Replication Study of Candidate Genes Associated With Type 2 Diabetes Based On Genome-Wide Screening

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OBJECTIVE—The present study was conducted to confirm possible associations between candidate genes from genome-wide association studies and type 2 diabetes in Japanese diabetic patients and a community-based general population. A total of 11 previously reported single-nucleotide polymorphisms (SNPs) from the *TCF7L2*, *CDKAL1*, *HHEX*, *IGF2BP2*, *CDKN2A/B*, *SLC30A8*, and *KCNJ11* genes were analyzed.

RESEARCH DESIGN AND METHODS—Candidate SNPs were genotyped in 506 type 2 diabetic patients and 402 control subjects and meta-analyzed with six previous association studies in Japanese patients. Associations with fasting plasma insulin levels were investigated in a general population sample (n = 1,963, 61 ± 13 years).

RESULTS—In our case-control subjects, susceptibility to type 2 diabetes was replicated in *TCF7L2* (rs12255372), *CDKAL1* (rs7756992, rs7754840), *HHEX* (rs7923837), *IGF2BP2* (rs4402960 and rs1470579), *CDKN2A/B* (rs10811661), and *SLC30A8* (rs13266634). In addition to these polymorphisms, meta-analysis confirmed the association of type 2 diabetes susceptibility with KCNJ11 rs5219, *TCF7L2* rs7903146, and *HHEX* rs1111875. The *TCF7L2* rs12255372 polymorphism showed the highest odds ratio (OR) for type 2 diabetes (OR 1.714 [1.298–2.263]). Odds ratio of other polymorphisms ranged from 1.13 to 1.41. The risk allele of *CDKAL1* rs7756992 was significantly associated with lower insulin levels in type 2 diabetic patients after adjustment for other confounding factors.

CONCLUSIONS—Type 2 diabetes susceptibility of seven candidate genes was confirmed in Japanese. Conservation of susceptible loci for type 2 diabetes was independent of ethnic background. ***Diabetes*** **58:493–498, 2009**

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Received 18 December 2007 and accepted 17 November 2008.

Published ahead of print at http://diabetes.diabetesjournals.org on 25 November 2008. DOI: 10.2337/db07-1785.

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A great number of studies in various populations have suggested an association between several single-nucleotide polymorphisms (SNPs) and type 2 diabetes. For example, transcription factor 7-like 2 (*TCF7L2*) is a highly reliable predisposing gene for type 2 diabetes (1–3). In addition, recent genome-wide association studies (GWASs) have provided new susceptible loci for type 2 diabetes (4–10). A GWAS in French subjects, for example, identified rs13266634, a nonsynonymous SNP (R325W) on the solute carrier family 30 member 8 (*SLC30A8*) gene, as a polymorphism involved in type 2 diabetes susceptibility (4). The study also reported an association between type 2 diabetes and rs1111875, as well as rs7923837, located in the hematopoietically expressed homeobox gene (*HHEX*). These associations were replicated in three independent GWASs in various populations (5–7).

Additional susceptible SNPs were independently identified in the insulin-like growth factor 2 mRNA-binding protein 2 gene (*IGF2BP2*, rs4402960, and rs1470569) (5,6). Involvement of SNPs rs10811661, located upstream of cyclin-dependent kinase inhibitor genes *CDKN2A* and *CDKN2B*, and "rs7754840/rs7756992," located in the CDK5 regulatory subunit-associated protein 1-like 1 gene (*CDKAL1*), has also been suggested (5,6,8,9). A recent population-based study in Danish subjects replicated the susceptible association of *HHEX* rs111875, *CDKN2A/B* rs10811661, and *IGF2BP2* rs4402960 with type 2 diabetes (10).

Findings from previous GWASs, however, cannot be extrapolated to other populations with different lifestyles and environmental backgrounds. In particular, the genetic background for type 2 diabetes development in East Asians, who show lower basal insulin secretion and a marked decrease in insulin release in response to development of glucose tolerance (11), appears to be different from that in Caucasians or individuals of European origin. Further, SNP frequency differences are suggested to be an additional factor influencing type 2 diabetes susceptibility.

Here, based on a recent GWAS (4–10), we conducted a replication study of candidate SNPs associated with type 2 diabetes in Japanese diabetic subjects, as well as in a general Japanese population sample.

