorts. However, since rs13278062 has been reported to associate with the transcriptional activity of TNF-related apoptosis inducing ligand (TRAIL) receptors (TRAILR1),[39] which are known to be involved in apoptosis and inflammation,[40] rs13278062 might have a functional role in developing AMD.

In the present study, we focused on just two SNPs that were identified as the "peak" associations in the two novel regions reported by Arakawa et al.[27] The failure to replicate the 4q12 association might be due to the limited attempt to replicate only one SNP. However, in the original study,[27] they found that rs1713985 represents a linkage disequilibrium block that spans from 57.421 to 57.611 million bases (Mb) on 4q12. In addition, they showed a successful replication for the observed association in the discovery phase using the additional cohort of 708 AMD cases. Thus, if there were true associations between variants at 4q12 and developing AMD, the association of rs1713985 should be replicated in our cohorts that include the same population.

In conclusion, this study provides an Asian population-wide replication study for associations of rs13278062 at *TNFRSF10A-LOC389641* and rs1713985 at *REST-C4orf14-POLR2B-IGFBP7* with the development of neovascular AMD; we confirmed the significance of rs13278062 for Asian neovascular AMD but found no association for rs1713985. Thus, the results suggest that only one locus (*TNFRSF10A-LOC389641*) of the two suggested loci confers increased risk of developing AMD in Asian populations. Because most of the reported genetic risk factors for developing AMD in Asians are similar to those for developing Caucasian AMD, a replication study using a Caucasian cohort would be needed to confirm the significance of *TNFRSF10A-LOC389641* on chromosome 8p21.

References

1. de Jong PT. Age-related macular degeneration. *N Engl J Med.* 2006;355:1474–1485.
2. Jakobsdottir J, Conley YP, Weeks DE, Mah TS, Ferrell RE, Gorin MB. Susceptibility genes for age-related maculopathy on chromosome 10q26. *Am J Hum Genet.* 2005;77:389–407.
3. Rivera A, Fisher SA, Fritsche LG, et al. Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. *Hum Mol Genet.* 2005; 14:3227–3236.
4. Edwards AO, Ritter R, 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science.* 2005;308:421–424.
5. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A.* 2005;102:7227–7232.
6. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science.* 2005;308:419–421.
7. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science.* 2005;308:385–389.
8. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet.* 2006;38:458–462.
9. Baird PN, Robman LD, Richardson AJ, et al. Gene-environment interaction in progression of AMD: the CFH gene, smoking and exposure to chronic infection. *Hum Mol Genet.* 2008;17: 1299–1305.
10. Seitsonen SP, Onkamo P, Peng G, et al. Multifactor effects and evidence of potential interaction between complement factor H Y402H and LOC387715 A69S in age-related macular degeneration. *PLoS One.* 2008;3:e3833.
11. Anderson DH, Radeke MJ, Gallo NB, et al. The pivotal role of the complement system in aging and age-related macular degeneration: hypothesis re-visited. *Prog Retin Eye Res.* 2010; 29:95–112.
12. Rohrer B, Long Q, Coughlin B, et al. A targeted inhibitor of the alternative complement pathway reduces angiogenesis in a mouse model of age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2009;50:3056–3064.
13. Smailhodzic D, Klaver CC, Klevering BJ, et al. Risk alleles in CFH and ARMS2 are independently associated with systemic complement activation in age-related macular degeneration. *Ophthalmology.* 2012;119:339–346.
14. Kawasaki R, Yasuda M, Song SJ, et al. The prevalence of age-related macular degeneration in Asians: a systematic review and meta-analysis. *Ophthalmology.* 2010;117:921–927.
15. Lavanya R, Jeganathan VS, Zheng Y, et al. Methodology of the Singapore Indian Chinese Cohort (SICC) eye study: quantifying ethnic variations in the epidemiology of eye diseases in Asians. *Ophthalmic Epidemiol.* 2009;16:325–336.
16. Laude A, Cackett PD, Vithana EN, et al. Polypoidal choroidal vasculopathy and neovascular age-related macular degeneration: same or different disease? *Prog Retin Eye Res.* 2010;29: 19–29.
17. Mori K, Horie-Inoue K, Gehlbach PL, et al. Phenotype and genotype characteristics of age-related macular degeneration in a Japanese population. *Ophthalmology.* 2010;117:928–938.
18. Gotoh N, Nakanishi H, Hayashi H, et al. ARMS2 (LOC387715) variants in Japanese patients with exudative age-related macular degeneration and polypoidal choroidal vasculopathy. *Am J Ophthalmol.* 2009;147:1037–1041. 1041 e1031-1032.
19. Nakata I, Yamashiro K, Yamada R, et al. Significance of C2/CFB variants in age-related macular degeneration and polypoidal choroidal vasculopathy in a Japanese population. *Invest Ophthalmol Vis Sci.* 2012;53:794–798.