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Journal of Human Genetics (2009) 54, 236–241

www.nature.com/jhg

ORIGINAL ARTICLE

Construction of a prediction model for type 2 diabetes mellitus in the Japanese population based on 11 genes with strong evidence of the association

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Prediction of the disease status is one of the most important objectives of genetic studies. To select the genes with strong evidence of the association with type 2 diabetes mellitus, we validated the associations of the seven candidate loci extracted in our earlier study by genotyping the samples in two independent sample panels. However, except for *KCNQ1*, the association of none of the remaining seven loci was replicated. We then selected 11 genes, *KCNQ1*, *TCF7L2*, *CDKAL1*, *CDKN2A/B*, *IGF2BP2*, *SLC30A8*, *HHEX*, *GCKR*, *HNF1B*, *KCNJ11* and *PPARG*, whose associations with diabetes have already been reported and replicated either in the literature or in this study in the Japanese population. As no evidence of the gene–gene interaction for any pair of the 11 loci was shown, we constructed a prediction model for the disease using the logistic regression analysis by incorporating the number of the risk alleles for the 11 genes, as well as age, sex and body mass index as independent variables. Cumulative risk assessment showed that the addition of one risk allele resulted in an average increase in the odds for the disease of 1.29 (95% CI=1.25–1.33, $P=5.4\times10^{-53}$). The area under the receiver operating characteristic curve, an estimate of the power of the prediction model, was 0.72, thereby indicating that our prediction model for type 2 diabetes may not be so useful but has some value. Incorporation of data from additional risk loci is most likely to increase the predictive power.
Journal of Human Genetics (2009) **54**, 236–241; doi:10.1038/jhg.2009.17; published online 27 February 2009

Keywords: gene–gene interaction; genome-wide association study; prediction model; single nucleotide polymorphism (SNP); type 2 diabetes mellitus

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Received 22 December 2008; revised 25 January 2009; accepted 5 February 2009; published online 27 February 2009

INTRODUCTION

Genome-wide association studies (GWASs) have identified novel susceptibility genes for type 2 diabetes mellitus in Caucasians.[1–5] *TCF7L2*, *CDKAL1*, *CDKN2A/B*, *IGF2BP2*, *SLC30A8* and *HHEX* have been widely replicated as susceptibility genes for type 2 diabetes in Asian populations[6–12] as well as in populations of European ancestry.[13,14] We recently identified *KCNQ1* as a novel susceptibility gene, as well as seven other candidate susceptibility loci in a multistage GWAS for type 2 diabetes in the Japanese population, in which a total of 1612 cases and 1424 controls and 100 000 single nucleotide polymorphisms (SNPs) were included.[15] *KCNQ1* was found to confer risk of type 2 diabetes with a relatively large effect size in Asian populations (odds ratio (OR) for Japanese, Chinese and Korean individuals of 1.42),[15] which was similar to that demonstrated earlier for *TCF7L2* in the Japanese population.[6]

Follow-up of GWASs includes analysis of second-tier genes, meta-analysis for specific populations, as well as analysis of gene–gene or gene–environment interactions. A large-scale meta-analysis[16] and an analysis of gene–gene interaction for susceptibility genes[17] have been performed for type 2 diabetes in populations of European ancestry.

In this study, we attempted to confirm in independent subject panels of Japanese and Hong Kong Chinese individuals the associations of the seven candidate susceptibility loci that we identified in addition to *KCNQ1* in our GWAS of type 2 diabetes.[15] However, as described in this article, we failed to replicate the associations of the seven loci with diabetes. We then attempted to extract genes with strong evidence of the associations with diabetes, and selected 11 genes, including *KCNQ1*. As we did not detect any gene–gene interaction between the 11 genes, we then attempted to construct a prediction model for this disease by using the data from the 11 genes, as well as age, gender and body mass index (BMI) as independent variables to obtain a comprehensive understanding of the genetic background of diabetes in the Japanese population.

MATERIALS AND METHODS

Validation of the results from a multistage GWAS in the Japanese population

Study subjects. We assembled two independent subject panels for our replication study: replication-Japanese and replication-Chinese. The 1000 cases and 1000 controls for the replication-Japanese panel were recruited by the Study Group of the Millennium Genome Project for Diabetes Mellitus. The inclusion criteria for diabetic patients were (i) an age at disease onset of 30–60 years and (ii) the absence of antibodies to GAD. Types of diabetes other than type 2 were excluded on the basis of clinical data. The criteria for controls included (i) an age of >50 years, (ii) no past history of a diagnosis of diabetes and (iii) an HbA_{1c} content of <5.8%.

For the replication-Chinese panel, subjects of southern Han Chinese ancestry, who resided in Hong Kong, were recruited. The cases consisted of 1416 individuals with type 2 diabetes selected from the Prince of Wales Hospital Diabetes Registry;[5,18] 626 of these subjects had early-onset diabetes (age at diagnosis of <40 years) and a positive family history, whereas the remaining 790 patients were randomly selected from the registry. Patients with classic type 1 diabetes with acute ketotic presentation or a continuous requirement for insulin within 1 year of diagnosis were excluded. The controls consisted of 1577 subjects with normal glucose tolerance (fasting plasma glucose concentration of <6.1 mmol l^{-1}); 596 of these individuals were recruited either from the general population participating in a community-based screening program for cardiovascular risk or from hospital staff, whereas the remaining 981 subjects were recruited from a population-based screening program for cardiovascular risk in adolescents.[19] The clinical characteristics of the subjects in each panel are summarized in Supplementary Table 1A. The study protocol was approved by the local ethics committee of each institution. Written informed consent was obtained from each subject.

Study design and statistical analysis. For the validation of the results from our earlier multistage GWAS,[15] seven SNPs (rs2250402, rs2307027, rs3741872, rs574628, rs2233647, rs3785233 and rs2075931) were genotyped in the two panels either by sequence-specific primer–PCR analysis followed by fluorescence correlation spectroscopy[20] or by real-time PCR analysis with TaqMan probes (Applied Biosystems, Foster City, CA, USA). Differences in allele frequency between cases and controls for each SNP were evaluated by χ^2 with one degree of freedom. Meta-analysis was performed by the Mantel–Haenszel method (fixed-effects models) with the 'meta' package of the R-Project (http://www.r-project.org). A *P*-value of <0.05 was considered statistically significant.

Examination of gene–gene interaction and construction of a prediction model

Study subjects. In total, 2424 cases and 2424 controls of the Japanese population obtained by combining the second and third screening panels in our original study[15] and the replication-Japanese panel of this study were included in this analysis (analysis-panel). The criteria for the second and third screening panels were described in the earlier report.[15] The clinical characteristics of the subjects are summarized in Supplementary Table 1B.

Selection of the loci included in this study. Prediction of the phenotypes on the basis of genetic polymorphisms should include the genetic data from the loci with strong evidence of the association. Starting from 15 genes described in earlier reports, we selected 11 genes with strong evidence of the association on the basis of the data in the literature and on the results of the replication experiments in this study. Process of the selection of the 11 genes will be described in detail in Results.

Statistical methods. Multiplicative gene–gene interaction was evaluated for each pair of the 11 genes using an interaction term in addition to the terms for the pair of the genes in the logistic regression model. The genotypes for each locus were coded by 0, 1 and 2. Correction for multiple testing was performed by Bonferroni's method.

As there was no evidence for the presence of gene–gene interactions, we attempted to construct a phenotype prediction model by incorporating the number of risk alleles for the 11 loci as an independent variable in addition to age, gender and BMI. The Cochran–Armitage test was used to examine the trend of the increase in the odds by increasing the number of the risk alleles. To construct a prediction model, the log of odds was expressed by the linear combination of the independent variables. Coefficients for the variables were estimated by the logistic regression analysis after making disease (cases) or nondisease (controls) as the dependent variable. Using the coefficients estimated by the logistic regression analysis, we constructed a phenotype prediction model. To evaluate the prediction model, receiver operating characteristic (ROC) curves[21] for the sensitivity and specificity of the prediction model with or without adjustment for age, sex and BMI were generated, and the area under the curve (AUC) was calculated from the ROC curve.

RESULTS

Validation of the results from a multistage GWAS in the Japanese population

We identified earlier 10 loci associated with type 2 diabetes by three-staged GWAS starting from 100 000 SNPs. Among the 10 loci, 3 SNPs were located in an intron of *KCNQ1*, and the association of this gene with diabetes was confirmatory.[15] To validate the other seven loci for the association with type 2 diabetes, we analyzed them in two independent replication panels of Japanese and Han-Chinese individuals (Table 1, Supplementary Table 2). Only one SNP, rs2250402, which is located in *EIF2AK4*, was found to be significantly associated in the replication-Japanese panel (P=0.039, OR=1.17, 95% CI=1.01–1.36). However, neither this SNP (P=0.41, OR=1.05) nor any of the other six SNPs showed such an association in the replication-Chinese panel. Meta-analyses for these SNPs showed that rs2307027 in *KRT4* and rs3785233 in *A2BP1* yielded *P*-values of <0.05 and ORs between 1.12 and 1.13 (Table 1). When the original second and third screening

Table 1 Association study for the candidate susceptibility genes for type 2 diabetes selected by multistage screening in the Japanese population

SNP ID	*Chr*	*Gene*	*Risk allele*	*Panel*	*RAF (DM)*	*RAF (NC)*	*P*	*OR*	*95% CI*
rs2250402	15	*EIF2AK4*	C	Replication-Japanese	0.23	0.20	0.04	1.17	1.01–1.36
				Replication-Chinese	0.24	0.23	0.41	1.05	0.93–1.19
				Meta-analysis			0.05	1.10	1.00–1.20
rs2307027	12	*KRT4*	C	Replication-Japanese	0.18	0.17	0.17	1.12	0.95–1.32
				Replication-Chinese	0.14	0.13	0.16	1.11	0.96–1.29
				Meta-analysis			0.05	1.12	1.00–1.25
rs3741872	12	*FAM60A*	C	Replication-Japanese	0.25	0.24	0.18	1.11	0.96–1.28
				Replication-Chinese	0.23	0.22	0.21	1.08	0.96–1.22
				Meta-analysis			0.07	1.09	0.99–1.20
rs574628	20	*ANGPT4*	G	Replication-Japanese	0.60	0.61	0.46	0.95	0.84–1.08
				Replication-Chinese	0.65	0.65	0.59	1.03	0.93–1.15
				Meta-analysis			0.96	1.00	0.92–1.08
rs2233647	6	*SPDEF*	G	Replication-Japanese	0.86	0.87	0.70	0.97	0.81–1.16
				Replication-Chinese	0.94	0.93	0.54	1.07	0.87–1.31
				Meta-analysis			0.90	1.01	0.88–1.16
rs3785233	16	*A2BP1*	C	Replication-Japanese	0.18	0.16	0.19	1.12	0.95–1.32
				Replication-Chinese	0.13	0.12	0.10	1.14	0.97–1.34
				Meta-analysis			0.04	1.13	1.01–1.27
rs2075931	1	Intergenic	A	Replication-Japanese	0.67	0.66	0.85	1.01	0.89–1.16
				Replication-Chinese	0.73	0.74	0.27	0.94	0.84–1.05
				Meta-analysis			0.48	0.97	0.89–1.06

Abbreviations: Chr, chromosome; OR, odds ratio for risk allele frequency.
Assignment of risk alleles was based on the original study.[15] Numbers of cases versus control subjects in the replication-Japanese and replication-Chinese panels were 1000 versus 1000 and 1416 versus 1577, respectively. RAF (DM) and RAF (NC) denote risk allele frequencies in cases and controls, respectively. *P* values were calculated for allele frequency. Meta-analysis was performed by the Mantel–Haenszel method (fixed-effects models). *P*-values for the test of heterogeneity among panels joined in the Mantel–Haenszel tests were all >0.05.

panels were included in the meta-analyses, these two loci, as well as the SNPs in *EIF2AK4* (rs2250402) and *FAM60A* (rs3741872), gave *P*-values of <0.001 and ORs between 1.15 and 1.18 (Supplementary Table 3). However, the *P*-values did not reach the proposed significance of GWAS ($=5\times10^{-7}$).

