A Missense Mutation of Pax4 Gene (R121W) Is Associated With Type 2 Diabetes in Japanese

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Pax4 is one of the transcription factors that play an important role in the differentiation of islet β-cells. We scanned the Pax4 gene in 200 unrelated Japanese type 2 diabetic patients and found a missense mutation (R121W) in 6 heterozygous patients and 1 homozygous patient (mutant allele frequency 2.0%). The mutation was not found in 161 nondiabetic subjects. The R121W mutation was located in the paired domain and was thought to affect its transcription activity through lack of DNA binding. Six of seven patients had family history of diabetes or impaired glucose tolerance, and four of seven had transient insulin therapy at the onset. One of them, a homozygous carrier, had relatively early onset diabetes and slowly fell into an insulin-dependent state without an autoimmune-mediated process. This is the first report of a Pax4 gene mutation that exhibits loss of function and seems to be associated with type 2 diabetes. This work provides significant implications for the Pax4 gene as one of the predisposing genes for type 2 diabetes in the Japanese. *Diabetes* 50:2864–2869, 2001

Type 2 diabetes has been thought to be a polygenic disorder. Although several gene mutations have been found in type 2 diabetic patients, the main susceptibility genes common to different ethnic backgrounds for type 2 diabetes have not been found. Recently, gene mutations of the transcription factors, which were thought to play an important role in regulating β-cell differentiation, have been reported to be responsible for the early onset of type 2 diabetes with strong family history (mature-onset diabetes of the young) (1–4). Pax4 is a member of the Pax family and was first reported as a transcription factor related to the segmentation of *Drosophila* (5). Gene-targeting experiments for Pax4 revealed that the Pax4 was essential for the differentiation of insulin-producing β-cells (6,7). These findings suggest that Pax4 may be a susceptibility gene for type 2 diabetes. Thus, we scanned the Pax4 gene for mutation and identified a missense mutation in Japanese type 2 diabetic patients.

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EMSA, electrophoretic mobility shift assay; ICA, islet cell antibody; ivTT, in vitro translation and transcription; OGTT, oral glucose tolerance test; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism; TK, thymidine kinase.

RESEARCH DESIGN AND METHODS

We randomly enrolled 200 unrelated Japanese type 2 diabetic patients attending to Wakayama University Hospital in Wakayama Prefecture, located in the middle part of Japan, and 161 nondiabetic subjects without family history of diabetes (>60 years of age). Nondiabetic subjects were confirmed to have plasma glucose <6.1 mmol/l and HbA_{1c} <5.8%. Informed consent was obtained from all participants. The study was approved by the Ethical Committee of Wakayama University of Medical Science and was in accordance with the principle of the Declaration of Helsinki. As a second screening for the R121W mutation, we screened 193 type 2 diabetic patients in Okinawa Prefecture, located in the southern part of Japan.

Mutation screening. Using genomic DNA obtained from peripheral leukocytes, all nine exons of the Pax4 gene (8) were amplified by polymerase chain reaction (PCR) based on fluorescent end-labeling primers (Table 1) and analyzed by single-strand conformational polymorphism (SSCP) method at two different temperatures (4 and 22°C) with a DNA sequencer (ALFred; Pharmacia Biotech, Tokyo). When an aberrant band was scanned by SSCP analysis, nucleotide sequence was determined by the dideoxy chain termination method using ABI prism 310 (PE Biosystems, Tokyo).

