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A single-nucleotide polymorphism in *ANK1* is associated with susceptibility to type 2 diabetes in Japanese populations

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To identify a novel susceptibility locus for type 2 diabetes, we performed an imputation-based, genome-wide association study (GWAS) in a Japanese population using newly obtained imputed-genotype data for 2 229 890 single-nucleotide polymorphisms (SNPs) estimated from previously reported, directly genotyped GWAS data in the same samples (stage 1: 4470 type 2 diabetes versus 3071 controls). We directly genotyped 43 new SNPs with *P*-values of $<10^{-4}$ in a part of stage-1 samples (2692 type 2 diabetes versus 3071 controls), and the associations of validated SNPs were evaluated in another 11 139 Japanese individuals (stage 2: 7605

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type 2 diabetes versus 3534 controls). Combined meta-analysis using directly genotyped data for stages 1 and 2 revealed that rs515071 in *ANK1* and rs7656416 near *MGC21675* were associated with type 2 diabetes in the Japanese population at the genome-wide significant level ($P < 5 \times 10^{-8}$). The association of rs515071 was also observed in European GWAS data (combined P for all populations = 6.14×10^{-10}). Rs7656416 was in linkage disequilibrium to rs6815464, which had recently been identified as a top signal in a meta-analysis of East Asian GWAS for type 2 diabetes ($r^2 = 0.76$ in stage 2). The association of rs7656416 with type 2 diabetes disappeared after conditioning on rs6815464. These results indicate that the *ANK1* locus is a new, common susceptibility locus for type 2 diabetes across different ethnic groups. The signal of association was weaker in the directly genotyped data, so the improvement in signal indicates the importance of imputation in this particular case.

INTRODUCTION

Genome-wide association studies (GWAS) for type 2 diabetes have been conducted extensively and have successfully identified over 40 susceptibility loci, mostly in European populations (1,2). The first round of GWAS for type 2 diabetes reported in 2007 confirmed five new loci—*HHEX*, *SLC30A8*, *CDKAL1*, *CDKN2A-CDKN2B* and *IGF2BP2* (3–7)—in addition to three previously reported loci—*TCF7L2* (8), *PPARG* (9) and *KCNJ11* (10). The Wellcome Trust Case Control Consortium/United Kingdom Type 2 Diabetes Genetics Consortium (WTCCC/UKT2D) study also identified a strong association between *FTO* variants and type 2 diabetes, although the effect of the *FTO* variants was mostly mediated through an increased body weight (11). After the first round of European GWAS, additional studies combined individual GWAS data to increase the sample size and make common variants with lower effect sizes detectable. These studies have so far identified more than 30 additional susceptibility loci for type 2 diabetes (12–14). Additionally, some of them have been shown to confer similar susceptibility to type 2 diabetes in non-European populations (15–19). However, the integration of all this information can explain only ~10% of type 2 diabetes heritability (1,2,13), suggesting that most of the genetic factors for the condition remain to be identified, especially in non-European populations. Cumulative evidence suggests that Asians may be more genetically susceptible to type 2 diabetes than populations of European ancestry (20). Also, there are significant interethnic differences in the risk allele frequency or in effect sizes at several loci, which may affect the power to detect the associations in these populations (2,20). Therefore, it is considered to be relevant to perform GWAS for type 2 diabetes using non-European populations as well as European populations to uncover the missing heritability of type 2 diabetes.

In 2008, two Japanese GWAS simultaneously identified the *KCNQ1* locus as a strong susceptibility locus for type 2 diabetes (21,22). Recently, we performed a larger scale Japanese GWAS, identifying the additional loci *UBE2E2* and *C2CD4A-C2CD4B* (23). The associations of *KCNQ1* and *C2CD4A-C2CD4B* with type 2 diabetes were consistently observed among European populations, underlining the importance of examining non-European populations through GWAS. This will help us to identify not only ethnicity-specific loci, but also common-susceptibility loci among different ethnic groups.

Here, we show the results of an imputation-based GWAS as an extended analysis of our previous Japanese GWAS for 459 359 directly genotyped single-nucleotide polymorphisms (SNPs). We obtained imputed-genotype data for 2 229 890 SNPs estimated from 459 359 directly genotyped SNPs in our previous report (23). The analysis using over 2 million of newly obtained imputed SNPs data and subsequent *in silico* replication study in European GWAS data provide the evidence that the *ANK1* locus is a novel common-susceptibility locus for type 2 diabetes across different ethnic groups.

