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involvement of *FGD4* in CMT disease, a congenital peripheral neuropathy, provides strong evidence for the biologic significance of this finding. The fact that a common *FGD4* SNP is associated with an increased risk of paclitaxel-induced sensory peripheral neuropathy in patients with both European and African ancestry makes it of potentially broad clinical significance. Additional SNPs in *EPHA5* and *FZD3* were also identified as potential risk factors for the onset and severity of sensory peripheral neuropathy. Additional samples for extension and validation of these findings are currently being collected in ongoing CALGB clinical trials of paclitaxel in the setting of metastatic breast cancer.

Disclosure of Potential Conflicts of Interest

The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute. No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE

A genome-wide association study identifies SNP in *DCC* is associated with gallbladder cancer in the Japanese population

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Gallbladder cancer (GC) is a relatively uncommon cancer with higher incidence in certain areas including Japan. Because of the difficulty in diagnosis, prognosis of GC is very poor. To identify genetic determinants of GC, we conducted a genome-wide association study (GWAS) in 41 GC patients and 866 controls. Association between each single-nucleotide polymorphism (SNP) with GC susceptibility was evaluated by multivariate logistic regression analysis conditioned on age and gender of subjects. SNPs that showed suggestive association ($P<1\times10^{-4}$) with GC were further examined in 30 cases and 898 controls. SNP rs7504990 in the *DCC* (*deleted in colorectal cancer*, 18q21.3) that encodes a netrin 1 receptor achieved a combined *P*-value of 7.46×10^{-8} (OR=6.95; 95% CI=3.43–14.08). Subsequent imputation analysis identified multiple SNPs with similarly strong associations in an adjacent genomic region, where loss of heterozygosity was reported in GC and other cancers. Reduced expression of *DCC* was indicated to be associated with the poorly differentiated histological type, increased proliferation and metastasis through loss of adhesiveness. However, due to the limited sample size investigated here, further replication study and functional analysis would be necessary to further confirm the result of the association.
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Keywords: *DCC*; GWAS; gallbladder cancer; SNP

INTRODUCTION

Gallbladder cancer (GC) is the most common malignancy of the biliary tract and the fifth most common cancer of the gastrointestinal tract.[1] With an overall 5-year survival rate of <5%, GC is a highly lethal malignancy with very poor prognosis.[2] Most of GCs were diagnosed at a very late stage because of the lack of symptoms and non-specific symptoms of early-stage tumors.[1,3] Although the incidence of GC is relatively rare compared with other cancers, higher incidences of GC have been reported in certain geographical regions including India, Pakistan, Ecuador, Korea and Japan.[4] In addition, the prevalence of GC is known to be three times higher in women than in men.[5] Although several clinical risk factors of GC such as gallstones, cholecystitis, porcelain gallbladder, gallbladder polyps, anomalous panreatobiliary duct junction and obesity have been indicated,[1,6] etiology of GC is largely unknown. Only few somatic genetic changes including mutations in K-ras, TP53 and $p16^{Ink4}$/*CDKN2* as well as loss of heterozygosity at several chromosomal regions harboring known or putative tumor-suppressor genes have been reported in GCs.[5,7] Hence, identification of novel genetic factors associated with susceptibility to GC should provide new insights into pathogenesis and novel therapeutic interventions of GC. Here, we report a genome-wide association study (GWAS) that aims to identify genetic factors associated with GC susceptibility.

MATERIALS AND METHODS

Subjects

In the GWAS, 41 patients who were diagnosed to have GC and 866 control subjects were examined. All cases were registered into Biobank Japan supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan (http://www.biobankjp.org/). Controls consisted of healthy volunteers from Osaka-Midosuji Rotary Club. In the replication study, 30 GC patients and 898 controls were investigated. All cases and controls in the replication study were obtained from Biobank Japan. All control subjects do not have medical history of GC or gallstones, cholecystitis and other known confounding diseases for GC. Among the 71 cases, 45 were adenocarcimas and one was a squamous cell carcinoma, and the histological information for the remaining ones was not obtained. All subjects had given written informed consent to participate in the study in accordance with the process approved by Ethical Committee at the Institute of Medical Science of the University of Tokyo and Center for Genomic Medicine of RIKEN. Demographical information of subjects was summarized in Supplementary Table 1.

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Genotyping and quality control

All cases and controls in the GWAS were genotyped by using the Illumina HumanHap550 Genotyping BeadChip (San Diego, CA, USA). QC of genotyping data was performed whereby subjects with a call rate of <98%, single-nucleotide polymorphisms (SNPs) with a call rate of <99% or minor allele frequency of <0.01, as well as SNPs with a Hardy–Weinberg equilibrium test's *P*-value of $<1\times10^{-6}$ were excluded from the subsequent statistical analysis. In the replication study, cases and controls were genotyped by using the Illumina HumanHap610 Genotyping BeadChip (San Diego).

Statistical analysis

Association of each SNP with susceptibility to GC was evaluated by logistic regression analysis conditioned on age and gender of subjects. The significance of association was evaluated based on the minimum *P*-value among the additive, dominant and recessive model of inheritance. SNPs that showed a minimum *P*-value of $<1\times10^{-4}$ in the GWAS were considered as showing suggestive association with GC and were examined in additional subjects. QC and statistical analysis were performed by using the PLINK statistical software (v1.06) (http://pngu.mgh.harvard.edu/~purcell/plink/).[8]

Imputation

Imputation analysis was performed based on genotype of Japanese (JPT) individuals in the Phase II HapMap database (release 24) by using software MACH v1.0 (http://www.sph.umich.edu/csg/yli/mach/index.html).[9] SNPs located in the genomic region within 1500 kb upstream and downstream of the marker SNP, which showed the strongest association with GC, was imputed by implementing 50 Markov Chain iterations. As a QC measure, imputed SNPs with an imputation quality score of <0.3 were excluded from the subsequent association analysis. Pair-wise linkage disequilibrium (r^2) between each SNP with the marker SNP was estimated by using the PLINK statistical software (v1.06); whereas regional association plot was generated by the R program v2.10.0 (http://www.r-project.org/). Possible functional consequences of SNPs were predicted *in silico* by using the SNPinfo web server (http://manticore.niehs.nih.gov/cgi-bin/snpinfo/snpfunc.cgi). eQTL analysis was performed based on data available from the Sanger Institute GENEVAR project[10] for lymphoblastoid cell lines from the four HapMap populations.

RESULTS

In this GWAS, 425 706 SNPs with a total genotyping rate of >99% in 41 GC patients and 866 controls were analyzed after QC of the genotyping data. A genomic inflation factor (λ) in the quantile–quantile plot (Supplementary Figure 1a) was calculated to be 0.9903, implying low possibility of false-positive associations resulting from the population stratification or cryptic relatedness. The Manhattan plot (Supplementary Figure 1b) indicated that none of the genotyped SNPs achieved genome-wide significant association ($P<1\times10^{-7}$) with GC. However, 130 SNPs showed suggestive association with a minimum *P*-value of $<1\times10^{-4}$. These SNPs were examined in the additional 30 GC patients and 898 controls, and the genotyping results are shown in Supplementary Table 2. Among these SNPs, SNP rs7504990 that is located in the *DCC* (*deleted in colorectal cancer*) achieved a minimum *P*-value of 9.67×10^{-5} under the recessive model of inheritance in the replication study. As illustrated in Table 1, this SNP achieved a *P*-value of 7.46×10^{-8} (OR=6.95; 95% CI=3.43–14.08) in the combined study. Genotype AA the risk genotype, was found to be enriched in cases than in the controls, with a genotype frequency of 0.19 versus 0.04. Interestingly, the SNP that showed the second lowest *P*-value, SNP rs4078288, is also located in the *DCC* (Table 1). To further characterize the association of the *DCC* region with the GC risk, genotypes of SNPs located in the genomic region within 1500 kb upstream and downstream of the marker SNP rs7504990 were imputed. Association analysis for the imputed and genotyped SNPs with GC susceptibility was then conducted. Figure 1 illustrates the regional plot for association results of the investigated

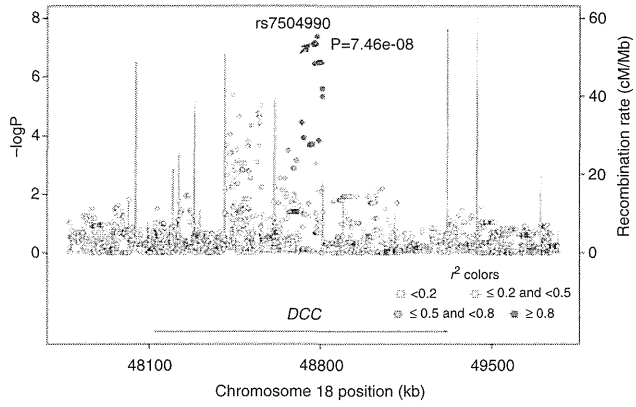

Figure 1 Regional plot for associations of SNPs located within 1500 kb upstream and downstream of the marker SNP, rs7504990, with gallbladder cancer (GC). The $-\log_{10}(P)$ values of SNPs were plotted against relative chromosomal locations. Diamond and circle signs represent genotyped and imputed SNPs, respectively. All SNPs are color-coded as red (0.8–1.0), orange (0.5–0.8), yellow (0.2–0.5) and white (<0.2), according to their pairwise r^2 to the marker SNP. The marker SNP is indicated by an arrow and the combined *P*-value of the marker SNP is represented by a blue diamond sign. SNP positions followed NCBI build 36 coordinates. Estimated recombination rates (cM/Mb) were plotted in dark blue line.

SNPs. We observed that multiple SNPs in the genomic region adjacent to the marker SNP also showed associations, as strong as or stronger than the marker SNP, with GC susceptibility. SNPs that showed *P*-values of $<1\times10^{-6}$ with GC susceptibility were listed in Supplementary Table 3. All these SNPs are located in intronic regions of the *DCC*. Online prediction by using the SNPinfo web server (http://manticore.niehs.nih.gov/cgi-bin/snpinfo/snpfunc.cgi) revealed that none of these SNPs was likely to alter transcription factor binding site. In addition, none of them was linked to exonic SNPs of the *DCC* with an r^2 value of 0.8 or over. We examined eQTL data for all SNPs in Supplementary Table 3 that showed strong association with GC ($P<1\times10^{-6}$) and found that none of them potentially altered expression of *DCC* in the four investigated HapMap populations.