Plasmid construction. Full-length human Pax4 cDNA was isolated from a human placenta 5′-Stretch Plus cDNA library (Clontech, Palo Alto, CA) by PCR using *Pfu* polymerase (Stratagene, La Jolla, CA). For the transient luciferase assay, the human Pax4 cDNA was subcloned into an expression vector, pcDNA3.1:pcDNAPax4(Wild) (the pcDNA3.1 was from Invitrogen, Leek, the Netherlands). We recently showed that Pax4 functioned as a repressor of transcription and that Pax4 inhibited Pax6-induced transcriptional activation by competing for the same binding sites (9). Thus, the mouse Pax6 expression plasmid, pcDNAPax6, which is described elsewhere (9), was used with pcDNAPax4 in the luciferase assay. For a reporter plasmid, five copies of the consensus sequence for Pax6-binding element (P6CON) (10) was inserted into the thymidine kinase (TK) gene promoter with firefly luciferase gene (5×P6CON-TK-Luc) (9). Mutagenesis was performed by Quick Change Site-Directed Mutagenesis Kit (Stratagene) using *Pfu* polymerase and the restriction enzyme *Dpn*I to generate mutant cDNA:R121W (arginine at codon 121 to tryptophan). For electrophoretic mobility shift assay (EMSA), the mouse Pax4 cDNA (9) (GenBank accession no. AB010558) was subcloned into pcDNA3.1(His):pmHisPax4(Wild) [the pcDNA3.1(His) was from Invitrogen]. We used this His-tagged fusion protein for supershift assay, because anti–Pax4-specific antibodies could not be obtained. The pmHisPax4(Wild) was subjected to mutagenesis to generate mutant (R129W) Pax4, which corresponded to the R121W of human Pax4:pmHisPax4(Mutant).

Luciferase assay. The reporter plasmid (100 ng 5 × P6CON-TK-Luc) was cotransfected with 50 ng pcDNAPax6 and 50 ng pcDNAPax4(Wild) or pcDNAPax4(Mutant) into COS7 cells (8×10^4 per well in 24-well tissue culture dishes) by Effectene transfection reagent (QIAGEN, Tokyo). The cells were cultured for 48 h, and the transient luciferase activity was measured. The transfection efficiencies were normalized using *Renilla* luciferase activity cotransfected with each experiment.

EMSA. To obtain the wild-type and mutated Pax4 protein, pmHisPax4(Wild) or pmHisPax4(Mutant) were subjected to in vitro translation and transcription (ivTT) system using T7 polymerase and TNT-coupled wheat germ extract system (Promega, Madison, WI). [^{35}S]-labeled methionine was used with SDS-PAGE to check the amount of protein yielded from each plasmid. For EMSA analysis, rat glucagon promoter G3 element (GluG3: 5′-TTTTCACGC CTGACTGAGATTGAAGGGTGTATTT-3′) (11,12), which included P6CON se-

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TABLE 1
Sequences of primers used to amplify exons of the human Pax4 gene

Exon (bp)	Forward (5′-3′)	Reverse (5′-3′)	Size of PCR product (bp)	Anealing temperature (°C)
1 (131)	AGGTGGTGTGTGGATACCTC	CAGGCTCTTGCCTTCAGAG	241	62
2 (216)	CCATCATGCCTCACCTGTC	GCCTCTTTTCCAGCCCCAGTG	298	62
3 (76)	CCTGAGTCTGAGCACCATCTC	GATTTGGCTGTGATTAGCCC	165	58
4 (126)	CTGACCAGAGGAATCACCATC	CCCTGTGTCACACTGAGGAC	233	58
5 (83)	GAGACCCATGCCTTGCTCCTC	GGCCCAGACTCTTCCTCCTTG	194	58
6 (70)	GATCAGCAGGTGACAGGCAGC	GATGACTGAGCGGGCAGATG	173	58
7 (56)	AGTGGCTGACTTTCCTAGAAC	GAGCCCATGAGCCCTTCAGTC	226	58
8 (142)	CTCTACAGGAGGCATCACTG	GAGGTTGAGTCAGTCGACCCT	259	58
9 (141)	GTCCCCACAGGCCACTTGCC	TGGGCAGGATGGTATTAGATCTTCTCTATG	205	58

quence in the 5′ region, was used as a probe. The ivTT product (5 μl) was preincubated with 1.5 μl poly(dI-dC) in a buffer containing 10 mmol/l Tris, pH 7.4, 50 mmol/l NaCl, 25 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol, 1 mmol/l EDTA, 1 mg/ml bovine serum albumin, and 10% glycerol in a final volume of 20 μl at room temperature for 20 min. After the addition of the double-stranded oligonucleotide probes (5′ end–labeled by [γ-^{32}P]), the samples were incubated at room temperature for 15 min, and then electrophoresis was performed on 6% nondenaturing polyacrylamide gel in 0.5 × Tris-borate-EDTA buffer at 4°C. For supershift assay, 2 μl of the ivTT reaction mixture and 3 μl anti-HisG antibodies (Invitrogen) (1:5,000) or phosphate-buffered saline were preincubated at 4°C for 1 h before probe addition. Mouse serum, which does not recognize His-tagged epitope, was used as a negative control for the specific antibody.

