A Novel *PAX4* Mutation in a Japanese Patient with Maturity-Onset Diabetes of the Young

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Maturity-onset diabetes of the young (MODY) is a genetically and clinically heterogeneous type of diabetes mellitus, characterized by early onset (often before 25 years of age) and absence of pancreatic autoimmunity markers. Paired-homeodomain transcription factor 4 (*PAX4*) functions as a transcriptional repressor and is involved in the differentiation of insulin-secreting β -cells. Here we identified a novel *PAX4* mutation in a Japanese patient with MODY. A 15-year-old, non-obese boy was admitted to our hospital because of polyuria and polydipsia. Laboratory evaluation showed an elevated fasting glucose level; however, islet cell antibodies and glutamic acid decarboxylase antibodies were not detected in the patient's serum. The proband's father had been diagnosed as having type 2 diabetes at age of 30 years. We therefore analyzed several candidate genes of MODY, and identified a novel mutation of a 39-base heterozygous deletion in exon 3 (c.374-412 del39) of *PAX4* in the proband and his father. This mutation may cause exon 3 skipping that results in a frameshift, thereby producing a premature stop codon in exon 5. As this mutant *PAX4* lacks a part of the homeodomain that is critical for binding to the target gene, this mutant was thought to lose the transcriptional repressor function. As expected, luciferase-reporter assays revealed that the mutant *PAX4* could not repress the activities of insulin and glucagon gene promoters, unlike the wild-type *PAX4* that repressed the promoter activities. The present study demonstrates that a novel mutation of *PAX4* is likely to be associated with diabetes in this Japanese family.

Keywords: Maturity onset of diabetes of young (MODY); *PAX4*; mutation

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Maturity-onset diabetes of the young (MODY) is a genetically and clinically heterogeneous type of diabetes mellitus characterized by early onset (often before 25 years of age) and absence of pancreatic autoimmunity markers (Fajans et al. 2001). Mutations of six distinct genes have been implicated in the six different types of diabetes (MODY1-6). One of the six genes encodes the glycolytic enzyme glucokinase (*GCK*) (associated with MODY2) and the other five encode transcription factors: hepatocyte nuclear factor (*HNF4A*) (MODY1), *HNF1A* (MODY3), insulin promoter factor 1 (*IPF1*) (MODY4), *HNF1B* (MODY5) and *NEUROD1* (MODY6) (Malecki et al. 1992; Vionnet et al. 1992; Yamagata et al. 1996a, 1996b; Horikawa et al. 1997; Stoffers et al. 1997). However, as genetic defects of these genes are not frequently seen among Japanese MODY patients (Nishigori et al. 1993; Furuta et al. 2002; Kagami-Takasugi et al. 2006), mutations in other genes may be responsible for the development of MODY.

The paired homeodomain transcriptional factor, *PAX4*,

functions as a transcriptional repressor through a paired domain and homeodomain (Sosa-Pineda et al. 1997; Smith et al. 1999; Sosa-Pineda 2004; Brun and Gauthier 2008). In heterozygous *Pax4* knockout mice, mature β -cell and δ -cells did not differentiate and proliferate, and thus *PAX4* is thought to be essential for the commitment of progenitor cells to the different islet cell lineages. In addition, *PAX4* is likely to be important for regeneration of β -cells in adult life (Brun and Gauthier 2008).

It was reported that a *PAX4* mutation was associated with type 2 diabetes (Shimajiri et al. 2001). Thereafter, two possible pathogenic mutations of *PAX4* in two Thai patients with MODY were described (Plengvidhya et al. 2007). These findings suggest that *PAX4* mutation may be responsible for MODY.

Here we analyzed *PAX4* in a Japanese family with MODY, and have identified a novel 39-base deletion mutation (c.374-412 del39).

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Clinical report

A 15-year-old, non-obese boy was admitted to our hospital because of polyuria and polydipsia. On physical examination, his height was 161.5 cm and body weight was 47.2 kg. His blood pressure was 115/67 mmHg. Laboratory evaluation showed that the fasting glucose level was 642 mg/dl and urine ketone bodies were positive. Ketoacidosis was not observed (pH of venous blood 7.36). Other biochemical findings were within normal limits. The HbA1c level was 14.5% (NGSP) (Seino et al. 2010). Islet cell antibodies (ICAs) and glutamic acid decarboxylase (GAD) antibodies were not detected in the serum. Urinary C-peptide concentrations measured on two different days were lower than normal range (13.1 μ g/day and 18.8 μ g/day, normal range 20.1–155 μ g/day). Blood C-peptide levels (fasting /postprandial) were also low (1.08 /1.25 ng/ml) despite the elevated blood glucose levels (244/342 ng/ml). Abdominal computed tomography revealed a normal-sized pancreas and kidneys. Since his capacity of insulin secretion was thought to be decreased, insulin therapy was started at this time. His most recent insulin requirement and HbA1c level were 0.5 unit/kg and 7.4% (NGSP), respectively. The proband's father had also been diagnosed as having type 2 diabetes at age 30 yr. Data on the father at the time that he had been diagnosed, were not available. At this time, the father's weight was 85 kg and his BMI was 32 kg/m². The proband's father's diabetes has been well controlled by only diet therapy without insulin administration.

