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A genome-wide association study in the Japanese population confirms 9p21 and 14q23 as susceptibility loci for primary open angle glaucoma

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Primary open angle glaucoma (POAG) is one of leading causes of adult blindness worldwide. To identify genetic variants associated with susceptibility to POAG, we conducted a genome-wide association study (GWAS) using 1394 cases and 6599 controls. Subsequently, we analyzed 33 single nucleotide polymorphisms (SNPs) which showed suggestive association ($P < 1 \times 10^{-4}$) by GWAS, using an additional set of 1802 cases and 7212 controls. In addition to confirmation of the association of the chromosome 9p21 locus [rs1063192, $P = 5.2 \times 10^{-11}$, odds ratio (OR) = 0.75], and 14q23 (rs10483727, $P = 9.49 \times 10^{-8}$, OR = 0.79) with POAG in Caucasians reported recently, we identified a suggestive-associated locus on 2q21 (rs7588567, $P = 3.89 \times 10^{-7}$, OR = 0.85). For these described SNPs, minor alleles are suspected to have a protective effect from the disease. An linkage disequilibrium block containing rs10483727 includes the *SIX6* gene that was implicated to have a critical role in eye development, and genes in both represented loci (*SIX6* on chromosome 14q23, and *CDKN2A-CDKN2B* on chromosome 9p21) are known to be expressed in human ocular tissues, including the retina. Our GWAS results should contribute to better insight into the genetic basis of POAG.

INTRODUCTION

Glaucoma is one of leading causes of adult visual impairment and irreversible blindness worldwide (1). It is a complex, chronic neurodegenerative optic neuropathy causing progressive degeneration of retinal ganglion cells (RGCs) and their axons along with the supporting glia cells and vasculature, finally resulting in a thinning of the neuro-retinal rim of the optic nerve and visual-field defect. Primary open angle glaucoma (POAG; MIM # 137760) is the most common form of the disease.

A large cohort study in Tajimi, Japan, implicated the prevalence of glaucoma and POAG to be ~5.0 and 3.9%, respectively, in individuals of >40 years old (2). POAG is considered to have a multifactorial etiology and was reported that black race, older age, elevated intra ocular pressure, central corneal thickness, myopia and systemic diseases such as diabetes and hypertension are factors increasing the risk of the disease (3). In addition, the family history of POAG is a well-known risk factor as first-degree relatives of patients have 3–9-folds higher risk than the general population (4). Among more than 20 genetic loci that have been suggested by linkage analyses (5), three genes encoding myocilin (*MYOC*) (6), optineurin (*OPTN*) (7) and WD-repeat domain 36 (*WDR36*) (8) have been identified as causes of the disease, although mutations in these genes were found to contribute to a small subset of the cases (9). Recently, genome-wide association studies (GWASs) for POAG in the Japanese population as well as for those in individuals with European ancestry were reported (10–13). Variants reported in two Japanese studies did not reach to the genome-wide significant level and have not been replicated in other populations. Single nucleotide polymorphisms (SNPs) in caveolins 1 and 2 (*CAV1-CAV2)* on chromosome 7q31 found in Caucasians are not polymorphic in the Japanese population. Furthermore, variants in *TMCO1* (transmembrane and coiled-coiled domains 1) on 1q24 and *CDKN2B-AS* on 9p21 have not been studied in other populations.

The aim of this study is to identify the genetic variations contributing to the susceptibility of the POAG in the Japanese population, incorporating a total of 3196 cases and 13 811 controls.

