moderate or severe atrophy in the corpus, and 247 (49%) had intestinal metaplasia in the corpus.

H pylori was successfully eradicated from 203 (75%) patients in the eradication group (88 [72%] newly diagnosed patients, 115 [77%] patients in post-resection follow-up). *H pylori* infection resolved in 12 (5%) patients in the control group during follow-up. When patients lost to follow-up are excluded, median length of follow-up was 1076 (range 34–1277) days in the eradication group and 1041 (48–1270) days in the control group. Overall, 133 (52%) patients in the eradication group and 122 (45%) patients in the control group received endoscopic examinations according to the protocol; 167 (66%) patients in the eradication group and 157 (63%) in the control group received endoscopic examination at the end of 3 years' follow-up. There was no significant difference between groups in terms of the number of endoscopies done (data not shown); each patient underwent a mean of 3·1 (SD 1·1) endoscopic procedures. By the end of the study, patients had been followed up for 639 patient-years in the eradication group and for 593 patient-years in the control group.

During 3 years of follow-up after endoscopic treatment of primary gastric cancer, metachronous gastric cancer developed in 33 participants—nine in the eradication group and 24 in the control group. The characteristics of these patients are shown in table 2. There were no differences between the two groups with regard to sex, age, location, histological type, depth of invasion, or diameter of metachronous cancers (table 2).

In the full intention-to-treat population, after adjustment for stratification, the odds ratio for metachronous cancer was 0·353 (95% CI 0·161–0·775, p=0·009) in favour of *H pylori* eradication. In the modified intention-to-treat population, the incidence of metachronous cancer was 14·1 cases per 1000 person-years in the eradication group and 40·5 cases per 1000 person-years in the control group (hazard ratio 0·339, 95% CI 0·157–0·729, p=0·003). The cumulative incidence of gastric cancer differed between the eradication and control groups. The risks in the two groups remained in proportion over time (figure 2).

We also analysed the effect of eradication by stratum; risk ratios were 0·46 [95% CI 0·16–1·33] in the newly diagnosed and 0·27 [0·09–0·79] in the post resection stratum. No interaction between the strata and the allocation was noted (p=0·48). The length of time between resection and randomisation had no effect on the incidence of metachronous carcinoma (data not shown). Residual cancer recurred in 18 participants (eight in the eradication group and ten in the control group) because of incomplete resection.

In the assessment of the robustness of the results, 11 extra metachronous cancers were to be expected if all participants had been followed up for the full 3 years. In the worst-case scenario, six of these extra cases would be in the eradication group (15 in total) and five in the control group (29 in total). The difference in incidence between the two groups was statistically significant (p=0·04).

	Eradication group (N=9)	Control group (N=24)	p value
Sex			0·597*
Male	7 (77·8%)	21 (87·5%)	
Female	2 (22·2%)	3 (12·5%)	
Age (years)	70 (71–73)	71 (65·74)	0·584†
Location			0·160‡
Upper	1 (11·1%)	3 (12·5%)	
Middle	6 (66·7%)	8 (33·3%)	
Lower	2 (22·2%)	13 (54·2%)	
Histology			1·000*
Intestinal type	9 (100%)	23 (95·8%)	
Diffuse type	0 (0·0%)	1 (4·2%)	
Depth of invasion			1·000*
Mucosa	8 (88·9%)	23 (95·8%)	
Submucosa	1 (11·1%)	1 (4·2%)	
Diameter of carcinoma (mm)	8 (7–15)	7 (5–10)	0·383†

Data are n (%) or median (IQR). *Fisher's exact test. † Wilcoxon rank sum test. ‡Goodness of fit test.

Table 2: **Characteristics of patients who developed metachronous carcinoma**

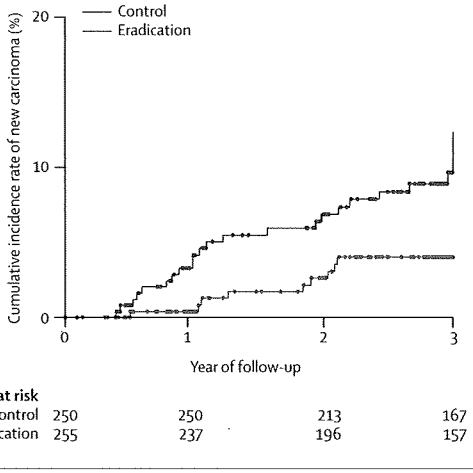

Figure 2: **Kaplan-Meier analysis of cumulative incidence rate of new carcinoma**

No severe or moderate adverse events associated with eradication treatment were seen over the 3 years of follow-up. One individual dropped out of the eradication group because of an adverse event (drug eruption), which resolved within a few days of halting treatment. The only adverse events that occured with a frequency of more than 5% were soft stools (32 [12%] patients in the eradication group) and diarrhoea (19 [7%] patients in the eradication group).

Discussion

The results of this multi-centre, open-label, randomised controlled trial suggest that treatment to eradicate *H pylori* reduces the risk of developing new gastric carcinoma in

patients who have a history of such disease and are thus at high risk for developing further gastric cancers.

Although randomisation was used to reduce potential bias at baseline, one patient in each group voluntarily left the study at allocation, and 21 patients in the control group and 16 in the eradication group left the study during follow-up. However, baseline characteristics were not different between the two groups, except for the time between endoscopic resection and random allocation in patients who enrolled post-resection (although not statistically significant at the 5% level). Therefore, no bias was expected at baseline in terms of patients who were included in final analyses. Because this is an open study, there could have been an observation bias toward finding metachronous cancers, the primary endpoint. Nevertheless, there was no significant difference in size or depth of invasion of metachronous cancers between the two groups (table 2). Thus, the effect of observation bias seems to be negligible. For ethical reasons, the managing committee advised that eradication therapy be given to patients in the control group, as well as to patients in the eradication group who had not been successfully eradicated, after the results of final analyses were assessed.

A prospective randomised trial of *H pylori* eradication with incidence of gastric cancer as the primary endpoint has been attempted before in Japan. However, because of the low number of participants enrolled, the endpoint was changed to examine the reversibility of precancerous gastritis. This experience revealed the difficulty in doing properly designed intervention studies for gastric cancer: such trials would require enrolment of thousands of patients and have to run for decades.[29,30] A theoretical model estimated that in high-risk countries, a sample size of 17 625 middle-aged individuals per group and follow-up period of 10 years would be required to demonstrate a 50% reduction in the expected increase of gastric cancer incidence after *H pylori* eradication.[31] Gastric cancer prevention studies designed to assess the long-term effects of *H pylori* eradication have found that, after receiving informed consent, few participants are prepared to enter the placebo group.[32,33] In this study, we targeted metachronous gastric cancers after endoscopic treatment of early stage gastric cancer. Eligible patients were at high risk for metachronous gastric cancer, thus reducing the number of participants needed and shortening the follow-up period. Furthermore, since the participants knew that they were at high risk for metachronous cancer, they were not reluctant to frequently visit clinics for endoscopic examinations.

In Japan, mucosal gastric cancer is usually resected by endoscopic treatment. Because only a small part of the gastric mucosa is resected, metachronous gastric cancer after endoscopic treatment often develops at another site of the stomach. In one study with 143 patients with early gastric cancer, during a median follow-up period of 57 months after endoscopic treatment, 20 (14%) patients reported metachronous cancers and 16 (11%) reported synchronous cancer.[23] Another study reported that carcinomas existed at sites other than the main lesion in 4·8% of 839 patients who had undergone surgical resection of the stomach.[34] Similar results have been shown in other Japanese studies.[35,36] The incidence of new gastric cancer after endoscopic treatment seems to be consistent with the frequency of undiagnosed gastric cancers in resected stomachs. The high incidence of metachronous gastric cancer may be the result of occult gastric cancers that were not detectable at the time of endoscopic treatment, but had grown enough to be diagnosed during the follow-up.

Risk for gastric cancer is directly related to the degree of atrophy. In a study from China, a benefit with *H pylori* eradication was seen only among those with low baseline risks (without atrophy).[37] This study also showed that *H pylori* eradication was prophylactic in the highest risk group (ie, those with moderate or severe atrophy and a history of early gastric cancer). In the current study, the risk of subsequent cancer was reduced from about 4000 per 100 000 individuals per year to 1400 per 100 000 individuals per year.

