a subsequent 35 cycles of reaction at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min; the reaction conditions with KOD FX were an initial denaturation step of 94°C for 2min and a subsequent 35 cycles of reaction at 98°C for 10 sec, 60°C for 30 sec, and 68°C for 1 min. After purification, each PCR product was subjected to cycle sequencing with BigDye Terminator cycle sequencing FS (Applied Biosystems, Foster City, CA) using each forward and reverse primer. Reaction products were purified by ethanol precipitation and sequenced by ABI PRISM 3130 sequencer (Applied Biosystems).

Estimation of haplotype frequencies and evaluation of pattern of LD in KLF11

Haplotypes comprising tag SNPs and haplogenotypes were inferred by the expectation-maximization method by Haploview (http://www.broad.mit.edu/personal/jcbarret/haploview) and PHASE 2.1.1 (http://www.stat.washington.edu/stephens/software.html), respectively.

Mutation screening and genotyping of frequent polymorphisms in KLF11

We examined all of the coding regions and the putative promoter region of *KLF11* in 182 early-onset T2DM, 96 of the 553 late-onset T2DM patients (56 males and 40 females; age 63.2 ±11.0yr, BMI, 24.5±5.4kg/m^2; HbA1c 7.9±1.6 %) and 96 of 563 control subjects (35 males and 61 females; age 67.6 ± 5.8age yr; BMI, 22.8 ± 2.8 kg/m^2; HbA1c 4.9 ± 0.3 %). We performed an additional screening for R29Q and S124F mutations in all late-onset T2DM patients and controls.

Association study was performed for tag SNPs (SNP2,-3,-5,-6) in the promoter region with 96 subjects each from 552 late-onset T2DM and 563 controls by direct sequencing. As it was extremely difficult to amplify the promoter region due to its high GC content, only 96 subjects from each group were examined. Association study for tag SNP 13 (rs6432053) was performed in the 552 late-onset T2DM patients and 563 controls by TaqMan assay (Applied Biosystems) on an ABI PRISM 7900HT sequence detector (AppliedBiosystems). Thermal cycling conditions followed the manufacturer's instructions.

Cell lines

MIN6-m9 cells were maintained in DMEM containing 25 mM glucose, 10% heat-inactivated FBS, 50mM 2-mercaptoethanol, 100mg/l streptomycin sulfate, and 60.5mg/l penicillin G under a humidified condition of 5% CO2-95% air at 37°C [21]. HepG2 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, 100mg/l streptomycin sulfate, and 60.5mg/l penicillin G under a humidified condition of 5% CO2-95% air at 37°C.

Identification of the minimal promoter region of human KLF11

To establish the promoter activity of the 5' flanking region of *KLF11*, we designed luciferase expression vectors including a series of 5' deletion fragments. Three different length fragments, from -1389 to +162 bp, -896 to +162 bp and -250 to +162bp relative to the transcription start site, were prepared by PCR and inserted into the firefly luciferase reporter vector, pGL4.12-Basic (Promega, Madison, WI). The MIN6-m9 cells or HepG2 cells were seeded in 6-well culture plates. The confluency was 50-70% at the time of transfection. The reporter constructs (500 ng) were transfected to cells by using a ExGEN 500 *in vitro* Transfection Reagent (Fermentas, St. Leon-Rot, Germany). Transcriptional activity was normalized with a co-transfected control thymidine kinase (TK)-regulated Renilla luciferase vector, pRL-TK 17 ng (Promega). Transactivation activity was measured using Dual-Luciferase Reporter Assay system (Promega).

Subcloning of human KLF11 and variants

A cDNA identical to *KLF11* was retrieved from a human islet cDNA library and subcloned in pENTR/D-TOPO (Invitrogen, Carlsbad, CA) after amplification with Pfu (Stratagene, La Jolla, CA) and transferred for expression to pcDNA6.2-DEST (Invitrogen). The R29Q and S124F mutations were introduced by site-directed mutagenesis (Stratagene, La Jolla, CA) with pENTR/d-TOPO wild-type *KLF11* as template and confirmed by sequencing.

Functional analysis of KLF11 mutant proteins

The reporter constructs for insulin, catalase1, or Smad7 promoter-pGL3 were prepared by cloning the human insulin (-365~+40 bp) [18], catalase1 (-734~+11 bp) [10], or Smad7 (-836~+74 bp) [11] gene promoter into the pGL3-Basic vector (Promega, Madison, WI). The MIN6-m9 cells ($1x10^5$ cells/well) were seeded in 6-well culture plates. The confluency was 50-70% at the time of transfection. Constructed plasmids, pcDNA6.2 wild-type hKLF11, or pcDNA6.2 mutant-type hKLF11 and each reporter construct were transfected to MIN6-m9 cells using ExGEN 500 *in vitro* Transfection Reagent (Fermentas, St. Leon-Rot, Germany). Transcriptional activity was normalized with a co-transfected control thymidine kinase (TK)-regulated Renilla luciferase vector, pRL-TK (Promega). Transactivation activity was measured using Dual-Luciferase Reporter Assay system. (Promega)

Statistical analyses

Statistical difference in allele frequencies between late-onset T2DM and control subjects was assessed by χ^2 test, and other categorical clinical variables were compared using *t* test or logistic regression analysis adjusted for relevant covariates. Statistical analysis was performed with StatView 5.0 software (SAS Institute, Inc., Cary, NC). Comparison of estimated haplotype frequencies was performed by conducting separate one-degree of freedom tests for a series of 2 x 2 contingency tables testing the frequency of each specific haplotype vs all others between cases and controls, and omnibus testing of differences in haplotype frequency profiles between the cases and controls (statistical significance assessed empirically via permutation testing with PHASE 2.1.1 software). The present study had about 33-50% power to detect an OR of 1.20 when the frequency of a risk allele was 10-20% and *P*-value was less than 0.05 under a multiplicative model with 553 patients and 563 controls, while it had only 10-13% power to detect an OR of 1.20 when the frequency of a risk allele was 10-20% and *P*-value was less than 0.05 under a multiplicative model with 96 each of patients and controls.