Selection of polymorphisms for the prediction model

To construct a reliable prediction model for diabetes, polymorphisms with strong evidence of association should be used. From the previous literature, we selected 15 genes (including one intergenic marker), that is, *SLC30A8*, *HHEX*, *LOC387761*, *EXT2*, *CDKN2A/B*, *GCKR*, *IGF2BP2*, *CDKAL1*, *FTO*,[1–5] *TCF7L2*,[22] *KCNJ11*,[23] *PPARG*,[24] *WFS1*,[25] *HNF1B*[26] and *KCNQ1*,[15] as candidate genes to be included in both gene–gene interaction analysis and construction of a prediction model. Starting from 23 SNPs in these 15 genes, we selected 11 SNPs in 11 genes according to the following process. There is sufficient evidence of the associations of *KCNQ1* and *TCF7L2* genes with diabetes as supported by replication studies in the Japanese population.[6,15,27] In addition, *SLC30A8*, *HHEX*, *CDKN2A/B*, *IGF2BP2* and *CDKAL1* associated with the disease in the European population were found in our earlier study to be associated with the disease in the Japanese population as well.[7–9]

To further extract genes with strong evidence of the association with diabetes, we attempted to replicate the associations reported earlier using our own data (analysis panel with 2424 cases and 2424 controls). For the 19 SNPs in *SLC30A8*, *HHEX*, *LOC387761*, *EXT2*, *CDKN2A/B*, *GCKR*, *IGF2BP2*, *CDKAL1*, *FTO*, *TCF7L2*, *KCNJ11*, *PPARG* and *KCNQ1*, we extracted genotyping data from our earlier studies[6–9,15,27–29] and, if necessary, genotyped additional subjects to obtain a data set for 2424 cases and 2424 controls of the Japanese population (analysis panel). The SNPs in *WFS1* (rs6446482, rs734312) and *HNF1B* (rs7501939, rs4430796) were genotyped for this study in the same individuals. SNPs with *P*-values for the test of deviation from the Hardy–Weinberg equilibrium of <0.01 were excluded for further analysis. When two SNPs were located in the same genomic region, the one with the lower *P*-value for the association test was selected for further analysis. *GCKR*, for which we earlier reported the marginal association with type 2 diabetes,[7] was found to be associated with the disease in this enlarged Japanese panel ($P=1.7\times10^{-5}$; Supplementary Table 4). *KCNJ11* and *PPARG*, which have been included in the genes associated with diabetes in Caucasians, showed marginal associations ($P=0.066$ and $P=0.075$, respectively; Supplementary Table 4) in our panel. Two SNPs in *WFS1* and two SNPs in *HNF1B* were newly genotyped in the analysis panel. Although no association was apparent between *WFS1* and type 2 diabetes, both SNPs in *HNF1B* exhibited *P*-values of <0.05 (Supplementary Table 4). From these data, we included 11 SNPs in 11 genes as described above for the source of genotype data to be analyzed in both the examination of gene–gene interaction and the prediction of phenotypes.

Gene–gene interaction

We evaluated multiplicative gene–gene interaction for each pair of the 11 loci as described in Materials and methods. Two combinations, rs1801282 (*PPARG*) ×rs1470579 (*IGF2BP2*) (nominal $P=0.0025$) and rs1801282×rs3802177 (*SLC30A8*) (nominal $P=0.018$), showed *P*-values of less than 0.05 (Supplementary Figure 1). However, these *P*-values were not significant when Bonferroni's correction for multiple testing was applied (significance level, $0.05/55=9.1\times10^{-4}$). Although *PPARG* and *IGF2BP2* are located on the same chromosome (3p25 and 3q28, respectively), it is unlikely that loci on different arms of the same chromosome show significant linkage disequilibrium. *SLC30A8* is located on a different chromosome (8q24.11) from

PPARG. The reason why nominal *P*-values of these combinations showed less than 0.05 may be because of the low minor allele frequency of rs1801282.

Cumulative risk assessment for type 2 diabetes on the basis of susceptibility genes

As there was no evidence of gene–gene interaction between 11 SNPs of 11 genes, *SLC30A8*, *HHEX*, *CDKN2A/B*, *GCKR*, *IGF2BP2*, *CDKAL1*, *TCF7L2*, *KCNJ11*, *PPARG*, *KCNQ1* and *HNF1B*, they were included in the prediction model as independent variables with the additive effect (additive effect in the liability and multiplicative effect in the odds) without interaction terms. Effective numbers of cases and controls whose genotypes for the 11 loci were successfully obtained were 2316 and 2370, respectively. The Cochran–Armitage trend test gave a *P*-value of 4.7×10^{-56} for the trend in the increase in the odds for cases relative to controls with an increasing number of risk alleles for the 11 susceptibility loci (Supplementary Table 5). We then estimated ORs for type 2 diabetes in subjects with different numbers of risk alleles on the basis of the multiplicative model by logistic regression analysis with adjustment for age, sex and BMI. The ORs for type 2 diabetes in subjects with 7–18 risk alleles in comparison with those harboring 0–6 risk alleles are shown in Figure 1. An increase of one risk allele resulted in an average increase in the odds of 1.29 (95% CI=1.25–1.33, $P=5.4\times10^{-53}$, logistic regression analysis).

To predict disease status for type 2 diabetes in a given individual, we constructed a prediction model on the basis of the number of risk alleles or the liability value calculated from the number of risk alleles as well as age, sex and BMI. The coefficients to calculate the liability value were estimated with the logistic regression model. To estimate the predictive power of the model, we generated ROC curves as described in Materials and methods. The AUC was 0.63 when only the number of risk alleles was used for the prediction. When age, sex and BMI were also included, the AUC increased to 0.72 (Figure 2). Meanwhile, an AUC value for the ROC curve based on only age, sex and BMI was 0.68, which was better than that based on only the number of risk alleles (data now shown). The model incorporating age, sex and BMI as well as the number of risk alleles thus showed moderate power for the prediction of type 2 diabetes. The best accuracy was 0.66 at the threshold between non-diabetic and diabetic status of 0.52 (non-diabetic status=0, diabetic status=1), for which the specificity and the sensitivity were 0.71 and 0.61, respectively.

DISCUSSION

By the validation of the results from our multistage GWAS, we detected only marginal associations of *EIF2AK4*, *KRT4* and *A2BP1* with type 2 diabetes in meta-analyses with two subject panels of Japanese or Chinese individuals. Relations of *KRT4* (keratin 4 gene) and *A2BP1* (ataxin-2-binding protein 1 gene, also known as *FOX1*) to glucose or lipid metabolism are unknown. Deletion of *EIF2AK4* (eukaryotic translation initiation factor 2 alpha kinase 4 gene, also known as *GCN2*) in mice resulted in liver steatosis during leucine deprivation as a result of unrepressed expression of lipogenic genes.[30] The functionally related gene, *EIF2AK3* (also known as *PERK* or *PEK*), has been shown to cause diabetes mellitus both in humans (Wolcott–Rallison syndrome, OMIM604032) and in rodent models.[31,32] Taken together, *EIF2AK4* may be a good candidate for the diabetes susceptibility gene. The sample size required for a statistical power of 0.80 with equal numbers of cases and controls is 10 505 when the frequency of the risk allele, OR and type I error probability are assumed to be 0.20, 1.10 (the value for *EIF2AK4* in the meta-analysis in Table 1) and 0.05, respectively. Further studies of these genes in other Asian populations as well as in other ethnic groups are needed for confirmation of their association with type 2 diabetes. Given this uncertainty, we did not include these genes in the assessments of cumulative risk and gene–gene interaction.

Among tens of type 2 diabetes susceptibility genes identified by recent GWASs in Caucasians, the associations of six genes, that is, *TCF7L2*, *CDKAL1*, *CDKN2A/B*, *IGF2BP2*, *SLC30A8* and *HHEX*, have been replicated in Asian populations as well as in populations of European ancestry. A recent meta-analysis in Japanese subjects also supported the associations.[12] In this study, we performed replication study, and, on the basis of the results, we added five more genes, that is, *KCNJ11*, *PPARG*, *GCKR*, *KCNQ1* and *HNF1B*, for the cumulative risk assessment for type 2 diabetes. Thus, the SNPs of *HNF1B*, which were earlier associated with type 2 diabetes in Chinese as well as in Caucasians,[26] showed the association with the disease in the Japanese

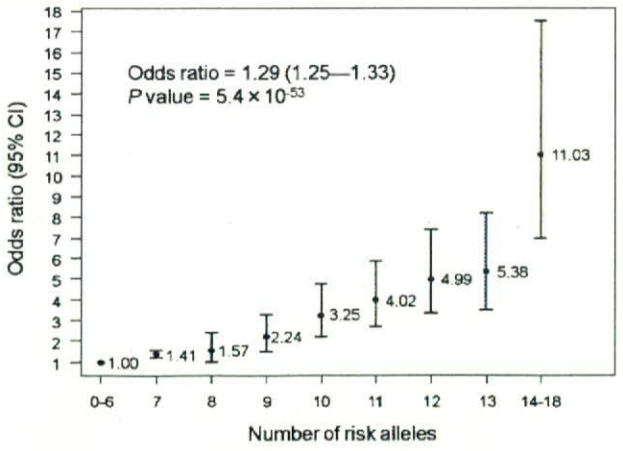

Figure 1 Odds ratios for subjects with different numbers of risk alleles for 11 susceptibility loci for type 2 diabetes. The cumulative effect of the 11 loci on type 2 diabetes was tested by counting the number of risk alleles associated with type 2 diabetes with a logistic regression model with adjustment for age, sex and BMI. The ORs for subjects with each number of risk alleles are expressed relative to individuals with 0–6 risk alleles.

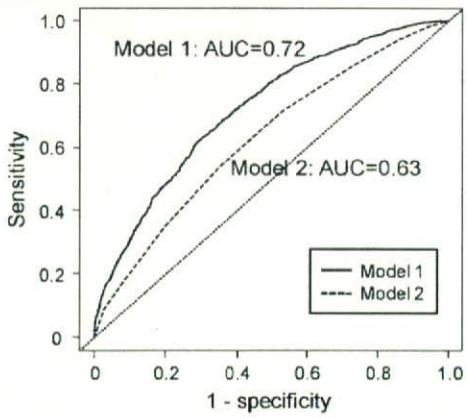

Figure 2 ROC curves for the prediction model on the basis of the number of risk alleles for 11 susceptibility loci for type 2 diabetes. The prediction model for type 2 diabetes was constructed using the logistic regression model, and ROC curves for the model were generated. In model 1, the number of risk alleles was used as an independent variable together with age, sex and BMI as covariates, whereas only the number of risk alleles was used as an independent variable in model 2.

population in this study. In addition, the *C* allele of rs780094 in *GCKR* was associated with increased risk of type 2 diabetes in this study, which is consistent with a recent study in Caucasians.[33] The associations of *KCNJ11* and *PPARG* with diabetes were marginal in this study; however, they were included for the prediction model, as the associations were replicated in some studies of Caucasians.

Our gene–gene interaction analysis showed no significant interaction for any of the 55 possible pairs of genes when corrected for multiple testing. When the significance level was set at 0.05, two pairs were judged to be significant. However, such gene–gene interactions were not supported from the functional point of view. A large-scale study may provide more convincing evidence for such interactions.