Methods

DNA amplification and sequence analysis

The ethical committee of Hokkaido University School of Medicine approved this study. Written informed consent to participate in the study was obtained from the patient's parents and the patient. Genomic DNA was extracted from peripheral leukocytes. Each exon and exon-intron boundaries of *HNF4A*, *HNF1A*, *IPF1*, *HNF1B*, *NEUROD1*, *GCK* and *PAX4* were amplified by polymerase chain reaction (PCR). After amplification, the PCR products were purified and sequenced directly using an ABI PRISM Dye Terminator Cycle Sequencing Kit and an ABI 373A automated fluorescent

sequencer (PE Applied Biosystems, Foster City, CA). Primers for the PCR reactions are shown in Table 1.

mRNA analysis

In order to analyze the effect of the mutation on mRNA expression, a mini-gene construct was produced. The normal and mutant alleles of the *PAX4* gene from exon 1 to exon 4 were amplified from the proband's genomic DNA with a set of primers 5'-ATGAACCAGCTTGGGGGG-3' in exon 1 (codon 1 to codon 6 in exon 1, primer 1) and 5'-CAGTGCCTCTGCTTGGCT-3' in exon 4 (codon 177 to codon 172, primer 2). Thereafter, the PCR products were cloned into the pCR 3.1 TA cloning vector (Invitrogen, Carlsbad, CA). We selected several clones and verified the DNA sequence of the normal and mutant alleles. Each of the normal and mutant alleles was cloned into the expression vector, pCDNA3 (Invitrogen, Carlsbad CA), using the appropriate restriction enzyme. The normal and mutant constructs were designated as WT-gPAX4 and MT-gPAX4, respectively.

In order to analyze the mRNA expression of WT-gPAX4 and MT-gPAX4, COS7 (African green monkey SV40-transformed kidney fibroblast) and MIN6 cells (immortalized insulin-secreting pancreatic β cells) were transiently transfected using Lipofectamine 2000 (Promega, Madison, MI) with either WT-gPAX4 (1.0 μ g) or MT-gPAX4 (1.0 μ g). Total RNA extraction and RT-PCR were done using FastPure RNA kit and PrimeScript RT-PCR kit (Takara, Tokyo, Japan). The obtained cDNA from WT-gPAX4 or MT-gPAX4 was purified and sequenced directly.

Wild-type and mutant *PAX4* cDNA construction and plasmid construction

Human *PAX4* cDNA was amplified from PCR Ready First Strand DNA of normal human placenta (BioChain Institute, Inc) by PCR and subcloned into a pcDNA 3.1 expression vector. The mutant *PAX4*-cDNA was created by the PrimeSTAR mutagenesis kit (Takara, Tokyo, Japan). The wild and mutant *PAX4* cDNAs were designated as WT-cPAX4 and MT-cPAX4, respectively.

The human insulin and glucagon promoters were amplified by PCR and subcloned into pGL3 reporter vectors (Promega, Madison, MI) and were designated as pGL3-insulin and pGL3-glucagon, respectively.

Luciferase assay

MIN6 cells were obtained from American Type Cell Culture (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum.

Table 1. Sequences of primers used to amplify *PAX4*.

Exon	Forward (5'-3')	Reverse (5'-3')
1	AGGTGGTGTGTGGATACCTC	CAGGCTCTTGCCTTCAGAG
2	CCATCATGCCTCACCTGTC	GCCTCTTTTCCAGCCCCAGT
3	CCTGAGTCTGAGCACCATCTC	GATTTGGCTGTGATTAGCCC
4	CTGACCAGAGGAATCACCATC	CCCTGTGTCACACTGAGGAC
5	GAGACCCATGCCTTGCTCCTC	GGCCCAGACTCTTCCTCCTTG
6	GATCAGCAGGTGACAGGCAGC	GATGACTGAGCGGGCAGATG
7	AGTGGCTGACTTTCCTAGAAC	GAGCCCATGAGCCCTTCAGTC
8	CTCTACAGGAGGCATCACTG	GAGGTTGAGTCAGTCGACCCT
9	GTCCCCACAGGCCACTTGCC	TGGGCAGGATGGTATTAGATCT

