Assay system (Promega Corp., WI, USA) and the assay was reported at least 3 times. Luciferase activity was expressed as mean ± S.D. and the significance of differences was tested by one-way ANOVA followed by Sheffe's post-hoc test. P-value less than 0.05 are considered as significant.

3.3 Screening of a novel candidate gene using data from genome-wide scan

This part of the study was conducted by Miss Watip Boonyasrisawat, a PhD candidate under the Thailand Research Fund-Royal Golden Jubilee (TRF-RGB) Scholarship, in Dr.Alessandro Doria's laboratory at the Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts, USA.

Eight MODY families that diabetes not caused by mutation of known MODY genes was included in this study. B lymphoid kinase (*BLK*) gene was screened in 2 persons per family through the bi-directional direct sequencing to sequence all exons, exon/intron boundaries and promoter region. The fragments that showed a DNA sequence difference in probands would be further examine by typing the DNA samples of unrelated non-diabetic controls. Family members might also be genotyped to determine whether the specific variant could account for segregation of diabetes in the family. If so, it would be likely diabetes-causing allele. If no variant could account for segregation of diabetes in a family, SNPs within and around the gene would be examined the disease-causing variant in an intron or distant regulatory element in the 5' or 3' flanking region.

(1) Bi-directional sequencing

Exons, exon/intron boundaries and 5' regulatory regions were amplified by PCR on a Thermocycler. Primers are shown in Appendix I, Table 27. PCR products were purified using a Qiaquick Gel Extraction Kit (Qiagen). Purified PCR samples were sequenced using both forward and reverse primers using the BigDye Terminator Cycle Sequencing v2.0 kit (Applied Biosystems) according to the manufacturer's protocol. After ethanol precipitation, sequencing products were resuspended in formamide, denatured at 95°C, and then electrophoresed on an ABI 3100 Genetic Analyzer using POP-4 polymer and the rapid POP-4 sequencing module (30 sec injection time, 1.0 kV injection voltage, 20 min collection time, 15.0 kV EP voltage, 50°C temperature). Sample quality and heterozygous sequence differences were determined for each sample

individually using the SEQUENCING ANALYSIS 3.4.1 software (applied Biosystems), and then imported into SEQUENCHER 3.0 (Gene Codes Corp.) where traces were aligned and analyzed for homozygous sequence differences in both the forward and reverse directions. Lastly, the position of all identified sequence differences was determined using the UCSC genome database (http://genome.ucsc.edu/).

RESULTS

1. Studies of six known MODY genes and new candidate genes

The clinical characteristics of studied probands compared with non-diabetic control subjects are shown in Table 3.

Table 3 Clinical characteristics of MODY probands and non-diabetic controls**.**

	MODY patients*	**Non-diabetic controls***
Age (years)	33.54 ± 11.91	26.91 ± 5.55
Age at onset (years)	26.15 ± 8.88	-
Duration (years)	6.79 ± 9.00	-
BMI (kg/m^2)	27.13 ± 6.64	20.32 ± 2.32
Waist (cm)	85.67 ± 12.14	71.00 ± 7.70
Hip (cm)	98.36 ± 7.29	90.61 ± 5.88
Waist/Hip ratio	0.87 ± 0.10	0.79 ± 0.05
Systolic BP (mmHg)	119.60 ± 12.50	ND
Diastolic BP (mmHg)	76.89 ± 10.38	ND
FPG (mg/dl)	210.24 ± 78.48	84.24 ± 5.45
HbA1c (%)	9.23 ± 3.17	ND
Serum creatinine (mg/dl)	3.04 ± 14.52	ND
Total Cholesterol (mg/dl)	214.49 ± 54.05	ND
Triglyceride (mg/dl)	229.53 ± 171.07	ND
LDL (mg/dl)	130.24 ± 44.60	ND
HDL (mg/dl)	43.94 ± 9.40	ND

*Data are shown as mean ± SD.

1.1 Studies of six known MODY genes

(1) Screening for nucleotide variations by PCR-SSCP and direct sequencing

The promoters, exons and exon-intron boundaries of six known MODY genes (*HNF-4α, GCK, HNF-1α, IPF-1, HNF-1β* and *NeuroD1* genes) were screened for

nucleotide variations in the 51 MODY probands and 15 non-diabetic controls by PCR-SSCP followed by direct sequencing.

A total of thirty seven nucleotide variations (five in the *HNF-4α* gene, five in the *GCK* gene, nineteen in the *HNF-1α* gene, two in the *IPF-1* gene, two in the *HNF-1β* gene and four in the *NeoroD1* gene) were identified. Twelve were novel variations. The novel variations are presented in Figures 3-7 and all results are shown in Tables 4-9.

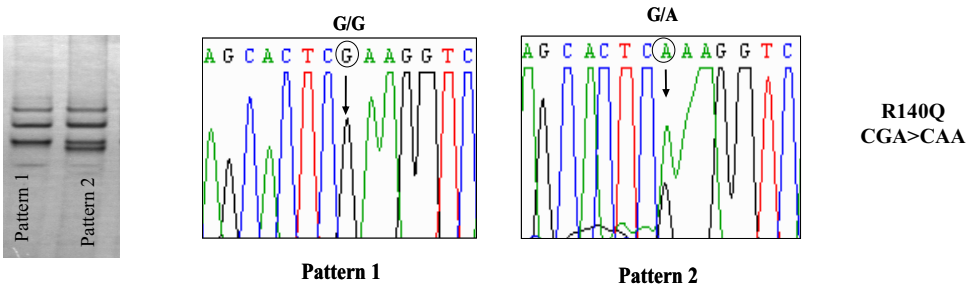


Figure 3 SSCP analysis and chromatograms of DNA sequencing results presenting novel variations identified in *HNF-4α* genes.

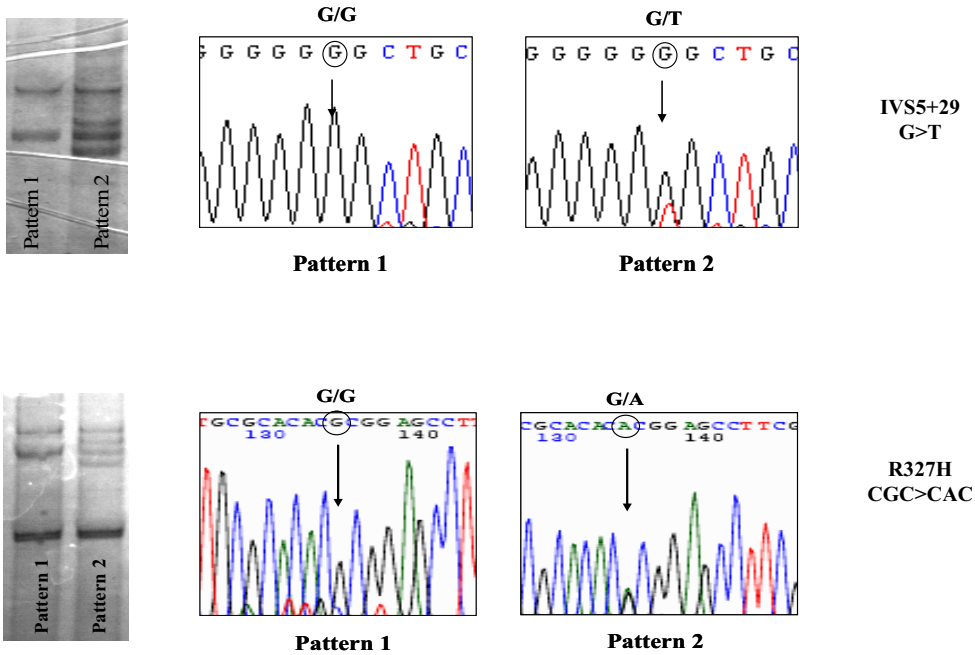
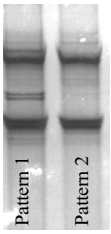
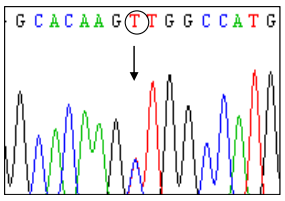


Figure 4 SSCP analysis and chromatograms of DNA sequencing results presenting novel variations identified in *GCK* genes.

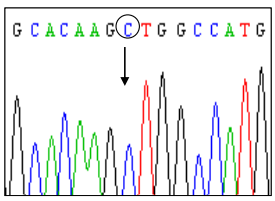


C/T



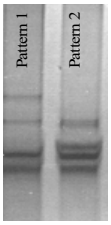
Pattern 1

C/C

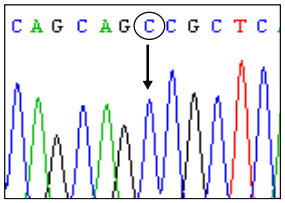


Pattern 2

L281L
CTG>TTG

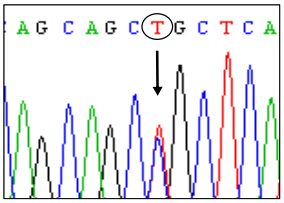


C/C



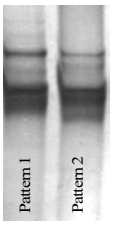
Pattern 1

C/T

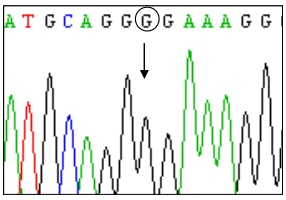


Pattern 2

P475
CCG>CTG

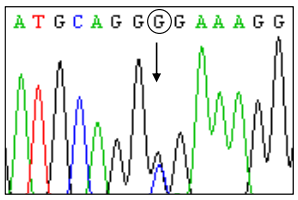


G/G



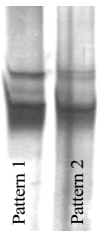
Pattern 1

G/C

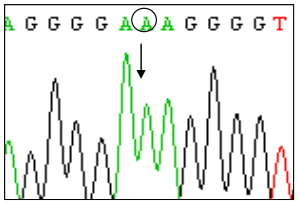


Pattern 2

IVS7+65
G>C

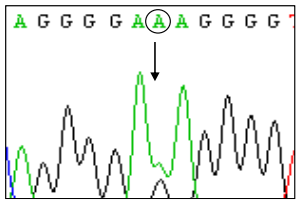


A/A



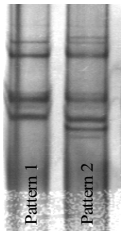
Pattern 1

A/G

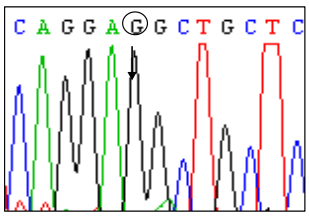


Pattern 2

IVS7+68
A>G

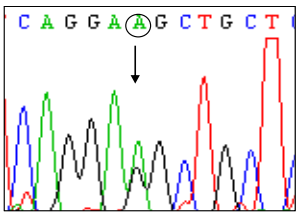


G/G



Pattern 1

G/A



Pattern 2

IVS8-19
G>A

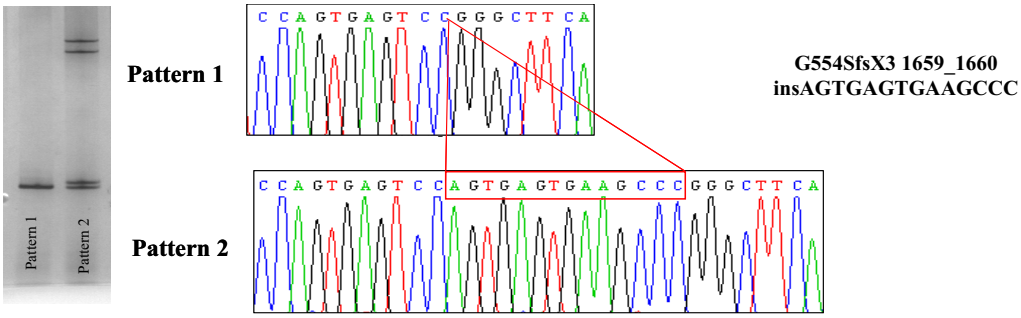


Figure 5 SSCP/hetrduplex analysis and chromatograms of DNA sequencing results presenting novel variations identified in *HNF-1α* genes.

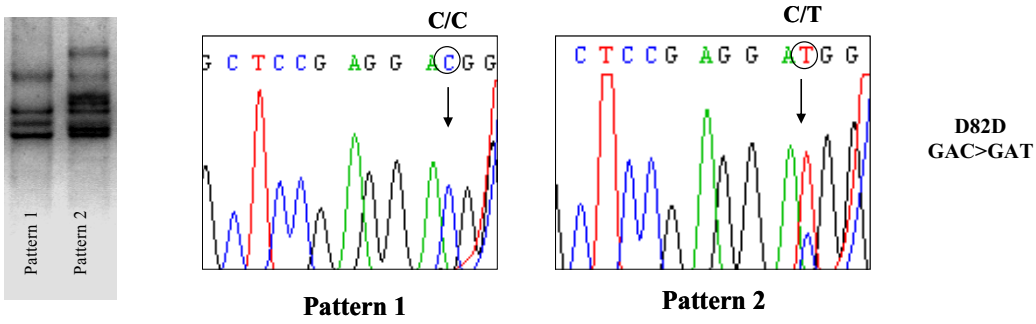


Figure 6 SSCP analysis and chromatograms of DNA sequencing results presenting novel variations identified in *HNF-1β* genes.

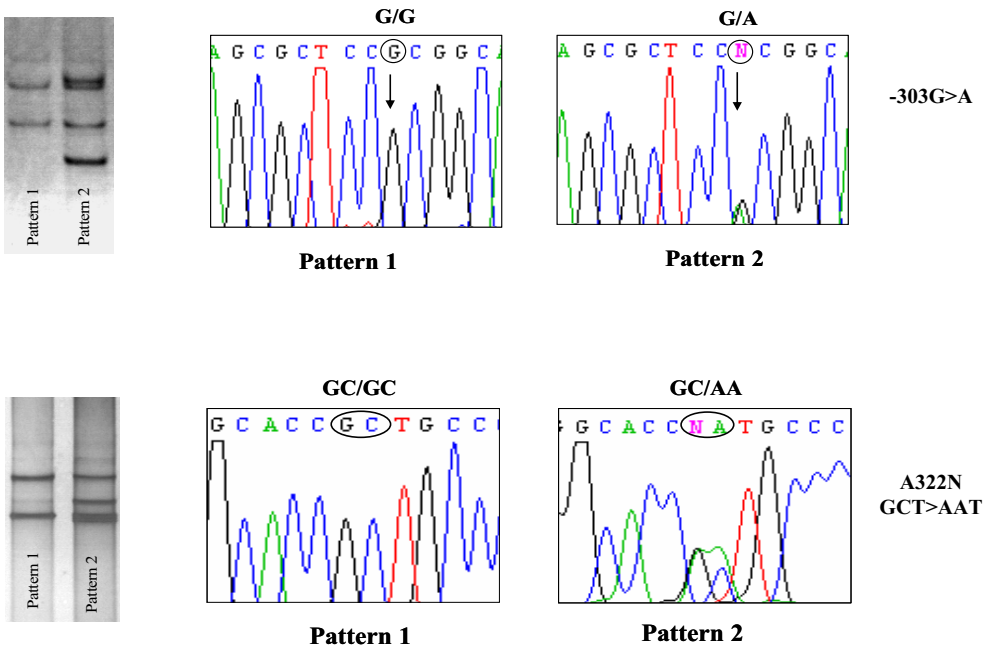


Figure 7 SSCP analysis and chromatograms of DNA sequencing results presenting novel variations identified in *NeuroD1* genes.

(2) Genotype and allele frequencies of nucleotide variations identified in six known MODY genes by PCR-SSCP analysis

Tables 4-9 summarizes nucleotide variations of *HNF-4α, GCK, HNF-1α, IPF-1, HNF-1β* and *NeuroD1* genes that were identified in MODY probands and non-diabetic controls by PCR-SSCP analysis whereas G554SfsX3 1659_1660 insAGTGAGTGAAGCCC variant of the *HNF-1α* gene was identified by heteroduplex analysis. The frequencies of these nucleotide variations between 51 MODY probands and 15 non-diabetic controls are compared. The T139I, R140Q and R312H variants of the *HNF-4α* gene, -194A>G, -30G>A, IVS5+29G>T and R327H variants of the *GCK* gene, R203C, L281L, IVS5+9C>G, L459L, P475L, IVS7+65G>C, IVS7+68A>G, IVS8-19G>A and G554SfsX3 1659_1660insAGTGAGTGAAGCCC variants of the *HNF-1α* gene, D82D variants of the *HNF-1β* gene, -303G>A and A322N variants of the *NeuroD1* gene were not detected in 15 non-diabetic controls, while remaining variations of the six known MODY gene were found in both groups without significant difference allele frequencies (p>0.05).

Table 4 Genotype and allele frequencies of nucleotide variations identified in the *HNF-4α* gene by PCR-SSCP analysis.

Location	Neucleotide change	Designation	Sample	Genotype Frequency			Allele Frequency	
				A/A	A/C	C/C	A	C
Promoter	A >C	-639A>C	MODY	25	19	7	0.68	0.32
			Control	9	4	2	0.73	0.27
				C/C	C/T	T/T	C	T
Intron 1	C >T	IVS1-5C>T	MODY	38	13	0	0.87	0.13
			Control	12	3	0	0.90	0.10
				C/C	C/T	T/T	C	T
Exon 4	ACT >ATT	T139I	MODY	49	2	0	0.98	0.02
			Control	15	0	0	1.00	0.00
				G/G	G/A	A/A	G	A
Exon 4	**CGA >CAA**	**R140Q**	MODY	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00
				G/G	G/A	A/A	G	A
Exon 8	CGT >CAT	R312H	MODY	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00

*** Novel variation is indicated in bold.**

Table 5 Genotype and allele frequencies of nucleotide variations identified in the *GCK* gene by PCR-SSCP analysis.

Location	Neucleotide change	Designation	Sample	Genotype Frequency			Allele Frequency	
				A/A	A/G	G/G	A	G
Promoter	A>G	-194A>G	Proband	45	6	0	0.94	0.06
			Control	15	0	0	1.00	0.00
				G/G	G/A	A/A	G	A
Promoter	A>G	-30G>A	Proband	47	4	0	0.96	0.04
			Control	15	0	0	1.00	0.00
				G/G	G/T	T/T	G	T
Intron 5	**G/T**	**IVS5+29G>T**	Proband	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00
				G/G	G/A	A/A	G	A
Exon 8	**CGC >CAC**	**R327H**	Proband	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00
				C/C	C/T	T/T	C	T
Intron 9	C >T	IVS9+8C>T	Proband	12	35	4	0.58	0.42
			Control	4	9	2	0.57	0.43

*** Novel variations are indicated in bold.**

Table 6 Genotype and allele frequencies of nucleotide variations identified in the *HNF-1α* gene by PCR-SSCP analysis.

Location	Neucleotide change	Designation	Sample	Genotype Frequency			Allele Frequency	
				C/C	C/G	G/G	C	G
Exon 1	CTC >CTG	L17L	Proband	28	18	5	0.73	0.27
			Control	6	9	0	0.70	0.30
				A/A	A/C	C/C	A	C
Exon 1	ATC>CTC	I27L	Proband	30	16	5	0.75	0.25
			Control	6	8	1	0.67	0.33
				G/G	G/A	A/A	G	A
Intron 1	G >A	IVS1-42G>A	Proband	25	20	6	0.69	0.31
			Control	5	10	0	0.67	0.33
				T/T	T/A	A/A	T	A
Intron 2	T >A	IVS2-51T>A	Proband	17	24	10	0.57	0.43
			Control	6	7	2	0.63	0.37
				C/C	C/T	T/T	C	T
Exon 3	CGT >TGT	R203C	Proband	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00
				C/C	C/T	T/T	C	T
Exon 4	**CTG >TTG**	**L281L**	Proband	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00
				G/G	G/C	C/C	G	C
Exon 4	GGG>GGC	G288G	Proband	50	1	0	0.99	0.01
			Control	14	1	0	0.97	0.03
				C/C	C/G	G/G	C	G
Intron 5	C >G	IVS5+9C> G	Proband	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00
				G/G	G/T	T/T	G	T
Intron 5	G >T	IVS5 -42G>T	Proband	38	13	0	0.87	0.13
			Control	10	5	0	0.83	0.17
				C/C	C/T	T/T	C	T
Exon 7	CTG >TTG	L459L	Proband	27	17	7	0.70	0.30
			Control	4	9	2	0.57	0.43
				G/G	G/A	A/A	G	A
Exon 7	CTG >CTA	L459L	Proband	49	2	0	0.98	0.02
			Control	15	0	0	1.00	0.00
				C/C	C/T	T/T	C	T
Exon 7	**CCG >CTG**	**P475L**	Proband	50	1	0	0.99	0.01
			Control	15	0	0	1.00	.000
				G/G	G/A	A/A	G	A
Exon 7	AGC>AAC	S487N	Proband	27	17	7	0.70	0.30
			Control	4	9	2	0.57	0.43
				G/G	G/A	A/A	G	A
Intron 7	G >A	IVS7 +7G>A	Proband	25	19	7	0.68	0.32
			Control	4	9	2	0.57	0.43
				G/G	G/C	C/C	G	C
Intron 7	**G >C**	**IVS7+65G>C**	Proband	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00
				A/A	A/G	G/G	A	G
Intron 7	**A >G**	**IVS7+68 A>G**	Proband	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00
				G/G	G/A	A/A	G	A
Intron 8	**G >A**	**IVS8-19G>A**	Proband	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00
				N/N	N/M	M/M	N	M
Exon 9	**Insertion 14 nt**	**G554SfsX3 1659_1660insAGTGAGTGAAGCCC**	Proband	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00
				T/T	T/C	C/C	T	C
Intron 9	T >C	IVS9-24T>C	Proband	24	20	7	0.67	0.33
			Control	4	9	2	0.57	0.43

*** Novel variations are indicated in bold.**

Table 7 Genotype and allele frequencies of nucleotide variations identified in the *IPF-1* gene by PCR-SSCP analysis.

Location	Neucleotide change	Designation	Sample	Genotype Frequency			Allele Frequency	
				N/N	N/M	M/M	N	M
Promoter	G deletion	-10delG	Proband	21	30	0	0.71	0.29
			Control	7	8	0	0.73	0.27
				G/G	G/A	A/A	G	A
Enhancer	G >A	-1768G>A	Proband	21	24	6	0.65	0.35
			Control	6	6	3	0.60	0.40

Table 8 Genotype and allele frequencies of nucleotide variations identified in the *HNF-1β* gene by PCR-SSCP analysis.