RESEARCH DESIGN AND METHODS

Case and control subjects. Basic clinical characteristics of subjects are summarized in Supplemental Table 1 (located in an online appendix at http://dx.doi.org/10.2337/db07-1785). All type 2 diabetic subjects (n = 506) were inpatients or outpatients evaluated by diabetes specialists at Ehime University Hospital and Ehime Prefectural Hospital in Japan. Diabetes was diagnosed based on the 1998 American Diabetes Association criteria (12). Nondiabetic control subjects (n = 402) were chosen based on the absence of a history of diabetes in the subject and among first-degree relatives, as well as

either normal glucose tolerance, confirmed by a 75-g oral glucose tolerance test, or A1C levels <5.6 with fasting plasma glucose levels <110 mg/dl. All case and control subjects were native Japanese. Selection criteria details have been described in a previous study (13). A total of 139 type 2 diabetic patients and 136 control subjects were overlapped with the previous meta-analysis for TCF7L2 polymorphisms (14).

General population. The general population subjects were selected from residents of a community of 11,000 inhabitants in Ehime Prefecture, a largely rural area located in western Japan (15). Subjects were recruited through a community-based annual medical checkup process for self-employees, including farmers and foresters, employees of small companies, and elderly without fixed employment. The sample population consisted of 1,963 middle-aged to elderly residents (Supplemental Table 2). Overnight fasting plasma samples for the measurement of plasma insulin concentrations were available for all sample subjects. Baseline clinical characteristics were obtained from personal health records evaluated during the medical checkup. All study procedures were approved by the ethics committee of the Ehime University Graduate School of Medicine, and informed consent was obtained from each participating subject.

Genotyping. Genomic DNA was extracted from peripheral blood (QIAamp DNA blood kit; QIAGEN, Hilden, Germany). All SNPs were analyzed by TaqMan probe assay (Applied Biosystems, Foster City, CA) using commercially available primers and probes purchased from the Assay-on-Demand system (Supplemental Table 3).

Statistical analysis. Data are expressed as means ± SD. Linkage disequilibrium was assessed using the Haploview software (Broad Institute, Cambridge, MA) (16). Frequency differences in each genotype were assessed by the χ^2 test. The pooled odds ratios for allele frequency with those of six other association studies in Japanese (17–22) were estimated using the fixed-effects model (Mantel-Haenszel method). Differences in plasma insulin levels among genotypes (ANOVA and multiple regression analysis [additive model] adjusted for age, sex, and BMI) were assessed using a commercially available statistical software package (SPSS Version 14.0; SPSS, Chicago, IL). Current treatment of hyperglycemia was further adjusted in type 2 diabetic patients when appropriate. Null hypotheses were rejected at a level of significance of $P < 0.05$.

RESULTS

Table 1 summarizes the association between 11 candidate SNPs and type 2 diabetes in case-control subjects. The T allele of *TCF7L2* (rs12255372) was significantly associated with type 2 diabetes. A tendency to association was also observed with SNP rs7903146, which was in linkage disequilibrium with rs12255372 ($D' = 0.854$, $r^2 = 0.421$). However, the risk allele frequency of these SNPs was considerably low, which is in agreement with previous reports in Japanese subjects (17,18). The post hoc calculated statistical power of these SNPs (allele frequency) was 36.1% and 25.4% for rs12255372 and rs7903146, respectively, with a 5% type 1 error rate.

In addition to *TCF7L2* polymorphisms, a significant association was observed between type 2 diabetes and polymorphisms in *CDKAL1* (rs7756992 [power: 51.0%], rs7754840 [52.9%]; $D' = 0.920$; $r^2 = 0.648$), *HHEX* (rs7923837 [30.4%]), *IGF2BP2* (rs4402960 [31.3%], rs1470579 [51.1%]; $D' = 0.997$; $r^2 = 0.918$), *CDKN2A/B* (rs10811661 [32.6%]), and *SLC30A8* (rs13266634 [10.7%]), but not *HHEX* (rs1111875 [8.5%]). Further, a marginally significant association was observed between type 2 diabetes and the *KCNJ11* polymorphism (rs5219 [21.8%])). Compared with control subjects of European descent, risk allele frequencies in Japanese control subjects were higher in the *CDKAL1* gene (rs7756992 G allele, 0.470 vs. 0.258) and lower in the *HHEX* (rs1111875 C allele, 0.288 vs. 0.598; rs7923837 G allele, 0.177 vs. 0.597), *CDKN2A/B* (rs10811661 T allele, 0.555 vs. 0.850), *SLC30A8* (rs13266634 C allele, 0.568 vs. 0.699), and *KCNJ11* (rs5219 T allele, 0.372 vs. 0.464) genes (4,6,8). In contrast, no significant frequency differences were observed in the *CDKAL1* rs7754840 (C allele, 0.399 vs. 0.360) and *IGF2BP2* rs4402960 (T allele, 0.287 vs. 0.304) polymorphisms.

