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BRIEF REPORT

PAX4 Mutations in Thais with Maturity Onset Diabetes of the Young

Nattachet Plengvidhya, Suwattanee Kooptiwut, Napat Songtawee, Asako Doi, Hiroto Furuta, Masahiro Nishi, Kishio Nanjo, Wiwit Tantibhedhyangkul, Watip Boonyasrisawat, Pa-thai Yenchitsomanus, Alessandro Doria, and Napatawn Banchuin

Division of Endocrinology and Metabolism, Departments of Medicine (N.P.), Immunology and Immunology Graduate Program (N.P., N.S., W.T., W.B., N.B.), Physiology (S.K.), and Research and Development (N.S., P.Y.), Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, 10700 Thailand; The First Department of Medicine (A.Doi, H.F., M.N., K.N.), Wakayama Medical University, Wakayama 641-8509, Japan; and Section on Genetics and Epidemiology (A.Dor.), Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts 02215

Context: Six maturity onset diabetes of the young (MODY) genes have been discovered to date but account for a small proportion of MODY among Asians, suggesting the existence of other MODY genes in this racial group.

Objective: The aim of this study was to investigate whether or not genetic variants in *PAX4*, a crucial transcription factor in β-cell development, contribute to MODY in Thais.

Design and Methods: We screened *PAX4* coding sequences in 46 MODY probands without mutation in known MODY genes and in 74 nondiabetic controls using PCR-single-stranded conformational polymorphism analysis followed by direct sequencing. Genotyping of variants identified was done by PCR-restriction fragment length polymorphism analysis.

Results: Eight sequence differences were identified. Two novel variations (R164W and IVS7–1G>A) were found in two different probands. Neither was found in the 74 nondiabetic controls and additional 270 healthy subjects of Thai origin. R164W segregated with diabetes in the family of the proband and *in vitro* studies showed that it impairs the repressor activity of PAX4 on the insulin and glucagon promoters. The remaining six variants were previously described and observed in both groups. One of them, R192H, was three times more frequent in MODY probands than in 342 nondiabetic controls (minor allele frequency = 0.196 *vs.* 0.064; $P < 0.00001$). The same variant was associated with a younger age at diagnosis among 254 Thai subjects with adult-onset type 2 diabetes (44.6 ± 15 *vs.* 49.7 ± 11 yr; $P = 0.048$).

Conclusions: We have identified two possible pathogenic mutations of *PAX4,* R164W, and IVS7–1G>A. For one of these, we have shown evidence of segregation with diabetes and a functional impact on PAX4 activity. Single-nucleotide polymorphism R192H might influence the age at onset of diabetes. **(*J Clin Endocrinol Metab* 92: 2821–2826, 2007)**

MATURITY ONSET DIABETES of the young (MODY) is a genetically heterogeneous form of diabetes characterized by an early onset, frequent insulin-independence at the beginning of the disease, absence of ketosis, and an autosomal dominant pattern of inheritance (1). Six different MODY genes have been identified to date. One codes for the glycolytic enzyme glucokinase (MODY2) (2), the other five for transcription factors expressed in pancreatic β-cells (3–7). The observation of forms of familial diabetes that fit the MODY criteria but are unlinked to any of the six known MODY genes suggests the existence of additional MODY genes (8). Such forms of MODY are frequent in Asians, among whom they could account for 60–80% of MODY cases (9, 10). Indeed, we found that only one of 47 MODY probands that we recently recruited in Bangkok had a mutation in a known MODY gene (*HNF-1A* R203C), indicating that mutations in unidentified genes are responsible for the vast majority of MODY cases in Thailand (11). These genes may code for transcription factors involved in β-cell development and function.