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RESULTS

To screen genetic variants contributing to the susceptibility of POAG in the Japanese population, we conducted a GWAS using BioBank Japan samples of 1394 POAG cases and 6599 control subjects, who genotyped with Illumina HumanOmniExpress BeadChip (see Materials and Methods, Supplementary Material, Table S1). Following applying stringent quality control (QC) filtering (see Materials and Methods), 602 216 SNPs were selected for further association analysis with POAG. Principal component analysis (PCA) indicated that all cases and controls were of Asian ancestry (Supplementary Material, Fig. S1A) and >99% of the cases and controls were clustered within either of the two known major clusters of the Japanese population (14) (Supplementary Material, Fig. S1B). Since quantile–quantile plot analysis using all the samples revealed the genomic inflation factor (λ_{GC}) of 1.30, we applied a logistic regression model incorporating age, gender and the first two eigenvectors as covariates for the analysis, in addition to apply the genomic control method (15) ($\lambda_{GC} = 1.00$; Supplementary Material, Fig. S2A).

The association analysis indicated that one SNP, rs7588567, on chromosome 2q21 was within the GWAS-significance level ($P = 4.79 \times 10^{-8}$), in addition to another 57 SNPs to be possibly associated with POAG ($P < 1 \times 10^{-4}$) as shown in Supplementary Material, Figure S2B. After examining the linkage disequilibrium (LD) of these SNPs, we further selected 33 SNPs for replication studies (Supplementary Material, Table S2) using an additional set of independent 1802 POAG cases and 7212 controls collected from BioBank Japan (Supplementary Material, Table S1). Among 33 examined SNPs, 2 SNPs, rs1063192 in the *CDKN2B* gene on chromosome 9p21 and rs10483727 on 14q23, were significantly replicated following the Bonferroni correction of multiple testing (P-value < 0.0015), and both indicated stronger statistical association following combined analyses of the GWAS and the replication study [$P_{meta} = 5.2 \times 10^{-11}$, odds ratio (OR) = 0.75 and $P_{meta} = 9.49 \times 10^{-8}$, OR = 0.79, respectively] (Fig. 1 and Table 1). No significant heterogeneity was observed following combining the GWAS and replication data for both SNPs (Table 1). For these two SNPs, the minor alleles are considered to be protective from the disease and risk alleles seem to have the additive effect in the development of the disease. The SNP rs7588567 on 2q21 revealed no association in the replication analysis ($P = 0.07$), and indicated a suggestive association following meta-analysis ($P = 3.89 \times 10^{-7}$), although a significant evidence of heterogeneity was observed between the GWAS and replication sets for this SNP (Table 1).

Since the disease-mix controls in our GWAS included cases of intracranial aneurysm (IA), one of diseases being associated with the 9p21 locus (16), we performed the analysis of the SNP rs1063192 after exclusion of IA samples from the controls, and found stronger association of this SNP with the POAG ($P = 8.00 \times 10^{-12}$; Supplementary Material, Table S3), implying that our results are unlikely to be false-positive.

A recent report suggested imputation analysis using the 1000 Genomes Project resource is sufficient to capture most of SNPs with frequency >5%, and provides a relevant method for fine-mapping of the GWAS data (17). Therefore, we performed imputation-based fine-mapping for the promising loci; 9p21, 14q23 and 2q21 using the data of the East-Asian populations in the 1000 Genomes Project as a reference (Materials and Methods). For the 9p21 locus, a comparative evidence of association to rs1063192 was observed for rs10120806 in *CDKN2B-AS* (Fig. 1A and Supplementary Material, Table S4), confirming *CDKN2B-AS* is another possible candidate for the disease in the 9p21 region as reported (13). Several highly linked SNPs in the 14q23 region revealed comparative associations with rs10483727, including the missense variant rs33912345 in the *SIX6* gene and rs6573307 which is located within the 32 kb distance from *PPM1A* (protein phosphatase, Mg2+/Mn2+ dependent, 1A) (Fig. 1B and Supplementary Material, Table S4).