In order to assay insulin and glucagon gene promoter activity, MIN6 cells were plated in 6-well plates to 70% confluence and transiently transfected using Lipofectamine 2000 with the empty expression vector (1.0 μ g), WT-cPAX4 (1.0 μ g), MT-cPAX4 (1.0 μ g), or WT-cPAX4 (0.5 μ g) plus MT-cPAX4 (0.5 μ g) together with each reporter vector (0.5 μ g) (pGL3-insulin or pGL3-glucagon) according to the previous report (Plengvidhya et al. 2007). Cell extracts were prepared 48 hours after transfection and luciferase assays were performed. Luciferase measurements were divided by β -galactosidase activity to control for transfection efficiency. Transfections were performed in triplicate within a single experiment, and the experiment was repeated three times. The mean of each triplicate was expressed as a percentage of the empty vector control to allow comparison of data from different experiments. The significance of differences between the wild type and mutant was assessed by one-way ANOVA followed by Scheffer's *post hoc* test. A *P*-value of < 0.05 was considered significant.

Fluorescence analysis and microscopy

COS7 cells transfected with either WT-PAX4-GFP or MT-PAX4-GFP were placed onto glass coverslips and fixed in 4% (vol/vol) formaldehyde/PBS 24hr after transfection. Cells were permeabilized in 0.1% Triton/PBS and then examined under a Fuji fluorescence microscope (Fuji, Tokyo, Japan).

Results

Gene sequencing results

Sequence analysis of genomic *PAX4* demonstrated a heterozygous 39-base deletion mutation in exon 3 (c.374-412 del39) (Fig. 1A). His father also had this heterozygous mutation. One hundred fifty normal Japanese subjects did not have this deletion.

mRNA analysis

RT-PCR analysis after transfection of WT-gPAX4 demonstrated a 531 bp cDNA as expected, whereas RT-PCR analysis of MT-gPAX4 yielded a shorter cDNA fragment (Fig. 2A) in COS7 cells. Sequence analysis of this shorter cDNA fragment demonstrated the lack of exon 3 (Fig. 2B). The exon 3 skipping changed the open reading frame, resulting in a premature stop codon at codon 206 in exon 5. The same results were obtained when using MIN6 cells.

Functional analysis

Cotransfection of WT-cPAX4 with insulin and glucagon promoters repressed the luciferase reporter gene relative to the empty vector; however, MT-cPAX4 lacked the repressor activity, indicating loss-of-function (Fig. 3A, B). Co-transfection of MT-cPAX4 and an equivalent amount of WT-cPAX4 did not affect the activity of WT-cPAX4, which

Fig. 1. Sequence analysis of *PAX4*.

(A) Sequence analysis of genomic DNA from the patient. Sequence analysis of the *PAX4* gene demonstrated a 39-base deletion (c. 374-412 del39). Note that after mutation, double bands are present (arrow). (B) Schematic representation of human the *PAX4* protein. The black box represents paired domain and the shaded box represents homeodomain. Two previously-reported pathogenic missense mutations (arrowheads) and the mutation found in this study (arrow) are displayed. The deletion mutation found in the present study produces a premature stop codon at codon 206 (asterisk) in the homeodomain.

Fig. 2. RT-PCR analysis of the mutation.

(A) RT-PCR analysis of PAX4 after transfection of WT-gPAX4 demonstrated a 531 bp cDNA as expected, whereas RT-PCR analysis after transfection of MT-gPAX4 yielded a shorter cDNA fragment. (B) Sequence analysis of this shorter cDNA demonstrated the lack of exon 3. The sequence of the cDNA from WT-gPAX4 is also shown.

excluded dominant negative effects (Fig. 3A, B).

Next, we analyzed the subcellular localization of WT-cPAX4 and MT-cPAX4. Both WT-cPAX4 and MT-cPAX4 proteins were localized to the nucleus (Fig. 3C).