Results

Identification of polymorphisms in KLF11

Twelve of the random controls were examined to detect genetic variations in the entire region of *KLF11* including all 4 exons. A total of 17 polymorphisms, four of which are novel, were found as shown in Table 1; the locations of these polymorphisms are shown in Fig. 1 in relation to the genomic structure of *KLF11*. The additional two variants, R29Q and S124F were found by screening a large number of type 2 diabetic patients. Two coding variants, R29Q and S124F, are novel. In the 1552 bp (from -1389 to +162 bp) region of the *KLF11* promoter, a total of seven polymorphisms including four novel ones were identified.

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Evaluation of the pattern of LD in KLF11

Sixteen polymorphisms with frequencies of more than 10% were used to define haplotypes and to evaluate the pattern of LD. As shown in Fig. 2, the single, large LD block appears in this region. Haplotype combinations were estimated with 96 control subjects. Four major haplotypes comprising tag SNPs (SNP-2, -3, -5, -6, -and -13) were inferred in the entire *KLF11* region.

Mutation screening of KLF11 and association study in T2DM patients

All exons and the putative promoter region (-1389 to +162 bp) of *KLF11* were examined in 182 early-onset T2DM patients, 96 late-onset T2DM patients, and 96 control subjects. A total of 3 cSNPs (R29Q, S124F and V395V), two of which, R29Q and S124F, are novel, were found (Table 2). R29Q mutation was found in five T2DM (3 from late-onset and 2 from early-onset) patients and in one control (Fisher's *P*-value = 0.245). S124F mutation was found in one early-onset T2DM patient and was not found in controls. An association study using tag SNPs in T2DM patients was performed, but no association of *KLF11* variants with T2DM was found (Table 3A&B). No haplotypes were found to be susceptible to T2DM by χ^2 test. Using permutation testing (100 permutations), no significant differences in haplotype frequencies between control and T2DM were found (Table 4).

Functional analysis of KLF11 mutant proteins

The results of alignment by CLUSTALW show that R29 of *KLF11* is conserved among human, mouse, and rat, while S124 is not conserved (Fig. 3). The transcriptional activity of the R29Q and S124F mutants of KLF11 were therefore compared with that of wild type KLF11. KLF11, catalase1, or Smad7 expression in MIN6-m9 cells was first confirmed, and all of these genes were endogenously expressed in both MIN6-m9 cells (data not shown). Wild-type KLF11 reduced all of these promoter activities to approximately 0.5-fold. Significantly reduced activity of R29Q mutant repression was found in catalase1 promoter, and reduced S124F mutant repression activity was found both in insulin and catalase1 promoter. However, the differences in activity between wild-type and these mutants of KLF11 were very small (Fig. 4).

Identification of the minimal promoter region of human KLF11

To identify the region essential for basal promoter activity of *KLF11*, the relative luciferase activity of the three sizes of 5' deletion reporter constructs was evaluated. As shown in Fig. 5, the shortest promoter construct comprising each of the four major haplotypes had basal activity both in MIN6-m9 cells and HepG2 cells, suggesting that the basal promoter is involved in the region between -250 and +162 bp. The second longest and longest promoter constructs had stronger activity in HepG2 cells, suggesting that the essential enhancer element for promoter activity of *KLF11* is incorporated in the region between -896 and -250 bp, while the second longest and longest promoter constructs had similar activity in MIN6-m9 cells. One of the promoter polymorphisms, SNP-4, is located at the sixth Sp1 binding site from the transcription start site, and is not incorporated in one of the major haplotypes, 1-3-1-1 (Table 4, Fig. 6). A significant difference in promoter activity between this haplotype and the other three major haplotypes was not detected in the promoter assay in MIN6-m9 cells. One of the major haplotypes, 2-2-2-2, showed the highest transcriptional activity with the longest and the second longest constructs (1.43 ± 0.046 fold, *P*=0.0064; 1.28 ± 0.018 fold, *P*=0.0069) (Fig. 5).

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Discussion

Genetic analysis of *KLF11* reveals two rare variants, A347S and T220M, which segregate in families with early-onset type 2 diabetes. In addition, analysis of 1,696 type 2 diabetic patients and 1,776 controls found one frequent polymorphic Q62R variant that significantly associates with late-onset type 2 diabetes in North European populations [17]. Florez et al. reported that KLF11 Q62R polymorphism is not associated with late-onset type2 diabetes in 8,676 subjects of northern-European ancestry, and both A347S and T220M rare variants also are not found [22]. Recently, Ma et al. reported that neither Q62R nor any other common variant in KLF11 was associated with late-onset type 2 diabetes in the Pima population [23]. Normoglycemic R62 carriers showed a significantly decreased plasma insulin level at 60 and 120 min after oral glucose load, leanness, and increased insulin sensitivity [17]. These characteristics clearly differ from those of Caucasian patients with T2DM, which are often caused by impaired insulin action mainly due to obesity. Obesity and insulin resistance are closely associated with type 2 diabetes in Caucasians, while type 2 diabetes in Japanese is characterized primarily by pancreatic beta-cell dysfunction [24, 25]. Thus, *KLF11* is a candidate diabetogenic gene in Japanese. Tanahashi et al. reported identification of eight variants, but were unable to find any missense mutation or association of common *KLF11* variants in late-onset Type2 diabetes in 1818 Japanese subjects [26].

In the present study, the minimal promoter region identified in this study, 5'UTR, coding region, 3'UTR, and flanking introns were screened for mutations in unrelated Japanese subjects with 182 early-onset and 96 late-onset T2DM patients. Our analysis revealed a novel missense mutation, R29Q (G>A), in exon2 in two early-onset type 2 diabetes patients and three late-onset T2DM patients in the heterozygous state and S124F in exon3 in one early-onset type 2 diabetes patient, but none of the A347S, T220M, Q62R, and promoter variant -1659G>C mutations previously identified in Northern Europeans in *KLF11*.