However, except for the *CDKAL* polymorphisms, statistical significance was not reached in the observed associations using Bonferroni's correction, possibly due to limited statistical power. To further clarify type 2 diabetes susceptibility, seven association studies in Japanese subjects (17–22), including our present data, were meta-analyzed (Fig. 1). Type 2 diabetes susceptibility was confirmed in all analyzed polymorphisms, both before and after Bonferroni's adjustment. Further, two SNPs (rs1111875 in *HHEX* and rs5219 in *KCNJ11*), which were not replicated in our data, were confirmed as susceptible polymorphisms for type 2 diabetes.

To further clarify the pathophysiological significance of the susceptibility of these seven genes for type 2 diabetes, associations with plasma insulin levels were evaluated in a community-derived population sample (Table 2). Although differences in plasma insulin levels among the *CDKAL1* rs7756992 genotype did not reach statistical significance, probably due to the limited statistical power (post hoc calculated statistical power: 28.3% for type 2 diabetic patients, 31.8% for control subjects), multiple regression analysis involving the genotype as an additive model showed significantly lower insulin levels in type 2 diabetic subjects with risk genotypes after adjusting for age, sex, and BMI. The association of *CDKAL1* rs7756992 remained significant after further adjustment for the current treatment of hyperglycemia ($n = 67$, $P = 0.021$). The risk allele of *CDKAL1* rs7754840 also tended to be associated with lower insulin levels. However, no significant associations were observed in other SNPs.

DISCUSSION

In the present study, we replicated the associations of several candidate genes derived from a recent GWAS (4–10). However, several conflicting results were observed with other replication studies in Japanese diabetic patients. Horikoshi et al. (21) and Furukawa et al. (20) observed a markedly strong association between type 2 diabetes and variants of *HHEX* rs1111875, whereas no association was observed in our study. However, results of our meta-analysis (Fig. 1) clearly indicate the type 2 diabetes susceptibility of all candidate genes, including *HHEX* rs1111875. Conservation of susceptible loci for type 2 diabetes was independent of ethnic background.

However, the attributable risk of these SNPs susceptible for type 2 diabetes was different from that of European ancestries. For example, the pooled odds ratios of the *TCF7L2* gene polymorphisms were slightly higher than those in European ancestries (6,23), whereas the risk allele frequencies were considerably lower. Alternatively, for *HHEX* gene polymorphisms, odds ratios were slightly higher in Japanese (7). Very recently, genome-wide screening in a Japanese population identified the *KCNQ1* gene polymorphism as a new susceptible loci for type 2 diabetes (24). These authors reported that the risk alleles of rs2237892 and other SNPs in linkage disequilibrium with rs2237892 were associated with an increased risk of type 2 diabetes. However, apparent associations of the *KCNQ1* SNPs were not observed in previous GWAS in populations of European descent (4–10). This discrepancy may be due mainly to the differences in allele frequencies of the susceptible SNPs.

It has been suggested that several candidate SNPs

TABLE 1
Association of candidate SNPs with type 2 diabetes in case and control subjects