RESULTS

We successfully obtained the new information of 2 229 890 imputed SNPs with a quality score (proper_info) of >0.40, minor allele frequency (MAF) of >0.01, and Hardy–Weinberg equilibrium (HWE) P-value of $>1 \times 10^{-6}$ by using IMPUTE with previously reported GWAS data (459 359 directly genotyped SNPs) (stage 1) (23) and from 89 HapMap samples (44 JPT and 45 CHB in HapMap phase 2).

Among 2 229 890 imputed SNPs, we found that the *KCNQ1* locus appeared as the top signal, although the association did not attain genome-wide significance levels (rs2237896; $P = 5.9 \times 10^{-8}$, rs2283228; $P = 8.7 \times 10^{-8}$). We did not observe evidence for population stratification in stage-1 samples [Supplementary Material, Fig. S1, genomic inflation score (λ_{GC}) = 1.07347]. We also identified 330 SNPs with P-values between 1×10^{-7} and 1×10^{-4} that were derived from 70 distinct loci, including 7 already confirmed loci and 27 loci evaluated in the prior analysis (23; Supplementary Material, Table S1). Two hundred and sixty-six SNPs within these 34 (7 + 27) loci and 23 proxies ($r^2 > 0.8$) out of the remaining 66 SNPs in 36 loci were excluded from further analysis in order to focus on identifying novel T2D susceptible loci. Therefore, we selected 43 SNPs within the 36 loci and directly genotyped these SNPs using a part of stage-1 samples (2692 type 2 diabetes versus 3071 controls); the remaining 1778 type 2 diabetes samples were not available for the direct genotyping. In this analysis, we successfully obtained information for 40 SNPs. Among them, 10 were excluded from stage-2 analysis because an association study using directly genotyped data showed that they were not associated with type 2 diabetes ($P \geq 0.01$, Supplementary Material, Table S2).

In stage-2 analysis (7605 type 2 diabetes versus 3534 controls), four SNPs were associated with type 2 diabetes ($P < 0.01$), although none of the SNPs showed an association with a genome-wide significance level.

Next, we performed the combined meta-analysis by using directly genotyped data for stages 1 and 2 using the Mantel–Haenszel procedure. In this combined analysis, we found that two SNPs—rs515071 in *ANK1* and rs7656416 near *MGC21675*—were significantly associated with type 2 diabetes in the Japanese population [rs515071: $P = 1.37 \times 10^{-8}$, odds ratio (OR) = 1.18, 95% CI 1.12−1.25, rs7656416: $P = 1.37 \times 10^{-8}$, OR = 1.15, 95% CI 1.10−1.21, Table 1, Supplementary Material, Table S3]. We identified additional two SNPs associated with type 2 diabetes, but the association did not attain genome-wide significance level (Supplementary Material, Table S3, rs1327796: $P = 3.17 \times 10^{-6}$, rs10993738: $P = 4.61 \times 10^{-6}$). The association of these SNPs with type 2 diabetes was not affected by adjusting for age, sex or body mass index (BMI; Supplementary Material, Table S4). We then searched for data on the top SNP—rs515071 in *ANK1*—in publicly available, European GWAS studies. Analysis of these data showed that rs515071 was also associated with type 2 diabetes in European populations [$P = 0.0129$, OR = 1.1, 95 % CI 1.02–1.19, combined data for WTCCC/UKT2D and the Diabetes Genetics Initiative (DGI), Table 2]. The association of rs515071 with type 2 diabetes was further strengthened in a larger European GWAS meta-analysis data [13; $P = 8.54 \times 10^{-4}$, OR = 1.09, 95% CI 1.03–1.14, DIAGRAM Diabetes Genetics Replication and Meta-analysis (DIAGRAM) Table 2]. The effect direction of rs515071 was consistent throughout all studies. In the previously reported Japanese GWAS data using directly genotyped SNPs, the best *P*-value for directly genotyped SNPs in this region was $>1 \times 10^{-4}$ (rs6989203, $P = 4.65 \times 10^{-4}$), whereas the *P*-value of the top imputed signal for this region (rs515071) in the present study (stage 1) was 2.69×10^{-5} (Supplementary Material, Tables S1 and S2).