Western blotting. The expression levels of the wild-type and mutant Pax4 were confirmed as similar by SDS-PAGE and Western immunoblot assay using anti-Xpress antibody (Invitrogen) in extracts of the cells used for the luciferase assay.

Statistics. Data were expressed as the means ± SE. Statistical analysis was carried out by χ^2 test or Fisher's extraction test and Student's t test. $P < 0.05$ was considered significant.

RESULTS

Mutation detection. We identified six kinds of missense mutations and a silent mutation (Table 2). Among them, the mutant allele frequency (2.0%; 8 of 400) of the R121W located in the paired domain was significantly higher in the diabetic group than in nondiabetic group ($P < 0.01$). The mutation was detected in six diabetic patients as the heterozygous state and in one patient as the homozygous state (Table 3). None of the 161 nondiabetic subjects (322 alleles) had the mutation estimated by PCR/restriction fragment–length polymorphism using *Cac8I*. Detected mutations at codon 31, 183, 192, and 321 in the diabetic group were also seen in the same frequency in the nondiabetic group.

Clinical characteristics of R121W carriers. Six patients (patients 1, 2, 4, 5, 6, and 7) had a family history of diabetes or impaired glucose tolerance (Table 3). The family history of patient 3 had not been clarified because his parents died when he was young. The diabetic state of six heterozygous patients varied from very mild diabetes treated with diet alone (patients 1 and 2) to severe diabetes requiring insulin treatment (patient 6). However, four of seven carriers were treated with insulin (patients 6 and 7) or had past history of insulin treatment (patients 3 and 5), and all of the four patients had past history of transient (couple of months) insulin treatment at the onset. Their onset age also varied.

The R121W homozygous proband. Patient 7 was a 44-year-old woman. She was referred to our hospital at the age of 29 because of weight loss, general fatigue, polyuria, and thirst. At that time, her fasting plasma glucose was 15.2 mmol/l, HbA_{1c} was 12.6%, urine ketone bodies were negative, and islet cell antibodies (ICAs) were negative.

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TABLE 2
Mutations of the human Pax4 gene in a Japanese population

Exon	Nucleotide change	Designation	Genotype frequency*: Type 2 (n = 200)	Genotype frequency*: Nondiabetic subjects (n = 161)	Mutant allele frequency (%): Type 2	Mutant allele frequency (%): Nondiabetic subjects	Significance†
1	CGG→CAG	R31Q	R/R, R/Q, Q/Q 194, 6, 0	R/R, R/Q, Q/Q 153, 8, 0	1.5	2.5	NS
3	CGG→TGG	R121W	R/R, R/W, W/W 193, 6, 1	R/R, R/W, W/W 161, 0, 0	2.0	0	$P < 0.01$
4	CAA→CAG	Q173Q	12	Not tested	—	—	—
5	CGT→TGT	R183C	R/R, R/C, C/C 195, 5, 0	R/R, R/C, C/C 160, 1, 0	1.3	0.3	NS
	CGT→AGT	R192S	R/R, R/S, S/S 190, 10, 0	R/R, R/S, S/S 152, 9, 0	2.5	2.8	NS
	CGT→CAT	R192H	R/R, R/H, H/H 179, 21, 0	R/R, R/H, H/H 141, 20, 0	5.3	6.2	NS
9	CCC→CAC	P321H	H/H, H/P, P/P 93, 86, 21	H/H, H/P, P/P 78, 70, 13	68.0	70.2	NS

*Data for genotype frequency are presented as the genotype(s) and corresponding n values. †Statistical significance of the mutant allele frequency between type 2 diabetic and nondiabetic subjects estimated by χ^2 or Fisher's extraction test.