PAX4, a paired-homeodomain transcription factor, functions as a transcription repressor through a pair homeobox and homeodomain (12, 13). Such action plays a critical role in pancreatic β-cell development and function (14). PAX4 first appears in the endocrine progenitor cells at embryonic d 9.5 and is later selectively expressed in β-cells (13), where it is required to maintain the expression of Pdx1 and Nkx 6.1, two essential modulators of pancreatic β-cell development (14). Heterozygous *PAX4* knockout (KO) mice have few mature β- and δ-cells, and numerous, abnormally clustered α-cells, suggesting that PAX4 is a critical regulator of the commitment of progenitor cells to the different islet cell lineages (15). Remarkably, the abnormalities of *PAX4* KO mice resemble those of mice with a targeted disruption of insulin-promoter-factor 1, a known MODY gene (16). Pax4 also appears to be important for the regeneration of β-cell in adult life, as

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Abbreviations: KO, Knockout; MODY, maturity onset diabetes of the young; SNP, single-nucleotide polymorphism; SSCP, single-stranded conformational polymorphism.

suggested by the finding that *PAX4* mutations impair the ability of β-cells to proliferate (17). On this basis, we investigated whether sequence variants in *PAX4* contribute to MODY in the Thai population or not.

Subjects and Methods

Study subjects

The study included 46 diabetic probands of MODY families recruited at the Diabetic Clinic, Siriraj Hospital, Bangkok, Thailand. The inclusion criteria were: 1) the proband and at least one first-degree relative diagnosed with type 2 diabetes before age 35 yr, 2) two or more generations affected by diabetes, 3) diabetes treatment with diet and/or oral agents, 4) no history of diabetic ketoacidosis, and 5) absence of glutamic acid decarboxylase antibody. Mutations in any of the six known MODY genes were excluded by PCR-single-stranded conformational polymorphism (SSCP) analysis followed by direct sequencing. Nondiabetic subjects were 74 healthy staff members in the Department of Immunology and Department of Research and Development at Mahidol University, Bangkok, Thailand. All of them had fasting plasma glucose levels less than 100 mg/dl and had no family history of diabetes in first-degree relatives. A total of 270 additional nondiabetic subjects were healthy blood donors without a history of diabetes. The study was approved by the Faculty of Medicine Siriraj Hospital Research Ethics Committee. All study subjects signed a consent form before their enrollment.

Mutation screening and sequence analysis of PAX4

PAX4 exons and exon-intron boundaries were screened for nucleotide variants in the 46 MODY probands and 74 nondiabetic subjects by PCR followed by SSCP analysis. PCR products showing a mobility shift were directly sequenced by the ABI Prism BigDye 228 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Fluorescent signals were detected with the ABI Collection software and analyzed by the Sequencer Navigator software and Chromas program version 1.4.4 (Conor McCarthy, Griffith University, Queensland, Australia).

Genotyping of PAX4 variants

The R164W, IVS7–1G>A, and R192H variants were genotyped in an additional 270 nondiabetic subjects by PCR-restriction fragment length polymorphism analysis using the enzymes *Hae*III, *Bsr*I, and *Mse*I (Fermentas Inc., Hanover, MD), respectively. DNA fragments were separated on 12% polyacrylamide gels or 2% agarose gels, and genotype and allele frequencies compared between MODY probands and nondiabetic subjects by χ^2 tests with Yates' correction for continuity or Fisher exact tests (Statistical Package for the Social Sciences; SPSS, Inc., Chicago, IL). P values < 0.05 were considered significant.

