

Identification of Minimal Promoter and Genetic Variants of Kruppel-like Factor 11 Gene and Association Analysis with Type 2 Diabetes in Japanese

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Abstract. Genetic analysis of the KLF11 gene revealed two rare variants, A347S and T220M, segregating in families with early-onset type 2 diabetes, and one frequent polymorphic Q62R variant significantly associated with type 2 diabetes in Northern Europeans. Furthermore, it has been reported that over-expression of KLF11 has a deleterious effect on insulin promoter activity. Thus, an altered expression level of KLF11 may contribute to the occurrence of type 2 diabetes. To investigate the contribution of KLF11 to type 2 diabetes in Japanese, we surveyed the 5' flanking region of *KLF11* by reporter assay and identified the minimal promoter region of the gene. The promoter region from –250 to +162 bp including five Sp1 binding sites showed basal promoter activity both in MIN6-m9 and HepG2 cells. We also examined the entire region of *KLF11* to detect genetic variants. A total of 19 polymorphisms, six of which are novel, were identified, but none of them showed association with the occurrence of type 2 diabetes. Two of the identified polymorphisms, R29Q and S124F, are novel coding variants. Functional analyses of these variants were performed, and similarly reduced effects on transcriptional activities of insulin, catalase1, and the Smad7 gene were found. We conclude that variants of *KLF11* are not a major factor in the occurrence of type 2 diabetes in Japanese. The promoter region of *KLF11* identified in the present study should be useful in further elucidation of the transcriptional regulation mechanism of the gene and genetic analyses of type 2 diabetes.

Key words: KLF11, SNP, association study, promoter, type 2 diabetes

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KRUPPEL-LIKE transcription factor (KLF)11 (also known as TIEG2) is a member of the Sp1-like transcription factor family, which is defined by the presence of three conserved DNA-binding C-terminal zinc finger domains and variant N-terminal domains [1–4]. In contrast with Sp1, one of the best-characterized transcriptional activators, KLF11 behaves as a potent transcriptional repressor. KLF/Sp1-like transcription

regulation may participate in many aspects of cellular function, including cell proliferation, apoptosis, differentiation, and neoplastic transformation [5–8].

The KLF11 gene is located at chromosome 2p25 [9], and is ubiquitously expressed in human tissues with an abundance in pancreas and muscle [4]. KLF11 has elicited significant attention due to its role as a negative regulator of exocrine cell growth by decreasing growth and increasing apoptosis via a mechanism that involves down-regulation of the oxidative stress genes SOD2 and catalase1 [10], which also are expressed in pancreatic islets, and an increased susceptibility to oxidative insult [11]. The Smad-regulated transcriptional pathway plays a central role in TGF- β

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induced cell growth inhibition [12]. Smad signaling activity is potentiated by KLF11 in normal epithelial cell lines through termination of the negative feedback loop imposed by Smad7, which requires binding to GC-rich promoter boxes of the Smad7 promoter [13], and the TGF- β signaling pathway is a major regulator of endocrine cell fate [14–16].

The role of KLF11 within the endocrine pancreas remains to be elucidated. Recently, Neve *et al.* reported that KLF11 binds to the insulin promoter and up-regulates its activity in beta-TC3 cells. Genetic analysis of *KLF11* revealed two rare variants (Ala347Ser and Thr220Met) that segregate with diabetes in families with early-onset type 2 diabetes (T2DM) and significantly impair its transcriptional activity [17]. On the other hand, Niu *et al.* reported that over-expression of hKLF11 inhibits the activity of human insulin promoter in INS-1E and beta-TC3 cells in a dose-dependent and glucose-independent manner [18]. Furthermore, it has been reported that a *KLF11* promoter variant has a deleterious effect on insulin sensitivity via STAT3-mediated up-regulation of *KLF11* [19]. Thus, an altered expression level of KLF11 may contribute to the occurrence of type 2 diabetes.