Although this study was open label, we believe that the use of a placebo would have made little difference, since endoscopists can predict whether or not a patient has undergone eradication therapy on the basis of the severity of redness in gastric mucosa and mucous status changes after eradication of *H pylori*.[38,39] Furthermore, the trial was designed to be open label to increase the feasibility of enrolling sufficient numbers of participants; Japanese individuals feel strong anxiety when they do not know whether they are being given active drugs or not, and thus often refuse to join placebo controlled trials.

Since participants in this study had a history of gastric cancer, one would expect that they differ from the general population in terms of specific genotypes and environmental factors. Although this could limit the generalisability of the results, we believe that our data add to those from previous studies showing a causal relationship between *H pylori* infection and gastric cancer, and also support the use of *H pylori* eradication to prevent the development of gastric cancer.[40]

Contributors

KF, MK, KI, NU, SO, ST, and KA conceived and designed this study with the Japan Gast Study Group. MK was the coordinating principal investigator for the study. SK and SH analysed and interpreted the results. MK and SK drafted the report. MA was responsible for the overall planning and conduct of the study. All authors were members of the steering committee. All authors have seen and approved the final version of the manuscript.

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Conflict of interest statement

We declare that we have no conflict of interest.

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ARTICLE

Common variants at the *GCK*, *GCKR*, *G6PC2–ABCB11* and *MTNR1B* loci are associated with fasting glucose in two Asian populations

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Abstract

Aims/hypothesis To test fasting glucose association at four loci recently identified or verified by genome-wide association (GWA) studies of European populations, we performed a replication study in two Asian populations.

Methods We genotyped five common variants previously reported in Europeans: rs1799884 (*GCK*), rs780094 (*GCKR*), rs560887 (*G6PC2–ABCB11*) and both rs1387153 and rs10830963 (*MTNR1B*) in the general Japanese (n=4,813) and Sri Lankan (n=2,319) populations. To identify novel

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variants, we further examined genetic associations near each locus by using GWA scan data on 776 non-diabetic Japanese samples.

Results Fasting glucose association was replicated for the five single nucleotide polymorphisms (SNPs) at $p<0.05$ (one-tailed test) in South Asians (Sri Lankan) as well as in East Asians (Japanese). In fine-mapping by GWA scan data, we identified in the *G6PC2–ABCB11* region a novel SNP, rs3755157, with significant association in Japanese ($p=2.6\times10^{-8}$) and Sri Lankan ($p=0.001$) populations. The strength of association was more prominent at rs3755157 than that of the original SNP rs560887, with allelic heterogeneity detected between the SNPs. On analysing the cumulative effect of associated SNPs, we found the per-allele gradients ($\beta=0.055$ and 0.069 mmol/l in Japanese and Sri Lankans, respectively) to be almost equivalent to those reported in Europeans.

Conclusions/interpretation Fasting glucose association at four tested loci was proven to be replicable across ethnic groups. Despite this overall consistency, ethnic diversity in the pattern and strength of linkage disequilibrium certainly exists and can help to appreciably reduce potential causal variants after GWA studies.

Keywords Asians · Association study · Ethnicity · Fasting plasma glucose · Polymorphisms

Abbreviations

CEU	Utah residents with northern and western European ancestry from the Centre d'Etude du Polymorphisme Humain collection
FPG	Fasting plasma glucose
GWA studies	Genome-wide association studies
JPT	Japanese in Tokyo
LD	Linkage disequilibrium
RAF	Risk allele frequency
SNP	Single nucleotide polymorphism

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Introduction

Fasting plasma glucose (FPG) levels are associated with the future risk of type 2 diabetes and cardiovascular diseases [1, 2] and are tightly regulated despite considerable variation in food intake [3]. It has been reported that genetic effects explain 54.8% of the variance of glucose levels in a European population [4]. Recent progress in complex-trait genetics has allowed the identification of loci regulating FPG levels [5–20].

Several loci influencing FPG levels have been identified or verified by genome-wide association (GWA) studies of Europeans; these include glucokinase (*GCK*) [5–7], glucokinase regulatory protein (*GCKR*) [8–10, 13], glucose-6-phosphatase catalytic subunit 2 (*G6PC2*), the ATP-binding cassette, subfamily B (*MDR/TAP*), member 11 (*ABCB11*) [14–17], and melatonin receptor 1B (*MTNR1B*) [16–18].

All the associations were originally identified in populations of European ancestry. While some studies have shown reproducible associations [9, 11, 12, 19, 20], it remains to be further defined to what degree loci discovered in Europeans will show an association in populations of different ancestries. In addition, to localise the variant(s) responsible for an association signal, we need to generate a comprehensive list of potential causal variants in the regions of interest, i.e. to conduct fine-mapping after GWA studies. As discussed elsewhere [21], this fine-mapping will be challenging and genetic information from populations of different ancestries is expected to be useful [21–24].

Apart from assessing the previously identified variants in two Asian populations, Japanese of East Asian ancestry and Sri Lankan of South Asian ancestry, we also explored index single nucleotide polymorphism (SNP) markers, which either tag the SNPs attaining a locus-wise significance level in the GWA scan of Japanese or were previously reported in Europeans. This was done to advance the fine-mapping of the associated loci [5–20].

Methods

Study populations

A replication study of the previously identified variants was performed in the general Japanese and Sri Lankan

populations (Electronic supplementary material [ESM] Table 1, ESM Study samples for continuous traits), using 5,456 Japanese samples (including 4,813 non-diabetic participants) consecutively enrolled in a population-based setting as described elsewhere [25] and 3,012 Sri Lankan samples (including 2,319 non-diabetic participants) who had participated in the baseline survey of the Ragama Health Study [26] in Sri Lanka. Complementary to this replication study, we organised genetic studies of FPG levels as part of an ongoing GWA scan for cardiometabolic disorders among the Japanese population (ESM Study samples for continuous traits). We used 776 population-based, non-diabetic Japanese samples for preliminary screening of association with FPG levels. Then, the association signals were examined in the general populations mentioned above. In addition to quantitative trait analysis, type 2 diabetes associations were tested for index SNPs at *G6PC2–ABCB11* in a Japanese case–control study panel comprising 5,629 cases and 6,406 controls as previously reported [27], and in a Sri Lankan case–control study panel (ESM Study samples for type 2 diabetes case control studies). All participants from these different studies provided written informed consent and the local Ethics Committees approved the protocols.

Type 2 diabetes was diagnosed according to the WHO criteria as described in ESM Study samples for type 2 diabetes case–control studies.

SNP genotyping and quality control

In the replication study, samples were genotyped using the TaqMan assay (Life Technologies Japan, Tokyo, Japan) for five SNPs from four gene loci previously identified in European-descent populations [5–10, 13–18]. These included *GCK* (rs1799884), *GCKR* (rs780094), *G6PC2–ABCB11* (rs560887) and *MTNR1B* (rs1387153 and rs10830963).

In the GWA scans, genotyping was performed with a bead array (Infinium HumanHap550; Illumina, San Diego, CA, USA) as described elsewhere [27] (ESM Fig. 1, ESM SNP genotyping, ESM Quality control of the GWA scan data). After the GWA scan, three additional SNPs in the *G6PC2–ABCB11* region, rs483234, rs3755157 and rs853778, were genotyped with the TaqMan assay for follow-up.

Statistical analysis

SNP association analysis SNPs were tested for association with FPG levels by using linear regression analysis in the additive genotype model (ESM SNP-based association analysis). A p value of <0.05 was considered statistically significant. For an association to be considered significant, it had to involve the same risk allele as that reported in Europeans and was accordingly assessed with a one-tailed test. To assess the proportion of variance for FPG that could be explained by a SNP, we calculated the coefficient of determination R^2. The per-allele gradients, which correspond to the increase in FPG levels by additional 'high FPG' alleles of associated SNPs, were calculated in the linear regression model (including age, sex and BMI as covariates) as previously reported [14, 18] (ESM Evaluation of cumulative effect of multiple loci on FPG). We used PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/), the R software (version 2.8.1; www.r-project.org) and the rmeta package (http://cran.r-project.org) for association test and meta-analysis (websites accessed 15 October 2009).