TABLE 3
Clinical characteristics of the Pax4 gene mutation (R121W) in type 2 diabetic patients

Patient	Age (years) and sex	Diabetic family members	Maximal BMI (kg/m^2) before diagnosis	Age at diagnosis (years)	HbA_{1c} (%)	Treatment
1	51, F	Sister	29.4	43	7.0	Diet
2	71, M	Mother, sister	26.7	49	6.1	Diet
3	62, M	?	17.8	49	8.1	OHA
4	61, F	Sister	32.4	47	6.8	OHA
5	50, M	Parents, sister	22.0	32	8.8	OHA
6	66, F	Mother	21.8	25	8.2	Insulin
7	44, F*	Mother†	22.2	29	7.3	Insulin

*Homozygote; †impaired glucose tolerance. OHA, oral hypoglycemic agents.

After 1 month of insulin treatment, insulin was changed to sulfonylureas because her glucose levels were fairly controlled. However, she transitioned to an insulin-requiring state within a year after diagnosis. The required insulin dose had gradually increased. At the time of the study, she had been receiving intensive insulin therapy (four times a day) for the previous 5 years. At the age of 28 (1 year before the diagnosis), she delivered an overweight baby (5,200 g) with a cleft lip. She had no history of ketosis. Abdominal computed tomography scan and ultrasonography revealed no evidence of organic abnormalities of the pancreas and other organs. She had no complications of diabetes. Fasting plasma glucagon (40 pmol/l) and somatostatin (18 pmol/l) concentrations were within normal limits.

Family study of the R121W homozygous proband. The pedigree and results of a 75-g oral glucose tolerance test (OGTT) are shown in Fig. 1*A* and Table 4, respectively. Her parents were cousins carrying the R121W mutation in the heterozygous state. Her younger sister was also a heterozygous carrier. These heterozygotes had normal glucose tolerance or impaired glucose tolerance, in contrast to the homozygous proband with severe diabetes. However, the early response of insulin relative to the increment of glucose at 30 min during an OGTT, which was estimated as the insulin-to-glucose ratio, was low in these three heterozygous carriers compared with 24 subjects with normal glucose tolerance (121.4 ± 3.8) (Table 4).

Family study of a R121W heterozygous proband. We detected 12 heterozygous carriers in the second screening of the other residents. The mutant allele frequency of R121W in the second screening (3.1%, 12 of 386) was similar to that of the first screening. There were no homozygous carriers in the second screening. The family study and OGTT were performed in one heterozygous proband's family. The pedigree and OGTT results are shown in Fig. 1*B* and Table 4, respectively. The proband was a 49-year-old woman treated with insulin. She had a 20-year-old daughter and an 18-year-old son. Although both had normal glucose tolerance, the son, who had a heterozygous mutation, had a low early insulin response relative to glucose during the OGTT, as observed in the other heterozygous carriers (Table 4). In contrast, the daughter, who had no mutation, had an almost normal insulin-to-glucose ratio at 30 min. The insulin-to-glucose ratio of the four heterozygotes (34.6 ± 1.8) was significantly ($P < 0.001$) decreased from that of nondiabetic subjects.

Luciferase assay. To assess the R121W mutant Pax4 function, we compared the transcription activity between the wild-type and the mutant R121W using a luciferase reporter system in COS7 cells. As shown in Fig. 2, basal activity was increased approximately fourfold by Pax6 (*lane 2*), and the suppressive effect of wild-type Pax4 was obvious (*lane 3*), which suppressed activity to nearly basal levels. Mutant Pax4 lacked this suppressive effect against Pax6 activation by almost 92% (*lane 4*).