In this study, we surveyed the 5' flanking region of *KLF11* and identified the minimal promoter region of the gene, which should be useful in further genetic and functional analyses of type 2 diabetes. We also examined all of the regions of *KLF11* in twelve Japanese subjects to detect genetic variants, evaluated the pattern of linkage disequilibrium to infer haplotypes in the gene, and performed association studies with type 2 diabetes patients.

Material and Methods

Subjects

A total of 182 Japanese subjects with clinical diagnosis of early-onset type 2 diabetes (70 males and 112 females; onset age 11.9 ± 3.1 yr, BMI, 23.9 ± 6.2 kg/m²; onset HbA1c $8.8 \pm 3.1\%$, HbA1c $7.1 \pm 2.3\%$) were screened for mutations by direct sequencing of PCR products. Patients with glutamic acid decarboxylase (GAD) antibodies and other types of diabetes were excluded on the basis of clinical data.

A total of 553 Japanese patients with late-onset T2DM [310 males and 243 females; age at testing,

61.1 ± 10.6 yr; BMI, 23.9 ± 4.1 kg/m²; glycosylated hemoglobin (HbA1c), $7.7 \pm 3.5\%$] and 563 controls (224 males and 339 females; age at testing 67.4 ± 6.0 yr; BMI, 22.9 ± 2.9 kg/m²; HbA1c, $5.0 \pm 0.4\%$) were examined for association study. The diagnosis of T2DM was based on medical records or 75 g oral glucose tolerance test according to the criteria of the Japan diabetes Society [20]. Informed consent was obtained from all of the diabetic subjects and volunteer controls. The study was approved by the ethics committee of Gifu University.

SNP identification in *KLF11*

Genomic DNA was extracted from samples of whole blood using QIAamp DNA blood kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Twelve of the random control samples (24 alleles) were used to detect SNPs in *KLF11*. Primers for PCR experiments were designed by Primer3 (available from <http://www.genome.wi.mit.edu/cgi-bin/primer/preimer3/www.cgi>) on the basis of the genomic contig sequence (GenBank ID: NT_005334.15, nt 5016199–5029771 bp) of the *KLF11* region. The mixture for PCR was 20 μ l in 10 ng template DNA, 0.5 mM of each dNTP, 2.5 pmol of each forward and reverse primer, 0.5 U ExTaq polymerase (Takara, Kyoto, Japan), and 2 μ l of 10 \times PCR buffer or 0.4 U KOD FX (TOYOBO, OSAKA, JAPAN) and 10 μ l of 2 \times PCR buffer for KOD FX. The reaction conditions with Ex Taq polymerase were an initial denaturation step of 94°C for 1 min and 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 2 min 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 ± 11.0 yr, BMI, 24.5 ± 5.4 kg/m²; HbA1c $7.9 \pm 1.6\%$) and 96 of 563 control subjects (35 males and 61 females; age 67.6 ± 5.8 yr; BMI, 22.8 ± 2.8 kg/m²; HbA1c $4.9 \pm 0.3\%$). 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 (Applied Biosystems). 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, 50 mM 2-mercaptoethanol, 100 mg/l streptomycin sulfate, and 60.5 mg/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, 100 mg/l streptomycin sulfate, and 60.5 mg/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 +162 bp 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 (1×10^5 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 or logistic regression analysis adjusted for relevant covariates, and other categorical clinical variables were compared using *t* test. 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 2×2 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 frequen-

cy 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.

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.

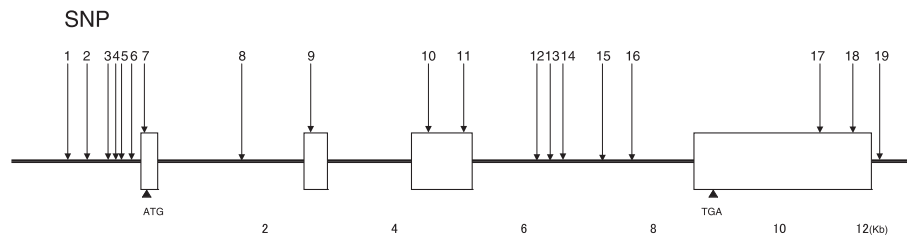


Fig. 1. Polymorphisms of *KLF11* identified in this study. The locations of the polymorphisms described in the text are shown. Nucleotide indicates the location of the SNP relative to the A of ATG of the initiator Met of *KLF11*.

