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Association Between the Cholesteryl Ester Transfer Protein Gene and Polypoidal Choroidal Vasculopathy

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Submitted: January 4, 2013
Accepted: August 8, 2013

Citation: Nakata I, Yamashiro K, Kawaguchi T, et al; for the Nagahama Study Group. Association between the cholesteryl ester transfer protein gene and polypoidal choroidal vasculopathy. *Invest Ophthalmol Vis Sci.* 2013;54:6068–6073. DOI:10.1167/iovs.13-11605

Purpose. To determine whether genetic variants in the lipid-associated genes are related to the risk of developing polypoidal choroidal vasculopathy (PCV) in a Japanese population.

Methods. Five hundred eighty-one patients with PCV and 793 controls were enrolled in the study. Association analysis of allele and genotype frequencies was performed for the following single-nucleotide polymorphisms (SNPs) that are associated with high-density lipoprotein cholesterol levels in blood: rs493258 at the hepatic lipase gene (*LIPC*), rs3764261 at the cholesteryl ester transfer protein gene (*CETP*), and rs12678919 at the lipoprotein lipase gene (*LPL*). A further model adjusting for age-related maculopathy susceptibility 2 (*ARMS2*) A69S, complement factor H (*CFH*) I62V, age, sex, and smoking status was used to confirm the independent association of these SNPs with other covariates.

Results. *CETP* rs3764261 was significantly associated with the development of PCV; the frequency of the minor allele *A* was higher in the PCV cases (24.0%) than in the control subjects (18.5%) ($P = 0.0025$; odds ratio [OR], 1.41; 95% confidence interval, 1.13–1.75). Furthermore, we found an independent association of *CETP* variants with age, sex, smoking status, and genetic background of *ARMS2* A69S, *CFH* I62V, *LIPC* rs493258, and *LPL* rs12678919 ($P = 0.0013$; OR, 1.50). *LIPC* rs493258 and *LPL* rs12678919 did not show significant associations with the development of PCV ($P > 0.05$).

Conclusion. *CETP* variants are associated a risk of developing PCV among the Japanese population.

Keywords: PCV, lipid, *CETP*, case-control study

Polypoidal choroidal vasculopathy (PCV) is characterized by aneurysmal dilations with interconnecting vessels that are best demonstrated by indocyanine green angiography.[1–3] Clinically, PCV is classified into a specific subtype of age-related macular degeneration (AMD), and the incidence of PCV in Asian populations has been reported to be higher than that in Caucasians.[4–6] Controversies exist about the pathogenesis of PCV; whether this condition represents inner choroidal vascular abnormalities or a particular variety of choroidal neovascularization (CNV) remains undetermined. However, because there are apparent differences in the demographic risk profile, clinical course, and visual prognosis, PCV is thought to be a distinct clinical entity.[7] For example, the response to treatment, particularly in photodynamic therapy for PCV, is completely different from that for typical AMD and CNV.[8,9]

Cholesterol and lipids are reported to accumulate underneath the retinal pigment epithelium (RPE) with age. When sufficient debris, including lipids, accumulates and forms a mound between the RPE cell and its basement membrane, it can be seen clinically as drusen. Because many population-based studies have shown the association between drusen and the progression of AMD, drusen is thought to be one of the determinants of both early and late AMD. In fact, an association between high-density lipoprotein (HDL) cholesterol level and the development of AMD has been reported in several studies.[10–12]

Previous studies[13–15] showed that the prevalence of drusen under RPE was reported to be lower in PCV than in AMD. Therefore, the absence of drusen was thought to be one of the criteria necessary to diagnose PCV.[6,15,16] However, the results of a clinical study[16] suggested that drusen is frequently seen in PCV eyes, and several studies[6,17,18] reported that drusen were observed in 20% to 27% of unaffected, fellow eyes in patients with unilateral PCV. Therefore, whether drusen has a functional role in the development of PCV remains controversial.

While previous investigations showed a lower prevalence of drusen among patients with PCV, lipid deposits that distribute from the inner retina to the outer retina are known to be the paramount features of PCV (Figure). Some recent investiga-

