

GWAS of Folate Metabolism With Gene–environment Interaction Analysis Revealed the Possible Role of Lifestyles in the Control of Blood Folate Metabolites in Japanese: The J-MICC Study

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ABSTRACT

Background: The present genome-wide association study (GWAS) aimed to reveal the genetic loci associated with folate metabolites, as well as to detect related gene–environment interactions in Japanese.

Methods: We conducted the GWAS of plasma homocysteine (Hcy), folic acid (FA), and vitamin B₁₂ (VB₁₂) levels in the Japan Multi-Institutional Collaborative Cohort (J-MICC) Study participants who joined from 2005 to 2012, and also estimated gene–environment interactions. In the replication phase, we used data from the Yakumo Study conducted in 2009. In the discovery phase, data of 2,263 participants from four independent study sites of the J-MICC Study were analyzed. In the replication phase, data of 573 participants from the Yakumo Study were analyzed.

Results: For Hcy, *MTHFR* locus on chr 1, *NOX4* on chr 11, *CHMP1A* on chr 16, and *DPEP1* on chr 16 reached genome-wide significance ($P < 5 \times 10^{-8}$). *MTHFR* also associated with FA, and *FUT2* on chr 19 associated with VB₁₂. We investigated gene–environment interactions in both studies and found significant interactions between *MTHFR* C677T and ever drinking, current drinking, and physical activity >33% on Hcy ($\beta = 0.039, 0.038$ and $-0.054, P = 0.018, 0.021$ and <0.001 , respectively) and the interaction of *MTHFR* C677T with ever drinking on FA ($\beta = 0.033, P = 0.048$).

Conclusion: The present GWAS revealed the folate metabolism-associated genetic loci and gene–environment interactions with drinking and physical activity in Japanese, suggesting the possibility of future personalized cardiovascular disease prevention.

Key words: genome-wide association study; folate metabolism; gene–environment interaction; cardiovascular disease prevention

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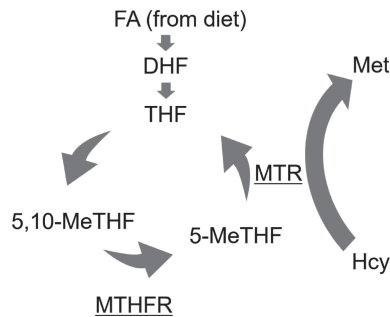


Figure 1. Homocysteine metabolism pathway. Chr, Chromosome; FA, Folic acid; DHF, Dihydrofolate; THF, Tetrahydrofolate; 5,10-MeTHF, 5,10-Methylene THF; 5-MeTHF, 5-Methyl THF; Hcy, Homocysteine; Met, Methionine; MTHFR, Methylene tetrahydrofolate reductase; MTR, 5-Methyltetrahydrofolate-homocysteine methyltransferase.

INTRODUCTION

Folic acid (FA) is involved in the transfer of one-carbon units of thymidylate, purines, and methionine.¹ Intake of FA is essential for DNA synthesis, stability, repair, and normal cell division, especially during rapid growth such as embryonic development and cancer.¹ The lack of FA in pregnancy is associated with an increased risk of neural tube defects.^{2,3}

Homocysteine (Hcy) is an established blood risk marker of atherosclerosis and subsequent cardiovascular diseases (CVDs). The high concentrations of Hcy are associated with risks of stroke, coronary heart disease, venous thrombosis, peripheral arterial disease, and atherosclerosis.⁴⁻⁷ The increased risk of dementia and Alzheimer's disease is also associated with elevated Hcy.⁸ The heritability of Hcy is estimated to be 47–70%,⁹⁻¹² and gene mutations in Hcy metabolic enzymes are well-known to be related to the levels of Hcy.

Vitamin B₁₂ (VB₁₂) works as a coenzyme for methionine synthase in the formation of Hcy to methionine. The enzyme 5-methyltetrahydrofolate reductase (*MTHFR*) produces 5-methyltetrahydrofolate from 5,10-methyltetrahydrofolate, which is transferred to Hcy by methionine synthase to form methionine and tetrahydrofolate (Figure 1). In Hcy metabolism, FA deficiency and lack of VB₁₂ may lead to homocysteinemia, and subsequent vascular inflammation and increased risk of CVDs.¹³ The conditions of the lack of VB₁₂ also lead to megaloblastic anemia and neurodegeneration, and to cognitive decline. Moreover, it leads to an increased risk of neural tube defects.¹⁴

Several studies have reported associations between genetic polymorphisms related to the folate metabolizing pathway and Hcy metabolism. The associations of *MTHFR*, methionine synthase, thymidylate synthase, and serine hydroxymethyltransferase genes with the risk of several types of cancers, including colorectal cancer, breast cancer, and malignant lymphoma, were found by the candidate single-nucleotide polymorphism (SNP)-based association studies.¹⁵⁻¹⁸

In addition, one recent genome wide association study (GWAS) has revealed several functional variants that affect folate metabolisms in humans, including genomic variants in *betaine-homocysteine S-methyltransferase*, *folate hydrolase 1*, and *cystathionine beta-synthase* genes.¹⁹ Several GWAS on folate metabolisms have also been conducted, but few studies have

examined the interaction between these SNPs and lifestyles,²⁰ prompting us to investigate these associations to find a way for possible individualized prevention of human disorders related to folate metabolism, such as CVDs, in the future.

This study aimed to investigate the SNPs associated with blood FA, VB₁₂, and Hcy levels in Japanese using the GWAS data of the Japan Multi-Institutional Collaborative Cohort (J-MICC) Study and to detect the interactions between genetic polymorphisms and lifestyle variables, such as smoking, alcohol intake, and physical activity (PA), on blood Hcy concentrations, FA, and VB₁₂ among Japanese.