As no confirmatory evidence for gene–gene interaction was observed, we treated the 11 genes as independent variables in the prediction model. The addition of one risk allele was estimated to increase the odds by an average of 1.29 according to the multiplicative model. This value is similar to that (1.24) estimated for type 2 diabetes in Caucasians.[17] Two earlier cumulative risk assessments for type 2 diabetes in Asian populations with relatively small numbers of associated loci yielded values of 1.17 and 1.24 for the fold increase in risk for each additional risk allele.[11,34] In our prediction model for type 2 diabetes, the AUC for the ROC curve was lower than that in the earlier study[17] based on 15 loci in Caucasians (0.72 and 0.86, respectively). However, the number of loci in our study (11 loci) was lower than that in the study for Caucasians. The inclusion of additional loci in our model should improve its ability to predict type 2 diabetes in Asian populations. Several reports of the prediction of type 2 diabetes using ~18 loci were recently described for populations of European ancestry.[35–38] A prediction based on 18 loci gave an AUC value of 0.80 for the ROC curve,[35] whereas the corresponding values for a population-based prospective study were 0.68,[36] 0.615[37] and 0.75.[38] They concluded that genetic variations associated with diabetes had a small effect on the ability to predict the development of type 2 diabetes as compared with clinical characteristics alone. In fact, the AUC value (0.72) based on both the genetic variations and the clinical characteristics was slightly better than that based on only the clinical characteristics (0.68). We admit that the evidence of the association with diabetes is a little weaker for *KCNJ11* and *PPARG* in the Japanese population than for the other nine genes. If *KCNJ11* and *PPARG* were excluded from the analysis, the AUC for the ROC curve in the prediction model incorporating age, sex and BMI remained unchanged at 0.72, probably because of the relatively large effects of *KCNQ1* and *TCF7L2*.

Finally, our prediction model for type 2 diabetes achieved limited success even though it has some value. Given that GWASs for diabetes in Asians have not been as extensive as those in Caucasians, many risk loci for diabetes in Asians remain most likely to be undiscovered. Considering that the average increase in OR conferred by each additional risk allele was similar between Caucasians and Japanese, incorporation of data from additional risk loci is most likely to increase the predictive power.

ACKNOWLEDGEMENTS

We thank all the subjects who joined this project; Sumio Sugano and Shoji Tsuji for support and helpful discussion throughout the project; and Megumi Yamaoka-Sageshima for technical assistance. This work was supported by a grant from the Program for Promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency (PMDA) of Japan; a grant from the National Institute of Biomedical Innovation (NIBIO) of Japan; grants from the Ministry of Health, Labour and Welfare of Japan; a Grant-in-Aid for Scientific Research on Priority Areas (C), 'Medical Genome Science (Millennium Genome Project)', 'Applied Genomics', and 'Comprehensive Genomics', from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and a grant from the Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER, Tokushima, Japan). The Hong Kong diabetes case–control study was supported by the Hong Kong Research Grants Committee Central Allocation Scheme CUHK 1/04C.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)

nature genetics

Variants in *KCNQ1* are associated with susceptibility to type 2 diabetes mellitus

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We carried out a multistage genome-wide association study of type 2 diabetes mellitus in Japanese individuals, with a total of 1,612 cases and 1,424 controls and 100,000 SNPs. The most significant association was obtained with SNPs in *KCNQ1*, and dense mapping within the gene revealed that rs2237892 in intron 15 showed the lowest *P* value (6.7×10^{-13}, odds ratio (OR) = 1.49). The association of *KCNQ1* with type 2 diabetes was replicated in populations of Korean, Chinese and European ancestry as well as in two independent Japanese populations, and meta-analysis with a total of 19,930 individuals (9,569 cases and 10,361 controls) yielded a *P* value of 1.7×10^{-42} (OR = 1.40; 95% CI = 1.34–1.47) for rs2237892. Among control subjects, the risk allele of this polymorphism was associated with impairment of insulin secretion according to the homeostasis model assessment of β-cell function or the corrected insulin response. Our data thus implicate *KCNQ1* as a diabetes susceptibility gene in groups of different ancestries.

In Japan, the prevalence of type 2 diabetes mellitus is increasing rapidly, and more than 10% of individuals over 40 years of age are affected. Relatively few diabetic individuals in Japan are obese, and impairment of insulin secretion often develops before the onset of diabetes[1]. As part of a national project designated the Millennium Genome Project in Japan, in 2002 we began a multistage genome-wide association study (GWAS) to identify disease-associated SNPs for type 2 diabetes mellitus using 100,000 SNPs from a collection of

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Received 3 March; accepted 6 June; published online 17 August 2008; doi:10.1038/ng.207

Table 1 Positive SNPs identified in the third screening

				Panel 1 (187 cases)				Panel 2 (752 cases, 752 controls)				Panel 3 (672 cases, 672 controls)			
dbSNP ID	Risk allele	Chr.	Gene	RAF(DM)	RAF(NC)	OR (95% CI)	*P* value	Control	RAF(DM)	RAF(NC)	OR (95% CI)	*P* value	RAF(DM)	RAF(NC)	OR (95% CI)	*P* value
rs151290	C	11	*KCNQ1*	0.63	0.57	1.30 (1.03–1.65)	0.027	ODG	0.62	0.55	1.34 (1.16–1.55)	7.4×10^{-5}	0.61	0.54	1.36 (1.16–1.58)	1.1×10^{-4}
rs163184	G	11	*KCNQ1*	0.51	0.43	1.33 (1.06–1.67)	0.015	JDC	0.49	0.44	1.22 (1.06–1.41)	0.0064	0.48	0.42	1.27 (1.09–1.48)	0.0021
rs2237895	C	11	*KCNQ1*	0.45	0.35	1.53 (1.22–1.93)	2.8×10^{-4}	JDC	0.42	0.33	1.49 (1.28–1.73)	1.4×10^{-7}	0.42	0.33	1.45 (1.24–1.70)	3.4×10^{-6}
rs2250402	C	15	*EIF2AK4*	0.20	0.27	1.45 (1.09–1.93)	0.011	JDC	0.24	0.21	1.20 (1.01–1.43)	0.035	0.26	0.21	1.34 (1.11–1.60)	0.0018
rs2307027	C	12	*KRT4*	0.14	0.22	1.68 (1.20–2.36)	0.0024	ODG	0.20	0.17	1.23 (1.02–1.47)	0.031	0.21	0.16	1.37 (1.12–1.67)	0.0017
rs3741872	C	12	*FAM60A*	0.29	0.23	1.37 (1.06–1.76)	0.015	ODG	0.29	0.24	1.29 (1.09–1.52)	0.0024	0.28	0.23	1.28 (1.07–1.52)	0.0060
rs574628	G	20	*ANGPT4*	0.56	0.64	1.38 (1.09–1.74)	0.0066	ODG	0.65	0.61	1.17 (1.01–1.36)	0.037	0.64	0.59	1.28 (1.10–1.50)	0.0018
rs2233647	G	6	*SPDEF*	0.92	0.86	1.87 (1.07–3.27)	0.026	ODG	0.88	0.86	1.24 (1.00–1.54)	0.047	0.89	0.86	1.29 (1.02–1.62)	0.033
rs3785233[a]	C	16	*A2BP1*	0.20	0.17	1.20 (0.90–1.61)	0.22	ODG	0.19	0.16	1.25 (1.03–1.51)	0.023	0.19	0.16	1.23 (1.01–1.50)	0.039
rs2075931	A	1		0.71	0.64	1.37 (1.07–1.75)	0.013	ODG	0.68	0.65	1.17 (1.01–1.37)	0.038	0.68	0.64	1.18 (1.00–1.38)	0.048

P values were calculated for allele data. For panel 1, two control groups (ODG, other disease group; JDC, Japanese database control) were used for association studies and the lower *P* values are listed. RAF(DM) and RAF(NC), risk allele frequencies in cases and controls, respectively. OR, odds ratio for risk allele.
[a]This SNP was selected for the second stage on the basis of the recessive model (OR = 2.59, CI = 1.20–5.58, $P = 0.012$).

standard Japanese SNPs[2] (which we refer to as the JSNP Genome Scan (JGS)), as part of the multi-disease collaborative genome scan (**Supplementary Fig. 1** online).

Among 100,000 SNPs genotyped by multiplex PCR–based Invader analysis in the first stage of the study, 82,343 autosomal polymorphisms passed our typing quality control in 187 individuals with diabetes (**Supplementary Table 1** online). We then carried out two separate association analyses to compare the 187 individuals with diabetes with two different control groups, which we considered as population controls: one to compare allele frequencies with reference data for 752 individuals representing the general Japanese population deposited in the JSNP database (referred to as the 'JSNP database control' (JDC)), and one to compare allele or genotype frequencies with those of the 752 individuals in the initial panels for the other four disease groups (Alzheimer's disease, gastric cancer, hypertension and asthma) of the national project (referred to as the 'other disease group' (ODG)). The combination of two types of association analysis resulted in the selection of 2,880 SNPs for the second stage of the study. An independent case-control panel (panel 2) was analyzed, and 201 positive SNPs ($P < 0.05$) were selected for the third stage (see **Supplementary Table 2a** online). Ten SNPs yielded a *P* value of <0.05 at the third stage using another case-control panel (panel 3; **Table 1** and **Supplementary Table 2b**). These SNPs showed variable *P* values in the first stage, suggestive of a limited power of the study design. The most significant association ($P = 3.4 \times 10^{-6}$) was obtained with rs2237895, which is located in intron 15 of *KCNQ1*. Another two SNPs (rs151290 and rs163184) were also located in the same intron, yielding *P* values of 1.1×10^{-4} and 0.0021, respectively. Panels 2 and 3 combined (panel 2+3) were analyzed for these 10 SNPs, yielding even lower *P* values for all the SNPs (**Supplementary Table 2b**). The genotype-based Cochran-Armitage trend test gave *P* values similar to those based on the allele data (**Supplementary Table 2b**).

We further analyzed *KCNQ1*, which was the only gene that yielded positive results according to the standard criterion (*P* value of $< 5 \times 10^{-7}$) recently proposed for GWAS[3]. The three SNPs of *KCNQ1* that passed the third scan (rs151290, rs163184 and rs2237895) were in moderate linkage disequilibrium (LD) with each other (**Fig. 1**). The SNP with the lowest *P* value, rs2237895, yielded D' and r^2 values of 0.54 and 0.12 with rs151290 and 0.83 and 0.46 with rs163184, respectively. We isolated 49 additional SNPs of *KCNQ1* from dbSNP of NCBI and typed them together with the three originally positive SNPs in panel 2+3 (**Fig. 1**). Among these 52 SNPs, rs2237892, which is also located in intron 15, showed the strongest association with diabetes ($P = 6.7 \times 10^{-13}$), with OR =1.49 and 95% CI = 1.34–1.66; the *P* value for the trend test was 1.7×10^{-12} (**Table 2**). The D' and r^2 values for rs2237895 and rs2237892 were 0.95 and 0.30, respectively.

We also sequenced all the exons and the 47-kb genomic region corresponding to intron 15 of *KCNQ1* in 24 Japanese individuals and identified 212 variations, including three synonymous and two nonsynonymous (P448R and G643S) polymorphisms (**Supplementary Table 3a** online). We then genotyped ten of the newly identified SNPs of intron 15 and the two nonsynonymous polymorphisms in panel 2+3. None of these SNPs showed a stronger association with diabetes than did rs2237892 (**Fig. 1** and **Supplementary Table 3b**).