Location	Neucleotide change	Designation	Sample	Genotype Frequency			Allele Frequency	
				C/C	C/T	T/T	C	T
Exon 1.2	**GAC >GAT**	**D82D**	Proband	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00
				C/C	C/T	T/T	C	T
Intron 8	C >T	IVS8-22C>T	Proband	47	4	0	0.96	0.04
			Control	14	1	0	0.97	0.03

*** Novel variation is indicated in bold.**

Table 9 Genotype and allele frequencies of nucleotide variations identified in the *NeuroD1* gene by PCR-SSCP analysis.

Location	Neucleotide change	Designation	Sample	Genotype Frequency			Allele Frequency	
				G/G	G/A	A/A	G	A
Pro	**G >A**	**-303G>A**	Proband	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00
				A/A	A/G	G/G	A	G
Pro	A >G	36A>G	Proband	35	14	2	0.82	0.18
			Control	10	3	2	0.77	0.23
				G/G	G/A	A/A		
Exon 2.1	GCC >ACC	A45T	Proband	37	14	0	0.86	0.14
			Control	9	5	1	0.77	0.23
				GC/GC	GC/AA	AA/AA	GC	AA
Exon 2.3	**GCT >AAT**	**A322N**	Proband	50	1	0	0.99	0.01
			Control	15	0	0	1.00	0.00

*** Novel variations are indicated in bold.**

(3) Genotyping of possible pathogenic mutations by PCR-RFLP/mismatch PCR-RFLP analysis

The ten nucleotide variations in promoter and coding regions (excluded silent variations) that were not detected in 15 non-diabetic controls, including T139I, R140Q and R312H variants of the *HNF-4α* gene, -194A>G, R327H variants of the *GCK* gene, R203C, P475L, and G554SfsX3 1659_1660insAGTGAGTGAAGCCC variants of the *HNF-1α* gene, -303G>A, A322N variants of the *NeuroD1* gene were genotyped in additional 50 non-diabetic controls by PCR- RFLP whereas R312H variant of *HNF-4α* gene was genotyped by mismatch PCR-RFLP. The primers for PCR-RFLP/mismatch PCR-RFLP are shown in Appendix I, table 37.

The R312H variant of the *HNF-4α* gene, R327H variant of the *GCK* gene, R203C, P475L, and G554SfsX3 1659_1660insAGTGAGTGAAGCCC variants of the *HNF-1α* gene, -303G>A, A322N variants of the *NeuroD1* gene were not identified in additional 50 non-diabetic controls, while the T139I and R140Q variants of the *HNF-4α* gene and -194A>G variant of the *GCK* gene were detected in 1, 2 and 4 non-diabetic controls respectively. The result of additional genotyping are summarized in Table 10

Table 10 Additional genotyping for identification of possible pathogenic mutations in six known MODY genes by PCR-RFLP/mismatch PCR-RFLP analysis.

Gene	Location	Neucleotide change	Designation	Sample	Genotype Frequency			Allele Frequency	
					C/C	C/T	T/T	C	T
HNF-4α	Exon 4	ACT >ATT	T139I	MODY	49	2	0	0.98	0.02
				Control	49	1	0	0.99	0.01
					G/G	G/A	A/A	G	A
	Exon 4	**CGA >CAA**	**R140Q**	MODY	50	1	0	0.99	0.01
				Control	48	2	0	0.98	0.02
					G/G	G/A	A/A	G	A
	Exon 8	**CGT >CAT**	**R312H**	MODY	50	1	0	0.99	0.01
				Control	50	0	0	1.00	0.00
					A/A	A/G	G/G	A	G
GCK	Promoter	A>G	(-194)A>G	Proband	45	6	0	0.94	0.06
				Control	46	4	0	0.96	0.04
					G/G	G/A	A/A	G	A
	Exon 8	**CGC >CAC**	**R327H**	Proband	50	1	0	0.99	0.01
				Control	50	0	0	1.00	0.00
					C/C	C/T	T/T	C	T
HNF-1α	Exon 3	CGT >TGT	R203C	Proband	50	1	0	0.99	0.01
				Control	50	0	0	1.00	0.00
					C/C	C/T	T/T	C	T
	Exon 7	**CCG >CTG**	**P475L**	Proband	50	1	0	0.99	0.01
				Control	50	0	0	1.00	.000
					N/N	N/M	M/M	N	M
	Exon 9	**Insertion 14 nt**	**G554SfsX3 1659_1660insAGTGAGTGAAGCCC**	Proband	50	1	0	0.99	0.01
				Control	50	0	0	1.00	0.00
					G/G	G/A	A/A	G	A
NeuroD1	Promoter	G >A	**-303G>A**	Proband	50	1	0	0.99	0.01
				Control	50	0	0	1.00	0.00
					GC/GC	GC/AA	AA/AA	GC	AA
	Exon 2.3	**GCT >AAT**	**A322N**	Proband	50	1	0	0.99	0.01
				Control	50	0	0	1.00	0.00

*** Novel variations are indicated in bold.**

(4) Analysis of segregation of possible pathogenic mutations with diabetes in the families

The variations that were found only in diabetic patients were subjected for test of family segregation with diabetes as described below.

The R203C mutation of the *HNF-1α* gene was analyzed in family members of proband M43 by direct sequencing. The pedigree of the proband is shown in Figure 8a.

The G554SfsX3 1659_1660insAGTGAGTGAAGCCC of the *HNF-1α* gene was analyzed in family members of proband M27 by heteroduplex analysis. The pedigree of the proband is shown in Figure 8b.

The A322N of the *NeuroD1* gene was analyzed in family members of proband M50 by PCR-RFLP. The pedigree of the proband is shown in Figure 8c.

Family members of other four probands who carried R312H variant of the *HNF-4α* gene, R327H variant of the *GCK* gene, P475L variant of the *HNF-1α* gene, -303G>A variant of the *NeuroD1* gene were not available for analysis.

Table 11 summarizes possible pathogenic mutations that were identified in six known MODY genes.

Table 11 Summary of family studies of possible pathogenic mutations identified in six known MODY genes.

Gene	Location	Nucleotide change	Designation	Family	Linkage to disease
HNF-4α	Exon 8	CGT >CAT	R312H	M19	ND
GCK	**Exon 8**	**CGC >CAC**	**R327H**	**M19**	ND
HNF-1α	Exon 3	CGT >TGT	R203C	M43	Yes
	Exon 7	**CCG >CTG**	**P475L**	**M22**	ND
	Exon 9	**Insertion 14 nt**	**G554SfsX3 1659_1660insAGTGAGTGAAGCCC**	**M27**	Yes
NeuroD1	**Promoter**	**G >A**	**-303**	**M36**	ND
	Exon 2.3	**GCT >AAT**	**A322N**	**M50**	No

*** Novel variations are indicated in bold.**

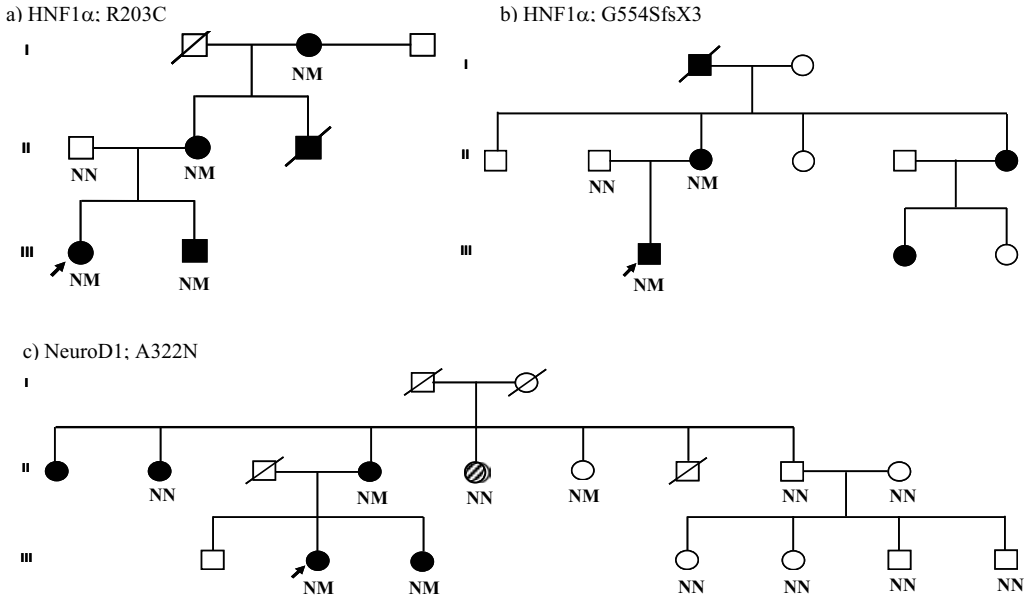


Figure 8 (a) Pedigree of family whose proband M43 carries the *HNF-1α* mutation (R203C)**.** (b) Pedigree of family whose proband M27 carries the *HNF-1α* mutation (G554SfsX3 1659_1660insAGTGAGTGAAGCCC). (c) Pedigree of family whose proband M50 carries the *NeuroD1* mutation (A322N). Symbols are indicated as: ○ and □, normal fasting glucose; ⊘ , impaired fasting glucose; ● and ■, diabetes. Genotypes indicated under symbols are: NN, normal homozygote; NM, heterozygote.

(5) Multiple alignment of possible pathogenic mutations identified in six known MODY genes

Alignment of HNF-4α protein sequence from nine species (human, chimpanzee, dog, mouse, rat, chicken, drosophila, mosquito and worm), GCK protein sequence from four species (human, chimpanzee, mouse and rat), HNF-1α protein sequence from five species (human, dog, mouse, rat and chicken) and NeuroD1 protein sequence from seven species (including human, chimpanzee, dogs, mouse, rat, chicken and worm) were performed by BioEdit Sequence Alignment Editor Version 7.0.1. Arginine at position 312 of the HNF-4α, arginine at position 327 of the GCK, arginine at position 203 and proline at position 475 of the HNF-1α, alanine at position 322 of the NeuroD1 were conserved in all studied species. The results were summarized in Figures 9a-9e.

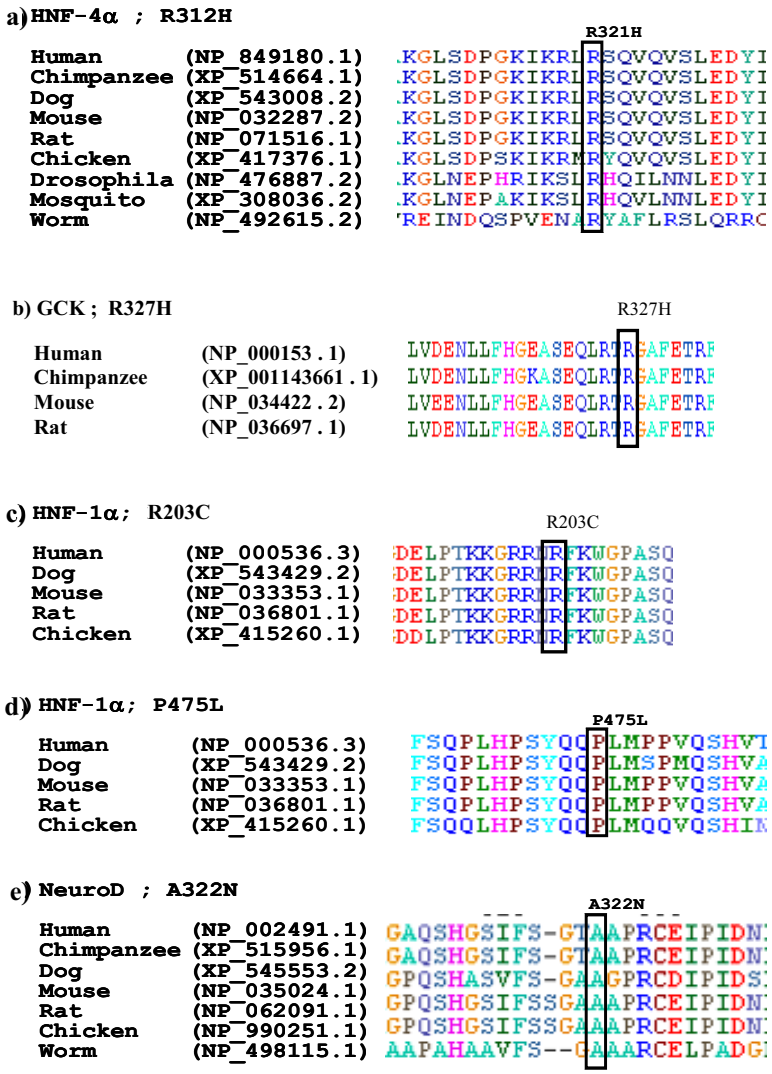


Figure 9 Multiple sequence alignment of six known MODY proteins from different species. Amino acid sequences around variations are shown. The arginine residue at codon 312 of HNF-4α (a), the arginine residue at codon 327 of GCK (b), the arginine residue at codon 203(c) and codon 475(d) of HNF-1α and the alanine at codon 322 of NeuroD1(e) are conserved among different species. The sequences of protein of different species were obtained from GenBank database. ClustalW multiple alignment was operated by BioEdit Sequence Alignment Editor Version 7.0.1.

1.2 Studies of new candidate genes

(1) Screening for nucleotide variations by PCR-SSCP and direct sequencing

The coding regions and intron-exon boundaries of the *Pax4*, *Nkx6.1*, and *Nkx2.2* genes were screened for variations in 46 MODY probands without mutations in known MODY genes and in 74 non-diabetic controls by PCR-SSCP followed by direct sequencing .

A total of fifteen nucleotide variations (eight in the *Pax4* gene, four in the *Nkx6.1* gene, and three in the *Nkx2.2* gene) were identified. The novel variations are presented in Figures 10-12.

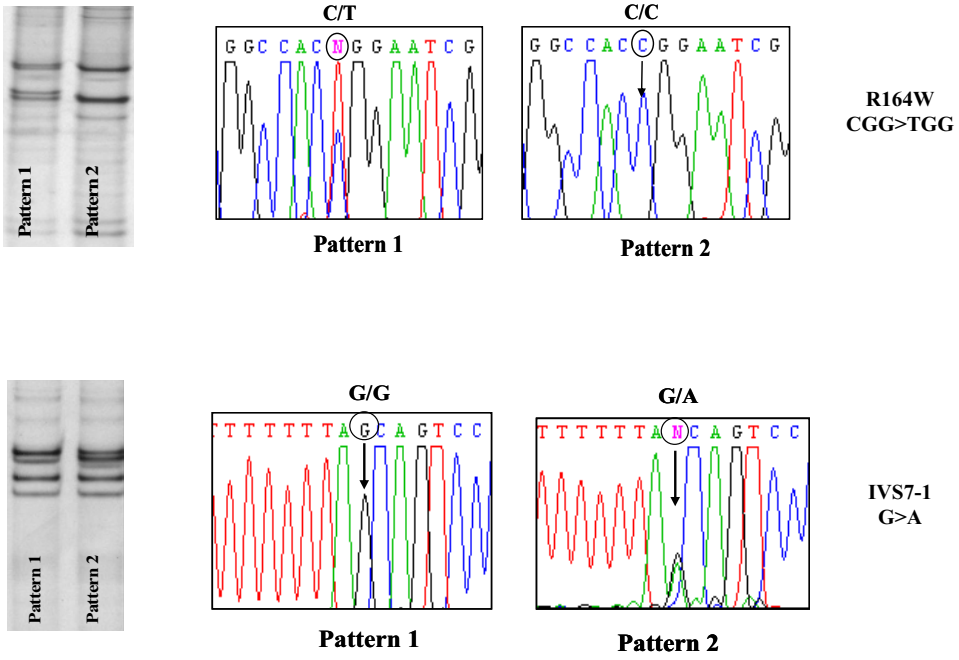


Figure 10 SSCP analysis and chromatograms of DNA sequencing results presenting novel variations identified in *Pax4* genes.

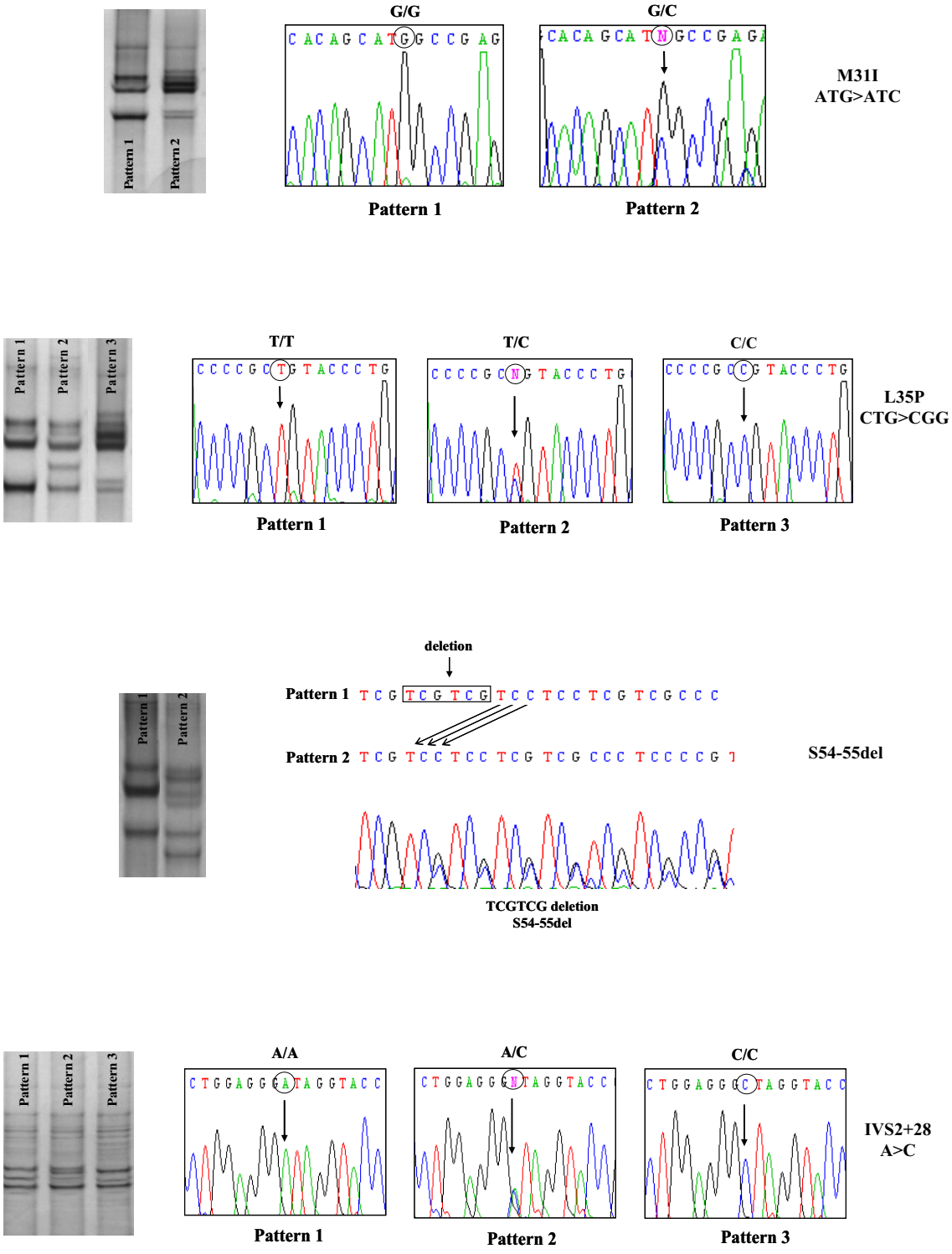


Figure 11 SSCP analysis and chromatograms of DNA sequencing results presenting novel variations identified in *Nkx6.1* genes.

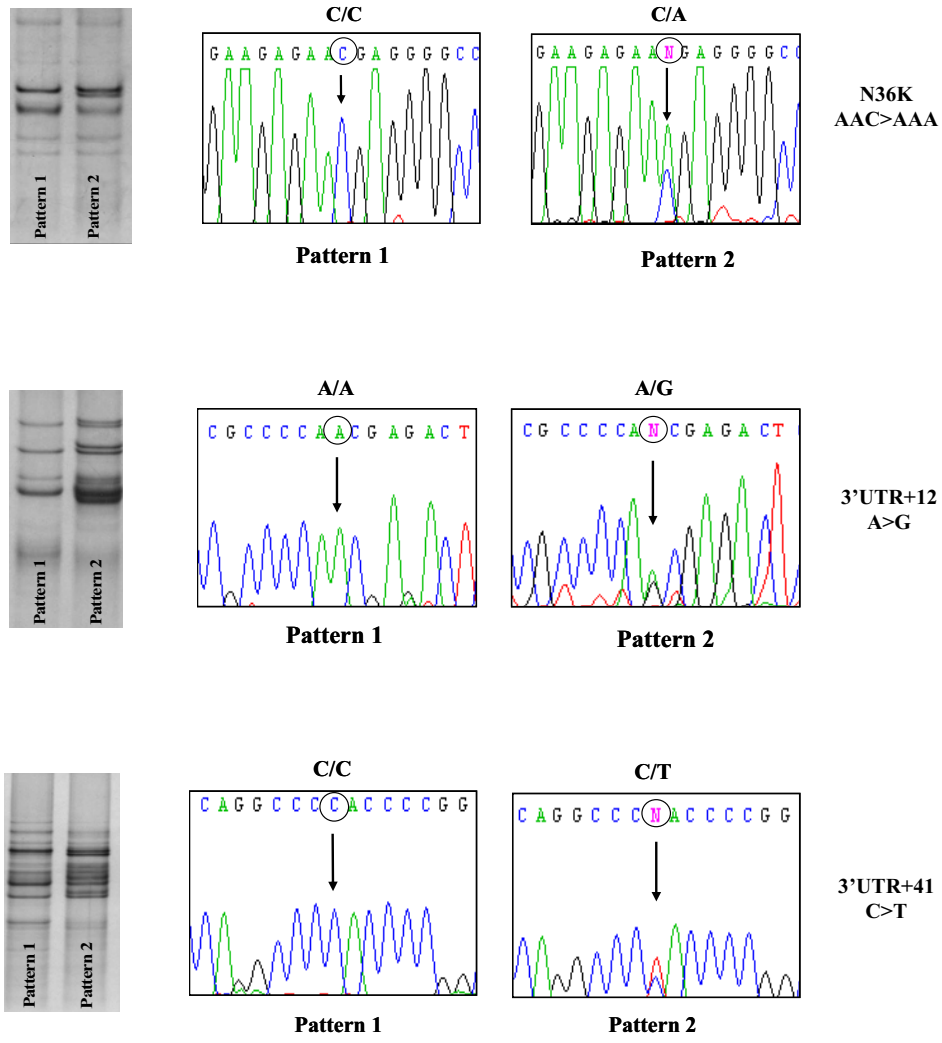


Figure 12 SSCP analysis and chromatograms of DNA sequencing results presenting novel variations identified in *Nkx2.2* genes.