We examined about 13 kb covering the entire coding region of *KLF11* and identified a total of 19 genetic variations including 6 novel variants. We defined haplotypes based on the LD pattern estimated using the 16 most frequent SNPs, the frequencies of which were more than 10%, and performed an association study with T2DM. No association of *KLF11* variants with T2DM was found and no haplotypes were found to show susceptibility to T2DM. We examined one variant, rs4073397, located 21kb upstream from the coding region and outside of the LD block of the *KLF11* region, that shows a marginal association with T2DM in Japanese (26), but we could not replicate the association in this study with power similar to that of the former report ($P = 0.268$ and 0.576 for allele and genotype analysis, respectively; OR 1.2; RAF 0.5; α=0.05; 1-β= 63.9%).

We searched for consensus sequences of transcription factor binding sites in the promoter region by using TFSEACH. As shown in Fig. 6, no TATA-box and ten Sp1 binding sites were found, but there were no differences in haplotype frequencies of the *KLF11* promoter region between T2DM patients and controls. It is of interest that one of the promoter polymorphisms, SNP-4, is located at the sixth Sp1 binding site from the transcription start site and that this binding site is not incorporated in one of the major haplotypes, but significantly reduced promoter activity of this haplotype was not detected by promoter assay in MIN6-m9 cells. One of the major haplotypes comprising all of the minor alleles showed the highest transcriptional activity with the longest and the second longest constructs. No association of the haplotype with occurrence of T2DM was found, but the findings are inconclusive due to the low statistical power of this study. In any case, the effect of the KLF11 expression level itself on the onset of type 2 diabetes should be slight, if any at all.

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RT-PCR demonstrated endogenous KLF11 mRNA expression in whole rat pancreas, human pancreas, and mouse MIN6-m9 cells (data not shown). We therefore examined whether the KLF11 mutant affects transcriptional regulation of the human insulin gene. We found, contrary to Neve's report (17), that wild-type KLF11 inhibited insulin promoter activity as Niu et al. reported [18], but no difference in activity between wild and KLF11 mutants was found. Inhibition by wild-type KLF11 was similar in the three beta cell lines, INS-1E, beta-TC3 in the previous study, and MIN6-m9 in this study, indicating stable performance of KLF11 inhibition of insulin promoter activity in rodent beta cells.

KLF11 is involved in the TGF-β pathway, which plays a critical role in the development and homeostasis of exocrine and endocrine pancreas via Smad signaling [29]. KLF11 may affect pancreatic beta cell function by modulating the expression of free radical scavengers such as superoxide dismutase (SOD) 2 and catalase1, recently identified as KLF11 target genes [10]. Oxidative stress is generally believed to be involved in the progression of pancreatic beta cell dysfunction found in T2DM [30, 31]. Since a decreased expression level of these antioxidant enzyme genes might contribute to the occurrence of type 2 diabetes, we examined the effects of the KLF11 R29Q and S124F variants on the expression levels of these target genes.

We then examined whether or not KLF11 mutants affect transcriptional regulation of the catalase1 and Smad7 genes. A significant but small difference in promoter activity between wild-type and R29Q mutant was found in catalase1 only. R29Q mutation is located 12 amino acids upstream of SID (mSin3A interaction domain) /R1 domain, which determines repression activity of KLF11 [10, 32–35]. This mutant of KLF11, although showing somewhat different activities in the catalase1 promoter, showed similar transcriptional activity compared with wild-type KLF11. Although it is inconclusive whether or not R29Q mutation contributes to the development of type 2 diabetes because of the small sample number in this study (Fisher's P-value = 0.245), our results suggest that the mutation does not contribute to the development of type 2 diabetes through alteration of the expression level of these target genes. No unusual clinical characteristics were found in subjects with R29Q mutation.

S124F mutation is located between the SID/R1 and R2 domain, and is not conserved among rat, mouse, and human, i.e., proline for rat and mouse and serine for human. Similar data were obtained from S124F mutant of KLF11, which showed somewhat different activities in insulin and catalase1 promoters and similar transcriptional activity compared with wild-type KLF11, suggesting little biological effect. No useful clinical information was available for the patient with S124F mutant.

In this study, we focused on the effects of KLF11 on beta cell function. In addition, the caveolin-1 gene, which is highly expressed in adipose tissue, is repressed by KLF11 in a cholesterol-dependent manner [36]. The findings that caveolin-1 binding to the insulin receptor stimulates both kinase activity and recruitment of the insulin receptor to lipid rafts at the plasma membrane, and that insulin receptor mutations impairing calveolin binding result in T2DM [37] indicate that calveolin 1 is essential for insulin signaling and suggest that KLF11 might affect insulin sensitivity as well.

In conclusion, we identified two novel missense variants of *KLF11* and the minimal promoter region of *KLF11*, but neither of the variants nor the haplotypes identified in this study was associated with the occurrence of type 2 diabetes in Japanese, although the findings are inconclusive due to the low statistical power of the study. Further investigation with a larger sample number is required to determine the magnitude of the contribution of the R29Q and S124F mutations to the development of diabetes and the role of KLF11 in normal pancreatic β-cell function. Identification of the minimal promoter region of *KLF11* should be useful in further elucidation of its transcriptional regulation mechanism.

