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.

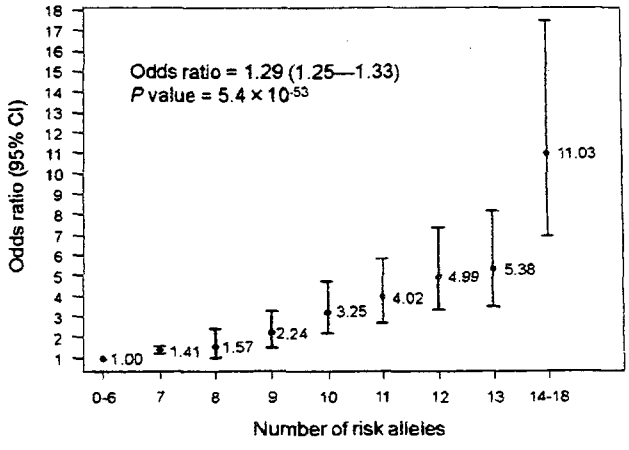

Figure 1 Odds ratios for subjects with different numbers of risk alleles for 11 susceptibility loci for type 2 diabetes. The cumulative effect of the 11 loci on type 2 diabetes was tested by counting the number of risk alleles associated with type 2 diabetes with a logistic regression model with adjustment 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.

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

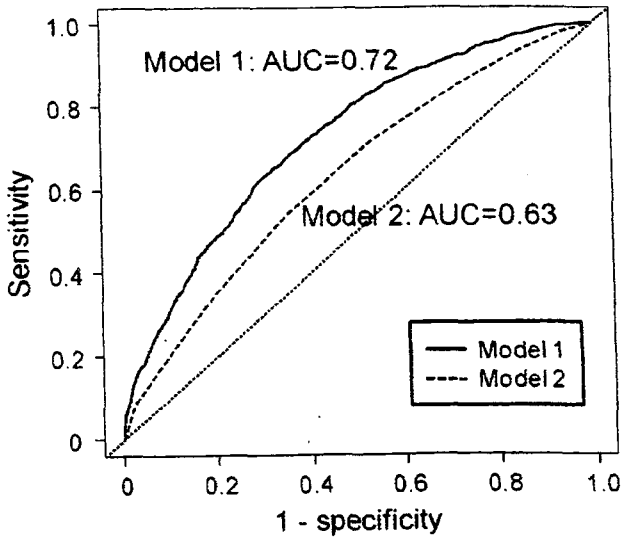

Figure 2 ROC curves for the prediction model on the basis of the number of risk alleles for 11 susceptibility loci for type 2 diabetes. The prediction model for type 2 diabetes was constructed using the logistic regression model, and ROC curves for the model were generated. In model 1, the number of 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.

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 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.

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Glucose induces FGF21 mRNA expression through ChREBP activation in rat hepatocytes

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ABSTRACT

Fibroblast growth factor 21 (FGF21) has beneficial effects of improving the plasma glucose and lipid profiles in diabetic rodents. Here, we investigated carbohydrate response element binding protein (ChREBP) involvement in the regulation of FGF21 mRNA expression in liver. Glucose stimulation and adenoviral overexpression of dominant active ChREBP increased FGF21 mRNA. Consistently, adenoviral expression of dominant negative Mlx inhibited glucose induction of FGF21 mRNA. Furthermore, deletion studies of mouse FGF21 gene promoter (−2000 to +65 bp) revealed a glucose responsive region between −74 and −52 bp. These findings suggest that FGF21 expression is regulated by ChREBP.

1. Introduction

Fibroblast growth factor 21 (FGF21) belongs to the fibroblast growth factor (FGF) family involved in cell growth, cell differentiation, and embryonic development [1]. FGF21 has been found to have multiple beneficial effects in treatment of metabolic syndrome including obesity as well as diabetes mellitus [2]. Transgenic mice with FGF21 overexpression are resistant to diet-induced obesity and glucose intolerance [3]. Treatment with FGF21 induces energy expenditure and improves glucose intolerance, hypertriglycemia and hepatic steatosis in ob/ob mice [4]. In human, serum FGF21 levels in diabetic or obese individuals are higher than normal, and are significantly correlated with adiposity, plasma fasting insulin and the triglyceride concentration [5]. Furthermore, Akt signaling and PPAR- increases FGF21 mRNA expression in muscle and adipose tissue, respectively [6]. These findings suggest that FGF21 is increased in adaptation to increased body weight and energy intake.