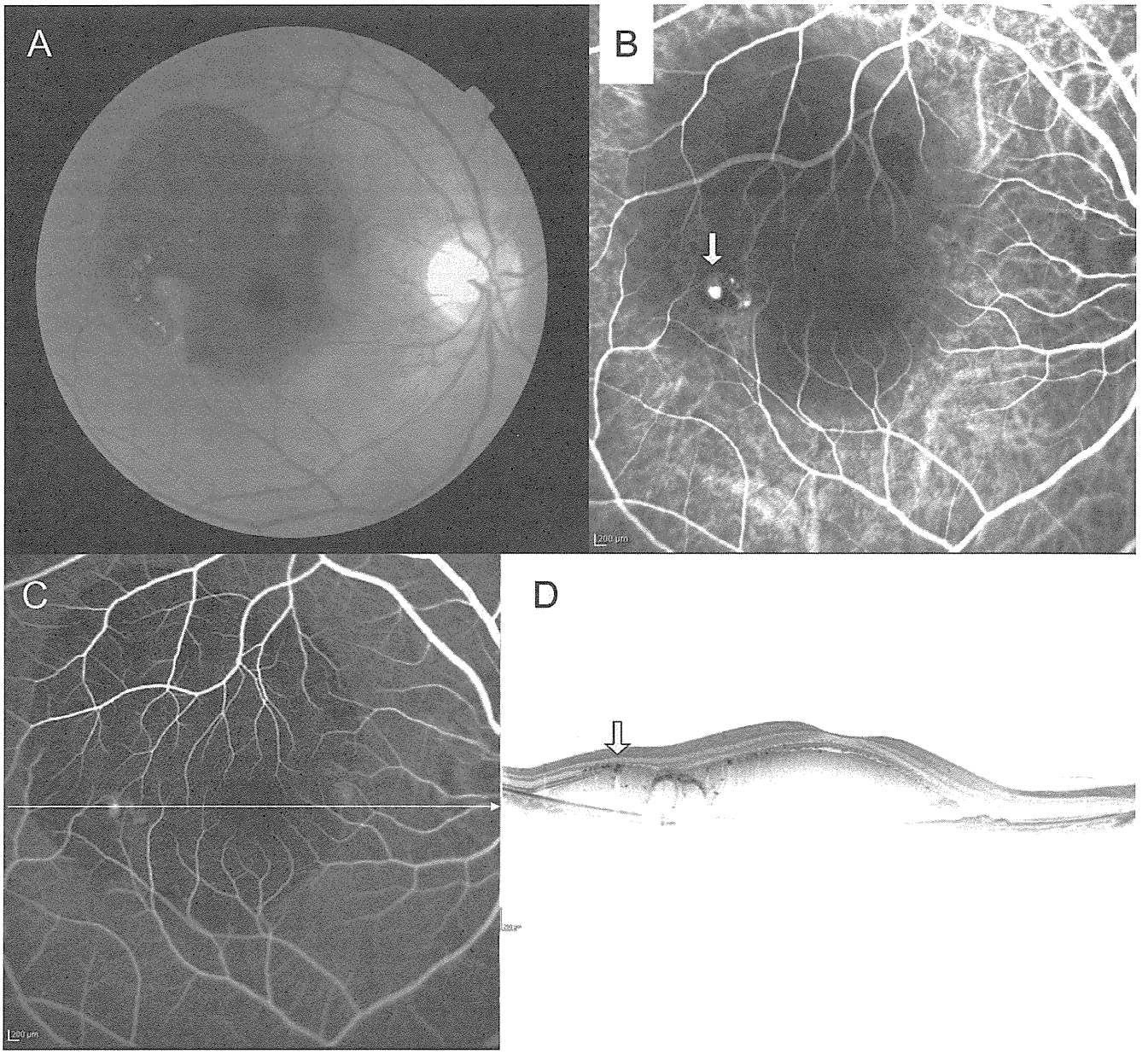

FIGURE. A 64-year-old woman with a typical case of PCV in the right eye. (**A**) Fundoscopic examination shows massive subretinal hemorrhage, lipid deposits, and reddish orange nodules. (**B**) Indocyanine green angiography demonstrates a small branching vascular network terminating in polypoidal lesions (*white arrow*). The speckle noise-reduced spectral-domain optical coherence tomography image of a horizontal section corresponding to the arrow indicated in fluorescein angiography (**C**) shows hyperreflective foci, indicating lipids ([**D**], *arrowhead*), in the outer retina beside the polyp ([**D**], *white arrow*).

tions, including a study[19] in a large cohort of Caucasians, showed significant associations between the lipid-associated genes and the development of AMD. These discoveries of genetic variants in the lipid pathway provided new insight into the pathogenesis of AMD. However, there are limited reports evaluating the association between the lipid-associated genes and the development of PCV. Although several genes are thought to be involved in regulating susceptibility to the development of PCV,[20–23] almost all are identical to those involved in the development of AMD, including the age-related maculopathy susceptibility 2 and high-temperature requirement factor A1 genes (*ARMS2/HTRA1*) locus[24,25] and the complement factor H gene (*CFH*).[26–29] Considering that several studies[13–15] reported a difference in the clinical features of drusen between AMD and PCV, there could be different roles of the lipid-associated genes in these subtypes. Thus, we aimed in this study to determine whether genetic variants in the lipid-associated genes, including variants affecting HDL cholesterol levels, are related to the risk of developing PCV in a Japanese population.

METHODS

All procedures in this study adhered to the tenets of the Declaration of Helsinki, and the ethics committee of each institution involved approved the study protocols. All patients were fully informed about the purpose and procedures of this study, with each patient providing written consent.

TABLE 1. Characteristics of the Study Population

Variable	Cases, $n = 581$	Controls, $n = 793$	P Value
Age, y			
Mean ± SD	72.59 ± 8.13	65.99 ± 4.33	<0.0001
Range	48-92	60-75	
Sex, *n* (%)			
M	420 (72.3)	326 (41.1)	<0.0001
F	161 (27.7)	467 (58.9)	
Smoking status, *n* (%)			
Never	200 (38.5)	509 (64.3)	<0.0001
Former	195 (37.6)	176 (22.3)	
Current	124 (23.9)	106 (13.4)	

Five hundred eighty-one patients with PCV were recruited from the departments of ophthalmology at Kyoto University Hospital, Fukushima Medical University Hospital, and Kobe City Medical Center General Hospital. The diagnosis of PCV was based on indocyanine green angiography, which showed a branching vascular network terminating in polypoidal swelling (Figure), and was confirmed by three retina specialists (KY, AT, AO); a fourth specialist (NY) was consulted when the diagnosis could not be agreed on by the initial three reviewers. Patients who had both typical CNV and polypoidal lesions were excluded from this study. The control group consisted of 793 unrelated individuals 60 years or older recruited in the Nagahama Prospective Genome Cohort for Comprehensive Human Bioscience (the Nagahama Study).[30] Fundoscopic photographs of both eyes confirmed the absence of any signs of AMD (large drusen or pigment change) using the Age-Related Eye Disease Study[31] severity scale, with grading by two independent ophthalmologists (IN, YAK), followed by grading by a senior reviewer (KY).