DISCUSSION

Through the current GWAS and the subsequent replication study, we report here that SNP rs7504990 in the *DCC* showed genome-wide significant association with GC susceptibility ($P_{Combined}=7.46\times10^{-8}$; OR=6.95; 95% CI=3.43–14.08) in the Japanese population. Subsequent imputation and association analysis revealed additional SNPs located adjacent to the marker SNP rs7504990 in the *DCC* to be associated with GC susceptibility. Regional association plot indicated that SNPs showing strong association with GC are located in one linkage disequilibrium block that was not disrupted by recombination event. Results from subsequent *in silico* analysis and eQTL analysis are concordant, whereby none of these SNPs potentially alter transcription factor binding site nor possibly alter the expression of *DCC*.

DCC encodes a netrin 1 receptor, which is a transmembrane protein that is a member of the immunoglobulin superfamily of cell adhesion molecules.[11] Loss of heterozygosity and microsatellite instability in the chromosome 18q21 region that contains the *DCC* have been observed in multiple cancer types, particularly cancers in the digestive organ including colon,[12] stomach,[13] esophagus,[14] pancreas,[15] and gallbladder.[16] In addition, reduced or loss of the *DCC* expression has been associated with the poorly differentiated histological type, increased proliferation and metastasis through the loss of adhesiveness.[17]

Table 1 SNPs in *DCC* associated with GC

Chromosome no.	Chromosome location	SNP	A1	Stages	Cases p(11)	Cases p(12)	Cases p(22)	Cases MAF	Controls p(11)	Controls p(12)	Controls p(22)	Controls MAF	Minimum P	OR	L95	U95
18	48771774	rs7504990	A	GWAS	7	11	23	0.30	28	297	533	0.21	9.84E-05	6.99	2.63	18.58
				Replication	6	12	8	0.46	38	272	541	0.20	9.67E-05	8.13	2.83	23.30
				Combined	13	23	31	0.37	66	569	1074	0.21	7.46E-08	6.95	3.43	14.08
18	48801249	rs4078288	G	GWAS	7	11	23	0.30	27	291	539	0.20	5.00E-05	7.71	2.87	20.67
				Replication	5	13	8	0.44	38	270	543	0.20	8.70E-04	6.63	2.18	20.17
				Combined	12	24	31	0.36	65	561	1082	0.20	3.19E-07	6.66	3.22	13.77

Abbreviations: A1, minor allele; CHR: chromosome; GC, gallbladder cancer; L95, lower boundary of 95% confidence interval; MAF, minor allele frequency; OR, odds ratio; p(11), number of subjects with homozygous genotypes for minor allele; p(12), number of subjects with heterozygous genotypes; p(22), number of subjects with homozygous genotypes for major allele; SNP, single-nucleotide polymorphism; U95, upper boundary of 95% confidence interval.

Although the pathophysiological role of *DCC* in gallbladder carcinogenesis has not been clarified, *DCC* was demonstrated to induce apoptosis in the absence of its ligand netrin-1.[18] Furthermore, enforced expression of netrin 1 in mouse gastrointestinal tract has been found to induce spontaneous formation of hyperplastic and neoplastic lesions, highlighting the potential role of *DCC* as a tumor-suppressor gene.[19]

Our finding that SNPs in *DCC* were associated with GC susceptibility supports findings of several recent studies, which also pointed out a tumor-suppressing role of the gene in gallbladder carcinogenesis. For instances, previous reports have revealed the incidences of loss of heterozygosity at *DCC* in GC to be as high as 30–45%.[16,19,20] Chromosomal loss in human genome often implies that the affected region may harbor a tumor-suppressor gene, where the loss of which could lead to carcinogenesis. Nevertheless, both *in silico* and eQTL analysis indicated that SNPs in *DCC* showed no association with expression of the gene, implying other unknown mechanisms might be in action. Subsequent fine-mapping and resequencing for this region and functional analysis would be necessary to clarify association between *DCC* and GC.

In the current study, because of the limited number of subjects examined, we might not have enough statistical power to detect other genetic variants with modest or weak effects on susceptibility to GC. Hence, some SNPs of clinical importance might be missed in this study. Further replication study involving a larger number of samples and functional analysis of the *DCC* is urgently needed for validation of the association of *DCC* with the risk of GC.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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A genome-wide association study identifies four genetic markers for hematological toxicities in cancer patients receiving gemcitabine therapy

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Objective **Genetic factors are thought to be one of the causes of individual variability in the adverse reactions observed in cancer patients who received gemcitabine therapy. However, genetic factors determining the risk of adverse reactions of gemcitabine are not fully understood.**

Patients and methods **To identify a genetic factor(s) determining the risk of gemcitabine-induced leukopenia/neutropenia, we conducted a genome-wide association study, by genotyping over 610 000 single nucleotide polymorphisms (SNPs), and a replication study in a total of 174 patients, including 54 patients with at least grade 3 leukopenia/neutropenia and 120 patients without any toxicities.**

Results **We identified four loci possibly associated with gemcitabine-induced leukopenia/neutropenia [rs11141915 in *DAPK1* on chromosome 9q21, combined $P=1.27\times10^{-6}$, odds ratio (OR)=4.10; rs1901440 on chromosome 2q12, combined $P=3.11\times10^{-6}$, OR=34.00; rs12046844 in *PDE4B* on chromosome 1p31, combined $P=4.56\times10^{-5}$, OR=4.13; rs11719165 on chromosome 3q29, combined $P=5.98\times10^{-5}$, OR=2.60]. When we examined the combined effects of these four SNPs, by classifying patients into four groups on the basis of the total number of risk genotypes of these four SNPs, significantly higher risks of gemcitabine-induced leukopenia/neutropenia were observed in the patients having two and three risk genotypes ($P=6.25\times10^{-10}$, OR=11.97 and $P=4.13\times10^{-9}$, OR=50.00, respectively) relative to patients with zero or one risk genotype.**

Conclusion **We identified four novel SNPs associated with gemcitabine-induced severe leukopenia/neutropenia. These SNPs might be applicable in predicting the risk of hematological toxicity in patients receiving gemcitabine therapy. *Pharmacogenetics and Genomics* 00:000–000 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine) is a deoxycytidine analogue that is used for the treatment of patients with various solid tumors, including pancreatic and non-small-cell lung cancers, as a single agent or in combination with platinum agents [1,2]. Dose-limiting adverse drug reactions (ADRs) of gemcitabine are known to be emesis and hematological toxicities, including neutropenia, leukopenia, anemia, and thrombocytopenia [1]. The use of gemcitabine is often limited by these unpredictable dose-limiting toxicities. A large interindividual variation has been noted in the toxicities of gemcitabine, and the frequency of severe leukopenia/neutropenia was reported to be 13–35% [3,4].

Gemcitabine is transported into cells by centrative nucleoside transporters (CNT1 and CNT3; also known as solute carrier (SLC) 28A1 and SLC28A3, respectively) and an equilibrative nucleoside transporter (ENT1; SLC29A1) [5–7], activated by intracellular phosphorylation by deoxycytidine kinase (dCK) to form gemcitabine monophosphate [8], and incorporated into DNA as its triphosphate to inhibit DNA synthesis. It has also been

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suggested that the diphosphate and triphosphate of gemcitabine inhibit ribonucleotide reductase [9]. Gemcitabine is rapidly metabolized to the inactive metabolite 2′,2′-difluorodeoxyuridine by cytidine deaminase (CDA), and excreted into the urine [10]. A number of genetic polymorphisms have been reported in these enzymes and transporters; therefore, genetic variations in these genes may influence the pharmacokinetics and pharmacodynamics of gemcitabine, resulting in differences in the toxicity and efficacy of gemcitabine among individuals. Several case–control association studies focusing on gemcitabine-metabolizing enzymes and transporters as candidates have been carried out [11–14]; however, useful genetic markers to predict toxicities of gemcitabine have not been identified yet. In this study, to identify genetic loci related to the gemcitabine-induced hematological toxicities, we carried out a genome-wide association study (GWAS) by genotyping over 610 000 single nucleotide polymorphisms (SNPs), and identified the loci that are likely to be associated with risk of severe leukopenia/neutropenia in the patients treated with gemcitabine monotherapy.

Materials and methods

Patients

Most of the patients were registered in the BioBank Japan Project (*http://www.biobankjp.org/*) [15], which was started in 2003 by a collaborative network of 66 hospitals across Japan, with the goal of collecting genomic DNA, serum, and clinical information from 300 000 patients who have at least one of the 47 diseases. Among the patients registered in BioBank from June 2003 to December 2008, 461 patients received gemcitabine treatment. Of them, 52 patients (11.3%) experienced grade 3 or 4 leukopenia/neutropenia (ADR group), whereas 86 patients revealed no adverse events (non-ADR group), and the remaining patients had grade 1 or 2 leukopenia/neutropenia and/or other ADRs. We selected 79 patients treated with gemcitabine monotherapy, consisting of 21 ADR patients and 58 non-ADR patients, for the GWAS (Table 1). In a replication study, 33 ADR and 62 non-ADR patients were collected from Sapporo Medical University Hospital, Wakayama Medical University Hospital, Kure Kyosai Hospital, and Hakodate Kyoukai Hospital, as well as BioBank Japan Project from January 2009 to December 2010. Clinical information including drug use and ADRs was collected from medical records. The grades of toxicities were given according to the National Cancer Institute – Common Toxicity Criteria, version 2. As a general control population, we used healthy volunteers from the Midousuji Rotary Club, Osaka, Japan. All participants were of Japanese origin and provided written informed consent. This project was approved by the Institutional Review Board in the Institute of Medical Science, The University of Tokyo, Wakayama Medical University, Kure Kyosai Hospital, and Sapporo Medical University.

Genotyping and quality control

In the GWAS, 79 patients were genotyped using the Illumina Human610-Quad BeadChip (Illumina, San Diego, California, USA). We applied SNP quality control (call rate of ≥ 0.99 in both ADR and non-ADR groups, and a Hardy–Weinberg equilibrium P-value of $> 1.0 \times 10^{-6}$ in the non-ADR group); 470 064 SNPs in autosomal chromosomes passed the quality control filters. SNPs with a minor allele frequency of less than 0.01 were also excluded from further analysis. Of the SNPs analyzed in the GWAS, we selected 70 SNPs of the top 100 SNPs, after considering linkage disequilibrium (LD; $r^2 < 0.8$), for a replication study. In the replication study, we carried out a multiplex polymerase chain reaction-based Invader assay (Third Wave Technologies, Madison, Wisconsin, USA) on ABI PRISM 7900HT (Applied Biosystems, Foster City, California, USA) [16].