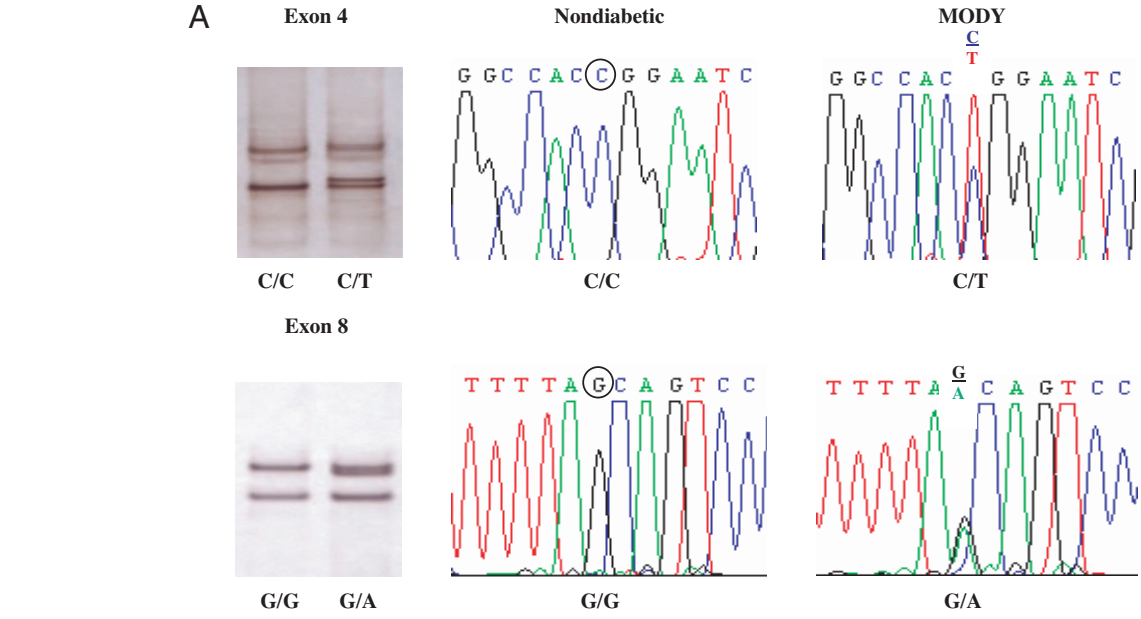
Functional study of PAX4 variant

Full-length human wild-type *PAX4* cDNA was amplified from PCR Ready First Strand cDNA of normal human placenta (BioChain Institute, Inc., PSA Vista, Singapore) by PCR using platinum *Pfx* DNA polymerase (Invitrogen, Leek, The Netherlands) and subcloned into a pcDNA 3.1 expression vector. The R164W mutation was introduced by site-directed mutagenesis (QuikChange Mutagenesis Kit; Stratagene, La Jolla, CA) to generate pcDNA3.1-*PAX4*-R164W. Human insulin and glucagons promoters were isolated by PCR using *Pfu* DNA polymerase (Stratagene) and separately subcloned into pGL3 reporter vectors to generate human insulin and glucagon promoter-firefly luciferase reporters. *PAX4* wild-type and mutant constructs (500 ng) were transfected into MIN6 or αTC-1.6 cells using the FUGENE 6 transfection reagent (Roche Diagnostics, Roche Applied Science, Indianapolis, IN) along with 100 ng pGL3-human insulin promoter, and 10 ng pRL-SV40 (to control for the transfection efficiency). After 24 h, the transactivation activity of the normal and mutant PAX4 proteins was measured by the Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI). The significance of differences among constructs was tested by one-way ANOVA followed by Scheffe's *post hoc* test. A P value < 0.05 was considered significant.