We next examined the possible association of *KCNQ1* with diabetes in several additional subject panels, including those of other ancestral groups, by genotyping rs2237892, rs2237895 and rs2074196, the three SNPs that showed the strongest association in the original study. Two independent Japanese panels revealed a strong association of these

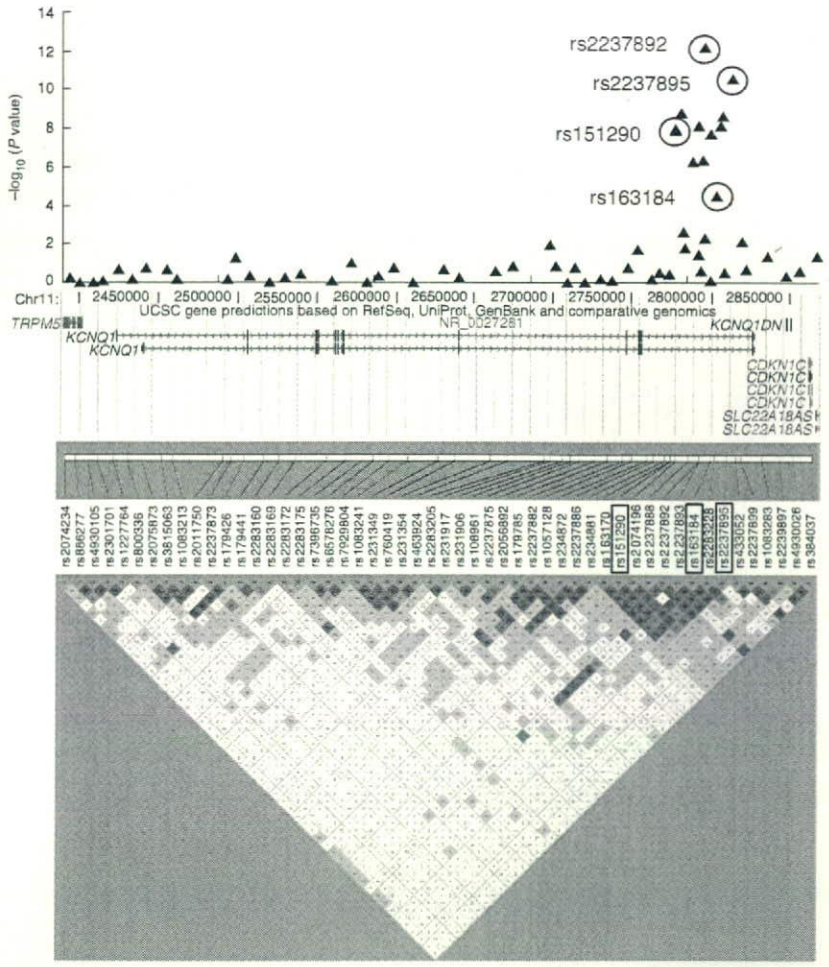

Figure 1 Dense mapping analysis of *KCNQ1*. The top panel shows the association $-\log_{10}$ (*P* value) in panel 2+3 for 64 SNPs of *KCNQ1*. The three blue circles represent the positive SNPs in the third screening. The red circle (rs2237892) indicates the SNP showing the most significant association with type 2 diabetes. The upper middle panel shows the physical position of *KCNQ1* and neighboring genes on chromosome 11 (UCSC Genome Browser). The lower middle panel shows the positions and rs numbers of the 52 previously identified SNPs. Blue rectangles indicate the positive SNPs in the third screening. The bottom panel shows a Haploview representation of LD (*D'*) based on genotyping data from control subjects in panel 2+3 ($n = 1,424$).

polymorphisms with diabetes (**Table 2** and **Supplementary Table 4** online); rs2237892, for example, showed allelic *P* values of 9.6×10^{-10} and 6.9×10^{-10} in the replication 1 and 2 panels, respectively. The three Japanese panels (panel 2+3 and replication 1 and 2), which included a total of 4,378 cases and 4,412 controls, yielded an allelic *P* value of 2.8×10^{-29} and OR of 1.43 (95% CI = 1.34–1.52) for rs2237892. The association was also reproduced in the replication 3 (Chinese) and replication 4 (Korean) panels; the allelic *P* values for rs2237892 in these two panels were 1.3×10^{-8} and 1.7×10^{-5}, respectively (**Table 2** and **Supplementary Table 4**). Meta-analysis of the Asian populations yielded a *P* value of 2.5×10^{-40} and OR of 1.42 (95% CI = 1.34–1.49) for rs2237892. We also examined rs2237892 and rs2074196 in the replication 5 panel (recruited from Sweden), with both SNPs showing a positive association ($P = 7.8 \times 10^{-4}$ and 0.017, respectively). With the inclusion of the replication 5 panel, meta-analysis with a total of 19,930 individuals (9,569 cases and 10,361 controls) yielded a *P* value of 1.7×10^{-42} and OR of 1.40 (95% CI = 1.34–1.47) for rs2237892 (**Table 2** and **Supplementary Fig. 2** online).

We next investigated the relation of rs2237892 to clinical phenotype. Among 1,424 individuals with diabetes in panel 2+3, no association was found between this SNP and clinical parameters such as body mass index (BMI) and the level of insulin resistance. Among the 948 control subjects in panel 2+3 whose fasting plasma glucose and insulin levels were available, homozygotes for the risk allele of rs2237892 (CC) showed a significantly lower homeostasis model assessment of β-cell function (HOMA-β)[4] than did those with the other genotypes (**Supplementary Table 5** online). Among nondiabetic subjects of the Botnia prospective cohort (**Supplementary Methods** online), the corrected insulin response (CIR) at the follow-up visit was significantly lower for individuals with the CC genotype of rs2237892 than for those with the other two genotypes in both an additive and recessive model for this SNP ($P = 0.024$ and 0.010, respectively; **Supplementary Table 5**). These results suggested that the risk allele of *KCNQ1* might contribute to diabetes susceptibility by impairing insulin secretion.

The multistage strategy for GWASs has an advantage in the effective elimination of a large number of false-positive results and has proved to be successful[5]. Indeed, we detected the association of several SNPs of *KCNQ1* with diabetes in the JGS, and this association was reproduced in two independent Japanese panels. *KCNQ1*, which encompasses 404 kb, is located at chromosome 11p15.5, not far from a candidate region at 11p13–p12 with suggestive evidence of linkage to type 2 diabetes in two independent studies of affected Japanese sibpairs[6,7]. We also reproduced the association of *KCNQ1* with diabetes in Chinese and Korean panels, establishing *KCNQ1* as a diabetes susceptibility gene for populations of East Asian descent. We further showed the association to be significant in individuals of European descent. Given that *KCNQ1* was not implicated as a diabetes susceptibility gene in two recent GWASs with individuals of European descent[8,9], we examined SNPs of *KCNQ1* in the available datasets (**Supplementary Fig. 3** and **Supplementary Table 6a,b** online). Within the LD block of *KCNQ1* that includes the SNPs associated with diabetes in Japanese, 11 SNPs in the WTCCC dataset[8] and 9 SNPs in the DGI dataset[9] had been typed, and none of them had been selected for further analysis. This apparent discrepancy may be due mainly to the allele frequencies of the causative SNPs (the minor allele frequency of rs2237892 was 0.28–0.41 and 0.05–0.07 in populations of East Asian and European descent, respectively). Indeed, in a recent meta-analysis of three GWASs (DGI, WTCCC and FUSION; see URLs section in Methods)[10], the risk alleles of both rs2237892 and rs2074196 identified in the present study were associated with an increased risk of type 2 diabetes ($P = 0.01$ and 0.02,

Table 2 Association study results for SNPs in *KCNQ1* and type 2 diabetes

SNP ID	Risk allele	Panel	RAF(DM)	RAF(NC)	P_{allele}	OR	95% CI		P_{trend}	Meta-analysis OR (95% CI) *P* value
rs2074196	G	2+3 (dense mapping)	0.63	0.55	1.7×10^{-9}	1.39	1.25	1.54	1.8×10^{-9}	
		Replication 1 (Japanese)	0.61	0.54	1.4×10^{-7}	1.32	1.19	1.46	2.1×10^{-7}	
		Replication 2 (Japanese)	0.62	0.55	4.7×10^{-7}	1.31	1.18	1.46	6.2×10^{-7}	
		All Japanese (4,378 cases, 4,412 controls)	**0.62**	**0.55**	**4.6×10^{-21}**	**1.34**	**1.26**	**1.42**	**9.8×10^{-21}**	**1.34 (1.26–1.42), $P = 4.8 \times 10^{-21}$**
		Replication 3 (Chinese)	0.71	0.63	1.2×10^{-9}	1.40	1.26	1.56	9.8×10^{-10}	
		Replication 4 (Korean)	0.66	0.58	3.0×10^{-5}	1.39	1.19	1.62	2.1×10^{-5}	
		All Asian (6,552 cases, 6,621 controls)	**0.64**	**0.57**	**9.9×10^{-32}**	**1.35**	**1.28**	**1.42**	**2.1×10^{-31}**	**1.36 (1.29–1.42), $P = 7.9 \times 10^{-33}$**
		Replication 5 (European)	0.96	0.95	0.017	1.23	1.04	1.46	0.017	
		All	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	**1.35 (1.28–1.41), $P = 8.6 \times 10^{-34}$**
rs2237892	C	2+3 (dense mapping)	0.69	0.60	6.7×10^{-13}	1.49	1.34	1.66	1.7×10^{-12}	
		Replication 1 (Japanese)	0.66	0.59	9.6×10^{-10}	1.39	1.25	1.54	1.6×10^{-9}	
		Replication 2 (Japanese)	0.68	0.60	6.9×10^{-10}	1.41	1.26	1.57	1.1×10^{-9}	
		All Japanese (4,378 cases, 4,412 controls)	**0.68**	**0.59**	**2.8×10^{-29}**	**1.43**	**1.34**	**1.52**	**1.7×10^{-28}**	**1.43 (1.34–1.52), $P = 3.0 \times 10^{-29}$**
		Replication 3 (Chinese)	0.72	0.65	1.3×10^{-8}	1.38	1.24	1.55	4.2×10^{-9}	
		Replication 4 (Korean)	0.69	0.61	1.7×10^{-5}	1.41	1.21	1.65	1.0×10^{-5}	
		All Asian (6,552 cases, 6,621 controls)	**0.69**	**0.61**	**2.0×10^{-39}**	**1.41**	**1.34**	**1.48**	**2.5×10^{-39}**	**1.42 (1.34–1.49), $P = 2.5 \times 10^{-40}$**
		Replication 5 (European)	0.95	0.93	7.8×10^{-4}	1.29	1.11	1.50	7.2×10^{-4}	
		All	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	**1.40 (1.34–1.47), $P = 1.7 \times 10^{-42}$**
rs2237895	C	2+3 (dense mapping)	0.41	0.33	3.1×10^{-11}	1.44	1.30	1.61	4.0×10^{-11}	
		Replication 1 (Japanese)	0.38	0.33	4.5×10^{-5}	1.25	1.12	1.38	4.7×10^{-5}	
		Replication 2 (Japanese)	0.41	0.34	5.8×10^{-8}	1.35	1.21	1.50	5.5×10^{-8}	
		All Japanese (4,378 cases, 4,412 controls)	**0.40**	**0.33**	**1.3×10^{-20}**	**1.34**	**1.26**	**1.43**	**1.7×10^{-20}**	**1.34 (1.26–1.43), $P = 1.4 \times 10^{-20}$**
		Replication 3 (Chinese)	0.40	0.34	3.5×10^{-5}	1.25	1.12	1.39	3.4×10^{-5}	
		Replication 4 (Korean)	0.35	0.30	3.2×10^{-3}	1.27	1.08	1.49	2.7×10^{-3}	
		All Asian (6,552 cases, 6,621 controls)	**0.39**	**0.33**	**2.7×10^{-25}**	**1.31**	**1.24**	**1.38**	**2.7×10^{-25}**	**1.31 (1.25–1.38), $P = 6.1 \times 10^{-26}$**
		Replication 5 (European)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	

RAF(DM) and RAF(NC), risk allele frequencies in cases and controls, respectively. P_{allele} values were calculated for allele data. OR, odds ratio for risk allele. P_{trend} values were calculated by the Cochran-Armitage trend test. Meta-analysis was performed by the Mantel-Haenszel method (fixed-effects models). n.a., not applicable.

respectively). These results provide further support for *KCNQ1* as a general susceptibility gene for diabetes, and they also highlight the need to extend GWAS to different populations.

