

FIG. 3. Effects of TE treatment on glucose oxidation in Wistar (A) and GK (B) islets. Islets were cultured without (control [C]; open bars) or with TE (black bars) for 12 h. After cultured islets were preincubated with 2.8 mmol/L glucose for 30 min, glucose oxidation during incubation for 90 min in the presence of 2.8 and 16.7 mmol/L glucose was measured in islets. Data are shown as means \pm SE of 12 determinations from three (A) and four (B) experiments. * $P < 0.01$ vs. corresponding 2.8 mmol/L glucose; † $P < 0.01$ vs. 16.7 mmol/L glucose in control.

coupling in β -cells (30,31). However, regulation of UCP2 expression may not play an important role in the mechanism, as the UCP2 expression level was not affected in GK islets by TE treatment. Western blot analysis revealed that the expression of respiratory chain proteins, including complex I, III, IV, and V, was not affected by TE treatment. In addition, the relative mRNA levels of nuclear factors, including *NRF-1*, *NRF-2*, and *TFAM*, which affect mitochondrial biogenesis, also were not altered by TE treatment (data not shown). These results indicate that the regulation of mitochondrial mass is not involved in the mechanism.

TABLE 4
ATP production by mitochondria fraction from islets

Experimental conditions	Wistar rat		GK rat	
	C	TE	C	TE
0.5 mmol/L succinate	2.86 \pm 0.42	2.58 \pm 0.15	2.43 \pm 0.27	2.18 \pm 0.21
0.5 mmol/L succinate + 1 μ mol/L antimycin A	0.40 \pm 0.03	0.46 \pm 0.02	0.36 \pm 0.00	0.36 \pm 0.00
1 mmol/L succinate + 1 μ mol/L rotenone	3.96 \pm 0.48	3.55 \pm 0.26	2.92 \pm 0.22	2.71 \pm 0.48
1 mmol/L glutamate + 1 mmol/L malate	0.92 \pm 0.23	0.86 \pm 0.14	0.87 \pm 0.13	0.90 \pm 0.14
1 mmol/L pyruvate + 1 mmol/L malate	1.32 \pm 0.38	1.17 \pm 0.29	1.27 \pm 0.22	1.23 \pm 0.22
0.5 mmol/L TMPD + 2 mmol/L ascorbate	5.42 \pm 0.41	4.91 \pm 0.15	6.27 \pm 0.62	5.94 \pm 0.54
1 mmol/L G3P	0.92 \pm 0.14	3.03 \pm 0.17†	0.69 \pm 0.05	1.50 \pm 0.12†

Islets were cultured with or without TE for 12 h. Mitochondrial suspension was obtained from control (C) or TE-treated islets (TE). Mitochondrial ATP production is indicated as the ratio to ATP production from adenylate kinase to normalize the mass of the intact mitochondria obtained, which was determined from the same sample in parallel incubation. Data are given as the mean \pm SE of three different experiments. † $P < 0.01$ vs. control without TE.

ATP production is driven by the H^+ gradient across the mitochondrial membrane generated by the transport of high-energy electrons in the respiratory chain. These electrons are derived from NADH and $FADH_2$, derived from the Krebs cycle in the matrix and/or transferred from the cytosol by the shuttle system. To determine which step in mitochondrial carbohydrate metabolism is affected in TE-treated islets, ATP production in the presence of various substrates and inhibitors was measured using mitochondrial fractions from the cultured islets. In the presence of G3P, ATP production was increased by TE treatment in both the Wistar and GK mitochondrial fraction. Transfer of a reduced equivalent from cytosol to mitochondria by the glycerol phosphate shuttle may well play a more important role in mitochondrial ATP production in islets than in other tissues, since the activity of mGPDH, a key enzyme in the glycerol phosphate shuttle is ~ 60 -fold higher in islets than in other tissues (24). In addition, decreased activity is observed in diabetic islets (32). Although an increase in the activity of mGPDH by TE treatment was detected, the increase was similar in the mitochondrial fraction from both GK and Wistar islets. Therefore, an improvement of mGPDH activity alone may not account for the improvement in glucose-induced ATP elevation in GK islets by TE treatment.

TE treatment did not affect the activity of glucokinase, a rate-limiting enzyme in glycolysis (26). In contrast, glucose oxidation at high glucose, which reflects Krebs cycle velocity, was improved in GK islets by TE treatment. These results suggest that coupling between glycolysis and mitochondrial oxidation, which plays an important role in glucose-induced IS (33), is improved by TE treatment. Lactate production weakens coupling between glycolysis and mitochondrial oxidation by decreasing the pyruvate supply to mitochondria. In several studies, the over-expression of LDH-A in β -cells attenuated glucose-induced IS (34–36) with an increase in lactate production (34,37). In addition, lactate release from β -cells increases in parallel with a decrease in the glucose oxidation rate (36). These findings support the hypothesis that regulation of lactate production may play an important role in the improvement of metabolism-secretion coupling by TE treatment. In the current study, lactate production in GK islets was higher compared with that in control, as shown in a previous study (38), and it was reduced by TE treatment. The protein expression level of LDH-A, an enzyme that catalyzes the reaction from pyruvate and NADH to lactate and NAD^+ , was 8.6-fold higher in GK islets compared with

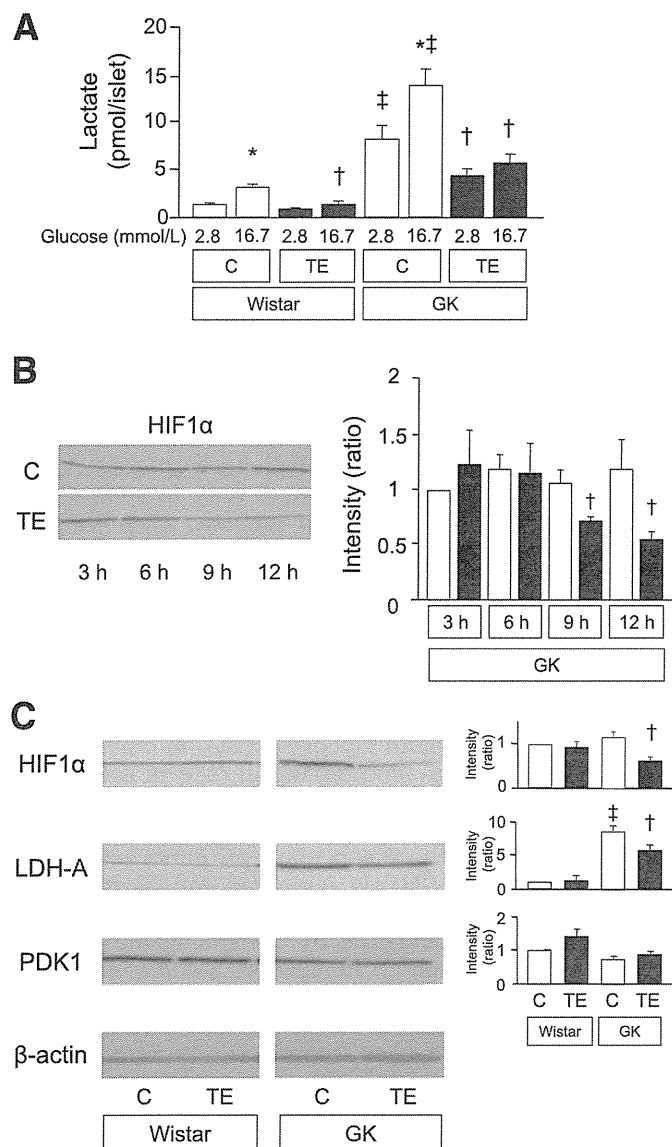


FIG. 4. Effects of TE treatment on lactate production and HIF1 α signaling. Islets were cultured without (control [C]; open bars) or with TE (black bars) for 12 h. **A:** Effects of TE treatment on lactate production in Wistar and GK islets. After cultured islets were preincubated under 2.8 mmol/L glucose for 30 min, 20 islets were batch incubated in the medium containing 2.8 and 16.7 mmol/L glucose for 60 min at 37°C, and released lactate in the medium was measured. Values are means \pm SE of 13 determinations from three (Wistar) and four (GK) experiments. **B:** Time course of HIF1 α protein levels in GK and Wistar islets during TE treatment using whole-islet lysates. Islets were cultured without (C; open bars) or with TE (closed bars) for indicated hours. The bar graphs are expressed relative to 3 h-cultured control values corrected by β -actin level. The values are means \pm SE of determinations from three different experiments. Representative blot panels are shown. **C:** Effect of TE treatment on protein levels of HIF1 α and HIF1 α downstream target proteins in whole lysate of Wistar and GK islets. Representative blot (left) and quantification data (right) are shown. The bar graphs are expressed relative to Wistar without TE treatment values corrected by β -actin level. The values are means \pm SE of determinations from three different experiments. * P < 0.01 vs. corresponding 2.8 mmol/L glucose; † P < 0.01 vs. corresponding control without TE; ‡ P < 0.01 vs. corresponding Wistar.

control Wistar islets, and was reduced by \sim 30% after TE treatment. In nondiabetic pancreatic β -cells, low LDH activity together with high mGPDH activity may act as a device to transfer NADH in the cytosol to the mitochondria and reduce lactate production by decreasing the NADH level in cytosol (39). In GK islets, enhanced LDH activity

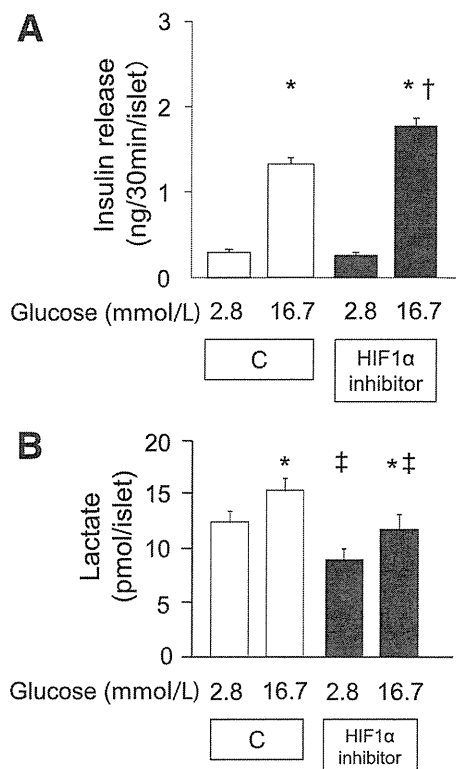


FIG. 5. Effects of HIF1 α inhibitor on IS and lactate production in GK islets. Islets were cultured without (control [C]; open bars) or with 1 μ mol/L HIF1 α inhibitor (black bars) for 12 h. **A:** Insulin secretion. After cultured islets were preincubated with 2.8 mmol/L glucose for 30 min, IS during incubation for 30 min in the presence of 2.8 and 16.7 mmol/L glucose was measured in islets. Data are shown as means \pm SE of 25 determinations from three experiments. * P < 0.01 vs. corresponding 2.8 mmol/L glucose; † P < 0.01 vs. 16.7 mmol/L glucose in control. **B:** Lactate production. After cultured islets were preincubated under 2.8 mmol/L glucose for 30 min, islets were batch incubated in the medium containing 2.8 and 16.7 mmol/L glucose for 60 min, and released lactate in the medium was measured. Values are means \pm SE of 12 determinations from three experiments. * P < 0.01 vs. corresponding 2.8 mmol/L glucose; ‡ P < 0.01 vs. corresponding control without HIF1 α inhibitor.

and reduced mGPDH activity might produce lactate overproduction and reduce mitochondrial oxidation by decreasing the supply of pyruvate and reducing equivalents to mitochondria and by an increase in the availability of cytosolic NADH to produce lactate from pyruvate as well. In this case, reduced LDH activity and increased mGPDH activity by TE treatment may well have decreased lactate overproduction and ameliorated metabolism-secretion coupling in GK islets.

These metabolic profiles in GK islets resemble the Warburg effect, characteristic aerobic metabolism in cancer cells by which lactate is overproduced from glucose even under nonhypoxic conditions (40). HIF1 α is a transcription factor that functions as a master regulator of oxygen homeostasis and upregulates the expression of *LDH* (41). HIF1 α activity is controlled at the level of protein stability. When oxygen is present, two proline residues in the oxygen-dependent degradation domain of HIF1 α are hydroxylated by the prolylhydroxylase enzymes (PHDs) (42). These hydroxylated motifs allow capture by the von Hippel-Lindau protein that forms the recognition component of an E3 ubiquitin ligase complex, which targets the hydroxylated HIF1 α for polyubiquitination and proteasomal degradation (43). In the current study, TE treatment showed a time-dependent decrease in HIF1 α protein levels

in GK islets and 50% reduction compared with that in nontreated GK islets at 12-h culture, which could reduce LDH-A protein in TE-treated GK islets. The Warburg effect is usually thought of as a high rate of glycolysis. Since TE treatment did not affect glucokinase activity, which determines glycolytic velocity in β -cells, alteration in the HIF1 α level in the pathophysiological range may not affect glycolytic velocity in β -cells. In this respect, lactate overproduction in GK islets differs from the typical Warburg effect.

HIF1 α downregulates the expression of GLUT2 and glucokinase and upregulates PDK-1, which inactivates pyruvate dehydrogenase and suppresses metabolism in the Krebs cycle (44) in islets of von Hippel-Lindau gene (*vhl*)-deficient mice (45). On the contrary, TE treatment did not affect the glucokinase activity and protein level of PDK-1 in GK islets. This discrepancy is not fully understood, but one possibility is that the expression level of HIF1 α may be higher than the pathophysiological level in *vhl*-deficient islets. This is supported by the fact that glucose-induced IS is impaired severely and almost abolished in *vhl*-deficient islets. The transcript level of *LDH-A* in *vhl*-deficient islets is about 20-fold higher compared with control islets, which is much higher than that of *PDK-1* (~5-fold) (46). This suggests that the expression of *LDH-A* is more readily affected by HIF1 α expression than that of *PDK-1*.