We have reported that ChREBP, a glucose activated transcription factor, regulates lipogenic enzyme gene expression and is involved in the development of metabolic syndrome [7,8]. Transactivity of ChREBP is increased in genetically obese mice, and gene deletion of ChREBP improves metabolic disorders such as fatty liver and glucose intolerance [9]. Recently, some groups have reported that overexpression of dominant negative Mlx, which forms a heterodimer with ChREBP, inhibits glucose mediated FGF21 gene expression in rat hepatocytes [10]. These findings suggest that glucose activation of ChREBP might be involved in FGF21 mRNA expression.

In this study, we tested whether glucose stimulation or activation of ChREBP could increase FGF21 mRNA expression in rat hepatocytes We also performed a deletion study of mouse FGF21 gene promoter and identified the glucose responsive region. Our findings demonstrate that glucose stimulation via ChREBP activation induces FGF21 gene expression in rat primary hepatocytes and that FGF21 has an important role in glucose and lipid homeostasis.

2. Materials and methods

2.1. Materials, tissue materials, hepatocyte isolation, and Taqman PCR analysis

The protocols for all animal experiments were approved by the Institutional Animal Care and Use Committee of Gunma University

Abbreviations: FGF21, fibroblast growth factor 21; ChREBP, cabohydrate response elemnt binding protein; LPK, liver type pyruvate kinase; FASN, fatty acid synthase

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Medical School (code nos. 08-025 and 08-026). Rat primary hepatocytes were isolated and cultured from 6 weeks age male Wistar rats (SLC) as previously described [11,12].

2.2. Construction of plasmid and adenovirus vectors

pcDNA-daChREBP, Ad-daChREBP, Ad-dnMlx, and pGL4 TK RLuc were used previously [11,12]. The series of pGL3-mFGF21 vectors were constructed as follows: a fragment representing −2000, −1205, −1012, −797, −317, −197, −100, −67, and −40 bp (position −2000/+65, −1205/+65, −1012/+65, −797/+65, −317/+65, −197/+65, −100/+65, −67/+65, and −40/+65 relative to the transcription start site of mouse FGF21 of the native 5′ sequence flanking the mouse FGF21 gene were cloned upstream of the luciferase gene in pGL3 basic vector. The series of pGL3 promoter mFGF21 vectors were constructed as follows: a fragment representing −100/−30, −90/−40, −67/−30, −100/−67 bp (position −100/−30, −90/−40, −67/−30, −100/−67 relative to the transcription start site of mouse FGF21 gene, respectively) of the native 5′ sequence flanking the mouse FGF21 gene were cloned upstream of TK promoter in pGL3 promoter vector. A fragment 3XFGF21 E1(−101/−80 bp) or 3XFGF21 E2 (−74/−52 bp) was cloned upstream of TK promoter in pGL3 promoter vector. Mouse FGF21 cDNA expressing adenovirus (Ad-FGF21) was constructed as follows: Mouse FGF21 full-length cDNA was cloned using Prime Star DNA polymerase reagents. PCR fragment was ligated into pENTR vector (Invitrogen). Recombination of adenovirus and pENTR FGF21 vector was performed to produce Ad-FGF21 vectors according to manufacture's protocol. All plasmid and adenovirus vectors were verified by sequencing analysis.

2.3. Treatment with recombinant adenovirus in rat hepatocytes

Rat primary hepatocytes were cultured in six-well plates in 2 ml DMEM medium. After 2, 10, and 50 m.o.i. of adenovirus bearing GFP, dominant active ChREBP (daChREBP) dominant negative Mlx (dnMlx), or FGF21 was infected into hepatocytes for 2 h, media were removed and infected hepatocytes were incubated in media with 2.5 or 25 mM glucose concentration for 18 h. Cells were then collected and used for RNA extraction, cDNA synthesis and RT-PCR analysis as previously described [11,12].

2.4. Mammalian transfection and reporter assay

Rat primary hepatocytes were cultured in six-well plates in 2 ml DMEM without antibiotics. The cells were transfected with Lipofectamine2000 (10 μl), the series of pGL3-mFGF21 (3.6 μg), and the pGL4 TK RLuc vector (0.4 μg) [12]. After 24 h of incubation at 2.5 or 25 mM glucose concentration, the cells were collected and used to measure luciferase activity (Dual Luciferase assay system; Promega, Madison, WI) according to manufacture's protocol. To determine glucose dependency on the glucose response region in mouse FGF21 gene promoter, cells were transfected with 3.1 μg of pGL3 promoter 3XFGF21 E1, 3XFGF21 E2, or 3XLPK ChoRE vectors, 0.4 μg of pGL4-RLuc-TK vectors and 0.5 μg of pcDNA6.2 empty vector or daChREBP vector. After 24-h incubation with various glucose concentrations, the cells were collected for measurement of luciferase activity.