TABLE 1. Summary of the *PAX4* variants

Location	Codon	Nucleotide change	Designation	Genotype frequency						Allele frequency		*P* value
				MODY (n = 46)			Nondiabetic[a]			MODY	Nondiabetic	
Exon 1	31	CGG > CAG	R31Q	G/G	G/A	A/A	G/G	G/A	A/A			
				45	1	0	74	0	0	G 0.99	G 1.00	NS[b]
				0.98	0.02	0.00	1.00	0.00	0.00	A 0.01	A 0.00	
Exon 4	164	CGG > TGG	R164W	C/C	C/T	T/T	C/C	C/T	T/T			
				45	1	0	344	0	0	C 0.99	C 1.00	NS[b]
				0.98	0.02	0.00	1.00	0.00	0.00	T 0.01	T 0.00	
	173	CAA > CAG	Q173Q	A/A	A/G	G/G	A/A	A/G	G/G			
				44	2	0	73	1	0	A 0.98	A 0.99	NS[b]
				0.96	0.04	0.00	0.99	0.01	0.00	G 0.02	G 0.01	
Exon 5	183	CGT > TGT	R183C	C/C	C/T	T/T	C/C	C/T	T/T			
				45	1	0	73	1	0	C 0.99	C 0.99	NS[b]
				0.98	0.02	0.00	0.99	0.01	0.00	T 0.01	T 0.01	
	192	CGT > AGT	R192S	C/C	C/A	A/A	C/C	C/A	A/A			
				45	1	0	72	2	0	C 0.99	C 0.98	NS[b]
				0.98	0.02	0.00	0.97	0.30	0.00	A 0.01	A 0.02	
	192	CGT > CAT	R192H	G/G	G/A	A/A	G/G	G/A	A/A			
				30	14	2	300	40	2	G 0.800	G 0.940	<0.00001[c]
				0.67	0.29	0.04	0.88	0.12	0.00	A 0.196	A 0.064	
Intron 7	nt-1	AG > AA	IVS7-1 G>A	G/G	G/A	A/A	G/G	G/A	A/A			
				45	1	0	344	0	0	G 0.99	G 1.00	NS[b]
				0.98	0.02	0.00	1.00	0.00	0.00	A 0.01	A 0.00	
Exon 9	321	CCC > CAC	P321H	C/C	C/A	A/A	C/C	C/A	A/A			
				5	23	18	10	39	25	C 0.36	C 0.40	NS[c]
				0.11	0.50	0.39	0.14	0.52	0.34	A 0.64	A 0.60	

[a] The genotyping of *PAX4* variants was done in 74 nondiabetic controls except for R164W and IVS-1G>A, which were done in 344 nondiabetic controls. R192H was genotyped in 342 nondiabetic controls. $P < 0.05$ was considered statistically significant. NS, Nonsignificant.
[b] Fisher's exact test.
[c] χ^2 test.