Haplotype analysis In the *G6PC2–ABCB11* region, we selected SNPs attaining a locus-wise significance level (p<0.002 by Bonferroni's correction for 23 SNPs genotyped in the relevant region) or reported in European studies, inferring the haplotypes using PLINK [28] and PHASE [29] software (http://depts.washington.edu/ventures/UW_Technology/Express_Licenses/PHASEv2.php). We then tested which haplotypes were strongly associated with the trait. In parallel, haplotypes were inferred from the genotype data of the SNPs in HapMap (www.hapmap.org) Utah residents with northern and western European ancestry from the Centre d'Etude du Polymorphisme Humain collection (CEU) and Japanese in Tokyo (JPT) categories using HaploView software (www.broad.mit.edu/mpg/haploview/) [30] and in South Asians from the Human Genome Diversity Panel (http://hagsc.org/hgdp/files.html) [31]. Haplotype-tagging SNPs were selected and characterised in the large study panels.

Stepwise regression analysis for testing of independent associations To test the most likely explanation for the signal of association among the index SNPs and their genotyped correlates, we performed stepwise linear regression analysis for FPG levels by forward selection (ESM Index SNPs showing an independent association). If two SNPs simultaneously included in the model each attained significance (p<0.05), they could have independent associations. Further, when two haplotype classes that are distant in the phylogeny have an opposite effect and are tagged by two SNPs showing independent associations, the haplotype classes are presumably linked to different causative variants, thus implying allelic heterogeneity (ESM Haplotype explaining index association).

Cross-population filtering of causal variants To appreciably narrow the location of potential causal variants, we closely inspected subsets of SNPs and haplotypes shared by multiple ethnicities. We partitioned all the HapMap SNPs

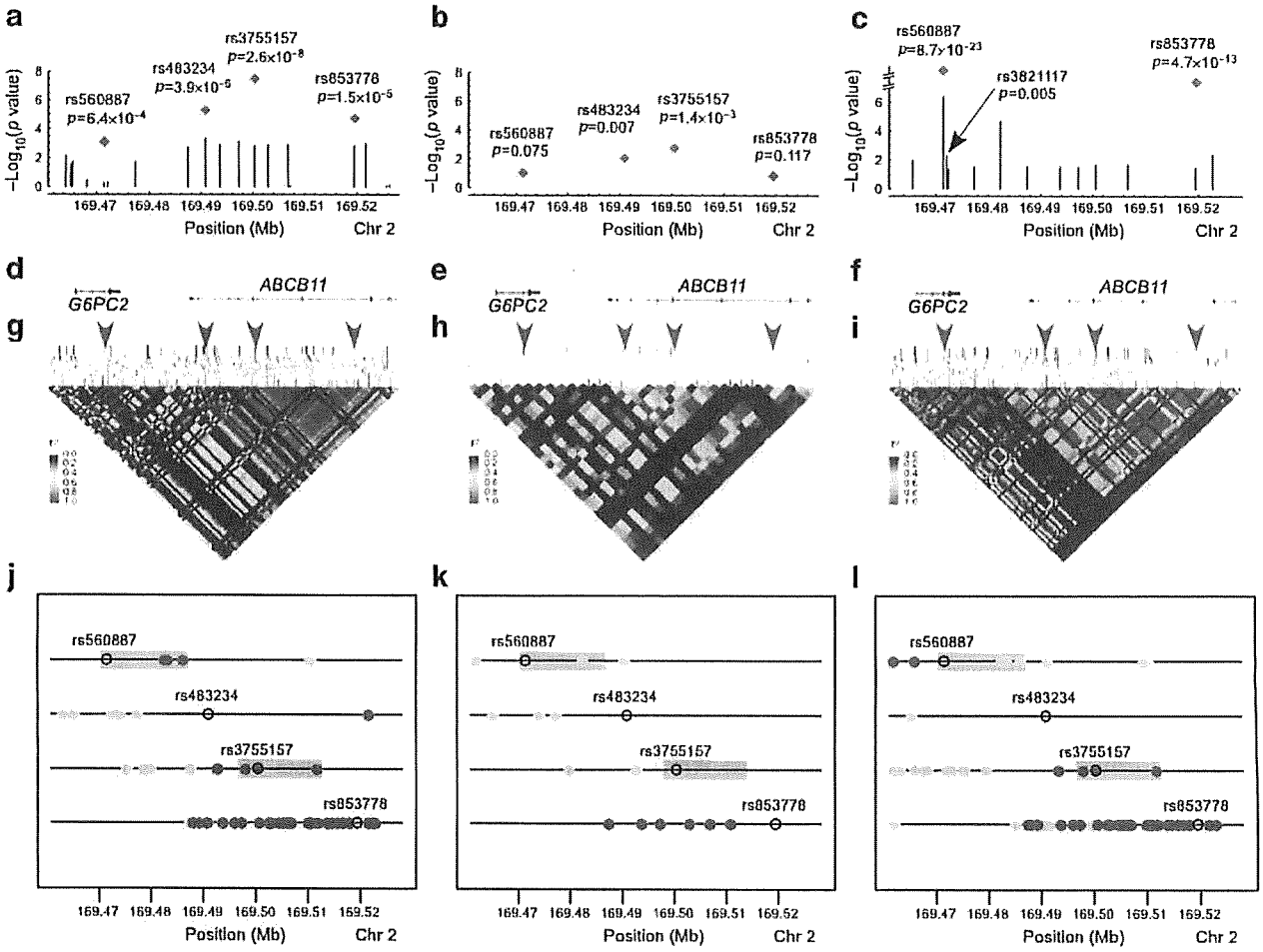

Fig. 1 Plots of FPG association, and LD and SNP partitioning for the *G6PC2–ABCB11* region in Japanese (**a**, **d**, **g**, **j**), Sri Lankan (**b**, **e**, **h**, **k**) and Europeans (**c**, **f**, **i**, **l**). Association results for Europeans are drawn from the published studies [14, 17]. **a**–**c** Bar graphs of all genotyped SNPs that passed the quality control (ESM Table 6) in the Japanese GWA scan (**a**) and those in the published European GWA scan [14] (**c**) with $-\log_{10}$ (p values) for FPG plotted against chromosome position in Mb. Red diamonds, p values for the genotypes of general populations (n=4,813 in Japanese, n=2,319 in Sri Lankan) and meta-analysis data (>12,000 in the ENGAGE consortium [17]). **d**–**f** Genomic location of *G6PC2* and *ABCB11* genes with intron and exon structure (NCBI Build 36) in the relevant populations. **g**–**i** WGAViewer (http://people.genome.duke.edu/~dg48/WGAViewer/whatis.php) [36] plot of LD (r^2) for all SNPs across the regions for the HapMap populations JPT (**g**) and CEU (**i**), and for the Human Genome Diversity Panel, ethnic groups belonging to South Asia (**h**). **j**–**l** SNPs in the LD block were partitioned into four subsets using the extent of LD with lead SNPs and/or haplotype-tagging SNPs, i.e. rs560887, rs483234, rs3755157 and rs853778 (see Methods). Red circles, SNP with $0.8 \leq r^2 \leq 1.0$ to the index SNP; orange circles, SNP with $0.6 \leq r^2 < 0.8$ to the index SNP; green bars, intervals where causative variants are most likely to be located

located in the *G6PC2–ABCB11* region (Fig. 1) so that SNPs in the strongest linkage disequilibrium (LD) with one index SNP (e.g. rs3755157) rather than with other index SNPs (e.g. rs560887, rs483234 and rs853778) were grouped into a bin of rs3755157 correlates. We then narrowed target intervals by investigating a subset (or subsets) of variants that could show a consistent pattern of trait association across different ethnic groups (ESM Narrowing target intervals in fine-mapping).