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References

1. Bieker JJ (2001). Kruppel-like factors: three fingers in many pies. *J Biol Chem* 276: 34355-34358.
2. Lomberk G, Urrutia R (2005). The family feud: turning off Sp1 by Sp1-like KLF proteins. *Biochem J* 392: 1-11.
3. Turner J, Crossley M (1999). Mammalian Kruppel-like transcription factors: more than just a pretty finger. *Trends Biochem Sci* 24: 236-240.
4. Cook T, Gebelein B, Mesa K, Mladek A, Urrutia R (1998). Molecular cloning and characterization of TIEG2 reveals a new subfamily of transforming growth factor-beta-inducible Sp1-like zinc finger-encoding genes involved in the regulation of cell growth. *J Biol Chem* 273: 25929-25936.
5. Kaczynski J, Cook T, Urrutia R (2003). Sp1- and Kruppel-like transcription factors. *Genome Biol* 4: 206.
6. Black AR, Black JD, Azizkhan-Clifford J (2001). Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer. *J Cell Physiol* 188 143-160.
7. Asano H, Li XS, Stamatoyannopoulos G (1999). FKLF, a novel Kruppel-like factor that activates human embryonic and fetal beta-like globin genes. *Mol Cell Biol* 19 3571-3579.
8. Ou XM, Chen K, Shih JC (2004). Dual functions of transcription factors, transforming growth factor-beta-inducible early gene (TIEG). 2 and Sp3, are mediated by CACCC element and Sp1 sites of human monoamine oxidase (MAO) B gene. *J Biol Chem* 279: 21021-21028.
9. Scohy S, Gabant P, Van Reeth T, Hertveldt V, Dreze PL, Van Vooren P, Riviere M, Szpirer J, Szpirer C (2000). Identification of KLF13 and KLF14 (SP6), novel members of the SP/XKLF transcription factor family. *Genomics* 70: 93-101.
10. Fernandez-Zapico ME, Mladek A, Ellenrieder V, Folch-Puy E, Miller L, Urrutia R (2003). An mSin3A interaction domain links the transcriptional activity of KLF11 with its role in growth regulation. *EMBO J* 22: 4748-4758.
11. 11. Robertson RP, Harmon J, Tran PO, Poitout V (2004). Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. *Diabetes* 53:Suppl 1 S119-124.
12. Massague J, Blain SW, Lo RS (2000). TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* 103: 295-309.
13. Ellenrieder V, Buck A, Harth A, Jungert K, Buchholz M, Adler G, Urrutia R, Gress TM (2004). KLF11 mediates a critical mechanism in TGF-beta signaling that is inactivated by Erk-MAPK in pancreatic cancer cells. *Gastroenterology* 127: 607-620.
14. Kim SK, Hebrok M (2001). Intercellular signals regulating pancreas development and function. *Genes Dev* 15: 111-127.
15. Sanvito F, Herrera PL, Huarte J, Nichols A, Montesano R, Orci L, Vassalli JD (1994). TGF-beta 1 influences the relative development of the exocrine and endocrine pancreas in vitro. *Development* 120: 3451-3462.

16. Smart NG, Apelqvist AA, Gu X, Harmon EB, Topper JN, MacDonald RJ, Kim SK (2006). Conditional expression of Smad7 in pancreatic β cells disrupts TGF-β signaling and induces reversible diabetes. *Plos Biol* 4: e39.
17. Neve B, Fernandez-Zapico ME, Ashkenazi-Katalan V, Dina C, Hamid YH, Joly E, Vaillant E, Benmezroua Y, Durand E, Bakaher N, Delannoy V, Vaxillaire M, Cook T, Dallinga-Thie GM, Jansen H, Charles MA, Clement K, Galan P, Hercberg S, Helbecque N, Charpentier G, Prentki M, Hansen T, Pedersen O, Urrutia R, Melloul D, Froguel P (2005). Role of transcription factor KLF11 and its diabetes-associated gene variants in pancreatic beta cell function. *Proc Natl Acad Sci U S A* 102: 4807-4812.
18. Niu X, Perakakis N, Laubner K, Limbert C, Stahl T, Brendel MD, Bretzel RG, Seufert J, Path G (2007). Human Kruppel-like factor 11 inhibits human proinsulin promoter activity in pancreatic beta cells. *Diabetologia* 50:1433-1441.
19. Gutiérrez-Aguilar R, Froguel P, Hamid YH, Benmezroua Y, Jørgensen T, Borch-Johnsen K, Hansen T, Pedersen O, Neve B. (2008). Genetic analysis of KLF11 variants in 5,864 danish individuals; potential effect on insulin resistance and modified stat3 binding by promoter variant -1659G>C. *J Clin Endocrinol Metab* 93: 3128-3135.
20. Kuzuya T, Nakagawa S, Satoh J, Kanazawa Y, Iwamoto Y, Kobayashi M, Nanjo K, Sasaki A, Seino Y, Ito C, Shima K, Nonaka K, Kadowaki T. (2002). Report of *the Committee on the classification and diagnostic criteria of diabetes mellitus. Diabetes Res. Clin. Pract.* 55: 65-85.
21. Minami K, Yano H, Miki T, Nagashima K, Wang CZ, Tanaka H, Miyazaki JI, Seino S (2000). Insulin secretion and differential gene expression in glucose-responsive and -unresponsive MIN6 sublines. *Am J Physiol Endocrinol Metab* 279: E773-781.
22. Florez JC, Saxena R, Winckler W, Burtt NP, Almgren P, Bengtsson Bostrom K, Tuomi T, Gaudet D, Ardlie KG, Daly MJ, Altshuler D, Hirschhorn JN, Groop L (2006). The Kruppel-like factor 11 (KLF11) Q62R polymorphism is not associated with type 2 diabetes in 8,676 people. *Diabetes* 55: 3620-3624.
23. Ma L, Hanson RL, Que LN, Mack JL, Franks PW, Infante AM, Kobes S, Bogardus C, Baier LJ (2008). Association anlysis of KLF11 variants with type 2 diabetes in Pima Indians. *J Clin Endocrinol Metab* 93: 3644-3649.
24. Fukushima M, Suzuki H, Seino Y (2004). Insulin secretion capacity in the development from normal glucose tolerance to type 2 diabetes. *Diabetes Res Clin Pract* 66: Suppl 1 S37-43.
25. Matsuoka K (2000). Genetic and environmental interaction in Japanese type 2 diabetics. *Diabetes Res Clin Pract* 50: Suppl 2 S17-22.
26. Tanahashi T, Shinohara K, Keshavarz P. Yamaguchi Y, Miyawaki K, Kunika K, Moritani M, Nakamura N, Yoshikawa T, Shiota H, Inoue H and Itakura M. (2008). The association of genetic variants in Kruppel-like factor 11 and Type 2 diabetes in the Japanese population. Diabetic Medicine 25: 19-26.
27. Tonooka N, Tomura H, Takahashi Y, Onigata K, Kikuchi N, Horikawa Y, Mori M, Takeda J (2002). High frequency of mutations in the HNF-1alpha gene in non-obese patients with diabetes of youth in Japanese and identification of a case of digenic inheritance. *Diabetologia* 45: 1709-1712.
28. Rosenbloom AL, Joe JR, Young RS, Winter WE (1999). Emerging epidemic of type 2 diabetes in youth. *Diabetes Care* 22: 345-354.
29. Cook T, Urrutia R (2000). TIEG proteins join the Smads as TGF-beta-regulated transcription factors that control pancreatic cell growth. *Am J Physiol Gastrointest Liver Physiol* 278: G513-521.