Alternative splicing has been found to generate several variants of *KCNQ1* mRNA (see Accession codes section in Methods), but we do not know whether the identified candidate SNPs in intron 15 affect the splicing pattern of the primary transcript. Although neighboring genes seem to be located outside the LD block containing rs2237892, we are not able to exclude completely the possibility that the SNPs identified in the present study affect the expression of other causative genes. We did not find any microRNA harboring rs2237892 in the miRBase database.

KCNQ1 encodes the pore-forming subunit of a voltage-gated K^+ channel (KvLQT1) that is essential for the repolarization phase of the action potential in cardiac muscle[11]. Mutations in this gene are associated with cardiac diseases such as hereditary long QT syndrome (Romano-Ward syndrome[12] and Jervell and Lange-Nielsen syndrome[13]) and familial atrial fibrillation[14]. This K^+ channel is also expressed in other tissues, including brain, adipose tissues and pancreas[15,16]. The lower HOMA-β or CIR apparent for CC homozygotes of rs2237892 among Japanese and Europeans in the present study may reflect a functional role for this channel in insulin-producing cells. We examined the abundance of *Kcnq1* mRNA by reverse transcription and real-time PCR analysis in the islets of 12-week-old diabetic KK-Ay mice, which manifested both hyperglycemia and hyperinsulinemia. The amount of the mRNA was significantly increased ($P = 0.0004$) by a factor of 1.6 compared with that in the islets of C57BL6 control mice (data not shown). The KCNQ1 protein was previously shown to be expressed in insulin-secreting INS-1 cells, and the KCNQ1 blocker 293B was found to stimulate insulin secretion in the presence of tolbutamide[17]. It is also possible that fine-tuning of the membrane potential by this channel might modulate the survival of pancreatic β cells in the long term. Further studies are necessary to elucidate the precise mechanism by which the risk allele of *KCNQ1* confers susceptibility to diabetes.

We may have missed a substantial number of susceptibility genes in our screening, given that the strategy we adopted seven years ago lacks sufficient analytical power[18] relative to that now achievable as a result of recent progress in genomic studies. The genomic coverage of the SNP set was not robust, in part because the IMS-JST Japanese SNP (JSNP) database was designed to focus on 'gene-centric' SNPs[2]. Several comprehensive studies based on new platforms for GWASs have recently been described, with about ten genes being found to be

reproducibly associated with type 2 diabetes in individuals of European ancestry[8,9,19–23]. None of these genes showed a positive association in our JGS typing data. Given that some of these genes were recently shown to confer susceptibility to diabetes in Japanese[24–26], the lack of association in our study might be due to the limited sample size of the first scan or to weak LD between the SNPs we used and the causative variants; actually, some genes were totally missed in our JGS (**Supplementary Table 6c**).

In summary, with a comprehensive multistage SNP association study in Japanese, we have identified *KCNQ1* as a previously unreported susceptibility gene as well as several other candidate genes for type 2 diabetes mellitus. Replication studies further confirmed the association of *KCNQ1* with diabetes in individuals of East Asian and European descent. Our findings may provide new insight into the pathophysiology of diabetes as well as a basis for the development of new therapeutic agents.

METHODS

Study participants. We assembled three independent subject panels for multistage genome-wide screening. Panel 1 consisted of 188 cases only, panel 2 of 752 cases and 752 controls and panel 3 of 672 cases and 672 controls. The inclusion criteria for diabetic patients were as follows: (i) age of disease onset of 40 to 55 years, (ii) maximum BMI of <30 kg/m^2, (iii) insulin treatment not initiated until at least three years after diagnosis and (iv) absence of antibodies to glutamic acid decarboxylase. Most Japanese diabetic individuals have a BMI of <30 kg/m^2, and we aimed to focus on the most common subtype of type 2 diabetes in Japan. The criteria for controls in panels 2 and 3 were as follows: (i) age of >60 years, (ii) no past history of diagnosis of diabetes and (iii) hemoglobin A_{1c} content of $<5.6\%$. The cases in the three panels and the controls in panels 2 and 3 were recruited at 11 core facilities located in various regions of Japan. Panels 2 and 3 were assembled simultaneously. Genomic DNA was extracted from peripheral blood by standard methods. We also obtained clinical information such as BMI, blood biochemistry (including plasma glucose and insulin levels) and family history of diabetes. The replication panels are described in **Supplementary Methods**. The clinical characteristics of subjects in each panel are summarized in **Supplementary Table 1**. The study protocol was approved by the local ethics committee of each institution, and written informed consent was obtained from all participants.

Study design. The general design and power for the multistage screening in the Millennium Genome Project (**Supplementary Fig. 1**), referred to as the JSNP Genome Scan (JGS), have been described previously[18]. In the first stage, 188 individuals with each disease (panel 1 for diabetes) were genotyped for 100,000 SNPs in the IMS-JST JSNP database (see URLs section below)[2]. The coverage of the nucleotide sequences of the RefSeq NM exonic regions (as defined by 5′ UTR + CDS (coding sequences) + 3′ UTR) achieved by the JSNP 'gene-centric' genome-wide LD mapping is estimated to be ~35%, if we assume an average extent of LD of 10 kb for each SNP with a minor allele frequency (MAF) of $>15\%$. We also previously evaluated the power of the first two stages of the JGS by a simulation experiment[18]. For example, this analysis would yield a sensitivity of ~13% for SNPs with an odds ratio of 1.5 and a disease-associated genotype frequency of 30%.

One subject did not yield a genotype call for any SNP in the first stage. We then carried out two separate association analyses to compare the 187 diabetic individuals with two different control groups, which we referred to as JDC and ODG, respectively. We did not detect significant population stratification among individuals of the initial panels of the five disease groups by standard methods such as genomic control[27] (inflation factor = 1.06 with 1,025 SNPs selected for genomic control analysis). The genotype-based analysis was done with dominant and recessive models. First, SNPs whose MAF was $>10\%$ in the database and which showed either a genotype OR of >1.5 or an allele OR of >1.3 in either association analysis were selected. If multiple SNPs in the same gene with positive association were in strong LD ($r^2 > 0.9$), only one SNP was chosen for the next step to avoid redundancy. A total of 2,880 SNPs for each disease was then selected for the second screening in order of P value; for diabetes, 2,343 and 1,111 SNPs were selected by the association analyses with ODG and JDC, respectively, with 574 SNPs being selected by both analyses.

In the second stage, an independent case-control panel (panel 2) was analyzed, generating valid data for 2,827 SNPs after a quality check. Thirty-eight SNPs gave no results for all the samples in panel 2, whereas five and three SNPs yielded no data for all case or control samples, respectively, by multiplex PCR-based Invader analysis, and seven probes were not annotated on the updated human genome. The call rate for the 2,827 SNPs was 0.993. A total of 201 positive SNPs ($P < 0.05$) was selected for the third stage of the study on the basis of allelic data (**Supplementary Table 2a**). In the third stage, another case-control panel (panel 3) was typed; one SNP could not be typed by SSP-PCR-FCS analysis (see below) for any of the subjects in panel 3, with the call rate for the other 200 SNPs being 0.990. The ten positive SNPs ($P < 0.05$; **Table 1**) were also then analyzed in the combined panels 2 and 3 (panel 2+3, 1,424 cases and 1,424 controls). Panel 2 was genotyped again for these ten SNPs by SSP-PCR-FCS analysis, and the concordance rate with the Invader method used in the second screening was 0.992. The possibility of stratification in panels 2 and 3 was assessed by typing of 28 diabetes-unrelated SNPs followed by (i) comparison of allele and genotype frequencies by the χ^2 test, (ii) principal component analysis or (iii) STRUCTURE analysis (see URLs section below). None of these analyses showed evidence of stratification among cases and controls of panels 2 and 3 (data not shown).

The list of SNPs used for the initial screening and the allele and genotype frequency data for the first and the second stages of the JGS for the five diseases studied in the Millennium Genome Project of Japan, including diabetes, have been deposited in the Genome Medicine Database of Japan (GeMDBJ, see URLs section below).

Dense SNP mapping for *KCNQ1*. We first selected 49 additional SNPs of *KCNQ1* from the dbSNP database of NCBI, with an average interval of ~10 kbp, and typed these polymorphisms in panel 2+3 together with the three positive SNPs originally included in the JGS. We sequenced 24 control Japanese subjects for the gene, including all the exons and the putative promoter region (4 kbp upstream from the transcription start site), in order to comprehensively identify genetic variants in Japanese. We also sequenced the regions surrounding the positive SNPs of *KCNQ1*, spanning 47 kbp (intron 15). Ten of the SNPs identified in the 47-kbp region were selected on the basis of LD and MAF ($>10\%$). These 10 SNPs and the two identified nonsynonymous variants were genotyped in panel 2+3. A total of 64 SNPs was thus genotyped for *KCNQ1*, including 18 SNPs in the 35.6-kbp region between rs151290 and rs2237895, with an average interval of 2 kbp (see **Supplementary Table 3b**).

Typing methods. In the first and second stages of the study, genotyping was done by the multiplex PCR-based Invader assay (Third Wave Technologies) as previously described[28]. In the third stage and for dense mapping, genome-wide amplified DOP degenerate oligonucleotide–primed (DOP)-PCR templates were genotyped by sequence-specific primer (SSP)-PCR analysis followed by fluorescence correlation spectroscopy (FCS)[29]. Some SNPs included in dense mapping were therefore re-genotyped in panel 2 by the SSP-PCR-FCS method. Some SNPs were genotyped by real-time PCR analysis with TaqMan probes (Applied Biosystems). For replication panels, we applied either SSP-PCR-FCS or the TaqMan method.

Statistical analysis. In the first screening, we performed two case-control evaluations as described above. We examined allele or genotype (dominant or recessive models) data in 2×2 contingency tables for comparison with ODG, as well as allele data in 2×2 contingency tables for comparison with JDC (for which genotype data were not available). In the second and third screening and dense mapping, we analyzed allele data in 2×2 contingency tables by the χ^2 test. LD and haplotype analyses were done with Haploview 3.31 software[30]. A P value of <0.05 was considered statistically significant. For ten positive SNPs in the JGS, rs2237892 and rs2074196, genotype-based analyses were also performed by the Cochran-Armitage trend test. Meta-analysis was done by the Mantel-Haenszel method (fixed-effects models) with the "meta" package of the R Project; the P values for heterogeneity among panels joined in the Mantel-Haenszel tests were all >0.05.

URLs. Genome Medicine Database of Japan, https://gemdbj.nibio.go.jp/dgdb/; DGI, WTCCC and FUSION, http://www.well.ox.ac.uk/DIAGRAM/; miRBase database, http://microrna.sanger.ac.uk/sequences/; IMS-JST JSNP database, http://snp.ims.u-tokyo.ac.jp/; STRUCTURE analysis, http://pritch.bsd.uchicago.edu/software.html.

Accession codes. GenBank: *KCNQ1* mRNA, NM_000218.2 and NM_181798.1.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank all the participants in the project; S. Sugano and S. Tsuji for support and helpful discussion throughout the project; H. Sakamoto, K. Yoshimura and N. Nishida for genotyping and quality control of the data; M. Yamaoka-Sageshima, K. Nagase, D. Suzuki and A. Berglund for technical assistance; and staff of Mitsui Knowledge Industry Inc. (Tokyo) for help with bioinformatics. This work was supported by a grant from the Program for Promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency (PMDA) of Japan; a grant from the National Institute of Biomedical Innovation (NIBIO) of Japan; grants from the Ministry of Health, Labour, and Welfare of Japan; a Grant-in-Aid for Scientific Research on Priority Areas (C), "Medical Genome Science (Millennium Genome Project)," "Applied Genomics" and "Comprehensive Genomics" from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; and a grant from New Energy and Industrial Technology Development Organization (NEDO). The replication 2 study was supported by a grant from Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER, Tokushima, Japan). The Hong Kong diabetes case-control study was supported by the Hong Kong Research Grants Committee Central Allocation Scheme CUHK 1/04C. The Korean case-control study was supported by a grant from the Korea Health 21 R&D Project of the Ministry of Health and Welfare of the Republic of Korea (00-PJ3-PG6-GN07-001 to K.S.P.). The replication 5 study and Botnia prospective study were supported by Swedish Research Council (Linne grant), Sigrid Juselius Foundation, Folkhaelsan Research Foundation, European Foundation for the Study of Diabetes and Swedish Diabetes Research Foundation.