(2) Genotypes and allele frequencies of nucleotide variations identified in new candidate genes

A total of fifteen nucleotide variations (eight in the *Pax4* gene, four in the *Nkx6.1* gene, and three in the *Nkx2.2* gene) were identified and genotyping in 74 non-diabetic controls by PCR-SSCP analysis. The frequencies of these nucleotide variations between MODY patients and non-diabetic controls are shown in Tables 12-14. The R31Q, R164W, IVS7-1G→A variants of the *Pax4* gene, M31I variant of the *Nkx6.1* gene, and 3' UTR+12A→G variant of the *Nkx2.2* gene were not detected in 74 non-diabetic controls, while Q173Q, R183C, R192S variants of the *Pax4* gene and N36K variant of the *Nkx2.2* gene were detected in 1, 1, 2 and 4 non-diabetic controls respectively. By using statistical analysis, R192H variant of the *Pax4* gene showed high frequencies with statistically significant difference between both groups of subjects ($p<0.01$), while P321H variant of the *Pax4* gene and IVS2+28A→C variant of the *Nkx6.1* gene showed high frequencies with no statistically significant difference ($p>0.05$).

Table 12 Genotype and allele frequencies of nucleotide variations identified in the *Pax4* gene by PCR-SSCP analysis.

Location	Nucleotide change	Designation	sample	Genotype frequency			Allele frequency	
				G/G	G/A	A/A	G	A
Exon1	CGG → CAG	R31Q	MODY	45	1	0	0.99	0.01
			Control	74	0	0	1.00	0.00
				C/C	C/T	T/T	C	T
Exon4	**CGG → TGG**	**R164W**	MODY	45	1	0	0.99	0.01
			Control	74	0	0	1.00	0.00
				A/A	A/G	G/G	A	G
	CAA → CAG	Q173Q	MODY	44	2	0	0.98	0.02
			Control	73	1	0	0.99	0.01
				C/C	C/T	T/T	C	T
Exon5	CGT → TGT	R183C	MODY	45	1	0	0.99	0.01
			Control	73	1	0	0.99	0.01
				C/C	C/A	A/A	C	A
Exon5	CGT → AGT	R192S	MODY	45	1	0	0.99	0.01
			Control	72	2	0	0.98	0.02
				G/G	G/A	A/A	G	A
Exon5	CGT → CAT	R192H	MODY	31	13	2	0.82	0.18
			Control	65	9	0	0.94	0.06
				G/G	G/A	A/A	G	A
Intron7	**G → A**	**IVS7-1 G→A**	MODY	45	1	0	0.99	0.01
			Control	74	0	0	1.00	0.00
				C/C	C/A	A/A	C	A
Exon9	CCC → CAC	P321H	MODY	5	23	18	0.36	0.64
			Control	10	39	25	0.40	0.60

*** Novel variations are indicated in bold.**

Table 13 Genotype and allele frequencies of nucleotide variations identified in the *Nkx6.1* gene by PCR-SSCP analysis.

Location	Nucleotide change	Designation	sample	Genotype frequency			Allele frequency	
				G/G	G/C	C/C	G	C
Exon1	**ATG → ATC**	**M31I**	MODY	45	1	0	0.99	0.01
			Control	74	0	0	1.00	0.00
				T/T	T/C	C/C	T	C
Exon1	**CTG → CCG**	**L35P**	MODY	45	1	0	0.99	0.01
			Control	73	0	1	0.98	0.02
				A/A	A/G	G/G	A	G
Exon1	**TCGTCG deletion**	**S54-55del**	MODY	46	0	0	1.00	0.00
			Control	73	1	0	0.99	0.01
				C/C	C/T	T/T	C	T
Intron2	**A → C**	**IVS2+28 A→C**	MODY	18	23	5	0.64	0.36
			Control	30	37	7	0.66	0.34

*** Novel variations are indicated in bold.**

Table 14 Genotype and allele frequencies of nucleotide variations identified in the *Nkx2.2* gene by PCR-SSCP analysis.

Location	Nucleotide change	Designation	sample	Genotype frequency			Allele frequency	
				C/C	C/A	A/A	C	A
Exon1	**AAC → AAA**	**N36K**	MODY	45	1	0	0.99	0.01
			Control	70	4	0	0.97	0.03
				A/A	A/G	G/G	A	G
3' UTR	**A → G**	**3'UTR+12 A → G**	MODY	45	1	0	0.99	0.01
			Control	74	0	0	1.00	0.00
				C/C	C/T	T/T	C	T
3' UTR	**C → T**	**3'UTR+41 C → T**	MODY	46	0	0	1.00	0.00
			Control	73	1	0	0.99	0.01

*** Novel variations are indicated in bold.**

(3) Additional genotyping of possible pathogenic mutation of *Pax4* by PCR-RFLP analysis

The R31Q, R164W and IVS7-1G→A variants of the *Pax4* gene were genotyped in additional 270 non-diabetic controls, and the R192H variant in additional 268 non-diabetic controls by PCR-RFLP analysis. The primers for PCR-RFLP are shown in Appendix I, Table 38. The R164W and IVS7-1G→A were not detected in additional 270 non-diabetic controls, while the R31Q was detected in 3 non-diabetic controls. The R192H variant showed high frequencies in the 46 MODY probands compared to 268 additional non-diabetic controls that added to the control group ($p<0.0001$). The results are showed in Table 15.

Table 15 Genotype and allele frequencies of possible mutations of new candidate genes by PCR-RFLP analysis.

Gene	Variation	Group	Genotype frequency			Allele frequency		*P*-value
Pax4	R31Q		GG	GA	AA	G	A	
		MODY	45	1	0	0.99	0.01	NS
		Controls	267	3	0	0.99	0.01	
	R164W		CC	CT	TT	C	T	
		MODY	45	1	0	0.99	0.01	NS
		Controls	270	0	0	1.00	0.00	
	R192H		GG	GA	AA	G	A	
		MODY	31	13	2	0.82	0.18	<0.00001
		Controls	236	30	2	0.94	0.06	
	IVS7-1G>A		GG	GA	AA	G	A	
		MODY	45	1	0	0.99	0.01	NS
		Controls	270	0	0	1.00	0.00	

(4) Analysis of segregation of *Pax4* R164W with diabetes in family

The R164W mutation of the *Pax4* gene was analyzed in family members of proband by direct sequencing. The pedigree of the probands is shown in Figure 13. Both her 51-year-old father and her 28-year-old sister who had type 2 diabetes, and her 13-year-old brother with impaired glucose tolerance were heterozygous for this mutation. However, this mutation was not present in other two sisters (27 and 21-year-old) who also had impaired glucose tolerance. Family members of probands with IVS7-1G→A were not available for analysis.

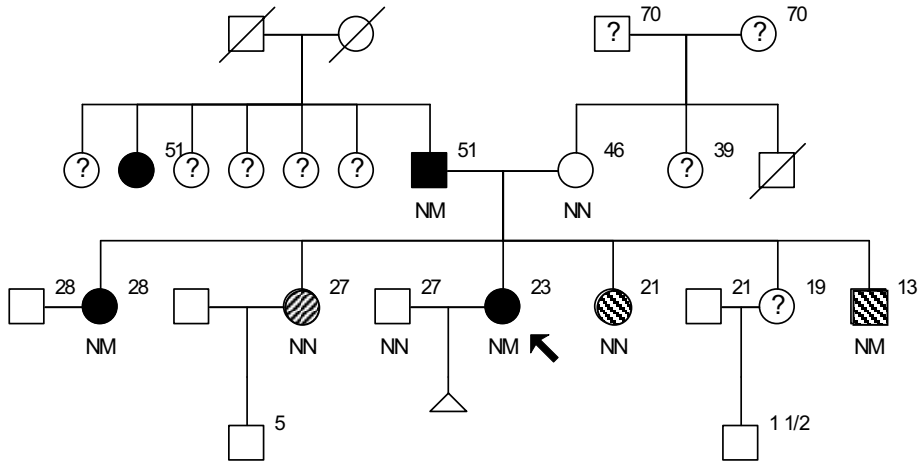


Figure 13 Pedigree of family whose proband carries the *Pax4* mutation (R164W)**.** Symbols are indicated as: ○and □, normal fasting glucose; ▨ and ▨ impaired fasting glucose; ● and ■, diabetes; ○ and □ with "?", unknown. Genotypes are indicated as: NN, normal homozygote; NM, heterozygote). The arrow indicates the proband. Ages (in years) are shown on the upper left side of each symbol.

(5) Multiple alignment of possible pathogenic mutation identified in new candidate genes

Pax4 protein sequence from six species, including human, chimpanzee, mouse, rat, chicken, fruit fly and mosquito were aligned by BioEdit Sequence Alignment Editor Version 7.0.1. In Figure 14(a,b) arginines position 31 and 164 are conserved in all compared species.

Nkx6.1 protein sequence from six species, including human, mouse, rat, hamster, zebra fish and worm were aligned by BioEdit Sequence Alignment Editor Version 7.0.1. In Figure 14c methionine at position 31 is conserved in human, mouse, rat, hamster and zebra fish, except for round worm.

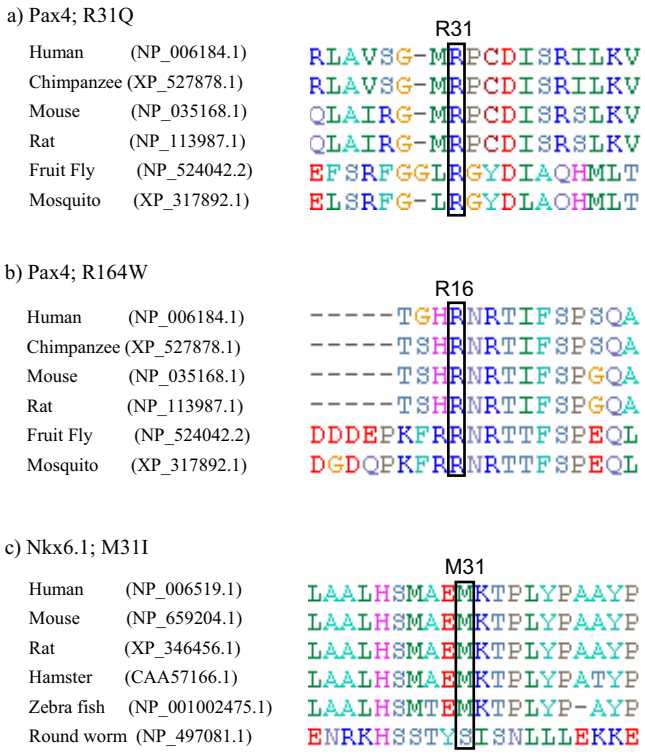


Figure 14 Multiple sequence alignment of the Pax4 and Nkx6.1 proteins from different species. Amino acid sequences around R31 and R164 of Pax4 and M31 of Nkx6.1 are shown. The arginines residues at codon 31 (a) and codon 164 (b) of Pax4 and the methionine at codon 31 of Nkx6.1(c) are conserved in most species. The sequences of Pax4 and Nkx6.1 proteins of different species were obtained from GenBank database. ClustalW multiple alignment was operated by BioEdit Sequence Alignment Editor Version 7.0.1.

2. Functional studies

Pax4 represses the activity of the insulin and glucagons promoters (59, 83). To assess whether the R164W variant affects such function, we transiently transfected MIN 6 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% (Figure 15a). 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 (Figure 15b). 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.

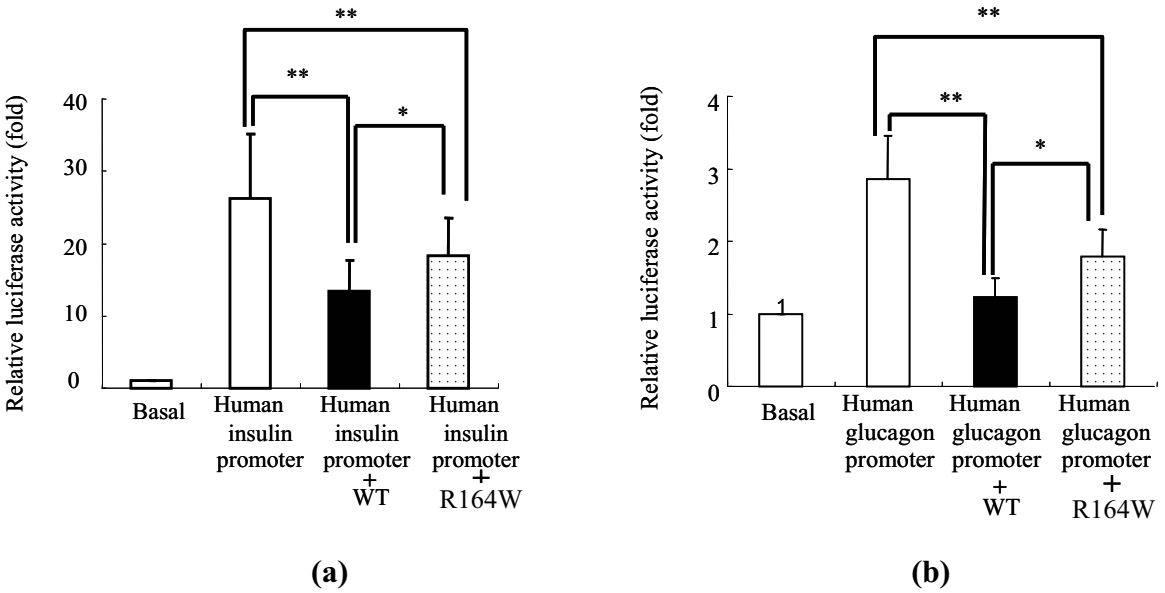


Figure 15 Effect of *Pax4* mutation on luciferase activity in MIN6 and α-TC1.6 cells. MIN 6 and α-TC1.6 cells were transfected with 0.5 mg of human wild-type Pax4 and R164W mutant and 0.5 mg of human insulin (a) and glucagon (b) promoter reporter genes, respectively, together with 10 ng of pRL-SV40 internal control vector. Data are expressed as mean ± SD, N = 6, three times. *$p < 0.01$ and ** $p < 0.001$.

3. Screening of a novel candidate gene using data from genome-wide scan

After screening for mutations in six known MODY genes (*HNF-4α, GCK, HNF-1α, IPF-1, HNF-1β* and *NeuroD1* genes) and new candidate genes (*Pax4, Nkx6.1* and *Nkx2.2* genes), we chose eight families that did not have mutation in these genes for mutation screening of a novel candidate gene, *BLK*. A total of *BLK* gene region including exons, exon-intron junction, 5'and 3' flanking regions and conserved regions were screened for mutations by bi-directional sequencing**.** The results were summarized in Table 16. All SNPs that were found in Thai patients are located in intron and not at spice site. SNPs that were found in Caucasian samples were not found in Thai samples.

Table 16 SNPs of *BLK* gene identified in Thai MODY patients from selected 8 families.

Family number	Sample number	SNPs number	Location
8	8_7	rs936550, rs4841556	Intron 6
8	8_15	rs936550, rs4841556	Intron 6
20	20_21	rs936550, rs4841556	Intron 6
20	20_22	rs4841556	Intron 6
21	21_6	rs4841556	Intron 6
21	21_9	rs4841556	Intron 6
26	26_4	rs4841556	Intron 6
26	26_7	rs4841556	Intron 6
39	39_4	rs4841556	Intron 6
39	39_13	rs4841556	Intron 6
46	46_3	rs11250146	Intron 6
46	46_12	rs2255108	Intron 6
48	48_3	rs11776201	Intron 12
48	48_4	rs11776201	Intron 12
50	50_11	rs11250148	Intron 11
50	50_12	rs11250148	Intron 11

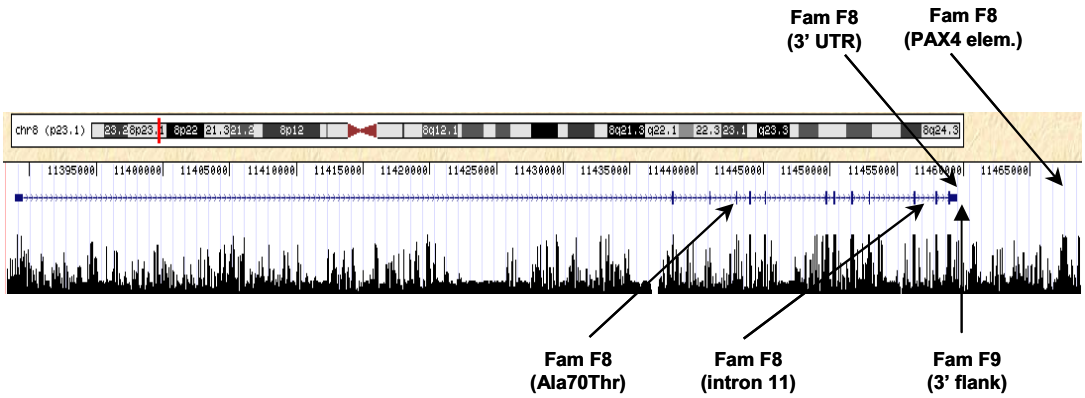


Figure 16 Locations of SNPs of *BLK* gene.

DISCUSSION

Fifty one unrelated Thai early onset type 2 diabetic probands which twenty one of them were in concordance with classic MODY criteria were screened for sequence variations of all known MODY genes. By using PCR-SSCP analysis, seven mutations that might be potentially classified as pathogenic mutations were identified (Table 11). Five of them have not been reported previously. These possible pathogenic mutations are either non-synonymous mutations or mutation located in regulatory region, which were absent in 135 chromosomes of non-diabetic control subjects.

The R312H of the *HNF-4α*/MODY1 gene was found in one proband, whose family members could not be recruited to test segregation with diabetes. The missense mutation R312H in exon 8 was previously identified in two MODY families of Caucasian origin, by Ellard et al (unpublished data). HNF-4α is a nuclear receptor that essential for development in organisms ranging from insects to mammals, and regulates many essential genes related to nutrient transport and metabolism (84). Arginine at position 312 of HNF-4α is conserved across 9 species, from worm to human (Figure 9a). This amino acid residue is located in helix 9 of ligand-binding domain (LBD) of human HNF-4α, which involves in not only ligand binding but also protein dimerization (85), alteration of polar positive charged arginine to polar uncharged histidine could therefore potentially affect many aspects of receptor function, such as DNA binding protein stability, ligand binding and interaction with co-regulatory molecules.

One novel missense mutation, R327H was found in exon 8 of *GCK*/MODY2 gene. In our study, patient carried R327H exhibited clinical characteristics of MODY2 including the presence of mild hyperglycemia (135 mg/dl) and upper normal range of HbA1C level (6.7%), and diabetes successfully managed by diet alone. The effect of this mutation is difficult to predict, base on its location or the nature of amino acid change (86-88). Therefore, both enzymatic activity of the mutant protein and the affinity of mutant enzyme to glucose should be further investigated.

Sequence variations of *HNF-1α*/MODY3 gene were more common among Thai MODY patients as compared to those of other known MODY genes. One of the three identified mutations, R203C in exon 3, was previously reported in 2 families, one from Denmark (89) and another from Japan (41). However, the familial segregation study has not been performed yet. Herein, for the first time, we demonstrated the segregation of this

heterozygous mutation with diabetes within family. Arginine residue at position 203 is conserved among 5 species, including chicken, rat, mouse, dog, and human and located within DNA binding domain (DBD), alteration of arginine to cystein should therefore affect transactivation activity. Nevertheless, functional analysis of mutant protein (41) revealed biphasic activity, in which decreasing of activity was observed at low DNA concentration, whereas gaining of function was performed by high level of transfected mutant DNA. In addition, mutant protein showed weak but positive signal of nuclear localizing defects, which could be involved, at least in part, in biphasic effect on transactivation activity. Thus, the precise mechanism of R203C in contribution to etiology of MODY3 should be further elucidated. The other two identified mutations, P475L in exon 7 and Ser553fsX3 in exon 9, were novel. Both of them belong to transctivation domain. Proline at position 475 is conserved across 5 species. Substitution of a distinctive cyclic structure of proline residue, whose secondary imino group is usually held in a rigid conformation that reduces the structural flexibility of polypeptide region, with leucine might affect conformation of protein. Fourteen nucleotide insertions at codon 553 result in addition of 2 amino acids followed by stop codon. The truncated mutant polypeptide is 77 amino acids shorter than wild-type. The influence of Ser553fsX3 on intrinsic transctivation of HNF-1α is currently under investigation.

Two novel mutations, -303G>A and A322N, of *NeuroD1*/MODY6 gene were identified in this study. Nucleotide change at position -303 is located in highly conserved promoter region of human and mouse *NeuroD1* gene, which was identified as necessary region for basal transcriptional activation (90). Therefore, nucleotide alteration in such promoter region might affect expression level of this transcription factor. Another mutation, A322N, is located in a region that associated with the co-activators CBP and p300. Alteration from hydrophobic and non-polar of alanine residue to be polar residue of asparagine might affect stability of protein, and might influence on association with co-activator proteins. Interestingly, two probands carried -303G>A and A322N exhibited more hyperglycemia (271 and 320 mg/dl, repectively), compared to probands carried mutations in other MODY genes. However, segregation study could not established association of A332N with diabetes in the family (Figure 8c). Thus, the roles of this mutation in glucose homeostasis remain to be explored.

Identification of these possible pathogenic mutations in known MODY genes were accounted for a small proportion of classic MODY patients being studies. The genetic

defects that might contribute to etiology of diabetes in other 44 probands, is an excellent resource for discovering as yet unidentified MODY genes.