We also examined the associations of SNPs in *TMCO1*, *MYOC*, *OPTN, WDR36* and *CAV1-CAV2* genes that were previously reported as candidates for POAG. Although the reported SNPs in *CAV1-CAV2* in the Icelandic population (rs4236601 and rs1052990) (12) are not polymorphic in the Japanese population, other SNPs in this locus were replicated in a comparable OR (top SNP rs7795356; $P = 0.0073$, OR = 1.31) (Supplementary Material, Table S5). Rs4656461 (the reported variant in *TMCO1*) did not pass the QC criteria in this GWAS, and no proxy SNP was included. For other genes, no SNP revealed significant association with POAG ($P > 0.05$) as summarized in Supplementary Material, Table S5. In addition, using our GWAS set, the results of SNPs reported in the Japanese population by Nakano *et al.* (10) (rs7081455), and Meguro *et al.* (11) (rs735860), were not replicated ($P = 0.16$, 0.93, respectively) in association with POAG.

DISCUSSION

We performed a GWAS and replication analyses for POAG in the Japanese population, and identified two susceptibility loci.

For the most significantly associated locus on chromosome 9p21, a SNP rs1063192 is located within a gene encoding cyclin-dependent kinase inhibitor 2B (*CDKN2B*) which is also known as *p15*. Notably, an LD including this SNP contains three additional genes; *CDKN2A* (also known as *p16*), *p14/ARF* (encoded by alternate reading frame) as well as *CDKN2B-AS* (CDKN2B antisense RNA, and is also known as *ANRIL*) that is transcribed in the antisense direction of p14, p15 and p16, and emerged as a possible candidate for the disease using a 1000 Genomes imputation-based mapping in this study (Fig. 1A). *CDKN2A* and *CDKN2B* are well-known tumor suppressor genes involved in the retinoblastoma (Rb) pathway, and *ANRIL* is a long non-coding RNA which is thought to regulate the transcription of the *p14/p15/p16* genes. This region on chromosome 9p21 was implicated as a hotspot locus showing the association with various diseases, including IA (16,18), coronary artery disease (19,20), type 2 diabetes (21–23), glioma (24) and endometriosis (25). The SNP rs1063192 was reported as being associated with glioma, but the C allele that was indicated to be a risk allele for glioma is considered to be protective from POAG in this study. The association of rs1063192 in