2.5. Data presentation and statistical methods

All data are expressed as means ± S.D. The listed n values represent the number of single experiments performed (each experiment was duplicated). Comparisons between two groups were performed by student t-test and comparison between multiple groups was performed by Tukey–Kramer test. A value of $P < 0.05$ was regarded as significant.

3. Results

3.1. The role of ChREBP in glucose mediated FGF21 gene expression

As FGF21 mRNA is expressed mainly in islets and liver (Fig. S1A), we examined FGF21 mRNA expression in liver of diabetic model mice. FGF21 mRNA levels in 6 weeks age male STZ mice and ob/ob mice were 1.1- and 2.5-fold higher than that in 6 weeks age C57BL/6J mice, respectively (Fig. S1B). To verify that glucose increased FGF21 mRNA expression, we examined the effect of glucose and ChREBP on FGF21 mRNA expression. High glucose stimulation increased FGF21 mRNA expression in a time dependent manner (Fig. 1A). These findings suggest that a metabolite in the glycolytic and pentose pathway induces FGF21 mRNA expression. Adenoviral overexpression of daChREBP increased FGF21 mRNA expression in a dose-dependent manner (Fig. 1B). Furthermore, Mlx is an obligate heterodimer partner of ChREBP that is required for binding and activation of glucose-regulated gene expression [11]. In accord with Fig. 1B, dnMlx successfully inhibited glucose induction of FGF21 mRNA gene expression in rat primary hepatocytes (Fig. 1C). Similarly, siRNA against ChREBP inhibited not only ChREBP mRNA but also glucose mediated FGF21, liver type pyruvate (LPK), and fatty acid synthase (FASN) gene expression in rat primary hepatocytes (Fig. S1C). Thus, transactivation of ChREBP induces FGF21 mRNA expression in hepatocytes.

3.2. Identification of glucose response region in mouse FGF21 gene promoter

Deletion studies of mouse FGF21 gene promoter showed that a region between −100 and −67 bp is critical for the glucose response to FGF21 mRNA induction (Fig. 2A). We tested in detail putative glucose response elements in mouse FGF21 promoter (between −200 and +0 bp) (Fig. 2B). Fig. 2B shows that a region between −90 and −40 bp is critical for the glucose response to FGF21 mRNA expression. We also determined which putative E-Box (E1: −101/−80 bp and E2: −74/−52 bp) functions as the glucose response element (Supplementary Fig. S2). In accord with Fig. 2A and B, the E2 element (−74/−52 bp) is shown to possesses glucose responsiveness or ChREBP mediated activation of FGF21 mRNA expression (Fig. 2C). We also tested the glucose response to luciferase activity in pGL3-3XFGF21 E2 vector. Compared with pGL3 FGF21 −2 kbp vector, luciferase activities were similarly dependent on the glucose concentration (Fig. 2D).

3.3. Effect of FGF21 overexpression on ChREBP and expression of its target genes in rat hepatocytes

FGF21 lowers plasma glucose and increases glucose uptake in muscle and adipose tissue, but its role in liver is unclear. Because the FGF21 signaling cascade requires both FGF receptors (FGFRs) and beta-Klotho [13,14], we confirmed that all of the FGFRs were present in both mouse liver and rat hepatocytes and beta-Klotho was detected in mouse livers (Fig. S3A and B) [14]. We then tested whether FGF21 affects glucose mediated LPK and FASN mRNA expression in rat hepatocytes. Glucose induction of LPK and FASN mRNA expression in rat hepatocytes infected in a dose-dependent manner with Ad-FGF21 were unchanged as compared with those in untreated hepatocytes (Fig. 3).