We targeted three single-nucleotide polymorphisms (SNPs) of three genes reported to be associated with HDL cholesterol levels in blood, including rs493258 at the hepatic lipase gene (*LIPC*), rs3764261 at the cholesteryl ester transfer protein gene (*CETP*), and rs12678919 at the lipoprotein lipase gene (*LPL*).[32] Genomic DNA was prepared from peripheral blood using a DNA extraction kit (QuickGene-610L; Fujifilm, Minato, Tokyo, Japan). All case samples were genotyped using the Taqman SNP assay with an ABI PRISM 7700 system (Applied Biosystems, Foster City, CA). Controls were genotyped using Human610-Quad BeadChips and HumanOmni2.5 BeadChips (Illumina, Inc., San Diego, CA). *ARMS2* A69S (rs10490924) and *CFH* I62V (rs800292) were also genotyped in the same manner. Fasting serum samples from the control subjects were analyzed for HDL cholesterol level, measured using a direct assay system with the selective inhibitory method on an automatic analyzer (LABOSPECT 008; Hitachi, Ltd., Tokyo, Japan). We did not have HDL cholesterol data for the case samples.

Information on smoking status was obtained via a self-reported questionnaire with three categories of never smoker, former smoker, and current smoker. The never smokers were those who had smoked fewer than 100 cigarettes in the past, current smokers were those who had smoked in the past year, and former smokers were those who had quit smoking more than 1 year earlier.

TABLE 3. Logistic Regression Analysis, Including Major Factors Associated With PCV

Variable	P Value*	OR (95% CI)
Age	<0.0001	1.18 (1.16-1.21)
F:M sex	<0.0001	3.16 (2.20-4.52)
ARMS2 rs10490924 (*G/T*)	<0.0001	2.27 (1.86-2.77)
CFH rs800292 (*A/G*)	<0.0001	1.77 (1.43-2.19)
LIPC rs493258 (*G/A*)	0.689	1.05 (0.82-1.35)
CETP rs3764261 (*C/A*)	0.0013	1.50 (1.17-1.92)
LPL rs12678919 (*A/G*)	0.948	0.99 (0.72-1.35)
Smoking (never, former, or current)	0.0107	1.35 (1.07-1.69)

* A logistic regression model was used for covariate adjustment.

Deviations in genotype distributions from the Hardy-Weinberg equilibrium (HWE) of the controls were assessed with the HWE exact test. Statistical differences in the observed allelic distribution were identified using logistic regression analyses with age and sex adjustments, under the assumption of an additive genetic effect where the genotypes of each SNP are coded numerically as 0, 1, and 2 for the number of minor alleles carried. A linear regression analysis was performed to assess the association between HDL cholesterol level and genotype. R software (http://www.r-project.org/ in the public domain) was used for statistical analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Demographics of the study population are given in Table 1. Genotype and allele frequencies of the three SNPs were analyzed in 581 patients with PCV and compared with those of 793 age-matched individuals without any signs of AMD or PCV. The genotyping of all evaluated SNPs had a success rate exceeding 99.4%.

Table 2 gives details of genotype and allele frequencies and summary statistics. The distributions of the genotypes for all evaluated SNPs were in HWE ($P > 0.05$). We found that *CETP* rs3764261 was significantly associated with the development of PCV; the frequency of the minor allele *A* in the patients with PCV (24.0%) was higher than that in the controls (18.5%) ($P = 0.0025$; odds ratio [OR], 1.41; 95% confidence interval [CI], 1.13-1.75). This significant association remained even after a correction for multiple testing ($P = 0.0075$). *LIPC* rs493258 and *LPL* rs12678919 did not show significant associations with the development of PCV ($P > 0.05$).

Next, we conducted a logistic regression analysis that included the effects of the most robust Japanese variants associated with AMD and PCV, *ARMS2* A69S (rs10490924) and *CFH* I62V (rs800292), as well as age, sex, smoking status, *LIPC*

TABLE 2. Distribution of Genotypes and Results of the Association Tests

		Allele		Cases, $n = 581$				Controls, $n = 793$				Association Results*	
Gene	SNP	1	2	11	12	22	MAF	11	12	22	MAF	P Value	OR (95% CI)
LIPC	rs493258	*G*	*A*	32	185	354	0.22	37	259	497	0.21	0.706	1.04 (0.84-1.30)
CETP	rs3764261	*C*	*A*	332	210	33	0.24	528	237	28	0.19	0.0025	1.41 (1.13-1.75)
LPL	rs12678919	*A*	*G*	439	135	3	0.12	602	179	12	0.13	0.883	1.02 (0.77-1.35)

MAF, minor allele frequency.
* Adjusted for age and sex.

rs493258, and *LPL* rs12678919 in the regression model. Table 3 gives the results of the logistic regression analysis. *CETP* rs3764261 remained significant for the development of PCV even after including the effects of these covariates ($P = 0.0013$; OR, 1.50; 95% CI, 1.17–1.92).

Finally, we investigated the role of *CETP* rs3764261 in blood HDL cholesterol level using fasting serum samples from 793 control subjects. The mean ± SD HDL cholesterol level of the control samples was 61.3 ± 16.1 mg/dL. In this analysis, we found that the *A* allele of rs3764261 was associated with the following increases in HDL cholesterol: 59.3 mg/dL for the *CC* genotype, 64.8 mg/dL for the *CA* genotype, and 67.2 mg/dL for the *AA* genotype ($P < 0.0001$).

Discussion

Plasma CETP was first described as a high-molecular-weight protein stimulating the transfer of cholesteryl ester between lipoproteins in plasma of hypercholesterolemic rabbits.[33] Other studies demonstrated various roles of CETP in the lipid pathway: CETP facilitates the transfer of triglycerides and phospholipids[34]; it is an important component of reverse cholesterol transport, which is chiefly characterized by the transport of cholesterol from peripheral tissues to the liver; and it regulates the concentration of HDL cholesterol.[35,36]

After the discovery of the association between HDL cholesterol level and cardiovascular diseases,[37] studies[38,39] evaluated the functional role of the lipid-associated genes that can affect the HDL cholesterol level. Among those genes, the *A* allele of *CETP* rs3764261 was associated with an increase in HDL cholesterol by 5.6 mg/dL among the Japanese population.[40] Herein, we confirmed the role of rs3764261 in increased HDL cholesterol levels among 793 healthy Japanese individuals.