Imputation

Imputation was performed by referring to the genotype data of Japanese (JPT) individuals, as deposited in the Phase II HapMap database [17] using MACH v1.0 (*http://www.sph.umich.edu/csg/yli/mach/index.html*). Genotypes

Table 1 Patients' characteristics

Stage	Platform	Source	No. of samples	Female (%)	Age (mean ± SD)	Cancer types, *N*			
						Pancreatic	Lung	Bile duct	Others
GWAS									
ADR	Illumina HumanHap610-Quad	BioBank Japan	21	45.0	64.8 ± 10.9	12	6	1	2
Non-ADR	Illumina HumanHap610-Quad	BioBank Japan	58	41.8	64.0 ± 8.7	23	19	10	1
Replication study									
ADR	Invader assay	BioBank Japan, Sapporo Medical University, Wakayama Medical University, Kure Kyosai Hospital	33	35.5	64.2 ± 9.9	28	3	4	3
Non-ADR	Invader assay	BioBank Japan, Sapporo Medical University, Wakayama Medical University, Kure Kyosai Hospital	62	30.2	64.9 ± 9.0	36	7	17	2

ADR, adverse drug reaction; GWAS, genome-wide association study.

of SNPs that are located in the genomic region within 500 kb upstream or downstream of the marker SNP, which showed the strongest association, at each locus were imputed using genotype data of the GWAS. In the process of imputation, 50 Markov chain iterations were implemented. Imputed SNPs with an imputation quality score of r^2 less than 0.3 were excluded from the subsequent analysis.

Statistical analysis

In the GWAS and the replication study, Fisher's exact test was applied to three genetic models: an allele frequency model, a dominant-inheritance model, and a recessive-inheritance model. Odds ratios (ORs) and confidence intervals (CIs) were calculated for the genetic model with the lowest *P*-value, using a nonrisk allele or a nonrisk genotype as a reference. We used a significance level of 1.07×10^{-7} (0.05/470 064) in the GWAS and 7.14×10^{-4} (0.05/70) in the replication study for adjustment of multiple testing by the strict Bonferroni correction. For combination analysis, the genotype count of the replication study was added to that of the GWAS. The difference in the distribution of age was assessed by the Mann–Whitney *U*-test, and the differences in the sex and cancer types were evaluated by Fisher's exact test. For the prediction scoring system of severe leukopenia/neutropenia induced by gemcitabine, we assigned a score of 1 to individuals homozygous for the risk allele and 0 to individuals with the other genotypes (homozygous and heterozygous for the nonrisk allele), and summed up the scores for each gene to obtain individuals' scores. On the basis of this system, each patient was classified into any of the five groups (group 0, 1, 2, 3, or 4). False discovery rate values were calculated using the Benjamini–Hochberg method to evaluate the probability of false-positive associations [18]. Population stratification for the GWAS data was examined by principal component analysis (PCA) using EIGENSTRAT software v2.0. The four HapMap populations, namely Europeans (CEU), Africans (YRI), and East-Asians (Japanese and Han Chinese, denoted JPT + CHB), were used as reference groups in the PCA. All the statistical analyses were carried out using R statistical environment version 2.12.1 (*http://www.r-project.org/*) or PLINK version 1.06 [19]. Haploview software was used to analyze LD values and to draw the LD map [20].

Results

Toxicity of gemcitabine

A total of 174 patients, including 54 ADR patients and 120 non-ADR patients who were treated with gemcitabine monotherapy, were analyzed in this study (Table 1). The distributions of sex (percentage of female patients) were 45.0 and 41.8% in the ADR and non-ADR groups, respectively, in the GWAS ($P = 1.00$), and 35.5 and 30.2%, respectively, in the replication samples ($P = 0.636$). There was no significant difference in the age distributions (mean ± SD) between ADRs and non-ADRs (64.8 ± 10.9 vs. 64.0 ± 8.7, $P = 0.988$ in the GWAS; 64.2 ± 9.9 vs. 64.9 ± 9.0, $P = 0.527$ in the replication study). More than half of the patients had pancreatic cancer ($N = 99$, 56.9%). The remaining subjects were patients with lung cancer ($N = 35$, 20.1%), bile duct cancer ($N = 32$, 18.4%), and other cancers ($N = 8$, 4.6%). No significant difference in these cancer types was observed between ADR and non-ADR patients in both the GWAS and the replication study ($P = 0.159$ and 0.125, respectively).

Genome-wide association and replication studies

We conducted a GWAS of 21 ADRs and 58 non-ADRs in the Japanese patients who received gemcitabine monotherapy using Illumina Human610-Quad BeadChip (Illumina, San Diego, California, USA). After the standard quality control, association analysis was carried out for 470 064 SNPs by Fisher's exact test on the basis of three genetic models: allelic, dominant, and recessive. PCA in the GWAS and HapMap samples showed no evidence of population stratification between the ADR and non-ADR groups (Supplementary Fig. 1, SDC-1, *http://links.lww.com/FPC/A357*). The top 100 SNPs which revealed the smallest *P*-values showed possible associations (2.12×10^{-4} to 6.69×10^{-6}; Supplementary Table 1, SDC-2, *http://links.lww.com/FPC/A358* and Supplementary Fig. 2, SDC-3, *http://links.lww.com/FPC/A359*). To validate the results of the GWAS analysis, we carried out a replication study using 95 independent patients, including 33 ADR and 62 non-ADR patients. Of the top 100 SNPs, we selected and genotyped 70 SNPs since 30 SNPs were highly linked ($r^2 > 0.8$) to another SNP. In the replication study, we identified four SNPs with associations of *P* less than 0.05 (rs11141915 on chromosome 9q21, $P = 2.77 \times 10^{-3}$; rs1901440 on chromosome 2q21, $P = 1.82 \times 10^{-2}$; rs12046844 on chromosome 1p31, $P = 3.09 \times 10^{-2}$; rs11719165 on chromosome 3q29, $P = 4.61 \times 10^{-2}$; Table 2). A combined result of the two studies suggested possible associations with loci rs11141915 (combined $P = 1.27 \times 10^{-6}$, OR = 4.10, 95% CI: 2.21–7.62), rs1901440 (combined $P = 3.11 \times 10^{-6}$, OR = 34.00, 95% CI: 4.29–269.48), rs12046844 (combined $P = 4.56 \times 10^{-5}$, OR = 4.13, 95% CI: 2.10–8.14), and rs11719165 (combined $P = 5.98 \times 10^{-5}$, OR = 2.60, 95% CI: 1.63–4.14; Table 2), although none of them reached the genome-wide significance ($P = 1.07 \times 10^{-7}$) when we considered the number of SNPs analyzed in the GWAS. To further characterize the four loci, which were associated with gemcitabine-induced severe myelosuppression, we imputed genotypes of SNPs that were not genotyped in the GWAS but are located within 500 kb upstream or downstream of the four marker SNPs, and examined the associations of these SNPs. Although several SNPs were indicated to be possibly associated with the gemcitabine-induced myelosuppression (Fig. 1), no SNP showed a lower *P*-value than the marker SNP. Imputation analysis revealed that rs11141915 represented an associated region spanning 87 kb (chr.9: 89.39–89.48 Mb), which is located in the *DAPK1* gene,

Table 2 Summary of association results of the genome-wide association study and replication study

SNP	Chromosome	Chromosome location[a]	Gene	Allele 1/2 (risk)	Stage	ADR				Non-ADR				*P*-value			False discovery rate	Odds ratio (95% CI)[b]
						11	12	22	RAF	11	12	22	RAF	Allelic	Dominant	Recessive		
rs11141915	9	89425614	*DAPK1*	G/T (T)	GWAS	0	3	18	0.07	7	30	21	0.38	1.27×10^{-4}	1.80×10^{-1}	1.04×10^{-4}		7.94 (2.32–27.25)
					Follow-up	0	11	22	0.17	8	31	23	0.38	2.77×10^{-3}	4.73×10^{-2}	9.23×10^{-3}	0.342	3.05 (1.45–6.41)
					Combined	0	14	40	0.13	15	61	44	0.38	1.27×10^{-6}	6.11×10^{-3}	6.91×10^{-6}		4.10 (2.21–7.62)
rs1901440	2	134154429	No gene	A/C (C)	GWAS	11	3	7	0.40	31	27	0	0.23	4.42×10^{-2}	1.00×10^{-0}	4.01×10^{-5}		60.52 (5.45–632.87)
					Follow-up	20	8	5	0.27	42	19	1	0.17	1.30×10^{-1}	5.05×10^{-1}	1.82×10^{-2}	0.488	10.89 (1.22–97.64)
					Combined	31	11	12	0.32	73	46	1	0.20	1.44×10^{-2}	7.39×10^{-1}	3.11×10^{-6}		34.00 (4.29–269.48)
rs12046844	1	66010967	*PDE4B*	T/C (C)	GWAS	1	5	15	0.83	12	32	14	0.52	3.93×10^{-4}	1.67×10^{-1}	1.95×10^{-4}		7.86 (2.56–24.12)
					Follow-up	4	10	19	0.73	7	34	21	0.61	1.50×10^{-1}	1.00×10^{-0}	3.09×10^{-2}	0.542	2.65 (1.11–6.31)
					Combined	5	15	34	0.77	19	66	35	0.57	3.05×10^{-4}	3.43×10^{-1}	4.56×10^{-5}		4.13 (2.10–8.14)
rs11719165	3	196067377	No gene	T/C (C)	GWAS	2	10	9	0.33	26	27	5	0.68	1.15×10^{-4}	3.49×10^{-3}	1.21×10^{-3}		4.27 (2.01–9.05)
					Follow-up	8	16	9	0.48	24	31	7	0.64	4.61×10^{-2}	1.78×10^{-1}	8.12×10^{-2}	0.600	1.87 (1.02–3.42)
					Combined	10	26	18	0.43	50	58	12	0.66	5.98×10^{-5}	3.26×10^{-3}	3.66×10^{-4}		2.60 (1.63–4.14)

ADR, adverse drug reaction; CI, confidence interval; GWAS, genome-wide association study; RAF, risk allele frequency; SNP, single nucleotide polymorphism.
[a]On the basis of NCBI 36 genome assembly.
[b]Odds ratios were shown for the model with minimum *P*-values.

encoding death-associated protein kinase 1 (Fig. 1a). rs1901440 on chromosome 2q12 was located in an associated region consisting of a 141-kb LD block (spanning from 134.14 to 134.28 Mb), which contains no known genes (Fig. 1b). Imputation analysis of chromosome 1p31 identified a 63-kb associated region (chr.1: 66.05–66.11 Mb) represented by rs12046844. This region contains the 5′ region of the *PDE4B* gene, encoding phosphodiesterase 4B (Fig. 1c). The rs11719165 locus on chromosome 3q29 was in an associated region spanning 104 kb (chr.3: 196.04–196.14 Mb), which is an intergenic region (Fig. 1d).