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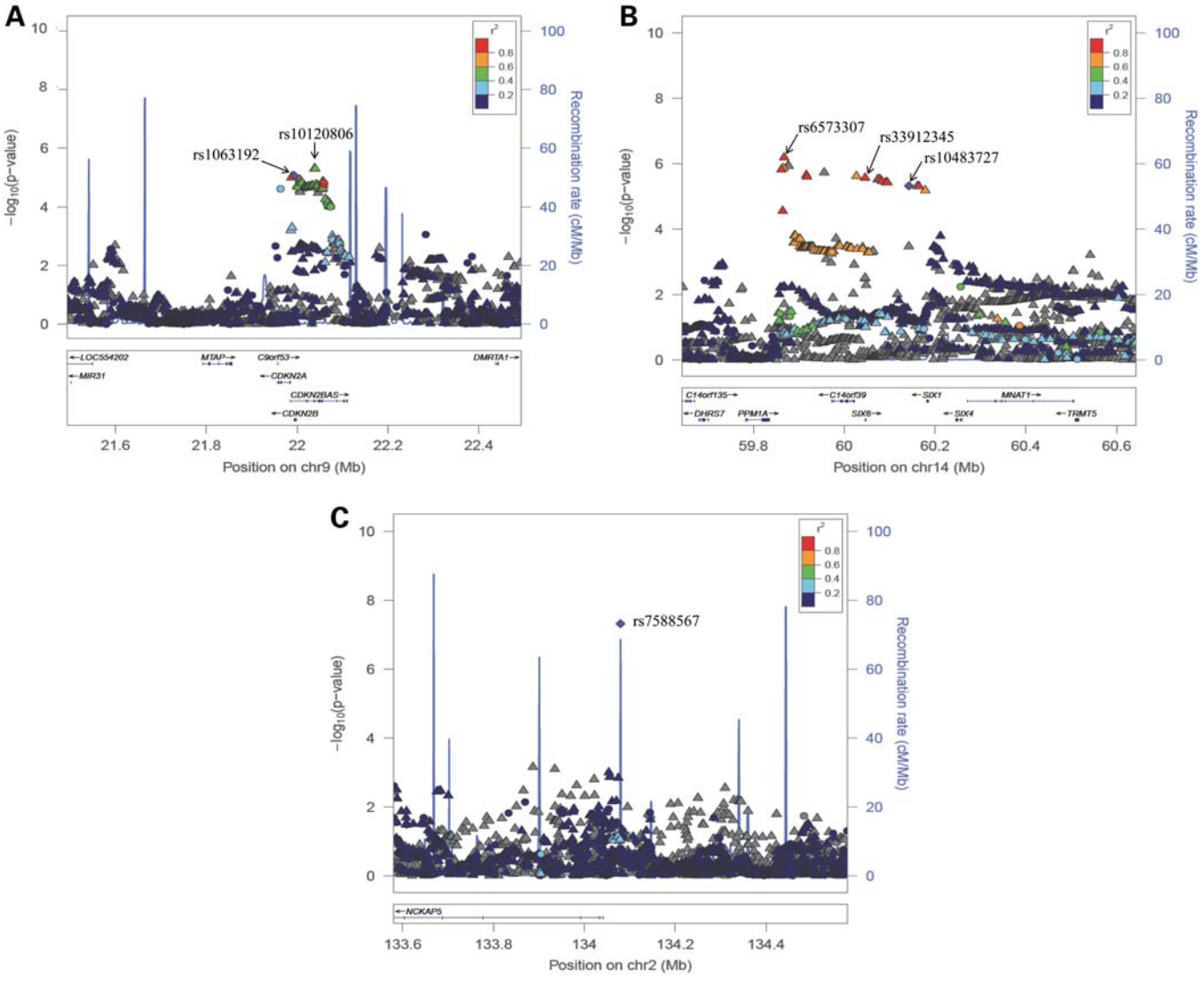

Figure 1. Regional plots for association of genotyped and imputed SNPs in the GWAS stage with POAG. SNPs plotted with their −log10 *P*-values of the logistic regression test (age, gender, first two eigenvectors and GC-adjusted) against their physical chromosomal positions (NCBI Build 36). Genotyped SNPs are indicated by circles, while imputed SNPs are indicated as triangles. The color scheme indicated the LD displayed as r^2 values between all SNPs and the top-ranked SNP in each plot. Top-ranked SNPs are shown as purple diamonds in the top of each chromosomal locus; (**A**) 9p21, (**B**) 14q23 and (**C**) 2q21.The blue lines represent the estimated recombination rates.

9p21 became stronger after exclusion of IA cases from the controls (Supplementary Material, Table S3); hence, our results represent most likely a true association of this region with POAG. A recent GWAS report for the optic disc parameters indicated significant association of rs1063192 with the vertical cup-disc ratio (VCDR) and POAG in the Caucasian population (26), which was supported by further reports (27,28). *CDKN2B* and *CDKN2A* genes are expressed in human ocular tissues including the RGCs, and were found to be upregulated in response to elevation of intraocular pressure (13). Although the SNP rs1063192 is located in a region that was predicted to be a binding site of a regulatory micro-RNA (miRNA) in the 3′UTR of *CDKN2B* in the SNPinfo database (29), further functional analysis for this region is required to identify the causative variant(s). However, our data at least provide further evidence for the association of the 9p21 region with POAG in Asians as well.