The study of new candidate genes, including *Pax4*, *Nkx6.1*, and *Nkx2.2* genes. All exons and conserved sequences located in the acceptor and donor splice sites were screened for mutations and polymorphisms in 44 MODY probands of families. The PCR-SSCP technique was used for such screening. The analysis of the sequence of the *Pax4* gene revealed eight nucleotide variations including three possible pathogenic mutations (R31Q, R164W, and IVS7-1G→A). These mutations were identified in three different probands. The novel missense mutation, R164W, is located within the homeodomain, another DNA binding domain of Pax4, which interacts by cooperative dimerization with palindromic binding sites consisting of inverted TAAT repeats (57). It is very likely to be a pathogenic mutation, not a rare polymorphism because *(i)* the mutation results in a replacement of arginine (a charged polar amino acid) by tryptophan (a nonpolar amino acid); *(ii)* the arginine at residue 164 may should an important role in the function of Pax4 as it is conserved in many species including human, chimpanzee, mouse, rat, fruit fly, and mosquito (Figure 14b); *(iii)* the mutation was not present in the 74 non-diabetic controls and in additional 270 non-diabetic subjects (688 normal chromosomes); *(iv)* the mutation cosegregated with diabetes in the family as it is inherited from an affected father to three affected offspring although cosegregation was not complete due to two members who were not a gene carrier have developed impaired glucose tolerance. Phenotypic variability in these two individuals is most likely due to phenocopies (the same phenotype being a result of environmental causes) or the consequence of interactions with other genes (modifier genes) and finally comparison of the transcription activity between wild-type and this mutant was done by luciferase reporter assay in both pancreatic α- and β-cell lines. *In vitro* expression studies demonstrated that the transactivation potential of the wild-type Pax4 protein still preserved its repressing function on the human insulin and glucagon promoter both in MIN6 and α-TC1.6 cells. In contrast, the recombinant R164W mutant protein show diminished ability to repress gene expression as shown in increasing transactivation activity of mutant protein to driven insulin and glucagon expression. These differences between wild-type and mutant protein function were not due to differences in transfection efficiencies on the expression of the transfected constructs (data not shown). This report provides the strong evidence that mutation of *Pax4*, R164W, show loss-of-function to repress its target gene *in vitro* and might perturb pancreatic β-cell function *in vivo*, therefore contributing to the development of MODY.

The novel splice site mutation, IVS7-1G→A, that disrupt the conserved AG dinucleotide of the splice acceptor site of intron 7 may result in abnormal mRNA processing such as exon skipping, intron retention, or usage of other splice acceptor splice site, which can be analyzed by RT-PCR using mRNA from human islet cells or lymphocytes if it has illegitimate transcription. It would be expected to generate the mutant truncated protein. Because family members of the proband who carried this mutation are not available for this study, the significance of this mutation in the contribution to diabetes in the family is not clear.

The missense mutation, R31Q, has previously been reported in Japanese patient with type 2 diabetic. However, it is thought to not be a disease-associated single nucleotide polymorphism (SNP) (67). This mutation is located within the paired domain, which is important as a DNA binding site, independently or in connection with the homeodomain. The mutation changes arginine (a charged polar amino acid) to glutamine (an uncharged polar amino acid). The arginine at residue 31 is conserved in many species including human, chimpanzee, mouse rat, fruit fly, and mosquito (Figure 14a). Like the IVS7-1G→A mutation, family members of the proband that carried the R31Q mutation are not available for this study. The other five previously reported single nucleotide polymorphisms (SNPs), Q173Q, R183C, R192S, R192H, and P321H (67), were also identified. Among these SNPs, the mutant allele frequency of the R192H polymorphism was significantly higher in the MODY group than in the non-diabetic controls group (minor allele frequency, MAF = 0.196 vs 0.064, $p<0.0001$). Thus, it would be possible that this polymorphism might increase risks for the development of diabetes in these MODY patients.

In summary of the *Pax4* gene, three possible pathogenic mutations, R31Q, R164W, and IVS7-1G→A, were identified in three different MODY probands. The R164W mutation showed incomplete cosegregation with diabetes in the family, suggesting that this mutation may be the cause of diabetes in this isolated family. Unlike the clearly defined mode of inheritance found in the monogenic forms of diabetes such as MODY, type 2 diabetes is likely to result from oligo- or polygenic inheritance. It could possibly be that a hypomorphic mutation in the MODY gene, such as R192H, may predispose to type 2 diabetes. This hypothesis needs to be tested in relatively large samples of type 2 diabetes using genetic case-control association study.

The analysis of the sequence of the *Nkx6.1* gene revealed four nucleotide variations including, missense M31I. The mutation, which is located within the

unidentified region, alters the side chain of a nonpolar amino acid by replacing sulphur group (methionine) to aliphatic group (isoleucine). This variation may play an important role in the function of Nkx6.1 as the methionine at residue 31 is conserved in many species including human, mouse, rat, hamster, and zebra fish. However, family members of the proband who carried this mutation are not available for this study, the significance of this mutation in the contribution to diabetes in the family is not clear. In addition to variation, the IVS2+28A→C polymorphism that is located within intronic region showed high frequency with no statistically significant difference between MODY group and non-diabetic controls group (p>0.05). A rare variation, L35P, was found in one heterozygous proband and in one homozygous non-diabetic control. An inframe deletion of serine at codon 54-55 of long serine stretches in the NH_2 terminal part of Nkx6.1 (S54-55del) was also found in one non-diabetic control as heterozygous state. To date, mutations in the *Nkx6.1* gene has not yet been analyzed in any ethnic populations. This is the first report of genetic variations in this gene.

The analysis of the sequence of the *Nkx2.2* gene revealed three nucleotide variations including, 3' UTR+12A→G. This mutation is located within the 3' untranslated region (UTR), which changes A to G at position 12 nucleotides downstream from the translation termination codon (TGA). The regulatory sequences in UTRs form regions with a high content of secondary structure (hairpins), which might serve as regulatory elements as they are recognized and bounded by regulatory proteins that protect mRNA from degradation by RNases (91). Thus, it would be assumed that 3' UTR+12A→G mutation may affect the stability of Nkx2.2 mRNA through alteration of the binding of proteins that regulate the newly synthesized RNA. Because family members of the proband who carried this mutation are not available for this study, the significance of this mutation in the contribution to diabetes in the family is not clear. In addition to a rare variant, N36K was found in one proband and four non-diabetic controls, and a rare variant, 3' UTR+41C→T, which changes C to T at position 41 nucleotides downstream from the translation termination codon (TGA) was found only in one non-diabetic control.

A total of thirty seven nucleotide variations of six known MODY genes (*HNF-4α, GCK, HNF-1α, IPF-1, HNF-1β* and *NeuroD1* genes) and fifteen of new candidate genes (*Pax4, Nkx6.1* and *Nkx2.2* gene) were identified in this study. However, PCR-SSCP technique can not detect all variations in the genes because sensitivity of this technique in a single run is generally about 80% if the fragments to be analyzed are shorter than 300 bp (92). The combination of SSCP and HA on a single gel may partially solve this problem

because of the fact that the detection modes of these two methods are different and thus the sensitivity of detection can be up to 90% in the fragments from 300-400 bp (92). In addition, the other screening methods such as DHPLC, DGGE, and ddF are suitable for searching the undetected variations in the genes as their sensitivity is higher than that of PCR-SSCP method (93).

Linkage analysis from the genome-wide scan provides evidence that certain chromosomal regions may linked to disease of interested and studying these particular regions may yield causative genes. However, genetic heterogeneity of MODY has made this approach not straightforward. The fact that we found no sequence variations of the *BLK* gene associated with diabetes in our MODY families supported this concept. This has emphasized the need of conducting genome-wide scan in our own patients that may reveal particular chromosomal region that associates with diabetes. Advance in genomic technology such as microarray genotyping may enable us to explore into this unknown territory.

CONCLUSIONS

Genetic variabilities of known MODY genes do not play important role in causing diabetes in early onset type 2 diabetes mellitus in Thai patients being studied. However, variations of *HNF-1α*/MODY3 gene are more common compared to other 5 genes. Functional study of impact of these mutations on protein function in appropriate systems will elucidate the role of these genes in glucose homeostasis as well as their impact on clinical characteristics of patients. Moreover, MODY families with unidentified contributing genes are valuable resource for discovering news MODY genes as demonstrated in *Pax4* gene. By exploring molecular genetics of these special forms of diabetes has emphasized the important role of genetic heterogeneity in association with certain disease. These has been clearly demonstrated in screening of novel candidate (*BLK*) gene using data from genome-wide scan in Caucasians. These families are precious resource for identifying new MODY gene in the era of rapid advance in genomic technology such as microarray genotyping.

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APPENDIX I

1. Instruments

Applied Biosystems 377 DNA sequencer, Applied Biosystems, Foster, USA.

Gel documentation system, UVItec, Cambridge, UK.

GeneAmp PCR system 2400, Applied Biosystems, Foster, USA.

GeneAmp PCR system 9600, Applied Biosystems, Foster, USA.

Horizontal gel electrophoresis apparatus, ATTO corporation, Tokyo, Japan.

Mini gel electrophoresis apparatus, Labnet international Inc., Edision, USA.

Power supplies, E143, 200-400 V, CONSORT, Turnhout, Belgium.

UV-transilluminator, FOTODYNE incorporated, Hartland, USA.

UV-visible spectrophotometer, Shimadzu UV-160A, Kyoto, Japan.

2. Enzymes

2.1 Restriction enzymes

AvaIII, Fermentas Inc.,Hanover, MD, USA

Bsp119I, Fermentas Inc.,Hanover, MD, USA

BsrI, Fermentas Inc.,Hanover, MD, USA

*EcoR*V, New England Biolabs. Beverly, USA.

FnuDII, Fermentas Inc.,Hanover, MD, USA

HaeIII, New England Biolabs. Beverly, USA.

MseI, Fermentas Inc.,Hanover, MD, USA

MspAII, Fermentas Inc.,Hanover, MD, USA

MwoI, Fermentas Inc.,Hanover, MD, USA

NheI, Fermentas Inc.,Hanover, MD, USA

NlaIII, New England Biolabs. Beverly, USA

NlaVI, Fermentas Inc.,Hanover, MD, USA

PvuII, New England Biolabs. Beverly, USA.

TaqI, Fermentas Inc.,Hanover, MD, USA

2.2 DNA polymerase

Immolase DNA polymerase, Bioline, MA, USA.

Taq DNA polymerase, Promega, Madison, USA.

Platinum *Pfx* DNA polymerase, Invitrogen, Carlsbad, CA, USA.

Pfu DNA polymerase Promega, Madison, USA.

2.3 Other enzyme

Proteinase K, Promega, Madison, USA.

3. DNA markers

100 bp DNA Ladder, New England, BioLabs, USA.

*Hae*III digested ϕx 174 DNA, Promega, Madison, USA.

Low molecular weight DNA ladder, New England, Biolabs, USA.

4. Primers

4.1 Primers for amplification of six known MODY genes.

Table 17 PCR primers for amplification of *HNF-4α* gene.

Fragment	Primer	Nucleotide sequence (5'→3')	No. of nucleotides	Tm (°C)	Product size (bp)
Promoter	HNF4A pro 1-L HNF4A pro 1-R	CCAAGGTCCCAGTTAGTAGACGGTAG GGCCCCAGATGCCAATCTTC	26 20	70 64	320
Promoter	HNF4A pro 2-L HNF4A pro 2-R	CCCAGGGGAACCGGGAAACT GCCCTAACTCACCGATGTTCA	20 21	66 64	340
Promoter	HNF4A pro 3-L HNF4A pro 3-R	GGGTGAGTCAAGGGTCAAAT CCTCTCTGCCTTCCTTTCAA	20 20	60 60	294
Ex1A	HNF4A Ex1A L HNF4A Ex1A R	GCCAAGACTCCCAGCAGATC ACCCAGGCACACCTGCCCCC	20 20	62 70	318
Ex1B	HNF4A Ex1B L HNF4A Ex1B R	GGACTCTGTTGTTGCCACTCAC GATGGGGCTCTTCCCTCCAG	22 20	68 66	210
Ex1C	HNF4A Ex1C L HNF4A Ex1C R	GGCATTCTGGGTGAAGGGAG CGTGCTGGGCTGAATCGCTG	20 20	64 66	179
Ex2	HNF4A Ex2 L HNF4A Ex2 R	TCTGTTCTTCCTGAAGCCTCACTC CAAGTGTGCCCATTTCCCAG	24 20	72 62	260
Ex3	HNF4A Ex3 L HNF4A Ex3 R	CCTAGTTCTGTCCTAAGAGG GTCATAAAGTGTGGCTACAG	20 20	60 58	253
Ex4	HNF4A Ex4 L HNF4A Ex4 R	CAGACACCCCCACCCCCTAC GAATGGAGGTGGAGGAGGTGAG	20 22	68 70	245
Ex5	HNF4A Ex5 L HNF4A Ex5 R	CGGACATCTCCAGCATTTTC CACTGCCCACTACTGCCCAC	20 21	60 62	234
Ex6	HNF4A Ex6 L HNF4A Ex6 R	GCGTCACTGAGTTGGCTACGG GCTAGGCATACCCTCCCTGGAG	22 22	68 72	210
Ex7	HNF4A Ex7 L HNF4A Ex7 R	CCCACAGGCACCAGCTATCTTG AGCGTTCTGGAGAGAGAGTCAGG	23 23	70 72	306
Ex8	HNF4A Ex8 L HNF4A Ex8 R	TTGCCCACCCTCTTCCATTG TCCCCACTCCAACCCCGCCC	20 20	62 70	300
Ex9	HNF4A Ex9 L HNF4A Ex9 R	TCTGCATCCCAGACTCTCCATC AGCCCCATCCTCACCCTTTG	22 20	68 64	246
Ex10	HNF4A Ex10 L HNF4A Ex10 R	CATTTACTCCCACAAAGGCT GACCACGTGATCACCAGGTG	20 20	58 64	277

Table 18 PCR primers for amplification of *glucokinase* gene.

Fragment	Primer	Nucleotide sequence (5'→3')	No. of nucleotides	Tm (°C)	Product size (bp)
Promoter	GCK pro 1-L GCK pro 1-R	TCTGGTTCAGATTTCAAGGAGA AAGCAGAGGAAAGGGAGCAG	22 20	62 62	265
Promoter	GCK pro2-L GCK pro 2-R	GGGATGTGAGATGGTCCCAGG CCAGGAGTGGCCTCAGCAGG	21 20	72 68	300
Promoter	GCK pro 3-L GCK pro 3-R	CTAATGACAGGATGGTCAGCCC CTCCTGGTTGTGTTGAGCTGTG	22 22	68 68	292
Promoter	GCK pro 4-L GCK pro 4-R	GGACACTAAGCCCCACAGCTCA CCCCTCAGCCTTCCCCAGTA	22 20	70 66	299
Ex1A	GCK Ex1A L GCK Ex1A R	TCCACTTCAGAAGCCTACTG TCAGATTCTGAGGCTCAAAC	20 20	60 58	195
Ex1B	GCK Ex1B L GCK Ex1B R	GCCCTTTCAGTGCAGAAGCC GCTCTCCCAGTGCAAAGTCCC	20 21	64 68	260
Ex1C	GCK Ex1C L GCK Ex1C R	AAGGACTGTCTCTGTACTGATGGCTC TTCTGAAGGGAGGTGGGAGG	26 20	78 64	236
Ex2	GCK Ex2 L GCK Ex2 R	TGCAGATGCCTGGTGACAGC CACAGCTGCTTCTGGATGAG	20 20	64 62	290
Ex3	GCK Ex3 L GCK Ex3 R	TAATATCCGGGCTCAGTCACC CTGAGATCCTGCATGGCCTTG	20 20	60 62	298
Ex4	GCK Ex4 L GCK Ex4 R	TAGCTTGGCTTGAGGCCGTG TGAAGGCAGAGTTCCTCTGG	20 20	64 62	272
Ex5	GCK Ex5 L GCK Ex5 R	TGCCTCCAGTATATGTTAGCAGCC TGCCAAGAAGCACAGAAGCTG	23 21	70 64	251
Ex6	GCK Ex6 L GCK Ex6 R	TCTCCTTGGCTTCCAGCACTG CAGGCTCTGCTCTGACATCACC	21 22	66 70	268
Ex7	GCK Ex7 L GCK Ex7 R	AGTGCAGCTCTCGCTGACAG CATCTGCCGCTGCACCAGAG	20 20	64 66	286
Ex8	GCK Ex8 L GCK Ex8 R	TGCCTGCTGATGTAATGGAC TGAGACCAAGTCTGCAGTGC	20 20	60 62	262
Ex9	GCK Ex9 L GCK Ex9 R	CACTCAGCGACCGCCCTACC CCCCACTTTACCAGGGAGAGAG	20 22	68 70	306
Ex10	GCK Ex10 L GCK Ex10 R	GTCGACTGCGTGCAGGGCGC TGTGGCATCCTCCCTGCGCT	20 20	70 66	263

Table 19 PCR primers for amplification of *HNF-1α* gene.

Fragment	Primer	Nucleotide sequence (5'➔3')	No. of nucleotides	Tm (°C)	Product size (bp)
Promoter	HNF1A pro 1-L HNF1A pro 1-R	TCCCATCGCAGGCCATAGCTC CCGTCTGCAGCTGGCTCAGTT	21 21	68 68	385
Promoter	HNF1A pro 2-L HNF1A pro 2-R	CCTCTGCCCTTGAGAAGAGC ACTTCAGCCCTGCAAAGTGC	20 20	64 62	300
Ex1	HNF1A Ex1 L HNF1A Ex1 R	GGCAGGCAAACGCAACCCACG GAAGGGGGGCTCGTTAGGAGC	21 21	70 70	483
Ex2	HNF1A Ex2 L HNF1A Ex2 R	CATGCACAGTCCCCACCCTCA CTTCCAGCCCCCACCTATGAG	21 21	68 68	390
Ex3	HNF1A Ex3 L HNF1A Ex3 R	GGGCAAGGTCAGGGGAATGGA CAGCCCAGACCAAACCAGCAC	21 21	68 68	304
Ex4	HNF1A Ex4 L HNF1A Ex4 R	CAGAACCCTCCCCTTCATGCC GGTGACTGCTGTCACTGGGAC	21 21	68 66	397
Ex5	HNF1A Ex5 L HNF1A Ex5 R	GGCAGACAGGCAGCTGGCCTA GCCTCCCTAGGGACTGCTCCA	21 21	68 70	346
Ex6	HNF1A Ex6 L HNF1A Ex6 R	TGGAGCAGTCCCTAGGGAGGC GTTGCCCCATGAGCCTCCCAC	21 21	70 70	322
Ex7	HNF1A Ex7 L HNF1A Ex7 R	GGTCTTGGGCAGGGGTGGGAT CTGCAATGCCTGCCAGGCACC	21 21	70 70	347
Ex8	HNF1A Ex8 L HNF1A Ex8 R	GAGGCCTGGGACTAGGGCTGT CTCTGTCACAGGCCAAGGGAG	21 21	70 70	229
Ex9	HNF1A Ex9 L HNF1A Ex9 R	CCTGTGACAGAGCCCCTCACC CGGACAGCAACAGAAGGGGTG	21 21	70 68	287
Ex10	HNF1A Ex10 L HNF1A Ex10 R	GTACCCCTAGGGACAGGCAGG ACCCCCCAAGCAGGCAGTACA	21 21	70 68	248

Table 20 PCR primers for amplification of *IPF-1* gene.

Fragment	Primer	Nucleotide sequence (5'→3')	No. of nucleotides	Tm (°C)	Product size (bp)
Promoter	IPF promoter-L IPF promoter-R	GCCTAGCCTCTTAGTGCG TGGGTCCTTGTAAAGCTG	18 18	58 54	312
Enhancer	IPF enhancer-L IPF enhancer-R	GCCGCAGACAATGGACTC AGATGCCCTTGCTGTCACC	18 19	58 60	182
Ex1-1	IPF Ex1-1 L IPF Ex1-1 R	GGCTCCAGCTCCCGACTC GGCGAGGGGGGGCACCTC	18 18	62 66	287
Ex1-2	IPF Ex1-2 L IPF Ex1-2 R	CGCTGGAGCAGGGCAGCC CGCTTGGAGGTAAGGCGG	18 18	64 60	304
Ex2-1	IPF Ex2-1 L IPF Ex2-1 R	TGGGGGCTGTGCGGGGCTC TGTCCTCCTCCTTTTTCCACTTC	19 23	68 68	266
Ex2-2	IPF Ex2-2 L IPF Ex2-2 R	CATGTTGAACTTGACCGAGAGACA C CGAGTGGTTGAAGCCCCTCAG	25 21	70 68	351

Table 21 PCR primers for amplification of *HNF-1β* gene.

Fragment	Primer	Nucleotide sequence (5'→3')	No. of nucleotides	Tm (°C)	Product size (bp)
Promoter	HNF1B pro 1-L HNF1B pro 1-R	CTGCAAGGCACTGGCTTAAC CTAACTTGCCATGATCGCCAC	20 21	62 64	280
Promoter	HNF1B pro 2-L HNF1B pro 2-R	TCCCCCTCCCCACCATCATTT GACGTGAGCTTGGACACCAT	20 21	66 62	293
Ex1-1	HNF1B Ex1-1 L HNF1B Ex1-1 R	CCTCACCCCCTTCTTTTTCC TCATAGTCGTCGCCGTCCTC	20 20	62 64	291
Ex1-2	HNF1B Ex1-2 L HNF1B Ex1-2 R	CGACACCAAGCCGGTCTTCCA GAGTGTGGTCGGGCGCAGTG	21 20	68 68	264
Ex2	HNF1B Ex2 L HNF1B Ex2 R	CTCCCACTAGTACCCTAACC GAGAGGGCAAAGGTCACTTCAG	20 22	62 68	291
Ex3	HNF1B Ex3 L HNF1B Ex3 R	AGTGAAGGCTACAGACCCTATC TTCCTGGGTCTGTGTACTTGC	22 21	66 64	365
Ex4	HNF1B Ex4 L HNF1B Ex4 R	CCCTCACTCACCATCTCCCCTCCA CCGAGGCAGTGAGGCCCAAC	24 20	76 68	301
Ex5	HNF1B Ex5 L HNF1B Ex5 R	TGCCGAGTCATTGTTCCAGG CCTCTTATCTTATCAGCTCCAG	20 22	62 64	276
Ex6	HNF1B Ex6 L HNF1B Ex6 R	CTGCTCTTTGTGGTCCAAGTCC GAGTTTGAAGGAGACCTACAG	22 21	68 62	288
Ex7	HNF1B Ex7 L HNF1B Ex7 R	ATCCACCTCTCCTTATCCCAG ACTTCCGAGAAAGTTCAGACC	21 21	64 62	341
Ex8	HNF1B Ex8 L HNF1B Ex8 R	TTTGCCTGTGTATGCACCTTG GCCGAGTCCATGCTTGCCAC	21 20	62 66	257
Ex9	HNF1B Ex9 L HNF1B Ex9 R	CTTTGCTGGTTGAGTTGGGC TTCCATGACAGCTGCCCAGAG	20 21	62 66	208

Table 22 PCR primers for amplification of *NeuroD1* gene.