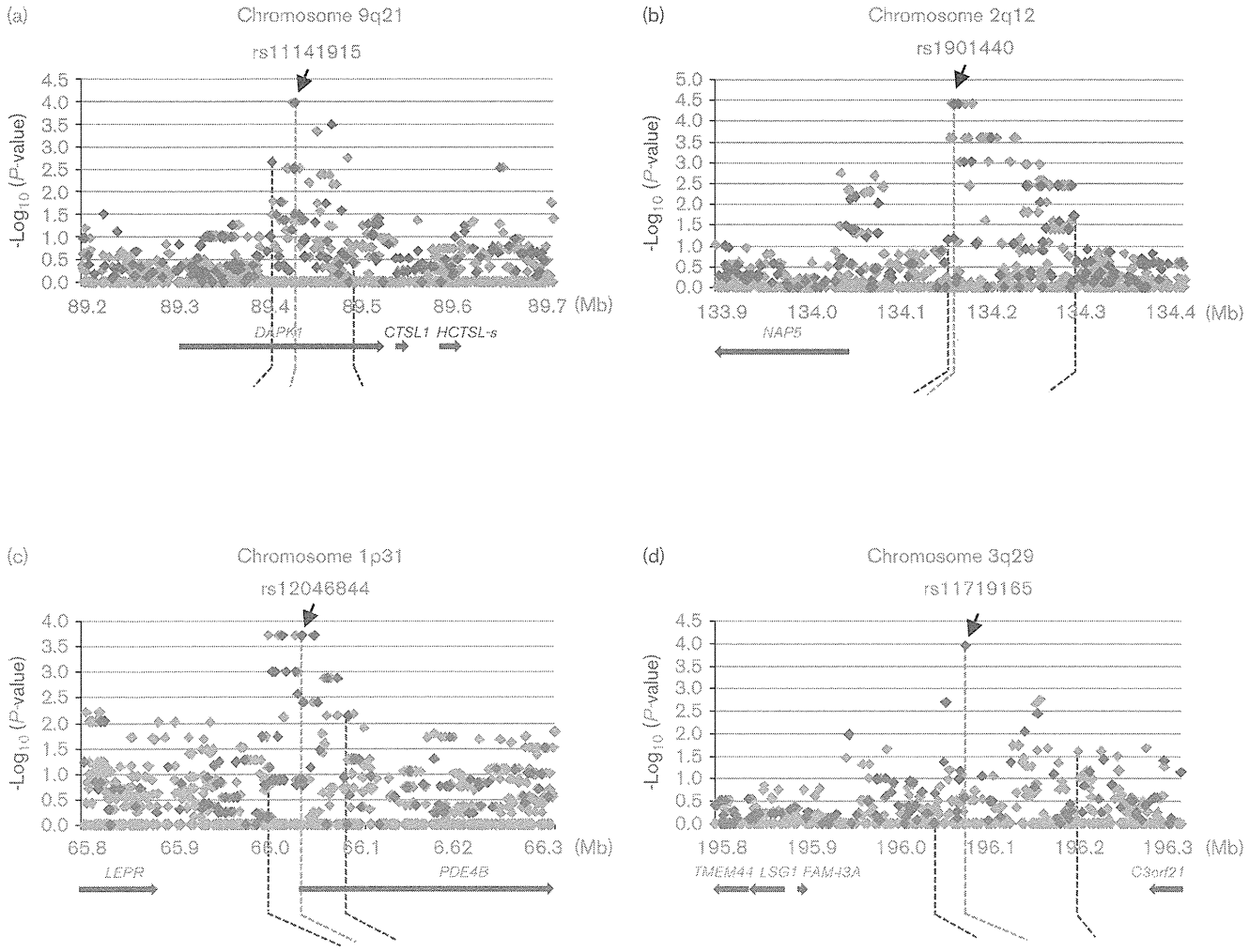
Predictive scoring system for gemcitabine-induced leukopenia/neutropenia

The four SNPs identified by the combined study were independent predictors of gemcitabine-induced toxicities when analyzed by multiple logistic regression ($P < 3.11 \times 10^{-3}$). Therefore, we investigated combined effects of the four loci on the risk of severe leukopenia/neutropenia using a scoring system. For the prediction scoring system, each patient was scored according to the total number of risk genotypes of the four SNP loci; a score of 1 was provided to individuals homozygous for the risk allele and 0 to those with the other genotypes, because the recessive-inheritance model revealed a lower *P*-value than the dominant-inheritance model in each of the four SNP loci. In this population, no patient had the four risk genotypes (score 4). The proportion of patients with gemcitabine-induced leukopenia/neutropenia was significantly increased in groups with higher prediction scores (trend test $P = 1.31 \times 10^{-14}$); the incidences of grade 3/4 leukopenia/neutropenia were 11.5% (13/113) in the combined group of scores 0 and 1, 60.9% (28/46) in the score 2 group, and 86.7% (13/15) in the score 3 group (Table 3). Correspondingly, the OR in the score 3 group was as high as 50.00 (95% CI: 10.13–246.90, $P = 4.13 \times 10^{-9}$) and that of the score 2 group was 11.97 (95% CI: 5.23–27.37, $P = 6.25 \times 10^{-10}$), compared with that in the group of scores 0 and 1. We also examined the distribution of prediction scores in the general control using the four SNPs, which could predict the risk of adverse events in patients treated with gemcitabine. In the general Japanese population, the frequencies of individuals with the scores 0, 1, 2, and 3 were 29.0, 45.3, 20.8, and 4.9%, respectively. This information would be useful to predict the number of patients classified into a group of high, moderate, or low risk before initiation of chemotherapy in the clinical setting.

Discussion

This study is the first GWAS that attempted to identify genetic variants associated with severe hematological toxicity induced by gemcitabine monotherapy, and identified four possible markers, rs11141915, rs1901440, rs12046844, and rs11719165, on chromosomal regions of 9q12, 2q12, 1p31, and 3q29, respectively. Furthermore, the combined analysis of the four SNP loci revealed that

Fig. 1



Regional association plots and linkage disequilibrium (LD) maps of the four loci associated with gemcitabine-induced severe hematological toxicity. *P*-value plots, genomic structures, and LD maps of chromosome regions 9q12 (a), 2q12 (b), 1p31 (c), and 3q29 (d). Blue, diamond-shaped dots represent $-\log_{10}$-transformed minimum *P*-values of single nucleotide polymorphisms genotyped using an Illumina Human610-Quad BeadChip in the genome-wide association study, and red, diamond-shaped dots show $-\log_{10}$-transformed minimum *P*-values of the imputed single nucleotide polymorphisms. Blue arrows indicate the position of known genes. The *D'*-based LD map (minor allele frequency ≥ 0.15) is drawn using genotype data of 79 patients enrolled in the genome-wide association study.

Table 3 Prediction scores of gemcitabine-induced sever leukopenia/neutropenia using rs11141915, rs1901440, rs12046844, and rs11719165

Score	ADR, *N* (%) (*N*=54)	Non-ADR, *N* (%) (*N*=120)		Odds ratio (95% CI) *P*-value	General control, *N* (%) (*N*=934)
0	4 (7.4%)	50 (41.7%)	]	1.00 (reference)	271 (29.0%)
1	9 (16.7%)	50 (41.7%)	]		423 (45.3%)
2	28 (51.9%)	18 (15.0%)		11.97 (5.23–27.37) 6.25×10^{-10}	194 (20.8%)
3	13 (24.1%)	2 (1.7%)		50.00 (10.13–246.90) 4.13×10^{-9}	46 (4.9%)
			(trend test)	9.91 (5.56–17.67) 1.31×10^{-14}	

ADR, adverse drug reaction; CI, confidence interval.

the number of risk genotypes of the SNPs has cumulative effects on the risk of gemcitabine-induced severe hematological toxicity.

Since this study was conducted with a small number of samples, the statistical power was calculated to be 12 and 43%, respectively, to detect an effect with an OR of 5.0 or greater for an allele with 30% frequency at the genome-wide significance level ($\alpha = 1.0 \times 10^{-7}$) and the significance level of possible association ($\alpha = 1.0 \times 10^{-5}$), suggesting that an SNP with a relatively small OR could easily be missed in our GWAS screening. In addition, the associations of SNPs detected in this study were marginal, which did not reach the genome-wide significance level; therefore, further replication studies are required.

The SNP showing the lowest *P*-value in this study, rs11141915 ($P = 1.27 \times 10^{-6}$, OR = 4.10), is located in intron 3 of the *DAPK1* gene. DAPK1 is a member of a serine/threonine kinase family that mediates the γ-interferon-induced cell death and also mediates apoptosis induced by tumor necrosis factor-α [21,22]. DAPK1 is reported to be expressed in bone marrow and peripheral blood [23,24]. It has also been reported that the expression of DAPK1 is associated with the resistance to an anticancer drug, irinotecan, in gastric cancer cell lines [25]. Although further analysis is required to clarify the functional importance of DAPK1 in gemcitabine-induced leukopenia/neutropenia, the difference in expression of this protein in the bone marrow might cause interindividual differences in toxicities induced by gemcitabine.

rs12046844 ($P = 4.56 \times 10^{-5}$, OR = 4.13; the third locus) was located in a 63-kb region containing the 5′ region of the *PDE4B* gene (Fig. 1c). PDE4B is a phosphodiesterase isozyme in various leukocytes, including neutrophils and monocytes, and plays a key role in the regulation of inflammatory cell activation [26,27]. The expression of PDE4B was increased in non-small-cell lung cancer cells that acquired gemcitabine resistance, and was restored by the treatment with bexarotene, a selective retinoid X receptor agonist, which has the potential of resensitizing gemcitabine-resistant tumor cells [28]. These lines of evidence suggest that PDE4B may regulate the sensitivity of cells to gemcitabine. The second locus (rs1901440 on chromosome 2q12) and the fourth locus (rs11719165 on chromosome 3q29) were in regions containing no reported genes (Fig. 1b and d). According to the UCSC database (*http://genome.ucsc.edu/*), several expressed sequence tags were mapped in these regions, but none of them has open reading frames. Hence, further studies will be required to clarify their functional associations with myelosuppression in patients treated with gemcitabine.