Recently, the SNP rs6815464, located 54 kb downstream of rs7656416, was shown to be associated with type 2 diabetes in East Asian genome-wide association meta-analysis (24). Therefore, we also genotyped rs6815464 in stage-2 samples, which did not overlap with the samples in the study for East Asian meta-analysis. We found that this SNP was also associated with type 2 diabetes and that these two SNPs were in modest linkage disequilibrium (LD; $r^2 = 0.76$ in our stage-2 samples, Supplementary Material, Fig. S2 and Table S5). Subsequent conditional analysis that included both rs7656416 and rs6815464 in the same logistic regression model revealed that conditioning the SNPs on each other removed their significance (before conditioning: rs7656416, $P = 1.01 \times 10^{-5}$; rs6815464; $P = 1.26 \times 10^{-5}$; after conditioning: rs7656416, $P = 0.22$; rs6815464, $P = 0.29$, Supplementary Material, Table S5).

We further examined the association of rs515071 and rs7656416 with quantitative metabolic traits among control participants. Rs515071-C, the risk allele for type 2 diabetes, was modestly associated with a decrease in BMI (beta = −0.012, S.E. 0.005, $P = 0.016$, adjusting age and sex, Table 3). Participants without a risk allele for diabetes (TT, $n = 115$) showed higher BMI than those with a homozygote

Table 1. Two SNPs significantly associated with type 2 diabetes in the Japanese population

Nearest gene	Stage 1[a] RAF		Stage 2 RAF		Stage 1 + 2[b]		Heterogeneity		Stage 1 + 2[c]
	Cases	Controls	Cases	Controls	Combined *P*	OR (95% CI)	Q	I^2	Combined *P*
Rs515071 **C** > T Ch 8 *ANK1*	0.826	0.804	0.822	0.794	1.37×10^{-8}	1.18 (1.12−1.25)	0.59	0	6.45×10^{-10}
Rs7656416 **C** > T Ch 4 *MGC21675*	0.706	0.675	0.705	0.675	1.37×10^{-8}	1.15 (1.10−1.21)	0.96	0	1.32×10^{-9}

Combined *P*-values with a fixed effect model are presented.
Ch, chromosome; RAF, risk allele frequency; OR, odds ratio;
Q, *P*-values for Cochran Q statistics; $I^2 = [Q \text{ statistics} - (K - 1)]/Q$ statistics, *K* is the number of the study.
[a]Direct genotyped data in 2692 type 2 diabetes and 3071 controls.
[b]Direct genotyped stage 1 + stage 2 data.
[c]Imputed data after GC correction in 4470 type 2 diabetes and 3071 controls (stage 1) + stage 2 data.

Table 2. Association of rs515071 in the *ANK1* locus with type 2 diabetes

	Sample size		CC/CT/TT (RAF)		*P*	OR (95% CI)	Q	I^2
	Cases	Controls	Cases	Controls				
1. Stage 1 (GWAS)	2692	3071	1804/757/80, −0.826	1905/950/104, −0.804	2.57×10^{-3}	1.16 (1.05–1.27)		
2. Stage 2	7605	3534	4806/2053/239, −0.822	2148/1116/145, −0.794	1.33×10^{-6}	1.20 (1.11–1.29)		
1 + 2, all Japanese	10 297	6605			1.37×10^{-8}	1.18 (1.12–1.25)	0.59	0
WTCCC/UKT2D	1924	2938	1167/669/86, −0.781	1696/1058/177, −0.759	0.012	1.13 (1.03–1.25)		
DGI	1464	1467	949/442/40, −0.818	933/450/47, −0.81	0.441	1.05 (0.92–1.20)		
3. WTCCC/UKT2D + DGI	3388	4405			0.0129	1.10 (1.02–1.19)	0.38	0
1 + 2 + 3	13 685	11 010			1.59×10^{-9}	1.15 (1.10–1.21)	0.4	0
4. DIAGRAM v2	22 570		N/A	N/A	8.54×10^{-4}	1.09 (1.03–1.14)		
1 + 2 + 4	39 472				6.14×10^{-10}	1.12 (1.08–1.17)	0.07	62.33

Combined *P*-values with a fixed effect model are presented.
RAF, risk allele frequency; OR, odds ratio; *P*; *P*-value is calculated with additive model;
Q, *P*-values for Cochran Q statistics, I^2 = [Q statistics – $(K - 1)$]/Q statistics, K = number of the study.