An increase in the basal HIF1 α level in GK islets was not found compared with that in Wistar islets. In contrast, basal levels of *LDH-A* in GK islets was prominently increased compared with that in Wistar islets, suggesting that regulators other than HIF1 α may play an important role in the enhancement of basal *LDH-A* in GK islets. However, our finding in the current study remains valid, as ROS-dependent regulation of *LDH-A* by HIF1 α , which plays an important role in lactate overproduction and impaired glucose-induced IS in GK islets, was established. In addition, the involvement of HIF1 α in lactate overproduction was shown clearly by the finding that HIF1 α inhibitor treatment restored high glucose-induced IS and suppressed lactate production in GK islets. ROS inhibits PHD activity and stabilizes HIF1 α (47,48). In GK islets, an excess amount of ROS may inhibit PHD activity and stabilize the HIF1 α protein. Thus, TE treatment may recover PHD activity by reducing the ROS level and decreasing the HIF1 α protein level to eventually ameliorate metabolism-secretion coupling in GK islets.

In conclusion, we show that 12-h suppression of endogenous ROS, including O₂⁻ and H₂O₂, improves impaired metabolism-secretion coupling in GK islets. Our results suggest that lactate overproduction plays an important role in impaired metabolism-secretion coupling in diabetic β -cells; ROS reduction improves mitochondrial metabolism by suppressing lactate overproduction through the inhibition of HIF1 α stabilization.

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M.S. researched data, contributed to the discussion, wrote the manuscript, and reviewed and edited the manuscript. S.F. contributed to the discussion, wrote the manuscript, and reviewed and edited the manuscript. Y.S., Y.N., E.M., G.Y., H.S., Y.T., and K.O. researched data. K.N. and N.I. contributed to the discussion, and reviewed and edited the manuscript. S.F. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

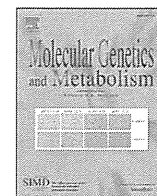
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REFERENCES

- Fujimoto S, Mukai E, Inagaki N. Role of endogenous ROS production in impaired metabolism-secretion coupling of diabetic pancreatic β cells. *Prog Biophys Mol Biol* 2011;107:304–310
- Fujimoto S, Nabe K, Takehiro M, et al. Impaired metabolism-secretion coupling in pancreatic β -cells: role of determinants of mitochondrial ATP production. *Diabetes Res Clin Pract* 2007;77(Suppl 1):S2–S10
- Bindokas VP, Kuznetsov A, Sreenan S, Polonsky KS, Roe MW, Philipson LH. Visualizing superoxide production in normal and diabetic rat islets of Langerhans. *J Biol Chem* 2003;278:9796–9801
- Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol* 2003;552:335–344
- Guichard C, Moreau R, Pessayre D, Epperson TK, Krause KH. NOX family NADPH oxidases in liver and in pancreatic islets: a role in the metabolic syndrome and diabetes? *Biochem Soc Trans* 2008;36:920–929
- Grankvist K, Marklund SL, Täljedal IB. CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. *Biochem J* 1981;199:393–398
- Tiedge M, Lortz S, Munday R, Lenzen S. Complementary action of antioxidant enzymes in the protection of bioengineered insulin-producing RINm5F cells against the toxicity of reactive oxygen species. *Diabetes* 1998;47:1578–1585
- Maechler P, Jornot L, Wollheim CB. Hydrogen peroxide alters mitochondrial activation and insulin secretion in pancreatic beta cells. *J Biol Chem* 1999;274:27905–27913
- Kominato R, Fujimoto S, Mukai E, et al. Src activation generates reactive oxygen species and impairs metabolism-secretion coupling in diabetic Goto-Kakizaki and ouabain-treated rat pancreatic islets. *Diabetologia* 2008; 51:1226–1235
- Mukai E, Fujimoto S, Sato H, et al. Exendin-4 suppresses SRC activation and reactive oxygen species production in diabetic Goto-Kakizaki rat islets in an Epac-dependent manner. *Diabetes* 2011;60:218–226
- Kajikawa M, Fujimoto S, Tsuura Y, et al. Ouabain suppresses glucose-induced mitochondrial ATP production and insulin release by generating reactive oxygen species in pancreatic islets. *Diabetes* 2002;51:2522–2529
- Nassar T, Kadery B, Lotan C, Da'as N, Kleinman Y, Haj-Yehia A. Effects of the superoxide dismutase-mimetic compound tempol on endothelial dysfunction in streptozotocin-induced diabetic rats. *Eur J Pharmacol* 2002; 436:111–118
- Dawson DA, Masayasu H, Graham DI, Macrae IM. The neuroprotective efficacy of ebselen (a glutathione peroxidase mimic) on brain damage induced by transient focal cerebral ischaemia in the rat. *Neurosci Lett* 1995;185:65–69
- Tang C, Han P, Oprescu AI, et al. Evidence for a role of superoxide generation in glucose-induced β -cell dysfunction in vivo. *Diabetes* 2007;56: 2722–2731
- Wang XD, Vatamaniuk MZ, Wang SK, Roneker CA, Simmons RA, Lei XG. Molecular mechanisms for hyperinsulinaemia induced by overproduction of selenium-dependent glutathione peroxidase-1 in mice. *Diabetologia* 2008;51:1515–1524
- Fujimoto S, Ishida H, Kato S, et al. The novel insulinotropic mechanism of pimobendan: direct enhancement of the exocytotic process of insulin secretory granules by increased Ca²⁺ sensitivity in β -cells. *Endocrinology* 1998;139:1133–1140
- Oliveira HR, Verlengia R, Carvalho CR, Britto LR, Curi R, Carpinelli AR. Pancreatic β -cells express phagocyte-like NAD(P)H oxidase. *Diabetes* 2003;52:1457–1463

18. Takasu N, Yamada T, Shimizu Y. Generation of H₂O₂ is regulated by cytoplasmic free calcium in cultured porcine thyroid cells. *Biochem Biophys Res Commun* 1987;148:1527–1532
19. Nabe K, Fujimoto S, Shimodahira M, et al. Diphenylhydantoin suppresses glucose-induced insulin release by decreasing cytoplasmic H⁺ concentration in pancreatic islets. *Endocrinology* 2006;147:2717–2727
20. Shimodahira M, Fujimoto S, Mukai E, et al. Rapamycin impairs metabolism-secretion coupling in rat pancreatic islets by suppressing carbohydrate metabolism. *J Endocrinol* 2010;204:37–46
21. Takehiro M, Fujimoto S, Shimodahira M, et al. Chronic exposure to β-hydroxybutyrate inhibits glucose-induced insulin release from pancreatic islets by decreasing NADH contents. *Am J Physiol Endocrinol Metab* 2005;288:E372–E380
22. Radu RG, Fujimoto S, Mukai E, et al. Tacrolimus suppresses glucose-induced insulin release from pancreatic islets by reducing glucokinase activity. *Am J Physiol Endocrinol Metab* 2005;288:E365–E371
23. Gardner RS. A sensitive colorimetric assay for mitochondrial alpha-glycerophosphate dehydrogenase. *Anal Biochem* 1974;59:272–276
24. MacDonald MJ. High content of mitochondrial glycerol-3-phosphate dehydrogenase in pancreatic islets and its inhibition by diazoxide. *J Biol Chem* 1981;256:8287–8290
25. Miwa I, Fukatsu H, Toyoda Y, Okuda J. Anomeric preference of glucose utilization in human erythrocytes loaded with glucokinase. *Biochem Biophys Res Commun* 1990;173:201–207
26. Meglasson MD, Matschinsky FM. Pancreatic islet glucose metabolism and regulation of insulin secretion. *Diabetes Metab Rev* 1986;2:163–214
27. Choi JH, Cho HK, Choi YH, Cheong J. Activating transcription factor 2 increases transactivation and protein stability of hypoxia-inducible factor 1α in hepatocytes. *Biochem J* 2009;424:285–296
28. Mysore TB, Shinkel TA, Collins J, et al. Overexpression of glutathione peroxidase with two isoforms of superoxide dismutase protects mouse islets from oxidative injury and improves islet graft function. *Diabetes* 2005;54:2109–2116
29. Lortz S, Tiedge M. Sequential inactivation of reactive oxygen species by combined overexpression of SOD isoforms and catalase in insulin-producing cells. *Free Radic Biol Med* 2003;34:683–688
30. Zhang CY, Baffy G, Perret P, et al. Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, β cell dysfunction, and type 2 diabetes. *Cell* 2001;105:745–755
31. Yoshihara E, Fujimoto S, Inagaki N, et al. Disruption of TBP-2 ameliorates insulin sensitivity and secretion without affecting obesity. *Nat Commun* 2010;1:127
32. Östenson CG, Abdel-Halim SM, Rasschaert J, et al. Deficient activity of FAD-linked glycerophosphate dehydrogenase in islets of GK rats. *Diabetologia* 1993;36:722–726
33. Malmgren S, Nicholls DG, Taneera J, et al. Tight coupling between glucose and mitochondrial metabolism in clonal β-cells is required for robust insulin secretion. *J Biol Chem* 2009;284:32395–32404
34. Zhao C, Rutter GA. Overexpression of lactate dehydrogenase A attenuates glucose-induced insulin secretion in stable MIN-6 β-cell lines. *FEBS Lett* 1998;430:213–216
35. Ainscow EK, Zhao C, Rutter GA. Acute overexpression of lactate dehydrogenase-A perturbs beta-cell mitochondrial metabolism and insulin secretion. *Diabetes* 2000;49:1149–1155
36. Alcazar O, Tiedge M, Lenzen S. Importance of lactate dehydrogenase for the regulation of glycolytic flux and insulin secretion in insulin-producing cells. *Biochem J* 2000;352:373–380
37. MacDonald MJ. Estimates of glycolysis, pyruvate (de)carboxylation, pentose phosphate pathway, and methyl succinate metabolism in incapacitated pancreatic islets. *Arch Biochem Biophys* 1993;305:205–214
38. Ling ZC, Efendic S, Wibom R, et al. Glucose metabolism in Goto-Kakizaki rat islets. *Endocrinology* 1998;139:2670–2675
39. Sekine N, Cirulli V, Regazzi R, et al. Low lactate dehydrogenase and high mitochondrial glycerol phosphate dehydrogenase in pancreatic β-cells. Potential role in nutrient sensing. *J Biol Chem* 1994;269:4895–4902
40. Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer* 2011;11:325–337
41. Gordan JD, Thompson CB, Simon MC. HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. *Cancer Cell* 2007;12:108–113
42. Jaakkola P, Mole DR, Tian YM, et al. Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* 2001;292:468–472
43. Maxwell PH, Wiesener MS, Chang GW, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 1999;399:271–275
44. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab* 2006;3:177–185
45. Cantley J, Selman C, Shukla D, et al. Deletion of the von Hippel-Lindau gene in pancreatic β cells impairs glucose homeostasis in mice. *J Clin Invest* 2009;119:125–135
46. Puri S, Cano DA, Hebrok M. A role for von Hippel-Lindau protein in pancreatic β-cell function. *Diabetes* 2009;58:433–441
47. Gerald D, Berra E, Frapart YM, et al. JunD reduces tumor angiogenesis by protecting cells from oxidative stress. *Cell* 2004;118:781–794
48. Kaelin WG Jr, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell* 2008;30:393–402



Exome sequencing identifies a new candidate mutation for susceptibility to diabetes in a family with highly aggregated type 2 diabetes

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ABSTRACT

The aim of this study was to investigate the genetic background of familial clustering of diabetes using genome-wide linkage analysis combined with exome sequencing. We recruited a Japanese family with a 3-generation history of diabetes. The family comprised 16 members, 13 having been diagnosed with diabetes. Nine members had been diagnosed before the age of 40. Linkage analysis was performed assuming an autosomal dominant model. Linkage regions were observed on chromosomes 4q34, 5q11–q13, and 12p11–q22 and the logarithm of odds (LOD) scores were 1.80. To identify the susceptibility variants, we performed exome sequencing of an affected family member. We predicted that the familial clustering of diabetes is caused by a rare non-synonymous variant, and focused our analysis on non-synonymous variants absent in dbSNP131. Exome sequencing identified 10 such variants in the linkage regions, 7 of which were concordant with the affection status in the family. One hundred five normal subjects and 67 lean diabetes subjects were genotyped for the 7 variants; the only variant found to be significantly more frequent in the diabetes subjects than in the normal subjects was the N1072K variant of the early endosome antigen 1 (EEA1) gene (0 in normal subjects and 4 in diabetes subjects, $p=0.022$). We therefore propose that the N1072K variant of the EEA1 gene is a candidate mutation for susceptibility to diabetes in the Japanese population.

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1. Introduction

In Japan, 11.2% of all adults suffer from diabetes mellitus, and its increasing prevalence is a serious concern [1]. Most of these patients have type 2 diabetes. While recent advances in genome-wide association studies (GWAS) have revealed over 60 type 2 diabetes susceptibility loci, all of them account for less than 20% of the genetic background of the disease [2–4]. GWAS is based on the common disease-common variant hypothesis, and rare disease variants with large effect size are frequently missed [5]. Approaches other than GWAS are necessary to elucidate the greater part of the genetic background of type 2 diabetes.

A family-based study is a promising approach to identify rare disease variants with large effect size [6]. Familial clustering of diabetes is frequently observed and the typical example of familial diabetes caused by rare disease variants is Maturity Onset Diabetes of the

Young (MODY). However, in most families in Japan, familial clustering cannot be attributed to mutations of the known 6 MODY genes (*HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, and *NEUROD1*) despite intensive linkage analyses and candidate-gene screening [7,8], and the genetic predisposition in such families has not been ascertained.

Recently, exome sequencing has become a powerful strategy for identifying causative genes for a number of Mendelian disorders [9]. As exome sequencing identifies thousands of exonic variants, a selection strategy to identify the particular variant causing the disorder is necessary.

In the present study, we combined linkage analysis and exome sequencing to identify the causal genetic variant in a family with highly aggregated diabetes and elucidated the genetic background of type 2 diabetes in Japan.

2. Material and methods

2.1. Family members

We recruited a Japanese family with a 3-generation family history of diabetes mellitus (Fig. 1). Medical history was gained from the family members and body mass index (BMI) and HbA_{1c} levels were measured in the year 2010 at entry to the study. All study participants

Abbreviations: LOD, Logarithm of Odds; EEA1, Early Endosome Antigen 1; GWAS, Genome-Wide Association Study; MODY, Maturity Onset Diabetes of the Young; HNF4A, Hepatocyte Nuclear Factor 4 Alpha; BMI, Body Mass Index; SNP, Single Nucleotide Polymorphism; CNV, Copy Number Variation; MAF, Minor Allele Frequency.