EMSA. EMSA was performed to determine the DNA binding activity of the mutant Pax4 (Fig. 3). Consistent with the results obtained in the luciferase assay, the R129W mutant Pax4 almost completely lacked binding activity to the probe. On the other hand, wild-type Pax4 clearly bound to the probe (long arrow, *lane 4*), and the band disappeared with the addition of the antibodies (*lane*

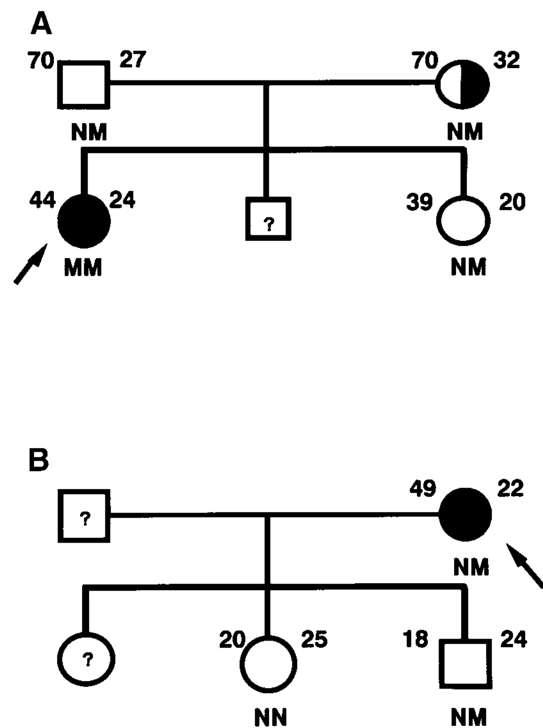

FIG. 1. Pedigrees of the Pax4 gene mutation (R121W) carriers. *A*: Homozygous carrier's pedigree. *B*: Heterozygous carrier's pedigree. Symbols indicate the state of glucose tolerance: □ and ○, normal glucose tolerance; ◑, impaired glucose tolerance; ■ and ●, diabetes; □ and ○ with "?", unknown. The genotype is indicated under the symbols: NN, wild-type; NM, heterozygote; MM, homozygote. An arrow indicates the proband. Age (in years) is shown on the upper left side of each symbol, and BMI (in kilograms per meter squared) is shown on the upper right side of the symbols.

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TABLE 4
Plasma glucose and serum insulin responses during 75-g OGTT in family members of subjects with the Pax4 gene mutation (R121W)

	Time (min)				Insulin-to-glucose ratio ($\times 10^{-9}$)*
	0	30	60	120	
Family members of the homozygous patient					
Father (heterozygote)					
Glucose (mmol/l)	4.7	8.1	8.6	5.9	36.5
Insulin (pmol/l)	26	150	258	246	
Mother (heterozygote)					
Glucose (mmol/l)	5.8	10.0	10.7	9.4	36.9
Insulin (pmol/l)	55	210	246	348	
Sister (heterozygote)					
Glucose (mmol/l)	4.9	8.5	7.2	6.6	20.3
Insulin (pmol/l)	35	108	234	204	
Family members of a heterozygous patient					
Daughter (wild-type)					
Glucose (mmol/l)	5.4	7.8	7.2	5.9	91.3
Insulin (pmol/l)	33	252	390	234	
Son (heterozygote)					
Glucose (mmol/l)	4.7	7.6	8.0	7.2	44.1
Insulin (pmol/l)	46	174	240	330	

*Insulin-to-glucose molar ratio was calculated by the increased insulin level and increased glucose level after 30 min during an OGTT.

5), although the binding complex could not be detected as a clear retarded band. There was no shift of the specific band with the addition of mouse serum (*lane 6*), indicating that the observation in *lane 5* was a specific effect caused by the antibodies.

DISCUSSION

We found a missense mutation (R121W) in the Pax4 gene that seemed to be associated with type 2 diabetes. This mutation was not found in the 161 (>60 years of age) nondiabetic control subjects, who were thought to have less chance of developing diabetes in the future. The mutant allele frequency (2.0%) was similar to that of the second screening in Okinawa, suggesting that the mutation is commonly distributed in Japanese type 2 diabetic patients. Furthermore, this allele frequency seemed to be considerably high compared with the other mutation previously reported in the type 2 diabetic patients.