Table 3. Association of rs515071-C and rs7656416-C with BMI[a]

	β (S.E.)	*P*-value	BMI (mean ± SD)		
rs515071					
Unadjusted	−0.013 (0.005)	0.008	CC ($n = 1527$)	CT ($n = 813$)	TT ($n = 115$)
Adjusted[b]	−0.012 (0.005)	0.016	23.0 ± 3.3	23.2 ± 3.4	24.0 ± 3.3[c]
rs7656416					
Unadjusted	−0.004 (0.004)	0.39	CC ($n = 1129$)	CT ($n = 1107$)	TT ($n = 265$)
Adjusted[b]	−0.004 (0.004)	0.37	23.0 ± 3.4	23.2 ± 3.3	23.1 ± 3.2

β, regression coefficient; *P*, *P*-values are calculated on linear regression analysis with additive model.
[a]Log-transformed BMI was used for the linear regression analysis.
[b]Adjusted for age and sex.
[c]$P < 0.05$ TT versus CC.

Table 4. Association of rs515071-C and rs7656416-C with quantitative glycemic traits

Adjustment	FPG (mmol/l)		HOMA-IR[a]		HOMA-β[a]	
	β (S.E.)	*P*-value	β (S.E.)	*P*-value	β (S.E.)	*P*-value
rs515071						
–	−0.022 (0.019)	0.26	−0.038 (0.024)	0.11	−0.026 (0.024)	0.29
Sex, age	−0.020 (0.019)	0.31	−0.034 (0.024)	0.15	−0.024 (0.024)	0.33
Sex, age, BMI[a]	−0.011 (0.019)	0.57	0.003 (0.02)	0.88	0.005 (0.022)	0.81
rs7656416						
–	0.015 (0.017)	0.38	0.006 (0.021)	0.79	−0.007 (0.021)	0.74
Sex, age	0.013 (0.017)	0.43	0.005 (0.021)	0.8	−0.006 (0.021)	0.77
Sex, age, BMI[a]	0.016 (0.017)	0.35	0.01 (0.018)	0.6	−0.001 (0.019)	0.95

β, regression coefficient; *P*, *P*-values are calculated on linear regression analysis with additive model.
[a]Values were log-transformed and used for the analysis.
Sample size: FPG, $n = 2347$; HOMA-IR, $n = 1623$; HOMA-β, $n = 1623$.

of the risk allele (CC, $n = 1527$; $P = 0.01$, 24.0 ± 3.3, compared with 23.0 ± 3.3). Meanwhile, no association was observed between rs7656416 and BMI.

We did not observe an association of rs515071 or rs7656416 with any glycemic traits, such as fasting plasma glucose (FPG), homeostasis model assessment of insulin resistance (HOMA-IR) or homeostasis model assessment of beta-cell function (HOMA-β) (Table 4).

DISCUSSION

By using imputation-based GWAS, we identified a novel susceptibility variant for type 2 diabetes at the *ANK1* locus. We also identified a strong signal at *MGC21675*, located in the same LD block as the *MAEA*, recently reported as a top signal in a meta-analysis of GWAS for East Asian type 2 diabetes.

Currently performed GWAS have examined ~1 000 000 directly genotyped SNPs and additional ~2 000 000 imputed SNPs. Those genotypes are estimated based on the degree of LD in directly genotyped alleles. The accuracy of imputed SNPs that pass the quality-control standards (proper_info for IMPUTE, r^2 for MACH) can widely be accepted in European populations. This bioinformatics technology significantly contributes to the identification of additional novel loci or SNPs more strongly associated with the disease. Approximately 40 loci have been identified and confirmed by examining more than 2 million directly genotyped and imputed SNPs in European populations. Recently, an East Asian study group identified eight novel loci by using the same strategy as the European GWAS meta-analysis (24). However, integration of all these data is still not sufficient to completely explain type 2 diabetes heritability. Thus, more efforts are necessary to identify additional susceptibility variants for the disease, especially among non-European populations.

In the present study, we identified the SNP rs515071 located at an intron of the Ankyrin1 gene as a novel susceptibility variant for type 2 diabetes. Because the analysis of European GWAS data has also shown a significant association between rs515071 and type 2 diabetes, rs515071 is likely a common locus for type 2 diabetes across multiethnic populations. This confirms the importance of extended analyses in multiethnic groups.