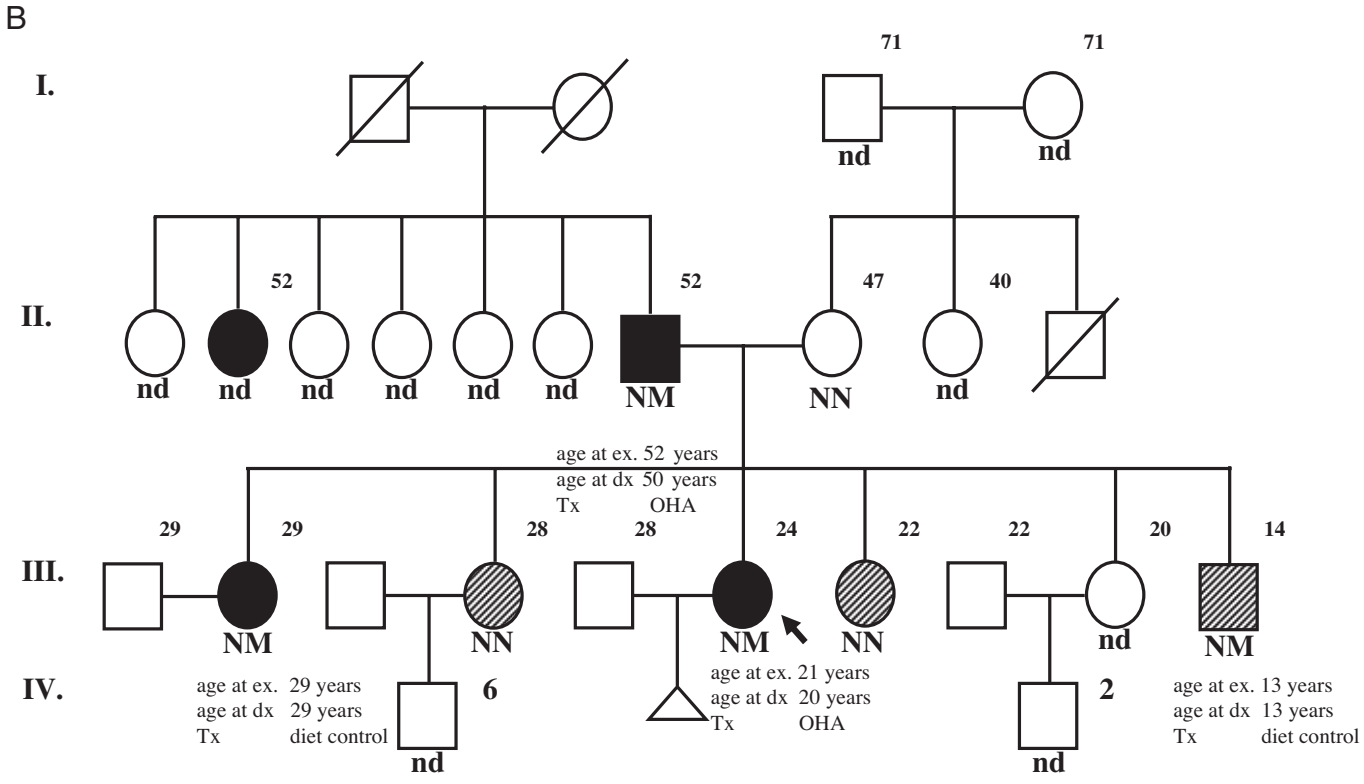


FIG. 1. A, PCR-SSCP and sequence analysis of *PAX4* from MODY probands compared with nondiabetic subjects. Abnormal SSCP pattern of 233-bp fragment of exon 4 (*upper panel*) and 260-bp fragment of exon 8 (*lower panel*) in probands from two families. Direct sequencing shows a C to T substitution (*upper panel*) in codon 164 resulting in R164W and a G to A substitution (*lower panel*) at splice acceptor of intron 7 (IVS7–1G>A). B, Pedigree of family whose proband carries the *PAX4* R164W mutation. *Symbols* indicate the state of glucose tolerance. ○ and □, Normal fasting glucose; *shaded circle* and *shaded square*, impaired glucose tolerance; ● and ■, diabetes; ○ and □ with "nd," unknown. The genotypes are indicated under the *symbol*. An *arrow* indicates the proband. Age (in years) is shown in the *upper right side* of each symbol. dx, Diagnosis; ex, examination; NN, normal homozygote; nd, not done; NM, heterozygote; OHA, oral hypoglycemic agent; Tx, treatment.

Results and Discussion

A total of eight sequence differences were identified (Table 1). Two were novel variants that were found in heterozygosity with the wild type in two different probands. One was a C to T substitution at codon 164 (CGG>TGG), resulting in the replacement of arginine with tryptophan (R164W). The other was a G to A substitution at the splice acceptor site of intron 7 (IVS7–1G>A) (Fig. 1A). Neither mutation was found in the 344 nondiabetic subjects of Thai origin. The R164W mutation segregated with diabetes, being present in the proband's 52-yr-old father, who was diagnosed as having diabetes at age 50 yr, and 29-yr-old sister, who both had type 2 diabetes, as well as in her 14-yr-old brother, who had impaired glucose tolerance (Fig. 1B). However, two sisters (28 and 22 yr old), who also had impaired glucose tolerance, did not carry the mutation and were probably phenocopies as observed in the case of other MODY genes (4, 7). No relatives were available for the segregation analysis of the IVS7–1G>A mutation with diabetes, but evaluation of the pedigree (data not show) revealed that two older sisters and one older brother had diabetes and early-onset renal failure (40 yr old), and all died at age 52–53 yr of end-stage renal disease. Her youngest sister was diagnosed as having diabetes at age 30 yr, was still alive, and already has diabetic retinopathy and nephropathy at age 40 yr.

The remaining six variants were previously described single-nucleotide polymorphisms (SNPs) that were observed in both MODY probands and nondiabetic controls (Table 1). The R192H variant was three times more frequent in the 46 MODY probands than in 342 nondiabetic controls (minor allele frequency = 0.196 *vs.* 0.064; $P < 0.00001$). We further genotyped the R192H in 254 Thai adult onset type 2 diabetes but found no significant difference in allele frequencies between patients and nondiabetic controls (Table 2). However, the mean age at diagnosis of heterozygotes was significantly lower than among major allele homozygotes (44.6 ± 15.0 *vs.* 49.7 ± 10.7 yr; $P = 0.048$).