Fragment	Primer	Nucleotide sequence (5'→3')	No. of nucleotides	Tm (°C)	Product size (bp)
Promoter	NeuroD pro 1-L NeuroD pro 1-R	GCTTTTCCCTTCCTTCCCTC ATATGGTCTTCCCGGTCCAG	20 20	62 62	229
Promoter	NeuroD pro 2-L NeuroD pro 2-R	ACAAAGGGGCCGGAATGGAG CAGTTAGTGATGCTAAGCGCGGG	23 20	64 72	305
Promoter	NeuroD pro 3-L NeuroD pro 3-R	TCAGGCGCATAGACCTGCTA ACACACTCTCGCAAACGCAC	20 20	62 62	285
Ex2-1	NeuroD Ex2-1 L NeuroD Ex2-1 R	CAAGCATTTGTACAGGTTTAG CTCCAGGCGAGCCTTAGTCATC	21 22	58 70	409
Ex2-2	NeuroD Ex2-2 L NeuroD Ex2-2 R	CCTCGAAACCATGAACGCAG GCTGTCCATGGTACCGTAAG	20 20	64 62	583
Ex2-3	NeuroD Ex2-3 L NeuroD Ex2-3 R	CCTGCAACTCAATCCTCGGAC CTGTAAGCACAGTGGGTTCG	21 20	66 62	561

4.2 Primers for amplification of new candidate genes

Table 23 PCR primers for amplification of *Pax4* gene.

Fragment	Primer	Nucleotide sequence (5'→3')	No. of nucleotides	Tm (°C)	Product size (bp)
Ex1	Pax4 Ex1L Pax4 Ex1R	AGGTGGTGTGTGGATACCTC CCAGGCTCTTGCCTTCAGAG	20 20	62 64	242
Ex2	Pax4 Ex2L Pax4 Ex2R	GCCCATCATGCCTCACCTGTC CTTTTCCAGCCCCAGTGTGGG	21 21	68 68	296
Ex3	Pax4 Ex3L Pax4 Ex3R	CCTGAGTCTGAGCACCATCTC GAGATTTGGCTGTGATTAGCCC	21 22	66 66	167
Ex4	Pax4 Ex4L Pax4 Ex4R	CTGACCAGAGGAATCACCATC CCCTGTGTCACACTGAGGAC	21 20	64 64	233
Ex5	Pax4 Ex5L Pax4 Ex5R	GAGACCCATGCCTTGCTCCTC GGCCCAGACTCTTCCTCCTTG	21 21	68 68	194
Ex6	Pax4 Ex6L Pax4 Ex6R	GATCAGCAGGTGACAGGCAGC AGATGACTGAGCGGGCAGATG	21 21	68 66	174
Ex7	Pax4 Ex7L Pax4 Ex7R	AGTGGCTGACTTTCCTAGAAC AGCCCATGAGCCCTTCAGTC	21 20	62 64	225
Ex8	Pax4 Ex8L Pax4 Ex8R	TCTCTACAGGAGGCATCACTG GAGGTTGAGTCAGTCGACCCT	21 21	64 66	260
Ex9	Pax4 Ex9L Pax4 Ex9R	TTTGAGAGGTGGGGTGGGAG GTAAGGACAATGGGCAGGATG	20 21	64 64	260

Table 24 PCR primers for amplification of *Nkx6.1* gene**.**

Fragment	Primer	Nucleotide sequence (5'→3')	No. of nucleotides	Tm (°C)	Product size (bp)
Ex1-1	Nkx6A Ex1-1L	TCGCTGCAAGGCTACGGTCTC	21	68	335
	Nkx6A Ex1-1R	TCGTTGATGCCGTGTGGGGTG	21	68	
Ex1-2	Nkx6A Ex1-2L	GGGCACCCACAACCCAGGCG	20	70	351
	Nkx6A Ex1-2R	GCGCTGGGGCTGAAGTAGAGC	21	70	
Ex1-3	Nkx6A Ex1-3L	CAGCCGCCGCCTCATCCCC	19	68	288
	Nkx6A Ex1-3R	GGAGTGGGCAGAACAGGCAC	20	66	
Ex2	Nkx6A Ex2L	TGCAGCCGCTTGTAACCGATTG	22	68	339
	Nkx6A Ex2R	CGACTGTTTGTTAGTTTGGGGTG	23	68	
Ex3-1	Nkx6A Ex3-1L	CGTTTAATTCCTCGCCCTTGCC	22	68	257
	Nkx6A Ex3-1R	TGCTGGACTTGTGCTTCTTCAAC	23	68	
Ex3-2	Nkx6A Ex3-2L	GAGGAAGAGGACGACGACTAC	21	66	286
	Nkx6A Ex3-2R	TAGCAAAGGGTCCCCGCAGG	20	66	

Table 25 PCR primers for amplification of *Nkx2.2* gene**.**

Fragment	Primer	Nucleotide sequence (5'→3')	No. of nucleotides	Tm (°C)	Product size (bp)
Ex1-1	Nkx2B Ex1-1L	ACGAATTGACCAAGTGAAGCTAC	23	66	240
	Nkx2B Ex1-1R	TCAGGGGCAGGCTCTGCACC	20	68	
Ex1-2	Nkx2B Ex1-2L	AACGATGAGGAGGGCTCTGTG	21	66	245
	Nkx2B Ex1-2R	CTGCAGGAATGGAGGGGACC	20	66	
Ex2-1	Nkx2B Ex2-1L	AGGGTGCTCCGAGTCTGGTG	20	66	327
	Nkx2B Ex2-1R	CGTGGGCGTGAGGCGGATG	19	66	
Ex2-2	Nkx2B Ex2-2L	GCAAGAAGCGAAAGCGGCGAG	21	68	301
	Nkx2B Ex2-2R	GCGGCTGCCAGGTCCTGGG	19	68	
Ex2-3	Nkx2B Ex2-3L	CCGTGCCCGTCTTGGTCAGG	20	68	350
	Nkx2B Ex2-3R	GGAGCCGAGAGTCAACTCGAC	21	68	

4.3 Primers for functional study

Table 26 Primers for amplification of human *Pax4* cDNA for cloning and site-direct mutagenesis.

Primer name	Nucleotide sequences (5>3')
NNPax4F	AGGTGGTGTGTGGATACCTC
NNPax4R	TGGGCAGGATGGTATTAGATCTTCTCTATG
*Eco*RV Pax4	GATATCATGAACCAGCTTGGGGGGCT
*Xba*I Pax4	GCTCTAGATCATTCCAAGCCATACAGTA
Pax4R164WF	GTACCCATCCAGGGACCGGCCACTGGAATCGG
Pax4R164WF	CCGATTCCAGTGGCCGGTCCCTGGATGGGTAC

4.4 Primers for *BLK* gene

Table 27 Primers for amplification of *BLK* gene.

Primer Name	Sequence (5'->3')	Region	PCR product (bp)	Tm
BLK_F1	GTTCTGGACATTGTTGTGAGCCC	5`UTR	597	61
BLK_R1	GTGAGTGGTGTCTGGAGTGGTGG			
BLK_F2	TACAGGATGCCACCCACCACTC	ex1-5`UTR	597	61
BLK_R2	GGAAAACTCGTGAGGAAGGACCA			
BLK_F3	TGGTCCTTCCTCACGAGTTTTCC	ex1-5`UTR	386	61
BLK_R3	TAAAGGAAGACGATAAAGAACCCG			
BLK_F4	CCACCCCACCTTTCTAACCAGC	ex1-coding	248	61
BLK_R4	GGGCAATCACTCACCAGGGG			
BLK_F5	CAGCAGTCTCAACCCCCAGG	ex2-coding	424	61
BLK_R5	CTTGGGTTTGATGATGCTGTGTG			
BLK_F6	GAAGCCTGTCCTCCTTGGTAGCC	ex3-coding	310	61
BLK_R6	CTGGGGGCAGGGCGATGG			
BLK_F7	CAAGCCCCTTCCTGCCTGCC	ex4-coding	381	61
BLK_R7	CTCATTTCCTGTCCCCTCTTTGC			
BLK_F8	GAGGGAGGCTGTGTGGGAATAC	ex5-coding	460	61
BLK_R8	CTCGGTGTTCCTGCTGACTGGG			
BLK_F9	GTGCCTTACTTCTCGTGTGTGTCTTC	ex6-7-coding	872	61
BLK_R9	GATGACACGCTATGAAAATGCTGAAC			
BLK_F10	GCTCTCTGTCTTCTGATTGGCTTCTTC	ex8-coding	468	61
BLK_R10	GAAATGCTGTCTGAACTGCTCCTG			
BLK_F11	CAGGGGCGGGTCACTTTGC	ex9-coding	538	61
BLK_R11	CCTACAGGAGATGTTTGTGGGCA			
BLK_F12	CAGTGTGGGCTCGGTCTTGG	ex10-coding	410	61
BLK_R12	GGAGGTCTCAGGGCACTACCATTC			
BLK_F13	GGTGAGGATGGAGGGTAGGGG	ex11-coding	330	61
BLK_R13	CCTGGGTCTCCGTCTTCGCTC			
BLK_F14	CCCCCCACCCACCGAGGAC	ex12-coding	562	61
BLK_R14	GTCGCTTACCAGTCCTGAACACCT			
BLK_F15	CGGAATCCAGTGGGCAGAGG	ex12-3`UTR	715	61
BLK_R15	GTAGTGGGAGCCGCCGACG			

5. PCR-SSCP conditions

5.1 PCR-SSCP conditions of six known MODY genes

Table 28 PCR-SSCP conditions for analysis of *HNF-4α* gene.

Fragment	Primer	Product size (bp)	PCR condition	SSCP condition		
			T. annealing (°C) and additive	Polyacrylamide gel	Duration of electrophoresis	Temp. (°C)
Promoter 1	HNF4A pro 1-L HNF4A pro 1-R	320	55	12% acrylamide with 5% glycerol	5.00 hr.	RT
Promoter 2	HNF4A pro 2-L HNF4A pro 2-R	340	60	10% acrylamide with 5% glycerol	5.00 hr.	RT
Promoter	HNF4A pro 3-L HNF4A pro 3-R	294	60	10% acrylamide with 5% glycerol	5.00 hr.	RT
Ex1A*	HNF4A Ex1A L HNF4A Ex1A R	318	65	15% acrylamide with 0% glycerol	4 hr.	RT
Ex1B	HNF4A Ex1B L HNF4A Ex1B R	210	55	10% acrylamide with 5% glycerol	3.30 hr.	RT
Ex1C*	HNF4A Ex1C L HNF4A Ex1C R	179	60	20% acrylamide with 0% glycerol	3.00 hr.	RT
Ex2*,[a]	HNF4A Ex2 L HNF4A Ex2 R	260	65	20% acrylamide with 0% glycerol	4.30 hr.	RT
Ex3	HNF4A Ex3 L HNF4A Ex3 R	253	56	12% acrylamide with 5% glycerol	4.30 hr.	RT
Ex4*	HNF4A Ex4 L HNF4A Ex4 R	245	65	10% acrylamide with 5% glycerol	5.00 hr.	RT
Ex5	HNF4A Ex5 L HNF4A Ex5 R	234	55	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex6	HNF4A Ex6 L HNF4A Ex6 R	210	55	20% acrylamide with 0% glycerol	3 hr.	RT
Ex7	HNF4A Ex7 L HNF4A Ex7 R	306	65	10% acrylamide with 10% glycerol	6 hr.	RT
Ex8	HNF4A Ex8 L HNF4A Ex8 R	300	55	10% acrylamide with 5% glycerol	5 hr.	RT
Ex9	HNF4A Ex9 L HNF4A Ex9 R	246	55	10% acrylamide with 10% glycerol	5 hr.	RT
Ex10	HNF4A Ex10 L HNF4A Ex10 R	277	55	10% acrylamide with 5% glycerol	4.30 hr.	RT

* PCR reaction contained 1 mM of $MgCl_2$.

[a] Amplification was performed with Immolase DNA polymerase.

Table 29 PCR-SSCP conditions for analysis of *GCK* gene.

Fragment	Primer	Product size (bp)	PCR condition	SSCP condition		
			T. annealing (°C) and additive	Polyacrylamide gel	Duration of electrophoresis	Temp. (°C)
Promoter 1	GCK pro 1-L GCK pro 1-R	265	55	10% acrylamide with 5% glycerol	5.00 hr.	RT
Promoter 2	GCK pro2-L GCK pro 2-R	300	70	15% acrylamide with 5% glycerol	5.00 hr.	RT
Promoter 3	GCK pro 3-L GCK pro 3-R	292	55	10% acrylamide with 5% glycerol	5.00 hr.	RT
Promoter 4	GCK pro 4-L GCK pro 4-R	299	55	10% acrylamide with 5% glycerol	5.00 hr.	RT
Ex1A	GCK Ex1A L GCK Ex1A R	195	55	10% acrylamide with 5% glycerol	4.00 hr.	RT
Ex1B	GCK Ex1B L GCK Ex1B R	260	60	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex1C	GCK Ex1C L GCK Ex1C R	236	60	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex2	GCK Ex2 L GCK Ex2 R	290	58	10% acrylamide with 5% glycerol	5.00 hr.	RT
Ex3	GCK Ex3 L GCK Ex3 R	298	60	10% acrylamide with 5% glycerol	5.00 hr.	RT
Ex4	GCK Ex4 L GCK Ex4 R	272	60	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex5	GCK Ex5 L GCK Ex5 R	251	55	10% acrylamide with 5% glycerol	3.30 hr.	RT
Ex6	GCK Ex6 L GCK Ex6 R	268	60	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex7	GCK Ex7 L GCK Ex7 R	286	60	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex8	GCK Ex8 L GCK Ex8 R	262	60	10% acrylamide with 5% glycerol	4.00 hr.	RT
Ex9	GCK Ex9 L GCK Ex9 R	306	60	10% acrylamide with 5% glycerol	4.00 hr.	RT
Ex10	GCK Ex10 L GCK Ex10 R	263	65	15% acrylamide with 5% glycerol	6.00 hr.	RT

Table 30 PCR-SSCP conditions for analysis of *HNF-1α* gene.

Fragment	Primer	Product size (bp)	PCR condition	SSCP condition		
			T. annealing (°C) and additive	Polyacrylamide gel	Duration of electrophoresis	Temp. (°C)
Promoter1[a]	HNF1A pro 1-L HNF1A pro 1-R	385	65	10% acrylamide with 5% glycerol	5.00 hr.	RT
Promoter2[a]	HNF1A pro 2-L HNF1A pro 2-R	300	63	10% acrylamide with 5% glycerol	5.00 hr.	RT
Ex1	HNF1A Ex1 L HNF1A Ex1 R	483	63	10% acrylamide with 5% glycerol	5.30 hr.	RT
Ex2	HNF1A Ex2 L HNF1A Ex2 R	390	63	10% acrylamide with 5% glycerol	4 hr.	RT
Ex3	HNF1A Ex3 L HNF1A Ex3 R	304	63	10% acrylamide with 5% glycerol	3.00 hr.	RT
Ex4	HNF1A Ex4 L HNF1A Ex4 R	397	63	10% acrylamide with 5% glycerol	5.30 hr.	RT
Ex5	HNF1A Ex5 L HNF1A Ex5 R	346	63	10% acrylamide with 5% glycerol	5.30 hr.	RT
Ex6	HNF1A Ex6 L HNF1A Ex6 R	322	63	10% acrylamide with 5% glycerol	5.30 hr.	RT
Ex7	HNF1A Ex7 L HNF1A Ex7 R	347	63	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex8	HNF1A Ex8 L HNF1A Ex8 R	229	63	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex9[a]	HNF1A Ex9 L HNF1A Ex9 R	287	67	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex10	HNF1A Ex10 L HNF1A Ex10 R	248	63	12% acrylamide with 5% glycerol	4.30 hr.	RT

[a] Amplification was performed with Immolase DNA polymerase.

Table 31 PCR-SSCP conditions for analysis of *IPF1* gene.

Fragment	Primer	Product size (bp)	PCR condition	SSCP condition		
			T. annealing (°C) and additive	Polyacrylamide gel	Duration of electrophoresis	Temp. (°C)
Promoter	IPF promoter-L IPF promoter-R	312	55	dbstand	3.30 hr.	RT
Enhancer	IPF enhancer-L IPF enhancer-R	182	55	20% acrylamide with 0% glycerol	2.30 hr.	RT
Ex1-1	IPF Ex1-1 L IPF Ex1-1 R	287	55	20% acrylamide with 0% glycerol	4.30 hr.	RT
Ex1-2	IPF Ex1-2 L IPF Ex1-2 R	304	60	12% acrylamide with 0% glycerol	4.00 hr.	RT
Ex2-1	IPF Ex2-1 L IPF Ex2-1 R	266	60	10% acrylamide with 5% glycerol	3.30 hr.	RT
Ex2-2	IPF Ex2-2 L IPF Ex2-2 R	351	60	10% acrylamide with 5% glycerol	5.00 hr.	RT

Table 32 PCR-SSCP conditions for analysis of *HNF-1β* gene.

Fragment	Primer	Product size (bp)	PCR condition	SSCP condition		
			T. annealing (°C) and additive	Polyacrylamide gel	Duration of electrophoresis	Temp. (°C)
Promoter 1	HNF1B pro 1-L HNF1B pro 1-R	280	60	10% acrylamide with 5% glycerol	4.30 hr.	RT
Promoter 2	HNF1B pro 2-L HNF1B pro 2-R	293	55	15% acrylamide with 5% glycerol	4.00 hr.	RT
Ex1-1	HNF1B Ex1-1 L HNF1B Ex1-1 R	291	56	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex1-2	HNF1B Ex1-2 L HNF1B Ex1-2 R	264	56/5%DMSO	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex2	HNF1B Ex2 L HNF1B Ex2 R	291	56	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex3	HNF1B Ex3 L HNF1B Ex3 R	365	56	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex4	HNF1B Ex4 L HNF1B Ex4 R	301	56	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex5	HNF1B Ex5 L HNF1B Ex5 R	276	56	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex6	HNF1B Ex6 L HNF1B Ex6 R	288	56	12% acrylamide with 5% glycerol	4.30 hr.	RT
Ex7	HNF1B Ex7 L HNF1B Ex7 R	341	56	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex8	HNF1B Ex8 L HNF1B Ex8 R	257	56	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex9	HNF1B Ex9 L HNF1B Ex9 R	208	56	10% acrylamide with 5% glycerol	3.30 hr.	RT

Table 33 PCR-SSCP conditions for analysis of *NeuroD1* gene.

Fragment	Primer	Product size (bp)	PCR condition	SSCP condition		
			T. annealing (°C) and additive	Polyacrylamide gel	Duration of electrophoresis	Temp. (°C)
Promoter 1	NeuroD pro 1-L NeuroD pro 1-R	229	55	10% acrylamide with 5% glycerol	5.00 hr.	RT
Promoter 2	NeuroD pro 2-L NeuroD pro 2-R	305	62	10% acrylamide with 5% glycerol	5.00 hr.	RT
Promoter 3	NeuroD pro 3-L NeuroD pro 3-R	285	50	10% acrylamide with 5% glycerol	5.00 hr.	RT
Ex2-1	NeuroD Ex2-1 L NeuroD Ex2-1 R	409	56	10% acrylamide with 5% glycerol	7.00 hr.	RT
Ex2-2	NeuroD Ex2-2 L NeuroD Ex2-2 R	583	56	10% acrylamide with 5% glycerol	7.00 hr.	RT
Ex2-3	NeuroD Ex2-3 L NeuroD Ex2-3 R	561	56	10% acrylamide with 5% glycerol	7.00 hr.	RT

5.2 PCR-SSCP conditions of new candidate genes

Table 34 PCR-SSCP conditions for analysis of *Pax4* gene.

Fragment	Primer	Product size (bp)	PCR condition	SSCP condition		
			T. annealing (°C) and additive	Polyacrylamide gel	Duration of electrophoresis	Temp. (°C)
Ex1	Pax4 Ex1L Pax4 Ex1R	242	58	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex2	Pax4 Ex2L Pax4 Ex2R	296	60	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex3	Pax4 Ex3L Pax4 Ex3R	167	60	10% acrylamide with 5% glycerol	3.00 hr.	RT
Ex4	Pax4 Ex4L Pax4 Ex4R	233	58	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex5	Pax4 Ex5L Pax4 Ex5R	194	60	12% acrylamide with 10% glycerol	6.30 hr.	4°C
Ex6	Pax4 Ex6L Pax4 Ex6R	174	60	12% acrylamide with 5% glycerol	5.00 hr.	RT
Ex7	Pax4 Ex7L Pax4 Ex7R	225	58	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex8	Pax4 Ex8L Pax4 Ex8R	260	58	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex9	Pax4 Ex9L Pax4 Ex9R	260	62 / 5%DMSO	10% acrylamide with 5% glycerol	4.30 hr.	RT

Table 35 PCR-SSCP conditions for analysis of *Nkx6.1* gene.

Fragment	Primer	Product size (bp)	PCR condition	SSCP condition		
			T. annealing (°C) and additive	Polyacrylamide gel	Duration of electrophoresis	Temp. (°C)
Ex1-1	Nkx6A Ex1-1L Nkx6A Ex1-1R	335	62 / 5%DMSO	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex1-2	Nkx6A Ex1-2L Nkx6A Ex1-2R	351	62 / 5%DMSO	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex1-3	Nkx6A Ex1-3L Nkx6A Ex1-3R	288	65 / 5%DMSO	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex2	Nkx6A Ex2L Nkx6A Ex2R	339	62	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex3-1	Nkx6A Ex3-1L Nkx6A Ex3-1R	257	62 / 5%DMSO	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex3-2	Nkx6A Ex3-2L Nkx6A Ex3-2R	286	62 / 5%DMSO	10% acrylamide with 5% glycerol	4.30 hr.	RT

Table 36 PCR-SSCP conditions for analysis of *Nkx2.2* gene.