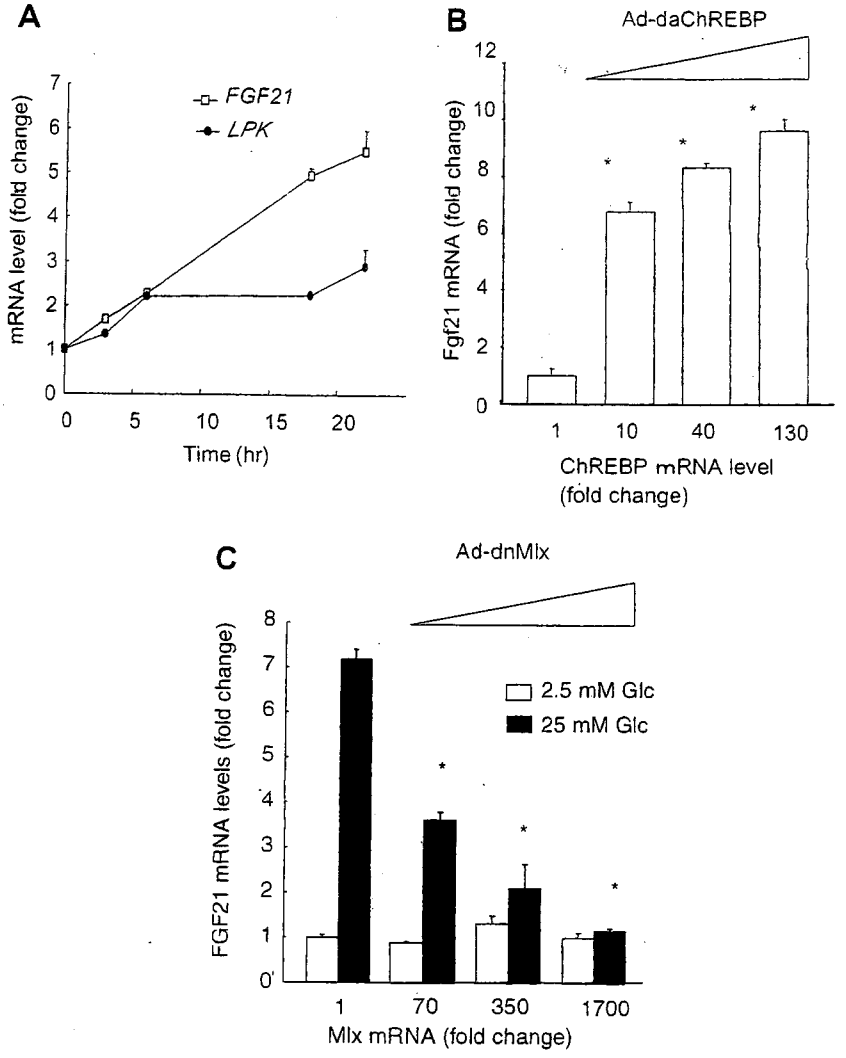

Fig. 1. (A) Glucose stimulates increased FGF21 mRNA expression in rat hepatocytes. Isolated hepatocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) including several concentrations of glucose for 24 h and collected at the indicated hours for Taqman RT-PCR analysis. (B) Adenoviral overexpression of dominant activ ChREBP increased FGF21 mRNA expression in rat hepatocytes. Isolated hepatocytes were infected with 2, 10, and 50 m.o.i. of Ad-daChREBP for 2 h. After culture in DME with 2.5 mM glucose concentration for 18 h, the cells were collected for Taqman RT-PCR analysis. Data represent means ± S.D. (C) Adenoviral overexpression of dominar negative Mlx increased FGF21 mRNA expression in rat hepatocytes. Isolated hepatocytes were infected with 2, 10, and 50 m.o.i. of Ad-dnMlx for 2 h. After culture in DME with 2.5 or 25 mM glucose concentration for 18 h, the cells were collected for Taqman RT-PCR analysis. Values represent means ± S.D. At most points, the error bars are to small to be shown.

4. Discussion

In this study, we show that FGF21 is a target gene of ChREBP in rat primary hepatocytes. Moreover, we identified a glucose response region located between −74 and −52 bp in the FGF21 promoter. Adenoviral delivery of FGF21 cDNA into rat primary hepatocytes was found not to directly affect glucose induction of LPK and FASN mRNA expression in rat hepatocytes. Thus, FGF21 is directly regulated by the glucose activated transcription factor ChREBP, and ChREBP is not directly regulated by FGF21.

As found in a previous study, FGF21 mRNA is most abundant in mouse islets and liver, in which ChREBP is also highly expressed (Fig. S1A) [2,15]. FGF21 mRNA is more highly upregulated in ob/ob mice, but is only slightly induced in STZ mice (Fig. S1B). In addition, ob/ob mice show hyperinsulinemia and hyperglycemia, while STZ mice show hypoinsulinemia and hyperglycemia (Fig. S1B). Considered together with our previous finding that ChREBP activation requires insulin action, glucose-stimulated FGF21 mRN expression may well require insulin action [12,16]. In accord wit the data shown in Fig. 1A, overexpression of daChREBP induce FGF21 mRNA expression and overexpression of dnMlx inhibite glucose-induced FGF21 mRNA expression (Fig. 1B and C). Mor over, siRNA against ChREBP suppressed glucose induction FGF21 mRNA expression in rat primary hepatocytes (Fig. S1C These findings indicate that glucose activation of ChREBP induce hepatic FGF21 mRNA expression. In fact, some groups have re ported that plasma FGF21 concentrations and hepatic FGF2 mRNA expression in obese and diabetic animal models are muc higher than those in control lean animals [5]. ChREBP is remark ably activated in livers from these diabetic animals, further sug gesting that glucose activation of ChREBP induces FGF21 mRN expression. Moreover, satiety signaling such as PPAR- and Akt sig naling induces FGF21 mRNA expression [6,16–18]. Thus, FGF2 gene expression is regulated by satiety signals such as glucos and insulin.