The variants of candidate genes, which are involved in the metabolism and transport of gemcitabine, such as *CDA*, *dCK*, *SLC28A1*, *SLC28A3*, and *SLC29A1*, as well as those that are target molecules of gemcitabine, including ribonucleotide reductase M1 subunit (*RRM1*), *RRM2*, and *RRM2B*, have been suggested to be associated with clinical outcomes and adverse events in gemcitabine therapy [11–14,29–32]. In our GWAS, no SNP in the pharmacokinetics-related candidate genes showed a significant association with the risk of severe leukopenia/neutropenia in the patients receiving gemcitabine monotherapy ($P \geq 1.07 \times 10^{-2}$). We also investigated the association between *CDA*3* (208 G > A; Ala70Thr), which was reported to be associated with higher gemcitabine concentration in plasma, and higher risks of gemcitabine-induced toxicity [12,13,33]. However, no significant association was observed between *CDA*3* and the risk of gemcitabine-induced leukopenia/neutropenia in this study (combined $P = 5.71 \times 10^{-1}$; Supplementary Table 2). For the pharmacodynamics-related candidate genes, we found possible associations between SNPs in *RRM1* ($P = 1.03 \times 10^{-3}$) and *RRM2B* ($P = 4.78 \times 10^{-4}$) in the GWAS samples. However, in the subsequent analysis of the replication sample set, we obtained no supportive results (Supplementary Table 2, SDC-4, *http://links.lww.com/FPC/A360*). Further validation studies using a large number of patients will be required to clarify the effects of these candidate SNPs on the risk of gemcitabine-induced severe leukopenia/neutropenia.

In conclusion, our GWAS using a total of 174 Japanese patients receiving gemcitabine monotherapy has identified four novel candidate loci, 1p31, 2q12, 3q29, and 9q12, which are associated with the risk of gemcitabine-induced grade 3 or 4 leukopenia/neutropenia. Furthermore, the combined analysis of the four SNP loci revealed that the number of risk genotypes of the SNPs was significantly associated with increasing risk of gemcitabine-induced severe hematological toxicity, although replication and validation studies are required. Detailed information about regimen and dosage of gemcitabine is not available in this study; however, further analysis considering this information could improve personalized selection of gemcitabine-based chemotherapy for patients with cancer.

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Conflicts of interest

There are no conflicts of interest.

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Genome-wide association study for intracranial aneurysm in the Japanese population identifies three candidate susceptible loci and a functional genetic variant at *EDNRA*

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Aneurysmal subarachnoid hemorrhage (aSAH) is the most serious subtype of stroke. Genetic factors have been known to play an important role in the development of intracranial aneurysm (IA), some of which further progress to subarachnoid hemorrhage (SAH). In this study, we conducted a genome-wide association study (GWAS) to identify common genetic variants that are associated with the risk of IA, using 1383 aSAH subjects and 5484 control individuals in the Japanese population. We selected 36 single-nucleotide polymorphisms (SNPs) that showed suggestive association ($P < 1 \times 10^{-4}$) in the GWAS as well as additional 7 SNPs that were previously reported to be associated with IA, and further genotyped an additional set of 1048 IA cases and 7212 controls. We identified an SNP, rs6842241, near *EDNRA* at chromosome 4q31.22 (combined *P*-value = 9.58×10^{-9}; odds ratio = 1.25), which was found to be significantly associated with IA. Additionally, we successfully replicated and validated rs10757272 on *CDKN2BAS* at chromosome 9p21.3 (combined *P*-value = 1.55×10^{-7}; odds ratio = 1.21) to be significantly associated with IA as previously reported. Furthermore, we performed functional analysis with the associated genetic variants on *EDNRA*, and identified two alleles of rs6841581 that have different binding affinities to a nuclear protein(s). The transcriptional activity of the susceptible allele of this variant was significantly lower than the other, suggesting that this functional variant might affect the expression of EDNRA and subsequently result in the IA susceptibility. Identification of genetic variants on *EDNRA* is of clinical significance probably due to its role in vessel hemodynamic stress. Our findings should contribute to a better understanding of physiopathology of IA.

INTRODUCTION

Intracranial aneurysms (IAs) are balloon-like dilations of the intracranial arterial wall in the brain. Rupture of IA causes subarachnoid hemorrhage (SAH), a serious subtype of stroke, which leads to fatality in ~50% of the cases and results in significant disability in 30% of the cases (1). The age- and sex-adjusted annual incidence and mortality rates of SAH were 23 and 9 per 100 000 for all ages, respectively, in Japan (1). The annual rupture risk of IA in Japan is relatively high at ~2.7% (2). The incidence of SAH is particularly higher in Finland and Japan than in the rest of the world (1,3–5).

Both environmental and genetic factors are known to be involved in the development of IA, and several studies have indicated that hypertension, hypercholesterolemia, cigarette smoking and female gender are risk factors for IA (6–10). Various Mendelian hereditary connective tissue disorders such as autosomal dominant polycystic kidney disease (11) and

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type-IV Ehlers–Danlos syndrome (12) are the inherited conditions that increase the risk of IA. Additionally, a positive family history for IA is considered to be an important risk factor, as the incidence of harboring IA for individuals who have at least two affected first-degree relatives was reported to range from 6 to 10% (6,13,14). With the high incidence of familial IA, several susceptible loci which include chromosomes 1p34.3–p36.13 (15), 7q11 (16), 19q13.3 (17) and Xp22 (18) were successfully identified to be linked to familial IA through the linkage analysis. All this evidence has strongly implied the role of genetic factors contributing to the physiopathology of IAs.

Since there is no clear symptom of IA, identification of risk factors, particularly genetic risk factors, that lead to an increase in the risk of the formation and rupture of IA, which result in aneurysmal subarachnoid hemorrhage (aSAH), is critically essential. With the current advances in biotechnology, it is now feasible to identify common genetic variants that are associated with polygenic diseases by means of genome-wide high-density SNP array. Two genome-wide association studies (GWAS) of multiple European populations have successfully identified common variants located on chromosomes 8q12.1, 9p21.3, 10q24.32, 13q13.1 and 18q11.2 that are associated with IA. Notably, these associated loci were successfully replicated in the Japanese population (19,20). Because of the complex linkage disequilibrium (LD) structures across different populations and potential interaction between genetic variants and environment factors, it is well known that the effect size of common genetic variants associated with the disease varies among different populations. Although a GWAS of IA in the Japanese population was reported previously, the association was not conclusive due to the lack of validation analysis as well as the insufficient statistical power of the study (21). Hence, we conducted an independent GWAS of IA with a larger samples size for the identification of genetic variants associated with IA in the Japanese population.

RESULTS

To identify genetic variants associated with susceptibility to IA in the Japanese population, we performed a GWAS, using 1383 aSAH patients and 5484 control (Supplementary Material, Table S1) subjects, with Illumina OmniExpress BeadChip Kits that contained 733 202 SNPs. After quality check of the SNP genotyping data, a total of 565 149 autosomal SNPs were used for association analysis (Supplementary Material, Table S2).

Principal component analysis (PCA) revealed that all the subjects participating in this study were clustered in the Asian population (Supplementary Material, Fig. S1). The detailed PCA analysis on the basis of the genotype information from the cases and controls classified the sample populations mostly into two major clusters consisting of the Ryuukyu cluster (southern islands of Japan) and the Hondo cluster (mainland cluster) (Supplementary Material, Fig. S2). To avoid influences of population substructure in the sample populations as well as age and gender biases, the association study was performed by logistic regression analysis with associated eigenvectors, age and gender as covariates.

A quantile–quantile (Q–Q) plot for this GWAS based on 565 149 SNPs is shown in Supplementary Material, Figure S3. The genomic inflation factor (λ_{GC}) of the test statistic in this study was 1.055. Since it is known that the λ_{GC} value increases with an increase of the sample size, we calculated the λ_{GC} value adjusted to a sample size of 1000—which was 1.031, indicating a low possibility of false-positive association by population stratification. We subsequently applied the genomic control method to adjust the *P*-values and used the adjusted *P*-values (P_{GC}) for further analysis. The Manhattan plot shown in Figure 1 indicated no SNP to have achieved genome-wide significance level with the threshold at a P_{GC} value of $<5 \times 10^{-8}$.

Six SNPs (rs10958409, rs9298506, rs1333040, rs11191514, rs1980781 and rs11661542) on chromosomes 8q11.23, 9p21.3, 10q24.32, 13q13.1 and 18q11.2, which were previously reported to be associated with IA in the European population, showed nominal association with *P*-values from 4.50×10^{-2} to 9.52×10^{-5} (Supplementary Material, Table S3). On chromosome 9p21.3, although the previously reported SNP, rs1333040, revealed a *P*-value of 3.79×10^{-2}, we identified another SNP, rs10757272, on this locus that showed stronger association with IA with a *P*-value of 7.75×10^{-4}. However, we observed no association with *P*-values of >0.05 for the five SNPs (rs7542311, rs358345, rs4628172, rs6461176, rs10217224) that were previously indicated in the GWAS in the Japanese population (Supplementary Material, Table S3).

To further validate a possible genetic variant(s) associated with IA, we selected a total of 64 SNPs showing suggestive association ($P_{GC} < 1 \times 10^{-4}$) with IA. After excluding SNPs that possess LD coefficient (r^2) of >0.8 within each LD block, we performed a replication study of 36 SNPs, using an independent set of samples consisting of 1048 IA patients and 7212 controls. In addition, we further analyzed seven previously reported SNPs, rs10958409, rs9298506, rs1333040, rs11191514, rs1980781 rs11661542 and rs10757272, that showed nominal association (*P*-value of <0.05) with IA in our first stage.

Among 43 SNPs in the replication study, two SNPs (rs6842241 on 4q31.22, rs10757272 on 9p21.3) were successfully replicated with Bonferroni-corrected *P*-value of $<1.16 \times 10^{-3}$ (0.05/43 independent tests) at the replication phase as shown in Table 1 and Supplementary Material, Table S4. The association of these two SNPs with IA was statistically significant, considering strict multiple testing with the Bonferroni correction. After evaluating the combined association of the discovery GWAS and replication stage using weighted inverse-variance meta-analysis, we identified an SNP, rs6842241, to have achieved the genome-wide significant level of association with IA in the Japanese population, yielding a combined *P*-value of 9.58×10^{-9} (OR = 1.25; 95% CI = 1.16–1.34) without any significant heterogeneity (*P* for heterogeneity = 0.606 with $I^2 = 0.0\%$). This SNP is located within the regulatory region of the *EDNRA* gene on the chromosome locus 4q31.22. We also identified another *EDNRA* intronic SNP, rs17612742 ($r^2 = 0.99$ with rs6842241), from the GWAS to be significantly associated with IA. Imputation analysis of this locus identified two additional SNPs, rs6841581 and rs1878406, with an r^2-value of >0.8 showing similar levels of association with rs6842241 (Fig. 2A).