PAX4 represses the activity of the insulin and glucagon promoters (12). To assess whether the R164W mutation affects such function, we transiently transfected MIN6 cells, which have characteristics similar to those of isolated islets, with allelic forms of the *PAX4* cDNA together with an insulin promoter-firefly luciferase reporter system. The wild-type PAX4 repressed the insulin promoter activity by about 50% (Fig. 2B). By contrast, the R164W mutant repressed the promoter by only 35% ($P < 0.01$ for mutant *vs.* wild-type). Similar results were obtained with a human glucagon promoter reporter system in α-TC1.6 cells (Fig. 2C). The PAX4 wild type repressed the promoter activity by 57%, whereas the R164W repressed it by only 35% ($P < 0.01$ for mutant *vs.* wild-type). These differences between wild type and mutant were not due to differences in transfection efficiencies or in the expression of the transfected constructs (data not shown).

Our results suggest that the R164W variant is likely to be a pathogenic mutation because: 1) it is extremely rare; 2) it segregates with diabetes in the proband's family; 3) it is placed in the homeodomain (Fig. 2A) responsible for PAX4 binding to target DNA sequences; 4) it concerns an amino acid residue conserved among species; 5) the mutation is rather severe, replacing a polar with a nonpolar amino acid; and 6) this amino acid substitution impairs the repressor activity of PAX4 on the insulin and glucagon promoters.

Given that the impairment of PAX4 repressor activity caused by the mutation is relatively small, we hypothesize that such abnormality causes diabetes through the interaction of multiple mechanisms, each of which is not sufficient by itself to lead to hyperglycemia. One of these may be the disruption of β-cell development in the embryo, resulting into reduced β-cell mass. Another mechanism may involve decreases insulin secretion in response to glucose in adult β-cell. Because heterozygous PAX4 KO mice have numerous, abnormally clustered α-cells (15), this mutation may similarly perturb the islet architecture in humans and affect the insulin to glucagon ratio, which is a critical determinant of gluconeogenesis and glycogenolysis.

The evidence supporting a pathogenetic role for the IVS7–1G>A variant is not as definitive because we did not have access to the proband's relatives to determine its segregation with diabetes and could not directly study the impact of this mutation because this would require access to islet mRNA from the proband. On the other hand, this mutation appears to be especially severe, abolishing the acceptor splice site of intron 7 and potentially leading to exon skipping, intron retention, or usage of another acceptor splice site. It would seem unlikely that a mutation having such predicted effects was silent. Indeed, the characteristic of diabetes in the pedigree suggests that this mutation may determine an especially severe form of diabetes characterized by an early onset of renal complications.

We also found that a relatively common polymorphism (R192H) was overrepresented in MODY probands compared with nondiabetic controls. Although highly significant, this result should be taken with caution because it concerns only 46 MODY cases. Furthermore, we failed to demonstrate an association between this polymorphism and later-onset type 2 diabetes, as previously shown in Japanese individuals (18). However, our data indicate that this variant may influence the age at onset rather than the overall risk of type 2 diabetes.

In conclusion, we have identified two possible pathogenic mutations of *PAX4,* R164W and IVS7–1G>A. For one of these, we have shown evidence of segregation with diabetes and a functional impact on PAX4 activity. We have also found that SNP R192H might also influence the risk of diabetes development, in particular for those forms characterized by an early onset.

