Molecular Genetics and Metabolism Reports 1 (2014) 350–361

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: http://www.journals.elsevier.com/molecular-genetics-and-metabolism-reports/

Association of genetic variants of the incretin-related genes with quantitative traits and occurrence of type 2 diabetes in Japanese

Mayumi Enya [1], Yukio Horikawa [*,1], Katsumi Iizuka, Jun Takeda

Department of Diabetes and Endocrinology, Gifu University Graduate School of Medicine, Gifu, Japan

ARTICLE INFO

Article history:
Received 13 June 2014
Received in revised form 26 July 2014
Accepted 26 July 2014
Available online 28 August 2014

Keywords:
Incretin
PCSK1
Polymorphism
Type 2 diabetes
Obesity

ABSTRACT

Background: None of the high frequency variants of the incretin-related genes has been found by genome-wide association study (GWAS) for association with occurrence of type 2 diabetes in Japanese. However, low frequency and rare and/or high frequency variants affecting glucose metabolic traits remain to be investigated.

Method: We screened all exons of the incretin-related genes (*GCG*, *GLP1R*, *DPP4*, *PCSK1*, *GIP*, and *GIPR*) in 96 patients with type 2 diabetes and investigated for association of genetic variants of these genes with quantitative metabolic traits upon test meal with 38 young healthy volunteers and with the occurrence of type 2 diabetes in Japanese subjects comprising 1303 patients with type 2 diabetes and 1014 controls.

Result: Two mutations of *GIPR*, p.Thr3Alafsx21 and Arg183Gln, were found only in patients with type 2 diabetes, and both of them were treated with insulin. Of ten tagSNPs, we found that risk allele C of SNP393 (rs6235) of *PCSK1* was nominally associated with higher fasting insulin and HOMA-R ($P = 0.034$ and $P = 0.030$), but not with proinsulin level, incretin level or BMI. The variant showed significant association with occurrence of type 2 diabetes after adjustment for age, sex, and BMI ($P = 0.0043$).

Abbreviations: GWAS, genome-wide association study; GLP-1, glucagon-like peptide 1; GIP, glucose-dependent insulinotropic peptide; GIPR, GIP receptor; GLP1R, GLP-1 receptor; DPP4, dipeptidyl peptidase 4; GCG, proglucagon gene; PCSK1, prohormone convertase (PC) enzymes. PC1/3; PCR, polymerase chain reaction; HbA1c, hemoglobin A1c; BMI, body mass index; SNP, single nucleotide polymorphism; IRI, immunoreactive insulin; CPR, c-peptide immunoreactivity; HOMA-R, homeostasis model assessment as an index of insulin resistance; HOMA-B, homeostasis model assessment as an index of insulin secretion; LD, linkage disequilibrium; OR, odds ratio.

* Corresponding author at: Department of Diabetes and Endocrinology, Gifu University Graduate School of Medicine, 1–1 Yanagido, Gifu-city, Gifu 501–1194, Japan. Fax: +81 58 230 6376.
E-mail address: yhorikaw@gifu-u.ac.jp (Y. Horikawa).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ymgmr.2014.07.009

Conclusion: Rare variants of *GIPR* may contribute to the development of type 2 diabetes, possibly through insulin secretory defects. Furthermore, the genetic variant of *PCSK1* might influence glucose homeostasis by altered insulin resistance independently of BMI, incretin level or proinsulin conversion, and may be associated with the occurrence of type 2 diabetes in Japanese.

1. Introduction

The development of type 2 diabetes involves reduced insulin secretion that cannot compensate for insufficient insulin action. Genes that affect insulin secretion are therefore candidate susceptibility genes for type 2 diabetes. In addition, pancreatic β-cell capacity of insulin secretion is intrinsically lower in Japanese than it is in Caucasian, and genetic factors are thought to contribute [1,2].

The incretin hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), are released after meal from intestinal L-cells and duodenal K-cells, respectively. They potentiate glucose-induced insulin secretion from pancreatic β-cells as well as play a role in β-cell differentiation and growth [3,4] through structurally related G protein-coupled receptors, the GIP receptor (GIPR) and GLP-1 receptor (GLP1R); however, they are rapidly degraded by the enzyme dipeptidyl peptidase 4 (DPP4). In addition, GIP promotes lipid accretion and resistin secretion from adipocytes, leading to progressive impairment of insulin action following long-term GIP administration in rodents fed a high-fat diet [5]. In contrast, GLP-1 induces satiety and sustained GLP1R activation is associated with weight loss in both preclinical and clinical studies, while elimination of GLP1R signaling leads to a reduced number of large islets and enhanced susceptibility to β-cell apoptosis in mice [6].

GLP-1 is post-translationally cleaved from the product of the proglucagon gene (*GCG*), which is expressed not only in L-cells but also in pancreatic α-cells and subsets of neurons in brainstem. Alternative processing of the prohormone is largely attributable to differential expression of prohormone convertase (PC) enzymes. PC1/3 (*PCSK1*) belongs to a family of calcium-dependent serine endoproteases and converts inactive prohormones into biologically active peptide hormones. Its numerous substrates are key hormones and neuropeptides in the regulation of energy metabolism and appetite, and include proopiomelanocortin, proglucagon, and proinsulin. Genetic variants of *PCSK1* have been reported to be associated with increased risk of obesity in several populations [7–10]. In addition, *PCSK1* expression has been shown in α-cells, indicating that GLP-1 is also produced in type 2 diabetic islets [11]. Increased GLP-1 production could represent an attempt to protect the β-cells from apoptosis [12]. The variants of the genes related to GLP-1 and GIP might therefore underlie susceptibility to type 2 diabetes through both defective insulin secretion and action in Japanese. However, none of the high frequency variants of the incretin-related genes has been identified by GWAS for association with occurrence of type 2 diabetes in Japanese.