Fragment	Primer	Product size (bp)	PCR condition	SSCP condition		
			T. annealing (°C) and additive	Polyacrylamide gel	Duration of electrophoresis	Temp. (°C)
Ex1-1	Nkx2B Ex1-1L Nkx2B Ex1-1R	240	62 / 5%DMSO	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex1-2	Nkx2B Ex1-2L Nkx2B Ex1-2R	245	62 / 5%DMSO	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex2-1	Nkx2B Ex2-1L Nkx2B Ex2-1R	327	62 / 5%DMSO	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex2-2	Nkx2B Ex2-2L Nkx2B Ex2-2R	301	62 / 5%DMSO	10% acrylamide with 5% glycerol	4.30 hr.	RT
Ex2-3	Nkx2B Ex2-3L Nkx2B Ex2-3R	350	65 / 5%DMSO	10% acrylamide with 5% glycerol	4.30 hr.	RT

6. PCR-RFLP/mismatch PCR-RFLP conditions

Table 37 PCR-RFLP/mismatch PCR-RFLP conditions for genotyping of possible mutations of six known MODY genes.

Gene	Variation	Method	Primer sequence (5'>3')	Product size(bp)	Size of restriction fragment (bp)	
					Major Allele	Minor Allele
HNF-4α	T139I	*Bsp119I* RFLP	CAGACACCCCCACCCCCTAC GAATGGAGGTGGAGGAGGTGAG	245	245	97, 148
	R140Q	*TaqI* RFLP	CAGACACCCCCACCCCCTAC GAATGGAGGTGGAGGAGGTGAG	245	97, 148	245
	R312H	*AvaIII* Mismatch RFLP	CAGGGAAGATCAAGCGGATGC GGTGAGGAAACTGAGCAGA	373	373	23, 350
GCK	(-194)A>G	*NheI* RFLP	GGGATGTGAGATGGTCCCAGG CCAGGAGTGGCCTCAGCAGG	299	134, 165	229
	R327H	*FnuDII* RFLP	TGCCTGCTGATGTAATGGAC TGAGACCAAGTCTGCAGTGC	262	16, 93, 153	93, 169
HNF-1α	R203C	*NlaIV* RFLP	GGGCAAGGTCAGGGGAATGGA CAGCCCAGACCAAACCAGCAC	304	1, 15, 135, 153	1, 135, 168
	P475L	*PvuII* RFLP	GGTCTTGGGCAGGGGTGGGAT CTGCAATGCCTGCCAGGCACC	347	39, 107, 201	39, 63, 107, 138
	G554SfsX3 1659_1660 insAGTGAGTGAAGCCC	Heteroduplex analysis	CCTGTGACAGAGCCCCTCACC CGGACAGCAACAGAAGGGGTG	287	287	Heteroduplex
NeuroD1	**-303**	*FnuDII* RFLP	ACAAAGGGGCCGGAATGGAG CAGTTAGTGATGCTAAGCGCGGG	305	61, 69, 99	99, 130
	A322N	*MspA1I* RFLP	CCTGCAACTCAATCCTCGGAC CTGTAAGCACAGTGGGTTCG	561	156, 405	561

*** Novel variation are indicated in bold.**

Table 38 PCR-RFLP conditions for genotyping of possible mutations of new candidate genes.

Gene	Variation	Method	Primer sequence (5'>3')	product size (bp)	Size of restriction fragment (bp)	
					Wild type	Variant
Pax4	R31Q	*Hae*III *RFLP*	AGGTGGTGTGTGGATACCTC CCAGGCTCTTGCCTTCAGAG	242	4, 35, 57, 68, 78	4, 68, 78, 92
	R164W	*Bsr*I *RFLP*	CTGACCAGAGGAATCACCATC CCCTGTGTCACACTGAGGAC	233	56, 177	40, 56, 137
	R192H	*Nla*III *RFLP*	GAGACCCATGCCTTGCTCCTC GGCCCAGACTCTTCCTCCTTG	194	10, 184	10, 88, 96
	IVS7(-1)G> A	*Mse*I *RFLP*	TCTCTACAGGAGGCATCACTG GAGGTTGAGTCAGTCGACCCT	260	260	59, 201

APPENDIX II

List of manuscripts, presentations, and theses

MANUSCRIPTS

1. Nattachet Plengvidhya., Watip Boonyasripiwat, Nalinee Chongjaroen, Prapaporn Jungtrakoon, Kanjana Leejinda, Wiwit Tantibhedhyangkul, Thaniya Sricharunrat, Jatuporn Sujjitjoon, Luksame Wattanamongkonsi, Sirirat Ploybutr, Sutin Sriussadaporn, Sathit Vannaseang, Napatawn Banchuin, Pa-thai Yenchitsomanas. **Mutations of maturity-onset diabetes of the young (MODY) gene in Thais with early-onset type 2 diabetes. (To be submitted to Human Genetic, Impact factor 2005: 4.331)**.
2. Nattachet Plengvidhya, Suwattnee Kooptiwut, Napat Songtawee, Asako Doi, Hiroto Furata,MasahiroNishi,KishioNanjo,Wiwit Tantibhedhyangkul, Watip Boonyasrisawat, Pa-thai Yenchitsomanus, Alessandro Doria, Napatawn Banchuin. ***PAX4*** **Mutations in Thais with Maturity-Onset Diabetes of the Young (MODY).**
   (Journal of Clinical Endocrinology and Metabolism, Impact factor 2005: 6.020, Manuscript under revision).
3. Suwattanee Kuptiwut, Jatuporn Sujjitjoon, Nattachet Plengvidhya, Napat Songtawee, Nalinee Chongjaroen, Prapaporn Jungtrakoon, Pa-thai Yenchitsomanas, Napatawn Banchuin. **Functional impact of novel *HNF-1α* mutation, G554SfsX3 1659_1660 insAGTGAGTGAAGCCC, found in Thai MODY patient. (Manuscript in preparation).**

PRESENTATIONS

Oral presentations

1. Napat Songtawee, Nattachet Plengvidhya, Watip Boonyasrisawat, Pa-thai Yenchitsomanus, Sathit Vannaseang, Sirirat Ploybutr, Napatawn Banchuin. A Study of Paired Box Gene (PAX) 4 in Thai Patients with Maturity Onset Diaetes of the Young (MODY). Oral presentation. Siriraj Scientific Congress, 18 March 2004, Faculty of Medicine Siriraj Hospital, Mahidol University.
2. Nattachet Plengvidhya, Napat Songtawee, Watip Boonyasrisawat, Pa-thai Yenchitsomanus, Sathit Vannaseang, Sirirat Ploybutr, Napatawn Banchuin. Genetic Variability of the *Pax4, Nkx6.1 and Nkx2.2* genes in Thai with Maturity Onset Diabetes of the Young (MODY). The 6th symposium on Molecular Diabetory In Asia (MDIA), December 2004.Grand Hotel Taipei.Taiwan.

Poster presentations

1. Napat Songtawee, Nattachet Plengvidhya, Watip Boonyasrisawat, Pa-thai Yenchitsomanus, Sathit Vannaseang, Sirirat Ploybutr, Napatawn Banchuin. Analysis of Paired Box Gene (PAX) 4 in Thai Patients with Maturity Onset Diabetes of the Young (MODY). Poster presentation. The 10th ASEAN conference in Medical Laboratory Technology, 26-30 April 2004, ASEAN Association of Medical Laboratory Technologist (AAMLT).
2. Nattachet Plengvidhya , Napat Songtawee , Wiwit Tantibhedhyangkul, Pa-thai Yenchitsomanus, Watip Boonyasrisawat, Nalinee Chongjaroen, Sirirat Plotbutr, Napatawn Banchuin. A Novel Mutation of *PAX4* Gene in a Thai MODY Family. Poster presentation. The 6th International Diabetes Federation Western Pacific Region Congress (IDFWPR 2005) 22-26th October 2005, Queen Sirikit National Convention Center, Bangkok, Thailand

THESES

1. **Mr. Napat Songtawee**

   Degree: M.Sc. (Immunology)

   Thesis title: A STUDY O F *PAX4*, *NKX6.1*, AND *NKX2.2* GENES IN THAI PATIENTS WITH MATURITY ONSET DIABETES OF THE YOUNG (MODY)

   Graduation: December, 2004

2. **Miss. Watip Boonyasrisawat**

   Degree: Ph.D. (Immunology)

   Thesis title: MOLECULAR PATHOGENESIS OF DIABETES MELLITUS IN THAI PATIENT

   Graduation: June, 2007 (expected)

2. **Miss. Jatuporn Sujjitjoon**

   Degree: M.Sc. (Immunology)

   Thesis title: FUNCTIONAL IMPACT OF NOVEL *HNF-1α* MUTATION, G554SfsX3 1659_1660insAGTGAGTGAAGCCC, FOUND IN THAI MODY PATIENT.

   Graduation: December, 2007 (expected)

3. **Miss. Titikan Chukijrungroat**

   Degree: M.Sc. (Physiology)

   Thesis title: FUNCTIONAL STUDY OF PAIRED BOX GENE 4 (*PAX4*) R192H POLYMORPHISM IN NOVEL MATURITY-ONSET DIABETES OF THE YOUNG (MODY) GENE

   Graduation: December, 2007 (expected)

APPENDIX III

List of Collaborators

1. **Alessandro Doria MD, PhD**

   Section on Genetic and Epidemiology, Joslin Diabetes Center,
   Harvard Medical School, Boston, Massachusetts, USA.
   (Memorandum of Understanding (MOU) in Scientific Collaboration has been signed and effective during Year 2006-2009)

2. **Hiroto Furuta MD, PhD**

   The First Department of Medicine, Wakayama University,
   Wakayama, Japan.

APPENDIX IV

Manuscripts, posters, and abstracts of oral presentations

Mutations of maturity onset diabetes of the young (MODY) genes in Thais with early-onset type 2 diabetes

Nattachet Plengvidhya[1], Watip Boonyasrisawat[2], Nalinee Chongjaroen[2], Prapaporn Jungtrakoon[2], Kanjana Leejinda[2], Wiwit Tantibhedhyangkul[2,] Thaniya Sricharunrat[2], Jatuporn Sujjitjoon[2], Luksame Wattanamongkonsil[3], Sirirat Ploybutr[1], Sutin, Sriussadaporn[1], Sathit Vannaseang[1], Napatawn Banchuin[2], Pa-thai Yenchitsomanus[4,*]

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Key words: Maturity onset diabetes of the young, MODY, early-onset type 2 diabetes, T2D, hepatocyte nuclear factor, glucokinase, insulin promoter factor, neurogenic differentiation

Abstract

Maturity onset diabetes of the young (MODY) is a special monogenic form of type 2 diabetes (T2D), characterized by young age at onset usually before 25 years and autosomal dominant inheritance. The disease transmitted in multiple (classically three) consecutive generations. To date, mutations in one of 6 different genes including *HNF-4α*, *GCK*, *HNF-1α*, *IPF-1*, *HNF-1β*, and *NeuroD11* are described as a cause of MODY. The aim of this study is to investigate the prevalence of MODY gene mutations in Thai patients with early-onset type 2 diabetes. Fifty-one unrelated probands with early-onset type 2 diabetes, 21 of whom were well-suited with classic MODY criteria were screened for nucleotide variations in promoters and exons, including exon-intron boundaries, of known MODY genes by using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP), followed by direct DNA sequencing. Mutations located in regulatory or coding regions that would affect gene expression or protein structure, which were absent in 135 chromosomes of non-diabetic controls, were classified as potentially pathogenic mutations. We found that mutations in known MODY genes account for a small proportion of both classic MODY (19%) and early-onset type 2 diabetes (10%). Five of these mutations are novel including *GCK* R327H, *HNF-1α* P475L, *HNF-1α* G554SfsX3, *NeuroD1* -303G>A and *NeuroD1* A322N. Mutation in *IPF-1* and *HNF-1β* was not identified in any studied probands. We conclude that defects in known MODY genes may not be a major cause of MODY in Thai patients. Thus, these families are precious resource for the discovery of new MODY genes.

Introduction

Maturity onset diabetes of the young (MODY) is a genetically heterogeneous form of diabetes characterized by an early onset (usually before 25 years), frequent insulin-independence at the beginning of the disease, absence of ketosis, and an autosomal dominant pattern of inheritance. MODY can result from mutations in any of six different genes. One encodes enzyme glucokinase (GCK/MODY2) whereas other five genes encode transcription factors, including hepatocyte nuclear factor (HNF)-4α (MODY1), HNF-1α (MODY3), insulin promoter factor-1 (IPF-1) (MODY4), HNF-1β (MODY5), and neurogenic differentiation 1/β-cell E-box transactivator 2 (NeuroD11/β2) (MODY6) (Fajans *et al*. 2001).

Glucokinase is an enzyme that functions as a glucose sensor, playing a key role in insulin secretion from pancreatic β-cells. Mutations in *glucokinase* (*GCK*) gene result in an impairment of β-cells sensitivity to glucose (Matschinsky 2002). However, the subjects with *GCK* mutations (MODY2) generally present mild-form of diabetes (Byrne *et al.* 1994; Vaxillaire *et al*. 1999), due to the presence of compensatory mechanism that increases the insulin secretion response (Sturis *et al.* 1994). In contrast, subjects carrying mutations in pancreatic transcription factors, *HNF-4α*, *HNF-1α* and *HNF-1β* (MODY1, MODY3, and MODY5, respectively), usually exhibit more severe hyperglycemia (Frayling *et al*. 2001). These three hepatocyte nuclear factors function together in regulation of insulin gene expression, as well as genes encoding proteins involved in glucose transporter and metabolism (Stoffel and Duncan 1997). IPF-1 (MODY4) is also a transcription factor that plays critical role in the development of pancreas and in the regulation of expression of the genes encoding insulin, glucokinase and glucose transporter (St-Onge *et al*. 1999). However, the clinical characteristics of MODY4 is in general much less severe than those seen in HNFs mutations, due to compensatory increased insulin sensitivity (Clocquet *et al*. 2000).

The prevalence of mutations in each MODY gene is different among various ethic groups. Mutations in *GCK* (MODY2) are the most common cause of MODY in France, accounting for more than 60% of studied families (Froguel *et al*. 1993; Velho *et al*. 1997), whereas the prevalence of this MODY subtype in United Kingdom (Beards *et al*. 1998) and Germany (Lindner *et al*. 1999) were only 11% and 8%, respectively. In general, *HNF-1α* (MODY3) mutations are the most common cause of MODY in Caucasians and the prevalence varied from 21% to 64% (Chevre *et al*. 1998; Costa *et al*. 2000; Doria *et al*. 1999; Frayling *et al*. 1997; Massa *et al*. 2001; Moises *et al.* 2001). The other four types of MODY are rare and have been described in a few families. Overall, sequence variations of these six genes accounted for 75-80% of MODY. Molecular genetic epidemiology indicated that other genes that cause MODY do exist in agree with the fact that there are at least 20-25% of MODY pedigrees (MODY-X) unlinked to known MODY genes. The estimated prevalence of MODY-X is 15-20% of European families (Chevre *et al*. 1998), and as many as 60-80% of Chinese (Ng *et al*. 1999) and Japanese families (Nishigori *et al*. 1998). Owing to different genetic background and lack of information on molecular defect of MODY in Thai and Southeast Asian populations, it is therefore very interesting to study the prevalence of MODY gene mutations in Thai patients.

Subjects and Methods

Subjects

Fifty-one unrelated probands with early-onset type 2 diabetes (T2D) were recruited at the Diabetic Clinics, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, according to the following criteria: *(i)* the proband and at least one first-degree relative diagnosed with type 2 diabetes before age 35, *(ii)* two or more generations affected by diabetes, *(iii)* diabetes treatment with diet and/or oral agents, *(iv)* no history of diabetic ketoacidosis (DKA), and *(v)* absence of anti-GAD antibody. Twenty-one probands were in concordance with strict MODY criteria (Frayling *et al*. 2003). Non-diabetic subjects were 65 healthy staff members in the Department of Immunology and Department of Research and Development, Faculty of Medicine Siriraj Hospital. All of them had fasting plasma glucose (FPG) level less than 100 mg/dl, and had no family history of diabetes in their first-degree relatives. This study was approved by our Institutional Ethics Committee. The subjects were informed for the purpose and extent of the study and signed a consent form before enrollment into this study.

Sample collection and laboratory assays

Fifteen milliliters of peripheral venous blood were collected from each subject into a sterile tube containing 20 μl of 20% EDTA. The red blood cell (RBC) lysis buffer was used to destroy RBC. Leukocytes were separated and stored in a 15-ml screw-capped tube at –70^{o}C. Standard phenol/chloroform extraction method was performed to prepare genomic DNA from the leukocytes. Plasma glucose was determined by glucose oxidase method. Glycosylated hemoglobin (HbA_1C), total cholesterol, triglyceride and HDL-cholesterol were assayed by standard methods. LDL-cholesterol were directly measured or calculated by Friedewald formula (LDL-cholesterol = total cholesterol-HDL-cholesterol-triglyceride/5). Anti-glutamic acid decarboxylase (GAD) antibody was assayed by a reagent kit (CIS Bio

International ORIS Group Gif-Sur-Yvette Cedex, France). Urine microalbumin and creatinine were measured by DCA 2000 Microalbumin/Creatinine Analyzer (Bayer Corporarion Elkhart, IN USA) using immunoturbidity method.

Screening for sequence variations of six MODY genes

All exons, flanking introns, 5' flanking and minimal promoter regions of *HNF-4α*, *GCK*, *HNF-1α*, *IPF-1*, *HNF-1β* and *NeuroD1* genes were amplified from genomic DNA sample of each proband by PCR. Twenty-five microlitres of PCR reaction contained 125 ng of DNA template, 1x PCR buffer (Promega Madison, USA or Bioline, USA), 5 mM dNTPs, 1.0-1.5 mM $MgCl_2$, 12.5 pmol each of forward and reverse primers, 0.25 units of either *Taq* or Immolase DNA polymerase. DNA amplifications were carried out in a DNA Thermal Cycler 2400 (Perkin-Elmer, California, USA), at 95°C for 30 s (denaturation), 55-70 °C for 30 s (annealing), and 72°C for 30 s (extension) for 35-40 cycles. In SSCP analysis, amplified products were mixed with loading dye buffer (95% formamide, 20 mM EDTA, 10 mM NaOH, 0.05% bromophenol blue, and 0.05% xylene cyanol FF), and denatured at 95°C for 10 min. The sample mixture was placed immediately on ice for 5 min, and then was load on to 10-20% polyacrylamide non-denaturing gel containing 0-10% glycerol. Electrophoresis was performed at room temperature in TBE (89 mM Tris–HCl pH 8.0, 89 mM boric acid, 2.5 mM EDTA) buffer, pH 7.4. The electrophoresis times varied from 2.5 to 6.5 h, depending on the sizes of the DNA fragments. SSCP pattern on the gel was visualized by silver staining. Briefly, gel was soaked with 40% methanol for 10 minutes, and incubated in 160 mM of nitric acid for 6 minutes. Then gel was rinsed by distilled water, and stained with 0.2% w/v silver nitrate ($AgNO_3$) solution for 20 minutes. After rinsing, the developer (3% w/v Na_2CO_3 and 0.0175% formaldehyde) was added into the gel. The reaction was stopped by adding 70 ml of 10% w/v citric acid, when DNA bands were revealed. PCR products showing a mobility shift on the SSCP gel were subjected to direct sequencing by mean of the ABI Prism

BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA). Fluorescent signals were detected with the ABI Collection software. Nucleotide sequences were determined by Sequencer Navigator software and analyzed with Chromas program version 1.4.4 (Conor McCarthy, Griffith University, Queensland, Australia).

Additional genotyping for identification of mutation

Identified sequence variations, which were present in at least one of 51 probands but absent in primary 15 non-diabetic controls, were then subjected to genotyping in additional 50 non-diabetic controls, by polymerase change reaction-restriction fragment length polymorphism (PCR-RFLP). In case that there was no change in site of digestion by any enzyme, new PCR primers containing introduced restriction sites were designed by using dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html), in order to perform mismatch-RFLP. Variation that was not presented in non-diabetic controls was classified as a potential pathogenic mutation and was subjected further for segregation test with diabetes in the families.

Statistical analysis

The χ^2-test with Yates' correction for continuity was used for testing the significance of difference and results were considered to be significantly different if $p<0.05$ (Statistics Package for Social Sciences, Chicago, IL, USA).

Results

The clinical characteristics of the probands were summarized in Table 1. The probands with classic MODY who were diagnosed at earlier ages than those of the patients with early-onset type 2 diabetes (T2D) had lower BMI and W/H ratio, although these were not reach statistically significant. The diastolic blood pressure was significantly lower in the classic MODY probands. Examination of laboratory parameters revealed that overall, glycemic control was poorer but lipid profiles were better in the classic MODY probands. The proportion of patients put on insulin therapy was slightly higher in the classic MODY probands compare to the early-onset T2D patients.

Among 37 variations that have been identified (Table 2), thirteen have not been previously reported. Seventeen of all variations were not identified in the group of 15 non-diabetic controls. These included *HNF-4α* R140Q and *GCK* R312H, -30G>A, IVS+29G>T, and R327H, *HNF-1α* R203C, L281L, IVS5+9C>G, L459L, P475L, IVS7+65G>C, IVS7+68A>G, IVS8-19G>A, and G554SfsX3, *HNF-1β* D82D, *NeuroD1* -303 G>A, and A322N. Some of these variations, excluding the silent and intronic changes unlikely to be non-pathogenic single nucleotide polymorphisms (SNPs), were then subjected to genotyping in additional 50 non-diabetic controls. Mutations which were not identified in additional non-diabetic controls were classified as possible pathogenic mutations (Figure 1 and Table 3). Two of these including *HNF-4α* R312H and *HNF-1α* R203C were previously reported in other population (Ellard *et al*, unplished data; Yamada *et al*. 1999). None of possible pathogenic mutation was found in *IPF-1* and *HNF-1β* genes.

Five missense mutations caused changes of amino acids which are conserved across several species (Figure 2). We investigated whether these mutations segregated with diabetes in the families (Figure 3). We found linkage between *HNF-1α* R203C and *HNF-1α* G554SfsX3 with diabetic phenotype, whereas *NeuroD1* A322N did not. Unfortunately,

families with probands carried other possible pathogenic mutations could not be recruited for segregation analysis at this time.