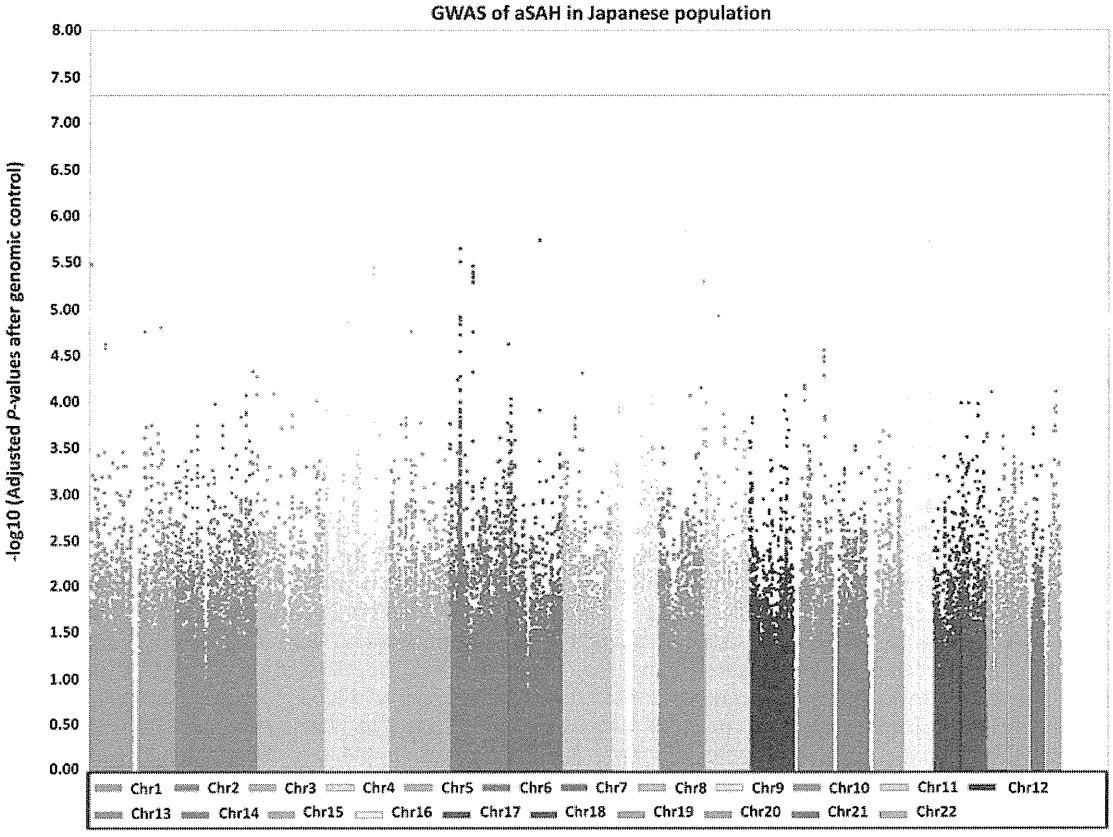

Figure 1. Manhattan plot of the GWAS of aSAH. A plot that utilized genomic-control adjustment *P*-values after eigenvectors, age and gender correction for 565 149 autosomal SNPs against their respective position on each chromosome.

Among the previously reported candidate loci (8q11.23, 9p21.3, 10q24.32, 13q13.1 and 18q11.2) associated with IA, the SNPs, rs1333040 and rs10757272 ($r^2 = 0.57$ with rs1333040), within the *CDKN2BAS* gene on a 9p21.3 region were replicated their significant association with IA in the Japanese population. In this study, rs10757272 revealed a combined *P*-value of 1.55×10^{-7} (OR = 1.21; 95% CI = 1.13–1.30) (Table 1). The other SNP, rs1333040, on the same locus was also successfully replicated, but the effect of this variant was less significant with a *P*-value of 5.56×10^{-5} (OR = 1.16, 95% CI = 1.09–1.25) than rs10757272 (Supplementary Material, Table S4). Imputation analysis on the 9p21.3 region indicated that most of the strongly associated SNPs were located at the 3′ end of *CDKN2BAS* (Fig. 2B).

After adjustment for known IA risk factors, which included smoking and hypertension, the association of rs6842241 and rs10757272 with IA remained strong (2.40×10^{-9}, OR = 1.26, 95% CI = 1.17–1.36; 1.32×10^{-8}, OR = 1.23, 95% CI = 1.24–1.33) (Supplementary Material, Table S5).

Among the SNPs that were previously reported, rs11191514 on 10q24.32 and rs1980781 on 13q13.1 revealed less significant association with IA in the Japanese population; the combined analysis of the two stages showed a *P*-value of 9.68×10^{-5} (OR = 1.17, 95% CI = 1.09–1.26) for rs11191514 on 10q24.32 and 9.20×10^{-5} (OR = 1.17, 95% CI = 1.09–1.26) for rs1980781 on 13q13.1.

Although the SNP rs671 located in *ALDH2* on chromosome 12q24.12 was marginally replicated with a *P*-value of 5.56×10^{-3}, the meta-analysis combining the two stages revealed stronger association with a *P*-value of 2.63×10^{-6} (OR = 1.24, 95% CI = 1.15–1.34). Owing to the functional relevance of this locus to IA, this SNP was considered to be significant in the susceptibility to IA in the Japanese population (Table 1).

Identification of novel genetic variants on the *EDNRA* gene revealed important insights into IA pathogenesis because of the biological function of this gene. Hence, we further investigated the role of genetic variants in the transcriptional level or the protein function. We identified four SNPs in an intron (rs17612742), or an upstream (rs6841581, 1878406 and rs6842241) of *EDNRA* to be significantly associated with IA through GWAS and imputation analysis. To examine the effect of these SNPs on the transcription, we performed electrophoretic mobility shift assays (EMSAs) and identified allelic differences in the binding affinity of a nuclear protein(s) from HEK293 cells, using the oligonucleotides corresponding to each allele of rs6841581 and rs1878406. The bands corresponding to the susceptible alleles G for rs6841581 and C for rs1878406 appeared to be strong, but those for protective alleles A and T appeared to be weak or undetectable (Fig. 3A). The different binding affinity between the two alleles for rs6841581 and those for rs1878406 were confirmed

Table 1. Association analysis of SNPs on chromosomes 4, 9 and 12 with IA

Chr.	SNP	Chrloc.	Gene	Stage	Allele		Case			Control			Risk allele frequency		P-value[a]	OR	95% confidence interval		P^{b}_{hetero}	I^2 (%)
					1	2	11	12	22	11	12	22	Case	Control			L95	U95		
4	rs6842241	148400819	*EDNRA*	GWAS	A	C	105	512	766	503	2356	2624	0.739	0.693	4.04E − 06	1.262	1.146	1.391		
				Replication	A	C	83	394	570	650	3110	3451	0.733	0.694	3.67E − 04	1.226	1.096	1.372		
				Combined	A	C	188	906	1336	1153	5466	6075	0.736	0.694	9.58E − 09	1.249	1.161	1.343	6.06E − 01	0.0
9	rs10757272	22088260	*CDKN2BAS*	GWAS	C	T	137	571	674	663	2474	2346	0.694	0.654	7.75E − 04	1.175	1.072	1.289		
				Replication	C	T	97	463	481	931	3253	3024	0.684	0.645	5.45E − 05	1.245	1.119	1.384		
				Combined	C	T	234	1034	1155	1594	5727	5370	0.690	0.649	1.55E − 07	1.213	1.133	1.300	8.88E − 01	0.0
Suggestive association																				
				GWAS	T	C	64	465	854	372	2083	3029	0.786	0.742	8.43E − 05	1.238	1.116	1.372		
12	rs671	112241766	*ALDH2*	Replication	T	C	67	343	638	491	2629	4091	0.772	0.750	5.56E − 03	1.180	1.050	1.326		
				Combined	T	C	131	808	1492	863	4712	7120	0.780	0.746	2.63E − 06	1.240	1.148	1.338	1.26E − 01	57.2

Chr., chromosome; Chrloc., chromosome location (bp); 11, homozygous genotype for allele 1; 12, heterozygous genotype; 22, homozygous genotype for allele 2; RAF, risk allele frequency; OR, odds ratio (calculated referred to risk allele).

[a]P-values of the GWAS were obtained from logistic regression analysis with eigenvectors, age and gender as covariates after genomic controls; P-values of the replication stage were obtained from logistic regression analysis with age and gender as covariates; P-values for combined were based on the weighted inverse-variance meta-analysis.

[b]P_{hetero} values were obtained from heterogeneity analysis based on Cochran's Q statistic and I^2 statistic (46).

by the competition assay in which non-labeled oligonucleotides were added at different concentrations; the increase of the non-labeled oligonucleotides inhibited the binding of the labeled oligonucleotides in a dose-dependent manner (Fig. 3B).

To identify whether these SNPs could affect the transcriptional activity of *EDNRA*, we performed reporter assays by inserting either of the oligonucleotides corresponding to the two alleles at the two SNP loci into luciferase-expressing vectors. Plasmids containing the susceptible allele G for the SNP rs6841581, which showed higher binding affinity to the nuclear protein(s), revealed significantly lower luciferase activity than the non-susceptible allele A (Fig. 3C), although no difference between the alleles was observed for the SNP rs1878406. It is notable that the non-susceptible allele A for rs6841581 revealed no enhancer activity in comparison with the mock (empty) vector, but plasmids containing the susceptible allele G revealed the suppressive effect on the activity (Fig. 3C). Taking together, our findings from the EMSA and reporter assays suggest that the 5′ flanking region including the SNP rs6841581 on *EDNRA* might function as a transcriptional repressor and that this SNP is likely to be a functional variant conferring IA susceptibility.