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nature genetics

SNPs in *KCNQ1* are associated with susceptibility to type 2 diabetes in East Asian and European populations

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We conducted a genome-wide association study using 207,097 SNP markers in Japanese individuals with type 2 diabetes and unrelated controls, and identified *KCNQ1* (potassium voltage-gated channel, KQT-like subfamily, member 1) to be a strong candidate for conferring susceptibility to type 2 diabetes. We detected consistent association of a SNP in *KCNQ1* (rs2283228) with the disease in several independent case-control studies (additive model $P = 3.1 \times 10^{-12}$; OR = 1.26, 95% CI = 1.18–1.34). Several other SNPs in the same linkage disequilibrium (LD) block were strongly associated with type 2 diabetes (additive model: rs2237895, $P = 7.3 \times 10^{-9}$; OR = 1.32, 95% CI = 1.20–1.45, rs2237897, $P = 6.8 \times 10^{-13}$; OR = 1.41, 95% CI = 1.29–1.55). The association of these SNPs with type 2 diabetes was replicated in samples from Singaporean (additive model: rs2237895, $P = 8.5 \times 10^{-3}$; OR = 1.14, rs2237897, $P = 2.4 \times 10^{-4}$; OR = 1.22) and Danish populations (additive model: rs2237895, $P = 3.7 \times 10^{-11}$; OR = 1.24, rs2237897, $P = 1.2 \times 10^{-4}$; OR = 1.36).

Type 2 diabetes affects more than 200 million individuals worldwide, and its prevalence is continuously increasing in many countries, including Japan. Although the precise mechanisms underlying the development and progression of type 2 diabetes have not been fully elucidated, a combination of multiple genetic and environmental factors is considered to contribute to the pathogenesis of the disease[1].

Recent development of SNP typing technology and collation of information regarding LD in the human genome have facilitated genome-wide association studies for investigating genes associated with disease susceptibility across the entire human genome. Recently, genome-wide association studies conducted by several independent research groups in Europe and the United States have identified multiple loci associated with susceptibility to type 2 diabetes, including *TCF7L2* (transcription factor 7-like 2), which had been originally identified by a large-scale association mapping prompted by prior evidence of linkage in that area[2], *SLC30A8* (solute carrier family 30 member 8), *HHEX* (haematopoietically expressed homeobox), *CDKAL1* (CDK5 regulatory subunit associated protein 1-like 1), *CDKN2A/B* (cyclin-dependent kinase inhibitor 2A/B) and *IGF2BP2* (insulin-like growth factor 2 mRNA-binding protein 2)[3–7].

Although these are considered to be loci convincingly associated with susceptibility to type 2 diabetes in populations of European descent, other genes related to susceptibility to the disease are probably still unidentified, particularly those for populations of other ancestries. In order to uncover genetic variants that increase the risk of type 2 diabetes, we conducted a genome-wide association study in Japanese individuals with type 2 diabetes and unrelated controls. We first genotyped 268,068 SNPs, which covered approximately 56% of common SNPs in the Japanese, in 194 individuals with type 2 diabetes and diabetic retinopathy (case 1) and in 1,558 controls (control 1) collected in the BioBank Japan. We compared the allele frequencies of 207,097 successfully genotyped SNPs and selected the

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Received 3 March; accepted 10 June; published online 17 August 2008; doi:10.1038/ng.208

Table 1 Association results for variants analyzed in Japanese individuals with type 2 diabetes and control individuals

		Type 2 diabetes		Control			
	Stage	Freq.	11/12/22	Freq.	11/12/22	*P* value	OR (95% CI)
rs13259803	1	0.02	182/9/0	0.06	761/87/8	0.006	0.38 (0.19–0.75)
TRPA1, Chr. 8	2	0.11	1,083/261/21	0.07	1,014/139/10	3.0×10^{-7}	1.70 (1.39–2.08)
(C>T)	3	0.12	2,526/694/33	0.11	1,052/256/22	0.58	1.04 (0.90–1.20)
rs612774	1	0.36	80/86/26	0.31	396/358/77	0.05	1.26 (1.0–1.59)
Chr. 7	2	0.34	607/588/163	0.27	458/323/66	3.1×10^{-6}	1.38 (1.21–1.58)
(T>G)	3	0.31	1,526/1,398/317	0.32	620/570/138	0.64	0.98 (0.89–1.08)
rs4712524	1	0.46	67/77/50	0.42	293/413/152	0.18	1.17 (0.94–1.46)
CDKAL1, Chr. 6	2	0.47	384/655/313	0.41	444/601/218	5.7×10^{-6}	1.29 (1.16–1.44)
(A>**G**)	3	0.46	980/1,525/744	0.42	439/653/228	0.0002	1.19 (1.09–1.31)
	Combined	0.47	1,431/2,257/1,107	0.42	1,176/1,667/598	3.3×10^{-10}	1.22 (1.15–1.31)
rs9295475	1	0.45	65/81/47	0.41	301/409/148	0.13	1.19 (0.95–1.49)
CDKAL1, Chr. 6	2	0.47	396/648/302	0.40	451/604/209	1.2×10^{-5}	1.28 (1.15–1.43)
(A>**G**)	3	0.46	995/1,527/728	0.41	451/644/225	0.0001	1.20 (1.09–1.31)
	Combined	0.46	1,456/1,986/1,077	0.41	1,203/1,657/582	4.1×10^{-9}	1.22 (1.14–1.30)
rs6769511	1	0.40	67/80/32	0.32	404/344/98	0.003	1.44 (1.14–1.82)
IGF2BP2, Chr. 3	2	0.38	533/619/204	0.32	528/535/110	2.4×10^{-5}	1.28 (1.14–1.44)
(T>**C**)	3	0.36	1,332/1,504/423	0.32	621/566/145	0.0004	1.19 (1.08–1.31)
	Combined	0.37	1,932/2,203/659	0.32	1,553/1,445/353	1.3×10^{-9}	1.23 (1.15–1.31)
rs9460546	1	0.46	67/77/50	0.42	293/414/152	0.18	1.17 (0.94–1.46)
CDKAL1, Chr. 6	2	0.47	391/650/307	0.41	444/604/218	3.0×10^{-5}	1.27 (1.13–1.41)
(T>**G**)	3	0.47	963/1,526/744	0.42	437/652/228	0.0001	1.20 (1.10–1.32)
	Combined	0.47	1,421/2,253/1,101	0.42	1,174/1,670/598	3.4×10^{-10}	1.23 (1.15–1.30)
rs4376068	1	0.37	82/81/30	0.29	440/343/76	0.003	1.42 (1.13–1.79)
IGF2BP2, Chr. 3	2	0.35	591/607/168	0.29	584/498/93	3.8×10^{-5}	1.28 (1.14–1.45)
(A>**C**)	3	0.33	1,466/1,417/371	0.30	658/552/117	0.001	1.18 (1.07–1.30)
	Combined	0.34	2,139/2,108/569	0.29	1,682/1,393/286	2.1×10^{-9}	1.23 (1.15–1.32)
rs2283228	1	0.65	80/84/24	0.58	266/436/134	0.01	1.34 (1.06–1.70)
KCNQ1, Chr. 11	2	0.64	557/638/167	0.59	370/547/178	5.7×10^{-5}	1.26 (1.13–1.42)
(**A**>C)	3	0.64	1,379/1,592/449	0.59	426/590/217	7.0×10^{-6}	1.24 (1.13–1.36)
	Combined	0.64	2,016/2,314/640	0.58	1,062/1573/529	3.1×10^{-12}	1.26 (1.18–1.34)
rs10836097	1	0.11	147/33/4	0.07	544/76/5	0.01	1.70 (1.15–2.51)
C11ORF41, Chr. 11	2	0.09	1,084/222/9	0.06	792/105/0	5.3×10^{-5}	1.62 (1.27–2.05)
(A>G)	3	0.09	2,658/534/37	0.09	1,041/213/11	0.86	1.01 (0.87–1.19)

The results include data from stage 1, stage 2 and replication study (3) for 9 SNPs. Risk allele frequency (Freq.) and genotype counts in individuals with type 2 diabetes and control subjects are shown. 11, homozygous of major allele; 12, heterozygous; 22, homozygous of minor allele. *P* values are for the additive model. Risk allele is denoted in boldface.

8,323 SNPs showing the lowest *P* values. We then attempted to genotype these 8,323 SNPs in 1,367 individuals with type 2 diabetes and diabetic retinopathy (case 2) and for 1,266 controls (control 2) (stage 2), and successfully obtained data for 6,731 SNPs (the *P* value distribution in the second test is shown in **Supplementary Fig. 1a** online). The results of principal component analysis[8] in the stage 1 and 2 samples and HapMap samples revealed that there was no evidence for population stratification between the case and control groups throughout the present tests (**Supplementary Fig. 1b,c**). We selected the 9 SNP loci showing *P* values <0.0001 (additive model in stage 2, **Table 1**) and genotyped a third set of cases and controls comprising 3,557 Japanese individuals with type 2 diabetes (cases 3,4,5) and 1,352 controls (controls 3,4). We evaluated the differences in the population structure among these three sets of case and two sets of control groups by Wright's *F* test. As the results indicated that there was no difference in the population structure among these groups (**Supplementary Table 1b** online), we combined these populations for the third test of case-control study. The third set of analysis identified the significant associations for six SNPs (**Table 1**), including the *CDKAL1* locus at 6p22.3 (rs4712524, rs9295475 and rs9460546), the *IGF2BP2* locus at 3q27.2 (rs6769511 and rs4376068) and the *KCNQ1* locus at 11p15.5 (rs2283228). The remaining three SNPs (rs13259803, rs612774 and rs10836097) had *P* values of >0.05 in the third test and were not further examined. *CDKAL1* and *IGF2BP2* were previously reported as susceptibility genes for type 2 diabetes in the Japanese population[9]. Therefore, we focused on the *KCNQ1* locus, which was highly associated with type 2 diabetes.