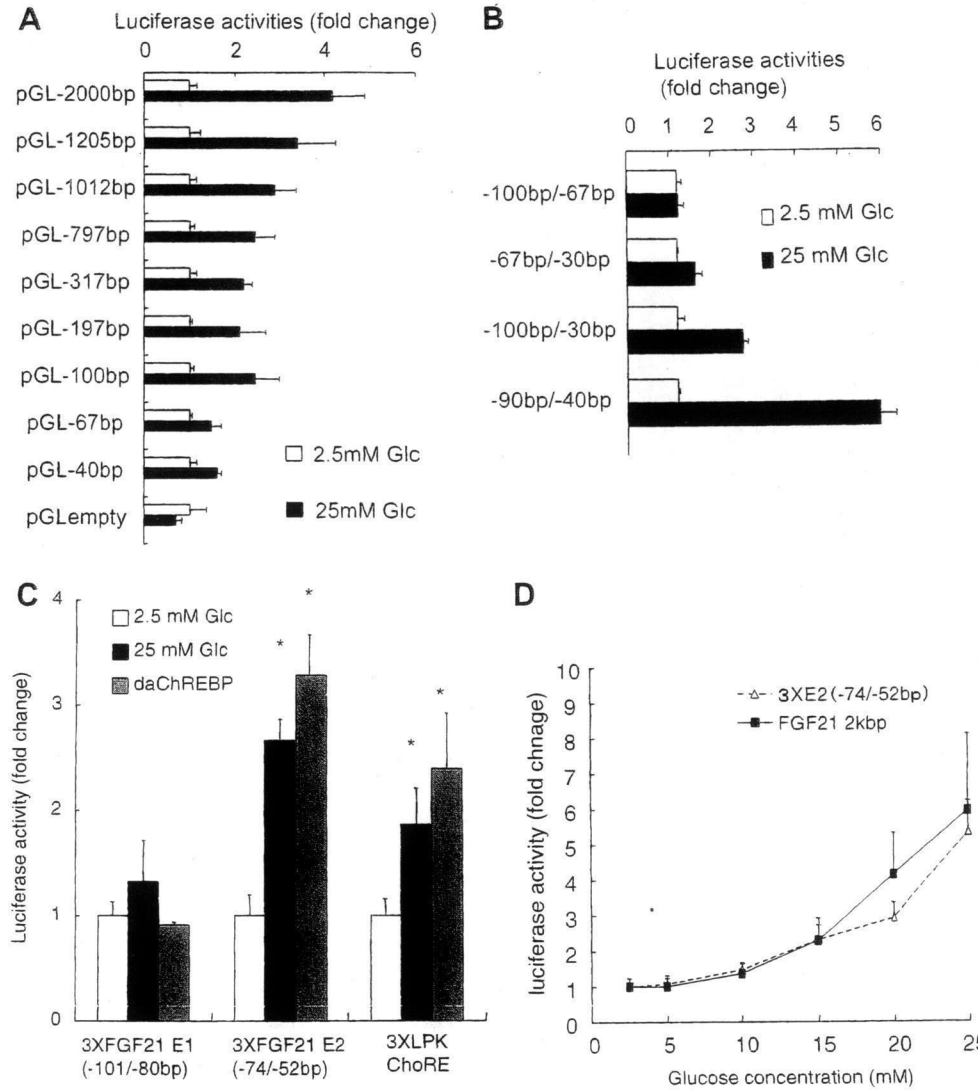

Fig. 2. (A) Deletion studies of mouse FGF21 gene promoter. Isolated hepatocytes were cultured in six-well dishes and transfected with the series of pGL3-mFGF21 vector (3.6 μg) and pGL4 TK RLuc (0.4 μg) using Lipofectamine2000 (10 μl). After 24 h incubation in DMEM including 2.5 or 25 mM glucose concentrations, luciferase activities were measured using Dual Luciferase assay kit. (B) Detailed analysis of the carbohydrate response region in mouse FGF21 promoter. Cells were transfected with the series of pGL3 promoter mFGF mutants (3.6 μg), and pGL4 TK RLuc (0.4 μg) using Lipofectamine2000 (10 μl). After 24 h incubation in DMEM including 2.5 or 25 mM glucose concentrations, luciferase activities were measured using Dual Luciferase assay kit. (C) Reporter analysis of pGL3 promoter 3XFGF21 E2 (−74/−52 bp). Rat primary hepatocytes were transfected with 3.1 μg of pGL3 promoter 3XFGF21 E2 (−74/52p), 3XFgf21 E1 (−101/−80 bp), or 3XLPK ChoRE vectors, 0.4 μg of pGL4-RLuc-TK vectors and 0.5 μg of pcDNA6.2 empty vector or pcDNA6.2 daChREBP vector. Cells were incubated at 2.5 or 25 mM glucose for 24 h and collected for the measurement of luciferase activities. (D) Glucose dependent activation of pGL3 promoter 3XFGF21 E2 (−74/−52 bp) vector and pGL3-mFGF21 −2 kbp. Cells were transfected with 3.6 μg of pGL3 promoter 3XFGF21 E2 (−74/−52 bp) vector or pGL3-mFGF21 −2 kbp and 0.4 μg of pGL4-RLuc-TK vector. Cells were incubated at several glucose concentrations for 24 h and collected for measurement of luciferase activities. Values represent means ± S.D. At most points, the error bars are too small to be shown. *$P < 0.05$ vs. Ad-GFP.

It has been reported that administration of FGF21 in livers of diet-induced obese mice reduces hepatic ChREBP-target gene expression [4,19]. The FGF21 signaling cascade requires the FGF21 receptor complex, FGFR, and an adapter-like molecule, beta-Klotho [14]. Beta-Klotho and FGFR1-4 are abundantly expressed in liver, adipose tissues, and pancreas [14,15,20–22]. In our experiments, all FGFRs were detectable in mouse