In this study, we screened all exons of the incretin-related genes (*GCG*, *GLP1R*, *DPP4*, *PCSK1*, *GIP*, and *GIPR*) to detect low frequency functional variants, and examined for association of high frequency genetic variants of the genes with metabolic quantitative traits and the occurrence of type 2 diabetes in Japanese. We also evaluated the plasma levels of incretin hormones after mixed meal in young healthy Japanese volunteers with reference to these genetic variants. This is the first comprehensive investigation of low and high frequency genetic variants of the incretin-related genes and their association with quantitative traits and occurrence of type 2 diabetes in Japanese.

2. Subjects and methods

2.1. Subjects

Type 2 diabetes was diagnosed in accordance with World Health Organization criteria. Other forms of diabetes were excluded based on the clinical data. Inclusion criteria for control subjects were as follows: 1) older than 60 years, 2) glycosylated hemoglobin A1c (HbA1c) values less than 6.6% [NGSP], 48 mmol/mol

[IFCC], and 3) no past history of type 2 diabetes. The sample set consisted of 1303 Japanese patients with type 2 diabetes (M/F, 745/558; age, 60.8 ± 10.6 years; BMI, 24.1 ± 3.9; HbA1c 8.4 ± 2.6% [NGSP], 68.2 ± 28.5 mmol/mol [IFCC]) and 1014 controls (M/F, 437/577; age, 70.8 ± 8.0 years; BMI, 22.3 ± 3.3; HbA1c 5.4 ± 0.4% [NGSP], 35.5 ± 4.3 mmol/mol [IFCC]). Screening for mutations in the coding regions of the incretin-related genes was performed with 96 patients with type 2 diabetes from the 1303 patients with type 2 diabetes described above (M/F, 53/43; age, 60.7 ± 13.7 years; onset age, 50.7 ± 12.8 years; duration 10.0 ± 8.0 years; BMI, 24.1 ± 5.4; HbA1c, 8.8 ± 2.2% [NGSP]). Two mutations, p.Thr3Alafsx21 and p.Arg183Gln, were assessed with a total of 555 Japanese patients with type 2 diabetes from the 1303 patients with type 2 diabetes described above (male/female, 311/244; age, 61.1 ± 10.6 years; onset age, 49.4 ± 12.7 years; duration 12.1 ± 9.0 years; BMI, 23.9 ± 4.0; HbA1c, 8.2 ± 3.5% [NGSP]) When a candidate missense mutation was detected, whether or not it was a rare polymorphism was determined by direct sequencing of 567 normal subjects from the 1014 controls described above (male/female 226/341; age, 67.4 ± 6.0 years; BMI, 23.0 ± 2.9 kg/m^2; HbA1c, 5.4 ± 0.4% [NGSP]). The plasma level of incretin after meal test was then measured in another 38 young healthy volunteers (male/female, 23/15; age, 30.8 ± 6.9 years; BMI, 20.9 ± 2.2, HbA1c 5.6 ± 0.19% [NGSP], 33.3 ± 2.0 mmol/mol [IFCC]). Stratification among cases and controls was not detected, as described previously [13]. (Table 1).

The study protocol was approved by the Institutional Review Board of Gifu University (no. 25–153). Written informed consent was obtained from all participants.

2.2. Mutation screening of incretin-related genes

For mutation screening of incretin-related genes, we examined all exons of six genes, *GCG*, *GLP1R*, *DPP4*, *PCSK1*, *GIP*, and *GIPR*, in 96 patients with type 2 diabetes by direct sequencing of the amplified polymerase chain reaction (PCR) products, using specific primer pairs and an ABI PRISM BigDye Terminator Cycle Sequencing FS ready Reaction Kit (Applied Biosystems, Foster City, CA). The PCR reaction conditions were an initial denaturation step of 94 °C for 1 min and a subsequent 35 cycles of reaction at 94 °C for 30 s, 55–64 °C for 30 s, and 72 °C for 1 min. The sequencing reactions were analyzed by automatic DNA sequencers (Applied Biosystems model 3130). When a candidate missense mutation was detected, whether or not it was a rare polymorphism was determined by direct sequencing of 567 normal subjects and also by protein functionality prediction programs such as SIFT (http://sift.bii.a-star.edu.sg/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), even though these programs disclose only whether these mutations are likely to be deleterious or not.

2.3. Association of the variants of incretin-related genes with quantitative traits including incretin level

We then measured plasma glucose, C-peptide, GLP-1 (active and total forms), and GIP (total form) at fasting and 30 min after ingestion of mixed meal (460 kcal, 56.5 g of carbohydrates, 18 g of protein, and 18 g of fat) in 38 young healthy volunteers. Plasma insulin and proinsulin were also measured at fasting.

Table 1
Clinical features of subjects examined in this study.

		N (% of male)	Age (yrs)	BMI (kg/m^2)	HbA1c (%) [NGSP] (mmol/mol) [IFCC]
Case–control subjects	Case for association	1303 (57.2)	60.8 ± 10.6	24.1 ± 3.9	8.4 ± 2.6 68.2 ± 28.5
	Case for exome	96 (55.2)	60.7 ± 13.7	24.1 ± 5.4	8.8 ± 2.2
		555* (56.0)	61.1 ± 10.6	23.9 ± 4.0	8.2 ± 3.5
	Control for association	1014 (43.1)	70.8 ± 8.0	22.3 ± 3.3	5.4 ± 0.4 35.5 ± 4.3
	Control for exome	567 (39.9)	67.4 ± 6.0	23.0 ± 2.9	5.4 ± 0.4
Young healthy volunteer		38 (60.5)	30.8 ± 6.9	20.9 ± 2.2	5.6 ± 0.2 33.3 ± 2.0

* For the two mutations, p.Thr3Alafsx21 and p.Arg183Gln.

Blood was drawn into a BD™ P700 tube (Becton, Dickinson and Company) containing a proprietary Dipeptidyl Peptidase-IV (DPP-IV) protease inhibitor. Plasma was collected after centrifugation and stored at −80 °C. The samples were measured by ELISA Kit (GLP-1 (Active) ELISA Kit; Millipore, Total GLP-1 Kit; Meso Scale Discovery, Human GIP (total) ELISA Kit; Millipore). Active GLP-1 was measured after preparation of solid phase extraction using Oasis HLB flangeless cart (Waters Corporation).