For the second locus in the 14q23 region, a SNP rs10483727 (combined $P = 9.49 \times 10^{-8}$, OR = 0.79) is located between two homologous genes belonging to the SIX/Sine oculis homeobox family; *SIX1* and *SIX6* (also known as *Optx2*), and is located ∼94 kb downstream from the *SIX6* gene. The comparative mapping analysis between the marker SNP and imputed SNPs from the 1000 genomes project revealed several SNPs with comparable associations in or around *SIX6* (Fig. 1B and Supplementary Material, Table S4), including the SNP rs33912345 ($r^2 =$ 0.92 with the SNP rs10483727), that is considered to be a possible functional candidate. Rs33912345 causes an amino acid substitution of codon 141 of the SIX6 protein which

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Table 1. Summary results of SNPs associated with POAG in GWAS and replication samples

SNP ID A1/A2	Chr:position	Locus, candidate	Stage ($N_{case}/N_{control}$)*	MAF Case	Control	P*	OR (95% CI)	P_{het}**
rs1063192 (C/T)	9:21,993,367	9p21.3, *CDKN2B*	GWAS (1393/6591)	0.169	0.206	5.68×10^{-5}	0.79 (0.71–0.86)	
			Replication (1800/7207)	0.161	0.214	1.73×10^{-7}	0.73 (0.64–0.82)	
			Meta-analysis	0.164	0.210	5.20×10^{-11}	0.75 (0.70–0.82)	0.16
rs10483727 (C/T)	14:60,142,628	14q23.1, *SIX1–SIX6*	GWAS (1394/6598)	0.198	0.238	4.68×10^{-5}	0.80 (0.72–0.89)	
			Replication (1798/7209)	0.200	0.226	5.00×10^{-4}	0.82 (0.73–0.92)	
			Meta-analysis	0.200	0.232	9.49×10^{-8}	0.79 (0.73–0.85)	0.21
rs7588567 (A/G)	2:134,079,502	2q21.2, *NCKAP5*	GWAS (1390/6569)	0.363	0.417	4.79×10^{-8}	0.78 (0.72–0.85)	
			Replication (1801/7141)	0.394	0.411	0.07	0.92 (0.83–1.00)	
			Meta-analysis	0.380	0.414	3.89×10^{-7}	0.85 (0.80–0.91)	0.0062

A1/A2, minor/major alleles; MAF, minor allele frequency.
*P-values in the GWAS stage were age, gender, first two eigenvectors and GC-adjusted; P-values in the replication stage were age and gender adjusted, and the inverse-variance method was used for meta-analyses.
**P-value of heterogeneity of the Breslow–Day test.

causes the loss of positive charge (a histidine residue with a positive charge is replaced with an asparagine residue with a negative charge), although the significance of this substitution on the protein function needs to be clarified. The *SIX6* mRNA was reported to be expressed in the retina, optic nerve and brain (30,31). Interestingly, mutations in this gene have been found in patients with defects in eye development (32). Furthermore, the SNP rs10483727 on 14q23 reported to be associated with VCDR (26) and POAG in the Caucasian population (27,28). The mapping analysis indicated that the association in this locus is extended to rs6573307 ($r^2 = 0.92$ with rs10483727) which is located within 32 kb distance from *PPM1A* (protein phosphatase, Mg2+/Mn2+ dependent, 1A) (Fig. 1B and Supplementary Material, Table S4). The protein encoded by this gene is a member of the PP2C family of Ser/Thr protein phosphatases. PP2C family members are known to be negative regulators of cell stress response pathways, and PPM1A is known to terminate TGF-β signaling through Smad phosphatase (33). As most of the known genes involved in the control of glaucoma-related traits belong to developmental and TGF-β signaling pathways (27,34), *PPM1A* constitutes another candidate in this region. Nonetheless, further in-depth functional analysis is needed to identify causative variant(s) in this region. Our data confirmed the 14q23 locus as a common susceptibility locus for POAG in different ethnic populations.