doi: 10.1507/endocrj.K08E-302

30. Rehman A, Nourooz-Zadeh J, Moller W, Tritschler H, Pereira P, Halliwell B (1999). Increased oxidative damage to all DNA bases in patients with type Ⅱ diabetes mellitus. *FEBS Lett* 448: 120-122.
31. Sakuaba H, Mizukami H, Yagihashi N, Wada R, Hanyu C, Yagihashi S (2002). Reduced β cell mass and expression of oxidative stress related DNA damage in the islet of Japanese type Ⅱ diabetic patients. *Diabetologia* 45: 85-96.
32. Ellenrieder V, Zhang JS, Kaczynski J, Urrutia R (2002). Signaling disrupts mSin3A binding to the Mad1-like Sin3-interacting domain of TIEG2, an Sp1-like repressor. *EMBO J* 21: 2451-2460.
33. Cook T, Gebelein B, Belal M, Mesa K, Urrutia R (1999). Three conserved transcriptional repressor domains are a defining feature of the TIEG subfamily of Sp1-like zinc finger proteins. *J Biol Chem* 274: 29500-29504.
34. Hassig CA, Fleischer TC, Billin AN, Schreiber SL, Ayer DE (1997). Histone deacetylase activity is required for full transcriptional repression by mSin3A. *Cell* 89 341-347.
35. Zhang JS, Moncrieffe MC, Kaczynski J, Ellenrieder V, Prendergast FG, Urrutia R (2001). A conserved alpha-helical motif mediates the interaction of Sp1-like transcriptional repressors with the corepressor mSin3A. *Mol Cell Biol* 21: 5041-5049.
36. Cao S, Fernandez-Zapico ME, Jin D, Puri V, Cook TA, Lerman LO, Zhu XY, Urrutia R, Shah V (2005). KLF11-mediated repression antagonizes SP1/sterol-responsive Element-binding protein-induced transcriptional activation of caveolin-1 in response to cholesterol signaling. *J Biol chem* 280: 1901-1910.
37. Cohen AW, Combs TP, Scherer PE (2003). Role of caveolin and caveolae in insulin signaling and diabetes. *Am.J.Physiol. Endocrinol.Metab.* 285: E1151-E1160.

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Table 1. Polymorphisms identified in *KLF11* region in this study

SNP No.	Position genome	db SNP ID	Variation	Location	Frequencies of minor allele
1	-1348	rs4669520	G>A	5' flanking	0.134
2	-1025	rs35035311	ins G	5' flanking	0.132
3	-530	novel	(CCG)*	5' flanking	4: 0.744
					2: 0.139
					5: 0.117
4	-499	novel	del (CCCCGCCG)	5' flanking	0.114
5	-446	novel	del/ins (CCCCCTCCG)	5' flanking	0.276
6	-278	novel	del/ins (GGCCGGGCACG)	5' flanking	0.138
7	-86		del/ins (GCC)	5' UTR	0.128
8	1467	rs6717092	C>G	Intron 1	0.136
9	2477	novel	G>A (R29Q)	Exon2	n.d
10	3992	novel	C>A (S124F)	Exon3	n.d
11	4806	rs11687357	T>A (V395V)	Exon3	0.133
12	5856	rs6432052	C>T	Intron 3	0.129
13	5992	rs6432053	T>C	Intron 3	0.268
14	6272	rs6721191	G>A	Intron 3	0.128
15	6741	rs4614909	T>A	Intron 3	0.263
16	8199	rs2487	T>C	Intron 3	0.135
17	10349	rs4669522	C>T	3' UTR	0.145
18	10644	rs7632	C>T	3' UTR	0.274
19	11224	rs6432055	C>T	3' flanking	0.259

*Triallelic variant with 2,4,5 CCG repeats. The nucleotide indicates the location of the SNP relative to the A of ATG of the initiator Met of *KLF11* (GenBank No. NT_005334.15). The frequencies of minor alleles in this table are observed in 96 random control samples except SNP 9, and 10. n.d; not detected.