METHODS

Study subjects

This study is part of the J-MICC study. The J-MICC study is one of the largest genome cohort studies in Japan, conducted at 13 independent universities and research institutions whose main goal is to detect gene–environment interactions mainly for cancer prevention.²¹ The recruitment for participants began in 2005 and ended in March 2014, with a total of 92,610 participants nationwide.²² At the baseline survey, the volunteer participants aged 35–69 completed a self-administered questionnaire and provide blood samples after informed consent. We collected blood samples in a 7-mL vacuum tube for serum and a 7-mL EDTA-2Na-containing vacuum tube for plasma and buffy coat. DNA was extracted from buffy coat and provided for genotyping. In the present study, we measured and analyzed plasma Hcy, FA, and VB₁₂ levels of 2,263 participants from eight independent study sites of the J-MICC study (Chiba, Aichi Cancer Center, Shizuoka, Daiko, Kyoto, Okazaki, Saga and Kagoshima), who participated in the study from the year of 2005 to 2012.

In the replication phase, we used a single data set consisting of 572 participants from the Yakumo Study.²³ The Yakumo Study is an epidemiological study conducted annually on health checkup examinees aged ≥ 39 years who resided in Yakumo-cho, Hokkaido, Japan since the year of 1982.^{23,24} We asked the 593 health check-up examinees residing in Yakumo-cho, Hokkaido to complete a self-administered questionnaire and provide blood samples after explanation of the aim and the procedure of the study, of whom 572 (96.5%) provided the consent for genetic testing and participated in the study.

Informed consent was obtained from all participants in this study. The protocol of this study was approved by the ethics committees of the Nagoya University Graduate School of Medicine (approval number: 253) and by each participating institution. All research procedures were conducted according to the Ethical Guidelines for Human Genome and Genetic Sequencing Research and the Ethical Guidelines for Medical and Health Research Involving Human Subjects in Japan.

Sample measurement and phenotype definition

In the J-MICC Study, the plasma sample of participants was stored at -80°C until measurements were performed. The serum Hcy levels were measured with EIA (enzyme immune assay) using JCA-RX20 autoanalyzer in a laboratory (SRL Inc., Hachioji, Japan) in Yakumo Study, and plasma Hcy levels were measured with LC-MS/MS (liquid chromatography-tandem mass spectrometry) in the J-MICC Study; serum FA and VB₁₂ levels in Yakumo Study and plasma FA and VB₁₂ levels in the J-MICC Study were measured with EIA in the same laboratory. Measurements of Hcy

by LC-MS/MS, or the measurements of Hcy, FA and VB₁₂ by EIA are shown to have high accuracy with CV (coefficients of variance) within 5%,^{25–28} suggesting that the results of these measurements are highly reliable. The clinically normal laboratory values are >4.0 ng/mL for plasma FA, 180–914 pg/mL for plasma VB₁₂, and 3.7–13.5 nmol/mL for plasma Hcy. In the Yakumo Study, the serum samples of the study participants were measured using the same methods.

Genotyping, imputation and quality control

DNA samples were automatically extracted from the buffy coat using a BioRobot M48 Workstation (QIAGEN group, Tokyo, Japan). Genotyping for the discovery phase has been performed using the Illumina HumanOmniExpressExome ver1.2 platform (Illumina, San Diego, CA, USA) at the RIKEN Center of the Integrated Medical Sciences (Yokohama, Japan). We estimated identity by descent sharing using the PLINK 1.9 software (<https://www.cog-genomics.org/plink2>) option ‘--genome’ to detect closely related pairs and principal component (PC) analysis²⁹ with a 1,000 Genomes reference panel (phase 3; <http://www.internationalgenome.org/category/phase-3/>) using Eigensoft v6.0.1³⁰ on an LD pruned SNP set. The LD pruned SNP set was obtained by removing high-LD SNPs with a genotype call rate <0.98, or a minor allele frequency (MAF) <0.01, or Hardy–Weinberg equilibrium exact test *P* value <1 × 10⁻⁶. The LD pruned SNP set was obtained using the PLINK option ‘--indep-pair-wise 50 5 0.2’. The identity-by-descent method identified 388 close relationship pairs (π -hat > 0.1875) and one sample from each of the 388 pairs was deleted. PC analysis with a 1,000 Genomes reference panel (phase 3) found 34 participants whose estimated ancestries were outside the Japanese population, who were identified by visual inspection after plotting the first and second PCs and excluded from the analyses. Finally, filtering for quality control resulted in 14,083 individuals and 575,802 SNPs. Genotype Imputation was Conducted Using SHAPEIT Version 2 (http://mathgen.stats.ox.ac.uk/genetics_software/shapeit/) and Minimac3 (genome.sph.umich.edu/wiki/Minimac3) software based on the 1,000 Genomes Project cosmopolitan reference panel (phase 3). After the genotype imputation, variants with MAF <0.05 and r^2 < 0.3 were excluded, leaving 6,288,024 variants for final analyses. In the discovery phase, the data for 2,263 subjects of 8 study areas (about 16% of the entire GWAS subjects), whose plasma folate levels were available, were used. In the replication phase, we determined the genotypes of all the discovered SNPs other than the SNP of *NOX4* (*NADPH Oxidase 4*) by the TaqMan real-time polymerase chain reaction (PCR), using the StepOnePlus™ real-time PCR system (ThermoFisher, Waltham, MA, USA). As the genotyping for *Nox4* rs2289125 was technically difficult and unsuccessful, we adopted *Nox4* rs10830278 as a surrogate marker, which was in tight linkage with rs2289125 ($D' = 0.9792$, $r^2 = 0.8685$). For the *NOX4* rs10830278 polymorphism, we determined the genotypes using PCR with confronting two-pair primers (PCR-CTPP).³¹ The primers used (and the thermal cycler conditions) for *NOX4* rs10830278 were as follows: F1: CTA TTA GGT TGA GCC ATA TAA AAT GGC TGA TT, R1: TCA TGT TGT CAC AAA TGG CAG GA, F2: ATG AGA TTA TAA AAG GGG CCA AGA ACT G and R2: TTG AAT CAT ATA GAT TGG TAG ATC AGA AAC AGT CAA AT (initial denaturation at 95°C for 10 min, followed by 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 5 min). The representative gel for the genotyping is shown in eFigure 1. We