Results

Association with FPG and metabolic traits at four loci

Significant (p<0.05 by one-tailed test) association was replicated for all five SNPs from four tested loci in the Sri Lankan and Japanese populations (Table 1). Together with the previous reports in Europeans and Chinese [8, 11, 12, 17, 18, 20], we performed meta-analysis of FPG associa-

Table 1 Association of SNPs with fasting plasma glucose level

SNP	Neighbouring gene(s)	Alleles		Japanese panel (n=4,813)			Sri Lankan panel (n=2,319)			Europeans	
		FPG+[a]	FPG−[a]	Allele frequency[b]	Per-allele effect[c]	p value	Allele frequency[b]	Per-allele effect[c]	p value	Allele frequency[d]	Per-allele effect[c,e]
Reported in European studies											
rs780094	*GCKR*	G	A	0.44	0.032 (0.012, 0.052)	0.002	0.80	0.074 (0.035, 0.113)	2.1×10^{-4}	0.62	0.067 (0.045, 0.090)
rs560887	*G6PC2–ABCB11*	G	A	0.97	0.103 (0.044, 0.162)	6.4×10^{-4}	0.91	0.050 (−0.005, 0.105)	0.075	0.67	0.064 (0.056, 0.072)
rs1799884	*GCK*	A	G	0.18	0.075 (0.049, 0.101)	1.1×10^{-8}	0.12	0.076 (0.028, 0.123)	0.002	0.20	0.062 (0.048, 0.076)
rs1387153	*MTNR1B*	T	C	0.41	0.058 (0.038, 0.078)	9.7×10^{-9}	0.38	0.036 (0.005, 0.068)	0.024	0.28	0.07 (0.05, 0.08)
rs10830963	*MTNR1B*	G	C	0.42	0.056 (0.036, 0.075)	2.9×10^{-8}	0.45	0.064 (0.033, 0.094)	3.6×10^{-5}	0.30	0.072 (0.062, 0.082)
Tested in addition[f]											
rs483234	*G6PC2–ABCB11*	A	G	0.51	0.046 (0.026, 0.065)	3.9×10^{-6}	0.42	0.043 (0.012, 0.074)	0.007	0.70	–
rs3755157	*G6PC2–ABCB11*	T	C	0.38	0.057 (0.037, 0.078)	2.6×10^{-8}	0.16	0.069 (0.027, 0.111)	0.001	0.07	–
rs853778	*G6PC2–ABCB11*	A	G	0.40	0.044 (0.024, 0.064)	1.5×10^{-5}	0.35	0.026 (−0.006, 0.058)	0.117	0.46	–

Type 2 diabetes participants were excluded from the analysis; FPG association of each SNP was tested using linear regression models with adjustment for BMI, age and sex

[a] FPG-increasing (+) and decreasing (−)

[b] Of FPG-increasing allele

[c] Effect (95% CI) (mmol/l)

[d] Of FPG-increasing allele in HapMap CEU panel

[e] Association results were drawn from the previous studies: rs560887, rs4607517 (in substitution for rs1799884, r^2 =1 in CEU) and rs10830963 [17]; rs1260326 (in substitution for rs780094, r^2 =0.93 in CEU) [8]; rs1387153 [18]

[f] In the *G6PC2–ABCB11* region

tions to compare the effect sizes among different ethnic groups (ESM Fig. 2). Among the four loci, significant cross-population heterogeneity was detected for rs1387153 (*MTNR1B*; p=0.03). The variance for FPG that was explained by the associated SNPs totalled 2% in both the Japanese and Sri Lankan populations (ESM Table 2). Per-allele gradients in the two Asian populations (β=0.055 and 0.069 mmol/l in Japanese and Sri Lankans, respectively) were almost equivalent to those reported in populations of European descent (β=0.07 mmol/l) [18] (ESM Fig. 3, ESM Evaluation of cumulative effect of multiple loci on FPG).

Besides FPG levels, we analysed the relationship of SNPs with lipid traits (ESM Tables 3 and 4). Notably, rs780094 (*GCKR*) significantly and consistently modulated triacylglycerol levels in both ethnic groups ($p=2.2\times10^{-10}$ in Japanese, $p=1.4\times10^{-4}$ in Sri Lankan populations), where glucose-increasing alleles were associated with lower triacylglycerol levels as previously reported [8, 9, 11, 13]. Furthermore, glucose-increasing alleles at rs1799884 (*GCK*) and rs10830963 (*MTNR1B*) were significantly associated with reduced beta cell function (HOMA-B; p=0.037 for rs1799884, $p=2.6\times10^{-4}$ for rs10830963 in the Sri Lankan population), with no appreciable effect on fasting insulin or insulin sensitivity (ESM Table 5).

Refinement of genetic association in the *G6PC2–ABCB11* region

In fine-mapping with Japanese GWA scan data, we identified in the *G6PC2–ABCB11* region a novel associated SNP rs3755157, which was proven to be independent of the SNPs previously reported by GWA studies in Europeans [14–17].

In our GWA scan, multiple and significant SNPs were found in the *G6PC2–ABCB11* region (p=0.0004 to 0.002; Fig. 1, ESM Table 6) but not in the other candidate regions (ESM Tables 7–9). We therefore performed a detailed investigation of the *G6PC2–ABCB11* region. With reference to the LD and haplotype data (ESM Tables 10–12), we chose four haplotype-tagging SNPs (rs3755157, rs483234, rs853778 and rs560887) for genotyping the general Japanese population (n=4,813), which resulted in concordant evidence of associations between the tests of individual SNPs and those of haplotypes (Tables 1 and 2, ESM Haplotype explaining index association). The most significantly associated haplotype, class 5 (frequency = 0.35, $p=2.8\times10^{-7}$) (Table 2), was almost unequivocally tagged by rs3755157, which showed the strongest association by SNP-based test in the general Japanese population ($p=2.6\times10^{-8}$; Table 1).

We then performed a stepwise linear regression (for FPG levels) to test whether one of the four haplotype-tagging SNPs was necessary and sufficient to explain the association signal (ESM Tables 13–17, ESM Fig. 4, ESM Index SNP showing an independent association). The FPG association remained significant (p<0.05) when two haplotype-tagging SNPs, rs3755157 and rs560887, were included in the regression model (ESM Table 13). This independent association had gone unnoticed among more significant associations of SNPs that were in strong LD with a leading SNP, rs560887, among Europeans [14, 15]. Thus the presence of a novel SNP, rs3755157, and of allelic heterogeneity (ESM Fig. 5)

Table 2 Fasting glucose association according to haplotypes in the *G6PC2–ABCB11* region

Haplotype class	Tested SNPs[a]				Japanese panel[b]			Sri Lankan panel[c]			Europeans
	rs560887	rs483234	rs3755157	rs853778	Frequency	Effect (mmol/l)	p value	Frequency	Effect (mmol/l)	p value	Frequency[d]
1	G[a]	G	C	G	0.48	−0.047	2.3×10^{-6}	0.56	−0.041	9.6×10^{-3}	0.26
2	G[a]	G	C	A[a]	0	–	–	0.02	−0.029	0.65	0
3	G[a]	A[a]	C	G	0.09	0.004	0.81	0.08	0.035	0.23	0.28
4	G[a]	A[a]	C	A[a]	0.01	0.048	0.31	0.09	0.030	0.28	0.07
5	G[a]	A[a]	T[a]	A[a]	0.35	0.054	2.8×10^{-7}	0.16	0.072	9.8×10^{-4}	0.08
6	G[a]	A[a]	T[a]	G	0.02	0.053	0.12	0	–	–	0
7	A	A[a]	C	A[a]	0.02	−0.098	2.5×10^{-3}	0.08	−0.078	8.1×10^{-3}	0.28

FPG association was tested with adjustment for BMI, age and sex

[a] Allele increasing fasting glucose

[b] 4,792 complete observations

[c] 2,306 complete observations

[d] Haplotype frequency estimated in the HapMap CEU panel

Table 3 Association of FPG-altering SNPs with type 2 diabetes in the *G6PC2–ABCB11* region

SNP	rs560887 (FPG-increasing allele: G)	rs483234 (FPG-increasing allele: A)	rs3755157 (FPG-increasing allele: T)
Japanese (JPN)[a]			
Frequency, cases (n=5,629)	0.974	0.508	0.380
Frequency, controls (n=6,406)	0.969	0.490	0.360
OR (95% CI)	1.20 (1.03–1.41)	1.07 (1.02–1.13)	1.09 (1.03–1.15)
p value for trend	0.019	0.0056	0.0017
Sri Lankan (SL)[b]			
Frequency, cases (n=599)	0.917	0.422	0.169
Frequency, controls (n=515)	0.896	0.408	0.159
OR (95% CI)	1.28 (0.95–1.73)	1.06 (0.89–1.26)	1.08 (0.86–1.36)
p value for trend	0.08	0.51	0.52
JPN and SL combined			
OR (95% CI)	1.22 (1.06–1.40)	1.07 (1.02–1.13)	1.09 (1.03–1.15)
European-descent			
OR (95% CI)	0.93 (0.89–0.97)[c]	1.05 (0.99–1.13)[d]	1.00 (0.91–1.09)[d]
p value	0.0017	0.12	0.93

Type 2 diabetes association was tested with the Cochran–Armitage trend test in the case–control analysis

[a] SNPs were genotyped in a Japanese case–control study panel independently of the general Japanese population [27]

[b] In the Sri Lankan population, 515 controls are part of those used for FPG association analysis, whereas 599 cases were independent participants

[c] Results for 18,236 cases and 64,453 controls from a previous study [17]

[d] Results for 4,549 cases and 5,579 controls from the DIAGRAM consortium [37]

has become evident in the *G6PC2–ABCB11* region for the first time, as a result of comparing the GWA scan data between European and Japanese populations.