Subsequently, we carried out LD mapping using 572 SNPs in the neighboring region of SNP rs2283228 and found that the LD block including this SNP extended approximately 40 kb (30 kb upstream and 10 kb downstream of this SNP; **Fig. 1a,b** and **Supplementary Fig. 2a,b** online). Therefore, we considered the critical region for susceptibility to type 2 diabetes to be present within this 40-kb LD block that corresponds to a part of intron 15 of *KCNQ1*. To further confirm the association, we genotyped the third set of 3,557 cases and 1,352 controls for the 32 additional SNPs within the block and found several SNPs to be significantly associated with type 2 diabetes in these populations ($P < 0.0001$, **Fig. 1c** and **Supplementary Table 2a,b** online). Among them, six SNPs showed stronger associations with type 2 diabetes than rs2283228 (rs2237897, $P = 6.8 \times 10^{-13}$; rs2237896, $P = 3.9 \times 10^{-12}$; rs2299620, $P = 8.3 \times 10^{-11}$; rs2237895, $P = 7.3 \times 10^{-9}$; rs2237892, $P = 1.3 \times 10^{-7}$; rs163171, $P = 4.8 \times 10^{-7}$;

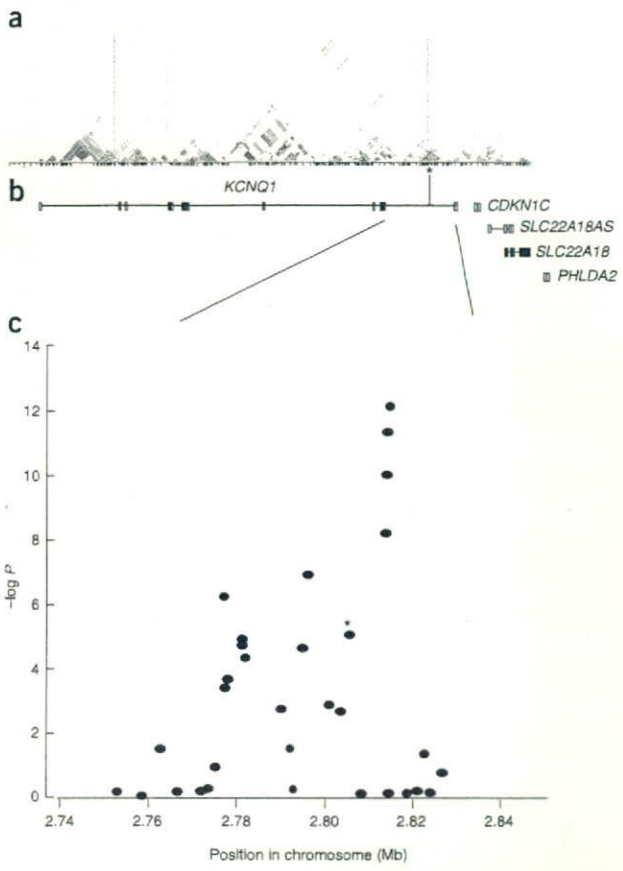

Figure 1 Schematic view of the association of type 2 diabetes with the variants in the *KCNQ1* region. (**a**) Pairwise correlation structure at a 0.5-Mb interval (2.4–2.9 Mb on chromosome 11, NCBI Build 36.2). The plot includes pairwise r^2 values from the HapMap release 22 for the JPT population. (**b**) Exon-intron structure of *KCNQ1* (exons shown as vertical bars). The asterisk indicates the SNP rs2283228 at intron 15. (**c**) Results of case-control association study for type 2 diabetes in 3,557 Japanese individuals with type 2 diabetes and 1,352 control individuals. The $\log_{10}$-transformed *P* values for an additive model are plotted on the *y* axis.

Fig. 1 and **Supplementary Table 2a**). The genotype distributions of these SNPs in the control subjects were within the Hardy-Weinberg equilibrium (HWE) ($P \geq 0.01$, **Supplementary Table 2b**). With 207,097 tests performed in the initial genome-wide association study, these associations, except that for rs163171, remained significant after Bonferroni adjustment[10]. We also found a significant interactive effect between rs2237897 and rs234844 by means of stepwise logistic regression analysis[11] (effect of addition; $P = 0.0015$, **Supplementary Table 3** online).

To further validate the association of the *KCNQ1* variants with type 2 diabetes, we examined the association of the above-mentioned SNPs with type 2 diabetes in populations of different ancestries. As shown in **Table 2**, rs2237895 and rs2237897 are significantly associated with the disease in the Singaporean population of East Asian descent and the Danish population of European descent (**Table 2** and **Supplementary Table 4** online), although there are significant differences in the allele frequencies within this locus between the East Asian and Danish populations (**Supplementary Table 5a** and **Supplementary Fig. 3a–d** online). A combined analysis of these three populations by using a meta-analysis after testing for heterogeneity gave *P* values of less than 1×10^{-16} for both rs2237895 and rs2237897 (**Supplementary Table 5b**).

To elucidate the possible involvement of *KCNQ1* in the pathogenesis of type 2 diabetes, we further examined the expression profile of the *KCNQ1* gene. Using reverse transcription polymerase chain reaction (RT-PCR), we found expression of *KCNQ1* in the human heart and pancreas and, to a lesser extent, in the placenta, lung, liver and kidney (**Supplementary Fig. 4** online). In addition, we also detected the expression of *KCNQ1* in a cultured insulin-secreting cell line, RIN-5F (**Supplementary Fig. 4**).

KCNQ1, located on 11p15.5, encodes the pore-forming α subunit of the $I_{Ks}K^+$ channel, which is expressed mainly in the heart and, to a lesser extent, in the inner ear, stomach, intestine, liver and kidney[12]. The $I_{Ks}K^+$ channel enables a K^+ current after electrical depolarization of the cell membrane in the heart[13,14]. Moreover, mutations in *KCNQ1* have been reported to cause long QT syndrome through the loss of function of the slowly activating K^+ channel in the heart. *KCNQ1* is also a known causative gene for Jervell and Lange-Nielsen syndrome, which is an autosomal recessive trait characterized by hearing loss and cardiac conduction abnormalities[13]. *KCNQ1*-knockout mice have been reported to show cardiac dysfunctions such as prolonged QT interval, hypochlorhydria, hypergastrinaemia, gastric hyperplasia and vacuolation of the parietal cells; the latter four effects suggest that *KCNQ1* also plays some roles in normal gastric acid secretion[12,15]. In the kidney, *KCNQ1* has been shown to enhance the Na^+-dependent transport in the proximal tubule, presumably by contributing to a favorable electrical gradient for Na^+ uptake[16]. *KCNQ1* is also expressed in the pancreas, including the pancreatic islets; however, the contribution of the *KCNQ1*-encoded protein to the molecular pathogenesis of type 2 diabetes remains to be elucidated. Subjects with a loss-of-function mutation in the gene or homozygous *KCNQ1* null mice have not been reported to show hyperglycaemia or glucose intolerance. Hence, we would expect that increased *KCNQ1* expression in the pancreatic β cells is probably responsible for the development of type 2 diabetes. However, a possibility is still present that a gene near *KCNQ1*, particularly *CDKN1C*, might be a true causal gene (**Fig. 1b** and **Supplementary Table 6** online); further studies are necessary to elucidate the underlying molecular mechanism of the gene and gene polymorphisms for conferring susceptibility to type 2 diabetes.

Our present study has missed most of susceptibility loci previously identified in populations with European descent, except for the *CDKAL1* and *IGF2BP2* loci. As we showed that previously identified loci associated with type 2 diabetes, such as *TCF7L2, CDKN2A/B, HHEX* and *KCNJ11*, were successfully replicated in our populations[9], a lack of study power to detect true association in the present stage 1 study seems to be responsible for the discrepancy between the present results and the results in the genome-wide association studies of European-descent populations. However, it is notable that the *KCNQ1* locus was missed in previous genome-wide association studies conducted by groups in Europe and the United States, as those studies are considered to be superior to our study in terms of study power or coverage rate of the human genome. This can probably be explained by the fact that ancestry-related differences were present in the allele frequencies or in the pattern of LD, and that uncovered regions still existed. Therefore, this emphasizes that studies extending genome-wide association analysis beyond the focus of the first rounds of studies, including a genome-wide association meta-analysis recently reported as well as the present study, are needed to search for additional genomic loci.

Table 2 Association results for rs2237897, rs2237895 and rs2283228 in three case-control groups from Japan, Singapore and Denmark

		Type 2 diabetes		Control			
	Population	Freq.	11/12/22	Freq.	11/12/22	*P*	OR (95% CI)
rs2237897							
(**C**>T)	Japanese	0.68	1,615/1,574/333	0.61	476/646/198	6.8×10^{-13}	1.41 (1.29–1.55)
	Singaporean	0.69	680/621/132	0.65	749/747/239	2.4×10^{-4}	1.22 (1.09–1.35)
	Dane	0.97	3,643/245/3	0.96	4,473/405/10	1.2×10^{-4}	1.36 (1.16–1.60)
rs2237895							
(A>**C**)	Japanese	0.41	1,225/1,633/577	0.34	567/580/152	7.3×10^{-9}	1.32 (1.20–1.45)
	Singaporean	0.38	574/709/214	0.35	801/848/232	8.5×10^{-3}	1.14 (1.03–1.26)
	Dane	0.45	1,082/1,854/750	0.40	1,578/2,203/714	3.7×10^{-11}	1.24 (1.16–1.32)
rs2283228							
(**A**>C)	Japanese	0.64	1,379/1,592/449	0.59	426/590/217	7.0×10^{-6}	1.24 (1.13–1.36)
	Singaporean	0.64	612/667/194	0.63	692/812/243	0.27	1.06 (0.96–1.17)
	Dane	0.94	3,413/469/15	0.92	4,154/706/29	3.1×10^{-4}	1.24 (1.10–1.39)

Risk allele frequency (Freq.) and genotype counts in individuals with type 2 diabetes and control subjects. 11, homozygous of major allele; 12, heterozygous; 22, homozygous of minor allele. *P* values for additive model. Risk allele is denoted in boldface.

In conclusion, we have replicated the association of *CDKAL1* and *IGF2BP2* with type 2 diabetes in a genome-wide association study. In addition, we have identified a previously unreported locus associated with type 2 diabetes. SNPs in the *KCNQ1* gene were significantly associated with type 2 diabetes in populations of both East Asian and European descent.

METHODS

Study participants. BioBank Japan: For the genome-wide association study, we selected case-control samples (cases 1 and 2, controls 1 and 2) from the subjects enrolled in the BioBank Japan. The subjects were recruited from several medical institutes in Japan, including Fukujuji Hospital, Iizuka Hospital, Iwate Medical University School of Medicine, Juntendo University, National Hospital Organization Osaka National Hospital, Nihon University, Nippon Medical School, Osaka Medical Center for Cancer and Cardiovascular Diseases, Shiga University of Medical Science, The Cancer Institute Hospital of Japanese Foundation for Cancer Research, Tokushukai Hospitals and Tokyo Metropolitan Geriatric Hospital. We selected type 2 diabetes cases from individuals registered as having type 2 diabetes and diabetic retinopathy (case 1, $n = 194$; case 2, $n = 1{,}367$, **Supplementary Table 1a**). Control groups comprised 1,558 (control 1) and 1,266 individuals (control 2) registered as individuals with diseases other than type 2 diabetes, including bronchial asthma, myocardial infarction, breast cancer, Basedow's disease, cerebral infarction, cerebral aneurism, osteoporosis, heart failure, unstable angina, pollinosis, arteriosclerosis obliterance, emphysema, atopic dermatitis, stomach cancer or liver cirrhosis.

RIKEN case-control study: DNA samples were obtained from peripheral blood samples of 1,630 individuals with type 2 diabetes recruited from the outpatient clinic of the Shiga University of Medical Science, Kawasaki Medical School (case 3: 978 men and 652 women; age, 61.5 ± 11.6 years; duration of diabetes, 10.9 ± 9.3 years; HbA1c, 7.4 ± 1.6%; fasting plasma glucose (FPG), 9.1 ± 3.5 mmol/l; and BMI, 23.7 ± 3.9 kg/m^2 (all values are expressed as mean ± s.d.), **Supplementary Table 1a**). Diabetes was diagnosed according to the World Health Organization (WHO) criteria. Type 2 diabetes is clinically defined as disease with a gradual adult onset. Subjects who tested positive for antibodies to glutamic acid decarboxylase (GAD) and those diagnosed with a mitochondrial disease (mitochondrial myopathy, encephalopathy, lactic acidosis or stroke-like episodes (MELAS)) or maturity-onset diabetes of young (MODY) were not included in the case group. Among the 1,630 subjects, 31 had already been enrolled in the Japanese Millennium Project for the study of type 2 diabetes and were therefore excluded from the present analysis. We also examined 1,064 control subjects who were enrolled from an annual health check-up conducted at either the Juntendo University or Keio University (control 3: 638 men and 426 women; age, 45.5 ± 9.5 years; HbA1c, 4.7 ± 0.4%; fasting plasma glucose, 5.1 ± 0.5 mmol/l; and BMI, 22.9 ± 3.0 kg/m^2 (all values are expressed as mean ± s.d.), **Supplementary Table 1a**). We further examined 1,304 independent subjects with type 2 diabetes and diabetic retinopathy who regularly attended outpatient clinics at the Shiga University of Medical Science, Tokyo Women's Medical University, Juntendo University, Kawasaki Medical School, Iwate Medical University, Toride Kyodo Hospital, Kawai Clinic, Osaka City General Hospital, Chiba Tokusyukai Hospital and Osaka Rosai Hospital (case 4: 774 men and 530 women; age, 61.1 ± 11.2 years; duration of diabetes, 17.4 ± 9.6 years; HbA1c, 7.6 ± 2.8%; fasting plasma glucose, 8.1 ± 2.8 mmol/l; and BMI, 23.7 ± 3.6 kg/m^2 (all values are expressed as mean ± s.d.), **Supplementary Table 1a**).