liver and rat hepatocytes and beta-Klotho was detected only in mouse liver (Fig. S3A and B). The sequence of rat beta-Klotho cDNA remains unknown and we could not check beta-Klotho mRNA expression in rat primary hepatocytes. We then tested whether FGF21 directly affects glucose activation of ChREBP-target gene expressions such as LPK and FASN. We found that FGF21 had no significant effects on the expression of these genes in rat hepatocytes (Fig. 3). GLUT1 gene expression, an FGF21 target, was also unchanged in rat hepatocytes infected by Ad-FGF21 (data not shown). Consistent with these data, some groups have reported that FGF21 does not act on mouse primary hepatocytes and rat hepatoma cell lines, even in the presence of beta-Klotho and FGFRs [14,23,24]. In contrast, some groups reported the opposite evidences that FGF21 is active in cells of hepatic origin [25]. The presence of beta-Klotho in rat primary hepatocytes remains unknown and it will be needed to further investigate the direct action of FGF21 in liver. In conclusion, these results indicate that FGF21 suppresses ChREBP transactivity at least indirectly by lowering plasma glucose concentrations in the whole body.

In conclusion, glucose induces FGF21 mRNA expression via transactivation of ChREBP, indicating that both FGF21 and ChREBP are involved in the regulation of glucose and lipid homeostasis in liver. Thus, FGF21 may well be a useful drug in the treatment of diabetes and obesity.

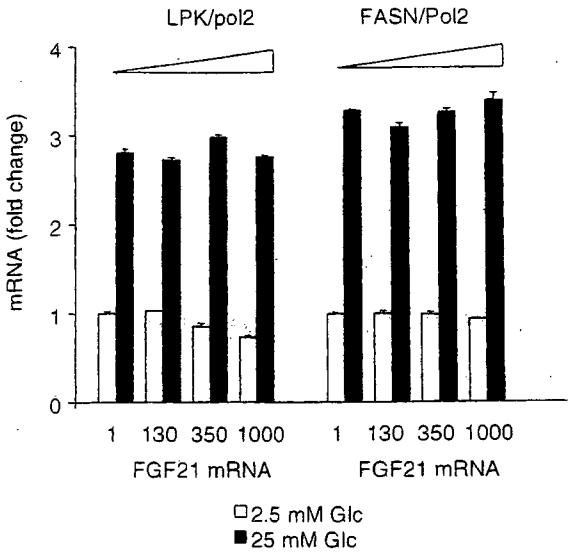

Fig. 3. Adenoviral overexpression of FGF21 did not inhibit LPK and FASN mRNA. Adenovirus expressing either GFP or FGF21 was transduced into rat hepatocytes at m.o.i. of 2, 10, and 50. As control, recombinant adenovirus expressing GFP was used at m.o.i. of 50. Two h after infection, the cells were cultured in DMEM including 2.5 or 25 mM glucose for an additional 18 h. Total RNA was extracted from hepatocytes, and RT-PCR analysis was performed. Values represent means ± S.D. At most points, the error bars are too small to be shown.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.07.053.