DISCUSSION

To identify genetic variants associated with IA in the Japanese population, we performed a GWAS and a replication study with a total of 2431 aSAH/IA subjects and 12 696 control individuals. Among the identified SNPs, we did not observe significant differences in odds ratio (risk) between the aSAH patients in the discovery GWAS phase and the IA patients in the replication stage, indicating that the identified SNPs are likely to be associated with the risk of IA development.

The SNP rs6842241, which is located 1.25 kb upstream from the *EDNRA* gene encoding endothelin receptor A, revealed the most significant association with the combined P-value of 9.58×10^{-9}. Identification of this genetic variant in the regulatory region of *EDNRA* is clinically interesting since endothelin-1 (EDN-1) and its receptors, EDNRA and EDNRB, have been known to play a significant role in IA pathophysiology. EDN-1 is a potent vasoconstrictor produced by the endothelial cells in the vasculature system. The effect of EDN-1 is mediated by two major receptor subtypes, EDNRA and EDNRB, which activate a G-protein(s) and their second messenger system. EDNRA is located predominantly on vascular smooth muscle cells of the cerebrovascular system (22) and mediates vasoconstriction and proliferation (23). Accumulated evidence strongly implies that the EDN-1/EDNRA and EDNRB pathways are critically important to maintain the balance of vasoconstriction and vasodilatation in response to the hemodynamic stress. Previous reports suggested correlation of the elevated level of EDN-1 in the cerebrospinal fluid and plasma in patients with aSAH having persistent cerebral vasospasm (24,25). Interestingly, *EDNRA* variants were previously shown to be associated with a few cerebrovascular diseases including migraine (26), myocardiac infarction (27,28) and cystic fibrosis pulmonary disease (29). Furthermore, *EDNRA* variants were also associated with essential hypertension (30) and it is overexpressed in the arteries of

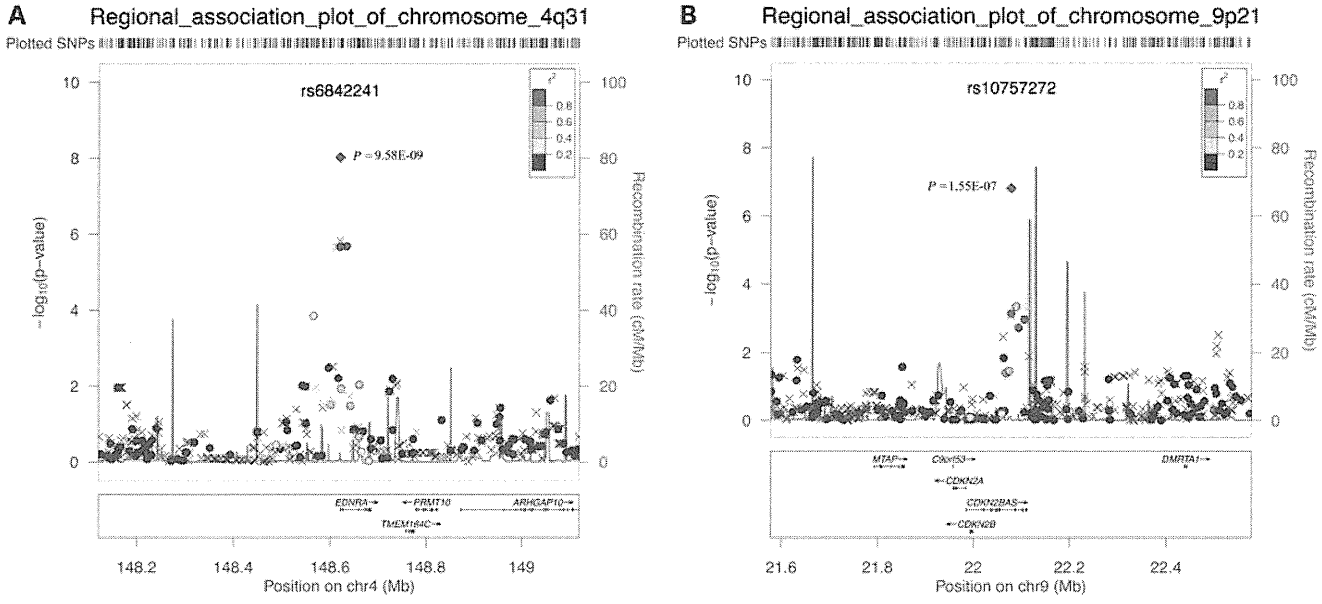

Figure 2. Regional association plots of two associated chromosome loci with IA after imputation analysis at 4q31 (**A**) and 9p21 (**B**) regions. The association of marker SNP is marked by a purple diamond before and after additional replication samples. SNPs from the GWAS are plotted as circles; imputed SNPs are plotted as crosses. The color intensity reflects the extent of LD with the marker SNP: red ($r^2 \geq 0.8$), orange ($0.6 \leq r^2 < 0.8$), green ($0.4 \leq r^2 < 0.6$), light blue ($0.2 \leq r^2 < 0.4$) and dark blue ($r^2 < 0.2$). Purplish blue lines represent local recombination rate. The SNP position is based on NCBI build 37.

hypertensive patients (31). The other three genes that reside at the same locus on chromosome 4q31.22 are *PRMT10* with an unknown function; *TMEM184C*, which is a possible tumor suppressor that may play some role in cell growth (32); and *ARHGAP10*, which is a Rho GTPase-activating protein 10 which stabilizes PAK and stimulates cell death (33).

With strong biological evidence for the role of *EDNRA* in IA pathogenesis, we carried out functional analysis of the identified SNPs from the GWAS and imputation analyses. It is well known that SNPs located in the transcriptional regulatory regions such as promoter and enhancer regions could affect the expression levels of the gene product through alteration of the binding affinity to a specific transcription factor(s) (34). The results of the EMSA and reporter assays indicated that the susceptible allele G of rs6841581 has higher affinity to the specific transcription factor(s) that might repress the transcriptional activity of *EDNRA*, compared with the non-susceptible allele A. We observed a different binding affinity to a nuclear protein(s) between the two alleles of rs1878406, and the reporter assay revealed no effect on the transcriptional activity between them. We suspect it might reflect that the 31 bp oligonucleotides may not be long enough to cover the enhancer- or repressor-binding regions. The other possibility is that a nuclear protein bound to this region has no stronger effect on the transcriptional regulation.

For SNPs on chromosome 9p21.3, we successfully validated the association of this locus with IA as reported previously. We identified a stronger association of an SNP, rs10757272 (combined $P = 1.55 \times 10^{-7}$), with IA than the SNP, rs1333040 (combined $P = 5.56 \times 10^{-5}$), that was previously identified in the study of the European population. Interestingly, the SNP rs10757272 was also shown to be associated with coronary artery disease (CAD) and platelet reactivity (a potential mechanism for increased vascular disease) in the European population (35,36). This finding suggests that rs10757272 or SNPs that are in high LD with it might be a common genetic risk factor(s) for multiple cardiovascular disorders. SNPs on a chromosome 9p21.3 region, which consist of $p15^{INK4b}$, $p16^{INK4a}$ and *CDKN2BAS*, have been indicated to be associated with several atherosclerotic vascular diseases such as CADs (37–39), stroke (40), myocardial infarction (38), abdominal aortic aneurysm (41) and IA (20). A recent study revealed that targeted deletion of the 9p21 CAD risk interval in a mouse model resulted in severely attenuated expression of two *CDKN2BAS*s' neighboring tumor-suppressor genes, $p15^{INK4b}$ and $p16^{INK4a}$, which subsequently affects the CAD progression by altering the dynamics of vascular cell proliferation (42). The findings of multiple vascular diseases associated with this locus have provided a new direction for the pathogenesis of these diseases.

Finally, although the association of rs671 (combined $P = 2.63 \times 10^{-6}$) on the *ALDH2* gene was marginally replicated in this study, the association of this locus remained to be of interest in the Japanese population. *ALDH2* belongs to the aldehyde dehydrogenase 2 family (mitochondrial), which is the second enzyme of the major oxidative pathway of alcohol metabolism. rs671 in this gene is the functional variant (Glu504Lys), and an A allele results in the inactivation of ALDH2, inducing 'alcohol flush'. The allelic frequency of this variant is uniquely high in the Asian population. In this study, the variant rs671A allele seemed to act as a protective allele, suggesting that individuals with the A allele might drink less amount of alcohol, which would result in the reduction of IA risk. Since alcohol drinking has been consistently indicated as one of the risk factors for IA, further validation of the association on this locus would be of medical importance.