In the *G6PC2–ABCB11* region, *G6PC2* and *ABCB11* are both biologically plausible candidate genes [15, 32, 33]. During fine-mapping, we attempted to partition the LD block into intervals, each containing SNPs strongly correlated with an index SNP, in the hope that correlation coefficients r^2 would reflect phylogenic closeness once the index SNPs were selected from a reasonably dense set of SNP markers. This partitioning approach helped to prioritise the target interval for fine-mapping, thereby reducing the potential candidate variants to manageable proportions. For the *G6PC2–ABCB11* region, the target intervals were estimated to be 14 kb (in the *ABCB11* gene) for rs3755157 and 14 kb (in the *G6PC2* gene and between the genes) for rs560887 when the LD threshold was set at $r^2 \geq 0.6$ in the HapMap data (Fig. 1, ESM Table 18, ESM Narrowing target intervals in fine-mapping).

Concordance of association for FPG levels and type 2 diabetes

In addition to the quantitative trait analysis of FPG, we performed case–control analysis of type 2 diabetes for three of four haplotype-tagging SNPs, rs3755157, rs483234 and rs560887, in the *G6PC2–ABCB11* region and confirmed a significant ($p<0.05$) association in a relatively large study panel comprising 5,629 cases and 6,406 controls. The strongest association was found for rs3755157 (OR 1.09, 95% CI 1.03–1.15, $p=1.7\times10^{-3}$; Table 3), which was in good agreement with the FPG association mentioned above. To confirm the consistency of associations with increases in FPG levels and the risk of type 2 diabetes, we examined the changes in risk allele frequency (RAF) between the diabetes subgroup and non-diabetic participants in quartiles of FPG levels stratified in the general populations for unbiased estimates (ESM Fig. 6, ESM Table 19). In the Japanese and Sri Lankan populations, the RAF in the diabetes subgroup reached the second highest quartile, supporting the concordant association for FPG levels and the risk of type 2 diabetes in the *G6PC2–ABCB11* region.

Discussion

The present study has proven that common variant loci influencing FPG levels are reproducible in two populations of Asian descent, Japanese (East Asians) and Sri Lankan

(South Asians). To our knowledge, this is the first study investigating the genetic associations with FPG and related metabolic traits at four candidate loci, *GCK*, *GCKR*, *G6PC2–ABCB11* and *MTNR1B*, in South Asians, who are known to have high prevalence of type 2 diabetes [34]. The combined impact of associated SNPs is almost equivalent across the ethnic groups despite some cross-population diversity in the effect size of individual loci (ESM Figs 2 and 3, ESM Table 2). Other novel aspects of the present study include a fine-mapping approach using Japanese GWA scan data and consistent associations of FPG and type 2 diabetes in the *G6PC2–ABCB11* region.

According to genome-wide patterns of SNPs examined in the Human Genomic Diversity Panel [31], much of sub-Saharan Africa, Europe, South and Central Asia (including Sri Lanka), and East Asia appear to be homogeneous and individuals from these populations can be distinguished from each other. Although limited in the number of examples, our study has provided evidence supporting the importance of human genetic diversity in complex disease studies. For instance, beside replicating FPG association at four candidate loci in two Asian populations, our data also clarified the genetic architecture of the *G6PC2–ABCB11* region with regard to ethnic diversity. Using the GWA scan data, we found a novel SNP, rs3755157, to be a leading SNP among Japanese and independent of a leading SNP, rs560887, in Europeans (ESM Table 13–15).

As a fine-mapping approach, we listed HapMap SNPs having the strongest r^2 (in the range of $r^2 \geq 0.6$) with each of the index SNPs in the *G6PC2–ABCB11* region (Fig. 1, ESM Table 18). We performed cross-population filtering, which appreciably decreased the number of potential causal variants from 79 to 8 in the *G6PC2–ABCB11* region (ESM Narrowing target intervals in fine-mapping). The novel SNP rs3755157 and its correlated SNPs are located in the 3′-side (introns) of the *ABCB11* gene. While four different mRNAs, two alternatively spliced variants and two unspliced forms, are known to be transcribed from the *ABCB11*, it is possible that the potential causal variant(s) will influence the selective production of any of the 3′-side mRNA variants or the alteration of mRNA expression. Thus, closer inspection of subsets of SNP haplotypes shared by multiple ethnicities may allow us to appreciably narrow the field of potential causal variants before starting in-depth resequencing and functional follow-up studies, as demonstrated for the *G6PC2–ABCB11* region. During the preparation of our manuscript, replication of the *G6PC2* association was also reported in a Chinese population [19], where four SNPs were selected from the HapMap database so as to tag common variations near the *G6PC2* gene. Although the index SNP (rs560887) originally detected in Europeans was not tested, three (of four) SNPs appeared to show significant association in the Chinese population, in agreement with our findings in Japanese.

Our data also verified concordance of association for FPG levels and type 2 diabetes risk by using a systematic study design; i.e. unbiased estimates with stratification of general populations plus large-scale case–control studies involving 12,035 Japanese and 1,114 Sri Lankan samples (Table 3, ESM Fig. 6). It has been debated whether the genetic determinants regulating FPG levels in physiological states differ from those increasing type 2 diabetes risk. Some studies report that carriers of glucose-increasing alleles at three loci (*MTNR1B*, *GCK* and *GCKR*) show a higher risk of type 2 diabetes [8, 17, 18], although there is no significant association between *G6PC2–ABCB11* variants and type 2 diabetes in populations of European descent [14, 17, 35]. In this context, our data not only supported the concordant association of *G6PC2–ABCB11* variants for FPG and type 2 diabetes in two Asian populations, but also indicated that genetic determinants regulating FPG levels could, at least in part, differ from those increasing type 2 diabetes risk (ESM Fig. 6, ESM Consistent association of fasting glucose and type 2 diabetes in the *G6PC2–ABCB11* region). It is likely that genetic susceptibility for FPG levels increases type 2 diabetes risk in the population at large, but that some diabetic patients will develop the disease independently of a predisposition to elevated FPG levels.

In summary, despite the overall reproducibility of FPG association across the populations, ethnic diversity in allele frequencies led to the discovery of allelic heterogeneity in the *G6PC2–ABCB11* region. The diversity in the LD pattern also helped to reduce the probable causative variants in the corresponding region. The prevalence of the phenomena described here in human complex trait genetics is another research area warranting investigation. For applicable cases, the use of ethnic diversity in genetic studies can constitute an efficient approach subsequent to GWA scan.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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ORIGINAL ARTICLE
Molecular Cardiology

Evaluation of Pharmacogenetic Algorithm for Warfarin Dose Requirements in Japanese Patients

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Background: Warfarin dosing is difficult to establish because of considerable interindividual variation. Thus, warfarin pharmacogenetics have attracted particular interest in relation to appropriate control of anticoagulation.

Methods and Results: The 200 eligible subjects were chosen from participants in a hospital cohort. Performance of a pharmacogenetic algorithm recently developed by the International Warfarin Pharmacogenetics Consortium (IWPC) was tested and compared with a clinical algorithm (without genotype data) by calculating the percentage of patients for whom the predicted dose deviated by less than 7 mg/week (1 mg/day) from the actual dose. The pharmacogenetic algorithm accurately identified a significantly ($P<0.05$) larger proportion of patients to achieve the target international normalized ratio than did the clinical algorithm (68% vs 36% for a low-dose group; and 21% vs 0% for a high-dose group). Also, an increase in warfarin dosage was found to be appropriate for the current status of alcohol drinking (4 mg/week, as against non-drinking) and smoking (3.3 mg/week, as against non-smoking).