also confirmed the genotyping results for rs10830278 using PCR-CTPP were completely replicated by TaqMan real-time PCR (primer-probe used: TaqMan SNP Genotyping, SNP ID: C_3223929_10).

Evaluation of lifestyle information

The lifestyle information was collected using a self-administered questionnaire by well-trained interviewers at the timing of baseline survey. The questionnaire consisted of items on smoking, alcohol consumption, PA, food intake, and medical history.^{32–34} Smoking habits were categorized as never, former, and current smokers, and pack-years of smoking were counted as well. Alcohol habits were categorized as never, former, and current drinkers, and the amount of alcohol consumption (g/day) was also estimated. Information on PA were collected at baseline survey of J-MICC Study using the questionnaire for the duration per episode in recent years and types of activity. The assigned intensity (in metabolic equivalents) was set based on International Physical Activity Questionnaire as follows^{35,36}: labor work, 4.5; walking, 3.0; mild exercise (not breathtaking), 3.4; breathtaking hard exercise (talkable), 7.0; breathtaking hard exercise (untalkable), 10.0; standing, 2.0; and sitting, 1.5. The total amount of PA was calculated as the sum of the products of duration and intensity for each type of activity in metabolic equivalents * hour/day. PA was dichotomized based on the distribution of the metabolic equivalents * hour/day, which is the well-known unit for metabolic equivalent, where subjects were coded as 1 if their PAs were more than or equal to 33 percentile, and coded as 0 otherwise.

Statistical analysis

We examined the associations of the SNPs with the quantitative traits of plasma FA, VB₁₂, and Hcy using the EPACTS software (<http://genome.sph.umich.edu/wiki/EPACTS>). The associations of SNPs with the natural logarithms of plasma FA, VB₁₂, and Hcy as continuous variables were tested using linear regression. For covariates to be adjusted, gender, age, and the first five principal components were included. All 6,288,024 variants with the MAF greater than (or equal to) 0.05 were considered. Manhattan and Q-Q Plots were generated using the ‘qqman’ function in R (<https://cran.r-project.org/web/packages/qqman/index.html>). Lead SNPs (or variants) were defined as those SNPs (or the variants) that reached the minimum *P*-values in each genetic locus, defined as the positions on the chromosome identified by the cytogenetic banding of the chromosome.³⁷ The β coefficient for the gene–environment interaction was estimated based on linear regression with the multiplicative product term for the interaction of the number of minor alleles and lifestyles as binary variables. The GxEs of GWAS identified SNPs with lifestyles of smoking, alcohol consumption and physical activity on blood levels of folate metabolites (Hcy, FA and VB₁₂) were examined.

For the analyses of GWAS, the genome-wide significance levels were set at $P < 5 \times 10^{-8}$, and suggestive levels were set at $P < 1 \times 10^{-6}$; for the rest of the analyses, statistical significance levels were set at $P < 0.05$, where adjustments for multiple comparisons were not applied due to the exploratory nature of the analyses.³⁸

RESULTS

Study characteristics and the genome-wide association study of folate metabolites

The characteristics of the study participants were shown in

Table 1. In the J-MICC Study, median plasma Hcy concentration were higher in men than in women (8.8; interquartile range [IQR], 7.2–10.8 nmol/mL vs 6.8; IQR, 5.8–8.2 nmol/mL, respectively; $P < 0.001$), but median plasma FA and VB₁₂ were higher in women than in men (855; IQR, 730–1,010 pg/mL vs 800; IQR, 700–945 pg/mL, respectively; $P < 0.001$).

At the first stage of GWAS, we found that multiple SNPs were associated with plasma Hcy, FA, and VB₁₂ levels at genome-wide significant level of $P < 5 \times 10^{-8}$ in six genomic regions (Table 2). The Manhattan plots, regional plots for the gene locus found to be genome-wide significant and Q-Q plots for the GWAS of Hcy, FA, and VB₁₂ levels are shown in Figure 2, Figure 3, and

eFigure 2, respectively. Plasma Hcy levels were significantly associated with three loci; *MTHFR* locus on chr 1 (lead SNP: rs1801133, also known as *MTHFR* C677T with $\beta = 0.09178$ and $P = 1.10 \times 10^{-28}$), *NOX4* locus on chr 11 (rs2289125 with $\beta = 0.06557$ and $P = 2.28 \times 10^{-16}$), *charged multivesicular body protein 1A (CHMP1A)* on chr 16 (rs71374191 with $\beta = 0.06382$ and $P = 5.84 \times 10^{-14}$), and *dipeptidase 1 (DPEPI)* on chr 16 (rs9673694 with $\beta = 0.06481$ and $P = 8.38 \times 10^{-14}$, and rs1126464 with $\beta = -0.06293$ and $P = 2.67 \times 10^{-13}$), both of which are considered as located on the same *DPEPI/CHMP1A* genomic locus.³⁹ Plasma FA levels were associated with *MTHFR* locus on chr 1 (lead SNP: rs1801133 with $\beta = -0.05960$ and

