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Genetic causes of MODY (Maturity-Onset Diabetes of the Young) — prevalence of mutations in the MODY genes in the Czech diabetic and nondiabetic populations

Ph.D. thesis summary

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1. INTRODUCTION

Diabetes mellitus is one of the longest known diseases. The first allusion dates back to the middle of the second millennium B.C. to the ancient Egypt. At present diabetes mellitus is one of the most world widely common illnesses.

MODY (maturity-onset diabetes of the young) was initially defined as a subtype of type 2 diabetes with polygenic background. It is now clear, that MODY is a group of heterogeneous monogenic disorders and it takes about 5 % of all diabetics. The revised ADA (American Diabetic Association) classification categorized MODY under "other specific forms of diabetes mellitus — genetic defects of β-cell function" (The Expert Committee 1999), which is characterized by mild, persistent fasting hyperglycaemia, low glucose-stimulated insulin secretion (GSIR), autosomal dominant inheritance and early onset (at least one affected family member with an onset before 25 years of age) ((Fajans 1990).

To date six subtypes of MODY resulting from mutations in six different genes were identified: MODY 1 = *HNF-4α*, MODY 2 = *GCK*, MODY 3 = *HNF-1α*, MODY 4 = *IPF-1*, MODY 5 = *HNF-1β* and MODY 6 = *NeuroD1* (Tab. 1) (Shih and Stoffel 2002).

The most common forms of the disease are MODY2 and MODY3, which account for 20-65 % of the all MODY subtypes in Europe (Frayling et al. 2001, Pruhova et al. 2003). Mutations in the MODY1 gene are less frequent and may account for 5 % of subject with MODY (Frayling et al. 2001, Pruhova et al. 2003), while MODY4-6 are very rare (Fajans et al. 2001, Frayling et al. 2001)

In 15-20 % of MODY any association with known MODY gene locus were detected suggesting the existence of other still unrecognised gene(s) that cause(s) MODYx (Chévre et al. 1998).

Tab. 1: Comparison of the different MODY subtypes

	MODY 1 *HNF-4α*	MODY 2 *GCK*	MODY 3 *HNF-1α*	MODY 4 *IPF-1*	MODY 5 *HNF-1β*	MODY 6 *NeuroD1*
Chromosomal location	20q12-q13.1	7p15-13	12q24.1	13q12.1	17cen-q21.3	2q32
Frequency	low (< 5 %)	high (15 – 60 %)	high (30 – 75 %)	very low (< 4 %)	very low (< 1 %)	very low
Onset of hyperglycaemia	adolescence, early adulthood (< 30 let)	childhood (from birth)	adolescence (< 25 let)	early adulthood (< 35 let)	adolescence (< 25 let)	adulthood (< 40 let)
Primary defect	pancreas/ liver/ kidney	pancreas/ liver/ gut/ brain	pancreas/ liver/ kidney	pancreas/ other?	pancreas/ kidney/ other?	pancreas/ gut/ brain
Function of the gene product	transcriptional factor	enzyme	transcriptional factor	transcriptional factor	transcriptional factor	transcriptional factor
Action at insulin gene	direct, indirect (through HNF-1α)	–	direct	direct	direct	direct
Mutations	misense, deletion	misense, deletion, large deletion	misense, deletion, inversion	misense, deletion	misense, deletion	misense, insertion
Severity of hyperglycaemia	progressive, may become severe	mild with minor deterioration with age	progressive, may become severe	middle, less penetrant than HNF-1α	may be severe	variable
Micro vascular complications	frequent	rare	frequent	little data	frequent, diabetic nephropathy	variable
Other complications		reduced birth weight	low renal threshold, aminoaciduria	pancreatic agenesis in homozygotes	renal cysts, proteinuria, renal failure	
Treatment	PAD, insulin	diet, PAD	PAD, insulin	PAD, insulin	insulin	PAD, insulin

1.1 HEPATOCYTE NUCLEAR FACTOR-4α (MODY1)

MODY1 is a rare form of MODY. The identification of mutations in the hepatocyte nuclear factor-4α (HNF-4α), the molecular genetic basis of MODY1, has been successful due to the largest and best studied MODY family known as "R-W pedigree", a Caucasian family of German/East Prussian ancestry which consists of 455 members in seven generations (Yamagata [b)] et al. 1996).