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*

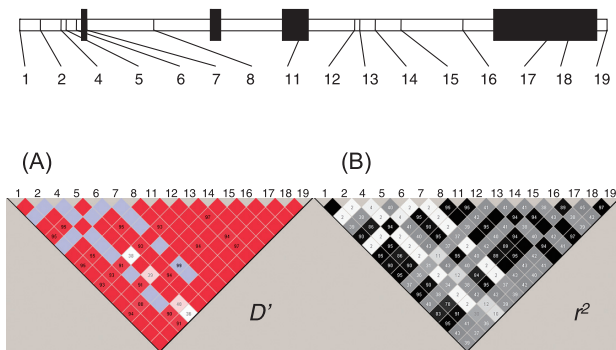


Fig. 2. Pairwise LD of *KLF11* evaluated by D' and r^2 . The panel shows a Haploview representation of LD (D' and r^2) based on genotyping data with 16 polymorphisms from control subjects (n = 96) (A) Pairwise combination with LD of $D' = 1$ and $\text{LOD} \geq 2$, $D' = 1$ and $\text{LOD} < 2$, $D'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

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

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	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
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.

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

SNP3	Frequencies of genotype						<i>P</i> *1
	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			<i>P</i> *2
	1	2	3	
Control	0.731	0.157	0.110	0.491
T2DM	0.732	0.125	0.142	

SNP3	Frequencies of genotype		<i>P</i> *3	Frequencies of genotype		<i>P</i> *4
	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	

SNP3	Frequencies of genotype		<i>P</i> *5	Frequencies of genotype		<i>P</i> *6
	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 variant was analyzed in a 2×6 contingency table with genotype frequencies.

*2 Triallelic variant was analyzed in a 2×3 contingency table with allele frequencies.

*3 Analyses in the recessive model of 22 genotype vs. others.

*4 Analyses in the recessive model of 33 genotype vs. others.

*5 Analyses in the dominant model of 22 + 12 + 23 genotypes vs. 11 + 13 + 33 genotypes.

*6 Analyses in the dominant model of 33 + 13 + 23 genotypes vs. 11 + 12 + 22 genotypes.

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

SNP 2 3 5 6 13	Haplotype frequencies		P-value
	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.

(A) R29Q(Exon2, G>A)

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MOUSE MHSPGSTGPGDGRAADIMDICESILERKRHDSERSTCSVLEQTDIEAVEALVCMSSWGQR
RAT   MHSPGSTGPGDARAADIMDICESILERKRHDSERSTCSILEQTDIEAVEALVCMSSWGQR
HUMAN MHTPDFAGPDDARAVIDIMDICESILERKRHDSERSTCSILEQTDMEAVEALVCMSSWGQR
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(B) S124F(Exon3, C>A)

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MOUSE TPVPSQVVNSKGCMTALPPSPAGGPRTLSKREPL——EPASGSSCRAVMTSVIRHTG
RAT   TPVPSQVINSQGCMTALPPSPTGGPRTLSKGEPP——EPSSESSCRAVMTSVIRHTG
HUMAN TPVSPQVTDSKACTATDVLQSSAVVARALSGGAERGLLGLEVPSSPCRAKGTSVIRHTG
      ***. **. :*. *. *: *: : *. : *. :* ** **

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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.