Table 1. Characteristics of participants in the Japan Multi-Institutional Collaborative Cohort (J-MICC)

	J-MICC						Yakumo							
	Total (n = 2,263)		Men (n = 1,036)		Women (n = 1,227)		P value	Total (n = 572)		Men (n = 212)		Women (n = 360)		P value
Age, years, mean SD	55.7	8.9	56.6	8.7	54.9	9.0		<0.001	64.4	10.2	66.6	10.4	63.1	
FA, ng/mL, median (IQR)	8.5	(7.4–10.1)	8.1	(7.1–9.4)	8.9	(7.7–10.5)	<0.001	5.8	(4.6–7.8)	5.3	(4.1–7.0)	6.1	(4.9–8.1)	0.018
Hcy, nmol/mL, median (IQR)	7.6	(6.2–9.4)	8.8	(7.2–10.8)	6.8	(5.8–8.2)	<0.001	7.5	(6.5–9.3)	8.7	(7.3–10.6)	7.0	(6.2–8.2)	<0.001
VB ₁₂ , pg/mL, median (IQR)	835	(715–975)	800	(700–945)	855	(730–1,010)	0.931	1,070	(829–1,280)	1,035	(804–1,245)	1,110	(845–1,305)	0.174
Smoking, n (%)														
Never	1,346	(59.5)	268	(25.9)	1,078	(87.9)		369	(64.5)	69	(32.5)	300	(83.3)	
Former	507	(22.4)	449	(43.3)	58	(4.7)	<0.001	119	(20.8)	91	(42.9)	28	(7.8)	<0.001
Current	408	(18.0)	318	(30.7)	90	(7.3)		84	(14.7)	52	(24.5)	32	(8.9)	
Former + current	915	(40.4)	767	(74.0)	148	(12.1)		203	(35.5)	143	(67.4)	60	(16.7)	
Pack-years, mean SD	16.8	28.9	33.5	34.7	2.8	9.4		32.7	20.0	39.0	19.9	22.5	15.6	
Drinking, n (%)														
Never	932	(41.2)	188	(18.2)	744	(60.6)	<0.001	302	(52.8)	51	(24.1)	251	(69.7)	<0.001
Former	39	(1.7)	24	(2.3)	15	(1.2)		20	(3.5)	13	(6.1)	7	(1.9)	
Current	1,290	(57.0)	823	(79.4)	467	(38.1)		250	(43.7)	148	(69.8)	102	(28.3)	
Former + current	1,329	(58.7)	847	(81.8)	482	(39.3)		270	(47.2)	161	(75.9)	109	(30.2)	
Alcohol consumption, g/day, mean SD	15.5	39.5	15.7	40.9	15.3	38.2		24.7	28.5	33.5	31.4	11.8	16.7	

FA, folic acid; Hcy, homocysteine; VB₁₂, vitamin B₁₂.

Table 2. The significant SNPs associated with plasma homocysteine (Hcy)/folic acid (FA)/vitamin B₁₂ (VB₁₂) level from Genome Wide Association Study (GWAS) of participants in the Japan Multi-Institutional Collaborative Cohort (J-MICC)

Hcy													
rsID	Genotyped/ imputed	Cytoband	Chr	Position	Gene	Function	Effect allele	Ref. allele	J-MICC		Yakumo		
									Beta	P value	Beta	P value	
rs1801133	genotyped	1p36.22	1	11856378	<i>MTHFR</i>	Nonsynonymous	A	G	0.09178	1.10×10^{-28}	0.02519	0.106	
rs2289125	imputed	11q14.3	11	89224453	<i>NOX4</i>	Utr5	C	A	0.06557	2.28×10^{-16}	0.00391	0.821	
rs71374191	imputed	16q24.3	16	89714866	<i>CHMP1A</i>	Intron	C	T	0.06382	5.84×10^{-14}	0.02870	0.013	
rs9673694	imputed	16q24.3	16	89694169	<i>DPEPI</i>	Intron	C	A	0.06481	8.38×10^{-14}	0.02264	0.166	
rs1126464	genotyped	16q24.3	16	89704365	<i>DPEPI</i>	Nonsynonymous	C	G	-0.06293	2.67×10^{-13}	0.02671	0.103	

FA													
rsID	Genotyped/ imputed	Cytoband	Chr	Position	Gene	Function	Effect allele	Ref. allele	J-MICC		Yakumo		
									Beta	P value	Beta	P value	
rs1801133	genotyped	1p36.22	1	11856378	<i>MTHFR</i>	Nonsynonymous	A	G	-0.05960	7.05×10^{-17}	-0.02260	0.339	

VB ₁₂													
rsID	Genotyped/ imputed	Cytoband	Chr	Position	Gene	Function	Effect allele	Ref. allele	J-MICC		Yakumo		
									Beta	P value	Beta	P value	
rs1047781	imputed	19q13.33	19	49206631	<i>FUT2</i>	Nonsynonymous	T	A	0.04298	4.30×10^{-8}	-0.11459	<0.001	

Hcy, homocysteine; *MTHFR*, 5-methyltetrahydrofolate reductase; *NOX4*, NADPH oxidase 4; *CHMP1A*, charged multivesicular body protein 1A; *DPEPI*, dipeptidase 1; FA, folic acid; VB₁₂, vitamin B₁₂; *FUT2*, fucosyltransferase 2.