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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 b-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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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)	$7.4 \cdot 10^{-5}$	0.61	0.54	1.36 (1.16–1.58)	$1.1 \cdot 10^{-4}$
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)	$2.8 \cdot 10^{-4}$	JDC	0.42	0.33	1.49 (1.28–1.73)	$1.4 \cdot 10^{-7}$	0.42	0.33	1.45 (1.24–1.70)	$3.4 \cdot 10^{-6}$
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 ($P = 3.4 \cdot 10^{-6}$) 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 $1.1 \cdot 10^{-4}$ 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 $<5 \cdot 10^{-7}$) 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 ($P = 6.7 \cdot 10^{-13}$), with OR = 1.49 and 95% CI = 1.34–1.66; the P value for the trend test was $1.7 \cdot 10^{-12}$ (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

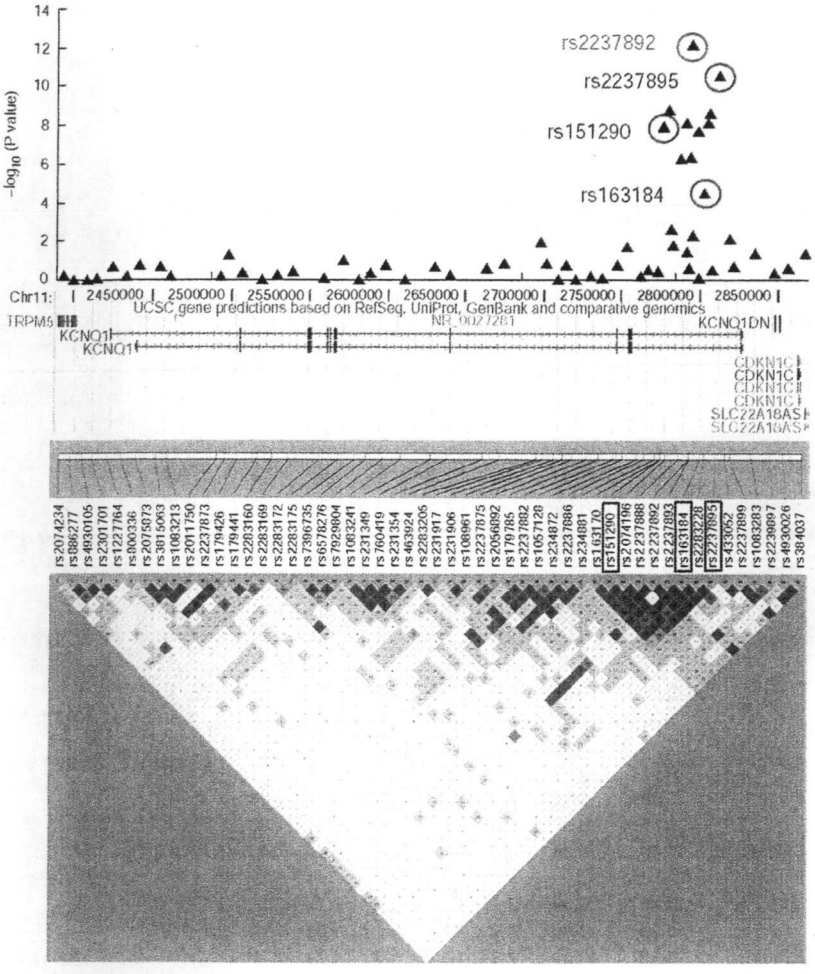

Figure 1 Dense mapping analysis of KCNQ1. The top panel shows the association $-\log_{10}$ (P value) in panel 2+3 for 64 SNPs of KCNQ1. The three blue circles represent the positive SNPs in the third screening. The red circle (rs2237892) indicates the SNP showing the most significant association with 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 • 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 b-cell function (HOMA-b)[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	$1.7 \cdot 10^{-9}$	1.39	1.25	1.54	$1.8 \cdot 10^{-9}$	
		Replication 1 (Japanese)	0.61	0.54	$1.4 \cdot 10^{-7}$	1.32	1.19	1.46	$2.1 \cdot 10^{-7}$	
		Replication 2 (Japanese)	0.62	0.55	$4.7 \cdot 10^{-7}$	1.31	1.18	1.46	$6.2 \cdot 10^{-7}$	
		All Japanese (4,378 cases, 4,412 controls)	0.62	0.55	$4.6 \cdot 10^{-21}$	1.34	1.26	1.42	$9.8 \cdot 10^{-21}$	1.34 (1.26–1.42), $P = 4.8 \cdot 10^{-21}$
		Replication 3 (Chinese)	0.71	0.63	$1.2 \cdot 10^{-9}$	1.40	1.26	1.56	$9.8 \cdot 10^{-10}$	
		Replication 4 (Korean)	0.66	0.58	$3.0 \cdot 10^{-5}$	1.39	1.19	1.62	$2.1 \cdot 10^{-5}$	
		All Asian (6,552 cases, 6,621 controls)	0.64	0.57	$9.9 \cdot 10^{-32}$	1.35	1.28	1.42	$2.1 \cdot 10^{-31}$	1.36 (1.29–1.42), $P = 7.9 \cdot 10^{-33}$