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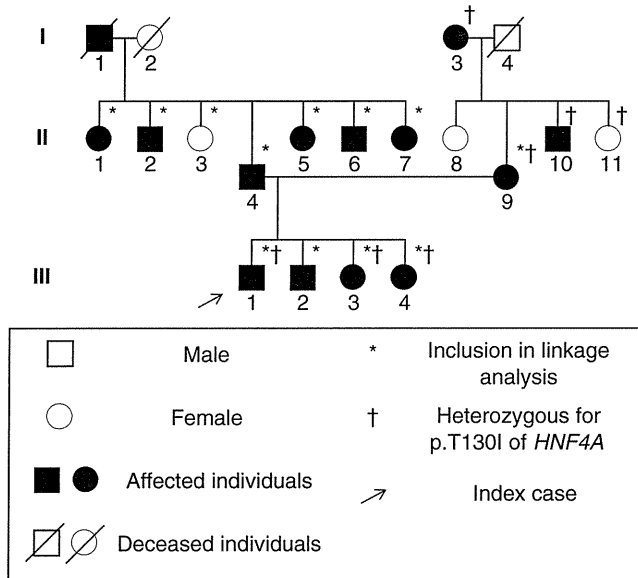


Fig. 1. A pedigree with familial aggregated diabetes mellitus.

from the family were negative for glutamic acid decarboxylase antibody. Affected status of the participants was determined in two ways. First, if participants had been diagnosed with diabetes and treated with oral antidiabetic drugs or insulin injection, they were regarded as affected. Second, if participants had not been treated with oral antidiabetic drugs or insulin injection, they underwent HbA_{1c} measurement. If their HbA_{1c} levels were $\geq 6.5\%$ [10], they were also regarded as affected. The clinical features of family members are shown in Table 1.

Deceased individual I-1 had diabetes according to the interview with family members. There was no information suggesting consanguineous marriage in the family. Some of the individuals in generation II had offspring and none of them except III-1, 2, 3, and 4, had diabetes according to the information from family members.

2.2. Normoglycemic controls and diabetes subjects

We selected normoglycemic controls from the participants in an annual medical check-up program performed in Japan. Nine-hundred ninety local residents (430 men, 560 women) were recruited in the program and consented to donate their DNA. From 2002 to 2007, participants underwent physical examination and blood tests including

Table 1
Characteristics of the family members.

ID	Age	Sex	BMI	HbA _{1c} (%)	Age when diagnosed	Current therapy
I-3	88	F	16.8	5.6	N/A	Oral drug
II-1	78	F	23.8	6.8	35	Oral drug
II-2	75	M	22.7	7.5	35	Oral drug
II-3	72	F	28.2	6.0		
II-4	68	M	23.3	9.2	35	Oral drug
II-5	65	F	24.6	6.8	65	Diet
II-6	62	M	22.4	8.1	30	Diet
II-7	60	F	23.3	7.6	35	Insulin 10 U/d
II-8	68	F	26.7	5.7		
II-9	66	F	23.9	7.8	44	Oral drug
II-10	63	M	20.5	7.4	N/A	Oral drug
II-11	61	F	21.2	5.4		
III-1	40	M	26.0	8.5	30	Oral drug
III-2	39	M	29.0	7.6	38	Oral drug
III-3	36	F	29.9	6.6	36	Diet
III-4	26	F	29.1	6.6	26	Diet

fasting plasma glucose and HbA_{1c} every year. Subjects defined as normoglycemic controls had the following characteristics: both HbA_{1c} and fasting plasma glucose below the screening standards for the diagnosis of diabetes (HbA_{1c} < 6.0% and fasting plasma glucose < 5.5 mmol/l [10]) during 5-year follow-up span, and age ≥ 55 . The number of subjects that satisfied the definition was 206 (81 men, 125 women). For genotyping, 105 normoglycemic controls were randomly selected (Supplementary Table 1), because genotyping of 210 normal chromosomes is necessary to achieve 80% power to detect a polymorphism present in 1% of the population [11]. We recruited lean diabetes subjects from participants in another medical check-up program performed in Japan. One hundred and thirty-eight participants had been diagnosed with diabetes and 68 were lean (BMI < 25). DNA was available from 67 of the lean diabetes subjects (Supplementary Table 1). Another, unrelated 64 diabetes subjects with family history had visited collaborating hospitals in Japan and donated their DNA. Genomic DNA was extracted from blood samples with a QIAamp DNA Blood Mini kit (Qiagen).

2.3. Linkage analysis

We performed a genome-wide linkage analysis using GENEHUNTER (version 2.1) software [12] with ABI Prism Linkage Mapping Set Version 2.5 (382 markers for 22 autosomes; Applied Biosystems) and other microsatellite fine markers designed according to information from the UniSTS map (<http://www.ncbi.nlm.nih.gov/genome/sts/>). A multipoint parametric linkage analyses for autosomes were performed assuming an autosomal dominant model because the 3-generation family history is suggestive of autosomal dominant mode of inheritance [6]. Since sensitivity analyses for the disease allele frequency by changing it from 0.01 to 0.00001 suggested negligible effects on LOD score, the disease allele frequency was set at 0.00001 and a phenocopy frequency of 0.00001 was assumed. Affected family members with known diabetes susceptibility variants were considered as having unknown phenotype. The purpose of including members assigned as unknown was to increase the accuracy of haplotype estimation in members assigned as affected, although inclusion did not increase the statistical power. Population allele frequencies for each microsatellite marker were assigned equal portions for individual alleles. We used a 2-stage design: first, all chromosomal regions were screened by genotyping at an approximately 10 cM density (screening), and the regions where LOD scores were highest were considered potentially interesting. Second, these regions were further finely mapped at approximately 1- to 2-cM densities (fine mapping). Haplotypes were constructed with GENEHUNTER.

2.4. Sanger sequencing

We directly sequenced the coding exons of 6 MODY genes (*HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, and *NEUROD1*) by Sanger method. Forward and reverse PCR primers for each exon were selected in an intronic sequence 50 bp away from the intron/exon boundaries. PCR products were run on 2% agarose gel, and the appropriate bands were excised and then purified with the use of the QIAquick Gel Extraction Kit (Qiagen). Sequencing results were analyzed on an ABI 3130 genetic analyzer (Applied Biosystems). Sanger sequencing was also used to confirm the single nucleotide polymorphisms (SNPs) identified in exome sequencing. Sequencing primer data will be provided on request.

2.5. Copy number variation (CNV) analysis

We selected CNVs that overlapped with MODY genes (*HNF1A*, *HNF1B*, and *GCK*, Supplementary Table 2). We performed real-time PCR to determine CNV status on normal control and family members. TaqMan probes designed by the manufacturer (Applied Biosystems, Supplementary Table 2), were used to target the specific regions. *RNase P* (Taqman Copy Number Reference Assay, Applied Biosystems)

was chosen as a reference gene. Every reaction was triplicated. CopyCaller Software v2.0 (Applied Biosystems) was used to determine the copy number status of each target region.

2.6. Targeted capture and massive parallel sequencing

We conducted exome analysis on one affected family member who was negative for mutations in the exons of the 6 MODY genes. We targeted all protein-coding regions as defined by RefSeq37. Approximately 210,000 coding exons from 3 µg of genomic DNA were captured using the Agilent SureSelect Human All Exon 50 Mb kit, following the manufacturer's protocols. The whole-exome DNA library was sequenced on an Illumina Genome Analyzer IIx in 76-bp paired-end reads using two channels. Sequence reads were mapped to the reference human genome (Ghr37/hg19) using the Burrows-Wheeler Aligner (<http://bio-bwa.sourceforge.net/>). Variant detection was performed with the CASAVA software (version 1.7; Illumina). Calls of variants were filtered to include only those with $\geq 8\times$ coverage, ≥ 40 consensus quality and ≥ 20 mapping quality.

2.7. Genotyping of SNPs

Genotyping of variants identified in the exome sequencing was performed in 105 normoglycemic controls and 67 lean diabetes subjects. The PCR-restriction fragment length polymorphism method for p.A80T of *CENPH*, p.R29C of *SREK1IP1*, and p.S33G of *SMAGP* and the Taqman method (Applied Biosystems) for p.F120L of *TMEM174*, p.P1818L of *NACA*, p.N1072K of *EEA1*, and p.E361G of *LRRIQ1* were used. Genotyping of the p.N1072K variant of *EEA1* was performed in another, unrelated 64 familial diabetes subjects.

2.8. RT-PCR analysis of the *TMEM174* gene product in mouse tissues

Pancreatic islets and tissues of the lung, the heart, the liver and the kidney were obtained from C57BL/6 mice. Complementary DNA was prepared by reverse transcriptase (Invitrogen) with an oligo(dT) primer (Invitrogen). Messenger RNA expression was examined by PCR using the LA Taq (TaKaRa) and the oligonucleotide primers. Thermal cycling conditions were denaturation at 95 °C for 10 min followed by 35 cycles at 95 °C for 15 s and 58 °C for 1 min. C- and N-terminal primers of target molecules were designed as follows: GAPDH forward, 5'-aaatggtgaaggtcggtgtg-3', and reverse, 5'-tcgttgatggcaacaatc-3'; and *TMEM174* forward, 5'-taccctcaagacaatgctgc-3', and reverse, 5'-tggaggagagaacatacgc-3'.

2.9. Statistical analysis

Frequencies of nucleotide changes in normoglycemic controls and lean diabetes subjects were compared by the Pearson's chi-squared test or the Fisher's exact test, wherever appropriate.

2.10. Ethics

Ethical approval for this study was given by the Institutional Review Board and Ethics Committee of Kyoto University School of Medicine, Kyoto University, Japan (approval number: G267; approval date: 9 October 2008). Written, informed consent was obtained from each participant. This study was carried out in accordance with the Declaration of Helsinki as revised in 2000.

3. Results

3.1. Characteristics of family members

A family with a 3-generation history of diabetes was enrolled in this study (Fig. 1, Table 1). Thirteen members (6 men, 7 women)

had previously been diagnosed with diabetes, 9 of them before the age of 40. Nine of the 13 family members with diabetes were lean (BMI < 25); none of them were both obese and late-onset (later than 40). Eight family members were treated with oral antidiabetic drugs and 1 with insulin.

3.2. Detection of a diabetes susceptibility mutation in the hepatocyte nuclear factor 4 alpha (*HNF4A*) gene

For the index case, we performed direct sequencing in entire coding exons of the 6 MODY genes. The detected missense SNPs were the p.S487N variant of *HNF1A* (rs2464196) and the p.T130I variant of *HNF4A* (rs1800961) (Supplementary Table 3). The p.T130I variant of *HNF4A* was not a cause of MODY but was associated with late-onset type 2 diabetes in a previous report [13], so we genotyped all family members for the variant. As a result, 6 members with diabetes and 1 member without diabetes were found to be heterozygous for the variant (Fig. 1). We hypothesized that the disease susceptibility variant for the 6 members with diabetes was the p.T130I variant of *HNF4A*. We detected no copy number losses in *HNF1A*, *HNF1B*, and *GCK* on the index case (Supplementary Table 4).

3.3. Linkage analysis

We performed linkage analysis to identify the susceptibility gene in the family members with diabetes and also negative for the p.T130I variant of *HNF4A*. Family members positive for the variant were assigned as having unknown phenotype in the linkage analysis. A total of 12 family members (7 assigned as having affected phenotype, 4 assigned as having unknown phenotype, and 1 assigned as having unaffected phenotype) were included in the linkage analysis, assuming an autosomal dominant model. The genome-wide linkage results in the screening are shown in Fig. 2. Regions of potential interest by the multipoint LOD score were observed on chromosomes 4, 5 and 12. After fine mapping, 4q34, 5q11–q13, and 12p11–q22 were revealed to be candidate regions (Supplementary Table 5, LOD = 1.80).

3.4. Candidate variants in exome sequencing

We searched candidate genetic variants in the implicated linkage region by performing exome sequencing in family member II-2, who was negative for mutations in the exons of the 6 MODY genes. Family member II-2 was also negative for copy number losses in *HNF1A*, *HNF1B*, and *GCK* (Supplementary Table 4). The exome DNA library of the family member was sequenced in 76 bp paired-end reads, using 2 channels of Genome Analyzer IIx. We generated approximately 153 million reads and 98.02% of the reads were mapped to the reference human genome (Ghr37/hg19). On target rate to the bait was 59.1% and coverage depth was ≥ 3 in 97.40% of the targeted region and ≥ 20 in 87.11% of the targeted region. We predicted that the familial clustering of diabetes is caused by a rare non-synonymous variant, and focused our analysis on

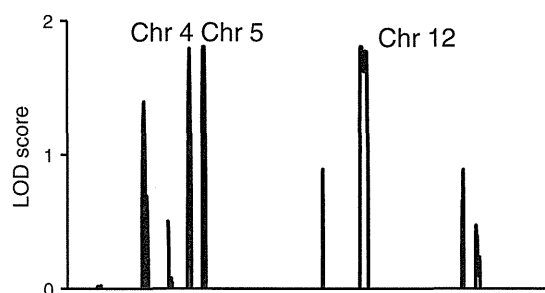


Fig. 2. Multipoint LOD scores in the genome-wide linkage analysis for the pedigree.

non-synonymous heterozygous or homozygous variants absent in the dbSNP131 (<http://www.ncbi.nlm.nih.gov/projects/SNP/>). In the implicated linkage region, we detected 10 such variants. All of these variants were heterozygous and were validated by Sanger method (Table 2). We genotyped all family members included in the linkage analysis for the 10 variants and 7 of them were detected in all affected family members without the p.T130I variant of *HNF4A*.

3.5. Allele frequencies of the candidate variants in cases and controls

To determine whether each of the 7 variants was rare or common in the general Japanese population without hyperglycemia, we genotyped 105 normoglycemic controls for the 7 variants. Minor allele frequencies ranged from 0.0% to 4.7% for the 7 variants (Table 3). We then genotyped 67 lean diabetes subjects to determine the frequency of the variants in a group predicted to be genetically predisposed to diabetes. The p.N1072K variant of *EEA1* was found to be significantly more frequent in the lean diabetes subjects than in the normoglycemic controls (0 in normoglycemic controls, 4 in lean diabetes subjects, $p = 0.022$, Fisher's exact test). Two of the 4 lean diabetes subjects had family history; the mother of one subject and a sibling of the other subject had diabetes, but DNA samples of their family members were unavailable. We also genotyped the p.N1072K variant of *EEA1* in another, unrelated 64 familial diabetes subjects and found 3 of them to be heterozygous for the variant; the minor allele frequency being 3/128 (2.3%). For 1 of the 3 subjects heterozygous for the variant, a DNA sample of a family member was available and the variant was concordant with the affection status in the family (Supplementary Fig. 1, Supplementary Table 6).