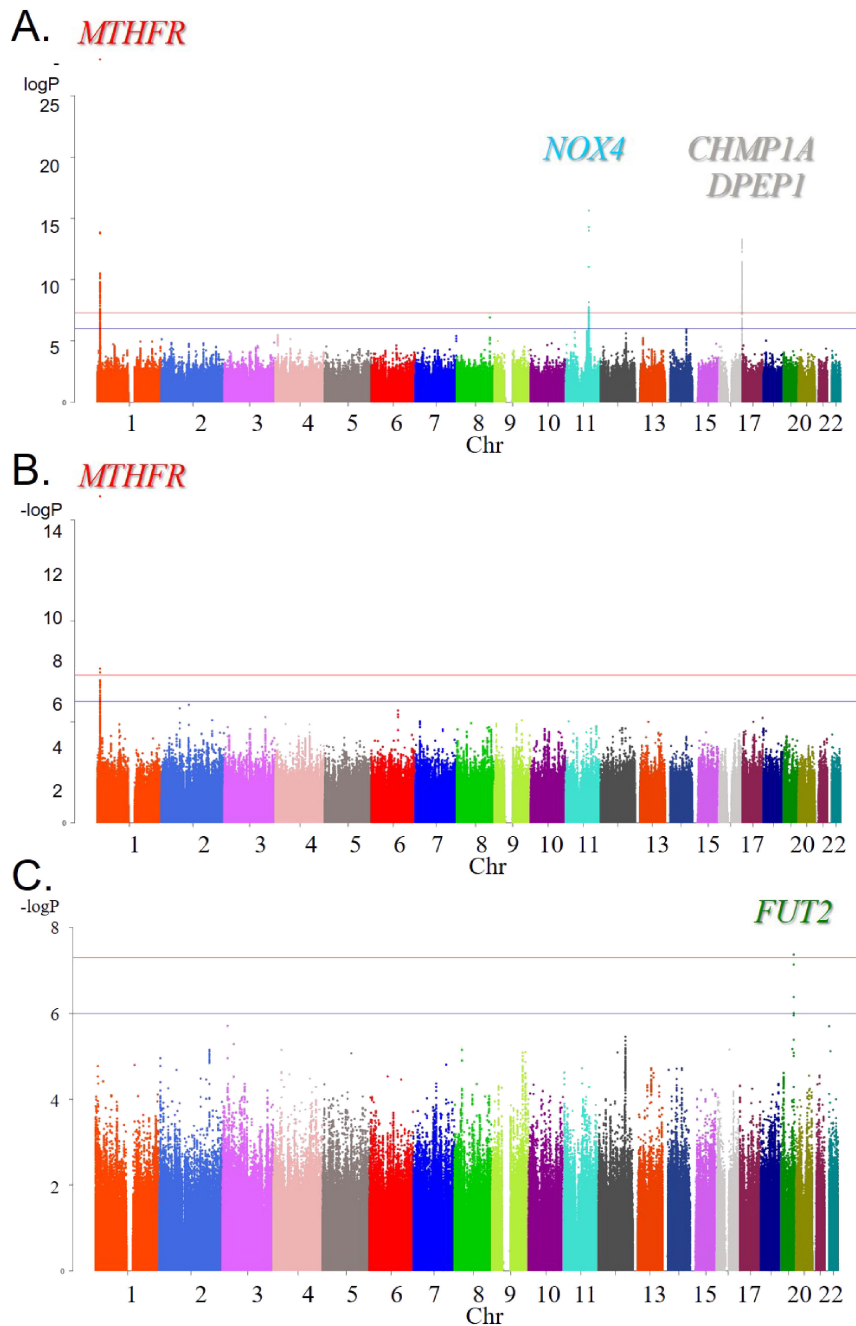


Figure 2. Manhattan Plots for the genome-wide association study (GWAS) of plasma folate metabolites of homocysteine (Hcy)/folic acid (FA)/vitamin B₁₂ (VB₁₂) in the Japan Multi-Institutional Collaborative Cohort (J-MICC) ([A] Hcy: $n = 2,192$; [B] FA: $n = 2,263$; [C] VB₁₂: $n = 2,260$).

$P = 7.05 \times 10^{-17}$), and plasma VB₁₂ levels were associated with fucosyltransferase 2- (*FUT2*) locus on chr 19 (rs1047781 with $\beta = 0.04298$ and $P = 4.30 \times 10^{-8}$) at genome-wide significant level of $P < 5 \times 10^{-8}$. In the independent dataset of Yakumo Study, most of the GWAS significant loci were in the same direction of effect (*MTHFR* rs1801133 with $\beta = 0.02519$ and $P = 0.106$, *NOX4* rs2289125 with $\beta = 0.00391$ and $P = 0.821$, *CHMP1A* rs71374191 with $\beta = 0.02780$ and $P = 0.013$, and *DPEPI* rs9673694 with $\beta = 0.02264$ and $P = 0.166$), except for the *DPEPI* rs1126464 ($\beta = -0.02260$ and $P = 0.339$) and *FUT2* rs1047781 ($\beta = -0.11459$ and $P < 0.001$) (Table 2). In addition, we also conducted the GWAS of folate metabolites adjusted for FA intake, which did not substantially affect the results (data not

shown). For the suggestively significant loci ($P < 1 \times 10^{-6}$), the *PVT1* locus on chromosome 8 associated with plasma Hcy had never been reported based on the information of GWAS catalogue (www.ebi.ac.uk/gwas/), suggesting that this SNPs was newly found (eTable 2).