Discussion

We identified a novel heterozygous 39-base deletion in the *PAX4* gene in a Japanese family with MODY. The authentic splice site of exon 3-intron 3 was still present in the mutant sequence; however, RT-PCR analysis demonstrated that this mutation led to exon 3 skipping and changed the open reading frame, resulting in a premature stop codon at codon 206 in exon 5. It is considered that exonic-cis elements, distinct from the classical splicing signals, are important for correct splice-site identification. These elements work as exonic splicing enhancers (ESEs) and are necessary for regulation of alternative splicing (Blencowe 2000). *In silico* analysis of ESE motifs in the wild type and mutant exon 3 revealed that the 39-base deleted region in exon 3 is located on several ESE sequence motifs and was predicted to influence ESEs (Table 2). Thus, this deletion probably disrupts these recognition sites such that exon inclusion may no longer be favored, resulting in exon skipping and the formation of aberrant transcripts. Indeed, several mutations of ESE sequences have been reported to cause exon skipping in human diseases (Moseley et al. 2002; Levit et al. 2010).

Our *in vitro* study revealed that this mutant PAX4 had reduced repression activity toward target genes compared with the wild type. The original papers demonstrated that PAX families' functions as a transcription repressor through a paired domain and homeodomain (Fujitani et al. 1999; Smith et al. 1999). As our mutant lacked the homeodomain, our *in vitro* results were consistent with these previous notions. Furthermore, our mutant did not demonstrate a dominant negative effect similar to the previously reported R121W and R164W mutations (Shimajiri et al. 2001; Plengvidhya et al. 2007). *In vivo*, the functional alteration induced by the deletion could be due to nonsense-mediated decay (NMD) (Rehwinkel et al. 2006). Degradation of mutant PAX4 via the NMD mechanism could prevent translation of the aberrant transcripts by eliminating mRNAs that contain a premature stop codon. Thus, the haploinsufficiency or reduced gene dosage of *PAX4* may have led to the development of diabetes in our family. However, it is noted that heterozygous *Pax4* knockout mice did not develop diabetes (Brun and Gauthier 2008). This may imply that humans are more sensitive than mice to the dosage of *PAX4*.

In the family in our study, the phenotypes of the proband and his father were different. Despite having the identical genotype of *PAX4*, the diabetes in the father has been controlled well, while his son requires insulin therapy. At the time of diagnosis of diabetes, the father had been obese,

Fig. 3. *In vitro* functional analysis of WT and MT-PAX4.

(A) WT-cPAX4 represses glucagon (A) and insulin (B) promoter activity, but MT-cPAX4 has reduced suppressive activity. Co-transfection of MT-cPAX4 and an equivalent amount of WT-cPAX4 does not affect the function of WT-cPAX4, suggesting no dominant negative effect. Data are expressed as mean $\pm$ s.d. ($n = 6$) from the analysis for three times. *, $P < 0.01$.

(C) Subcellular localization of WT-PAX4 and MT-PAX4. COS7 cells expressing WT-PAX4-GFP or MT-PAX4-GFP were visualized directly by GFP fluorescence. Both WT-PAX4 and MT-PAX4 are localized at the nucleus.

Table 2. Predictions of exonic splice enhancers.

WT-Exon 3			MT-Exon 3		
Motif	Site	Score		Site	Score
SRp40	ttacagg	6.01235		ttacagg	6.01235
SRp55	cacggc	3.88260	Deleted*	tacagg	2.34137
SF2/ASF	cacacgg	5.86231	Deleted*	attacagg	-2.44998
SF2/ASF (IgM-BRCA1)	cacacgg	4.96554	Deleted*	attacagg	-2.65315
SC35	gactaccg	4.74311	Deleted*	attacagg	-2.81196

*By the 39 bases deletion, MT-Exon 3 lacked these motifs.

but we did not have data on his insulin secretion. Two Japanese patients with heterozygous R121W mutation in *PAX4* were treated with diet alone, similar to the father in our study (Shimajiri et al. 2001). It may be speculated that the father had decreased insulin secretory response or insulin resistance, which improved after diet therapy. In contrast, the proband has continued to require insulin therapy. Shimajiri et al. (2001) also reported that two other patients with R121W mutation were treated with insulin transiently. Therefore, there is still a possibility that insulin may be able to be withdrawn from the proband and thus periodic evaluation of his insulin secretion capacity must be performed.

Similar to our familial case, in Thai patients, the phe-

notype varied in the same family (Plengvidhya et al. 2007). The detailed clinical history of this patient with IVS7-1G>A mutation was not described in the literature; however, diabetes in this pedigree seems to be severe and was characterized by early onset of renal complications. These variable phenotypes may thus be accounted for by the implication of other genetic and/or environmental factors (Peltonen and Mckusick 2001).

In conclusion, we identified a novel mutation of *PAX4*, which might be pathogenic. In addition, this family provides another example that there may be phenotypic heterogeneity within a MODY family.

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