The mutation was located at the COOH-terminal region in the paired domain, which was important as a DNA binding site independently or in connection with the homeodomain (13,14). The arginine residue at codon 121 is conserved among the Pax families (15). The structure of the human Pax6 revealed that R125, which corresponds to R121 of human Pax4 and R129 of mouse Pax4, made direct contact with the major groove of the DNA binding consen-

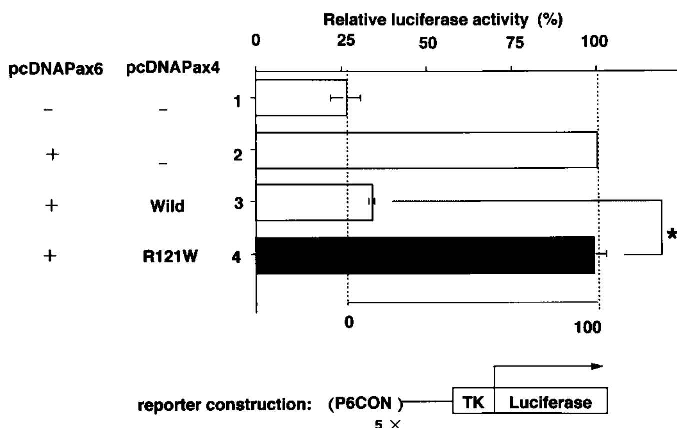

FIG. 2. Effects of the Pax4 mutation on Pax6 transcription activity in COS7 cells. The amount of reporter plasmids (5 × P6CON-TK-Luc) was 100 ng (*lanes 1–4*). The pcDNAPax6 was 50 ng (*lane 2*). The pcDNAPax4(Wild) (or R121W) was also 50 ng (*lanes 3* and *4*). The nonrecombinant plasmid (pcDNA 3.1) was cotransfected to adjust the total amount of DNA (200 ng) (*lanes 1* and *2*; 100 and 50 ng, respectively). *P < 0.01, n = 8.

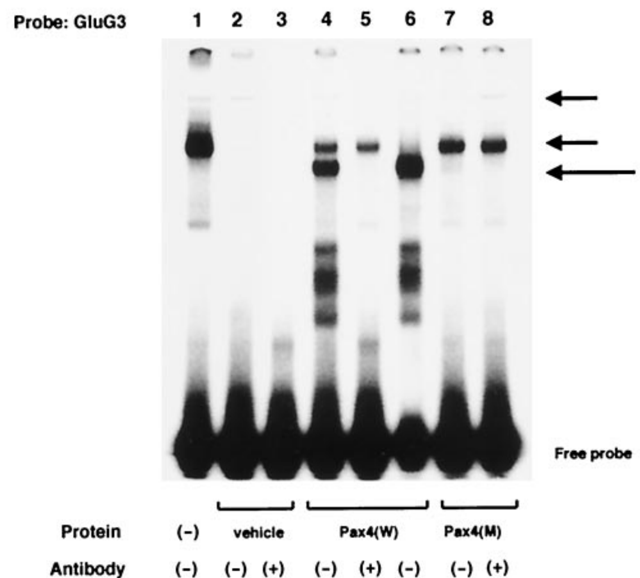

FIG. 3. Supershift assay of the Pax4 are shown. An equal amount of protein yielded by the ivTT system was applied in each lane. The mouse wild-type Pax4 (*lanes 4*, *5*, and *6*) and mouse mutant Pax4 (R129W) (*lanes 7* and *8*) were used as His-tagged fusion protein. Mouse R129W corresponds to human R121W. The anti-HisG antibodies were added in *lanes 3*, *5*, and *8*. Mouse serum was added in *lane 6*. Unprogrammed wheat germ cell extract (*lane 1*) and extract derived from nonrecombinant plasmid [pcDNA3.1(His)] (*lanes 2* and *3*) were applied as negative controls. The long arrow indicates specific binding of Pax4 with His-tagged fusion protein to the probe. The short arrows indicate nonspecific binding. The oligonucleotide probe was rat glucagon promoter G3 element (GluG3). Identical results were obtained in three independent experiments.