For the third possibly associated locus on 2q21.2, a SNP rs7588567 (combined $P = 3.89 \times 10^{-7}$, OR = 0.85) is located ~37 kb upstream to the *NCKAP5* (Nck-associated protein 5) gene (Fig. 1C). The expression of the NCKAP5 protein was detected in fetal and adult brain, leukocytes and fetal fibroblasts (35). It was shown to be interacting with an SH3 (Src homology region 3) domain of the adaptor protein Nck (35). Chromosome 2q21.2 locus was identified to be associated with optic disc area (34,36). The marker rs7588567 is located ~3.3 Mb forms the suggestive linkage marker (rs113906) described by Axenovich *et al*. (34). The association of 2q21.2 with POAG needs further validation, owing to the nature of the single-marker association presented here, which might be due to the high recombination rates in this locus (Fig. 1C).

We also replicated the association of *CAV1-CAV2* on chromosome 7q31 with POAG in the Japanese population. In our GWAS, rs7795356 ($P = 0.007$, OR = 1.31) (Supplementary Material, Table S5) indicated a comparable OR with the original report in the Icelandic population (12) and a USA cohort (37), although this SNP exhibited no linkage with the marker rs42336601 described in the Icelandic population by Thorleifsson *et al*. (12). Another SNP, rs959173 ($P = 0.027$, OR = 1.25; Supplementary Material, Table S5), was reported to be a part of a haplotype that was significantly associated with POAG (37). Although we could not genotype rs7795356 in our replication cohort due to difficulty in making a suitable Invader assay, these results at least provide an evidence for *CAV1-CAV2* as a candidate for POAG in Asians.

We estimated the cumulative risk effect of the two SNPs described above by counting the number of risk alleles of each SNP by assumption of the additive effect model and found that individuals with four risk alleles have 2.26 times higher risk for the disease than those with 0–1 risk alleles (Supplementary Material, Fig. S3).

In conclusion, we here reported results of the GWAS and replication study for POAG using a total of 3196 cases and 13 811 controls in the Japanese population. This report confirmed the 9p21 and 14q23 loci as common susceptibility loci for POAG among different ethnic populations. We showed that minor alleles of each of the representative SNPs are likely to be protective for the disease. Further in-depth functional analyses of these loci are needed to identify causative variants and clarify their contribution to the disease. Our data should give more insight for better understanding of the pathogenesis of POAG.

MATERIALS AND METHODS

Subjects

Characteristics of study groups are shown in Supplementary Material, Table S1. All case and control subjects were collected in BioBank Japan that was supported as the Japan leading project for personalized medicine from the Ministry of Education, Culture, Science, Sports, and Technology (38). For the GWAS, 1394 subjects who were diagnosed as

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POAG were selected. The diagnosis of POAG was judged by expert ophthalmologists in the 66 collaborating network hospitals according mainly to the glaucomatous optic neuropathy with corresponding glaucomatous visual-field deficit caused by the disease assessed using different perimeters. The following examinations were also included for diagnosis: slit-lamp microscopy, ophthalmoscopic examination of the optic disc and retinal nerve fibers layer, measuring of the VCDR, tonometry for measuring the intraocular pressure and gonioscopy for assessment of the iridocorneal angle which should necessarily to be open as inclusion criteria. Individuals with secondary, close angle or congenital glaucoma were not included in this study. We obtained control data for GWAS from the genome scanning of other five cohorts in BioBank Japan (Supplementary Material, Table S1). All control subjects were not known to have glaucoma according to their clinical records. For a replication study, another cohort of 1802 POAG cases was obtained also from BioBank Japan. Controls for the replication study were obtained from five different cohort sets as shown in Supplementary Material, Table S1. All participants provided a written informed consent for this study. The study was approved by the ethical committees at the Institute of Medical Science, the University of Tokyo, and the Center for Genomic Medicine, RIKEN, Yokohama Institute, Japan.