Gene (rs number)	Type 2 diabetes frequency	Risk allele	Hardy-Weinberg equilibrium (*P*) (control)	Allele		Dominant		Recessive		Additive (*P*)
				OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	
	Control									
TCF7L2 (rs12255372)	TT/TG/GG	T	0.721							
	1/33/453			2.082 (1.112–3.898)	0.022	2.059 (1.089–3.893)	0.026	–	–	0.065
	0/14/384									
TCF7L2 (rs7903146)	TT/TC/CC	T	0.501							
	2/45/434			1.589 (0.978–2.582)	0.061	1.549 (0.941–2.551)	0.085	–	–	0.132
	0/26/372									
CDKAL1 (rs7756992)	GG/GA/AA	G	0.053							
	155/217/119			1.307 (1.084–1.577)	0.005	1.081 (0.796–1.467)	0.618	1.887 (1.381–2.578)	6.8×10^{-5}	2.0×10^{-4}
	78/217/102									
CDKAL1 (rs7754840)	CC/CG/GG	C	0.189							
	117/225/149			1.321 (1.093–1.596)	0.004	1.209 (0.912–1.604)	0.187	1.866 (1.316–2.645)	4.6×10^{-4}	0.002
	57/203/137									
HHEX (rs1111875)	CC/CT/TT	C	0.593							
	44/211/235			1.086 (0.885–1.334)	0.430	1.141 (0.876–1.488)	0.328	1.018 (0.639–1.620)	0.942	0.602
	35/158/203									
HHEX (rs79203837)	GG/GA/AA	G	0.381							
	17/178/295			1.286 (1.015–1.630)	0.037	1.432 (1.085–1.891)	0.011	0.920 (0.454–1.866)	0.817	0.026
	15/111/273									
IGF2BP2 (rs4402960)	TT/TG/GG	T	0.972							
	66/196/231			1.239 (1.011–1.517)	0.039	1.175 (0.902–1.530)	0.232	1.714 (1.103–2.663)	0.016	0.050
	33/163/203									
IGF2BP2 (rs1470579)	CC/CA/AA	C	0.940							
	77/198/216			1.334 (1.091–1.630)	0.005	1.260 (0.967–1.643)	0.087	1.929 (1.263–2.947)	0.002	0.007
	35/165/198									
CDKN2A/B (rs10811661)	TT/TC/CC	T	0.394							
	189/222/85			1.227 (1.016–1.482)	0.034	1.116 (0.792–1.572)	0.531	1.454 (1.098–1.925)	0.009	0.031
	119/206/75									
SLC30A8 (rs13266634)	CC/CT/TT	C	0.395							
	162/259/72			1.103 (0.913–1.332)	0.311	1.439 (1.103–2.044)	0.042	0.983 (0.742–1.300)	0.902	0.090
	133/188/79									
KCNJ11 (rs5219)	TT/TC/CC	T	0.302							
	83/232/169			1.181 (0.974–1.432)	0.090	1.156 (0.878–1.523)	0.301	1.436 (0.983–2.099)	0.061	0.154
	50/195/152									

n = 908. Type 2 diabetes is defined by fasting blood glucose ≥126 mg/dl, or occasional blood glucose ≥200 mg/dl and/or current use of antidiabetic agents. Differences in genotype frequency between diabetic patients and normal control subjects, as well as deviations from the Hardy-Weinberg equilibrium in control subjects, were assessed using the χ^2 test.