HNF-4α is a member of the nuclear receptor superfamily and it is expressed in liver, kidney, gut and endocrine pancreas. The DNA-binding domain of HNF-4α consists of two zinc finger motifs that specifically interact with a DNA element found in many promoters and enhancers of target genes. These target genes encode serum proteins as well as enzymes involved in lipid, amino acid and glucose metabolism, insulin gene expression but also the tissue-specific transcription factor HNF-1α. HNF-4α also plays an important role in the early phases of embryogenesis and cooperates with other transcription factors in a network that is crucial for the function of pancreatic β-cells (Stoffel and Duncan 1997, Lausen et al. 2000).

Mutations in HNF-4α have been identified in both coding and regulatory regions, including the HNF-1α binding site in P2 promoter, DNA-binding domain and transactivating domain (Stoffel and Duncan 1997, Furuta et al. 1997, Lausen et al. 2000).

MODY1 is characterised by insulin secretory defects and major hyperglycaemia associated with microvascular complications. Progression of microvascular complication may be severe. Patients mostly require the insulin treatment (Hattersley 1998).

1.2 GLUCOKINASE

Glucokinase comes under the hexokinase gene family and plays a key role in the regulation and integration of glucose metabolism because it catalyses the initial step in these pathways the ATP (adenosine triphosphate)-dependent phosphorylation to form glucose-6-phosphate (G-6-P) (Matschinsky et al. 1993). GCK operates as a monomer.

GCK is expressed in pancreatic ß-cells and in hepatocytes but also in a variety of neural/neuroendocrine cells including the pancreatic α-cells, L- and K- gut enterocytes and certain rare neurons in the central nervous system, primarily in the hypothalamus (Schuit et al. 2001). Although the GCK from pancreas, liver and brain are similar in kinetic activity and are coded by the same gene with 12 exons on chromosome 7 (7p15.3-p15.1), their primary structures in the N-terminal are different due to different splicing of the RNA transcript. The enzymes contain 465 amino acids and the exon 1 is different due to the different promoter regions in the different tissues, the upstream promoter is functional in the pancreas and in the brain, the downstream promoter is used only in the liver (Sttofel

et al. 1992). The existence of alternative promoters allows tissue-specific regulation of the gene expression.

In the view of its crucial role in the regulation of glucose-stimulated insulin secretion, it is comprehensible that mutations in the *GCK* gene can cause both hyperglycaemia as well as hypoglycaemia. Genetic linkage studies have shown that *GCK* mutations are responsible for three different disorders of glucose regulation.

The first and most frequent are heterozygous inactivating *GCK* mutations known as a pathogenetic cause of the **maturity-onset diabetes of the young type 2 (MODY2)**. The most frequent cause of MODY2 is a missense mutation; to date more than 200 mutations with distinct enzymatic characteristics have been found. These mutations are also found in 5-6 % women diagnosed as gestational diabetics (Ellard et al. 2000).

In pancreatic β-cells, glucose metabolism and insulin secretion are strongly dependent on the enzymatic activity of GCK. Expressions studies have shown that the enzymatic activities of the GCK mutants detected in MODY2 families were impaired, resulting in decreased glycolytic flux in pancreatic β-cells. Reduction in insulin secretion for a given glucose level in these patients averages 60 % (Byrne et al. 1994). However, the impairment in enzymatic activity is less severe than predicted, suggesting the presence of compensatory mechanisms that increase the insulin-secretion response. According to animal models studies, this compensatory mechanism could be related to glucose-induced increased expression of the wild-type *GCK* gene allele (Sreenan et al. 1998). In addition to the β-cell dysfunction, abnormalities in liver glucose metabolism contribute to the pathogenesis of hyperglycaemia, the relative flux of the direct (GCK-dependent) pathway of hepatic glycogen synthesis was decreased and the indirect (gluconeogenic, GCK-independent) pathway was relatively more important for synthesizing glycogen in MODY 2 subjects (Velho et al. 1996).