Discussion

MODY is an unusual monogenic form of type 2 diabetes characterized by early-age onset and autosomal dominant inheritance. To date, mutations in any one of six genes are described as a cause of MODY, including *HNF-4α* (*MODY1*), *GCK* (*MODY2*), *HNF-1α* (*MODY3*), *IPF-1 (MODY4*), *HNF-1β (MODY5*), and *NeuroD11* (*MODY6*) (Fajans *et al*. 2001). The nature and position of different mutations in these genes could explain diverse phenotypic presentations of diabetes, ranging form mild hyperglycemia throughout life to progressive insulinopenia and increasing requirement for insulin replacement over time (Winter 2003).

Here, we have screened all 6 MODY genes in 30 unrelated early-onset type 2 diabetic probands and 21 probands whom well suited with classic MODY criteria. By using PCR-SSCP analysis, seven mutations that might be potentially classified as pathogenic mutations were identified (Table 3), in which five of them have not been reported previously. These possible pathogenic mutations are either non-synonymous mutations or mutation located in regulatory regions, which were absent in 65 non-diabetic control subjects.

Only one mutation within *HNF-4α* (MODY1) gene was found in one proband, whose family members could not be recruited to test segregation with diabetes. The missense R312H mutation in exon 8 of *HNF-4α* was previously identified in two Caucasian MODY families by Ellard *et al* (unplished data). *HNF-4α* is a nuclear receptor that essential for development in organisms ranging from insects to mammals, and regulates many essential genes related to nutrient transport and metabolism (Wang *et al*. 2000). Arginine at position 312 of HNF-4α is conserved across 9 species, from worm to human (Figure 2a). This amino acid residue is located in helix 9 of ligand-binding domain (LBD) of human HNF-4α, which involves in not only ligand binding but also protein dimerization (Bogan *et al*. 2000), alteration of polar positive charged of arginine to polar uncharged of histidine could therefore

potentially affect many aspects of receptor function, such as DNA binding protein stability, ligand binding and interaction with co-regulatory molecules.

One novel missense R327H mutation was found in exon 8 of *GCK* (MODY2) gene. In our study, patient carried *GCK* R327H exhibited clinical characteristics in respect to MODY2 phenotypes including the presence of mild hyperglycemia (135 mg/dl) and upper normal range of HbA1C level (6.7%) which were successfully controlled with diet alone. The effect of GCK mutation is difficult to predict, base on its location or the nature of amino acid change (Gidh-Jain *et al*. 1993; Mahalingam *et al*. 1999; Velho *et al.* 1997). Therefore, both enzymatic activity of the mutant protein and the affinity of mutant enzyme to glucose should be further investigated.

Sequence variations of *HNF-1α* (MODY3) gene were more common among Thai MODY patients as compared to those of other known MODY genes. One of the three identified mutations, *HNF-1α* R203C in exon 3, was previously reported in 2 families, one from Denmark (Johansen *et al*. 2005) and another from Japan (Yamada *et al.* 1999). However, the familial segregation study has not been performed in these two reports yet. Herein, for the first time, we demonstrated the segregation of this heterozygous mutation with diabetes within a family (Figure 3a). Arginine residue at position 203 of HNF-1α is conserved among 5 species, including chicken, rat, mouse, dog, and human (Figure 2c), and located within DNA binding domain (DBD), alteration of arginine to cystein should therefore affect its transactivation activity. Nevertheless, functional analysis of mutant protein (Yamada *et al*. 1999) revealed biphasic activity, in which decreasing of activity was observed at low DNA concentration, whereas gaining of function was performed by high level of transfected mutant DNA. In addition, mutant protein showed weak but positive signal of nuclear localizing defects, which could be involved, at least in part, in biphasic effect on transactivation activity. Thus, the precise mechanism of R203C in contribution to etiology of

MODY3 should be further elucidated. The other two identified mutations, *HNF-1α* P475L in exon 7 and *HNF-1α* G554SfsX3 in exon 9, were novel. Both mutations occur at transctivation domain of HNF-1α. Proline at position 475 is also conserved across 5 species (Figure 2d). Substitution of a distinctive cyclic structure of proline residue, whose secondary imino group is usually held in a rigid conformation that reduces the structural flexibility of polypeptide region, with leucine would affect conformation of protein. Fourteen nucleotide insertions at codon 554 result in addition of codons for 2 amino acids followed by a stop codon. The mutant polypeptide has 77-amino-acid truncation, comparing with the wild type protein. The effect of HNF-1α G554SfsX3 on intrinsic transctivation is currently being investigated in our laboratory.

Two novel mutations of *NeuroD11* (MODY6) gene, -303G>A and A322N, were identified in this study. Nucleotide change at position -303 is located in highly conserved promoter region of human and mouse *NeuroD11* gene, which was identified as necessary region for basal transcriptional activation (Miyachi *et al.* 1999). Therefore, nucleotide alteration in such promoter region might affect expression level of this transcription factor. Another mutation, A322N, is located in a region that associated with the co-activators CBP and p300. Alteration from hydrophobic and non-polar of alanine residue to be polar residue of asparagine might affect stability of protein, and might influence on association with co-activator proteins. Interestingly, two probands carried -303 G>A and A322N in *NeuroD11* exhibited more hyperglycemia (271 and 320 mg/dl, respectively), compared to probands carried mutations in other MODY genes. However, segregation study could not established association of A332N with diabetes in the family (Figure 3c). Thus, the role of this mutation in glucose homeostasis remains to be explored.

In summary, this is the first report on mutations of MODY genes in Thai patients with MODY and early-onset T2D. Identification of these possible pathogenic mutations in known

MODY genes accounts for a small proportion of both classic MODY (19%) and early-onset T2D (10%). We concluded that the genetic defects in known MODY genes are not a common cause of MODY and early-onset T2D in Thais. Our results are consistent with those of the Japanese (Nishigori *et al*. 1998), and the Chinese (Ng *et al*. 1999), in that genetic variability of known MODY genes contributed to diabetes in a small proportion of MODY families being studied. Thus, there are unidentified genes awaiting for discovery in the majority of MODY patients in Asian. Identification of these novel genes will facilitate better understanding of molecular mechanisms underlying pathogenesis of, not only MODY but also T2D which may lead to the development of more rationale preventative and therapeutic strategies.

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Figure legends

Figure 1 Mobility shifts of DNA fragments observed in the PCR-SSCP analysis and their sequencing profiles.

Figure 2 Multiple sequence alignments of parts of proteins encoded by *HNF-4α*, *GCK*, *HNF-1α*, and *NeuroD11* from different species, showing conserved amino acids where mutations occurred.

Figure 3 Pedigrees of families with the probands carried mutations: (a) *HNF-1α* R203C, (b) *HNF-1α* R203C, and (c) *NeuroD11* A322N.

Table 1 Clinical characteristics of the patients with MODY and early-onset T2D.

	MODY*	Early-onset T2D*	P value
Number**	12	17	-
Age (years)	21.75 ± 9.66	36.88 ± 7.03	<0.001
Age at onset (years)	16.00 ± 5.06	31.25 ± 5.83	<0.001
Duration (years)	5.75 ± 7.47	5.75 ± 5.32	0.556
BMI (kg/m^2)	25.32 ± 6.27	28.95 ± 7.16	0.175
Waist (cm)	81.13 ± 10.65	89.31 ± 13.27	0.091
Hip (cm)	95.96 ± 8.06	100.38 ± 5.20	0.090
Waist/Hip ratio	0.85 ± 0.08	0.89 ± 0.12	0.287
Systolic BP (mmHg)	116.25 ± 13.72	120.69 ± 9.21	0.312
Diastolic BP (mmHg)	70.42 ± 8.73	79.00 ± 9.24	0.020
FPG (mg/dl)	213.25 ± 83.88	197.06 ± 66.37	0.573
HbA1c (%)	10.02 ± 4.03	8.21 ± 2.01	0.131
Serum creatinine (mg/dl)	0.96 ± 0.89	0.72 ± 0.29	0.297
Total Cholesterol (mg/dl)	188.42 ± 55.15	218.06 ± 46.62	0.032
Triglyceride (mg/dl)	166.08 ± 133.54	244.25 ± 161.16	0.216
LDL (mg/dl)	121.24 ± 48.55	136.31 ± 43.33	0.279
HDL (mg/dl)	42.33 ± 9.93	45.44 ± 9.16	0.391
Current therapy:			
Diet	25%	23.08%	
OHA	66.67%	78.57%	
Insulin	33.33%	28.57%	
AHT	8.33%	28.57%	
AHL	16.67%	28.57%	

* Values are expressed as mean ± S.D., except for the current therapy which were present as percentages. ** The number of patients whose clinical characteristics were completely available. OHA, AHT, and AHL denote oral hypoglycemic agents, anti-hypertensive agents, and anti-hyperlipidemic agents, respectively.

Table 2 Sequence variations identified in Thai subjects, including 51 diabetic patients and either 15 or 65 non-diabetic controls

Gene	Location	Nucleotide change	Designation	Allele frequency
HNF-4α (MODY1)	Promoter	A>C	-639A>C	A 0.69, C 0.31
	Intron 1	C>T	IVS1-5 C>T	C 0.88, T 0.12
	Exon 4	ACT>ATT	T139I	C 0.99, T 0.01
	Exon 4	CGA>CAA	R140Q[a]	G 0.99, A 0.01
	Exon 8	**CGT>CAT**	**R312H**	**G 0.996; A 0.004**
GCK (MODY2)	Promoter	G>A	-30G>A	G 0.97, A 0.03
	Promoter	A>G	-194A>G	A 0.96, G 0.04
	Intron 5	G>T	IVS5+29 G>T[a]	G 0.99; T 0.01
	Exon 8	**CGC>CAC**	**R327H[a]**	**G 0.996; A 0.004**
	Intron 9	C>T	IVS9+8 C>T	C 0.58, T 0.42
HNF-1α (MODY3)	Exon 1	CTC>CTG	L17L	C 0.72, G 0.28
	Exon 1	ATC>CTC	I27L	A 0.73, C 0.27
	Intron 1	G>A	IVS1-42G>A	G 0.68, A 0.32
	Intron 2	T>A	IVS2-51 T>A	T 0.58, A 0.42
	Exon 3	**CGT>TGT**	**R203C**	**C 0.996; T 0.004**
	Exon 4	CTG>TTG	L281L	C 0.99; T 0.01
	Exon 4	GGG>GGC	G288G[a]	G 0.98, C 0.02
	Intron 5	C>G	IVS5+9 C> G	C 0.99; G 0.01
	Intron 5	G>T	IVS5-42 G>T	G 0.86, T 0.14
	Exon 4	CTG>CTA	L459L[a]	G 0.99; A 0.01
	Exon 7	CTG>TTG	L459L	C 0.67, T 0.33
	Exon 7	**CCG>CTG**	**P475L[a]**	C 0.99; T 0.01
	Exon 7	AGC>AAC	S487N	G 0.67, A 0.33
	Intron 7	G>A	IVS7+7 G>A	G 0.65, A 0.35
	Intron 7	G>C	IVS7+65 G>C[a]	C 0.99; G 0.01
	Intron 7	A>G	IVS7+68 A>G[a]	A 0.99; G 0.01
	Intron 8	G>A	IVS8-19 G>A[a]	G 0.99; A 0.01
	Exon 9	**Insertion 14 nt**	**G554SfsX3[a]**	**wt 0.996; ins 0.004**
	Intron 9	T>C	IVS9-24 T>C	T 0.64, C 0.36
IPF-1 (MODY4)	Promoter	G deletion	-10delG	G 0.71, delG 0.29
	Enhancer	G>A	-1768 G>A	G 0.64, A 0.36
HNF-1β (MODY5)	Exon 1	GAC>GAT	D82D[a]	C 0.99; T 0.01
	Intron 8	C>T	IVS8-22 C>T	C 0.96, T 0.04
NeuroD1	Promoter	A>G	-36A>G	A 0.81, G 0.19
	Promoter	**G>A**	**-303G>A[a]**	**G 0.996; A 0.004**
	Exon 2	GCC>ACC	A45T	G 0.84, A 0.16
	Exon 2	**GCT>AAT**	**A322N[a]**	**GC 0.996; AA 0.004**

Mutations which were not found in 130 non-diabetic chromosomes were classified as pathogenic mutations, as represented with bold characters.

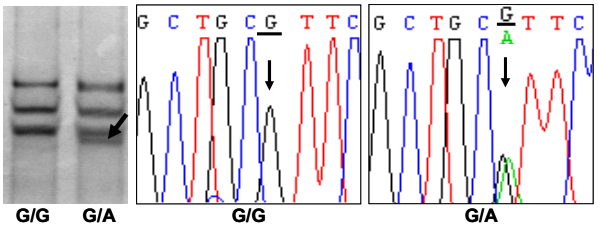
[a] Variations which were not previously reported

Table 3 Missense and 5'UTR mutations obtained from six MODY genes.

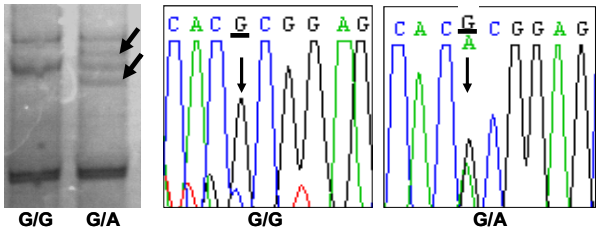
Gene	Location	Nucleotide change	Designation	Family	Subject	Linkage to disease
HNF-4α (MODY1)	Exon 8	CGT>CAT	R312H	F019	M19	ND
GCK (MODY2)	Exon 8	CGC>CAC	R327H[a]	F049	M49	ND
HNF-1α (MODY3)	Exon 3	CGT>TGT	R203C	F043	M43	Yes
	Exon 7	CCG>CTG	P475L[a]	F022	M22	ND
	Exon 9	Insertion 14	G554SfsX3[a]	F027	M27	Yes
NeuroD1 (MODY6)	Promoter	G>A	-303G>A[a]	F036	M36	ND
	Exon 2	GCT>AAT	A322N[a]	F050	M50	No

[a] Mutations that have not previously been reported.

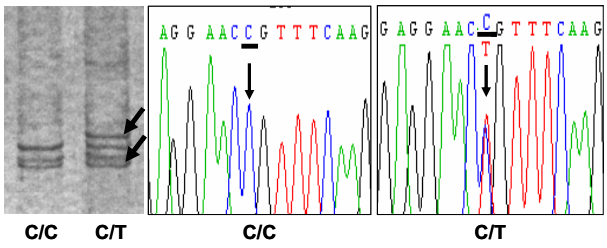
a) *HNF-4α* CGT>CAT (R312H)



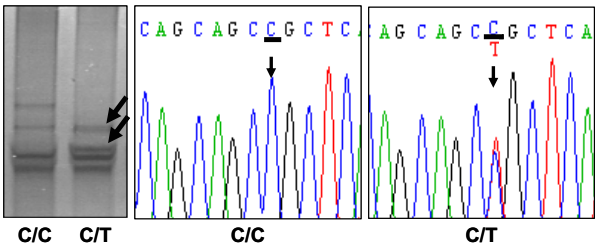
b) *GCK* CGC>CAC (R327H)



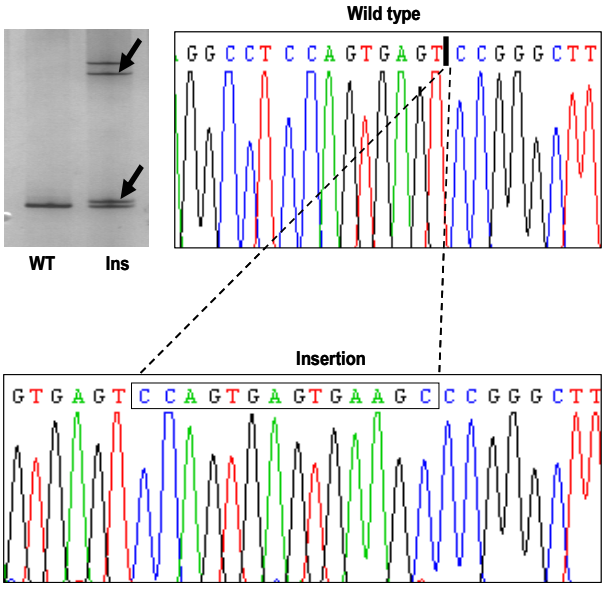
c) *HNF-1α* CGT>TGT (R203H)



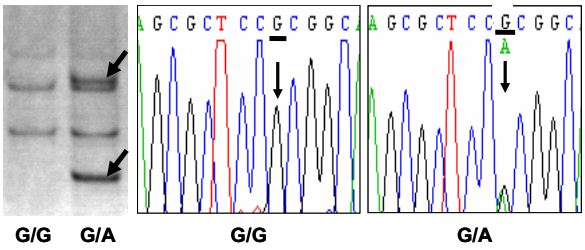
d) *HNF-1α* CCG>CTG (P475L)



e) *HNF-1α* G554SfsX3



f) *NeuroD1* -303G>A



g) *NeuroD1* GCT>AAT (A322N)

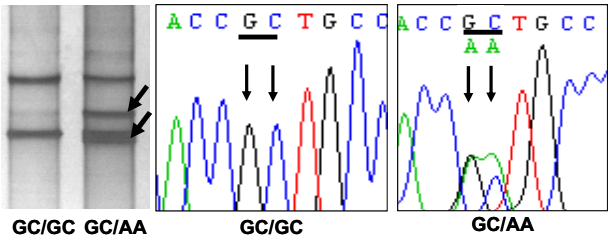
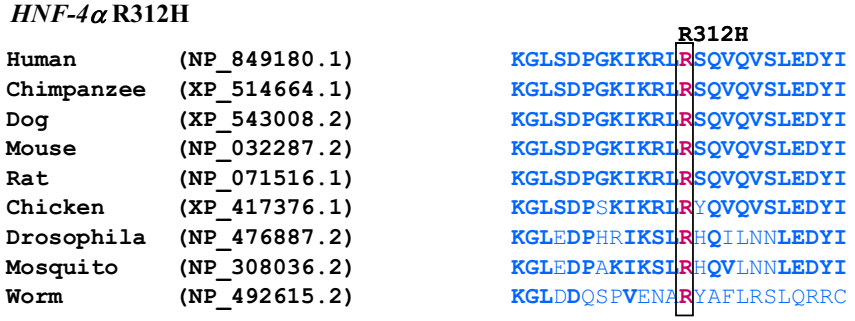
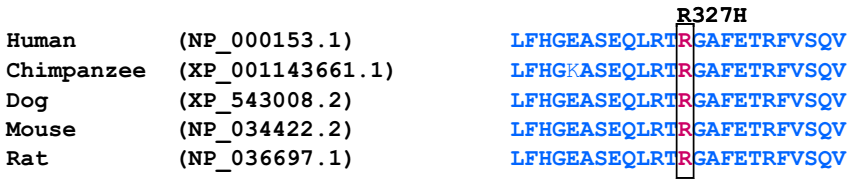


Figure 1

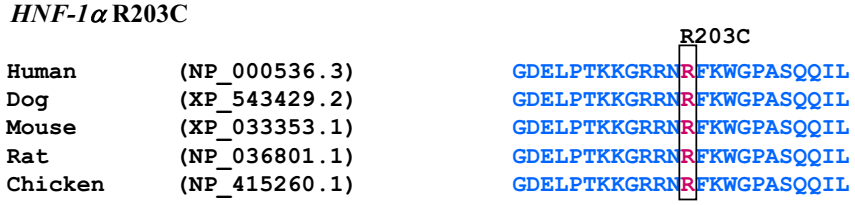
a) ***HNF-4α*** **R312H**



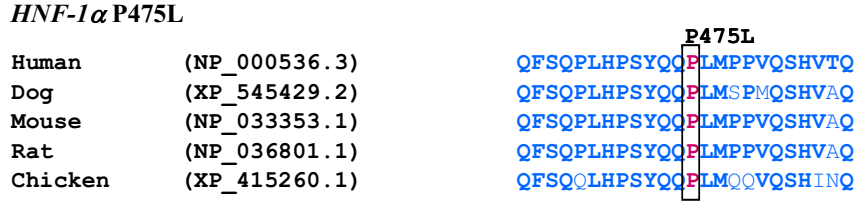
b) ***GCK*** **R327H**



c) ***HNF-1α*** **R203C**



d) ***HNF-1α*** **P475L**



e) ***NeuroD1*** **A322N**

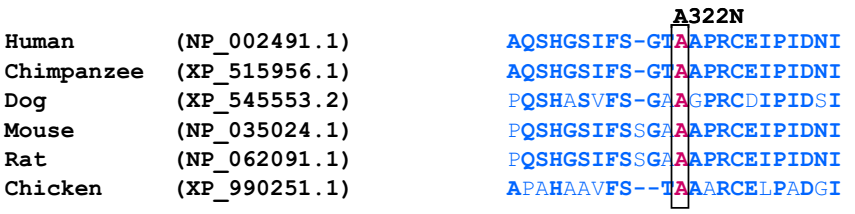
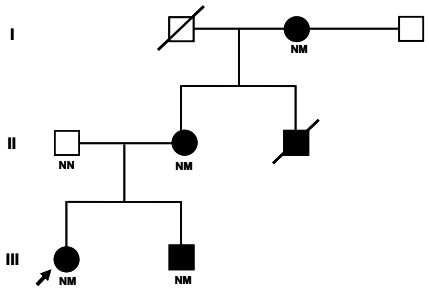
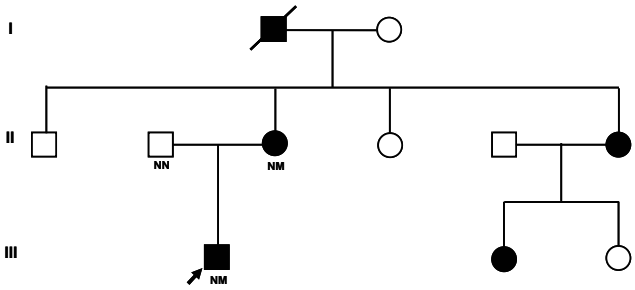


Figure 2

a) ***HNF-1α*** **R203C**



b) ***HNF-1α*** **G554SfsX3**



c) ***NeuroD1*** **A322N**

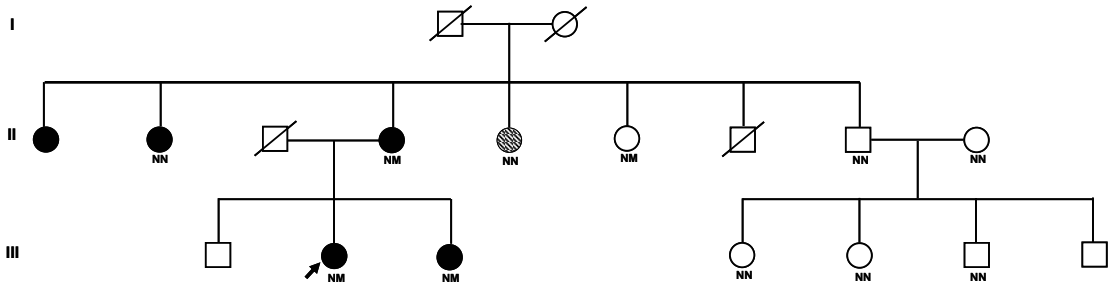


Figure 3

PAX4 Mutations in Thais with Maturity-Onset Diabetes of the Young (MODY)

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Abbreviations: PCR-SSCP, Polymerase chain reaction-single stranded conformational polymorphism; PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism

SNP, single-nucleotide polymorphism; BMI, Body mass index ; FPG, fasting plasma glucose; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Keywords: MODY, Pancreatic beta-cell transcription factors, PAX4, Single stranded conformational polymorphism, Thais

Word count: 1,835

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Context: Six 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 genetic variants in *PAX4*, a crucial transcription factor in β-cells 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 non-diabetic controls using PCR-SSCP analysis followed by direct sequencing. Genotyping of variants identified was done by PCR-RFLP.