Gene–environment interaction analyses

We also examined the gene–environment interactions of *MTHFR* *C677T* (rs1801133) with lifestyles (Table 3). Although the previously reported interaction of *MTHFR* *C677T* and smoking habits on plasma Hcy was only marginal ($\beta = 0.029$, $P = 0.089$), a significant interaction of *MTHFR* *C677T* and drinking habits on plasma FA, and the interaction of *MTHFR* *C677T* and PA on

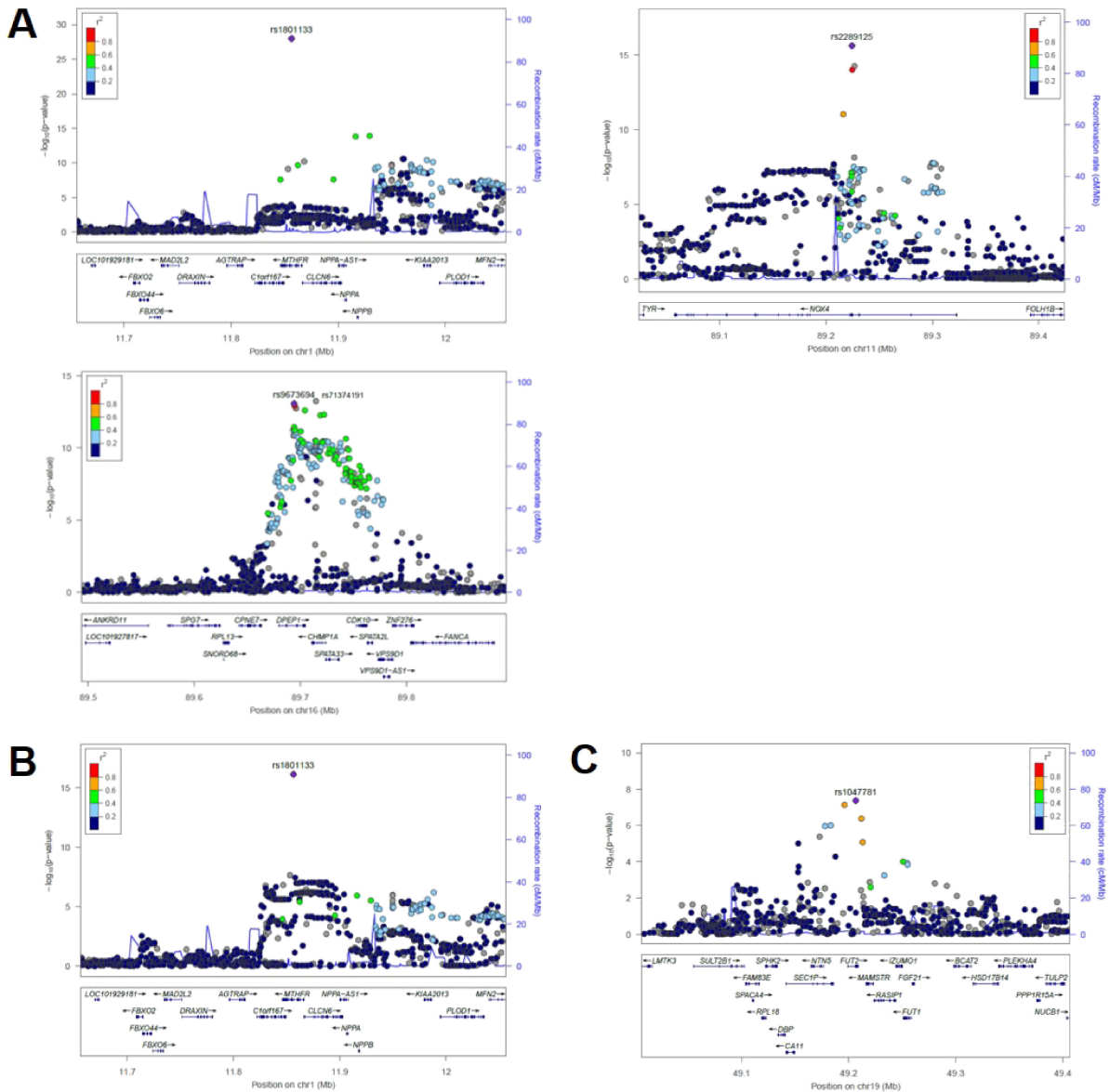


Figure 3. Regional Plots for the Genome Wide Association Study (GWAS) of plasma folate metabolites of homocysteine (Hcy)/ folic acid (FA)/vitamin B₁₂ (VB₁₂) in the Japan Multi-Institutional Collaborative Cohort (J-MICC) [**A**] Hcy: *MTHFR* locus on 1p36, *NOX4* locus on 11q14 and *DPEP1-CHMP1A* locus on 16q24; [**B**] FA: *MTHFR* locus on 1p36; [**C**] VB₁₂: *FUT2* locus on 19q13).

plasma Hcy were observed ($\beta = -0.040$, $P = 0.020$; $\beta = 0.035$, $P = 0.040$) (eFigure 3).

To validate these findings, we examined the corresponding interactions in an independent dataset from the Yakumo Study (Table 3). While only statistically marginal, we recapitulated the trend of the interactions of *MTHFR* 677 T/T with current drinking on FA and with PA on Hcy ($\beta = 0.074$, $P = 0.054$, and $\beta = -0.054$, $P = 0.100$, respectively).