In the present study comparing the allelic distributions of *CETP* variants in a sample of 581 patients with PCV and 793 control subjects, the *A* allele of *CETP* rs3764261 was significantly associated with a risk of developing PCV (OR, 1.41; 95% CI, 1.13–1.75), which indicates a higher level of HDL cholesterol in patients with PCV. In addition, the association of *CETP* variants remained significant even when we adjusted for the effects of other established risk factors for developing AMD and PCV (age, sex, smoking status, and genetic background of *ARMS2* A69S, *CFH* I62V, *LIPC* rs493258, and *LPL* rs12678919). Although the effect of *CETP* variants (OR, 1.50) was not as large as the effects of the major genes associated with AMD and PCV (ORs, 2.27 for *ARMS2* and 1.77 for *CFH*) in this regression analysis, we were able to confirm that *CETP* variants have a significant role in the development of PCV. Our findings for *CETP* rs3764261 were similar to the associations already documented in AMD among Caucasians,[41,42] which suggests that a higher HDL cholesterol level may be a risk factor in both PCV and Caucasian AMD. The hypothesis that a higher level of HDL cholesterol is associated with the development of PCV might appear contradictory to the fact that a lower level of HDL cholesterol is associated with an increased risk of cardiovascular disease. However, despite the well-known antiatherogenic properties of HDL cholesterol, some studies[10,11,43] found elevated levels of HDL cholesterol in Caucasian patients with AMD.

Recently, Zhang et al.[44] reported an investigation of lipid-associated SNPs for PCV and neovascular AMD in a Chinese population. In that article, they showed a significant association of *CETP* with PCV, while no association was found with neovascular AMD. Thus, they concluded that the HDL cholesterol pathway in the pathogenesis of PCV likely differs from that of neovascular AMD. However, the sample size evaluated in the their article was small (204 controls, 250 patients with PCV, and 157 patients with neovascular AMD), which suggests that the negative result of the association between *CETP* and neovascular AMD could have been due to insufficient power to detect the association. To confirm whether the observed association of *CETP* with PCV exists for neovascular AMD as well, we performed an additional analysis using another Japanese cohort of neovascular AMD cases ($n = 452$). In this evaluation, we found a significant association between *CETP* and neovascular AMD ($P = 0.0246$; OR, 1.35).

Adenosine triphosphate–binding cassette, subfamily A member 1 (ABCA1) is also known to be associated with the lipid pathway. Because *ABCA1* has been reported to be another susceptible gene for the development of AMD in Caucasians,[19] we also evaluated whether *ABCA1* rs1883025 has a significant role in the development of PCV but found no significant association with PCV ($P > 0.05$). In previous genome-wide association analyses for HDL cholesterol, the strongest and most consistently associated SNPs have been reported in the *CETP* locus.[45,46] Study[32] findings also suggest that *LIPC* rs493258 and *LPL* rs12678919 are associated with HDL cholesterol level in Caucasians, so the lack of association in the present study could be due to insufficient statistical power or racial/ethnic differences. Further study that includes a larger number of participants is needed to clarify the association between genetic variants of HDL cholesterol–associated genes and the development of PCV.

In the present study, there was a large sex difference between the PCV cases and the general population controls. It remains unknown why there is such a high prevalence of PCV among men. In a previous meta-analysis by Kawasaki et al.,[47] the prevalence of late AMD among Asian women was reported to be much lower than that among Asian men. In contrast, a male predominance was reported in PCV.[4] Considering the high prevalence of PCV among Asian populations, these results suggest that men are more likely to develop PCV. In our study, genetic factors had an enormous influence on whether participants developed PCV (Table 3). However, sex had the largest effect among all covariates on the development of PCV (OR, 3.16). A previous genetic study[23] among Japanese may provide insight into this question because the results suggested that differences in sex would affect phenotypic differences in AMD. Another limitation of the present study was the age difference between cases and controls. Although we enrolled only controls who were 60 years or older, the average age of the control cohort was still younger than that of the case cohort, which means that some of the young controls may develop PCV in the future. To exclude a potential confounder of genetic background with age, a logistic regression analysis adjusting for age and sex was performed in the present study. However, given that the prevalence of late AMD among the Japanese population is reported to be 0.5%,[48] the magnitude of statistical bias of the association analysis is negligible. In addition, considering that case-control association analyses among such subjects are less likely to be statistically significant, our positive results should be acceptable.

Overall, this study provides the first evidence to date that *CETP* variants have a significant role in the risk of developing PCV among the Japanese population. Our study also indicates the same role of HDL cholesterol in both PCV and Caucasian AMD, although the role of fatty acids in Japanese AMD is reported to be different from that in Caucasian AMD.[49] Further studies are needed to increase the understanding of the genetic backgrounds of PCV, as well as the molecular pathogenesis, particularly the role of lipids.

Acknowledgments

The authors thank the participants of the Nagahama Study, the Nagahama City Office, and the nonprofit organization Zero-ji Club for Health Promotion.

Supported by grants-in-aid from the following organizations: the Ministry of Education, Culture, Sports, Science, and Technology of Japan (2006-2012); Grants 19390442, 22791706, and 22791653 from the Japan Society for the Promotion of Science; the Japanese National Society for the Prevention of Blindness; and Takeda Science Foundation (2008-2012).