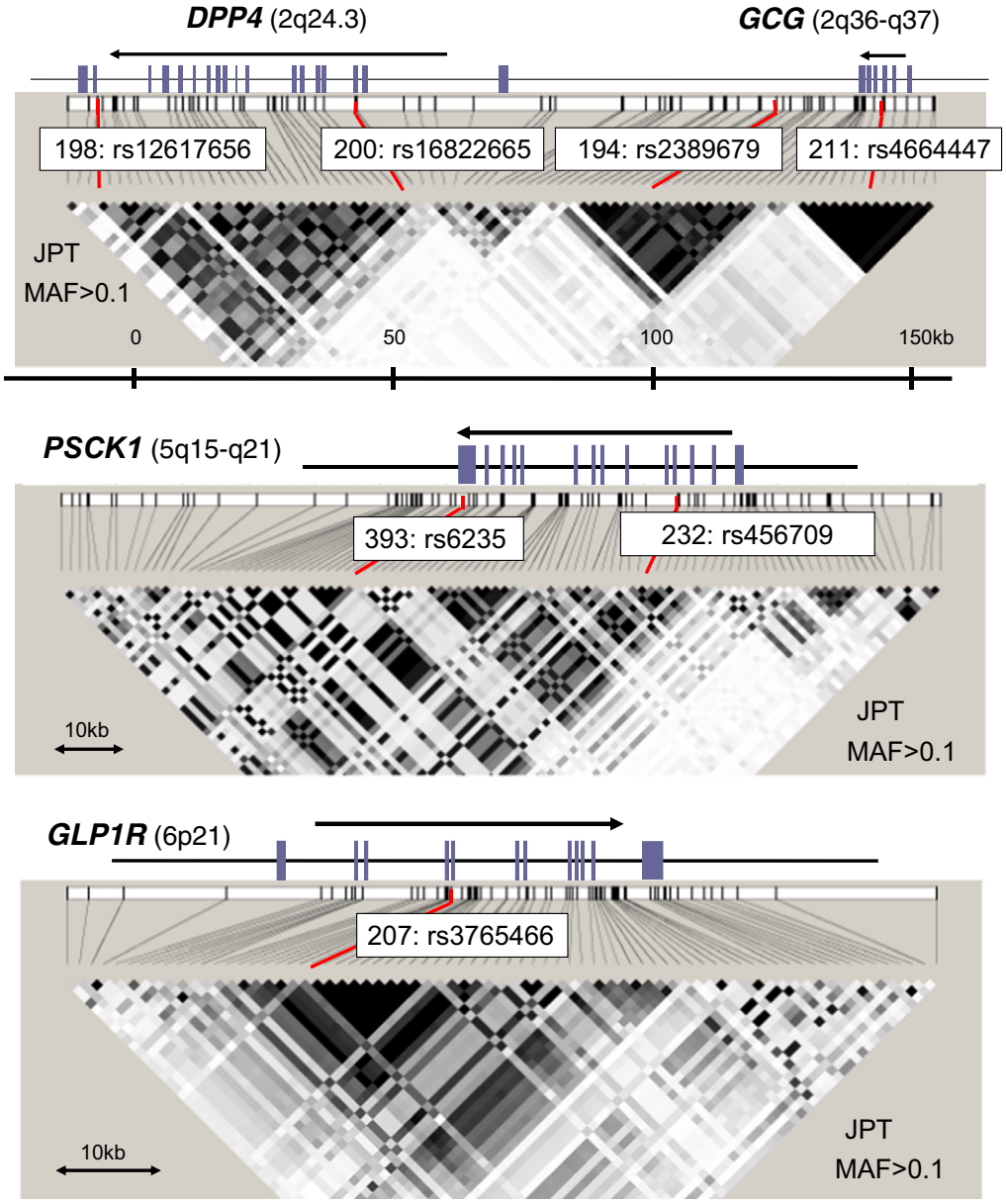

Fig. 1. The pattern of linkage disequilibrium in the incretin-related genes examined in this study. LD color schemes are designated as follows: $r^2 = 0$: white; $0 < r^2 < 1$: shades of gray; $r^2 = 1$: black.

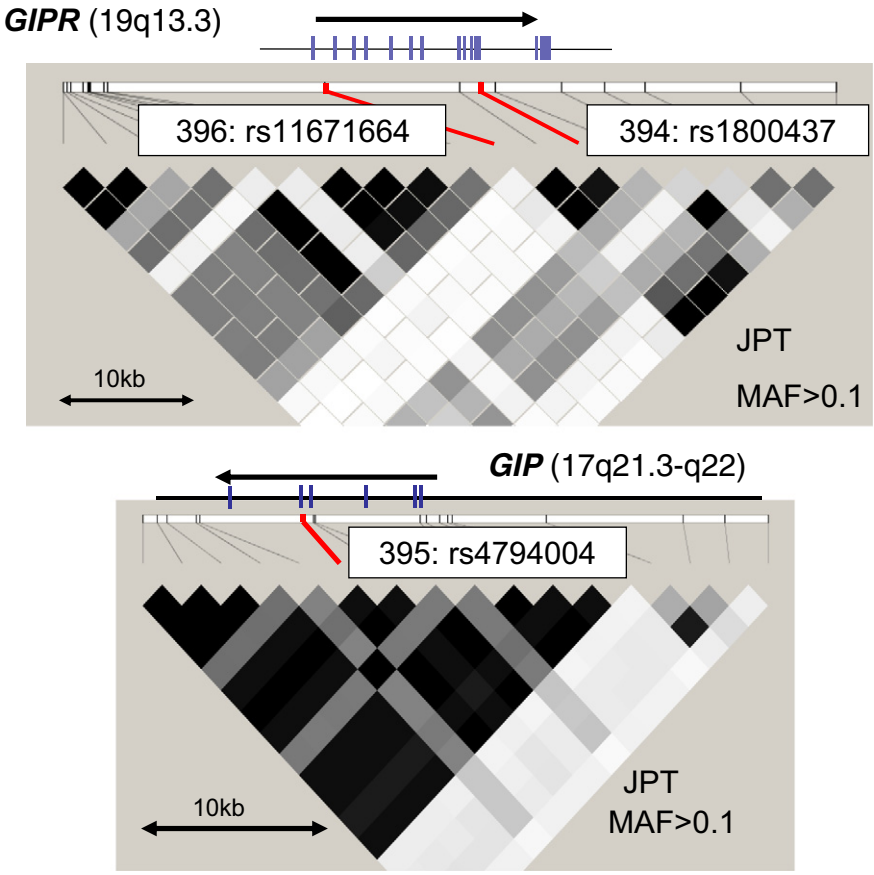

Fig. 1 (*continued*).