TABLE 2. Comparison of genotype and allele frequency of *PAX4* R192H between adult onset type 2 diabetic patients and nondiabetic controls

	Groups	Genotype frequency	*P* value	Allele frequency	*P* value
PAX4 R192H	T2DM (n=254)	GG=208 GA=45 AA=1	0.078	G=0.91 A=0.09	0.069
	Controls (n=342)	GG=300 GA=40 AA=2		G=0.94 A=0.06	

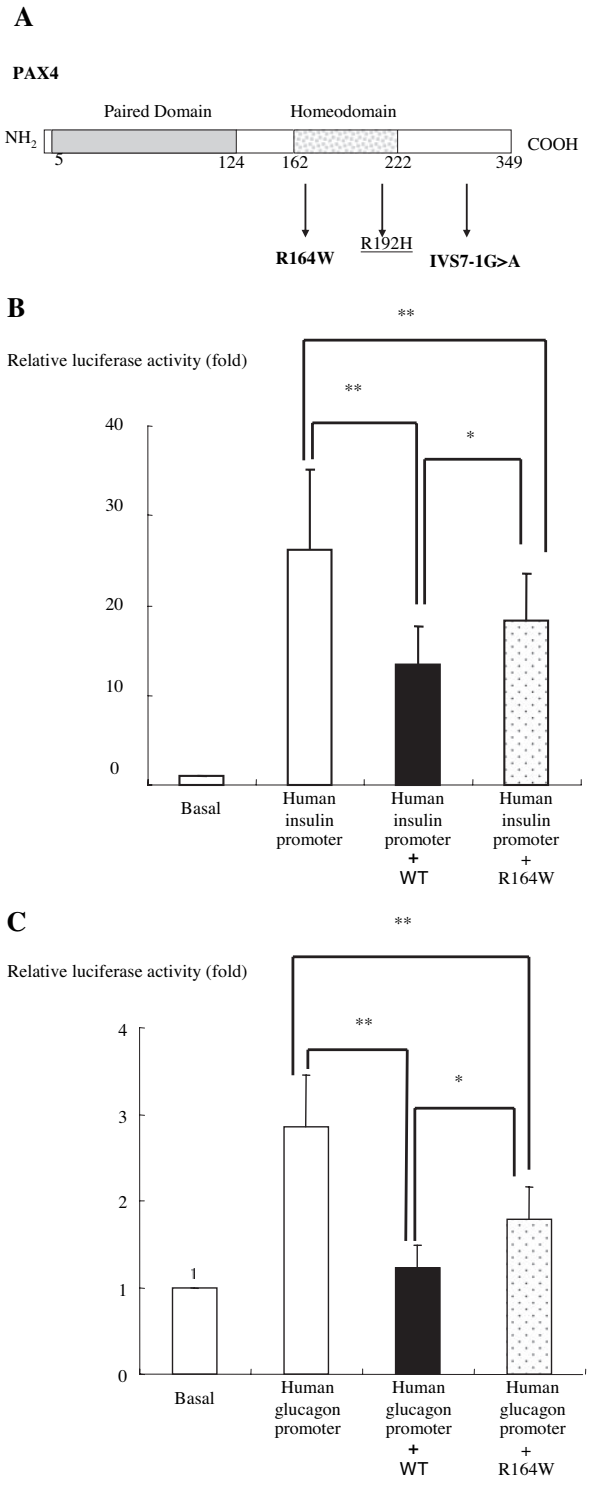


FIG. 2. A, Schematic representation of the PAX4 protein structure. The R164W and IVS7–1G>A variants that are expected to be pathogenic are shown in *bold letters*. The SNP R192H (*underlined*) showed high frequencies with significant difference between MODY probands and nondiabetic subjects. Effect of PAX4 mutation on luciferase activity in MIN6 and α-TC1.6 cells. MIN6 and α-TC1.6 cells were transfected with 0.5 mg human wild-type PAX4 and R164W mutant and 0.5 mg human insulin (B) and glucagon (C) promoter reporter genes, respectively, together with 10 ng pRL-SV40 internal control vector. Data are expressed as mean ± SD (n = 6) from the analysis for three times. *, $P < 0.01$. **, $P < 0.001$. WT, Wild-type.

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Address all correspondence and requests for reprints to: Nattachet Plengvidhya, M.D., Division of Endocrinology and Metabolism, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand. E-mail: sinpv.natpl@gmail.com.

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