Table 2. Frequencies of coding SNPs in *KLF11* in controls and in patients with T2DM

SNP No.	Position genome	db SNP ID	Nucleotide change	Exon	minor allele number		
					Controls (n=563)	Late-onset T2DM (n=553)	Early-onset T2DM (n=182)
9	2477	novel	G>A (R29Q)	2	1	3	2
10	3922	novel	C>A (S124F)	3	0	0	1
11	4806	rs11687357	T>A (V395V)	3	26 (n=96)	21 (n=96)	56

The nucleotide indicates the location of the SNP relative to the A of ATG of initiator Met of *KLF11*

Table 3A. Association study using tag SNPs in *KLF11* in patients with late-onset T2DM and controls

SNP		Frequencies of genotype			Frequencies of alleles		allele		genotype		dominant		recessive	
		Control	T2DM		Control	T2DM	χ^2	P	χ^2	P	χ^2	P	χ^2	P
2	W/W	0.747	0.773	W	0.868	0.882	0.159	0.689	0.237	0.888	0.208	0.647	0.006	0.936
	W/M	0.242	0.214	M	0.132	0.117								
	M/M	0.01	0.011											
5	W/W	0.564	0.492	W	0.724	0.725	0.0003	0.984	4.81	0.09	0.755	0.384	2.684	0.101
	W/M	0.320	0.464	M	0.276	0.274								
	M/M	0.115	0.042											
6	W/W	0.734	0.732	W	0.861	0.854	0.036	0.848	0.451	0.797	0.0005	0.982	0.436	0.508
	W/M	0.255	0.244	M	0.138	0.145								
	M/M	0.01	0.023											
13	W/W	0.486	0.505	W	0.707	0.698	0.224	0.636	0.517	0.772	0.042	0.839	0.517	0.472
	W/M	0.418	0.415	M	0.293	0.302								
	M/M	0.094	0.078											

Association study was performed for the SNPs (SNP2, 5, and 6) in the promoter region with 96 subjects each from 553 T2DM and 563 controls by direct sequencing, and for SNP (SNP13) in the 553 T2DM patients and 563 controls by TaqMan assay. M, Mutant; W, wild type.

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Table 3B. Association study using tag SNPs in *KLF11* in patients with late-onset T2DM and controls

	Frequencies of genotype						P^{*1}
SNP3	1 1	1 2	1 3	2 2	2 3	3 3	
Control	0.515	0.252	0.178	0.021	0.021	0.010	0.297
T2DM	0.559	0.178	0.166	0	0.071	0.023	

SNP3	Frequencies of allele			P^{*2}
	1	2	3	
Control	0.731	0.157	0.110	0.491
T2DM	0.732	0.125	0.142	

	Frequencies of genotype		P^{*3}	Frequencies of genotype		P^{*4}
SNP3	2 2	non 2 2		3 3	non 3 3	
Control	0.021	0.978	0.181	0.010	0.989	0.489
T2DM	0	1		0.023	0.976	

	Frequencies of genotype		P^{*5}	Frequencies of genotype		P^{*6}
SNP3	2 2 + 1 2 + 2 3	1 1 + 1 3 + 3 3		3 3 + 1 3 + 2 3	1 1 + 1 2 + 2 2	
Control	0.294	0.705	0.502	0.210	0.789	0.418
T2DM	0.250	0.750		0.261	0.738	

Association study was performed for SNP3.
Allele 1 indicates 4 CCG repeats. Allele 2 indicates 2 CCG repeats. Allele 3 indicates 5 CCG repeats.
*1 Triallelic varient was analyzed in a 2x6 contingency table with genotype frequencies.
*2 Triallelic varient was analyzed in a 2x3 contingency table with allele frequencies.
*3 Analyses in the recessive model of 2 2 genotype vs. others.
*4 Analyses in the recessive model of 3 3 genotype vs. others.
*5 Analyses in the dominat model of 2 2+1 2+ 2 3 genotypes vs. 1 1+ 1 3+3 3 genotypes.
*6 Analyses in the dominat model of 3 3+1 3+ 2 3 genotypes vs. 1 1+ 1 2+2 2 genotypes.

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Table 4. Major haplotypes and its combinations in *KLF11* and risk of late-onset T2DM

SNP	Haplotype frequencies		*P*-value
2 3 5 6 13	Control	T2DM	
1 1 1 1 1	0.59	0.57	0.463
1 1 2 1 1	0.127	0.125	0.504
1 3 1 1 2	0.113	0.129	0.985
2 2 2 2 2	0.119	0.113	0.640
Haplotype combination	Risk (O.R.)	95% C.I.	
1 1 1 1 1 / 1 1 1 1 1	1.29	0.71-2.35	
1 1 1 1 1 / 1 1 2 1 1	1.43	0.55-3.75	
1 1 1 1 1 / 1 3 1 1 2	0.60	0.26-1.36	
1 1 1 1 1 / 2 2 2 2 2	0.75	0.32-1.77	
1 1 2 1 1 / 1 1 2 1 1	0.32	0.03-3.19	
1 1 2 1 1 / 1 3 1 1 2	2.02	0.18-22.75	
1 1 2 1 1 / 2 2 2 2 2	0.19	0.02-1.67	
1 3 1 1 2 / 1 3 1 1 2	1.00	0.06-16.3	
1 3 1 1 2 / 2 2 2 2 2	6.38	0.75-54.13	

The haplotype frequencies were estimated from the genotype of 96 controls and 96 T2DM subjects. The O.R and 95% C.I. of each haplotype combination relative to the other haplotype combinations as a group are shown. The numbers 1 and 2 indicate major allele and minor allele (SNP 2, SNP 5, SNP 6, SNP 13). SNP 3; allele 1 indicates 4 CCG repeats, allele 2 indicates 2 CCG repeats, and allele 3 indicates 5 CCG repeats.

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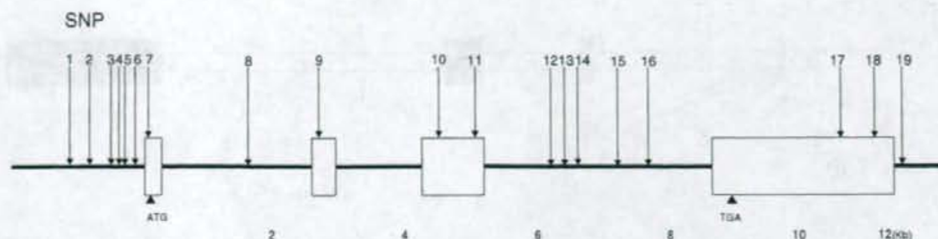

Fig. 1. Polymorphisms of *KLF11* identified in this study. The locations of the polymorphisms described in the text are shown. Nucleotide indicates the location of the SNP relative to the A of ATG of the initiator Met of *KLF11*.