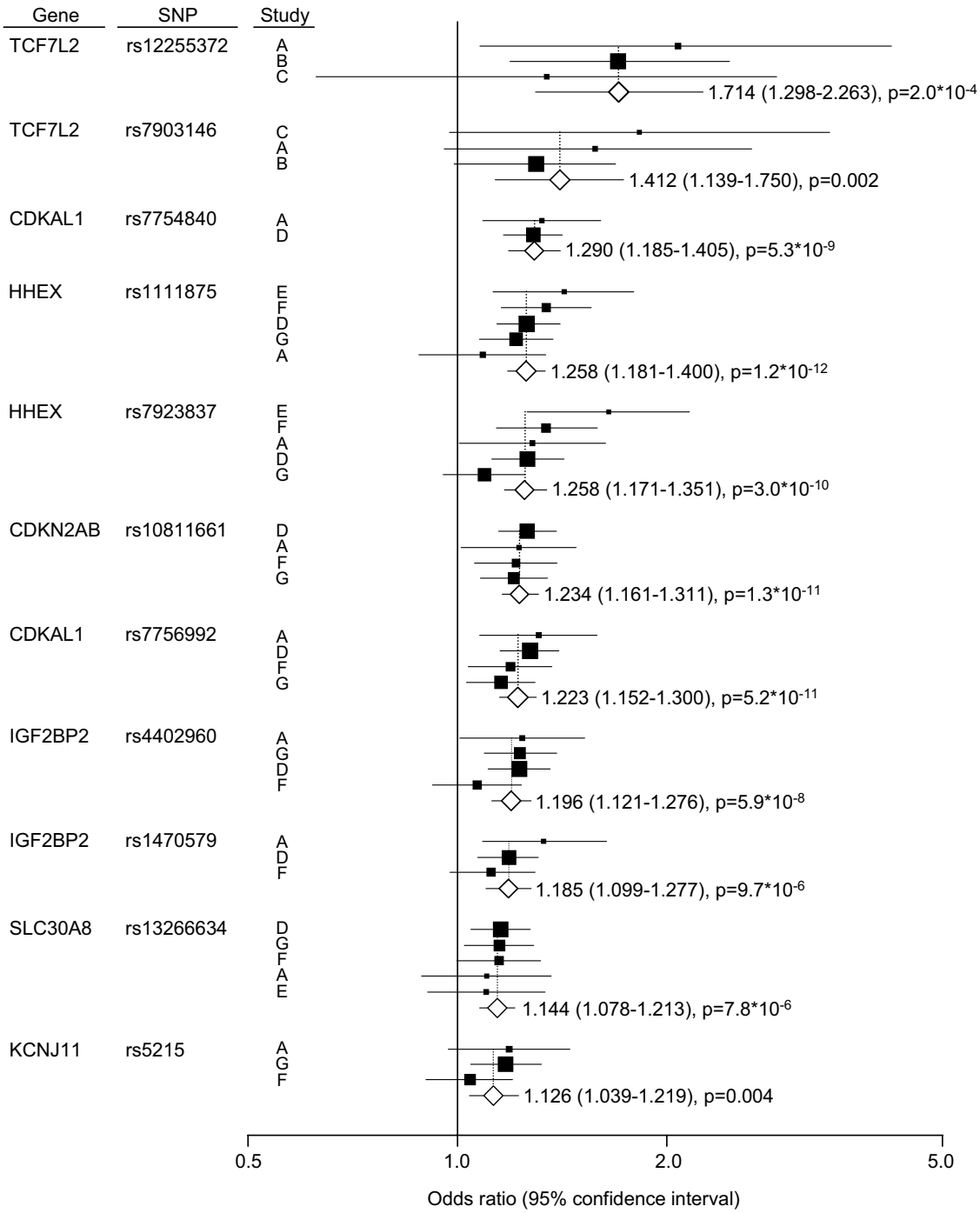

FIG. 1. Meta-analysis of type 2 diabetes genetic association studies in Japanese. Estimation of odds ratios and 95% CIs in each study are displayed as a closed square and horizontal line, respectively. Square size represents the study weighting. Combined odds ratio is represented as the diamond. Study A, present study; Study B, Hayashi et al. (18); Study C, Horikoshi et al. (17); Study D, Horikawa et al. (19); Study E, Furukawa et al. (20); Study F, Horikoshi et al. (21); Study G, Omori et al. (22).

identified from genome-wide screening contribute to diabetes susceptibility primarily through effects on insulin secretion. In our quantitative trait analysis in a general Japanese population, we observed lower basal plasma insulin levels in type 2 diabetic patients carrying the risk genotype of *CDKAL1* rs7756992 SNP. Although the function of the *CDKAL1* gene product is unknown, one study suggested that CDKAL1 has a role in the inhibition of cyclin-dependent kinase 5 (CDK5) activity in pancreatic β-cells (8), which prevents a decrease in insulin gene expression resulting from glucotoxicity. That study also observed reduced insulin secretion in response to glucose loading in homozygous carriers of the *CDKAL1* rs7756992 polymorphism risk allele (8). Pascoe et al. (25) also reported lower insulin secretion after glucose loading in risk allele carriers of another SNP of the *CDKAL1* gene. Our study is the first to show a possible association between this SNP and basal insulin levels in type 2 diabetic patients. This observation provides supporting evidence for the pathophysiological role of the *CDKAL1* gene products in the progression of type 2 diabetes, as well as the disease susceptibility of this genetic variant.

TABLE 2
Association of candidate SNPs with plasma insulin levels in the general population sample