2.4. Association study of the genetic variations with occurrence of type 2 diabetes

For association studies, we selected 10 tag-SNPs based on linkage disequilibrium (LD) pattern determined by HapMap data of 45 unrelated Japanese (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap24_B36/#search) using Haploview (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview) (Fig. 1). We first downloaded the SNP data of the Japanese from Hapmap Data Rel 27 Phase II + III and estimated LD blocks by Haploview program, and then selected each SNP from each LD block where r^2 of all possible combinations of SNPs in the database was more than 0.7. We also selected only SNPs in the neighboring regions of which there were no other SNPs, to avoid mistyping by TaqMAN assay. The ten SNPs consisted of one SNP of *GCG*, two SNPs of *DPP4*, one SNP of inter-region between *GCG* and *DPP4*, one SNP of *GLP1R*, two SNPs of *PCSK1*, one SNP of *GIP*, and two SNPs of *GIPR*. The SNP 393 (rs 6235) of *PCSK1*, Ser690Thr, was first reported to be associated with obesity in Caucasians [7] and the SNP394 (rs1800437) of *GIPR* is in strong linkage disequilibrium with the SNP rs10423928, which is reported to be associated with the plasma level of 2 h glucose and the index of insulin response during OGTT [14]. These SNPs were genotyped using Taqman SNP Genotyping Assays (Applied Biosystems, Foster City, CA) in Japanese subjects comprising 1303 patients with type 2 diabetes and 1014 controls.

Table 2
Missense and frameshift mutations of the incretin-related genes examined in this study.

Gene	Missense mutation	SNP ID	Location	MAF (T2DM)	MAF (control)
GLP1R	p.Pro7Leu	rs10305420	ex1	0.16	0.22
	p.Arg44His	rs2295006	ex2	0.017	0.011
	p.Arg131Gln	rs3765467	ex4	0.17	0.13
	p.Val194Ile	New	ex6	0.0057	0.011
	p.Leu260Phe	rs1042044	ex7	0.47	0.42
DPP4	p.Arg658Gln	rs138430103	ex22	0.0057	0.00090
GCG	n.d.				
PCSK1	p.Ser690Thr	rs6235	ex14	0.28	0.20
	p.Gln665Glu	rs6234	ex14	0.27	0.20
	p.Arg80Gln	rs1799904	ex2	0.024	0.016
GIP	p.Ser103Gly	rs271920	ex4	0.24	0.27
	p.Thr145Arg	New	ex5	0.0059	0
GIPR	p.Thr3Alafsx21	New	ex2	0.0072*	0
	p.Arg136Trp	rs13306402	ex6	0.012	0.011
	p.Arg183Gln	rs199583937	ex7	0.0018*	0
	p.Gly198Cys	rs13306398	ex7	0.029	0.014
	p.Ala207Gly	New	ex7	0.0057	0.0018
	p.Leu262His	rs200562041	ex8	0.0060	0.0057
	p.Glu354Gln	rs1800437	ex12	0.26	0.26
	p.Glu463Gln	rs13306395	ex14	0.011	0.016

DM: n = 96, CONT: n = 567.
n.d.: not detected.
* n = 555, for the two mutations, p.Thr3Alafsx21 and p.Arg183Gln.

2.5. Reporter assay of the region including the variant SNP232 (rs456709) of PCSK1

Approximately 500 bp of the region at which SNP232 (rs456709) is centered was amplified with template genomic DNA from subjects with the known genotype of the SNP, and the amplicons were ligated into pGL3-Promoter vector (Promega, Madison, WI). The constructs, which included either the C or T allele of SNP232, were then used to transfect MIN6m9 cells [15]. Specifically, 2 μg of purified DNA was co-transfected with 0.2 μg pRL-TK (Promega) DNA. The Dual-Luciferase Reporter Assay Kit (Promega) was used for reporter gene activities and the results are reported as the ratio of firefly to *Renilla* luciferase light units [16].

2.6. Statistical analysis

All data were first examined to determine whether they were distributed normally. Mann–Whitney *U* test and Student's *t*-test were performed for non-parametric and parametric analyses for comparison between the two groups, respectively. Differences in phenotypic values for genotype were examined using Kruskal–Wallis analysis or by ANOVA in co-dominant, dominant, and recessive models. Differences in allele frequencies of SNPs between type 2 diabetes patients and control subjects were compared using chi-square test. Logistic regression analysis under the additive model was performed with adjustment for age, sex, and BMI. Statistical significance was defined as $P < 0.05$ for single test and $P < 0.005$ ($= 0.05/10$) for multiple test by Bonferroni correction. Statistical analysis was performed with Statview 5.0 software (SAS Institute, Inc., Cary, NC).