The University of Tokyo study: In this study, we enrolled 654 subjects with type 2 diabetes who visited the outpatient clinic of the Department of Metabolic Diseases, Graduate School of Medicine, The University of Tokyo (Tokyo, Japan) or the outpatient clinic of the Hiroshima Atomic Bomb Casualty Council Health Management Center (Hiroshima, Japan) (case 5: 392 men and 262 women; age, 67.2 ± 8.4 years; duration of diabetes, 13.9 ± 10.2 years; HbA1c, 7.5 ± 3.6%; FPG, 9.5 ± 3.0 mmol/l; and BMI, 23.9 ± 3.3 kg/m^2 (all values are expressed as mean ± s.d.), **Supplementary Table 1a**). Exclusion criteria for cases were individuals positive for antibody to GAD or those with diabetes resulting from (i) liver dysfunction, (ii) steroids and other drugs that might raise glucose levels, (iii) malignancy or (iv) monogenic disorder known to cause diabetes. Control subjects were enrolled from an annual health check-up conducted at the Hiroshima Atomic Bomb Casualty Council Health Management Center (control 4: 112 men and 176 women; age, 71.6 ± 6.2 years; HbA1c, 5.2 ± 0.2%; FPG, 5.0 ± 1.1 mmol/l; and BMI 22.6 ± 3.2 kg/m^2 (all values are expressed as mean ± s.d.), **Supplementary Table 1a**). All subjects were not enrolled in the Japanese Millennium Project for the study of type 2 diabetes.

Singapore study: We selected Chinese individuals with type 2 diabetes from the Singapore Diabetes Cohort Study (SDCS). All individuals diagnosed with type 2 diabetes at primary care facilities of the National Healthcare Group Polyclinics (NHGPs) in Singapore had been invited to participate in the SDCS since 2004. Of the individuals approached, 91% agreed to participate in the study and formed a part of our SDCS case group. At the time of this genetic study, DNA samples from 1,498 Chinese individuals were available for analysis. The research protocol for SDCS was approved by both the National University of Singapore Institutional Review Board (NUS-012) and the National Healthcare Group Domain-Specific Review Board (C/05/118).

Nondiabetic controls were identified on the basis of the results of oral glucose tolerance test (OGTT) performed on the subjects belonging to the 1998 Singapore National Health Survey (NHS98), as detailed elsewhere[17]. The survey

protocol was based on the WHO-recommended model for field surveys of diabetes and other noncommunicable diseases and the WHO MONICA protocol for population surveys. A total of 1,881 nondiabetic Chinese individuals with 2-h OGTT values of <7.8 mmol/l were selected as controls. The research protocol for NHS98 was approved by the Singapore General Hospital Institutional Review Board (#54/2001).

Danish study: The Danish study population comprised the population-based Inter99 sample of middle-aged people sampled at Research Centre for Prevention and Health[18], individuals with type 2 diabetes sampled through the outpatient clinic at Steno Diabetes Center, a population-based group of middle-aged glucose-tolerant subjects recruited from Steno Diabetes Center, and the ADDITION study group sampled through Department of General Practice at University of Aarhus[19]. Detailed characteristics of study populations have been previously described[20]. In total, 4,085 individuals with type 2 diabetes and 5,302 glucose-tolerant control subjects were genotyped for the rs2283228, rs2237895 and rs2237897 polymorphisms using Taqman allelic discrimination (KBioscience). Discordance between 966 random duplicate samples was <0.5% and the genotyping success rate was >96%. All genotype groups obeyed Hardy-Weinberg equilibrium (all $P > 0.4$).

All the participants provided written informed consent. The protocol was approved by the ethics committee of the Institute of Physical and Chemical Research (RIKEN) and of each participating institution.

SNP genotyping. Using the standard protocols, we extracted the genomic DNA from the peripheral blood leukocytes. In the first stage, we genotyped 268,068 SNPs from autosomal chromosomes; these SNPs were selected as the tagging SNPs for the Japanese population from the JSNP[21] or HapMap database[22] by using high-density oligonucleotide arrays (Perlegen Sciences) as described previously. SNPs having call rate >90% and no extreme departure from HWE ($P \geq 10^{-6}$) were passed and used for the association study. In the second stage, genotyping was conducted using Affymetrix GeneChip SNP arrays. We carried out genotyping for the replication study using the multiplex-PCR invader assay[23] or the TaqMan assay, as described previously. The success rates of the multiplex-PCR invader assay and the TaqMan assay were >95%, and >99% agreement existed between the results of genotyping and direct sequencing.

Statistical analysis. Statistical methods for determining the associations and calculating the LD coefficients (r^2) have been described previously[24]. We performed HWE test according to a method previously described[25]. The cut-off value for the HWE test in the control groups was 0.000001 for the first stage and 0.01 for the second stage; the SNPs with P values less than the cut-off values of the HWE test were excluded from the analysis. We analyzed the differences between the case and control groups with regard to the genotype distribution or allele frequency in the genome-wide screening (first or second stage) by Fisher's exact test using dominant, recessive and allelic models with autosomal SNPs. The association of the candidate loci with type 2 diabetes in the replication study was evaluated by Armitage test for trend using an additive model, as described previously[3]. We applied stepwise logistic regression analysis to investigate the combinatorial effects of multiple SNPs[11].

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank all participating doctors and staff from collaborating institutes for providing DNA samples. We also thank the technical staff of the Laboratory for Endocrinology and Metabolism at the Center for Genomic Medicine for providing technical assistance. This work was partly supported by a grant from the Leading Project of Ministry of Education, Culture, Sports, Science and Technology Japan. The Danish study was supported by Lundbeck Foundation Centre of Applied Medical Genomics in Personalized Disease Prediction, Prevention and Care (LUCAMP), the European Union (EUGENE2, grant no. LSHM-CT-2004-512013) and the Danish Medical Research Council. Funding for Singapore arm of the study was supported by the National Medical Research Council (NMRC/1115/2007). We thank the staff of National Healthcare Group Polyclinics and National University of Singapore for their contributions towards the establishment of the Singapore Diabetes Cohort Study.

AUTHOR CONTRIBUTIONS

Principal investigators: S.M., H.U., Y.N. BioBank project planning and design: Y.N. Collected subjects and participated in the diagnostic evaluations: K.H., M. Horikoshi, T.B., H.H., M. Hayashi, Y.I., A.K., K.K., R. Kawamori, T. Kadowaki, R. Kikkawa. Oversaw a genotyping: M.K. Wrote the paper: S.M., H.U., Y.N., O.P. Statistical analyses: A.T., T. Kawaguchi, T.T., N.K. Principal investigator for Singapore study: D.P.K.N. Singapore study: S.N., E.S.T. Principal investigator for Danish study: O.P. Danish study: G.A., J.H., K.B.-J., T.J., A.S., T.L., T.H.

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Experimental Studies

Genetic Variations of Mrf-2/Arid5b Confer Risk of Coronary Atherosclerosis in the Japanese Population

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SUMMARY

A phenotypic change of smooth muscle cells (SMCs) is considered to be critical in the pathogenesis of atherosclerotic lesions such as coronary artery disease (CAD). Mrf-2/ARID5B, a member of the AT-rich interaction domain family of transcription factors, is highly expressed in the cardiovascular system and is believed to play essential roles in the phenotypic change of SMCs through its regulation of SMC differentiation. In addition, recent studies on gene-engineered mice suggested that this transcriptional factor is involved in obesity and adipogenesis, which are critical aspects for the pathogenesis of atherosclerosis. Thus, we hypothesized that genetic variations of the Mrf-2 gene might be associated with susceptibility to CAD.

We investigated 11 common genetic variations of Mrf-2 to determine whether they were associated with susceptibility to CAD in 475 CAD subjects and 310 control subjects. The prevalence of homozygotes for the minor allele G of SNP4 (rs2893880) and minor allele G of SNP6 (rs7087507) were significantly more frequent in the control subjects than in patients with CAD ($P = 0.0002$, rs2893880, $P = 0.0058$, rs7087507). Four nearby SNPs (SNP4 to SNP7) (rs2893880, rs10740055, rs7087507 and rs10761600) showed almost complete linkage disequilibrium, and haplotype analysis revealed that the haplotype G (rs2893880)-C (rs10740055)-G (rs7087507)-A (rs10761600) was also significantly negatively associated with susceptibility to CAD ($P = 0.049$). Moreover, these negative disease associations still existed after logistic regression analysis was taken into account to eliminate confounding conventional coronary risk factors.

The results implicate possible disease relevance of the polymorphisms in the Mrf-2 gene with susceptibility to CAD. However, a larger scale prospective study is needed to clarify these findings. (Int Heart J 2008; 49: 313-327)

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This study was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and a research grant from the National Institute of Biomedical Innovation, Japan. The study was also supported in part by Special Coordination Funds for Promoting Science and Technology by the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

Received for publication March 31, 2008.

Revised and accepted May 1, 2008.

Key words: Coronary artery disease, Atherosclerosis, Mrf-2, ARID5B, Polymorphism, Genetic risk factors

CORONARY artery disease (CAD) is a multifactorial disease and is the leading cause of mortality in industrialized countries. A multitude of environmental influences and genetic factors contribute to the development and outcome of CAD.[1] Single nucleotide polymorphisms (SNPs) are the most common genetic variations in the genome and some are reported to influence susceptibility to atherosclerosis, plaque destabilization, and thrombosis, all of which are fundamental aspects of CAD.[2,3]

Vascular smooth muscle is a critical cellular constituent of the blood vessel wall. Phenotypic changes in smooth muscle cells (SMCs) are considered to be critical in the pathogenesis of atherosclerotic lesions such as coronary artery disease (CAD). Our group previously investigated the mechanism of a phenotypic change by establishing an in vitro system in which a pluripotent neural crest cell line (MONC-1) can be induced to differentiate into SMCs. By using a differential mRNA display, we isolated a partial cDNA fragment of Mrf-2 (modulator recognition factor) as one of the genes robustly induced during the differentiation progress. Our group was the first to clone the full length of Mrf-2 and reveal the existence of at least two isoforms (α and β) which differ in the N-terminus but share a common DNA-binding domain.[4]

Mrf-2, which is also called as ARID5B or Desrt, is a member of the AT-rich interaction domain (ARID) family of transcription factors. The ARID defines a highly conserved sequence specific DNA binding domain and has been identified in proteins of yeast, Drosophila, and mammals.[5-7] ARID-encoding genes are involved in a variety of biological processes including embryonic development, tissue specific gene expression, proliferation control, and chromatin remodeling.[7-12]

We reported Mrf-2 as a nuclear protein, and its mRNA was highly expressed in the aorta, heart and lung.[4] We also demonstrated that Mrf-2 might also bind to the CArG box, a cis-acting element, which was reported to regulate the expression of many smooth muscle cell specific genes, including smooth muscle cell α-actin and SM 22α. In addition, overexpression of Mrf-2 in 3T3 fibroblast cells induced smooth muscle marker genes and retarded cell proliferation. These results suggested that Mrf-2 might be a potent key regulator for SMC phenotypic change via its regulation of SMC differentiation.

On the other hand, it was reported that targeted disruption of the Mrf-2 gene in mice caused growth retardation and significant reductions in lipid accumulation and weight gain in postnatal and adult life.[8,13,14] These observations sug-