3.6. Linkage reanalysis of the family with a 3-generation history of diabetes

We performed a linkage analysis in the family with a 3-generation history of diabetes of chromosome 12 using the g.93175846 genotype in *EEA1* together with the microsatellite markers. We reassigned the affection status: subjects heterozygous for the p.N1072K variant of *EEA1* (II-1, II-2, II-4, II-5, II-6, II-7, III-1, III-2, and III-4) were considered as having affected phenotype and subjects negative for the variant (II-9 and III-3) were considered as having unaffected phenotype. We found a LOD score of 2.70 at the locus of the p.N1072K variant of *EEA1* (Fig. 3).

4. Discussion

In the present study, we analyzed a family with highly aggregated diabetes mellitus by combining exome sequencing and a linkage analysis. We identified a candidate susceptibility variant of *EEA1* in the linkage region located on chromosome 12p11–q22. The region has not been reported in previous linkage analyses with familial diabetes in Japan.

Table 2
Non-synonymous nucleotide changes in the linkage regions.

Gene	Chr:position (GRCh37) nucleotide change	Amino acid change	Concordance
SREK1IP1	5:64037004 G>A	R29C	+
CENPH	5:68490521 G>A	A80T	+
TMEM174	5:72469428 T>C	F120L	+
SMAGP	12:51640553 T>C	S33G	+
KRT78	12:5323268 C>T	M424I	–
NACA	12:57109861 G>A	P1818L	+
LRIG3	12:59274465 G>A	R567W	–
TMEM19	12:72092800 C>T	P253L	–
LRRIQ1	12:85449653 T>C	E361G	+
EEA1	12:93175846 A>T	N1072K	+

Table 3

Allele frequencies of candidate variants in the normoglycemic controls and the lean diabetes subjects.

Gene	Amino acid change	Detected number of alleles					
		Controls (n = 105)			Diabetes (n = 67)		
		Major	Minor	MAF	Major	Minor	MAF
SREK1IP1	R29C	200	10	0.047	126	8	0.059
CENPH	A80T	208	2	0.009	132	2	0.014
TMEM174	F120L	210	0	0.000	134	0	0.000
SMAGP	S33G	208	2	0.009	134	0	0.000
NACA	P1818L	204	6	0.029	134	0	0.000
LRRIQ1	E361G	209	1	0.004	132	2	0.014
EEA1	N1072K	210	0	0.000	130	4 ^a	0.029

MAF = Minor Allele Frequency.

^a Fisher's exact test, $p < 0.05$.

The *EEA1* variant was detected in all affected family members without the p.T130I variant of *HNF4A*. We validated the candidate susceptibility variant of *EEA1* by performing a case–control study. The variant was significantly more frequent in the diabetes subjects than in the cohort of subjects that retained fasting normoglycemia for 5 years. It is important that the *EEA1* variant was not detected in 105 controls that are supposed to have protective genetic background against diabetes, because enough power to detect a polymorphism present in 1% of the control chromosomes was achieved by genotyping 105 individuals.

In several studies, exome sequencing has been performed to identify the cause of neonatal diabetes or familial diabetes and it has successfully identified the causal mutations in genes previously known to be involved in the pathogenesis of diabetes [14–17]. To our knowledge this is the first study to propose a new diabetes susceptibility gene by utilizing exome sequencing.

The *EEA1* protein is known to localize exclusively to early endosomes. *EEA1* is a membrane bound, endosome fusion promoting protein and an effector of the small GTPase Rab5 [18–20]. *EEA1* is a coiled-coil homodimer with an N-terminal C₂H₂ zinc finger and a C-terminal region containing a calmodulin binding motif and a FYVE domain [21]. It is reported that *EEA1* deficiency decreased insulin-stimulated glucose uptake and insulin-stimulated translocation of glucose transporter type 4 to the plasma membrane in human adipocytes [22]. Thus, the variant of *EEA1* could cause type 2 diabetes susceptibility by inducing insulin resistance. The Asn1024 residue of *EEA1* is conserved among mammals [23], suggesting that a substitution to lysine might affect the function of *EEA1*; however, the residue is located on the coiled-coil domain not directly involved in the interaction with Rab5 GTPase or endosomal membrane [21]. Further study is necessary to evaluate the functional significance of the variant.

Our study has other limitations. First, the p.T130I variant of *HNF4A* is not a cause of MODY and is reported to be found in non-diabetic individuals [13], so the possibility remains that the genetic cause of diabetes for

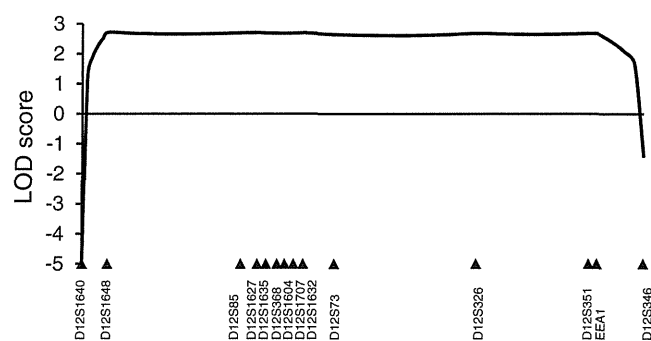


Fig. 3. Multipoint LOD scores for chromosome 12 after fine mapping and reassignment of the affection status.

members I-3, II-9, II-10, and III-3 in the family is not the p.T130I variant of *HNF4A*. Second, we focused only on nonsynonymous single nucleotide variants in the linkage region and eliminated a large number of insertion/deletions detected in the exome sequencing, most of which were not confirmed by the direct sequencing [24]. Third, in the linkage region, besides the p.N1072K variant of *EEA1*, p.F120L of *TMEM174* was also undetectable in the 105 control subjects and was considered rare. The p.F120L variant of *TMEM174* was not detected in the 67 diabetes subjects, but it might be an extremely rare diabetes susceptibility variant that is found specifically in this family. The PolyPhen-2 prediction score for the p.F120L variant of *TMEM174* was 0.990 and the variant was predicted to be probably damaging, and the p.T130I variant of *HNF4A* and the p.N1072K variant of *EEA1* were predicted to be benign with the prediction score of 0.001 and 0.191 respectively [25]. However, we thought that the *TMEM174* gene is less likely to be the cause of diabetes than the *EEA1* gene because the *TMEM174* gene is almost specifically expressed in the kidney [26], not in the pancreatic islets or the liver according to our RT-PCR analysis using mouse tissues (Supplementary Fig. 2). Fourth, the LOD score at the locus of the p.N1072K variant of *EEA1* in the linkage analysis was slightly lower than the genome-wide significance level. This lower-than-expected LOD score may be attributable to the difficulties inherent to the genetic heterogeneity of diabetes.

5. Conclusion

We propose that the *EEA1* gene is a candidate type 2 diabetes susceptibility gene. A family-based approach combined with exome sequencing may be a useful strategy to elucidate the complex genetic background of type 2 diabetes by identifying rare disease variants not detected by GWAS.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ymgme.2013.02.010>.

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References

- [1] International Diabetes Federation, IDF Diabetes Atlas, 5th ed., 2011.
- [2] M. Imamura, S. Maeda, Genetics of type 2 diabetes: the GWAS era and future perspectives, *Endocr. J.* 58 (2011) 723–739, (Review).
- [3] B.F. Voight, L.J. Scott, V. Steinthorsdottir, A.P. Morris, C. Dina, R.P. Welch, E. Zeggini, C. Huth, Y.S. Aulchenko, G. Thorleifsson, L.J. McCulloch, T. Ferreira, H. Grallert, N. Amin, G. Wu, C.J. Willer, S. Raychaudhuri, S.A. McCarrroll, C. Langenberg, O.M. Hofmann, J. Dupuis, L. Qi, A.V. Segrè, M. van Hoek, P. Navarro, K. Ardlie, B. Balkau, R. Benediktsson, A.J. Bennett, R. Blagieva, E. Boerwinkle, L.L. Bonnycastle, K. Bengtsson Boström, B. Bravenboer, S. Bumpstead, N.P. Burtt, G. Charpentier, P.S. Chines, M. Cornelis, D.J. Couper, G. Crawford, A.S. Doney, K.S. Elliott, A.L. Elliott, M.R. Erdos, C.S. Fox, C.S. Franklin, M. Ganser, C. Gieger, N. Grarup, T. Green, S. Griffin, C.J. Groves, C. Guiducci, S. Hadjadj, N. Hassanali, C. Herder, B. Isomaa, A.U. Jackson, P.R. Johnson, T. Jørgensen, W.H. Kao, N. Klopp, A. Kong, P. Kraft, J. Kuusisto, T. Lauritzen, M. Li, A. Lieverse, C.M. Lindgren, V. Lyssenko, M. Marre, T. Meitinger, K. Midtthjell, M.A. Morken, N. Narisu, P. Nilsson, K.R. Owen, F. Payne, J.R. Perry, A.K. Petersen, C. Platou, C. Proença, I. Prokopenko, W. Rathmann, N.W. Rayner, N.R. Robertson, G. Rocheleau, M. Roden, M.J. Sampson, R. Saxena, B.M. Shields, P. Shrader, G. Sigurdsson, T. Sparsø, K. Strassburger, H.M. Stringham, Q. Sun, A.J. Swift, B. Thorand, J. Tichet, T. Tuomi, R.M. van Dam, T.W. van Heften, T. van Herpt, J.V. van Vliet-Ostapchouk, G.B. Walters, M.N. Weedon, C. Wijmenga, J. Witteman, R.N. Bergman, S. Cauchi, F.S. Collins, A.L. Gloyn, U. Gyllenstein, T. Hansen, W.A. Hide, G.A. Hitman, A. Hofman, D.J. Hunter, K. Hveem, M. Laakso, K.L. Mohlke, A.D. Morris, C.N. Palmer, P.P. Pramstaller, I. Rudan, E. Sijbrands, L.D. Stein, J. Tuomilehto, A. Uitterlinden, M. Walker, N.J. Wareham, R.M. Watanabe, G.R. Abecasis, B.O. Boehm, H. Campbell, M.J. Daly, A.T. Hattersley, F.B. Hu, J.B. Meigs, J.S. Pankow, O. Pedersen, H.E. Wichmann, I. Barroso, J.C. Florez, T.M. Frayling, L. Groop, R. Sladek, U. Thorsteinsdottir, J.F. Wilson, T. Illig, P. Froguel, C.M. van Duijn, K. Stefansson, D. Altshuler, M. Boehnke, M.I. McCarthy, M. investigators, G. Consortium, Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis, *Nat. Genet.* 42 (2010) 579–589.
- [4] K. Miyake, W. Yang, K. Hara, K. Yasuda, Y. Horikawa, H. Osawa, H. Furuta, M.C. Ng, Y. Hirota, H. Mori, K. Ido, K. Yamagata, Y. Hinokio, Y. Oka, N. Iwasaki, Y. Iwamoto, Y. Yamada, Y. Seino, H. Maegawa, A. Kashiwagi, H.Y. Wang, T. Tanahashi, N. Nakamura, J. Takeda, E. Maeda, K. Yamamoto, K. Tokunaga, R.C. Ma, W.Y. So, J.C. Chan, N. Kamatani, H. Makino, K. Nanjo, T. Kadowaki, M. Kasuga, Construction of a prediction model for type 2 diabetes mellitus in the Japanese population based on 11 genes with strong evidence of the association, *J. Hum. Genet.* 54 (2009) 236–241.
- [5] W. Bodmer, C. Bonilla, Common and rare variants in multifactorial susceptibility to common diseases, *Nat. Genet.* 40 (2008) 695–701.
- [6] W. Liu, D. Morito, S. Takashima, Y. Mineharu, H. Kobayashi, T. Hitomi, H. Hashikata, N. Matsuura, S. Yamazaki, A. Toyoda, K. Kikuta, Y. Takagi, K.H. Harada, A. Fujiyama, R. Herzig, B. Kricsek, L. Zou, J.E. Kim, M. Kitakaze, S. Miyamoto, K. Nagata, N. Hashimoto, A. Koizumi, Identification of RNF213 as a susceptibility gene for moyamoya disease and its possible role in vascular development, *PLoS One* 6 (2011) e22542.
- [7] K. Yamagata, Regulation of pancreatic beta-cell function by the HNF transcription network: lessons from maturity-onset diabetes of the young (MODY), *Endocr. J.* 50 (2003) 491–499.
- [8] T. Yorifuji, R. Fujimaru, Y. Hosokawa, N. Tamagawa, M. Shiozaki, K. Aizu, K. Jinno, Y. Maruo, H. Nagasaka, T. Tajima, K. Kobayashi, T. Urakami, Comprehensive molecular analysis of Japanese patients with pediatric-onset MODY-type diabetes mellitus, *Pediatr. Diabetes* 13 (2012) 26–32.
- [9] M.J. Bamshad, S.B. Ng, A.W. Bigham, H.K. Tabor, M.J. Emond, D.A. Nickerson, J. Shendure, Exome sequencing as a tool for Mendelian disease gene discovery, *Nat. Rev. Genet.* 12 (2011) 745–755.
- [10] C.D. Saudek, W.H. Herman, D.B. Sacks, R.M. Bergenstal, D. Edelman, M.B. Davidson, A new look at screening and diagnosing diabetes mellitus, *J. Clin. Endocrinol. Metab.* 93 (2008) 2447–2453.
- [11] S. Ellard, C. Bellanné-Chantelot, A.T. Hattersley, E.M.G.Q.N.E.M. group, Best practice guidelines for the molecular genetic diagnosis of maturity-onset diabetes of the young, *Diabetologia* 51 (2008) 546–553.
- [12] L. Kruglyak, M.J. Daly, M.P. Reeve-Daly, E.S. Lander, Parametric and nonparametric linkage analysis: a unified multipoint approach, *Am. J. Hum. Genet.* 58 (1996) 1347–1363.
- [13] Q. Zhu, K. Yamagata, A. Miura, N. Shihara, Y. Horikawa, J. Takeda, J. Miyagawa, Y. Matsuzawa, T130I mutation in HNF-4alpha gene is a loss-of-function mutation in hepatocytes and is associated with late-onset Type 2 diabetes mellitus in Japanese subjects, *Diabetologia* 46 (2003) 567–573.
- [14] A. Bonnefond, E. Durand, O. Sand, F. De Graeve, S. Gallina, K. Busiah, S. Lobbens, A. Simon, C. Bellanné-Chantelot, L. Létourneau, R. Scharfmann, J. Delplanque, R. Sladek, M. Polak, M. Vaxillaire, P. Froguel, Molecular diagnosis of neonatal diabetes mellitus using next-generation sequencing of the whole exome, *PLoS One* 5 (2010) e13630.
- [15] S. Johansson, H. Irgens, K.K. Chudasama, J. Molnes, J. Aerts, F.S. Roque, I. Jonassen, S. Levy, K. Lima, P.M. Knappskog, G.I. Bell, A. Molven, P.R. Njølstad, Exome sequencing and genetic testing for MODY, *PLoS One* 7 (2012) e38050.
- [16] A. Bonnefond, J. Philippe, E. Durand, A. Dechaume, M. Huyvaert, L. Montagne, M. Marre, B. Balkau, I. Fajardy, A. Vambergue, V. Vatin, J. Delplanque, D. Le Guilcher, F. De Graeve, C. Lecoœur, O. Sand, M. Vaxillaire, P. Froguel, Whole-exome sequencing and high throughput genotyping identified KCNJ11 as the thirteenth MODY gene, *PLoS One* 7 (2012) e37423.
- [17] D.S. Lieber, S.B. Vafai, L.C. Horton, N.G. Slate, S. Liu, M.L. Borowsky, S.E. Calvo, J.D. Schmahmann, V.K. Mootha, Atypical case of Wolfram syndrome revealed through targeted exome sequencing in a patient with suspected mitochondrial disease, *BMC Med. Genet.* 13 (2012) 3.
- [18] F.T. Mu, J.M. Callaghan, O. Steele-Mortimer, H. Stenmark, R.G. Parton, P.L. Campbell, J. McCluskey, J.P. Yeo, E.P. Tock, B.H. Toh, EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine “fingers” and contains a calmodulin-binding IQ motif, *J. Biol. Chem.* 270 (1995) 13503–13511.
- [19] H. Stenmark, R. Aasland, B.H. Toh, A. D’Arrigo, Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger, *J. Biol. Chem.* 271 (1996) 24048–24054.
- [20] A. Simonsen, R. Lippé, S. Christoforidis, J.M. Gaullier, A. Brech, J. Callaghan, B.H. Toh, C. Murphy, M. Zerial, H. Stenmark, EEA1 links PI(3)K function to Rab5 regulation of endosome fusion, *Nature* 394 (1998) 494–498.
- [21] J.J. Dumas, E. Merithew, E. Sudharshan, D. Rajamani, S. Hayes, D. Lawe, S. Corvera, D.G. Lambright, Multivalent endosome targeting by homodimeric EEA1, *Mol. Cell* 8 (2001) 947–958.
- [22] R. Cheng, J. Qiu, X.Y. Zhou, X.H. Chen, C. Zhu, D.N. Qin, J.W. Wang, Y.H. Ni, C.B. Ji, X.R. Guo, Knockdown of STEAP4 inhibits insulin-stimulated glucose transport and GLUT4 translocation via attenuated phosphorylation of Akt, independent of the effects of EEA1, *Mol. Med. Rep.* 4 (2011) 519–523.
- [23] P.A. Fujita, B. Rhead, A.S. Zweig, A.S. Hinrichs, D. Karolchik, M.S. Cline, M. Goldman, G.P. Barber, H. Clawson, A. Coelmo, M. Diekhans, T.R. Dreszer, B.M. Gardine, R.A. Harte, J. Hillman-Jackson, F. Hsu, V. Kirkup, R.M. Kuhn, K. Learned,