We also examined these observed interactions in a combined data set of J-MICC and Yakumo Study. A significant interaction between *MTHFR* C677T and drinking habits on plasma FA ($\beta = 0.037$, $P = 0.019$ for the interaction of *MTHFR* C677T and ever drinking, $\beta = 0.037$, $P = 0.020$ for the interaction with current drinking), and the interaction between *MTHFR* C677T and PA on plasma Hcy were observed ($\beta = -0.043$, $P = 0.005$). (Table 3). In addition, the exhaustive examination of gene–environment interactions between the lead SNPs (and the surrogate,

Nox4 rs10830278) found in the present GWAS and the lifestyles of smoking habits, drinking habits, and PA revealed several statistically significant associations, although most of the interactions based on smoking, drinking, or PA as continuous variables failed to reach any statistical significance, which may worth verifying in future studies (eTable 1).

Associations of folate metabolites with the reported loci

We also examined the associations of each of folate metabolites (Hcy, FA, and VB12) with previously reported loci based on GWAS catalogue (trait ID: EFO_0004578, EFO_0005111 and EFO_0004620). As a result, statistically significant associations of rs1801133 (in the *MTHFR* gene on chromosome 1), rs12085006, and rs1999594 (both on chromosome 1) with blood Hcy levels and that of rs1801133 with blood FA levels were observed (eTable 3).

Table 3. The interactions of *MTHFR* C677T rs1801133 and lifestyles on plasma folate metabolites of homocysteine (Hcy)/folic acid (FA)/vitamin B₁₂ (VB₁₂) with lifestyle in the Japan Multi-Institutional Collaborative Cohort (J-MICC) and Yakumo study

	<i>MTHFR</i> rs1801133					
	J-MICC		Yakumo Study		Meta	
	Beta ^a	P-value ^a	Beta ^a	P-value ^a	Beta ^a	P-value ^a
Smoking						
Hcy						
ever	0.029	0.089	-0.012	0.706	0.020	0.184
current	0.030	0.185	-0.082	0.048	0.004	0.822
FA						
ever	0.016	0.270	0.035	0.486	0.018	0.209
current	0.015	0.445	0.009	0.887	0.014	0.440
VB12						
ever	-0.028	0.124	0.020	0.699	-0.022	0.186
current	-0.037	0.107	0.032	0.636	-0.030	0.171
Drinking						
Hcy						
ever	0.034	0.080	-0.004	0.903	0.024	0.156
current	0.033	0.151	-0.006	0.088	0.023	0.175
FA						
ever	0.035	0.040	0.058	0.220	0.037	0.019
current	0.033	0.129	0.074	0.054	0.037	0.020
VB12						
ever	-0.032	0.120	-0.023	0.636	-0.031	0.106
current	-0.034	0.086	-0.020	0.102	-0.032	0.095
PA						
Hcy						
PA >33 percentile	-0.040	0.020	-0.054	0.100	-0.043	0.005

MTHFR, 5-methyltetrahydrofolate reductase; J-MICC, Japan Multi-Institutional Collaborative Cohort; Hcy, homocysteine; FA, folic acid; VB₁₂, vitamin B₁₂; PA, physical activity.

^aAdjusted for age and sex.

DISCUSSION

Polymorphisms of *MTHFR*, *DPEP1*, *NOX4*, and *FUT2* have been previously reported to be associated with FA, VB₁₂, and Hcy levels.^{20,40,41} However, no GWAS of the folate metabolic pathways has been reported in Japanese. The present GWAS suggested that polymorphisms of *MTHFR*, *DPEP1*, *CHMP1A*, *FUT2*, and *NOX4* may play an important role in the regulation of plasma Hcy levels in Japanese.

Hcy remethylation requires a cosubstrate 5-methyltetrahydrofolate to form methionine. *MTHFR* catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate.⁴²⁻⁴⁴ The homozygotes of the thermolabile *MTHFR* 677T allele are shown to have reduced *MTHFR* enzyme activity,⁴⁵ which leads to higher blood Hcy levels and subsequent elevated risks of various vascular/atherosclerotic diseases.⁶

NOX4 has been previously associated with Hcy in three studies.⁴⁶⁻⁴⁸ *NOX4* is expressed in endothelial cells, cardiomyocytes, and vascular smooth muscle cells,⁴⁹ and elevated expressions were reported in patients with hypertension, atherosclerosis, heart failure, and stroke.⁵⁰ Upregulation of *NOX4* may also contribute to Hcy-mediated apoptosis of endothelial cells.⁵¹

DPEP1 is a kidney membrane enzyme that is highly expressed in the proximal convoluted tubules.⁵² Although their roles in Hcy

metabolism remain unclear, they may be involved in renal handling and metabolism of Hcy and cysteine, a precursor of Hcy. *DPEP1* was previously associated with Hcy in one analysis.⁴⁸

We also found a reported association between *CHMP1* polymorphism and plasma Hcy in humans.⁴⁷ *CHMP1A* is involved in protein transport, and is essential for the proliferation and maintenance of neural progenitor cells.⁵³

FUT2 (rs602662, rs492602, and the *FUT2* haplotype) was associated with VB₁₂ levels, which confirmed previously reported findings.^{19,54,55} Another non-synonymous SNP in the *FUT2* gene rs10447781 was strongly associated with VB₁₂ levels. *FUT2* variants reportedly reduce H-type antigen production and function and decrease the risk of VB₁₂ malabsorption due to *Helicobacter pylori* infection and associated gastritis.⁵⁶ In addition, *FUT2* variants increase the secretion of fucosylated glycoprotein of gastric intrinsic factors required for VB₁₂ absorption.⁵⁷

The functionality of *MTHFR* rs1801133 was already well demonstrated in previous reports⁴⁵; the rs2289125 SNP of *NOX4* is located in the open chromatin in most tissues and thus may associate with transcription regulation.⁴⁶ The rs1126464 SNP of *DPEP1* and the rs1047781 of *FUT2* is located in the non-synonymous region and may exert effect through alteration of *DPEP1* protein structure.