Conclusions: The IWPC pharmacogenetic algorithm has clinical application, particularly in identifying Japanese patients who require a low dosage of warfarin and are at greater risk of excessive anticoagulation. (*Circ J* 2010; **74:** 977–982)

Key Words: Anticoagulation; *CYP2C9*; Pharmacogenetics; *VKORC1*; Warfarin

Warfarin is the most commonly prescribed oral anticoagulant drug for the prophylaxis and treatment of thromboembolic disorders, but the appropriate dose can be difficult to establish because it can vary substantially (>10 fold) among patients, in part because of differences in each patient's age, diet, race and genotype.[1–3] Incorrect doses contribute to a high incidence of adverse effects (ie, bleeding and thromboembolic events) when the effectiveness of warfarin, expressed as the international normalized ratio of prothrombin time (PT-INR), is above or below the therapeutic range.

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During the initial dosing period (ie, the first few months), patients are at the greatest risk of overanticoagulation. To reduce this risk, a number of warfarin dosing algorithms[4,5] and regimens[6,7] have been proposed, mostly incorporating clinical factors, demographic variables, and molecular variations in 2 genes: the warfarin metabolic enzyme *CYP2C9* and the warfarin target enzyme, vitamin K epoxide reductase complex subunit 1 (*VKORC1*). Regarding genetic factors, of note is the fact that, in 2007, the US Food and Drug Administration (FDA) added pharmacogenetic information to the warfarin product label.[3] Along this line, the International Warfarin Pharmacogenetics Consortium (IWPC) has recently developed a pharmacogenetic dose algorithm for warfarin using a large data set (involving a total of 5,052 patients) from diverse ethnic groups.[8] The IWPC algorithm appears to provide better predictive accuracy than the one that uses only clinical variables or a fixed-dose (5 mg/day) strategy.

In general, patients of Asian descent require a lower maintenance dose of warfarin for a similar degree of anticoagulation than patients of European descent.[5,9] Moreover, it has been reported that compared with Europeans, the incidence of thromboembolism is low in Japan, despite the less intensive regimen;[10,11] which indicates that adjusted low-dose warfarin (eg, PT-INR 1.6–2.6) is optimal for prevention of thromboembolism in Japanese patients.[12,13]

Considering these racial differences in the anticoagulation therapy, we attempted to validate the IWPC pharmacogenetic dose algorithm for warfarin in Japanese patients under low-

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Table 1. Characteristics of Study Subjects

	n=200
M/F, n	136/64
Age at entry, years	67.8±10.3
Height, cm	160.3±14.6
Body weight, kg	61.4±12.7
Daily warfarin dose, mg	3.05±1.20
Primary reason for anticoagulation, n (%)	
Atrial fibrillation	119 (59.5)
Prosthetic valve replacement	27 (13.5)
Deep vein thrombosis/pulmonary embolism	12 (6)
Other	42 (21)
Amiodarone use, n (%)	11 (5.5)
VKORC1 rs9923231 genotype, n (%)	
G/G:A/G:A/A	3 (1.5): 31 (15.5): 166 (83)
CYP2C9 genotype, n (%)	
*1/*1:*1/*3:*3/*3	195 (97.5): 5 (2.5): 0 (0)
Alcohol drinking	
Never (%)	33.5
Ex-drinker (%)	18
Current drinker (%)	48.5
Smoking	
Never (%)	38
Ex-smoker (%)	49.5
Current smoker (%)	12.5

VKORC1, vitamin K epoxide reductase complex subunit 1.

dose treatment. Also, we examined the impact of alcohol intake and smoking on warfarin dose requirements, aiming at refinements of the algorithm.

Methods

Study Population

A total of 200 eligible subjects were chosen from participants in the Hospital-Based Cohort Study in the International Medical Center of Japan (IMCJ), which was designed to investigate clinical epidemiology, pharmacogenetics and genetic susceptibility of lifestyle-related disorders such as diabetes, hypertension and cardiovascular diseases.[14] We collected information on demographic characteristics, the primary indication for warfarin treatment, the stable therapeutic dose of warfarin, the treatment INR (the INR achieved with a stable warfarin dose), the use of concomitant enzyme inducers (carbamazepine, phenytoin, rifampin, or rifampicin) and amiodarone. Anticoagulation of patients was stably controlled with a target PT-INR of 1.6–2.6 for the prevention or treatment of thromboembolic diseases. Characteristics of the patients are shown in Table 1. We largely divided them into 3 categories of alcohol drinking (never-drinker; ex-drinker; current drinker) and 3 categories of smoking status (never-smoker; ex-smoker; current smoker). Participants were asked to report their daily alcohol consumption, using a structured questionnaire that ascertained the consumption of typical alcoholic beverages (beer, wine, Japanese sake, shochu and spirits). Alcohol intake was denoted in terms of servings of sake (1 gou [180 ml] of Japanese rice wine is considered equal to 22 g of ethanol). As a variable of smoking conditions, the Brinkman Index was calculated from [the number of cigarettes smoked daily]×[smoking period] in addition to the categorical smoking status. All subjects were Japanese and gave written informed consent for participation in the study. The ethics committee of IMCJ approved the study protocol.

Genotyping of *CYP2C9* and *VKORC1* SNPs

Among the genetic variants of *CYP2C9* used for the IWPC algorithm (*1, *2 and *3), the *CYP2C9*2* allele (I359L) has not been reported in Asian populations.[5,15,16] Accordingly, we genotyped the *3=rs1057910 polymorphism in relation to the wild-type allele *1, thereby determining *1/*1, *1/*3 and *3/*3 genotypes at the *CYP2C9* locus. At the *VKORC1* locus, on the other hand, the −1639 G→A=rs9923231 polymorphism was genotyped, following the IWPC algorithm.[8] Both SNPs were characterized with the use of TaqMan assays (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

First, we performed multiple regression analysis to test the effects of predictor variables on interindividual variability of warfarin dose, with the square root of the warfarin dose in mg/week being used as a dependent variable, which was in accordance with the IWPC study.[8] We then evaluated the potential clinical value of 2 algorithms (the IWPC pharmacogenetic algorithm and a clinical algorithm without including genotype data) by calculating the percentage of patients whose predicted dose of warfarin was within 7 mg/week (1 mg/day) of the actual stable therapeutic dose. The IWPC pharmacogenetic algorithm for Japanese was: 5.4952−(0.2614×[age in decades])+(0.0087×[height in cm])+(0.0128×[weight in kg])−(0.8677×[*VKORC1* A/G])−(1.6974×[*VKORC1* A/A])−(0.9357×[*CYP2C9**1/*3])−(2.3312×[*CYP2C9**3/*3])+(1.1816×[enzyme inducer status])−(0.5503×[amiodarone status])=Square root of weekly warfarin dose.[8] In addition, we calculated the percentage of patients for whom the predicted dose according to each algorithm was at least 7 mg/week higher than the actual dose (overestimation) or at least 7 mg/week lower than the actual dose (underestimation). Here, we adopted 7 mg/week (1 mg/day) as a difference that clinicians would be likely to define as clinically relevant. With consideration of warfarin dose distribution in the study sample (**Figure S1**), the performance of the IWPC algorithm was assessed in 3 dose groups: low dose (≤10.5 mg/week), high dose (≥31.5 mg/week), and intermediate doses (between 10.5 and 31.5 mg/week) for stable therapeutic anticoagulation. These thresholds of 10.5 mg and 31.5 mg/week bracket the usual maintenance dose of 17.5–24.5 mg/week (2.5–3.5 mg/day) in Japanese patients.[5,15,17,18] The overall performance was measured as the coefficient of determination, R^2, which was the square of the sample correlation "R" between the predicted and therapeutic doses. Besides assessing the potential benefit of using the pharmacogenetic algorithm instead of the clinical algorithm, we computed the number needed to genotype (NNG: the number of patients who must be genotyped in order for 1 patient to have an improved dose estimate).

Furthermore, we evaluated the effects of alcohol drinking and smoking on warfarin dose requirements by multiple regression analysis in which 3 numerical models (2 categorical and 1 continuous trait models) were tested for each behavior.

