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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

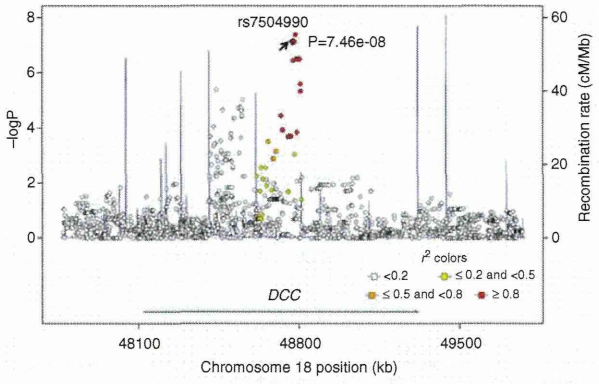

Figure 1 Regional plot for associations of SNPs located within 1500 kb upstream and downstream of the marker SNP, rs7504990, with gallbladder cancer (GC). The $-\log_{10}(P)$ values of SNPs were plotted against relative chromosomal locations. Diamond and circle signs represent genotyped and imputed SNPs, 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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Keywords: adverse drug reaction, myelosuppression, pancreatic cancer, single nucleotide polymorphism

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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).

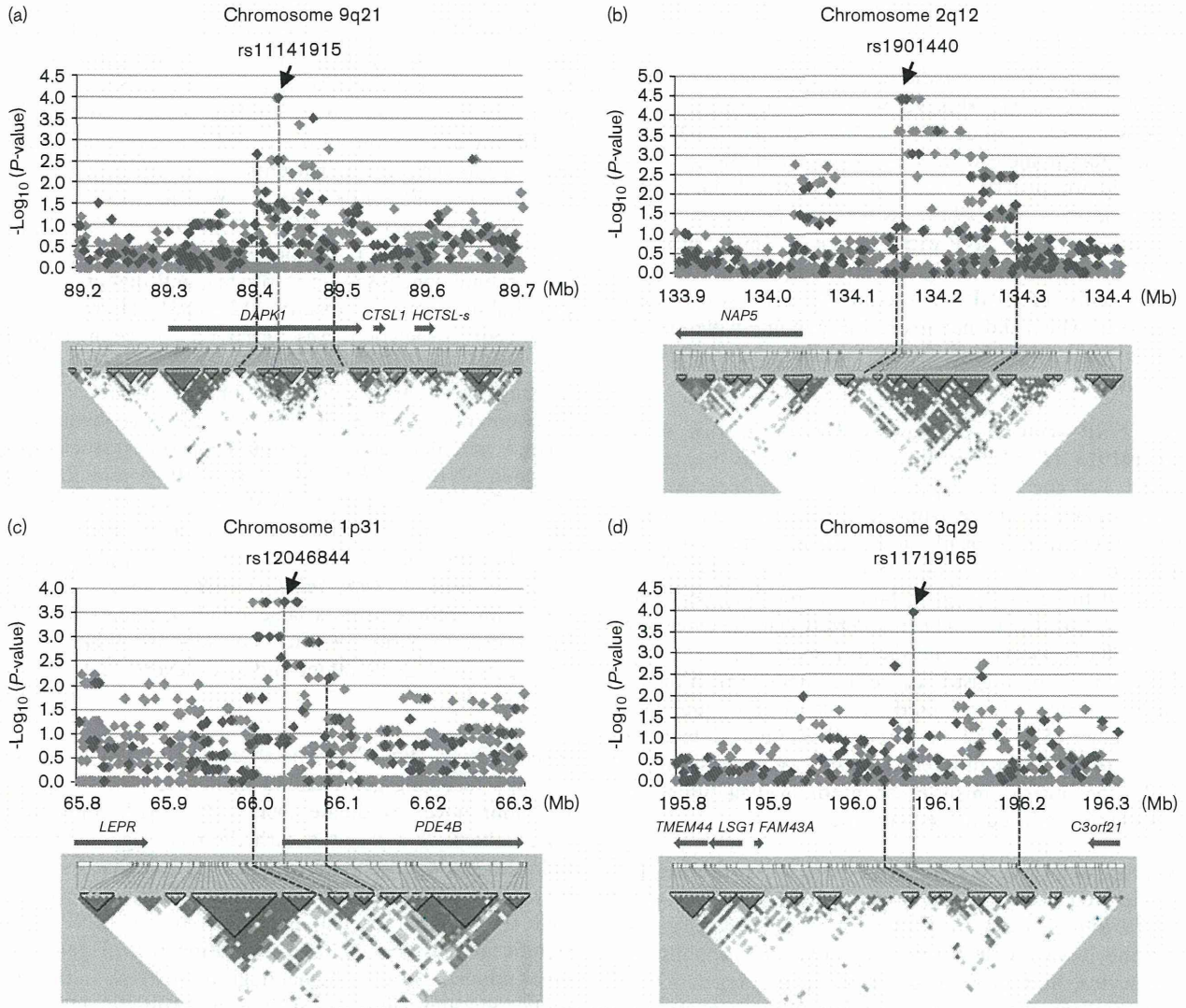
Predictive scoring system for gemcitabine-induced leukopenia/neutropenia

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.