3. Results

3.1. Mutation screening of the incretin related genes

Screening of all exons of the incretin-related genes (*GCG*, *GLP1R*, *DPP4*, *PCSK1*, *GIP*, and *GIPR*) by direct sequencing resulted in identification of eighteen missense mutations and one frameshift mutation in *GIPR*, p.Thr3Alafsx21, including four novel mutations, in 96 patients with type 2 diabetes (Table 2). Of the

eighteen missense mutations, two of them (GIPR, p.Arg183Gln; GIP, p.Thr145Arg) were not identified in control subjects. The Arg183Gln mutation of GIPR (rs199583937) is predicted to be deleterious by both SIFT and PolyPhen2 programs, but the Thr145Arg mutation of GIP is predicted to be benign and could be a rare polymorphism. There were no significant differences in the frequency of the other sixteen missense mutations. Regarding the two mutations, p.Thr3Alafsx21 and p.Arg183Gln, we assessed the additional diabetic subjects to further evaluate their frequency and contribution to T2DM in Japanese. The mutation p.Thr3Alafsx21 was found in four of 555 patients with type 2 diabetes, while the mutation p.Arg183Gln was found in one of 555 patients. All SNPs identified during the screening and the 10 tag-SNPs are listed in the Supplemental Table.

One of the patients with the novel frame-shift mutation (p.Thr3Alafsx21) of *GIPR* had died of heart attack at 68 years of age. He had been treated with insulin and received CABG operation at 62 years of age because of triple coronary vessel disease. His younger brother and sister had no overt diabetes. His mother died of heart attack in her forties and it is not known whether or not she had diabetes. There was no detailed clinical information regarding the other three patients. The patient with the missense mutation (p.Arg183Gln) of *GIPR* had overt diabetes at 46 years of age and showed an extremely reduced insulin secretory capacity of urinary CPR of 6.4 (μg/day) at 64 years of age without severe diabetic complications. However, because he was referred to another hospital, it was not possible to obtain the current clinical information.

3.2. Association of the variants of incretin-related genes with quantitative traits including incretin level

The ten tagSNPs were not associated with any of the incretin levels after mixed meal in 38 young healthy volunteers (data not shown). The minor C allele of SNP393 (rs6235) was nominally associated with higher fasting insulin, but not with proinsulin level, and also with HOMA-R ($P = 0.034$ and $P = 0.030$) (Tables 3a, 3b). These findings were not altered by adjustment for age, sex, and BMI ($P = 0.014$ and $P = 0.014$), but did not reach statistical significance by Bonferroni correction. The two variants of *PCSK1*, SNP232 (rs456709) and SNP393 (rs6235), were not associated with BMI in 1014 control subjects (Table 4), and were not further stratified by sex (data not shown).

Table 3a
Association of SNP393 (rs6235) with incretin level in healthy young volunteers.

		Average (±SD)			
		GG (n = 21)	GC (n = 16)	CC (n = 1)	*P*-value
Total GLP1 (pmol/L)	Fasting	5.3 (±2.4)	5.3 (±2.7)	7.3	0.73
	Postprandial	10.0 (±2.9)	10.3 (±4.2)	11.4	0.91
		4.7 (±2.6)	5.0 (±2.0)	4.1	0.90
Active GLP1 (pmol/L)	Fasting	0.9 (±0.3)	1.0 (±0.5)	1.42	0.30
	Postprandial	2.9 (±1.3)	3.1 (±1.2)	3.9	0.72
		2.1 (±1.2)	2.1 (±1.0)	2.4	0.94
Total GIP (pg/ml)	Fasting	49.6 (±27.1)	74.3 (±75.3)	31.8	0.62**
	Postprandial	505.2 (±188.6)	430.2 (±115.5)	347.9	0.35**
		455.6 (±188.1)	355.9 (±101.9)	316.0	0.22**
IRI (fasting)(pmol/L)		43.8 (±13.9)	55.6 (±18.1)	42.4	0.08
Proinsulin (fasting)(pmol/L)		6.2 (±3.1)	8.6 (±4.8)	9.7	0.26*
PI ratio (fasting)		0.16 (±0.11)	0.16 (±0.07)	0.23	0.68***
CPR (nmol/L)	Fasting	0.40 (±0.13)	0.43 (±0.13)	0.53	0.41
	Postprandial	1.6 (±0.53)	1.6 (±0.47)	2.2	0.53
		1.2 (±0.50)	1.2 (±0.37)	1.7	0.6
HOMA-R		1.5 (±0.49)	1.9 (±0.65)	1.47	0.066
HOMA-β		69.0 (±23.3)	84.3 (±29.0)	62.4	0.19

PI ratio: proinsulin/insulin ratio.
* Kruskal–Wallis analysis.
** Result from transformation from X to log(X + 1).
*** Result from transformation from X to sqr(x) + sqr(x + 1).

3.3. Association of genetic variations with occurrence of type 2 diabetes

All variants examined in this study were not deviated from Hardy–Weinberg equilibrium. The minor C allele of SNP393 (rs6235) of *PCSK1* was associated with increased risk of occurrence of type 2 diabetes only with adjustment for age, sex, and BMI by logistic regression analysis ($P = 0.0043$). The major C allele of SNP232 (rs456709) of *PCSK1* showed a tendency of association with increased risk of occurrence of type 2 diabetes in the dominant model ($P = 0.049$) and also by logistic regression analysis with adjustment for age, sex, and BMI ($P = 0.044$) (Table 5). The power (1-β) estimation of the present study for SNP232 (rs456709) and SNP393 (rs6235) at $\alpha = 0.05$ was 53% and 37%, respectively.

3.4. Reporter assay of the region containing SNP 232 (rs 456709) of PCSK1

SNP393 (rs6235) of *PCSK1* resulted in the missense mutation Ser690Thr, which has been reported not to affect its enzymatic activity [8]. Functional analysis of SNP232 (rs456709) of *PCSK1* was therefore performed. Since SNP232 (rs456709) is located at intron3 of *PCSK1* and is not in complete linkage disequilibrium with any of the missense mutations of *PCSK1*, we performed luciferase assay to determine whether or not it might affect transcriptional activity. The major C allele of SNP232 (rs456709) is conserved among human, mouse, and rat, and showed significantly lower transcriptional activity compared to that of the T allele ($P < 0.05$) (Fig. 2), but no transcriptional factors were detected at the region around SNPS 232 by the TF search program (www.cbrc.jp/research/db/TFSEARCH.html).