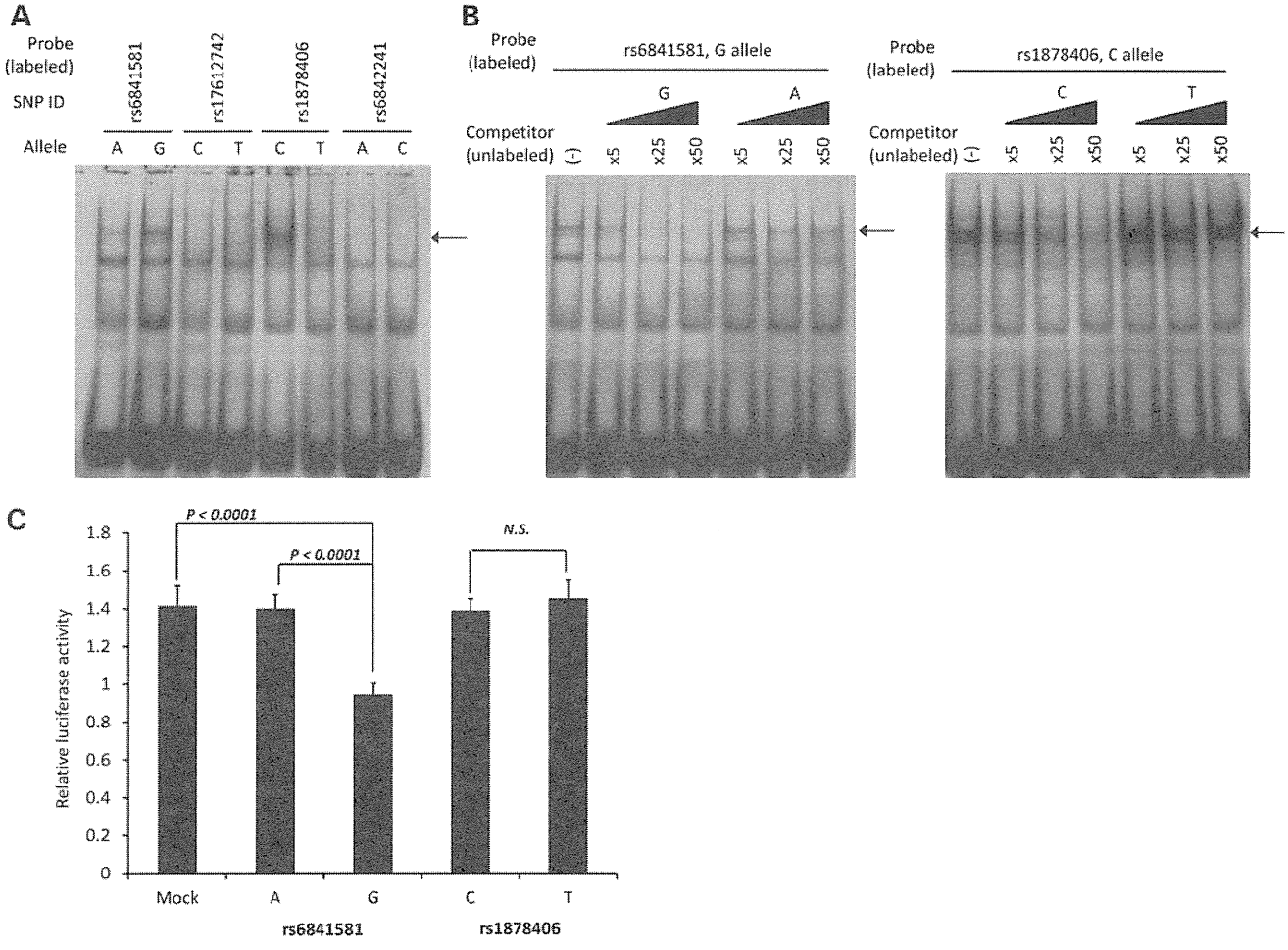

Figure 3. Functional analysis of SNPs at the *EDNRA* locus. (**A**) EMSA screening for variants associated with the binding of a nuclear protein(s) from nuclear extracts of HEK293 cells. The susceptible alleles G and C for rs6841581 and rs1878406, respectively, formed DNA–protein complexes, as pointed with arrows. (**B**) The specific interaction of labeled oligonucleotides corresponding to alleles G and C was completed in a dose-dependent manner using an unlabeled oligonucleotide with the G and C alleles, but not oligonucleotides corresponding to the A and T alleles of rs6841581 and rs1878406, respectively. (**C**) Differences in the transcriptional activity between the susceptible and non-susceptible alleles of rs6841581 and rs1878406 measured by dual-luciferase reporter assay. The values of the relative luciferase activity are shown with standard deviation after normalization with internal control *renilla* luciferase activity. The relative luciferase activity of the susceptible genotype G of rs6841581 was significantly lower than that of the non-susceptible allele A and mock (empty) vector ($P < 0.0001$, Student's *t*-test).

In conclusion, by a large-scale GWAS, we successfully identified two genomic loci, *EDNRA* (4q31.22) and *CDKN2BAS* (9p21.3), to be significantly associated with IA in the Japanese population. Owing to the complexity of detecting IA in the general population, the associations of genetic variants have not just served as a promising prediction tool to identify individuals who have a higher risk of IA, but have also provided a better understanding of the disease pathogenesis which subsequently leads to the development of clinical intervention for IA.

MATERIALS AND METHODS

Study population

All DNA samples for this study were recruited from the Biobank Japan Project that began in 2003 (http://biobankjp.org). The Biobank Japan Project has a collaborative network of 66 hospitals throughout Japan to collect DNAs and serum samples from nearly 300 000 cases with any of the 47 diseases we assigned. One of the major aims of this project was to identify common genetic variants that confer risk to common diseases, including metabolic diseases and cancers. As a discovery step of this study, we selected 1383 patients registered as aneurysmal SAH (aSAH) cases, which is caused by the rupture of IA. We selected aSAH patients for the discovery screening because aSAH is a severe form of IA in which the disease-associated variants are likely to be enriched. We verified the association results of the first set by utilizing an independent set of samples consisting of 1048 IA patients. Identification of IA in the case samples was done using computerized tomography angiogram, magnetic resonance angiogram or cerebral digital subtraction angiogram. We included 5484 and 7212 controls consisting of healthy volunteers from Midosuji Rotary Club, Osaka, Japan, Health Science Research Resource Bank and individuals in the Biobank

Japan who were registered not to have SAH/IA in the discovery and replication stages, respectively. The detailed demographic and clinical parameters of cases and controls are summarized in Supplementary Material, Table S1. Participants of this study provided written inform consent and this project was approved by the ethical committee from the Institute of Medical Sciences, the University of Tokyo and RIKEN Center of Genomic Medicine.

Genotyping and quality control

For the GWAS discovery stage, we genotyped both case and control samples using Illumina OmniExpress BeadChip that contained a total of 733 202 SNPs. We performed standard SNP quality control by excluding SNPs with a call rate of <0.99, those deviated from the Hardy–Weinberg equilibrium ($P \leq 1.0 \times 10^{-6}$), non-polymorphic ones and those on the X chromosome. The cluster plots of top 100 SNPs that revealed the strongest association with aSAH were checked by visual observation to exclude SNPs with ambiguous patterns. The number of excluded SNPs in each quality control process is summarized in Supplementary Material, Table S2. We utilized the identity-by-state method to evaluate cryptic relatedness for each sample; samples that possess an average estimate value of ≥ 1.7 were eliminated from subsequent analyses. Additionally, we examined population stratification by principal component analysis (PCA) using the EIGENSTRAT software v2.0 (http://genepath.med.harvard.edu/~reich/Software.htm). We first performed PCA, utilizing four populations in the HapMap database, which included Europeans (represented by Caucasian from UTAH, CEU), Africans (represented by Yoruba from Ibadan, YRI) and East Asians (represented by Japanese from Tokyo, JPT, and Han Chinese from Beijing, CHB) as reference populations for PCA. The top two principal components were utilized to produce a scatter plot for the identification of outliers who did not belong to the Asian cluster. To further investigate the population substructure in the sample population, we performed PCA using the genotype information of the case and control subjects in this study. The Q–Q plot that was generated between observed *P*-values against expected *P*-values and inflation factor (λ) values were used to evaluate the potential population substructure. Manhattan plot of the study was plotted using Haploview 4.1 (43).

For a replication study, a total of 36 SNPs that showed suggestive association with IA in the Japanese population ($P_{GC} < 1.0 \times 10^{-4}$) as well as 7 SNPs that were previously reported to be associated with IA (19,20) were selected for further evaluation with an independent set of 1048 IA cases and 7212 controls. We genotyped the cases with the multiplex-PCR Invader assay (44) and the control samples with Illumina OmniExpress BeadChip Kits. SNPs with a call rate of $<99\%$ and those that were deviated from the Hardy–Weinberg equilibrium ($P \leq 0.05$) were excluded for further analysis.

Statistical analysis

The case–control association was evaluated using logistic regression analysis with associated eigenvectors, age and gender as covariates in the discovery (GWAS) and replication phases of this study. The *P*-values obtained from the discovery phase were subsequently corrected using the genomic control method (45), and the corrected *P*-values were used for further analysis. Meta-analysis for the combined analysis of discovery and replication phases was performed using the weighted inverse-variance method implemented in the METAL software (http://www.sph.umich.edu/csg/abecasis/Metal/index.html). *P*-values for the heterogeneity test are evaluated with Cochran's Q statistic and I^2 statistic (46).

After the identification of candidate loci to be possibly associated with IA, imputation of the missing genotypes was performed with MACH 1.0 (http://www.sph.umich.edu/csg/abecasis/MACH/index.html). For imputation analysis, we included SNPs that were located <500 kb upstream or downstream of the marker SNP except SNPs that had a low genotyping rate ($<99\%$), showed deviations from Hardy–Weinberg equilibrium ($<1.0 \times 10^{-6}$) or had an MAF of <0.01. Genotype information from the Phase III HapMap database was used as reference. Using the MACH version 1.0 program, we estimated haplotypes, map crossover and error rates using 50 iterations of the Markov chain Monte Carlo algorithm. For imputation quality control, we excluded SNPs with r^2 values of <0.3. Regional association plots were generated using Locus Zoom (https://statgen.sph.umich.edu/locuszoom/genform.php?type=yourdata).

Cell line

A human embryonic kidney cell line, HEK293, was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). HEK293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA; ATCC) supplemented with 10% bovine serum (GIBCO) and 1% antibiotic/antimycotic solution (Sigma-Aldrich, St Louis, MO, USA). The cells were maintained at 37°C in atmospheres of humidified air with 5% CO_2.

Electrophoretic mobility shift assay

A nuclear fraction of HEK293 cells was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). Thirty-one base pairs of sense and antisense oligonucleotides corresponding to the genomic sequence which contained the SNPs of interest (rs6841581, rs17612742, rs1878406 and rs6842241) were synthesized (Supplementary Material, Table S6) and labeled with the DIG Gel Shift kit, second generation (Roche), according to the manufacturer's protocol. The reaction was started with pre-incubation of labeled oligonucleotide with poly[d(I-C)] and poly-L-lysine, and 10 μg of nuclear extract from HEK293, for 20 min at 25°C. For a competition assay, 5-fold, 25-fold or 50-fold excess of unlabeled oligonucleotide was added to nuclear extracts before adding the either of DIG-labeled probes. The protein–DNA complex was separated by electrophoresis on a 6% non-denaturing polyacrylamide gel with 0.5× Tris–borate EDTA buffer and transferred onto a nylon membrane. The protein complexes were visualized by autoradiography. All EMSAs were repeated twice to check for reproducibility.

Dual-luciferase reporter assays

To construct luciferase reporter plasmids containing the SNPs of interest (rs6841581 and rs1878406), *Sma*I and *Bgl*II restriction enzyme sites were added to the 31 bp oligonucleotides that were used as a probe in the EMSA assay and either of the annealed double-stranded oligonucleotides was inserted into the upstream of the luciferase reporter gene in the pGL3 promoter (Promega). The sequences of the constructs were verified using the ABI3730 Genetic Analyzer (Applied Biosystems). After 24 h incubation of HEK293 cells (2×10^4) on a 12-well plate, the cells were co-transfected with 400 ng of each reporter construct and 8 ng of the internal control pRL-TK (*renilla* luciferase), using the FuGene 6 transfection reagent (Roche). After 48 h incubation, the cells were lysed in passive lysis buffer and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). The results were normalized by *renilla* luciferase activity.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. The authors declare no competing financial interests.

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