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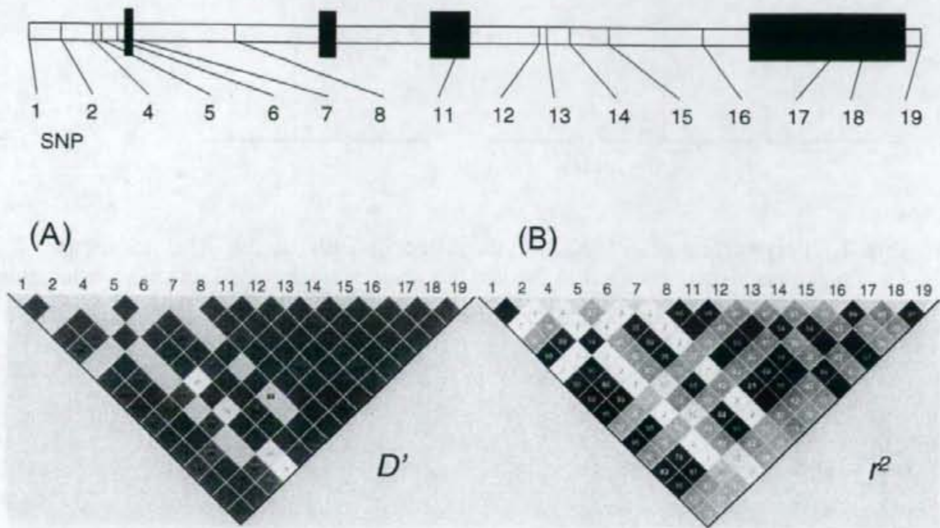

Fig. 2. Pairwise LD of KLF11 evaluated by D' and r2. The panel shows a Haploview representation of LD (D' and r2) based on genotyping data with 16 polymorphisms from control subjects (n = 96) (A) Pairwise combination with LD of D' = 1 and LOD ≧ 2, D' =1 and LOD < 2, D' <1 and LOD ≧ 2, D' <1 and LOD < 2 is shown with red, blue, pink and white. (B) Pairwise combination with LD of r2 =1, 0 <r2 <1, r2 =0 is shown in black, gray and white.

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(A) R29Q(Exon2, G>A)

```
MOUSE  MHSPGSTGPGDGRAADIMDICESILERKRHDSERSTCSVLEQTDIEAVEALVCMSSWGQR
RAT    MHSPGSTGPGDARAADIMDICESILERKRHDSERSTCSILEQTDIEAVEALVCMSSWGQR
HUMAN  MHTPDFAGPDDARAVDIMDICESILERKRHDSERSTCSILEQTDMEAVEALVCMSSWGQR
       **:*.  :**.*.**.**********************:*****:***************
```

(B) S124F(Exon3, C>A)

```
MOUSE  TPVPSQVVNSKGCMVTALPPSPAGGPRTLSKREPL------EPASGSSCRAVMTSVIRHTG
RAT    TPVPSQVINSQGCMATALPPSPTGGPRTLSKGEPP------EPSSESSCRAVMTSVIRHTG
HUMAN  TPVSPQVTDSKACTATDVLQSSAVVARALSGGAERGLLGLEPVPSSPCRAKGTSVIRHTG
       ***..** :*:.*.* :  *.:  .*:**            ** . *.***  ********
```

Fig. 3. The results of alignment of KLF11 among human, rat and mouse. A) Red indicates the novel mutation, R29Q, and blue indicates the region of SID. B) Red indicates the novel mutation, S124F, and blue indicates the region of R2. SID: mSin3A interaction domain; R2: transcriptional repression domain * completely conserved, highly conserved, moderately conserved.

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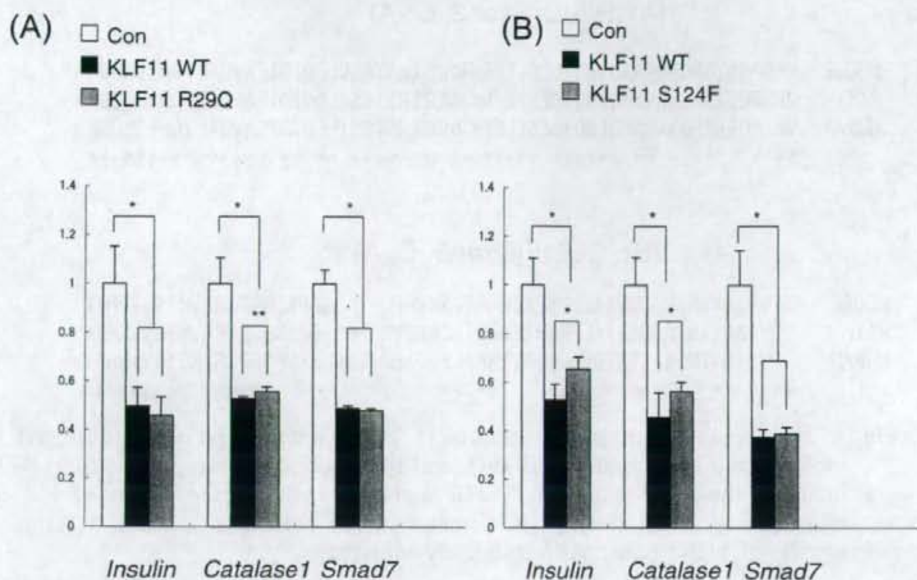

Fig. 4. Transactivation activity of mutant KLF11. Transcription activity with empty vector, wild type, and mutant KLF11 (500ng) was analyzed by co-transfection assay using reporter vector of Insulin, Catalase-1 and Smad7 promoter-PGL3 (500ng) and a Renilla luciferase (17ng) as internal control. (A) The results of R29Q mutant (n=6) (B) The results of S124F mutant (n=6) Data are expressed as means ± SD.
* P <0.005, ** P <0.05