4. Discussion

Genome-wide association study (GWAS) using high frequency variants have identified about 60 established susceptibility loci so far; however, at best, 10% of the observed heritability of type 2 diabetes has been captured [17]. Low frequency and rare variants in coding regions with a relatively larger effect on phenotypes remain to be discovered, and also high frequency variants that affect glucose metabolic traits other than the onset of type 2 diabetes need to be examined.

Table 3b
Association of SNP393 (rs6235) with incretin level in healthy young volunteers.
Dominant model for risk allele C.

		Average (±SD)		
		GG (n = 21)	GC + CC (n = 17)	*P*-value
Total GLP1 (pmol/L)	Fasting	5.3 (±2.4)	5.4 (±2.7)	0.88
	Postprandial	10.0 (±2.9)	10.3 (±4.1)	0.76
		4.7 (±2.6)	4.9 (±2.0)	0.77
Active GLP1 (pmol/L)	Fasting	0.87 (±0.29)	1.04 (±0.52)	0.37*
	Postprandial	2.9 (±1.3)	3.2 (±1.2)	0.55
		2.1 (±1.2)	2.1 (±0.94)	0.83
Total GIP (pg/ml)	Fasting	49.6 (±27.1)	71.8 (±73.6)	0.49**
	Postprandial	505.2 (±188.6)	425.3 (±113.6)	0.18**
		455.6 (±188.1)	353.6 (±99.2)	0.081**
IRI (fasting) (pmol/L)		43.8 (±13.9)	54.9 (±18.1)	0.034
Proinsulin (fasting) (pmol/L)		6.2 (±3.1)	8.6 (±4.7)	0.15*
PI ratio (fasting)		0.16 (±0.11)	0.16 (±0.071)	0.67***
CPR (nmol/L)	Fasting	0.40 (±0.13)	0.47 (±0.13)	0.22
	Postprandial	1.6 (±0.53)	1.7 (±0.47)	0.66
		1.2 (±0.50)	1.2 (±0.37)	0.89
HOMA-R		1.5 (±0.49)	1.9 (±0.64)	0.030
HOMA-β		69.0 (±23.3)	83.0 (±28.6)	0.10

PI ratio: proinsulin/insulin ratio.
* Mann–Whitney analysis.
** Result from transformation from X to log(X + 1).
*** Result from transformation from X to sqr(x) + sqr(x + 1).

Table 4
Association of the two variants of *PCSK1* with BMI.
n = 1014 controls.

SNP393	GG/GC/CC		GG + GC/CC		GG/GC + CC	
	ANOVA	ANCOVA[#]	Student's *t*-test	ANCOVA[#]	Student's *t*-test	ANCOVA[#]
P-value (rs6235)	0.17	0.54	0.67	0.28	0.10	0.66
SNP232	CC/CT/TT		CC + CT/TT		CC/CT + TT	
	ANOVA	ANCOVA[#]	Student's *t*-test	ANCOVA [#]	Student's *t*-test	ANCOVA [#]
P-value (rs456709)	0.54	0.50	0.98	0.22	0.30	0.44

[#] By ANCOVA with adjustment for age and sex.

We screened all exons of the incretin-related genes *GCG*, *GLP1R*, *GIP*, *GIPR*, *DPP4*, and *PCSK1*, and identified one novel frameshift mutation and one missense mutation in GIPR only in patients with type 2 diabetes. These two patients showed insulin secretory defects, suggesting a relationship of GIPR to insulin secretion and β-cell mass as previously reported [4,14], but we could not investigate the details due to insufficient clinical information. Further study is required to find low frequency functional variations residing in coding regions.

In this study, we identified two alleles of *PCSK1*, the major allele C of SNP232 (rs456709) and the minor allele C of SNP393 (rs6235), that show nominal and significant association with increased risk of the occurrence of type 2 diabetes, respectively. Although 21 SNPs of the *PCSK1* region were genotyped in the previous GWAS for type 2 diabetes in Japanese, SNP393 (rs6235) was not included. The closest SNP (rs3811942) is about 0.4 kb away from this variant, but was not in linkage disequilibrium (LD) ($r^2 = 0.047$). On the other hand, while SNP232 (rs456709) was genotyped in the previous GWAS for type 2 diabetes in Japanese, it did not show significant association with the onset of type 2 diabetes ($P = 0.724$) [13].

The minor alleles of the common non-synonymous variants rs6232 (N221D) and rs6235 (S690T) have been reported to be associated with an increased risk of obesity in several populations, and with the proinsulin level as well [18]. Recently, one variant (rs261967), which is located near the *PCSK1* gene, has been identified as a BMI-associated polymorphism in meta-analysis of GWAS with East Asians [19]. However, the variant (rs261967) is located 81.3 kb upstream of *PCSK1* and is not in LD with SNPs rs6232 or rs6235. In this study, we were unable to find a significant association of either SNP232 (rs456709) or SNP393 (rs6235) with BMI in Japanese controls, which also was the case after stratification by sex [20,21]. Bi-allelic mutations in *PCSK1* have been found to lead to human congenital PC1/3 deficiency, a syndrome characterized by obesity, and to abnormal glucose homeostasis with elevated circulating levels of certain prohormones [22,23]. Thus, the complexities of genetic background among races might significantly affect the clinical phenotype, especially BMI [24].

In addition, the minor C allele of rs6235 has been reported to affect the serum level of incretin hormones [20]. We therefore examined whether or not these two SNPs affect the plasma level of incretin after meal test. Neither the major risk C allele of SNP232 (rs456709) nor the minor C allele of SNP393 (rs6235) showed any association with the plasma incretin levels, although the risk allele C of SNP232 (rs456709) reduced the transcriptional activity. Considering that the minor C allele of SNP393 (rs6235) is associated with a higher fasting insulin level and HOMA-R in young healthy controls and also with the occurrence of type 2 diabetes, this risk allele might well be associated with insulin resistance, especially hepatic insulin resistance, at youth that evolves to decreased insulin secretion and diabetes later in life, partly due to the lower intrinsic β-cell capacity of insulin secretion in Japanese. On the other hand, the variant (rs6235) is associated with an increased risk of obesity rather than onset of type 2 diabetes in Caucasians, whose intrinsic capacity of insulin secretion is relatively high.