- C.H. Li, L.R. Meyer, A. Pohl, B.J. Raney, K.R. Rosenbloom, K.E. Smith, D. Haussler, W.J. Kent, The UCSC genome browser database: update 2011, *Nucleic Acids Res.* 39 (2011) D876–D882.
- [24] E. Karakoc, C. Alkan, B.J. O’Roak, M.Y. Dennis, L. Vives, K. Mark, M.J. Rieder, D.A. Nickerson, E.E. Eichler, Detection of structural variants and indels within exome data, *Nat. Methods* 9 (2012) 176–178.
- [25] I.A. Adzhubei, S. Schmidt, L. Peshkin, V.E. Ramensky, A. Gerasimova, P. Bork, A.S. Kondrashov, S.R. Sunyaev, A method and server for predicting damaging missense mutations, *Nat. Methods* 7 (2010) 248–249.
- [26] P. Wang, B. Sun, D. Hao, X. Zhang, T. Shi, D. Ma, Human TMEM174 that is highly expressed in kidney tissue activates AP-1 and promotes cell proliferation, *Biochem. Biophys. Res. Commun.* 394 (2010) 993–999.

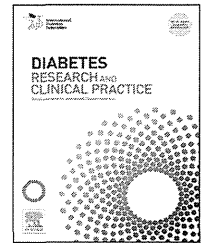


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A hospital-based cross-sectional study to develop an estimation formula for 2-h post-challenge plasma glucose for screening impaired glucose tolerance[☆]

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ABSTRACT

Aims: To create and validate an estimation formula for 2-h post-challenge plasma glucose (2-hPG) as an alternative to oral glucose tolerance test (OGTT) for impaired glucose tolerance (IGT) screening.

Methods: 380 Japanese subjects (57.6% males, aged 58.5 (14.0); mean (SD) years) undergoing OGTT were included in this hospital-based cross-sectional study mainly at Kyoto University Hospital between 2000 and 2011. We determined the main predictive variables of 2-hPG from clinical variables and separated the subjects randomly into two groups: a derivation group to construct an estimation formula of 2-hPG on the basis of predictive variables and a validation group to evaluate the accuracy of the formula.

Results: Fasting plasma glucose (FPG) and hemoglobin A1c (HbA1c) were highly correlated with 2-hPG measured by OGTT. Multiple linear regression analysis showed that estimated 2-hPG (e2-hPG) was calculated by the formula: $e2\text{-hPG} = 1.66 \times \text{FPG (mmol/l)} + 1.63 \times \text{HbA1c (\%)} - 10.11$ (R^2 , coefficient of determination = 60.2%). When the cut-off value was set to the diagnostic criteria of IGT, 7.8 mmol/l of e2-hPG, sensitivity, specificity, and negative predictive value (NPV) were 83.3%, 44.1%, and 74.3%, respectively. When the cut-off value was set lower (7.2 mmol/l), these values were 94.4%, 30.5%, and 85.7%, respectively. The area under the receiver operating characteristic (ROC) curve was 0.68.

Conclusions: This high-sensitive estimation formula may be a useful alternative to OGTT for IGT screening. For the levels ≤ 7.2 mmol/l, this formula may also be useful in cross-sectional study to identify people whose glucose tolerance is normal.

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1. Introduction

Impaired glucose tolerance (IGT) represents high risk not only for development of type 2 diabetes mellitus (DM) but also for cardiovascular disease [1–5]. A meta-analysis has shown that subjects with IGT have an annualized relative risk (95% confidence interval) for progression to DM of 6.35 (4.87–7.82) compared to those with normal glucose tolerance (NGT) [5]. Furthermore, it is known that lifestyle and pharmacological interventions for IGT are effective in preventing or delaying type 2 DM [6–9]. Collaborative Analysis of Diagnostic Criteria in Europe (DECODE), Collaborative Analysis of Diagnostic Criteria in Asia (DECODA), and Funagata Diabetes study have shown a strong association between postprandial hyperglycemia such as that seen in IGT and cardiovascular risk [3,10,11]. Cardiovascular mortality in subjects with IGT is similar to that in type 2 DM and much greater than that in impaired fasting glucose (IFG) [12]. Thus, detection of IGT is critical for preventing type 2 DM and reducing diabetic complications.

The “gold standard” method for diagnosing IGT defined by 1998 World Health Organization (WHO) criteria uses the level of 2-h post-challenge plasma glucose (2-hPG) during oral glucose tolerance test (OGTT) [13]. However, it is difficult to implement this test in a large population due to time and expense requirements [14]. For this reason, alternative methods for identifying IGT without OGTT have been investigated. Neither fasting plasma glucose (FPG) nor hemoglobin A1c (HbA1c) can be used singly to predict IGT due to the low detection rate [15–18]; however, combined use of FPG and HbA1c has been shown to be more effective [19]. Age, gender and body mass index (BMI) also have effects on the accuracy of IGT screening with single use of FPG and HbA1c [17,20–23]. At this point in time, there is no validated estimation formula to screen IGT that takes the predictive variables into account.

We screened for predictive variables of 2-hPG in hospital-based Japanese subjects and were able to develop an estimation formula for 2-hPG based on these predictive variables. We also validated the derived estimation formula for IGT screening which would be available for clinical settings.

2. Subjects and methods

2.1. Subjects

Three hundred eighty Japanese subjects not taking oral hypoglycemic agents and undergoing 75 g OGTT were recruited in this cross-sectional study at the Department of Diabetes and Clinical Nutrition, Kyoto University Hospital and other hospitals during the period of December 2000 through October 2011. Inclusion criteria were: family history of type 2 DM, past history of gestational diabetes, more than 20 years old, BMI > 25 kg/m², positive result of urine glucose test or hyperglycemia at examination for regular medical checkup. Exclusion criteria were: history of type 1 DM, endocrine diseases, operations such as gastrectomy and pancreatectomy, treatment with medications known to affect glucose

metabolism, and all conditions that might lead to misinterpretation of HbA1c, such as anemia. The study protocol was approved by Kyoto University Graduate School and Faculty of Medicine, Ethics Committee and conducted according to the Declaration of Helsinki. Informed consent was obtained from all subjects.

2.2. Measurements

Physical variables (age, gender, height, body weight, BMI) and laboratory variables (plasma glucose, immunoreactive insulin (IRI), HbA1c) were taken. Each standard OGTT was administered according to the National Diabetes Data Group recommendations [24]. Blood samples for determination of blood glucose levels were collected at 0, 30, 60, 90, and 120 min after oral administration of 75 g glucose. As the index of insulin secretion, we used the insulinogenic index, the change in the ratio of insulin to glucose level during the first 30 min of OGTT: (IRI 30 min – fasting immunoreactive insulin (F-IRI))/(PG 30 min – FPG) [25,26].

2.3. Laboratory examination

PG was measured by glucose oxidase method using the Hitachi Automatic Clinical Analyzer 7170 (Hitachi, Tokyo, Japan). IRI was measured by two-site radioimmunoassay (Insulin Ria-bead II, Dainabot, Tokyo, Japan). HbA1c was measured using high performance liquid chromatography (HPLC) and is expressed as a National Glycohemoglobin Standardization Program (NGSP) equivalent value calculated by the formula: HbA1c (NGSP value) (%) = 1.02 × HbA1c (Japan Diabetes Society value) (%) + 0.25 [27]. The HbA1c measurements in International Federation of Clinical Chemistry (IFCC) units (mmol/mol) were also calculated.

2.4. Definitions

According to the 1998 WHO diagnostic criteria [13], the subjects were classified into the following four subgroups: NGT; FPG mmol/l < 6.1 mmol/l and 2-hPG < 7.8 mmol/l, IGT; FPG < 7.0 mmol/l and 7.8 mmol/l ≤ 2-hPG < 11.1 mmol/l, IFG; 6.1 mmol/l ≤ FPG < 7.0 mmol/l and 2-hPG < 7.8 mmol/l, DM: 7.0 mmol/l ≤ FPG or 11.1 mmol/l ≤ 2-hPG.

As shown in the supplemental table, sensitivity was defined as the proportion of subjects with IGT by OGTT who were predicted to have a positive result by the estimation formula: $\{a/(a + c)\} \times 100$ (%). Specificity was defined as the proportion of subjects without IGT who were predicted not to have IGT; $\{d/(b + d)\} \times 100$ (%). Positive predictive value (PPV) was defined as the proportion of subjects predicted to have IGT who were truly IGT by OGTT; $\{a/(a + b)\} \times 100$ (%). Negative predictive value (NPV) was defined as the proportion of subjects predicted not to have IGT who were truly not IGT by OGTT; $\{d/(c + d)\} \times 100$ (%). A receiver operating characteristic (ROC) curve was constructed by plotting sensitivity against the false-positive rate (100 – specificity) (%) over a range of cut-off values.

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.diabres.2013.05.013>.

2.5. Statistical analyses

Statistical analyses were performed according to the following steps.

- (1) Background of the subjects: results were expressed as mean (standard deviation: SD or mean standard error: SE). Differences between the two groups were compared using the Student's t-test. *P* value < .05 (two-tailed) was considered as statistically significant.
- (2) The relationship between two variables: the relationship between measured 2-h post-challenge plasma glucose (m2-hPG) and clinical variables was evaluated by scatter plot and Pearson's correlation coefficient (*r*).
- (3) Derivation and validation: to avoid over-fitting of the estimation formula, two-step procedure was used. A total of 380 subjects were randomly divided into two groups at 1 to 1 ratio, a derivation group for constructing the estimation formula to screen IGT and a validation group without DM for evaluating the accuracy of the derived estimation formula.
- (4) Construction of the estimation formula in the derivation group: higher values of correlation coefficient demonstrated in the previous step were regarded as predictive

variables for m2-hPG, and the estimation formula of 2-hPG with these variables was then constructed by multiple linear regression analysis.

- (5) Evaluation of the derived estimation formula in the validation group: the diagnostic characteristics such as sensitivity, specificity, PPV, and NPV of this derived estimation formula were calculated. The performance of the estimation formula was assessed by calculating the area under the ROC curve based on sensitivity and specificity [28]. In addition, we determined the adequate cut-off value by considering these values.

All statistical analyses were performed using SAS version 9.2 (SAS Institute Inc, Cary, NC).