Disclosure: **I. Nakata**, None; **K. Yamashiro**, None; **T. Kawaguchi**, None; **N. Gotoh**, None; **H. Nakanishi**, None; **Y. Akagi-Kurashige**, None; **M. Miyake**, None; **A. Tsujikawa**, None; **A. Oishi**, None; **M. Saito**, None; **T. Iida**, None; **R. Yamada**, None; **F. Matsuda**, None; **N. Yoshimura**, None

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Appendix

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Annals of human genetics

doi: 10.1111/ahg.12034

Quantitative Variation in Plasma Angiotensin-I Converting Enzyme Activity Shows Allelic Heterogeneity in the *ABO* Blood Group Locus

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Summary

Angiotensin-I converting enzyme (ACE) occupies a pivotal role in cardiovascular homeostasis. Major loci for plasma ACE have been identified at *ACE* on Chromosome 17 and at *ABO* on Chromosome 9. We sought to characterise the genetic architecture of plasma ACE at finer resolution in two populations. We carried out a GWAS in 1810 individuals of Japanese ethnicity; this identified signals at *ACE* and *ABO* that together accounted for nearly half of the population variability of the trait. We conducted measured haplotype analysis at the *ABO* locus in 1425 members of 248 British families using haplotypes of three SNPs, which together tagged the alleles responsible for the principal blood group antigens A1, A2, B and O. Type O alleles were associated with intermediate plasma ACE activity compared to Type A1 alleles (in whom plasma ACE activity was ~36% lower) and Type B alleles (in whom plasma ACE activity was ~36% higher). We demonstrated heterogeneity among A alleles: A2 alleles were associated with plasma ACE activity that was very similar to the O alleles. Variation at *ACE* accounted for 35% of the trait variance, and variation at *ABO* accounted for 15%. A further 10% could be ascribed to polygenic effects.

Keywords: ABO blood group, angiotensin-I converting enzyme, genome wide association study, QTL

Introduction

The renin–angiotensin system plays a critical role in cardiovascular homeostasis, regulating blood pressure, arterial tone and renal salt excretion. The angiotensin I-converting enzyme (ACE) converts circulating angiotensin-I, which is

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biologically inactive, to the active angiotensin-II and degrades the vasodilator bradykinin. ACE occupies a pivotal position in the renin–angiotensin system: drugs which inhibit ACE, or which block the cellular receptor for the angiotensin-II generated by the action of ACE, are among the most widely prescribed agents in patients with coronary artery disease, hypertension and chronic renal disease (Yusuf et al., 2000). Plasma ACE activity is related to tissue ACE activity, and is strongly influenced by genetic factors, the largest such influence being due to polymorphic variation in the *ACE* (also known as *DCP1*) gene, which encodes ACE (Rigat et al., 1990; Keavney et al., 1998). Previous segregation and linkage analysis suggested the existence of a second major quantitative trait locus (QTL) influencing plasma ACE activity (McKenzie et al., 1995). A genome wide association study (GWAS) in a population of hypertensive patients of Han Chinese ancestry subsequently found this to be located at the *ABO* gene which encodes glycosyltransferases A and B (Chung et al., 2010). Global variation in the distribution of the alleles responsible for ABO blood groups is well described; we therefore sought to confirm the identity of the second principal locus influencing ACE activity and to estimate the proportions of phenotypic variance attributable to major gene effects in two additional populations from Japan and the United Kingdom.

Methods

Study Populations and ACE Phenotyping

The discovery cohort included 1830 volunteers recruited as a part of the Nagahama Prospective Genome Cohort for Comprehensive Human Bioscience (the Nagahama Study), a community-based prospective multiomics cohort study. The study has been described in detail elsewhere (Yoshimura et al., 2012); demographics of the cohort are summarised in Table S1. In brief, a total of 9809 volunteers from Nagahama City, Shiga Prefecture, Japan, were recruited for this study from 2008 to 2010. All participants completed a detailed health questionnaire. DNA, serum and plasma samples from all participants were obtained and stored for further analysis. Samples were kept on ice immediately after they were obtained from the participants and were promptly processed. Plasma was stored at −80°C. ACE activity was quantified by the method reported by Kasahara & Ashihara (1981). Patients receiving ACE inhibitor therapy were excluded from the analyses.

The replication cohort comprised 248 British families of Northern and Western European ancestry who participated in a quantitative genetic study of cardiovascular risk factors (Palomino-Doza et al., 2008). The population collection strategy has been previously described in detail (Gaukrodger et al., 2005). In brief, families were ascertained via a hypertensive proband between 1993 and 1996, and any sibship in the family (in the generation of the proband or his/her offspring) greater than three members quantitatively assessable for blood pressure was collected. Families were extended where additional hypertensives were encountered during collection; a total of 1425 individuals participated. Families underwent detailed cardiovascular phenotyping including a questionnaire, electrocardiographic and echocardiographic measurement and measurement of 24-hour ambulatory blood pressure using an automated monitor (Keavney et al., 2000; Mayosi et al., 2008). Demographics of the cohort are presented in Table S2. Blood was drawn into multiple anticoagulants, immediately put on ice and transported rapidly to a central facility for processing. ACE activity was assessed by HPLC using a synthetic substrate, as previously described (Chiknas, 1979).

Genotyping

The 1830 volunteers in the Japanese cohort were genotyped using the Infinium Human 610-Quad Bead Chip carrying 592,044 SNP markers on a Bead Station 500G Genotyping System (Illumina, Inc., San Diego, CA, USA). There were no subjects showing call rates lower than 0.99. Kinship analysis was performed using PLINK. Of the 20 pairs of samples showing high degrees of kinship (PI-HAT > 0.4), the sample with the lower call rate in each pair was removed. 165,591 SNPs were removed either due to call rate lower than 0.95, minor allele frequency of less than 0.05, or distorted Hardy-Weinberg equilibrium ($P < 10^{-7}$). Finally, the results of 426,453 SNP markers in 1810 subjects were used for the analysis.

Three SNPs at the ABO locus (rs505922, rs8176746 and rs8176750) were typed in the entire British family cohort using matrix-assisted laser desorption/ionisation—time of flight mass spectrometry (MALDI-TOF) on a Sequenom instrument (Sequenom, San Diego, CA, USA). ABO blood group in the British families was studied in a subset of 734 individuals by multiplex polymerase chain reaction (PCR). Two pairs of primers were used to amplify exons 6 and 7 of the *ABO* gene; the amplified fragments were digested with restriction endonucleases *Hpa*II and *KpnI* and separated by gel electrophoresis. This enabled us to call genotypes at the SNPs rs8176719, rs1053878, rs8176743 and rs8176472. As previously reported, genotypes at these SNPs, considered together, identify the A1, A2, B, O1 and O2 blood group alleles (Seltsam et al., 2003).