Since SNP232 (rs456709) and SNP393 (rs6235) are in strong linkage disequilibrium with each other in both Caucasian and Japanese ($D' = 1.0$ and $D' = 0.71$), the two variants might well interact. We therefore examined the effects of haplotypes comprising SNP393 (rs6235) and SNP232 (rs456709) on clinical features such as BMI and glucose and insulin level; none of these was significantly affected in Japanese (data not shown).

Table 5
Association of the ten tagSNPs of the incretin-related genes with onset of type 2 diabetes.

Gene	SNP ID	T2DM (n = 1303)					CONT (n = 1014)					Genotype	Allele	WW + WM/MM	WW/WM + MM	Odds Ratio (OR)		
		WW	WM	MM	MAF	HWE (*p*)	WW	WM	MM	MAF	HWE (*p*)	(*P*)	(*P*)	(*P*)	MM (*P*)	*P*#	OR	95%CI
DPP4	198	727	472	68	0.24	0.45	579	344	63	0.24	0.22	0.36	0.90	0.30	0.52	0.35	1.08	0.92–1.27
DPP4	200	727	478	76	0.25	0.83	597	337	66	0.23	0.05	0.19	0.37	0.51	0.16	0.084	1.15	0.91–1.35
Intergenic	194	496	560	181	0.37	0.26	406	429	127	0.35	0.42	0.49	0.23	0.34	0.32	0.31	1.08	0.93–1.24
GCG	211	710	464	70	0.24	0.61	597	336	48	0.22	0.93	0.19	0.08	0.44	0.072	0.41	1.07	0.91–1.26
PCSK1	232	366	628	254	0.46	0.86	267	473	240	0.49	0.29	0.14	0.075	0.049	0.34	0.044	1.15	1.00–1.32
PCSK1	393	745	449	72	0.23	0.69	624	321	48	0.21	0.42	0.15	0.052	0.37	0.054	0.0043	1.27	1.08–1.50
GLP1R	207	456	575	209	0.40	0.23	353	460	152	0.40	0.92	0.74	0.76	0.49	0.93	0.80	1.02	0.89–1.17
GIP	395	717	466	80	0.25	0.71	538	359	81	0.27	0.057	0.20	0.16	0.077	0.41	0.10	1.14	0.97–1.33
GIPR	396	457	586	219	0.41	0.19	337	439	194	0.43	0.020	0.28	0.17	0.11	0.47	0.45	1.05	0.92–1.21
GIPR	394	763	413	78	0.23	0.03	597	342	43	0.22	0.50	0.13	0.48	0.056	0.98	0.85	1.02	0.86–1.20

W: major allele; M: minor allele; MAF: Minor allele frequency; OR: ORs for risk alleles; #: adjusted by age, sex and BMI.

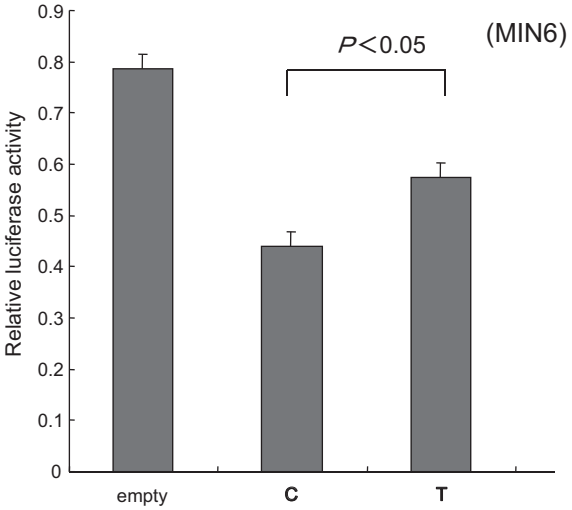

Fig. 2. Reporter assay of the region containing SNP 232 (rs 456709) of *PCSK1*. C: major allele (risk allele), T: minor allele.

Regarding limitations of the study, the sample number is too small to gain enough power to detect the effects of these variants on clinical phenotypes. In addition, these two SNPs of *PCSK1* have not been examined for their effects on the glucagon level. We did not attempt to measure the glucagon level in this study because of the many inconsistencies among the various commercial kits, often due to contamination of other excised products from proglucagon such as glicentin. Further studies investigating food intake and other hormones related to appetite such as α-MSH with larger sample size are required to elucidate the mechanism by which *PCSK1* increases the risk of occurrence of type 2 diabetes.

In conclusion, rare variants of *GIPR* may contribute to the development of type 2 diabetes in Japanese, possibly through insulin secretory defects. In addition, the variant of *PCSK1* might influence glucose homeostasis by altered insulin resistance independently of BMI and incretin level, and thus be associated with occurrence of type 2 diabetes rather than obesity in Japanese.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgmr.2014.07.009.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

We thank Dr Y. Hirota and S. Seino (Kobe University, Kobe, Japan) for providing some of the DNA samples, and H. Tsuchida, K. Yokoyama, and J. Kawada for their technical assistance. This work was supported by a Health and Labor Science Research Grant for Research on Rare and Intractable Diseases from the Japanese Ministry of Health, Labor and Welfare (ID. 12944234), a Grant-in-Aid for Scientific Research from the Japanese Ministry of Science, Education, Sports, Culture and Technology (ID. 13374321, 14499754), and a Strategic International Research Cooperative Program Grant from Japan Science and Technology Agency (ID. 10104011).

References

[1] M. Fukushima, H. Suzuki, Y. Seino, Insulin secretion capacity in the development from normal glucose tolerance to type 2 diabetes, Diabetes Res. Clin. Pract. 66 (2004) S37–S43.
[2] Y. Horikawa, K. Miyake, K. Yasuda, M. Enya, Y. Hirota, K. Yamagata, et al., Replication of genome-wide association studies of type 2 diabetes susceptibility in Japan, J. Clin. Endocrinol. Metab. 93 (2008) 3136–3141.
[3] J.A. Lovshin, D.J. Drucker, Incretin-based therapies for type 2 diabetes mellitus, Nat. Rev. Endocrinol. 5 (2009) 262–269.