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 differ-

ences 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 evalu-

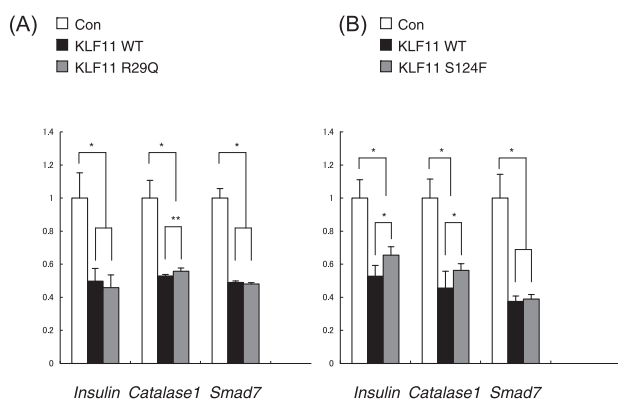


Fig. 4. Transactivation activity of mutant KLF11. Transcription activity with empty vector, wild type, and mutant KLF11 (500 ng) was analyzed by co-transfection assay using reporter vector of Insulin, Catalase-1 and Smad7 promoter-PGL3 (500 ng) and a Renilla luciferase (17 ng) as internal control. (A)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

ated. 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).

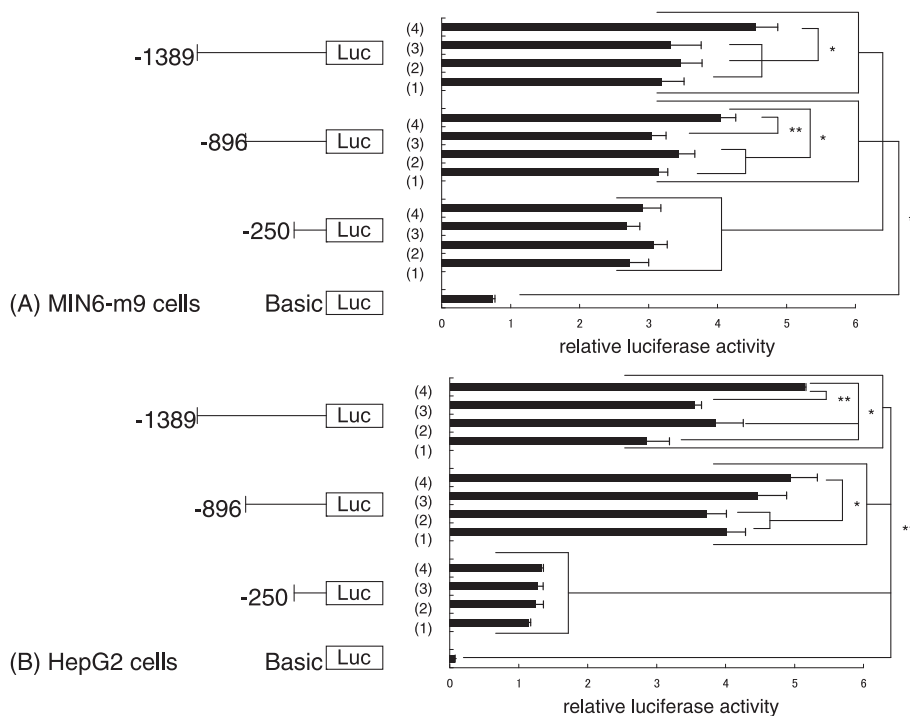


Fig. 5. Identification of *KLF11* promoter region using relative luciferase assays. The four haplotypes of the promoter region are indicated by (1) 1-1-1-1 (2) 1-1-2-1 (3) 1-3-1-1 (4) 2-2-2-2. The numerals 1 and 2 indicate major allele and minor allele (SNP 2, SNP 3, SNP 5, SNP 6). SNP 3, allele 1 indicates 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 \pm SD of a representative of experiments performed in triplicate. * P < 0.05, ** P < 0.005

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*.

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 type 2 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

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 exon 2 in two early-onset type 2 diabetes patients and three late-onset T2DM patients in the heterozygous state and S124F in exon 3 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 21 kb 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; $\alpha = 0.05$; $1-\beta = 63.9\%$).

We searched for consensus sequences of transcription factor binding sites in the promoter region by using TFSEARCH. 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.

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 [16, 27]. *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 [28, 29]. 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, 30–33]. 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 [34]. 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 caveolin binding result in T2DM [35] indicate that caveolin-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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