Statistical Methods

A quantitative linear regression analysis was first performed in the Japanese cohort to find the polymorphisms associated with ACE activity. SNP genotype imputation for SNPs within and

flanking the *ACE* (20 SNPs) and *ABO* (43 SNPs) loci was performed in the Japanese samples using the MaCH (version 1.0.10) computer program with 500 Markov sampler rounds and 200 haplotype states (Li et al., 2010). A forward-selection stepwise regression analysis was performed to identify a parsimonious subset of associated SNPs from the *ACE* and *ABO* loci in the Japanese population. This analysis was based on imputed SNP dosages using linear regression models based on marginal sums of squares and the *stepwise* procedure in Stata™ v10.1 (Stata Corp, College Station, TX, USA) using a $P < 0.01$ criterion for adding SNPs to the model. Variance component proportions (R^2) were calculated from a supplementary analysis of variance based on sequential sums of squares.

Haplotyping of *ABO* in the British samples was performed using PHASE (version 2.1.1) specifying a parent-independent multiallelic model for both SNP and blood group variation. (Stephens et al., 2001; Stephens & Scheet, 2005) Pedigree analysis was performed using the Pedigree Analysis Package (PAP version 5.0) to fit maximum likelihood models including polygenic variance components (Hasstedt, 1993) to extended families; missing data is efficiently incorporated into this analysis (Elston & Stewart, 1971). For the measured haplotype analysis, the PAP quantitative major gene subroutine *qmlprmv* was modified to parametrise an additive (codominant) genetic model. Likelihoods were maximised with simultaneous estimation of haplotype frequencies assuming Hardy–Weinberg equilibrium, haplotype-specific effects on ACE activity, covariate effects, polygenic effects and residual individual-specific random (i.e. environmental) effects and estimates of standard errors were calculated with the bundled quasi-Newton nonlinear optimisation function GEMINI (Lalouel, 1979). Variance component proportions were calculated by hand using a standard additive genetic variance formula.

Table 1 Forward selection stepwise regression analysis of plasma ACE activity and GWAS SNPs in the Japanese cohort. R^2 shows the proportion of variance explained by each variable.

Locus	Variable	Beta	SE	*F*-statistic	*P*-value	R^2
	Age	0.0363	0.0040	84.32	1.13E-19	0.0229
ACE	rs4362	−3.0450	0.0849	1286.30	9.60E-213	0.3494
ABO	rs495828	1.4762	0.0982	225.75	3.81E-48	0.0613
ABO	rs8176746	1.5698	0.1154	184.97	3.66E-40	0.0502

Table 2 *ABO* haplotype analysis using PHASE 2.1.1 in British families. Common haplotypes assessed in the measured haplotype analysis are shown in bold.

rs505922	rs8176746	rs8176750	Blood group allele	Conditional probability	Frequency
C	**G**	**G**	**A1**	**0.9235**	**0.1719**
C	G	G	A2	0.0049	0.0009
C	G	G	B	0.0139	0.0026
C	G	G	O1	0.0271	0.0050
C	G	G	O2	0.0307	0.0057
C	G	del	A1	0.1541	0.0096
C	**G**	**del**	**A2**	**0.8333**	**0.0517**
C	G	del	O1	0.0011	0.0001
C	G	del	O2	0.0115	0.0007
C	**T**	**G**	**B**	**1.0000**	**0.0513**
C	T	del	A2	0.2244	0.0004
C	T	del	B	0.7756	0.0014
T	G	G	A1	0.0068	0.0047
T	G	G	B	0.0055	0.0038
T	**G**	**G**	**O1**	**0.9618**	**0.6709**
T	G	G	O2	0.0259	0.0180
T	G	del	A1	0.0377	0.0000
T	G	del	O1	0.9623	0.0002
T	T	G	O1	1.0000	0.0007

Results

GWAS for ACE Activity

The SNPs with the strongest association with ACE activity genome-wide are presented in Table S3. Two loci, *ACE* on Chromosome 17, and *ABO* on Chromosome 9, showed genome-wide significant association ($P < 5 \times 10^{-8}$) with plasma ACE activity (Fig. S1). SNPs mapping to the *ACE* (20 SNPs) and *ABO* (43 SNPs) loci and their immediate upstream and downstream flanking regions (50 kb, respectively) were selected for fine-mapping analysis and any missing genotype data was imputed. Stepwise linear regression then identified three SNPs with independent significant effects ($10^{-213} < P < 10^{-40}$): rs4362 at *ACE*; and rs495828 and rs8176746 at *ABO*, that together accounted for nearly half of the population variation in the trait (Table 1), with the *ACE* locus accounting for 35% and the *ABO* locus accounting for 11%.

Association between Haplotypes Defining Blood Groups and ACE Activity

Haplotypes of rs505922, rs8176746 and rs8176750 showed strong associations with alleles defining the different blood groups in the subset of the British families (734 samples) where blood groups were available (conditional tagging probabilities range from 0.83 to 1.00; Table 2). Haplotypes of these three SNPs that occurred at a frequency of >0.05 in the population, and accurately tagged alleles responsible for A1, A2, B and O1 phenotypes, were taken forward to the

Table 3 Measured haplotype analysis of plasma ACE activity in British families. The *ABO* haplotype is defined by rs505922, rs8176746 and rs8176750; the most strongly tagged blood group allele is shown in parentheses.