[4] K. Prasadan, M. Koizumi, S. Tulachan, C. Shiota, N. Lath, J. Paredes, et al., The expression and function of glucose-dependent insulinotropic polypeptide in the embryonic mouse pancreas, Diabetes 60 (2011) 548–554.
[5] K. Miyawaki, Y. Yamada, N. Ban, Y. Ihara, K. Tsukiyama, H. Zhou, et al., Inhibition of gastric inhibitory polypeptide signaling prevents obesity, Nat. Med. 8 (2002) 738–742.
[6] D.J. Drucker, The biology of incretin hormones, Cell Metab. 3 (2006) 153–165.
[7] M. Benzinou, J.W. Creemers, H. Choquet, S. Lobbens, C. Dina, E. Durand, et al., Common nonsynonymous variants in PCSK1 confer risk of obesity, Nat. Genet. 40 (2008) 943–945.
[8] H. Choquet, J. Kasberger, A. Hamidovic, E. Jorgenson, Contribution of common PCSK1 genetic variants to obesity in 8,359 subjects from multi-ethnic American population, PLoS One 8 (2013) e57857.
[9] M. Villalobos-Comparán, H. Villamil-Ramírez, T. Villarreal-Molina, E. Larrieta-Carrasco, P. León-Mimila, S. Romero-Hidalgo, et al., PCSK1 rs6232 is associated with childhood and adult class III obesity in the Mexican population, PLoS One 7 (2012) e39037.
[10] Y.C. Chang, Y.F. Chiu, K.C. Shih, M.W. Lin, W.H. Sheu, T. Donlon, et al., Common PCSK1 haplotypes are associated with obesity in the Chinese population, Obesity (Silver Spring) 18 (2010) 1404–1409.
[11] G. Kilimnik, A. Kim, D.F. Steiner, T.C. Friedman, M. Hara, Intraislet production of GLP-1 by activation of prohormone convertase 1/3 in pancreatic α-cells in mouse models of β-cell regeneration, Islets 2 (2010) 149–155.
[12] P. Marchetti, R. Lupi, M. Bugliani, C.L. Kirkpatrick, G. Sebastiani, F.A. Grieco, et al., A local glucagon-like peptide 1 (GLP-1) system in human pancreatic islets, Diabetologia 55 (2012) 3262–3272.
[13] K. Yasuda, K. Miyake, Y. Horikawa, K. Hara, H. Osawa, H. Furuta, et al., Variants in KCNQ1 are associated with susceptibility to type 2 diabetes mellitus, Nat. Genet. 40 (2008) 1092–1097.
[14] R. Saxena, M.F. Hivert, C. Langenberg, T. Tanaka, J.S. Pankow, P. Vollenweider, et al., Genetic variation in *GIPR* influences the glucose and insulin responses to an oral glucose challenge, Nat. Genet. 42 (2010) 142–148.
[15] K. Minami, H. Yano, T. Miki, K. Nagashima, C.Z. Wang, H. Tanaka, et al., Insulin secretion and differential gene expression in glucose-responsive and -unresponsive MIN6 sublines, Am. J. Physiol. Endocrinol. Metab. 279 (2000) E773–E781.
[16] N. Yamada, Y. Horikawa, N. Oda, K. Iizuka, N. Shihara, S. Kishi, et al., Genetic variation in the hypoxia-inducible factor-1alpha gene is associated with type 2 diabetes in Japanese, J. Clin. Endocrinol. Metab. 90 (2005) 5841–5847.
[17] A.P. Morris, B.F. Voight, T.M. Teslovich, T. Ferreira, A.V. Segrè, V. Steinthorsdottir, et al., Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes, Nat. Genet. 44 (2012) 981–990.
[18] M. Heni, A. Haupt, S.A. Schäfer, C. Ketterer, C. Thamer, F. Machicao, et al., Association of obesity risk SNPs in PCSK1 with insulin sensitivity and proinsulin conversion, BMC Med. Genet. 11 (2010) 86, http://dx.doi.org/10.1186/1471-2350-11-86.
[19] W. Wen, Y.S. Cho, W. Zheng, R. Dorajoo, N. Kato, L. Qi, et al., Meta-analysis identifies common variants associated with body mass index in east Asians, Nat. Genet. 44 (2012) 307–311.
[20] A.P. Gjesing, M.A. Vestmar, T. Jørgensen, M. Heni, J.J. Holst, D.R. Witte, et al., The effect of PCSK1 variants on waist, waist–hip ratio and glucose metabolism is modified by sex and glucose tolerance status, PLoS One 6 (2011) e23907.
[21] T.J. Hsiao, Y. Hwang, H.M. Chang, E. Lin, Association of the rs6235 variant in the proprotein convertase subtilisin/kexin type 1 (PCSK1) gene with obesity and related traits in a Taiwanese population, Gene 533 (2014) 32–37.
[22] R.S. Jackson, J.W. Creemers, S. Ohagi, M.L. Raffin-Sanson, L. Sanders, C.T. Montague, et al., Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene, Nat. Genet. 16 (1997) 303–306.
[23] I.S. Farooqi, K. Volders, R. Stanhope, R. Heuschkel, A. White, E. Lank, et al., Hyperphagia and early-onset obesity due to a novel homozygous missense mutation in prohormone convertase 1/3, J. Clin. Endocrinol. Metab. 92 (2007) 3369–3373.
[24] Y. Horikawa, M. Enya, N. Fushimi, Y. Fushimi, J. Takeda, Screening of diabetes of youth for hepatocyte nuclear factor 1 mutations: clinical phenotype of HNF1β-related maturity-onset diabetes of the young and HNF1α-related maturity-onset diabetes of the young in Japanese, Diabet. Med. 31 (2014) 721–727.