ANK1 is located on chromosome 8p11.1 and encodes a member of the ankyrin family. The ankyrins act as adaptors among a variety of integral membrane proteins and the spectrin skeleton (25). Ankyrin1, the prototype of this family, was first discovered in the erythrocytes, but it has also been found in the brain and muscle cells. In humans, mutations to *ANK1* cause hereditary spherocytosis; therefore, *ANK1* has been considered pivotal in stabilizing the membrane structure of erythrocytes (25). Recently, variants in *ANK1*—rs4737009 and rs6474359—were shown to influence HbA1c levels in European, non-diabetic adults (rs4737009, $\beta = 0.027$, S.E. $= 0.004$, $P = 6.11 \times 10^{-12}$; rs6474359, $\beta = 0.058$, S.E. $= 0.011$, $P = 1.18 \times 10^{-8}$) (26). In the same report, the effect size of the *ANK1* variants has been shown to remain essentially unchanged after conditioning by either fasting or 2 h plasma glucose levels, and neither SNPs were associated with type 2 diabetes ($P = 0.069$, OR = 1.05, 95% CI 1.00–1.10). Therefore, the association of the *ANK1* variants with HbA1c was likely mediated by non-glycemic factors. Furthermore, *ANK1* variants may influence erythrocyte lifespan and lower HbA1c levels without affecting plasma-glucose levels.

In the present study, however, rs515071 in *ANK1* was significantly associated with susceptibility to type 2 diabetes and was found to be in weak LD with rs4737009 ($r^2 = 0$ in JPT, CEU) and rs6474359 ($r^2 = 0.22$ in JPT and 0 in CEU; Supplementary Material, Fig. S3). We further performed conditional analysis of rs515071 for type 2 diabetes susceptibility including two reported variants rs4737009 and rs6474359 into the same logistic model as co-valuables, and the results indicated that the association of rs515071 with type 2 diabetes was independent of these two SNPs (Supplementary Material, Table S8). In addition, we could not observe any significant association of these three SNPs with HbA1c levels in our stage-2 controls (Supplementary Material, Table S9). Taken together, we concluded that the association of rs515071 with type 2 diabetes is independent of already reported association signals for affecting HbA1c levels. The mechanisms by which the SNP in the *ANK1* contributes to susceptibility to type 2 diabetes are unknown.

We also examined the expression profile of *ANK1* in various tissues, clearly observing its expression in human islet, pancreas, skeletal muscle, adipose and liver tissues, along with the mouse pancreatic β-cell line, all of which are important organs for glucose metabolism (Supplementary Material, Fig. S4). The physiological or pathological role of ankyrin1 in pancreatic β-cell has not yet been reported. However, another member of the ankyrin family, ankyrin B (also termed ankyrin 2), is known to regulate K-ATP channel membrane trafficking and gating in excitable cells. In pancreatic β-cells, ankyrin B directly interacts with potassium inward rectifier 6.2 (Kir6.2)/sulphonylurea receptor ATP-sensitive potassium (K_{ATP}) channel and plays a key role in regulating ATP sensitivity (27). On the other hand, SNPs in the *ANK1* promoter have been reported to be associated with intramuscular fat in bovine or porcine tissues (28,29), suggesting that *ANK1* also contributes to the development of muscular insulin resistance (30). Therefore, *ANK1* may be one of the genes conferring susceptibility to type 2 diabetes, although a possibility still exists that other nearby genes confer the true causal effects. Although we did not observe a significant association between the *ANK1* SNP and glycemic traits (FPG, HOMA-IR or HOMA-β) in our limited, non-diabetic controls, the susceptibility allele for type 2 diabetes (rs515071-C) modestly reduced BMI. This might operate via the effects on lean body mass, because ankyrin1 appears to have a role in the organization of myofibrils during assembly and seems to cooperate with obscurin in mediating interactions between the sarcoplasmic reticulum and myofibrils (31,32). Because the association between rs515071 and type 2 diabetes was not affected by adjustment of BMI (Supplementary Material, Table S4), the effects of the variant on type 2 diabetes susceptibility and reducing BMI are probably independent of each other.