ABO haplotype (blood group allele)	Frequency	Mean	*SE*
TGG (O1)	0.647	9.295	0.084
CGG (A1)	0.212	5.989	0.231
CGdel (A2)	0.079	9.473	0.473
CTG (B)	0.063	12.808	0.528

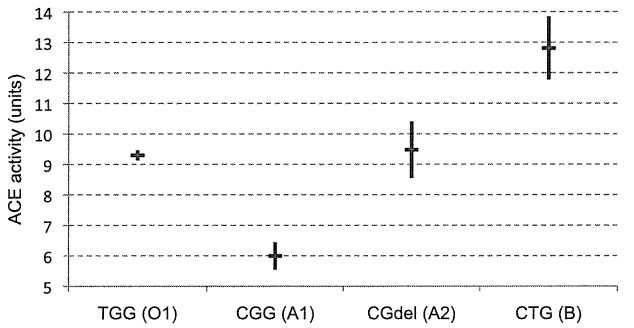

Figure 1 Measured haplotype analysis of plasma ACE activity in British families. The mean activities and 95% confidence intervals for each *ABO* haplotype are indicated by horizontal and vertical lines, respectively. *ABO* haplotypes are defined by rs505922, rs8176746 and rs8176750; the most strongly tagged blood group allele is shown in parentheses.

measured haplotype analysis of ACE activity in the total UK cohort. In the measured haplotype analyses, SNPs rs4295 and rs4392, previously shown in this cohort to tag the principal haplotype blocks influencing ACE activity at the *ACE* locus, were included as covariates. (Keavney et al., 1998) The measured haplotype analysis showed that the TGG haplotype of rs505922/rs8176746/rs8176750, which accurately tagged type O alleles and occurred at a frequency of ~65% in the population, was associated with an intermediate plasma ACE activity (Table 3). The CGG haplotype (frequency ~21%), which tagged type A1 alleles, was associated with a 36% lower plasma ACE activity than the TGG haplotype. The CTG haplotype (frequency ~6%), which tagged type B alleles, was associated with a 36% higher plasma ACE activity than the TGG haplotype. The CGdel haplotype (frequency ~8%), which tagged type A2 alleles, had a plasma ACE activity very similar to the TGG haplotype (Fig. 1). The measured haplotype analysis in the British families provided very similar estimates of variance components to the GWAS in the Japanese cohort: 35.4% of the variance was due to the *ACE* locus, and 13.0% to the *ABO* locus, with a further 9.6% attributable to polygenes and 42.1% to nongenetic residual variation.

Discussion

We have shown strong evidence for association between plasma ACE activity and genotypes at the *ABO* gene that define the major blood groups. Genome-wide analyses in a cohort of unrelated Japanese people confirmed that the strongest genetic influence on plasma ACE levels is located at the *ACE* gene itself on Chromosome 17 ($P = 1.55 \times 10^{-164}$ for rs4362) and demonstrated two further independent SNP effects at *ABO* (rs495828 and rs8176746). Stepwise regression suggested that the association at *ACE* accounted for 35% of the variability in the Japanese population and the two SNPs at *ABO* accounted for 11%. To confirm this finding, we typed SNPs at the *ABO* locus in a cohort of British families previously extensively genotypically characterised at the *ACE* locus. (McKenzie et al., 2001) Measured haplotype analysis in these families indicated that the haplotype characterizing group A1 was associated with the lowest plasma ACE level, the haplotypes characterizing groups O and A2 were associated with intermediate plasma ACE level, and the haplotype characterizing group B was associated with the highest plasma ACE level. The proportions of variance accounted for by the *ACE* and *ABO* loci in the British families were highly concordant with the Japanese cohort (35.4% and 13.0%, respectively) with an additional 9.6% attributable to polygenic effects. The *ACE* and *ABO* loci make the most substantial contribution to population variance in plasma ACE levels; also, there is appreciable heterogeneity in plasma ACE levels among the alleles specifying the two subgroups A1 and A2.

The *ABO* gene encodes a glycosyltransferase. Genetic variation in *ABO* results in the production of two differently named protein products: glycosyltransferase-A and glycosyltransferase-B. Glycosyltransferase-A transfers N-acetylgalactosamine to an acceptor glycoconjugate on the glycosphingolipid H-antigen, which is strongly present on the surface of red blood cells and more weakly present on a wide range of other cell types. Glycosyltransferase-B transfers D-galactose to the same position on the H-antigen. These glycosyltransferase activities define the blood group antigens A and B, respectively. AB heterozygotes have molecules with both A and B antigens present on the red cell surface. Mutations which inactivate the glycosyltransferase encoded by *ABO* result in nonmodification of the H-antigen, which characterises blood group O. A and B alleles are both dominant to O.

Previous studies have shown association between either *ABO* genotypes or ABO blood groups and plasma ACE activity. Cidl et al. found blood groups A and O to have similar levels of plasma ACE activity and groups AB and B to have progressively higher levels among 197 Caucasian subjects of Eastern European origin. (Cidl et al., 1996) Chung et al. performed a GWAS for plasma ACE activity among 1023 subjects with young-onset hypertension, replicating their findings in a

study of 428 hypertension pedigrees, all of self-reported Han Chinese ethnicity (Chung et al., 2010). By contrast with the findings of Cidl et al., these authors found plasma ACE activity in blood group A individuals to be 86% of that in the reference group O, and plasma ACE activity in blood group B individuals to be 114% of that in the reference group O. However, neither of these previous studies carried out measured haplotype analyses to determine the effect of the combination of SNPs defining the principal blood group antigens on plasma ACE levels, and neither conducted formal two-locus genetic analyses to determine the relative contribution of *ABO* haplotypes and *ACE* haplotypes on phenotypic variance. Moreover, the reason for the discordance between the two reports with respect to the relative levels of plasma ACE activity in type A and type O individuals remained unclear. Our findings illustrate significant differences in plasma ACE activity associated with the haplotypes defining the type A subgroups A1 and A2. The A1-defining haplotype had ~60% of the plasma ACE activity of the O-defining haplotype, whereas the plasma ACE activity of the A2-defining haplotype was not significantly different from the O-defining haplotype. In common with the previous studies, we found that the B-defining haplotype was associated with a ~40% higher plasma ACE activity than the O-defining haplotype.