Gene (rs number)	Risk allele (T)	Plasma insulin (μU/ml)						*P*	
		TT		TG		GG		ANOVA	Multivariate
TCF7L2* (rs12255372)									
Control		5.5 ± 2.7	(2)	6.2 ± 4.7	(52)	6.5 ± 4.8	(1,770)	0.661	0.749
Type 2 diabetes		—	(0)	4.2 ± 1.0	(3)	9.0 ± 7.3	(136)	0.256	0.368
TCF7L2* (rs7903146)	T	TT		TC		CC			
Control		6.8	(1)	6.5 ± 4.3	(111)	6.5 ± 4.8	(1,712)	0.967	0.336
Type 2 diabetes		—	(0)	5.1 ± 2.3	(8)	9.1 ± 7.4	(131)	0.130	0.226
CDKAL1 (rs7756992)	G	GG		GA		AA			
Control		6.3 ± 5.2	(428)	6.5 ± 4.6	(895)	6.6 ± 4.7	(501)	0.227	0.457
Type 2 diabetes		7.9 ± 7.0	(38)	8.8 ± 6.3	(75)	10.7 ± 10.0	(26)	0.269	0.020
CDKAL1 (rs7754840)	C	CC		CG		GG			
Control		6.1 ± 4.7	(315)	6.4 ± 4.8	(868)	6.7 ± 4.7	(641)	0.083	0.157
Type 2 diabetes		7.1 ± 5.1	(32)	9.0 ± 6.5	(67)	10.3 ± 9.4	(40)	0.154	0.097
HHEX (rs1111875)	C	CC		CT		TT			
Control		6.4 ± 4.6	(185)	6.4 ± 4.4	(755)	6.6 ± 5.1	(884)	0.867	0.883
Type 2 diabetes		9.8 ± 11.6	(15)	7.7 ± 5.2	(62)	9.9 ± 7.7	(62)	0.323	0.470
HHEX (rs7923837)	G	GG		GA		AA			
Control		6.1 ± 4.1	(112)	6.5 ± 4.9	(692)	6.5 ± 4.7	(1020)	0.818	0.749
Type 2 diabetes		7.3 ± 4.6	(9)	8.8 ± 6.9	(56)	9.1 ± 7.8	(74)	0.811	0.789
IGF2BP2 (rs4402960)	T	TT		TG		GG			
Control		6.2 ± 3.9	(215)	6.7 ± 5.2	(767)	6.3 ± 4.5	(842)	0.276	0.308
Type 2 diabetes		12.2 ± 10.0	(15)	7.8 ± 5.4	(72)	9.5 ± 8.4	(52)	0.186	0.843
IGF2BP2 (rs1470579)	C	CC		CA		AA			
Control		6.1 ± 3.8	(239)	6.8 ± 5.2	(781)	6.3 ± 4.5	(804)	0.118	0.314
Type 2 diabetes		11.6 ± 9.6	(17)	7.8 ± 5.4	(72)	9.5 ± 8.5	(50)	0.276	0.869
CDKN2A/B (rs10811661)	T	TT		TC		CC			
Control		6.4 ± 4.7	(591)	6.5 ± 4.6	(886)	6.6 ± 5.2	(347)	0.719	0.858
Type 2 diabetes		8.1 ± 8.0	(41)	8.9 ± 6.5	(72)	10.2 ± 8.0	(26)	0.346	0.169
SLC30A8 (rs13266634)	C	CC		CT		TT			
Control		6.5 ± 4.9	(671)	6.4 ± 4.4	(847)	6.6 ± 5.2	(306)	0.939	0.910
Type 2 diabetes		9.8 ± 7.1	(51)	7.9 ± 5.6	(68)	9.8 ± 11.5	(20)	0.151	0.332
KCNJ11 (rs5219)	T	TT		TC		CC			
Control		6.5 ± 4.8	(222)	6.3 ± 4.6	(836)	6.7 ± 4.9	(766)	0.088	0.156
Type 2 diabetes		6.8 ± 3.8	(28)	8.8 ± 7.2	(68)	10.3 ± 8.7	(43)	0.144	0.205

Data are means ± SD. $n = 1{,}963$. Number of subjects in each genotype is provided in parentheses. Statistical significance was assessed using log-transformed insulin value. Multiple regression analysis involving each genotype as an additive model adjusted for age, sex, and BMI. * T allele dominant model.

Several limitations of this study warrant mention. First, differences in linkage disequilibrium between Japanese and European subjects means that tracking of the causal variants may not be possible in SNPs based on the association study in European ancestries. Although our meta-analysis showed an association between type 2 diabetes susceptibility and the analyzed candidate SNPs, causal variants may also be strongly represented by other SNPs in Japanese subjects. Studies with multiple tag-SNPs at loci chosen based on the linkage disequilibrium pattern in Japanese may provide further clarification of this issue. Second, we did not investigate the class of antihyperglycemic drugs, including insulin treatment, in the general population sample. Although each drug may have affected fasting plasma insulin differently, association of the *CDKAL1* genotype with plasma insulin levels was statistically significant after further adjustment for current treatment for hyperglycemia.

In the present study, we replicated several genetic variants as risk markers for type 2 diabetes susceptibility in Japanese by performing a case-control analysis and meta-analysis. These findings may be useful in advanced clinical practice and public health genomics.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aids for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology of Japan; The Ministry of Health, Labor and Welfare of Japan; the Japan Arteriosclerosis Prevention Fund; and a Research Promotion Award from Ehime University.

No potential conflicts of interest relevant to this article were reported.

We greatly appreciate the support of Dr. Masaaki Ochi, Wataru Nishida, Yasunori Takata, and Yasuhisa Fujii and their help with sample collection.

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