We also found another significant signal at the rs7656416 locus, which was not identified in the previous Japanese GWAS. This site is located on chromosome 4p16.3 near *MGC21675* (alternatively, *C4orf42*), which encodes the hypothetical protein *LOC92070*. Recently, eight novel loci for type 2 diabetes were identified in an East Asian GWAS meta-analysis for type 2 diabetes, including rs6815464 at the *MAEA* locus. Rs6815464, located at 54 kb downstream of rs7656416, was identified as a top signal in the East Asian meta-analysis. Both SNPs are located in the same LD block. The association of these SNPs with type 2 diabetes disappeared after conditioning on one another in the present stage-2 samples, indicating that our results for rs7656416 simply replicated the previously identified association of this locus with type 2 diabetes. There are several genes in this locus, including *MGC21675*, macrophage erythroblast attacher (*MAEA*) and C-terminal binding protein-1 (*CTBP1*). Studies have not yet elucidated the roles of the proteins encoded by these genes in pancreatic β-cell or peripheral tissues involved in glucose metabolism. Further studies,

including fine mapping and functional analysis, are needed to clarify the mechanisms by which these variants confer susceptibility to type 2 diabetes.

Our present findings, identification of two loci for type 2 diabetes susceptibility with genome-wide significant levels, indicate that imputation-based genotype data are also useful in Japanese populations, because both loci, *ANK1* and *MGC21675-MAEA*, did not show strong evidence being associated with type 2 diabetes in a previously reported direct-genotyped GWAS data ($P > 10^{-4}$). However, in some cases, there are significant discrepancies between the imputation-based data and directly genotyped data (Supplementary Material, Table S10), probably resulting from insufficient sample number or no information for trio in the reference panel we used. Therefore, in contrast to the European data, association studies using imputation-based genotyped data should be performed with some caution in Japanese populations. Currently, the efforts to improve the quantity and quality of data for reference panels, and to improve the imputation programs themselves, are in progress and will further contribute to advances in genetic studies.

In summary, using imputation-based GWAS, we identified *ANK1* as a novel locus associated with type 2 diabetes at genome-wide significance levels in Japanese populations. The risk allele rs515071-C in *ANK1* was associated with type 2 diabetes susceptibility in European populations as well. Additionally, we showed that the association of the *MGC21675-MAEA* locus with type 2 diabetes also occurs among Japanese populations.

MATERIALS AND METHODS

Participants, DNA preparation and SNP genotyping

Stage-1 samples

For the GWAS, we selected case–control samples (4470 cases and 3071 controls) from subjects enrolled in the BioBank Japan, as previously reported (23). We selected type 2 diabetes cases from individuals registered as having type 2 diabetes. Control groups were healthy volunteers or individuals registered as individuals not having type 2 diabetes but with diseases other than type 2 diabetes, comprised of 13 distinct diseases.

Stage-2 samples

We selected another 7605 cases from the BioBank Japan or from subjects with type 2 diabetes who visited outpatient clinics at one of the nine different institutions: The University of Tokyo, Juntendo University, National Center for Global Health and Medicine, Hiranuma Clinic, St. Marianna University School of Medicine, the Hiroshima Atomic Bomb Casualty Council Health Management Center, Kawasaki Medical School, Toyama University Hospital or the Shiga University of Medical Science. We also examined 3534 controls enrolled during an annual health check-up at six institutions: The Hiroshima Atomic Bomb Casualty Council Health Management Center, The National Center for Global Health and Medicine, Keio University, Hiranuma Clinic, St. Marianna University School of Medicine or Toyama University Hospital. Diabetes was diagnosed according to World Health Organization (WHO) criteria. We excluded individuals who were positive for antibody to glutamic acid decarboxylase or those with diabetes due to (i) liver dysfunction, (ii) steroids and other drugs that might raise glucose levels, (iii) malignancy or (iv) a monogenic disorder known to cause diabetes. Clinical characteristics of stage-1 and -2 participants are shown in Supplementary Material, Table S6.

Genomic DNA was extracted from peripheral leukocytes using the standard phenol–chloroform procedure. Genotyping in stage-1 validation and stage 2 was performed using the multiplex-polymerase chain reaction (PCR)-invader assay (21,23).

The protocol was approved by the ethics committee of the Institute of Physical and Chemical Research (RIKEN), the University of Tokyo and each participating institution (Juntendo University, National Center for Global Health and Medicine, Keio University, St. Marianna University School of Medicine, Kawasaki Medical School, Toyama University and the Shiga University of Medical Science).