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sus sequence (16). These facts strongly suggest that the amino acid substitution at 121 might change the structure of the paired domain and affect its function. To assess the mutant Pax4 function, we first compared the transcription activity between wild-type and mutant Pax4 by luciferase assay in COS7 cells. The R121W mutant Pax4 lacked the inhibitory effect on transcription activity induced by Pax6 (Fig. 2). There was a sequence similar to the GluG3 sequence in the 5′ promoter region of the human preproglucagon gene. We also performed a luciferase assay using a promoter of the human preproglucagon gene as a reporter in αTC1 cells. The suppressive effect of the mutant Pax4 was impaired similarly to that observed in COS7 cells (data not shown). To further study mechanisms for the impairment of the inhibitory effect of the mutant Pax4, we performed an EMSA to estimate the DNA binding of the mutant, using mouse Pax4 instead of human Pax4 because binding affinity of human Pax4 to the oligonucleotide probes (P6CON and GluG3) was weak, with fuzzy bands seen in EMSA analysis. Consistent with the results obtained in the luciferase assay, the R129W mutant Pax4, which corresponded to R121W mutant human Pax4, did not bind to the probe (Fig. 3). These results support the notion that Pax4 recognizes consensus nucleotide sequences similar to those of Pax6 and acts as a suppressor of Pax6 by competing for these DNA binding sites (9,11,12). This speculation is not in conflict with the model of the differentiation lineage in the early embryonic stage of the pancreas (7).

Heterozygotes of the Pax4 knockout mice were reported to be indistinguishable from wild-type in terms of phenotype (6). In the present study, the onset age and diabetic state of the six heterozygous patients were multiform (Table 3), and heterozygous carriers (father and sister) in the family of the homozygote (patient 7) had normal glucose tolerance. Thus, the diabetogenic potential of the R121W mutation would not be strong on its own when remaining in the heterozygous state. Because type 2 diabetes is a multifactorial disease including polygene abnormality, the patients with more severe diabetes might be receiving an increased dose of type 2 diabetes polygenes and/or increased environmental factors.

Although the heterozygous carriers in patient 7's family had normal glucose tolerance or impaired glucose tolerance, they had a low insulin response relative to glucose at 30 min after oral glucose load, in contrast to the daughter without the mutation (Table 4). These findings suggest that the R121W mutation might influence insulin secretion even in a heterozygous state.

We unexpectedly found a patient carrying the homozygote of the R121W mutation. She had relatively early onset diabetes. She had a history of transient insulin therapy at the onset and had gradually fallen into diabetes requiring intensive insulin therapy to maintain a good metabolic control. Her clinical course was suggestive of slowly progressive type 1 diabetes without autoimmune-mediated process because ICA was negative at the onset. Homozygotes of Pax4-knockout newborn mice were reported to have few β-cells (6). The severity of diabetes in this patient may thus be associated with the homozygous state of the mutation through reduction of β-cells. However, the precise mechanism remains to be elucidated. She has a history of delivering an overweight baby 1 year before the onset of diabetes. Her gestation would have been acting as a strong environmental factor to cause diabetes. Four of seven patients (including the homozygote) carrying the R121W mutation have a past history of transient insulin therapy at the onset of diabetes. This evidence might reflect the reduced function of the mutant Pax4, a transcription factor related to β-cell differentiation, through a low reserve of β-cells or a lack of responsiveness of β-cells to the suddenly increasing insulin requirement of the periphery caused by a combination of environmental factors and other genetic factors for diabetes.

Pax4 has been reported to not be a major susceptibility gene for type 2 diabetes in Ashkenazi Jews assessed by sib-pair analysis (8). Another report also showed that there were some polymorphisms without cosegregation of diabetes in a French population (17). These negative findings against our data might be derived from ethnic or racial differences. Further examinations of the Pax4 gene mutation would be needed in other races.

In conclusion, we identified a missense mutation (R121W) of the Pax4 gene that seems to be associated with type 2 diabetes. This work provides significant implications for the Pax4 gene as one of the predisposing genes for type 2 diabetes in the Japanese.

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