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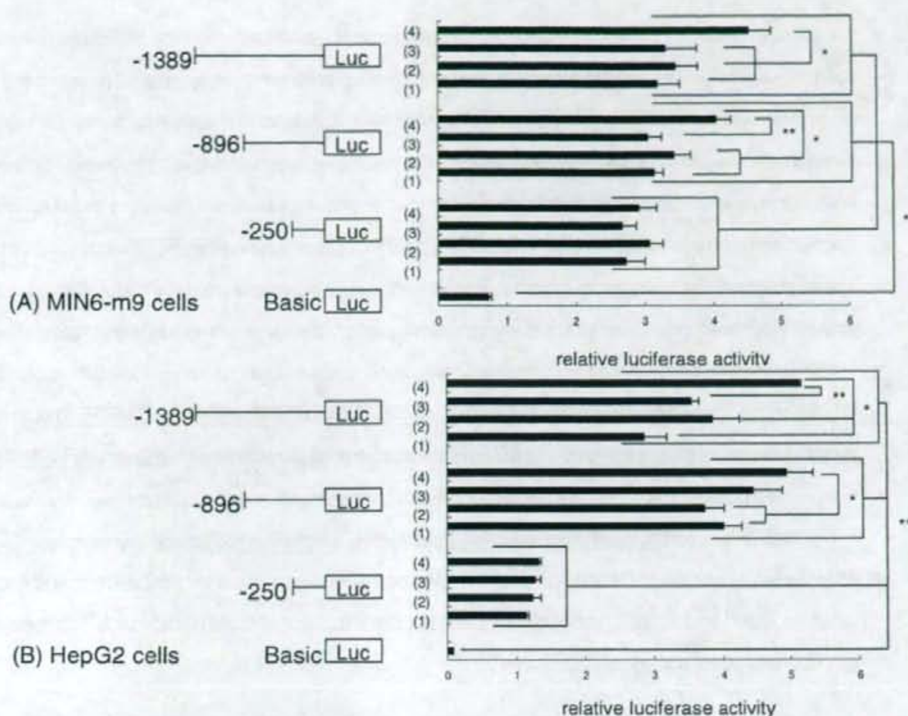

Fig. 5. Identification of *KLF11* promoter region using relative luciferase assays. The four haplotypes of the promoter region are indicated by (1) 1-1-1-1 (2) 1-1-2-1 (3) 1-3-1-1 (4) 2-2-2-2. The numerals 1 and 2 indicate major allele and minor allele (SNP 2, SNP 3, SNP 5, SNP 6). SNP 3, allele 1 indicates 4 CCG repeats, allele 2 indicates 2 CCG repeats, allele 3 indicates 5 CCG repeats. (A) The result in MIN6-m9 cells (n=3) (B) The result in HepG2 cells (n=3). Results are expressed as means ± SD of a representative of experiments performed in triplicate. * $P < 0.05$, ** $P < 0.005$

```
tttacacacagaacaccctgcgcccctctcagagcctccgggtcttcatgcgagcagtttatcacggttctttcgtcttaaaccctcttactatttcagcaaacaccctctcctc
-1389
agcctggtcaacccctctgcccaacccgagtgggccccgaaggaacaagtggggaccagagtcctgctcatgtgagtggtggggcccggcccgttcccctaagacca
                                                                                                   SNP 1
gaattctctcgctcagagcaaagccccggggtgccgggattctctctgctcccgctccctggatggagcttttaaaaaggaggattcctcactgcggcctctgccccttg

cactgagcgccaacccgcatcacagctcagcaaaggcgcgcgcagtgaacagacggaggaggggtggctcctcgctgagctgaaggctgcggcgacgaaaataa

acagcatggttcctgctctccacgaacctggcgctgcagacgtgggcaaaccacaccaagagacgcgagcaaccgaaatatagggaggaaaaaaaaaggtgccct
                                             -896                                  SNP 2
ggacagcgggctagatgtctcctgcaggctcgaggacttcccggag[ggggcggcg]tttctcggggcctgggaagaa[tgggcgggt]tcagcgggcgccttctgggtg

actgcccgtttcggcttcccctgaccccgtaacagagcggaagaggcccagagacgccagaggaggggcgtcagcgggacactcaggccaataagctctgactccc

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tccccgcaccgagtccggccccggccctgggatttccccagccgccgccgcc[gccccgcccc]tcc[gccccgccgc]cccgcc[gccccgccgc]cccctccgcccc
                                         SNP 3                                   SNP 4
cgccgcccctccgccccgccgcccctccgcccctccgccccgcttgcccctggcgccgctgcgacctttcccttactttccagggcctcag[gccccgcccc]tcgctc
                 SNP 5                                     -250
cgaccaaaacacgtgccgcgtcccgccctgcccgatcacgccgcggccaggctccgatcacgcggcccccgcggcgctcattggccggccgggcacgggccgg
                                                                                             SNP 6
gcacgaattggctggtggtggccg[gccccgcccc]ggca[ggggcgcggt]gtattttggttcgcctccgc[gccccgcccc]gccagccgctccctccgcggcc[gccccgc
ccc]tcccgcgccgcGAGGGCCGCGCCGGGGCAGAGCCGCGCGGGCGGGCGAGGCGCGTGCCGGCCGCAGGAGCTCC
       +1
GGGTTGCCGCCGCCGCCGCCGCCCGCAGCCCACGTGCGGCCGCTGCTGCGCCCGAGCTCACGCCCCGCGGCCG
            SNP 7
CTTTGTTGCTCCCGGCCGGCCTGCACGATG
                             *
```

Fig. 6. Nucleotide sequence of the promoter region of KLF11 from transcription start site to -1389 bp of 5'flanking region. Consensus sequences for Sp1 are boxed. Red color indicates the sites of polymorphisms. Large characters indicate 5' UTR region of KLF11 and +1 denotes a transcription start site. Asterisk indicates the A of ATG of the initiator Met of KLF11.