3. Results

3.1. Characteristics of the subjects

Clinical characteristics of the subjects are shown in Table 1. Results of age and HbA1c are shown as mean (SD); the others are shown as mean (SE). Because the number of IFG (*n* = 9) was too small for analysis, we compared physical and metabolic

Table 1 – Characteristics of the subjects.

	NGT	IFG	IGT	DM	Total
Number (%)	126 (33.2)	9 (2.4)	106 (27.9)	139 (36.6)	380 (100)
Male (%)	60 (47.6)	6 (66.7)	50 (47.2)	103 (74.1)	219 (57.6)
Age (year) [†]	54.5 (15.5)	61.4 (15.5)	57.0 (13.3)	63.2 (11.6)	58.5 (14.0)
BMI (kg/m ²) [‡]	23.5 (4.8)	26.1 (2.4)	25.9 (5.6) **vs. NGT vs. DM	24.4 (3.5)	24.6 (4.7)
HbA1c (%) [†]	5.9 (0.6)	6.5 (0.6)	6.2 (0.5) ***vs. NGT	7.0 (0.6) ***vs. NGT ***vs. IGT	6.4 (0.7)
HbA1c (mmol/mol)	40.9 (6.1)	47.5 (6.3)	44.0 (5.2) ***vs. NGT	53.1 (6.5) ***vs. NGT ***vs. IGT	46.0 (8.0)
F-IRI (pmol/l) [†]	36.0 (24.8)	50.7 (32.6)	43.7 (28.1) vs. NGT	41.7 (25.0) vs. NGT	40.6 (26.2)
2-hIRI (pmol/l) [†]	222.3 (155.6)	354.7 (282.4)	351.9 (217.9) ***vs. NGT	274.2 (180.5) vs. NGT vs. IGT	282.7 (194.6)
FPG (mmol/l) [†]	5.1 (0.5)	6.5 (0.2)	5.5 (0.6) ***vs. NGT	7.0 (1.2) ***vs. NGT ***vs. IGT	6.0 (1.2)
2-hPG (mmol/l) [†]	6.2 (1.1)	6.8 (0.7)	9.4 (0.9) ***vs. NGT	14.6 (2.8) ***vs. NGT ***vs. IGT	10.2 (4.0)
Insulinogenic index [‡]	65.1 (38.4)	31.2 (24.2)	38.5 (24.6) ***vs. NGT	15.8 (6.5) ***vs. NGT ***vs. IGT	33.8 (17.9)

Abbreviations: BMI, body mass index; HbA1c, hemoglobin A1c; F-IRI, fasting immunoreactive insulin; 2-hIRI, 2-h immunoreactive insulin; FPG, fasting plasma glucose; 2-hPG, 2-h post-challenge plasma glucose; NGT, normal glucose tolerance; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; DM, diabetes mellitus; SD, standard deviation; SE, standard error.

* *P* < .05.

** *P* < .01.

*** *P* < .001.

[†] Data are described means (SD).

[‡] Data are described means (SE).

variables among the three other groups (NGT, IGT, and DM). The total of mean age (SD) is 58.5 (14.0) years and 57.6% are males. Age of DM subjects is the highest ($P < .01$ vs. NGT) and BMI of IGT subjects is the highest ($P < .01$ vs. NGT and $P < .05$ vs. DM, respectively) among the three groups. HbA1c is significantly higher, while insulinogenic index is significantly lower in subjects with DM than in those with NGT and IGT. Glucose and plasma insulin levels during OGTT are given in the supplemental figure. For plasma glucose, subjects with NGT are those with 5.1 (0.5) mmol/l of FPG and 6.2 (1.1) mmol/l of 2-hPG (Supplemental Figure A). For subjects with IFG, FPG is 6.5 (0.2) mmol/l and for those with IGT, 2-hPG is 9.4 (0.9) mmol/l. For subjects with DM, FPG is 7.0 (1.2) mmol/l and 2-hPG is 14.6 (2.8) mmol/l. Early-phase insulin secretion shown in Supplemental Figure B is already decreased in the IGT stage as shown in the previous Japanese study [29].

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.diabres.2013.05.013>.

3.2. Relationship between two variables

We evaluated the relationship among all pairs of continuous variables using scatter plot and calculated Pearson's correlation coefficient. Using this procedure, we found the predictive variables for m2-hPG in this target population.

Fig. 1 shows the scatter plot of m2-hPG and FPG (Fig. 1A) and that of m2-hPG and HbA1c (Fig. 1B) in OGTT of all subjects. The correlation coefficient (r) between two variables is 0.74 and 0.67, respectively. Except for these two variables, there is no higher correlation coefficient than 0.5 between m2-hPG and the physical, metabolic variables.

3.3. Construction of the estimation formula in the derivation group

First, all subjects were randomly divided 1:1 into the derivation group and the validation group to avoid over-fitting.

At a stage prior to this, FPG and HbA1c were substantiated as main predictive variables for m2-hPG, then the estimation formula with these variables was constructed by using the multiple linear regression analysis in the derivation group. The obtained linear regression equation (estimation formula) is $e2\text{-hPG} = 1.66 \times \text{FPG (mmol/l)} + 1.63 \times \text{HbA1c (NGSP: \%)} - 10.11$, or $e2\text{-hPG} = 1.66 \times \text{FPG (mmol/l)} + 0.15 \times \text{HbA1c (mmol/mol)} - 6.61$. R^2 (coefficient of determination) is 60.2%. Moreover, we analyzed to determine whether inclusion of other variables known to affect 2-hPG improved the accuracy of this formula. Even though other variables such as BMI, age, gender, and IRI are included in the regression model, R^2 remains substantially unchanged (data not shown). FPG and HbA1c are thus the best predictors of 2-hPG based on the linear regression model and we concluded the estimation formula of 2-hPG shown in Fig. 2 in this derivation group.

3.4. Evaluation of the derived estimation formula in the validation group

The accuracy of this estimation formula: diagnostic characteristics such as sensitivity, specificity, PPV, NPV, and the area under the ROC curve were calculated in the validation group. Table 2 shows the results of sensitivity, specificity, PPV, and NPV for every 0.2 mmol/l of e2-hPG in this group. When the cut-off value is set to the diagnostic criteria of IGT, 7.8 mmol/l of e2-hPG, sensitivity, specificity and NPV of this formula are 83.3%, 44.1%, and 74.3%, respectively. When the cut-off value is ≤ 7.8 mmol/l of e2-hPG, sensitivity is retained more than 80%, and when lowered to 7.2 mmol/l of e2-hPG, sensitivity, specificity, and NPV are 94.4%, 30.5% and 85.7%, respectively. These results are plotted in Fig. 3. In the validation group, the ROC curves obtained by calculating sensitivity and specificity at possible cut-off points of estimated 2-hPG are also shown in Fig. 3. The performance of the estimation formula was assessed by calculating the area under the ROC curve, which is 0.68.

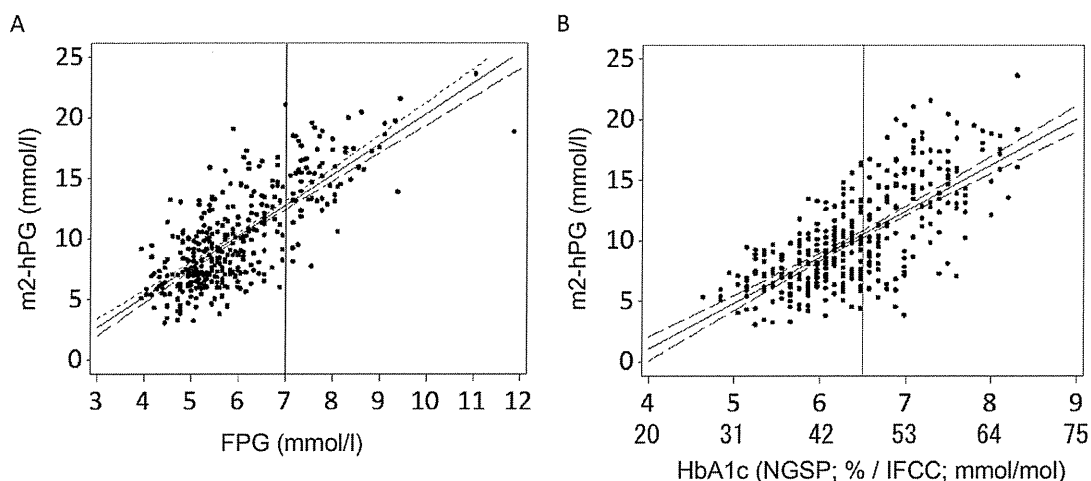


Fig. 1 – Scatter plot of measured 2-hPG (m2-hPG) and FPG in OGTT (A) and that of m2-hPG and HbA1c in OGTT (B); the correlation coefficient (r) = 0.74, 0.67, respectively.

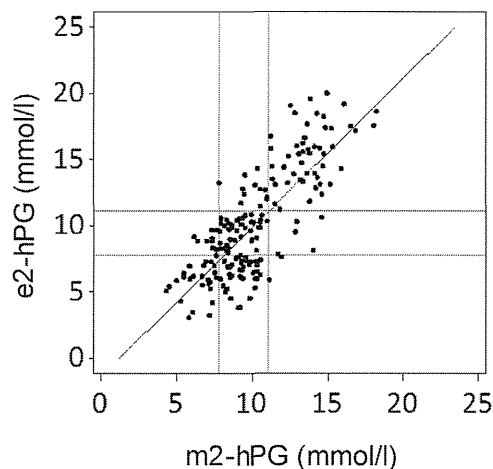


Fig. 2 – Scatter plot and the estimation formula of estimated 2h-PG (e2-hPG) in derivation group. $e2-hPG = 1.66 \times FPG (mmol/l) + 1.63 \times HbA1c (\%) - 10.11 (R^2 = 60.2\%)$. $e2-hPG = 1.66 \times FPG (mmol/l) + 0.15 \times HbA1c (mmol/mol) - 6.61 (R^2 = 60.2\%)$.

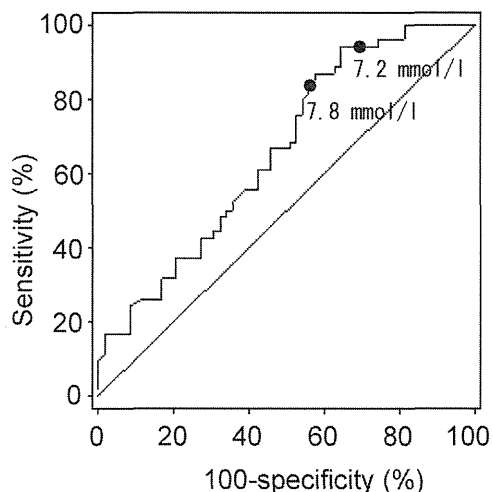


Fig. 3 – Receiver operating characteristic (ROC) curves obtained by calculating sensitivity and specificity at possible cut-off points of estimated 2-hPG in validation group. The horizontal axis shows the (100 – specificity) (%) and the vertical axis shows the sensitivity (%). When the cut-off value is set to the diagnostic criteria of IGT, 7.8 mmol/l of e2-hPG, sensitivity and specificity are 83.3% and 44.1%, respectively. When the cut-off value is set lowered to 7.2 mmol/l of e2-hPG, those are 94.4% and 30.5%, respectively. The area under the ROC curve is 0.68.

4. Discussion

In the present study, we found that the main predictive variables associated with m2-hPG are FPG and HbA1c from the data of 380 hospital-based Japanese subjects. We were able to develop a validated estimation formula for 2-hPG with these variables as an alternative to OGTT for IGT screening.

Studies have reported that lifestyle and pharmacological intervention for subjects with IGT can prevent or delay the onset of type 2 DM [6–9]. Detection and early intervention for these high-risk individuals is critical for preventing type 2 DM and reducing diabetic complications. Even though OGTT is the “gold standard” method for diagnosing IGT, in a large population that method is time-consuming and expensive [14].

Some studies have investigated alternative methods for evaluating IGT without performing OGTT [15–23]. The single

use of FPG or HbA1c is not suitable for IGT screening because of the low detection rate [15–18]. It was reported in a systematic review that for detecting IGT by HbA1c or FPG separately, that sensitivity is around 50% [16]. Indeed, in our result, with single use of FPG or HbA1c, R^2 was 56.2% and 48.3%, respectively (Fig. 1), while using the estimation formula with both FPG and HbA1c, R^2 was 60.2% (Fig. 2) for IGT screening. This result is compatible with the previous study recommending the combined use of these two variables to be more effective than single use for detecting IGT [19]. But this study did not elucidate the influence of each of the variables on 2-hPG.

Table 2 – Summary of sensitivity, specificity, PPV, and NPV for every 0.2 mmol/l of estimated 2-hPG (e-2hPG) in the validation group.

Cut-off value of e2-hPG (mmol/l)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
6.8	96.3	23.7	53.6	87.5
7.0	96.3	25.4	54.2	88.2
7.2	94.4	30.5	55.4	85.7
7.4	87.0	37.3	56.0	75.9
7.6	87.0	40.7	57.3	77.4
7.8	83.3	44.1	57.7	74.3
8.0	70.4	47.5	55.1	63.6
8.2	66.7	52.5	56.3	63.3
8.4	61.1	57.6	56.9	61.8
8.6	53.7	62.7	56.9	59.7
8.8	50.0	66.1	57.4	59.1

PPV, positive predictive value; NPV, negative predictive value.