Results: Eight sequence differences were identified. Two novel variations (R164W and IVS7-1G>A) were found in two different probands. None was found in the 74 non-diabetic controls and in additional 270 healthy subjects of Thai origin. The R164W segregated with diabetes in the family 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 were observed in both groups. One of them, the R192H, was three times as frequent in MODY probands than in the 74 non-diabetic controls (minor allele frequency [MAF]=0.185 vs. 0.061, $p<0.01$). A similar difference was observed when 268 additional healthy subjects of Thai origin were added to the control group (MAF=0.196 vs 0.064, $p<0.00001$).

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. SNP R192H might influence the risk of diabetes development.

MODY is a genetically heterogeneous form of diabetes characterized by an early onset (usually before 25 years), 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 (GCK; MODY2) (2), the other five for transcription factors expressed in pancreatic β-cells, namely HNF-4α (MODY1) (3), HNF-1α (MODY3) (4), IPF-1 (MODY4) (5), HNF-1β (MODY5) (6), and NeuroD1 (MODY6) (7). The observation of forms of familial diabetes that fit the MODY criteria but are not linked 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* R200Q), indicating that mutations in unidentified MODY 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 play a critical role in pancreatic β-cell development and function (14). Pax4 first appears in the endocrine progenitor cells at embryonic day 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 do not exhibit any obvious abnormalities and survive to adulthood, but 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 IPF1 – a known MODY gene (16). Pax4 also appears to be important for the regeneration of β-cell

in adult life, as suggested by the finding that Pax4 mutations impair the ability of β-cells to proliferate (17).

In this study, we investigated whether sequence variants in *PAX4* contribute to MODY in the Thai population. We screened this gene for mutations in 46 MODY probands and in non-diabetic controls by using PCR-SSCP analysis followed by direct sequencing of bands showing abnormal mobility.

Research Design and Methods

Study subjects

The study included 46 diabetic probands of MODY families recruited at the Diabetic Clinic, Siriraj Hospital, Bangkok, Thailand (Table1). The inclusion criteria were *(i)* the proband and at least one first degree relative diagnosed with type 2 diabetes before age 35, *(ii)* two or more generations affected by diabetes, *(iii)* diabetes treatment with diet and/or oral agents, *(iv)* no history of diabetic ketoacidosis (DKA), and *(v)* absence of anti-GAD antibody. Mutations in any of the six known MODY genes were excluded by PCR-SSCP analysis, followed by direct sequencing. Non-diabetic subjects were 74 healthy staff members in the Department of Immunology and Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. All of them had FPG level less than 100 mg/dl, and had no family history of diabetes in first-degree relatives. Approval for the study was granted by the Faculty of Medicine Siriraj Hospital Research Ethics Committee. All subjects were informed of the purpose and extent of the study, and signed a consent form before their enrollment to indicate their willingness to participate to this study.

Mutation screening and sequence analysis of *PAX4*

The exons and exon - intron boundaries of *PAX4* were screened for nucleotide variants in the 46 MODY probands and 74 non-diabetic subjects. DNA fragments were amplified by PCR using standard conditions. PCR primers were designed by means of the MacVector software version 4.5.3 (Kodak, Scientific Imaging Systems, CT, USA). The PCR products were screened for sequence variation by SSCP analysis using standard protocol. PCR products showing a mobility shift on the SSCP gel were subjected to directly sequenced by means of the ABI Prism BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA). Fluorescent signals were detected with the ABI Collection software. Nucleotide sequences were determined by the Sequencer Navigator software and analyzed by means of the Chromas program version 1.4.4 (Conor McCarthy, Griffith University, Queensland, Australia).

Genotyping of *PAX4* variants

The R164W and IVS7-1G→A variants were genotyped in additional 270 non-diabetic subjects, and the R192H variant in additional 268 non-diabetic subjects by PCR-RFLP using the enzymes *Hae*III, *Bsr*I, and *Mse*I (Fermentas Inc., Hanover, MD, USA), respectively. DNA fragments were separated on 12% polyacrylamide gels visualized by silver staining or 2% agarose gels visualized by ethidium bromide staining. Genotype and allele frequencies of each variant were compared in MODY probands and non-diabetic subjects by means of Chi-square tests with Yates' correction for continuity. For rare alleles Fisher exact test was used for comparison. P-values less than 0.05 were considered to be significant (Statistics Package for Social Sciences, IL, USA).

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., Singapore) by PCR using platinum *Pfx* DNA polymerase (Invitrogen, Leek, Netherlands). The cDNA was then subcloned into a pcDNA 3.1 expression vector for transient transfection. The R164W mutation was introduced by site-directed mutagenesis (QuickChange Mutagenesis Kit, Stratagene, La Jolla, CA, USA) to generate pcDNA3.1-Pax4-R164W. Human insulin and glucagons promoters were isolated by PCR using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) 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 MIN 6 or αTC-1.6 cells using the FUGENE 6 transfection reagent (Roche Diagnostics, Roche Applied Science, IN, USA) along with 100 ng of pGL3-human insulin promoter, and 10 ng of pRL-SV40 (to control for the transfection efficiency). The final amount of DNA in each transfection was adjusted to 500 ng by adding the appropriate amount of pcDNA3.1 DNA. After 24 h, the transactivation activity of the normal and mutant Pax4 proteins was measured by means of the Dual-Luciferase Reporter Assay System (Promega Corp., WI, USA). The mean ± SD of luciferase activity was calculated and the significance of differences was tested by one-way ANOVA followed by Sheffe`s post-hoc test. A *P* value smaller than 0.05 was considered as significant.

Results and Discussions

A total of eight sequence differences were identified (Table 2). Two were novel variants that were found in heterozygosis 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) (Figure 1a). Neither mutation was found in the 74 non-diabetic controls and in additional 270 non-diabetic subjects of Thai origin. The R164W mutation segregated with diabetes, being present in the proband's 52-year-old father who was diagnosed diabetes at age 50 years and 29-year-old sister, who both had type 2 diabetes, as well as in her 14-year-old brother, who had impaired glucose tolerance (Figure 1b). However, two sisters (28 and 22 years old), who also had impaired glucose tolerance, did not carry the mutation. No relatives were available for the segregation analysis of the IVS7-1G>A mutation with diabetes.

The remaining six variants were previously described SNPs that were observed in both MODY probands and non-diabetic control (R31Q, Q173Q, R183C, R192S, R192H and P321H) (Table 2). The R192H variant was three times as frequent in the 46 MODY probands than in the 74 non-diabetic controls (minor allele frequency [MAF]=0.185 vs. 0.061, $p<0.01$). A similar difference was observed when 268 additional non-diabetic subjects were added to the control group (MAF= 0.064 in 342 non-diabetic subjects, $p<0.00001$) for the comparison with MODY probands). As compared to non-carriers, minor allele carriers had a 3.8 fold increase in the odds of being in the MODY group rather than in the control group (95% CI= 1.9-7.6).

Pax4 represses the activity of the insulin and glucagons promoters (12). To assess whether the R164W mutation affects such function, we transiently transfected MIN 6 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 % (Figure 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 (Figure 2c). The Pax4 wild type repressed the promoter activity by 57%, whereas the

164W 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: *(i)* it is extremely rare, being not observed in 688 non-diabetic chromosomes; *(ii)* it segregates with diabetes in the proband's family; (iii) it is placed in the homeodomain, which is responsible for Pax4 binding to target DNA sequences; (iv) it concerns an aminoacid residue that is conserved across humans, chimpanzees, mice, rats, fruit flies, and mosquitos ; (v) the mutation is rather severe, causing the replacement of a polar with a non-polar amino acid; *(vi) in vitro* studies show that this aminoacid substitution impairs the repressor activity of Pax4 on the insulin and glucagon promoters. The evidence is not as abundant for the IVS7-1G>A variants. On the other hand, it abolishes the acceptor splice site of intron 7 potentially leading to exon skipping, intron retention, or usage of another acceptor splice site that further proof of its role as a pathogenic variant might not be necessary.

We also found that a relatively common polymorphism (R192H) was overrepresented in MODY probands as compared to non-diabetic controls. While highly significant, this result should be taken with caution. First, it concerns only 46 MODY cases. Second, a previous study from Japan has failed to demonstrate an association between this polymorphism and type 2 diabetes (18). It is possible that the R192H is simply a marker of other polymorphisms and that differences between Thais and Japanese in the linkage disequilibrium structure of this region are responsible for the different results in the two populations. However, the association between R192H and MODY should be replicated in another group of Thai subjects before it can be considered as genuine and the functional relevance of this SNP needs to be assessed by in vitro studies.

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.

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Table 1 Clinical Characteristics of MODY Probands and Non-diabetic Controls

	Non-diabetic controls (Mean ± SD)	MODY patients (Mean ± SD)
Age (years)	26.56 ± 9.25	33.49 ± 12.19
Age at onset (years)	-	28.00 ± 8.65
Duration (years)	-	5.58 ± 9.15
BMI (kg/m^2)	20.14 ± 3.37	26.31 ± 5.73
Waist (cm)	71.63 ± 9.30	84.98 ± 10.23
Hip (cm)	90.30 ± 7.83	98.77 ± 7.14
Waist/Hip ratio	0.79 ± 0.06	0.86 ± 0.08
Systolic BP (mmHg)	-	119.89 ± 14.23
Diastolic BP (mmHg)	-	79.67 ± 8.49
FPG (mg/dl)	83.85 ± 7.30	218.42 ± 80.79
HbA1c (%)	-	9.75 ± 3.44
Serum creatinine (mg/dl)	-	1.02 ± 3.73
Total Cholesterol (mg/dl)	-	215.30 ± 61.06
Triglyceride (mg/dl)	-	226.90 ± 146.80
LDL (mg/dl)	-	125.81 ± 47.65
HDL (mg/dl)	-	44.10 ± 12.85

Location	Codon	Nucleotide change	Designation	Genotype frequency						Allele frequency		*P* - value
				MODY ($n = 46$)			Nondiabetic[#]			MODY	Nondiabetic	
Exon1	31	CGG > CAG	R31Q	G/G	G/A	A/A	G/G	G/A	A/A	G: 0.99	G: 1.00	NS*
				45	1	0	74	0	0	A: 0.01	A: 0.00	
				0.98	0.02	0.00	1.00	0.00	0.00			
Exon4	164	CGG > TGG	R164W	C/C	C/T	T/T	C/C	C/T	T/T	C: 0.99	C: 1.00	NS*
				45	1	0	344	0	0	T: 0.01	T: 0.00	
				0.98	0.02	0.00	1.00	0.00	0.00			
	173	CAA > CAG	Q173Q	A/A	A/G	G/G	A/A	A/G	G/G	A: 0.98	A: 0.99	NS*
				44	2	0	73	1	0	G: 0.02	G: 0.01	
				0.96	0.04	0.00	0.99	0.01	0.00			
Exon5	183	CGT > TGT	R183C	C/C	C/T	T/T	C/C	C/T	T/T	C: 0.99	C: 0.99	NS*
				45	1	0	73	1	0	T: 0.01	T: 0.01	
				0.98	0.02	0.00	0.99	0.01	0.00			
	192	CGT > AGT	R192S	C/C	C/A	A/A	C/C	C/A	A/A	C: 0.99	C: 0.98	NS*
				45	1	0	72	2	0	A: 0.01	A: 0.02	
				0.98	0.02	0.00	0.97	0.30	0.00			
	192	CGT > CAT	R192H	G/G	G/A	A/A	G/G	G/A	A/A	G: 0.800	G: 0.940	<0.00001**
				30	14	2	300	40	2	A: 0.196	A: 0.064	
				0.67	0.29	0.04	0.88	0.12	0.00			
Intron7	nt-1	AG > AA	IVS7-1 G>A	G/G	G/A	A/A	G/G	G/A	A/A	G: 0.99	G: 1.00	NS*
				45	1	0	344	0	0	A: 0.01	A: 0.00	
				0.98	0.02	0.00	1.00	0.00	0.00			
Exon9	321	CCC > CAC	P321H	C/C	C/A	A/A	C/C	C/A	A/A	C: 0.36	C: 0.40	NS**
				5	23	18	10	39	25	A: 0.64	A: 0.60	
				0.11	0.50	0.39	0.14	0.52	0.34			

Table 2 Summary of the *PAX4* variants

[#]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.

* Fisher's exact test ** Chi-square test

Figure 1a 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 show 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)

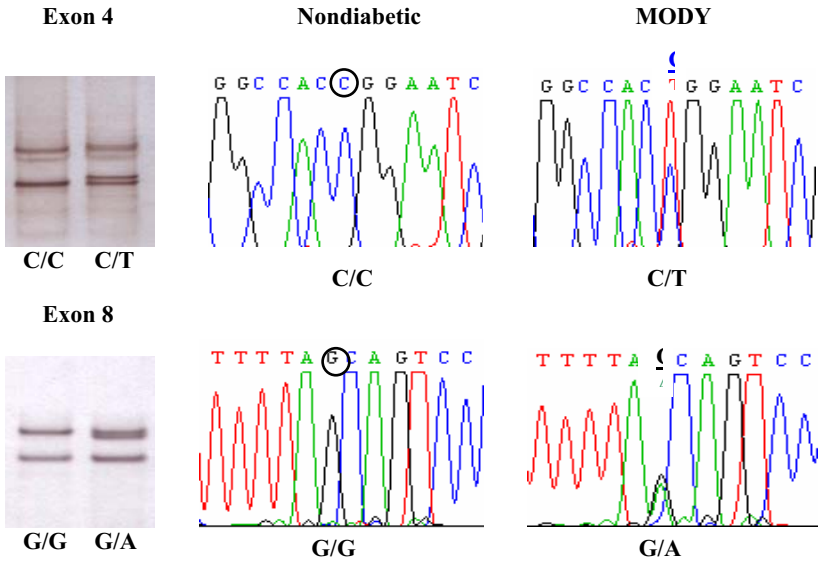

Figure 1b Pedigree of family whose proband carries the *PAX4* R164W mutation. Symbols indicate the state of glucose tolerance: ○ and □, normal fasting glucose; ⊘ and ▨ ,impaired glucose tolerance; ● and ■, diabetes; ○ and □ with "nd", unknown.

The genotypes are indicated under the symbol: NN, normal homozygote; NM, heterozygote; nd, not done. An arrow indicates the proband. Age (in years) is shown in the upper right side of each symbol. ex.; examination. dx; diagnosis. Tx; treatment. OHA; oral hypoglycemic agent.

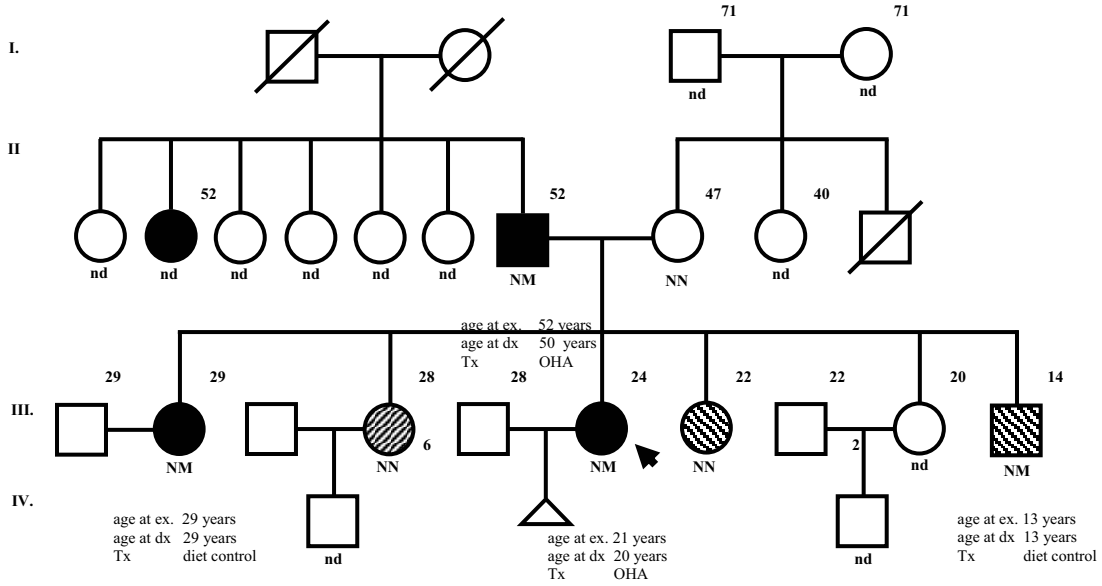

Figure 2a

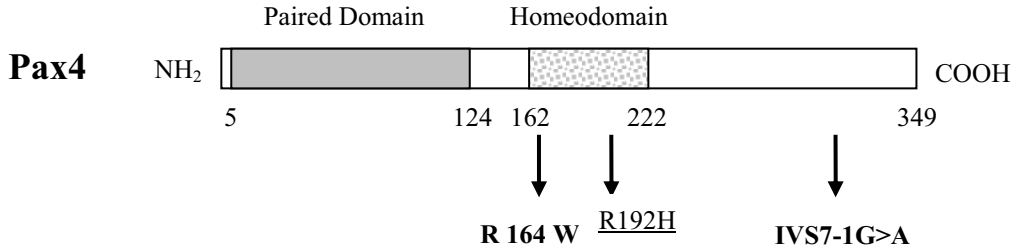

Figure 2b

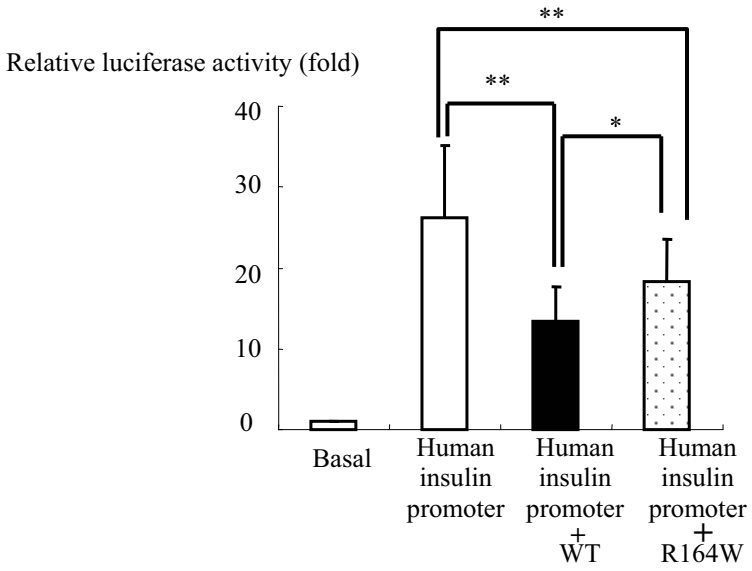

Figure 2c

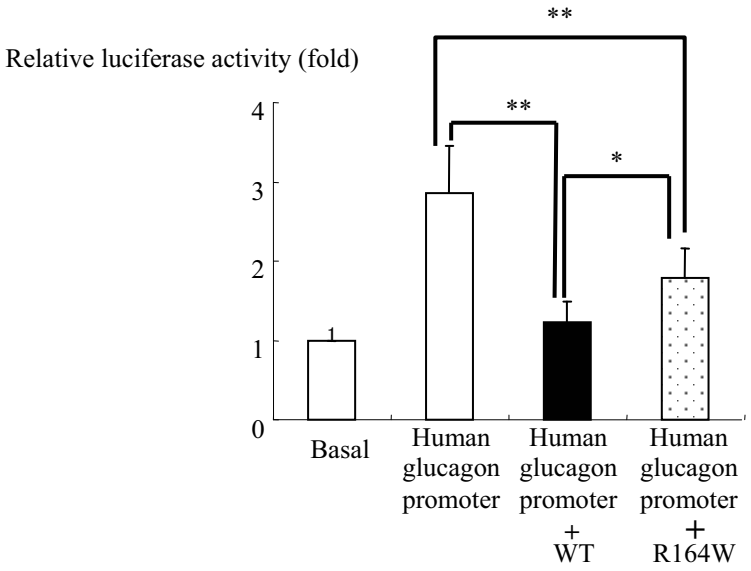

Figure 2a Schematic representation of the Pax4 protein structure. The R164W and IVS7-1G>A variants that are expected to be pathogenic are shown in bold letters. The SNP R192H (in underline) showed high frequencies with significant difference between MODY probands and non-diabetic subjects. Effect of Pax4 mutation on luciferase activity in MIN6 and α-TC1.6 cells. MIN 6 and α-TC1.6 cells were transfected with 0.5 mg of human wild type Pax4 and R164W mutant and 0.5 mg of human insulin **(2b)** and glucagon **(2c)** promoter reporter genes, respectively, together with 10 ng of pRL-SV40 internal control vector. Data are expressed as mean ± SD, N = 6, three times. *$p < 0.01$ and ** $p < 0.001$.