Some of the SNPs found in the present GWAS in association with blood Hcy, FA, and VB12 compared with previously found SNPs in the GWAS were partially the same (rs1801133 of *MTHFR* and rs2289125 of *NOX4*) but other SNPs were different SNPs in the same genes/genomic loci. For example, the and rs71374191 of the *DPEP1/CHMP1A* locus found in association with blood Hcy levels were different from those reported in European GWAS (rs7130284 of *NOX4* and rs154657 of *DPEP1*).⁴⁷ Although the present study found the rs1047781 of *FUT2* as the top significant SNP for VB₁₂, the two SNPs of rs602662 and rs492602 of *FUT2* are non-polymorphic in Japanese. The different SNPs found to be GWAS significant in association with blood folate metabolites may suggest the results of different evolutionary pressure between ethnicities and may lead to population-specific prevention strategies against diseases related to folate metabolism disorders, such as atherosclerosis and CVDs.^{58,59}

In previous studies, a positive interaction between *MTHFR* C677T and smoking habits on plasma Hcy was reported.^{18,60-63} The present study revealed the marginal interaction in the same direction, although it did not reach statistical significance. According to the Organisation for Economic Co-operation and Development (OECD) health statistics (<https://stats.oecd.org/>), the rate of male smokers is higher in Japan (around 30%) compared with those in other OECD member countries, such as United States or United Kingdom (around 10–20%). The interaction with smoking habits for *MTHFR* and vascular diseases risk may be explained by a similar mechanism via elevated Hcy.⁶³ Considering the different prevalence of smokers between countries, further investigation with sufficiently large sample sizes in each country's population is needed to clarify the interaction of *MTHFR* C677T with smoking habits on blood Hcy levels.

This study observed a positive interaction between *MTHFR* C677T and drinking habits on blood FA (ie, as the T allele of *MTHFR* C677T and drinking amount increase, they have multiplicative interactive effects on the increment of FA), and a negative interaction between *MTHFR* C677T and PA on blood Hcy (ie, as the T allele of *MTHFR* C677T and calories spent by

PA increase, they have multiplicative interactive effects on the reduction of Hcy) in Japanese. *MTHFR* 677T/T is a genotype similar to *ALDH2* Lys/Lys, and individuals with *MTHFR* 677T/T may have avoidant and deterrent responses to alcohol consumption due to excessive savings of Hcy in those with *MTHFR* 677T/T, which may explain the possible gene–environment interaction between drinking habits and *MTHFR* 677T allele on blood FA levels.⁶⁴ This might act against alcohol dependence and lead to attenuated FA reduction in those with *MTHFR* 677T/T genotype in drinkers, which is consistent with the results of previous studies.^{20,65} In the present study, however, no significant association between *MTHFR* 677T/T genotype and drinking behavior was observed. Considering that the recent meta-analysis revealed no association of *MTHFR* 677T/T and alcohol dependence, the effect of *MTHFR* genotype on drinking behavior might be relatively limited.⁶⁶ In addition, the reduced blood FA levels in subjects with *MTHFR* 677 T/T genotype in the present study are in accordance with the previous report,⁶⁷ underscoring the requirements for more folate intake in those with *MTHFR* 677 T/T genotype than in those with other genotypes. The influence of *MTHFR* polymorphism on the association between PA and blood Hcy levels has been an issue of interest to researchers in recent decades.^{65,68,69} This study might add some beneficial evidence for possible personalized prevention of atherosclerosis/CVDs based on genetic information in the near future. The detailed mechanisms of the reduction in plasma Hcy levels with exercise are not yet well described, which may be explained by mechanisms of protein turnover and the betaine pathway.^{70,71} The existence of different distributions of lifestyle factors, as well as different genotype distributions of SNPs, may lead to discovery of different GxEs and disease prevention measures specific to each population. Further investigations with sufficiently larger sample sizes in each population should be expected to clarify these associations.

The strength of the present study would be that this GWAS was conducted using only population-based cohort data from Japanese population with relatively large sample size, so it is possible that gene–environment interactions might be easier to detect without adjustment in Japanese population, where lifestyle components are relatively homogeneous.

Our study has several limitations. First, all lifestyle factors are self-reported and thus subject to bias, which is generally bias toward null. Second, we investigated the gene–environment interaction based only on the SNPs detected from GWAS results of folates (Hcy, FA, and VB₁₂) as outcomes. Although there is an alternative method to examine gene–environment interactions comprehensively based on GWAS data using the ProbABEL, the gene–environment interaction detection software in GWAS, we adopted a candidate SNP approach using GWAS detected SNPs because GWAS data using current folate measurement data ($n = 2,263$) are somewhat underpowered to detect robust gene–environment interactions. In addition, the cut-off level for the dichotomization of PA was determined to be 33% based on our sensitivity analysis for different cut-off levels of PA, which was mainly due to the exploratory context of the present study. Further investigations with sufficiently large sample sizes for this potentially intriguing interaction of *MTHFR* 677T/T with PA on blood Hcy levels should be warranted.

Conclusion

The present GWAS revealed the significance of folate

metabolism-associated SNPs in *MTHFR*, *DPEP1*, *CHMP1*, and *NOX4* genes also in Japanese. In addition, the interactions of these SNPs with lifestyles of smoking, drinking and PA on blood folate metabolite concentrations may pave the way for the possible personalized prevention of atherosclerotic diseases in Japanese, as well as in world populations. Further investigations are needed to confirm the results of the present study.

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Data availability: The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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SUPPLEMENTARY MATERIAL

Supplementary data related to this article can be found at <https://doi.org/10.2188/jea.JE20220341>.

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