Caucasians generally have higher BMI and insulin resistance is important in progression from NGT to IGT [30], while Asians including Japanese are generally less obese and have higher insulin sensitivity than Caucasians. Several previous studies reported that BMI is a helpful factor for IGT screening [17,20–23]. High BMI (>27 or >30 kg/m²) is associated with higher prevalence of IGT compared to that with BMI <25 kg/m². Hiltunen et al. reported that higher BMI (>30 kg/m²) was the best predictor of IGT in a Finland population-based study and Saydah SH et al. reported that BMI (>30 kg/m²) and age in addition to FPG or HbA1c can be used to improve the sensitivity for detecting IGT without OGTT among U.S. populations [20,23]. Contrary to these reports, we did not find a high correlation between 2-hPG and BMI ($r = 0.03$). The proportion of obesity of these studies is high, the ratio of BMI >27 kg/m² and >30 kg/m² are 53% and 24%, respectively [20,23], while in our study the mean (SD) of BMI was 24.6 (4.7) kg/m² and the ratio of obese subjects with BMI >30 kg/m² are only 1%. It was reported previously that the difference with or without the obesity (BMI >25 kg/m²) did not contribute to IGT screening in Japanese [21]. The BMI used in the study was similar to that in our study (BMI: 23–25 kg/m²). On the other hand, insulin secretion rather than insulin resistance is a more significant factor in progression from NGT to type 2 DM via IGT in Asian diabetes [29,31–35]. Indeed, in the present study insulin secretion is decreased gradually from NGT via IGT to type 2 DM (65.1, 38.5, 15.8, respectively) as in other previous reports in Asian diabetes [31,35] (Table 1). Taken together, the discrepancy of the correlation may be due to the ethnic differences such as BMI and insulin secretory capacity. The variables affecting 2-hPG during OGTT might differ among ethnic populations and require a different estimation formula and adequate cut-off value to identify IGT. It is also reported that age has an effect on the accuracy of IGT screening [22]. In our study, however, there was low correlation between 2-hPG and age ($r = 0.24$). The reason may be the different methods of analysis. We considered age from 20 to 89 years as a continuous variable rather than as a categorical one with relatively narrow age. In addition, gender was not an independent predictive variable for 2-hPG and even though included in the regression model, R² remains substantially unchanged in our study (data not shown).

Generally, the performance of a screening test depends on the cut-off value and it is rare to have both high sensitivity and specificity. The priority in this trade-off is determined by the characteristics of the condition to be diagnosed [36,37]. In the case of IGT, mis-identifying IGT subjects as normal (false negatives) is more critical than classifying healthy subjects as abnormal (false positives), since these subjects are at high risk for cardiovascular diseases as well as diabetes and its complications. Thus, a screening test for IGT should prioritize high sensitivity to decrease the false-negatives. As summarized in Table 2, when the cut-off value was set to the diagnostic criteria of IGT, 7.8 mmol/l of e2-hPG, sensitivity of this formula was 83.3%. However, even with this cut-off value, NPV still remained 74.3%. If the cut-off value was lowered to 7.2 mmol/l, NPV was up to 85.7% with 94.4% sensitivity. When a screening test has high sensitivity, subjects having a negative result can be judged as negative with high precision

[36–39]. The rule of SnNout states that if a screening test has high sensitivity (Sn), a negative result (N) rules out (Out) the target disorder, which describes IGT in our study. In accord with this theory, using our high-sensitive estimation formula for IGT screening and with the cut-off value set to 7.2 mmol/l of e2-hPG with 94.4% sensitivity, the subjects with e2-hPG ≤ 7.2 mmol/l were ruled out of the diagnosis of IGT. Thus, further OGTT to diagnose whether or not they have IGT may not be necessary for these low risk subjects.

Our study has several limitations. One is the high prevalence of type 2 DM subjects in our hospital-based study. It is reported that the prevalence of type 2 DM varies across studies, in hospital-based studies ranging from 10 to 44% and in community-based studies ranging from 6.2 to 7.4% [16]. While the proportion of subgroups in OGTT would affect the efficacy of the estimation formula, there are at present no established data of general population-based or hospital-based studies. Thus, no comparisons are possible between our results with previous findings. Another limitation is screening bias because it is a cross-sectional study. There are no definite inclusion criteria—they depend on the individual judgment of each doctor. Our results regarding sensitivity, specificity, and cut-off value are internally validated, but it remains to be determined whether these findings apply to other populations: generation-based or higher BMI like Caucasian and so on. Therefore further studies are required to validate in the broader population.

In conclusion, our high-sensitivity estimation formula based on FPG and HbA1c may be useful in screening for IGT. More than 80% sensitivity of this formula was preserved at ≤ 7.8 mmol/l of e2-hPG in this hospital-based study. In addition, when the levels of e2-hPG are ≤ 7.2 mmol/l, this estimation formula can be used to identify subjects with normal glucose tolerance.

Conflict of interest

The authors declare that they have no conflict of interest.

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REFERENCES

- [1] Blake DR, Meigs JB, Müller DC, Najjar SS, Andres R, Nathan DM. Impaired glucose tolerance, but not impaired fasting glucose, is associated with increased levels of coronary heart disease risk factors: results from the Baltimore Longitudinal Study on Aging. *Diabetes* 2004;53(8): 2095–100.
- [2] Rodriguez BL, Curb JD, Burchfiel CM, Huang B, Sharp DS, Lu GY, et al. Impaired glucose tolerance, diabetes, and cardiovascular disease risk factor profiles in the elderly, the Honolulu Heart Program. *Diabetes Care* 1996;19(6): 587–90.

- [3] Tominaga M, Eguchi H, Manaka H, Igarashi K, Kato T, Sekikawa A. Impaired glucose tolerance is a risk factor for cardiovascular disease, but not impaired fasting glucose: the Funagata diabetes study. *Diabetes Care* 1999;22(6):920–4.
- [4] Fuller JH, Shipley MJ, Rose G, Jarrett RJ, Keen H. Coronary-heart-disease risk and impaired glucose tolerance: the Whitehall study. *Lancet* 1980;1(8183):1373–6.
- [5] Gerstein HC, Santaguida P, Raina P, Morrison KM, Balion C, Hunt D, et al. Annual incidence and relative risk of diabetes in people with various categories of dysglycemia: a systematic overview and meta-analysis of prospective studies. *Diabetes Res Clin Pract* 2007;78(3):305–12.
- [6] Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med* 2002;346(6):393–403.
- [7] Tuomilehto J, Lindström J, Eriksson JG, Valle TT, Hämäläinen H, Ilanne-Parikka P, et al. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *N Engl J Med* 2001;344(18):1343–50.
- [8] Chiasson JL, Josse RG, Gomis R, Hanefeld M, Karasik A, Laakso M. Acarbose for prevention of type 2 diabetes mellitus: the STOP-NIDDM randomised trial. *Lancet* 2002;359(9323):2072–7.
- [9] Gillies CL, Abrams KR, Lambert PC, Cooper NJ, Sutton AJ, Hsu RT, et al. Pharmacological and lifestyle interventions to prevent or delay type 2 diabetes in people with impaired glucose tolerance: systematic review and meta-analysis. *BMJ* 2007;334(7588):299.
- [10] DECODE Study Group, the European Diabetes Epidemiology Group. Glucose tolerance and cardiovascular mortality: comparison of fasting and 2-hour diagnostic criteria. *Arch Intern Med* 2001;161(3):397–405.
- [11] Nakagami T, DECODA Study Group. Hyperglycaemia and mortality from all causes and from cardiovascular disease in five populations of Asian origin. *Diabetologia* 2004;47(3):358–94.
- [12] DeFronzo RA, Abdul-Ghani M. Assessment and treatment of cardiovascular risk in prediabetes: impaired glucose tolerance and impaired fasting glucose. *Am J Cardiol* 2011;108(Suppl. 3):3B–24B.
- [13] Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1. Diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998;15(7):539–53.
- [14] Report of the Expert Committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 1997;20(7):1183–97.
- [15] Pinelli NR, Jantz AS, Martin ET, Jaber LA. Sensitivity and specificity of glycated hemoglobin as a diagnostic test for diabetes and prediabetes in Arabs. *J Clin Endocrinol Metab* 2011;96(10):E1680–3.
- [16] Bennett CM, Guo M, Dharmage SC. HbA(1c) as a screening tool for detection of type 2 diabetes: a systematic review. *Diabet Med* 2007;24(4):333–43.
- [17] Mannucci E, Bardini G, Ognibene A, Rotella CM. Comparison of ADA and WHO screening methods for diabetes mellitus in obese patients. *American Diabetes Association. Diabet Med* 1999;16(7):579–85.
- [18] Motala AA, Omar MA. The value of glycosylated haemoglobin as a substitute for the oral glucose tolerance test in the detection of impaired glucose tolerance (IGT). *Diabetes Res Clin Pract* 1992;17(3):199–207.
- [19] Hu Y, Liu W, Chen Y, Zhang M, Wang L, Zhou H, et al. Combined use of fasting plasma glucose and glycated hemoglobin A1c in the screening of diabetes and impaired glucose tolerance. *Acta Diabetol* 2010;47(3):231–6.
- [20] Saydah SH, Byrd-Holt D, Harris MI. Projected impact of implementing the results of the diabetes prevention program in the U.S. population. *Diabetes Care* 2002;25(11):1940–5.
- [21] Gomyo M, Sakane N, Kamae I, Sato S, Suzuki K, Tominaga M, et al. Effects of sex, age and BMI on screening tests for impaired glucose tolerance. *Diabetes Res Clin Pract* 2004;64(2):129–36.
- [22] Nelson KM, Boyko EJ. Third National Health and Nutrition Examination Survey, Predicting impaired glucose tolerance using common clinical information: data from the Third National Health and Nutrition Examination Survey. *Diabetes Care* 2003;26(7):2058–62.
- [23] Hilltunen L, Kivelä SL, Läärä E, Keinänen-Kiukaanniemi S. Progression of normal glucose tolerance to impaired glucose tolerance or diabetes in the elderly. *Diabetes Res Clin Pract* 1997;35(2–3):99–106.
- [24] Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. *National Diabetes Data Group. Diabetes* 1979;28(12):1039–57.
- [25] Seltzer HS, Allen EW, Herron Jr AL, Brennan MT. Insulin secretion in response to glycemic stimulus: relation of delayed initial release to carbohydrate intolerance in mild diabetes mellitus. *J Clin Invest* 1967;46(3):323–35.
- [26] Seino Y, Ikeda M, Yawata M, Imura H. The insulinogenic index in secondary diabetes. *Horm Metab Res* 1975;7(2):107–15.
- [27] Kashiwagi A, Kasuga M, Araki E, Oka Y, Hanafusa T, Ito H, et al. International clinical harmonization of glycated hemoglobin in Japan: from Japan Diabetes Society to National Glycohemoglobin Standardization Program values. *J Diabetes Invest* 2012;3(1):39–40.
- [28] Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* 1982;143(1):29–36.
- [29] Fukushima M, Suzuki H, Seino Y. Insulin secretion capacity in the development from normal glucose tolerance to type 2 diabetes. *Diabetes Res Clin Pract* 2004;66(Suppl. 1):S37–43.
- [30] Saad MF, Knowler WC, Pettitt D, Nelson RG, Charles MA, Bennet P. A two-step model for development of non-insulin-dependent diabetes. *Am J Med* 1991;90:229–35.
- [31] Suzuki H, Fukushima M, Usami M, Ikeda M, Taniguchi A, Nakai Y, et al. Factors responsible for development from normal glucose tolerance to isolated postchallenge hyperglycemia. *Diabetes Care* 2003;26(4):1211–5.
- [32] Haffner SM, Howard G, Mayer E, Bergman RN, Savage PJ, Rewers M, et al. Insulin sensitivity and acute insulin response in African-Americans, non-Hispanic whites, and Hispanics with NIDDM: the Insulin Resistance Atherosclerosis Study. *Diabetes* 1997;46(1):63–9.
- [33] Matsumoto K, Miyake S, Yano M, Ueki Y, Yamaguchi Y, Akazawa S, et al. Glucose tolerance, insulin secretion, and insulin sensitivity in nonobese and obese Japanese subjects. *Diabetes Care* 1997;20(10):1562–8.
- [34] Mitrakou A, Kelley D, Mokan M, Veneman T, Pangburn T, Reilly J, et al. Role of reduced suppression of glucose production and diminished early insulin release in impaired glucose tolerance. *N Engl J Med* 1992;326(1):22–9.
- [35] Fukushima M, Usami M, Ikeda M, Nakai Y, Taniguchi A, Matsuura T, et al. Insulin secretion and insulin sensitivity at different stages of glucose tolerance: a cross-sectional

- study of Japanese type 2 diabetes. *Metabolism* 2004;53(7):831–5.
- [36] Fletcher RH, Fletcher SW, Wagner EH. *Clinical epidemiology. The essentials*, 3rd ed., Baltimore: Williams and Wilkins; 1996.
- [37] Hennekens CH, Buring JE, Mayrent SL. *Epidemiology in medicine*. Boston: Little Brown; 1987.
- [38] Sackett DL, Straus SE, Richardson WS, Rosenberg W, Haynes RB. *Evidence-based medicine. How to practice and teach EBM*. New York: Churchill Livingstone; 2000.
- [39] Sackett DL, Haynes RB, Guyatt GH, Tugwell P. *Clinical epidemiology, a basic science for clinical medicine*. Boston: Little Brown; 1992.

Tetrahydrobiopterin Has a Glucose-Lowering Effect by Suppressing Hepatic Gluconeogenesis in an Endothelial Nitric Oxide Synthase–Dependent Manner in Diabetic Mice

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Endothelial nitric oxide synthase (eNOS) dysfunction induces insulin resistance and glucose intolerance. Tetrahydrobiopterin (BH₄) is an essential cofactor of eNOS that regulates eNOS activity. In the diabetic state, BH₄ is oxidized to 7,8-dihydrobiopterin, which leads to eNOS dysfunction owing to eNOS uncoupling. The current study investigates the effects of BH₄ on glucose metabolism and insulin sensitivity in diabetic mice. Single administration of BH₄ lowered fasting blood glucose levels in wild-type mice with streptozotocin (STZ)-induced diabetes and alleviated eNOS dysfunction by increasing eNOS dimerization in the liver of these mice. Liver has a critical role in glucose-lowering effects of BH₄ through suppression of hepatic gluconeogenesis. BH₄ activated AMP kinase (AMPK), and the suppressing effect of BH₄ on gluconeogenesis was AMPK-dependent. In addition, the glucose-lowering effect and activation of AMPK by BH₄ did not appear in mice with STZ-induced diabetes lacking eNOS. Consecutive administration of BH₄ in *ob/ob* mice ameliorated glucose intolerance and insulin resistance. Taken together, BH₄ suppresses hepatic gluconeogenesis in an eNOS-dependent manner, and BH₄ has a glucose-lowering effect as well as an insulin-sensitizing effect in diabetic mice. BH₄ has potential in the treatment of type 2 diabetes. *Diabetes* 62:3033–3043, 2013

Nitric oxide (NO) is a biological messenger produced by NO synthase (NOS), which includes endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) isoforms. eNOS-derived NO is well-known to have a pivotal role in physiological regulation of endothelial function (1,2). eNOS dysfunction occurs in conditions of diabetes and is known to induce insulin resistance and glucose intolerance (3–5). Insulin resistance caused by eNOS dysfunction is thought to be induced by endothelial dysfunction, leading to decreased skeletal muscle blood flow and glucose uptake (4). On the other hand, glucose transport in isolated skeletal muscle is lower in eNOS-deficient (eNOS^{-/-}) mice, indicating that eNOS expressed in skeletal muscle also regulates its glucose

uptake (4). Moreover, eNOS^{-/-} mice are insulin resistant at the level of liver (5). These studies suggest that eNOS plays a central role in the regulation of glucose metabolism and insulin sensitivity and represents several therapeutic targets for type 2 diabetes.