Genome-wide imputation by the IMPUTE

We performed genome-wide imputation by using IMPUTE (https://mathgen.stats.ox.ac.uk/impute/impute.html) with previously reported GWAS data (459 359 directly genotyped SNPs; stage 1; 23) and from 89 HapMap samples (44 JPT and 45 CHB in HapMap phase 2). We successfully obtained the new information of 2 229 890 imputed SNPs with a quality score (proper_info) of >0.40, MAF of >0.01 and HWE P-value of $>1 \times 10^{-6}$.

Cell culture

Hepa1-6 and C2C12 were purchased from ATCC (Manassas, VA, USA). 3T3-L1 was purchased from Health Science Research Resources Bank (Sennan, Japan). MIN6-m9 cells were kindly provided by Prof. Susumu Seino (Kobe University, Kobe, Japan).

Hepa1-6 and C2C12 cells were maintained in Dulbecco's Modified Eagle Medium, containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The differentiation in C2C12 was induced by depleting FBS from 10 to 1% and subsequently culturing for 8 days. MIN6-m9 cells were cultured as previously described (33). 3T3-L1 cells were maintained and their differentiation was induced as described previously (34). Differentiated 3T3-L1 cells were harvested 8 days after initiating induction.

Quantitative reverse transcription-PCR

Each cell was harvested at the indicated time and the total RNA was extracted using the RNeasy Kit (Qiagen, Germantown, MD, USA). First-strand cDNAs were synthesized using the PrimeScript® II 1st Strand cDNA Synthesis Kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocol. We obtained human cDNAs from multiple tissues from CLONTECH, Inc. (Palo Alto, CA, USA). Human islet cDNA were kindly provided by Primary Cell Co., Ltd. (Sapporo, Japan).

The amount of first-strand cDNAs was quantified using SYBR premix Ex Taq II (Takara Bio Inc., Otsu, Japan) for amplification and Mx3000P multiplex quantitative PCR system (Stratagene, La Jolla, CA, USA) for detection. The thermal profile was 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Relative expressions of human ankyrin1 isoforms were normalized with GAPDH, and the expressions of mouse ankyrin1 were normalized with normalization factor derived from mouse Eef1g, Hmbs and Ppia, calculated using GeNorm software (http://medgen.ugent.be/~jvdesomp/genorm/).

The primers for quantitative PCR are described in Supplementary Material, Table S7.

Statistical analysis

Statistical methods for determining the associations and calculating the LD coefficients (r^2) have previously been described (23). We performed the HWE test according to a previously described method (35). The cut-off value for the HWE test in the control groups was 0.000001 for the first stage. The SNPs with *P*-values less than this were excluded from the analysis. We performed the imputation GWAS by SNPTEST (https://mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest.html) and used gene dosages for the analysis. As for directly genotyped data in Stage 1 validation and Stage 2, we analyzed the differences between the case and control groups in genotype distribution by using the Armitage test for trends, based on an additive model, as previously described (21,23). Combined meta-analysis was performed using the Mantel–Haenszel procedure with a fixed-effect model after testing for heterogeneity. We performed quantitative trait analyses for BMI, FPG, HbA1c, HOMA-β and HOMA-IR by using multiple linear regression analysis in an additive association model with or without adjusting for age, sex and log-transformed BMI. Because the Japanese samples studied here show the skewed distribution values for BMI, HOMA-IR and HOMA-β, we have analyzed the quantitative traits by using log-transformed BMI, HOMA-IR and HOMA-β. Tests for multiple comparisons were performed by analysis of variance followed by Scheffe's *post hoc* procedure.

AUTHORS' ROLES

M.Im., T.K., S.M., T.Y. and K.H. planned and coordinated the study; Y.N. and M.K. managed BioBank Japan; K.H., S.M., K.Y., M.H., M.N., H.F., H.W., H.M., M.O-I., M.Iw.. N.S., T.O., S.O., M.Iw., H.H., K.K., C.I., Y.T., K.T., A.K., R.K., M.K. and T.K. recruited and phenotyped or genotyped the patient cohorts; M.Im., T.M., A.T., T.T., N.K., S.M., K.H. and T.K. analyzed the genotyping data; M.Im. and S.M. wrote the manuscript; All authors contributed to the final version of the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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