Results

The characteristics of the 200 participants in the present study are summarized in Table 1. Among them, the most common indications for warfarin use were atrial fibrillation

Table 2. Effect Size of Predictors on Warfarin Dose Among Japanese Subjects

Predictor	Regression of warfarin dose		Effect in the IWPC algorithm
	Effect (95%CI)	P value	
Intercept	4.940 (3.404, 6.477)	1.6E-09	3.798*
Age in decades	−0.215 (−0.334, −0.095)	5.0E-04	−0.261
Height in cm	0.001 (−0.009, 0.012)	0.82	0.009
Weight in kg	0.012 (−0.001, 0.025)	0.07	0.013
VKORC1 rs9923231			
AG vs AA	0.862 (0.545, 1.178)	2.2E-07	0.830
GG vs AA	1.677 (0.714, 2.640)	7.3E-04	1.697
CYP2C9 rs1057910			
*1/*3 vs *1/*1	−0.714 (−1.466, 0.038)	0.06	−0.936
Amiodarone status	−0.475 (−0.972, 0.022)	0.06	−0.550

Predictor variables in the multiple regression are same as those in the pharmacogenetic dosing algorithm proposed by the IWPC; enzyme inducer status is not shown because none of the subjects was taking any of the enzyme inducers listed in the IWPC algorithm (ie, carbamazepine, phenytoin, rifampin or rifampicin).
A dependent variable is the square root of the warfarin dose in mg/week.
*Intercept used for patients of Asian race with AA genotype at *VKORC1* rs9923231 and *1/*1 genotype at *CYP2C9* rs1057910.
CI, confidence interval; IWPC, International Warfarin Pharmacogenetics Consortium; *VKORC1*, vitamin K epoxide reductase complex subunit 1.

Table 3. Percentage of Patients With Ideal, Underestimated or Overestimated Dose of Warfarin, as Estimated by the Pharmacogenetic and Clinical Algorithms, in Patients Requiring Low, Intermediate or High Actual Doses of Warfarin

Actual dose required	No. of patients	Patients classified by performance of prediction			Difference between 2 algorithms, P value
		Ideal dose (error within ≤7 mg/week), %	Underestimated, %	Overestimated, %	
≤10.5 mg/week	25				0.046
Pharmacogenetic algorithm		68	0	32	
Clinical algorithm		36	0	64	
>10.5 to <31.5 mg/week	151				0.613
Pharmacogenetic algorithm		80	19	1	
Clinical algorithm		79	18	3	
≥31.5 mg/week	24				0.050
Pharmacogenetic algorithm		21	79	0	
Clinical algorithm		0	100	0	

The clinical algorithm involves clinical and demographic variables (age, height, weight, and medication), and the pharmacogenetic algorithm involves the same set of variables plus genotypes in 2 genes (*CYP2C9* and *VKORC1*).
Underestimate is the case where the predicted dose (by either pharmacogenetic or clinical algorithm) is lower than the observed dose; overestimate is the opposite.
VKORC1, vitamin K epoxide reductase complex subunit 1.

(n=119, 59.5%), prosthetic valve replacement (n=27, 13.5%), and deep vein thrombosis or pulmonary embolism (n=12, 6%). The minor allele frequencies of rs1057910 (*CYP2C9*) and rs9923231 (*VKORC1*) were 0.013 and 0.093, respectively, which were comparable to those previously reported in Japanese patients[5,15,17,18] or HapMap JPT (http://hapmap.ncbi.nlm.nih.gov/): 0.016–0.049 for rs1057910 and 0.075–0.088 for rs9923231. Each SNP was in Hardy-Weinberg equilibrium (P>0.05).

The effects of predictor variables on warfarin dose were first examined in the ordinary regression model (Table 2). The effect sizes of individual variables thus estimated were almost comparable to those in the IWPC algorithm.[8] When applied to the Japanese patients' data, the IWPC pharmacogenetic algorithm identified significantly (P<0.05) larger proportions of patients who required 10.5 mg or less per week (low-dose group) or those who required 31.5 mg or more per week (high-dose group) to achieve the target PT-INR than did the clinical algorithm (68% vs 36% in low-dose group; and 21% vs 0% in high-dose group; Table 3). We depicted the fair performance of the IWPC pharmacogenetic algorithm in the plots comparing the predicted dose and actual dose of warfarin (R^2=0.28, P=1.5×10^{-15}) (Figure).

A significant benefit of using genetics was further verified with the NNG analysis (Table 4). The NNG can be computed using the number needed to treat (NNT) method; the NNT is the inverse of the absolute risk reduction (ARR). The ARR was calculated as the absolute difference between the event rate (ER) for the pharmacogenetic algorithm and the ER for the clinical algorithm (ER=ratio of the number of patients for which an algorithm estimates a poor dose (more or less than 7 mg/week than the actual therapeutic dose) over the total number of patients). Despite different criteria for a poor dose estimate (ie, the criteria in the IWPC study[8] were >20% above or below the actual therapeutic dose), the NNG was in good agreement between the studies: 13.3 in the present study and 13.2 in the IWPC study.

Our data on Japanese patients also indicated that both

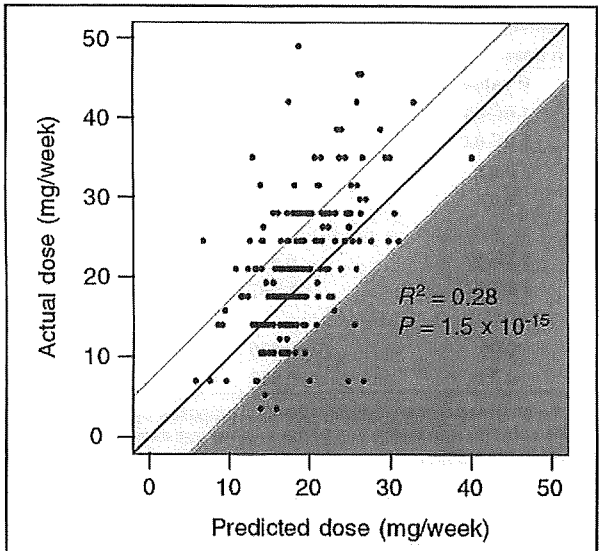

Figure. Predicted vs actual stable therapeutic warfarin dose (mg/week) for 200 Japanese patients in the present study. The diagonal solid (black) line indicates the perfect prediction, above and below which the gray lines indicate thresholds that the predicted dose according to the International Warfarin Pharmacogenetics Consortium pharmacogenetic algorithm is 7 mg/week lower than the actual dose (underestimation) or 7 mg/week higher than the actual dose (overestimation).

alcohol drinking and smoking significantly influence warfarin dose (Table 5). In the multiple regression model, the current status of drinking or non-drinking (ex-drinker+never-drinker) and that of smoking or non-smoking (ex-smoker+never-smoker) exerted approximate warfarin dose effects of 4 mg/week ($P=9.5\times10^{-5}$, $R^2=0.06$) and 3.3 mg/week ($P=0.03$, $R^2=0.02$), respectively. With these predictor variables being incorporated into the IWPC algorithm, its performance was augmented ($R^2=0.33$, $P=5.5\times10^{-19}$) (Figure S2).

Table 4. NNG Analysis: Clinical vs Pharmacogenetic Algorithm With ±7 mg/week Criterion

No. of events (n=200)	
Clinical >7 mg/week than actual	21
Clinical <7 mg/week than actual	51
Pharmacogenetic >7 mg/week than actual	10
Pharmacogenetic <7 mg/week than actual	47
Absolute risk reduction	0.075
NNG	13.3

NNG, number needed to Genotype.