The function of eNOS is regulated by multiple factors such as mRNA expression of eNOS, L-arginine, influx of Ca²⁺, and tetrahydrobiopterin (BH₄) (2,6,7). BH₄ is an essential cofactor for eNOS catalysis and functions as an allosteric modulator of arginine binding (7,8). Binding of BH₄ to eNOS elicits a conformational change that increases the affinity for binding of arginine-based ligands. BH₄ binding also plays a role in dimer formation of the active and stabilized form of eNOS (8). BH₄ is converted to 7,8-dihydrobiopterin (BH₂) by exposure to oxidative stress such as diabetes (8,9). Increase in BH₂ induces dysfunction of eNOS, as BH₂ is inactive for NOS cofactor function and competes with BH₄ for BH₄ binding (8,9). Furthermore, in states of diabetes and high glucose, de novo synthesis of BH₄, which is rate limited by GTP cyclohydrolase I (GTPCH I), is impaired (10–13). Thus, the availability of BH₄ is reduced and the function of eNOS is altered so that the enzyme produces superoxide anion (O₂⁻) rather than NO, a phenomenon called “eNOS uncoupling” (7,8,14). Supplementation of BH₄ can improve endothelial dysfunction by elevating the BH₄-to-BH₂ ratio, leading to recoupling of eNOS, and has been used in clinical trials with patients with atherosclerotic diseases for the expected vasodilatation effects of BH₄ through NO production (15). However, it is unclear whether BH₄ improves glucose metabolism and insulin sensitivity in diabetic conditions.

In the current study, we investigated the effects of BH₄ on blood glucose levels and insulin sensitivity in diabetic mice. Fasting blood glucose levels are regulated by the level of hepatic gluconeogenesis, elevation of which is the major cause of fasting hyperglycemia in diabetes (16,17). We demonstrate here that BH₄ lowers fasting blood glucose levels and suppresses gluconeogenesis in liver in an eNOS-dependent manner. In addition, BH₄ has an ameliorating effect on glucose intolerance as well as insulin resistance in diabetic mice. Using primary hepatocytes isolated from mouse liver, we have clarified the mechanism by which BH₄ suppresses hepatic gluconeogenesis. These data suggest that BH₄ has potential as a novel therapeutic approach to diabetes.

RESEARCH DESIGN AND METHODS

Male C57/BL6 (wild-type) mice and male heterozygous *Ins2^{Akita}* (diabetic Akita) mice, which exhibit hyperglycemia with reduced β-cell mass caused by a point

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mutation in the insulin 2 gene that leads to misfolded insulin and severe endoplasmic reticulum stress, were obtained from Shimizu (Kyoto, Japan) (18). Male eNOS^{-/-} mice in the C57/BL6 mice background were obtained from The Jackson Laboratory (Bar Harbor, ME). Male B6.V-Lepob/J (*ob/ob*) mice were obtained from Charles River Japan (Yokohama, Japan). Mice with streptozotocin (STZ)-induced diabetes were made by injection of STZ (120 mg/kg i.p.) to 7-week-old wild-type or eNOS^{-/-} mice. At 3 weeks after injection of STZ, the animals were confirmed to be diabetic by both high blood glucose levels (≥ 15 mmol/L) and other diabetic features, including polyuria, polydipsia, and hyperglycemia.

The mice were maintained in a temperature-controlled ($25 \pm 2^\circ\text{C}$) environment with a 12-h light/dark cycle with free access to standard laboratory chow and water. All experiments were carried out with mice aged 8–10 weeks. The animals were maintained and used in accordance with the Guidelines for Animal Experiments of Kyoto University. All experiments involving animals were conducted in accordance with the Guidelines for Animal Experiments of Kyoto University and were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

Preparations and cultures of mouse hepatocyte and aortic endothelial cell. Mouse hepatocytes were isolated by collagenase digestion as previously described (19). Primary hepatocytes were prepared by seeding in sixwell type 1 collagen-coated plates at a density of 1.5×10^6 cells in Dulbecco's modified Eagle's medium (DMEM) (low glucose, 5.6 mmol/L) containing 10% (vol/vol) FBS, 100 nmol/L regular insulin, 50 units/mL penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin. Hepatocytes were then cultured overnight in a humidified atmosphere (5% CO₂) at 37°C. As for mouse endothelial cells (ECs), the aorta was dissected and filled with collagenase type II solution. After incubation for 45 min at 37°C, ECs were removed from the aorta and collected by centrifugation at 1,200 rpm for 5 min. The EC was cultured in a sixwell collagen type I-coated dish for 1 week. **Glucose production via gluconeogenesis in hepatocytes.** Freshly isolated hepatocytes from mice fasted for 16 h were treated in 24-well plates (7.5×10^5 cells/well) in buffer A, which consisted of 0.5 mL Krebs-Ringer bicarbonate medium of 119.4 mmol/L NaCl, 3.7 mmol/L KCl, 2.7 mmol/L CaCl₂, 1.3 mmol/L KH₂PO₄, 1.3 mmol/L MgSO₄, and 24.8 mmol/L NaHCO₃ without glucose; 2% (wt/vol) BSA; 0.24 mmol/L 3-isobutyl-1-methylxanthine; and gluconeogenic substrates (1 mmol/L pyruvate plus 10 mmol/L lactate). Hepatocytes were treated with BH₄ (Schircks Laboratories, Jona, Switzerland), sodium nitroprusside (SNP), NG-nitro-L-arginine methyl ester (Sigma, St. Louis, MO), sepiapterin (Schircks Laboratories), erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (Wako, Osaka, Japan), and compound C (Sigma). Glucose production was measured by glucose oxidation method as previously described (19).

Immunoblotting analysis of hepatocytes. Western blotting was performed as previously described (19). Primary hepatocytes cultured overnight were incubated in buffer A treated with BH₄, SNP, sepiapterin, and EHNA. Hepatocytes were homogenized in lysis buffer. Cell lysates (50–150 μg protein/lane) were heated at 95°C for 5 min and subjected to electrophoresis on 6–10% (vol/vol) sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose membranes. For analysis of eNOS dimerization, the samples were not heated and the temperature was maintained at $<15^\circ\text{C}$ during electrophoresis. Primary antibodies used were anti-phosphorylated (phospho)-AMP kinase (AMPK α (Thr¹⁷²), anti-AMPK α , anti-phospho-acetyl-CoA carboxylase (ACC) (Ser⁷⁹), anti-ACC, anti-phospho-eNOS (Ser¹¹⁷⁷), anti-phospho-Akt (Ser⁴⁷³), anti-Akt (all at 1:1,000 dilution; Cell Signaling Technology, Danvers, MA), anti-eNOS polyclonal antibody (1:500 dilution; BD Transduction Laboratories, San Jose, CA), anti-CD31 monoclonal antibody (1:2,000 dilution; Dianova, Hamburg, Germany), anti-GTPCH I (1:3,000; kind gift from Prof. H. Ichinose, Tokyo Institute of Technology), anti-dihydrofolate reductase (DHFR), anti- α -antitrypsin (1:500; Santa Cruz, Delaware, CA), and anti- β -actin (1:5,000; Sigma). Secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit, -mouse, -rat, or -goat antibody (GE Healthcare, Buckinghamshire, U.K.). The fluorescent bands were visualized using a detection system (Amersham ECL Plus; GE Healthcare) and quantified by densitometry using Image J software from National Institutes of Health (Bethesda, MD).

Cell transfection and short interfering RNA. Stealth short interfering RNA (siRNA) of AMPK α 1 was purchased from Invitrogen (Carlsbad, CA). The sequences of siRNA for AMPK α 1 were 5'-UCUCUUUCUGAGGACCAUCUU AU-3' and 5'-AUAAGAUGGGUCCUCAGGAAAGAGA-3'. The sequences of control siRNAs were 5'-ACCAACAACAGUUUGGAAUAGGGA-3' and 5'-UCC CUAUCCCAAACUGUUGUUGU-3'. Isolated hepatocytes in DMEM (low glucose, 5.6 mmol/L) containing 10% (vol/vol) FBS and 100 nmol/L regular insulin were mixed with Opti-MEM containing siRNA and Lipofectamine RNAi MAX (Invitrogen) and were plated on wells and then incubated at 37°C in a CO₂ incubator. The final amounts of hepatocytes, DMEM, Opti-MEM, siRNA, and Lipofectamine RNAi MAX were 5.0×10^5 cells/mL, 75% (vol/vol), 25% (vol/vol), 50 nmol/L, and 0.2%, respectively. Medium was replaced with DMEM 6 h after transfection. Forty-eight hours after transfection, the medium was replaced with buffer A, the cells were incubated for 60 min with or without BH₄, and the glucose content of the supernatant was measured.

Nitrite/nitrate analysis. Primary hepatocytes and liver tissues were homogenized in buffer A, and the amount of nitrite/nitrate in the supernatant was determined by a fluorescence method.

Immunocytochemistry. The hepatocytes were incubated with rabbit polyclonal anti-nitrotyrosine antibody (1:100 dilution; Millipore, Billerica, MA). Cells were then incubated with goat anti-rabbit IgG fluorescein-conjugated secondary antibody (1:100 dilution, Alexa Fluor 488; Invitrogen). Fluorescence in cells was monitored as previously described (19).

Measurement of adenine nucleotide content. After primary isolated hepatocytes were incubated in buffer A with or without BH₄ and SNP for 30 min, treatment was stopped by rapid addition of 0.1 mL of 2 mol/L HClO₄, followed by mixing by vortex and sonication in ice-cold water for 3 min. Adenine nucleotide contents were measured by a luminometric method as previously described (19,20).

Isolation of total RNA and quantitative RT-PCR. Total RNA was isolated from livers of 10 week-old wild-type mice, wild-type mice with STZ-induced diabetes, and *ob/ob* mice using Trizol (Invitrogen) as previously described (21). The mouse sequence of forward and reverse primers to detect GTPCH I and DHFR, glucose 6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), and glyceraldehyde-3-phosphate dehydrogenase as an inner control are shown in Supplementary Table 1. SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA) was prepared for the quantitative RT-PCR run. The thermal cycling conditions were denaturation at 95°C for 10 min followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. mRNA levels were measured by real-time quantitative RT-PCR using ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

Biopterin analysis. Tissues or whole blood of wild-type mice and wild-type mice with STZ-induced diabetes was collected. For measurement of uptake of BH₄ in liver, BH₄ (20 mg/kg) dissolved with 0.9% (wt/vol) sterile saline was administered intraperitoneally to wild-type mice. After cervical dislocation, the mice were abdominally dissected and liver tissues were collected at 0, 30, 60, 120, and 180 min after injection. The organs were weighed, frozen immediately in liquid N₂, and then stored at -80°C . Total biopterin, BH₄, and BH₂ were measured as previously described (22).

Effect of BH₄ on blood glucose levels of wild-type mice with STZ-induced diabetes, eNOS^{-/-} mice with STZ-induced diabetes, and diabetic Akita mice. Blood glucose levels were measured in wild-type mice with STZ-induced diabetes, eNOS^{-/-} mice with STZ-induced diabetes, and diabetic Akita mice fasted for 16 h, and BH₄ (20 mg/kg) or metformin (250 mg/kg; Sigma) in 0.9% (wt/vol) sterile saline or 0.9% sterile saline alone was injected intraperitoneally. Blood glucose levels were measured again 2 h after injection.

Effect of BH₄ on blood glucose levels of *ob/ob* mice. Blood glucose levels and body weight of *ob/ob* mice were measured. The mice were divided into two groups shown in Supplementary Table 2, and 0.9% (wt/vol) sterile saline with or without BH₄ (10 mg/kg) was injected intraperitoneally twice a day for 10 days. Fed blood glucose levels were measured. After fasting overnight for 16 h, fasting blood glucose levels were measured.

Intraperitoneal glucose tolerance test. Wild-type mice were fasted overnight for 16 h, and glucose (2 g/kg) was injected intraperitoneally with BH₄ (20 mg/kg) in 0.9% (wt/vol) sterile saline or 0.9% sterile saline alone. After 10 days' treatment of saline with or without BH₄ (20 mg/kg), *ob/ob* mice were fasted overnight for 16 h, and glucose (1 g/kg) was injected intraperitoneally. Blood glucose levels and plasma insulin concentrations were measured at 0, 30, 60, 90, and 120 min after injection. Plasma insulin concentrations were determined by using an ELISA kit (Shibayagi, Gunma, Japan). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated with the following formula: [fasting insulin (mU/L) \times fasting plasma glucose (mmol/L)]/22.5.

Pyruvate tolerance test. Pyruvate, BH₄, and sepiapterin were dissolved with 0.9% (wt/vol) sterile saline. Wild-type, eNOS^{-/-}, and *ob/ob* mice were fasted overnight for 16 h, and pyruvate (1 g/kg) was injected intraperitoneally with or without BH₄ (20 mg/kg) and sepiapterin (20 mg/kg). Blood glucose levels were measured at 0, 30, 60, 90, and 120 min after injection.

Insulin tolerance test. After 10 days' treatment of saline with or without BH₄ (20 mg/kg), *ob/ob* mice were fasted for 6 h, and regular insulin (1 units/kg i.p.) was injected with 0.9% sterile saline. Blood glucose levels were measured at 0, 30, 60, 90, and 120 min after injection.

Statistics. Comparison between two groups was performed using unpaired Student *t* test (not noted) and paired Student *t* test. For more than two groups, one-way or two-way ANOVA followed by post hoc Bonferroni testing was performed. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Biopterin dynamics and effects of BH₄ on blood glucose levels in diabetic mice. In STZ diabetic wild-type mice, the content of BH₂ was increased and the