		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), $P = 8.6 \cdot 10^{-34}$
rs2237892	C	2+3 (dense mapping)	0.69	0.60	$6.7 \cdot 10^{-13}$	1.49	1.34	1.66	$1.7 \cdot 10^{-12}$	
		Replication 1 (Japanese)	0.66	0.59	$9.6 \cdot 10^{-10}$	1.39	1.25	1.54	$1.6 \cdot 10^{-9}$	
		Replication 2 (Japanese)	0.68	0.60	$6.9 \cdot 10^{-10}$	1.41	1.26	1.57	$1.1 \cdot 10^{-9}$	
		All Japanese (4,378 cases, 4,412 controls)	0.68	0.59	$2.8 \cdot 10^{-29}$	1.43	1.34	1.52	$1.7 \cdot 10^{-28}$	1.43 (1.34–1.52), $P = 3.0 \cdot 10^{-29}$
		Replication 3 (Chinese)	0.72	0.65	$1.3 \cdot 10^{-8}$	1.38	1.24	1.55	$4.2 \cdot 10^{-9}$	
		Replication 4 (Korean)	0.69	0.61	$1.7 \cdot 10^{-5}$	1.41	1.21	1.65	$1.0 \cdot 10^{-5}$	
		All Asian (6,552 cases, 6,621 controls)	0.69	0.61	$2.0 \cdot 10^{-39}$	1.41	1.34	1.48	$2.5 \cdot 10^{-39}$	1.42 (1.34–1.49), $P = 2.5 \cdot 10^{-40}$
		Replication 5 (European)	0.95	0.93	$7.8 \cdot 10^{-4}$	1.29	1.11	1.50	$7.2 \cdot 10^{-4}$	
		All	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.40 (1.34–1.47), $P = 1.7 \cdot 10^{-42}$
rs2237895	C	2+3 (dense mapping)	0.41	0.33	$3.1 \cdot 10^{-11}$	1.44	1.30	1.61	$4.0 \cdot 10^{-11}$	
		Replication 1 (Japanese)	0.38	0.33	$4.5 \cdot 10^{-5}$	1.25	1.12	1.38	$4.7 \cdot 10^{-5}$	
		Replication 2 (Japanese)	0.41	0.34	$5.8 \cdot 10^{-8}$	1.35	1.21	1.50	$5.5 \cdot 10^{-8}$	
		All Japanese (4,378 cases, 4,412 controls)	0.40	0.33	$1.3 \cdot 10^{-20}$	1.34	1.26	1.43	$1.7 \cdot 10^{-20}$	1.34 (1.26–1.43), $P = 1.4 \cdot 10^{-20}$
		Replication 3 (Chinese)	0.40	0.34	$3.5 \cdot 10^{-5}$	1.25	1.12	1.39	$3.4 \cdot 10^{-5}$	
		Replication 4 (Korean)	0.35	0.30	$3.2 \cdot 10^{-3}$	1.27	1.08	1.49	$2.7 \cdot 10^{-3}$	
		All Asian (6,552 cases, 6,621 controls)	0.39	0.33	$2.7 \cdot 10^{-25}$	1.31	1.24	1.38	$2.7 \cdot 10^{-25}$	1.31 (1.25–1.38), $P = 6.1 \cdot 10^{-26}$
		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-b 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 b 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 w^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 w^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.

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