Discussion

We have evaluated the IWPC pharmacogenetic algorithm in 200 Japanese patients under low-dose warfarin treatment. Although the target PT-INR (1.6–2.6) in the present study was slightly lower than the range (2.0–3.0) set in the IWPC study,[8] the performance of the tested algorithms in the 2 studies proved almost comparable: $R^2=0.28$ in the present study and $R^2=0.33$–0.34 for Asians in the IWPC study. Besides the reproducible performance in the whole study sample, of particular note is the fact that among patients in a low-dose group (≤10.5 mg/week), the percentage of overestimation was significantly smaller when the warfarin dose was predicted with the pharmacogenetic algorithm (32%) than with the clinical algorithm (64%) (Table 3), thus enabling us to appreciably reduce the risk of overanticoagulation. Furthermore, we demonstrated the substantial influence of alcohol drinking and smoking on warfarin dose requirements, which used to be anticipated but has not been evaluated in detail thus far.[2]

The incidence of major bleeding (eg, intracranial hemorrhage) has been reported as higher in Japanese patients (6.6% per year) than in European patients (1.6–2.5% per year) with adjusted standard-dose warfarin therapy: a target PT-INR of 2.2–3.5 in the Japanese and 2.0–4.5 in Europeans.[10–22] Because of such racial differences in bleeding tendency under warfarin, the necessity of customizing warfarin therapy has been argued.[10] Recently, a prospective study of 4,202 patients

Table 5. Effects of Alcohol Drinking and Smoking on Warfarin Doses in Different Regression Models

Model	Tested predictors	Approximate effect on warfarin dose in mg/week*		
		Effect (95%CI)	P value	R^2
Alcohol drinking				
Model 1	Stopped vs Yes	−4.1 (−6.6, −1.5)	0.003	0.06
	No vs Yes	−3.9 (−6.0, −1.7)	6.7E-04	
Model 2	Stopped/No vs Yes	−4.0 (−5.8, −2.1)	9.5E-05	0.06
Model 3	Alcohol unit (gou) per week†	0.6 (−0.05, 1.2)	0.070	0.01
Smoking				
Model 1	Stopped vs Yes	−3.5 (−6.4, −0.4)	0.03	0.02
	No vs Yes	−2.9 (−6.0, 0.4)	0.09	
Model 2	Stopped/No vs Yes	−3.3 (−6.1, −0.3)	0.03	0.02
Model 3	Brinkman index [amount per day×years]†	−0.02 (−0.1, 0.1)	0.56	0.001

Alcohol drinking was categorized into 3 groups: current drinker (yes), abstainer (stopped), and never-drinker (no), based on the self-reported questionnaire. Likewise, smoking status was categorized into 3 groups: current smoker (yes), ex-smoker (stopped) and never-smoker (no).
*Predictors in Table 2 were included as covariates in the tested regression model.
†The square root of the value was used for the regression analysis.
CI, confidence interval.

showed an optimal PT-INR of 3.0–3.5 in the Dutch,[23] whereas the Japanese Guidelines for Pharmacotherapy of Atrial Fibrillation[24–26] (JCS 2008) have set a PT-INR of 2.0–3.0 as the therapeutic range, except for the elderly (≥70 years of age), in whom a lower dose of warfarin (PT-INR 1.6–2.6) is recommended for prevention of thromboembolism and safety from bleeding complications.[27] The intensity of warfarin control (ie, optimal PT-INR) in the Japanese remains to be further defined according to the balance between risks and benefits under individual conditions. Among the primary indications for warfarin use, the optimal therapeutic range has been debated for patients with prosthetic valve replacement,[13] corresponding to 13.5% of the current subjects (Table 1). Partly because of the risk of eventual valve failure of bioprosthesis, and resultant reoperation, there seems to be a tendency for increased use of prosthetic valves in Japan, with its population's long life expectancy, as compared with the USA and Europe. Including patients with prosthetic valve replacement, because the target PT-INR is often set at 1.6–2.6 in outpatient clinics in Japan,[12] our findings obtained in equivalent clinical setting should encourage clinicians to apply the IWPC algorithm to their patients. Nevertheless, in cases where the optimal therapeutic range is set differentially according to the primary disease condition, some modification of the IWPC algorithm may be required.

We have found that the average dose of warfarin in the Japanese patients is 21 mg/week (3 mg/day), which is less than the standard dose (35 mg/week) in Europeans, and in the present study one-eighth (12.5%) of the participants were categorized into a low-dose group (≤10.5 mg/week). If a fixed dose of 3 mg/day is given to these patients without conscientious monitoring of PT-INR, there is a high risk of overanticoagulation, leading to fatal bleeding events. The use of the IWPC algorithm will enable clinicians to detect approximately two-thirds (68%) of the Japanese patients in this low-dose group, which is twice as large as the proportions (36%) attainable with the clinical algorithm (Table 3). Although the value of adding genotype to clinical (and demographic) information seems to be less modest, benefits also accrue to Japanese patients in the high-dose group (≥31.5 mg/week); 21% of the patients were identified with the pharmacogenetic algorithm, but none (0%) with the clinical algorithm (Table 3).

Since the US FDA changed the labeling of warfarin to suggest that clinicians consider using genetic tests to guide dosing,[3] warfarin pharmacogenetics has drawn substantial attention towards "personalized" patient care. Although more than 30 genes may contribute to the net warfarin effect, *CYP2C9* and *VKORC1* are known to exert the most influence.[28–30] The pharmacogenetic algorithm involving these polymorphisms, developed by the IWPC, can predict approximately one-third of all dosing variations at most.[8] The question of whether the knowledge of genetic information is cost-effective in reducing bleeding and thrombotic complications is under debate.[31] Many clinical factors influence warfarin dose requirements, including diet (in particular, the vitamin K content) and concomitant drug administration, besides a list of variables that have been incorporated into the IWPC algorithm. As the clinical factors often change in individual patients, appropriate alterations in warfarin dosing must be made, regardless of genetic information. In this respect, it is important to quantitatively evaluate the individual contribution of clinical factors, as has been performed for alcohol drinking and smoking in the present study, toward refining warfarin pharmacogenetic testing for not only initial but also maintenance dose requirements.

In summary, we report the usefulness of the IWPC pharmacogenetic algorithm in its clinical application, particularly for identifying Japanese patients who require a low dose of warfarin. However, it has to be kept in mind that considering the current limitations of its application to clinical medicine, this testing alone does not make conscientious PT-INR monitoring unnecessary. When the genotype cost falls to a more reasonable level, we expect that the use of pharmacogenetic-based initial dosing will become routine clinical practice.

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Supplementary files

Figure S1. Histogram of warfarin dose.

Figure S2. Actual dose vs predicted dose with information on alcohol drinking and smoking incorporated into the International Warfarin Pharmacogenetics Consortium (IWPC) pharmacogenetic algorithm.

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Long-term combination therapy of ezetimibe and acarbose for non-alcoholic fatty liver disease☆

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***Background/Aims*: Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome that is closely associated with multiple factors such as obesity, hyperlipidemia, type 2 diabetes mellitus and hypertension, making it difficult to treat NAFLD effectively using any monotherapy available to date. In this study, we propose a novel combination therapy for NAFLD comprising ezetimibe (EZ), a cholesterol absorption inhibitor, and acarbose (AC), an α-glucosidase inhibitor.**

***Methods*: C57BL/6J mice were divided into five treatment groups as follows: basal diet (BD), high-fat diet (HFD) only, HFD with EZ (5 mg/kg/day), HFD with AC (100 mg/kg/day), and HFD with both EZ and AC for 24 weeks.**

***Results*: Long-term combination therapy with EZ and AC significantly reduced steatosis, inflammation and fibrosis in the liver, compared with long-term monotherapy with either drug, in an HFD-induced NAFLD mouse model; the combination therapy also significantly increased the expression of microsomal triglyceride transfer protein (MTP) and peroxisome proliferators-activated receptor-α1 (PPAR-α1) in the liver, compared with either monotherapy, which may have led to the improvement in lipid metabolic disorder seen in this model.**

***Conclusions*: Combination therapy with EZ and AC for 24 weeks improved the histopathological findings in a mouse model of NAFLD.**

Keywords: **Ezetimibe; Acarbose; Combination therapy; Non-alcoholic fatty liver disease (NAFLD)**

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Abbreviations: NAFLD, non-alcoholic fatty liver disease; EZ, ezetimibe; AC, acarbose; BD, basal diet; HFD, high-fat diet; NASH, non-alcoholic steatohepatitis; ALT, alanine aminotransferase; HPLC, high-performance liquid chromatography; Chol, cholesterol; TG, triglyceride; CM, chylomicron; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; SREBP-1c, sterol regulatory element-binding protein-1c; SREBP-2, sterol regulatory element-binding protein-2; PPAR-α1, peroxisome proliferators-activated receptor-α1; MTP, microsomal triglyceride transfer protein; LDLR, low-density lipoprotein receptor; HMG CoA, 3-hydroxy-3-methylguataryl CoA.

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