SNP genotyping

For the GWAS stage, DNA from POAG cases and disease-mix controls were genotyped using Illumina HumanOmniExpress BeadChip (Illumina, CA, USA). The BeadChip contains 733 202 SNPs, among which 712 726 SNPs were located in autosomal chromosomes. Non-autosomal SNPs (20 476) and non-polymorphic loci in our samples (65 799 loci) were excluded from the subsequent analysis. A total of 602 216 SNPs in autosomal chromosomes passed the QC measures indicated as follows: call rate ≥ 0.99 in both cases and controls, exact *P*-value of the test of the Hardy–Weinberg equilibrium $\geq 1.0 \times 10^{-6}$ in controls, and minor allele frequency ≥ 0.01. Cluster plots of the top 100 SNPs showing the smallest *P*-values were checked by visual observation and 4 SNPs were excluded from further analysis because of ambiguous cluster plots. Genotype data were analyzed for screening of duplicate and closely related samples in the GWAS stage using identity-by-state approach in PLINK 1.0.6. To exclude individuals who do not belong to the Japanese ancestry, we applied EIGENSTRAT 3.0 to perform PCA using the case–control samples in this GWAS.

In replication analyses, we applied the multiplex polymerase chain reaction based Invader Assay method (39) for genotyping the selected SNPs in the case samples, and genotypes were called by visual inspection following application of QC measures of individual call rates >98%, and SNP call rates >99%. Control samples for the replication study were genotyped using the Illumina HumanOmniExpress BeadChip, and the same QC filters were applied.

Statistical analysis

In the GWAS and replication analyses, we used the logistic regression test to assess the statistical significance of each SNP (age, gender and the first two eigenvectors were incorporated as covariates) in addition to applying a genomic control method. We also applied the logistic regression model incorporating age and gender as covariates for the replication and combined analyses. The significance levels used were 1.5×10^{-3} (0.05/33) for the replication stage and 8×10^{-8} (0.05/602 216) in the combined analysis following the Bonferroni correction for multiple testing. Meta-analysis of the two sets of data was performed using the inverse-variance method and heterogeneity between GWAS and replication cohorts were tested using the Breslow–Day test. ORs and 95% confidence intervals were calculated using the minor allele genotype as a reference.

LD analyses were performed using PLINK 1.0.6, Haploview software version 4.2 and SNAP database, meta-analyses were performed using Metal software, and regional association plots were generated using Locus Zoom (see web resources).

Imputation analyses

Imputation for prediction of un-genotyped SNPs in the case–control GWAS samples on chromosomes 2, 9 and 14 using MACH program version 1.0.16 (see web resources). The references for the analyses were the observed genotypes in the GWAS stage, and the data of JPT + CHB in the 1000 Genomes Project, release 2010 June. Dosage score for the imputation analysis was obtained using MACH2DAT (40). QC for imputation was done by exclusion of SNPs with estimated square correlation (r^2) between imputed genotypes and actual genotypes of <0.5.

AUTHOR CONTRIBUTIONS

Y.N. obtained the fund and conceived the study; Y.N., W.O. and M.K. designed the study; M.K. supervised the selection of individuals and genotyping of the SNPs at the GWAS level, where W.O. selected SNPs for replication and performed the genotyping in the replication study; A.T. performed the quality assessment and statistical analysis for the GWAS data; W.O. and S.-K.L. participated in the experiment design and performed the analysis for the replication and imputation data; Y.N. and M.K. manage the samples at the BioBank Japan; W.O. summarized overall results and wrote the manuscript and Y.N. critically reviewed and edited it.

WEB RESOURCES

BioBank Japan Project, http://biobankjp.org/.

R statistical environment, http://www.r-project.org/.

EIGENSTRAT software, http://genepath.med.harvard.edu/~reich/EIGENSTRAT.htm.

The International HapMap Project, http://www.hapmap.org/.

MACH software, http://www.sph.umich.edu/csg/abecasis/MaCH/index.html.

Metal software, http://www.sph.umich.edu/csg/abecasis/Metal/index.html.

PLIKN software, http://pngu.mgh.harvard.edu/~purcell/plink/.

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Locus Zoom, http://csg.sph.umich.edu/locuszoom/.
SNAP, http://www.broadinstitute.org/mpg/snap/ldsearch.php.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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