The effects of the different *ABO* haplotypes on plasma ACE activity were assessed in a codominant (allelic association) model. This may explain the substantially smaller proportion of variation that could be accounted for by ABO blood grouping in the study of Cidl et al. (1996), when compared to the present study (since A and B alleles are dominant to O with respect to the determination of blood group). Differences in the proportions of A1 and A2 alleles in the previously studied populations may also contribute to the discrepancy in the results of previous studies: among Asian populations such as those studied by Chung et al. (2010), the A2 allele is extremely rare, while among European populations it comprises up to 25% of all A alleles. The A2 allele shows a weaker activity than the A1 allele in adding N-acetylgalactosamine to the acceptor glycoconjugate of the H antigen, which is present on red blood cells. At the DNA level, the A2 allele differs from the A1 in two ways: a single amino acid change (Pro156Leu) and a 1061delC mutation causing a frameshift that extends the reading frame by 64 nucleotides (Yamamoto et al., 1992).

Recently, several significant associations between the *ABO* locus and complex diseases have been reported. Association between ABO blood group and the risk of myocardial infarction (MI) was first observed decades ago; a GWAS approach recently confirmed strong association between non-O blood group and the risk of MI, although not of coronary artery disease without MI (Reilly et al., 2011). The likely principal mechanism is a differential susceptibility to thrombosis: people of blood group O have 25% lower plasma levels of von Willebrand factor and Factor VIII, both members of the blood coagulation cascade, and SNPs defining group O are associated with less coagulable blood on standard laboratory coagulation tests. (Tang et al., 2012) Congruent with this observation, *ABO* genotypes are the strongest risk factor genome-wide for venous thromboembolism (Qi et al., 2010). Nevertheless, other potential mechanisms whereby the pleiotropic *ABO* locus could contribute to MI risk have been identified through GWAS approaches. *ABO* genotypes are associated with plasma levels of lipids, and of other plasma factors associated with coronary artery disease including soluble ICAM-1, E-selectin and P-selectin (Barbalic et al., 2010; Qi et al., 2010; Pare et al., 2011). GWAS studies have also identified SNPs at *ABO* responsible for the long-standing epidemiological observations of association between blood group O and higher risk of duodenal ulceration; (Tanikawa et al. 2012) and lower risks of gastric and pancreatic cancer (Amundadottir et al., 2009). Plasma ACE activity has not, in general, been systematically studied as a risk factor for any of the conditions associated with ABO blood groups. Since the *ABO* haplotype defining type O in our study was associated with intermediate levels of plasma ACE activity compared with types A and B, it is unlikely that plasma ACE activity lies in the causal pathway for any of these conditions, in which type O lies at the upper or lower extreme of the risk distribution. Further research will be required to define the mechanism whereby *ABO* haplotypes are associated with plasma ACE activity, which remains uncertain. A plausible hypothesis is that it may involve differential affinities of receptors involved in the clearance of ACE, a heavily glycosylated protein, for the A B and H glycosylation motifs that depend upon *ABO* genotype.

Although evidence remains inconclusive, it has been suggested that blood group O protects against severe malaria, and that this may have constituted a selective advantage favouring the alleles responsible for the group O phenotype throughout human evolutionary history (Anstee, 2010). In this regard it is of interest that angiotensin-II has recently been shown to reduce erythrocyte invasion *in vitro* by *P. falciparum* in a dose-dependent manner, and that the deletion allele of the *ACE* insertion/deletion polymorphism, which is associated with higher plasma ACE activity, has been reported to be protective against cerebral malaria (Dhangadamajhi et al., 2010; Saraiva et al., 2011). Further research into the question of whether the major genetic effects on plasma ACE activity at the *ACE* and *ABO* loci contribute significantly to differential susceptibility to severe malaria would be of interest.

In conclusion, this study has confirmed the existence of two major genetic loci influencing plasma levels of ACE, and quantified the contribution of the two loci to the population variability of the trait in two populations. We have also demonstrated previously unrecognised heterogeneity among

alleles responsible for group A antigens with respect to their effect on plasma ACE.

Acknowledgements

The principal acknowledgement is to the participants in the Japanese and British cohorts. We are grateful to the Nagahama City Office and the nonprofit organisation "Zeoji Club" for their help in performing the Nagahama Study. The Nagahama Study is in part supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan; and by the Takeda Science Foundation. The collection and genotyping of the British families was funded by the Wellcome Trust and the British Heart Foundation. BK holds a British Heart Foundation Personal Chair. MF acknowledges support from the Wellcome Trust core award (090532/Z/09/Z) and the BHF Centre of Clinical Research Excellence in Oxford. ML acknowledges support from Genome Québec, le Ministère de l'Enseignement supérieur, de la Recherche, de la Science et de la Technologie (MESRST) Québec and McGill University, and from the Agence Nationale de la Recherche (ANR), France, for the Labex project "Medical Genomics." No author has a conflict of interest to declare in respect of this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Demographics of the Japanese discovery cohort.

Table S2 Demographics of the British replication cohort.

Table S3 Single-SNP analyses of GWAS SNPs at *ACE* and *ABO* loci in the Japanese cohort.

Figure S1 Manhattan plot of GWAS for plasma ACE in Japanese cohort, showing significant evidence of association at the *ACE* locus on Chromosome 17 and the *ABO* locus on Chromosome 9.

Received: 26 February 2013
Accepted: 16 May 2013

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

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

Introduction

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

Subjects and Methods

Study Subjects

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

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http://dx.doi.org/10.1016/j.ajhg.2013.05.024.

Table 1. Summary of Study Subjects

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

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

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

Genome Scanning

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

Quality Control of Genome Scanning

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

Replication Study

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

Combined Study and Association Study for Genotypes

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

Imputation of Genotypes

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

Calculation of Linkage Disequilibrium

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

Estimation of Interaction

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

In Silico Analysis of Association between the Gene Expression and rs6871626

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