Much less numerous are heterozygous activating missense mutations causing the **persistent hyperinsulinemic hypoglycaemia of infancy (PHHI)** which is characterized by an enhanced relative activity of GCK. In the β-cells it leads to a lowering of the threshold for glucose-stimulated insulin secretion. PHHI is a heterogeneous disorder also related to mutations in the other genes: most frequently, inactivating mutations in the sulfonylurea receptor *SUR1*, rare inactivating mutations in the β-cell potassium ATP channel inward rectifier *Kir6.2* (*KCNJ11*), or activating mutations it the mitochondrial enzyme glutamate dehydrogenase *GLUD1* has been detected (Gloyn 2003).

The third disease - the **permanent neonatal diabetes mellitus (PNDM)** arises owing to inactivating homozygous *GCK* mutations (Njølstad et al. 2001), to complete deficiency of the β-cell transcription factor insulin promoter factor 1 (*IPF-1*) (Stoffers et al. 1997 [b]) or to activating mutations in *Kir6.2* (*KCNJ11*) (Hattersley and Ashcroft 2005). It has more severe phenotype, for some mutations, severe type of PNDM is accompanied by marked

developmental delay, muscle weakness, and epilepsy disease (Hattersley and Ashcroft 2005). Infants with this disorder must be treated with insulin in order to survive within the first month of live, they are insulin dependent for live. Some PNDM patients may well respond to oral sulfonylurea treatment (Hattersley and Ashcroft 2005). It has been also suggested that homozygous absence of GCK results in markedly low birth weight and could increase intrauterine or neonatal death risk (Gloyn 2003).

The transition G>A at position -30 of the GCK β-cell-specific promoter (rs1799884) is common within the Caucasian as well as Asian populations. Higher frequency of the minor allele A was detected in subjects with gestational diabetes in French (Zouali et al. 1993), Scandinavian (Shaat et al. 2006) and UK populations (Zaidi et al. 1997), but also in subjects with metabolic syndrome and with impaired glucose tolerance (IGT) (Rose et al. 2005). The allele A was associated with fasting and postprandial levels of glucose and with impaired glucose-stimulated insulin secretion (GSIR). Generally the effect of A allele was pronounced under a recessive model of inheritance – the dosage effect was observed (Zaidi et al. 1997, März et al. 2004).

1.3 HEPATOCYTE NUCLEAR FACTOR-1α (MODY3)

MODY3 is the most common form of MODY in European populations. Its prevalence varies from 30 % in Germany, 40 % in French to 75 % in UK (Fajans et al. 2001).

HNF-1α is a transcription factor involved in the embryogenesis and regulation of liver, pancreatic β-cells, kidney and gut enterocytes (Wang et al. 1998). HNF-1α acts as homodimer or heterodimer with HNF-1β to target genes coding serum proteins with crucial role in glucose transport and metabolism. It binds to promoters of genes for *HNF-4α*, insulin and glucose transporter *GLUT2* (Wang et al. 1998, Fajans et al. 2001). HNF-1α positively regulates an enhancer of *IPF-1* gene (Ben-Shushan et al. 2001).

The gene of *HNF-1α* contains four domains: binding-site for HNF-4α, DNA- binding site and domains of dimerisation and transactivation. *HNF-1α* gene consists of 10 exons. Till this time more than 130 mutations in associations with MODY3 were identified (Hattersley 1998, Ellard et al. 2000, Bjørkhaug et al. 2003).

HNF-1α mutations have similar biochemical effect as *HNF-4α* mutations: impairment of GSIR but no decrease of insulin sensitivity suggesting defect of β-cell function no of insulin activity.

Onset of MODY3 mostly begins after adolescence in early adulthood. Patients are no obese and have hyperglycaemia without ketoacidosis (Isomaa et al. 1998, Timsit et al.

2005). They have decreased renal threshold for glucose reabsorption therefore they are more prone to glycosuria (Fajans et al. 2001). The progression of diabetic microvascular complications, generally retinopathy, is very often severe (Hattersley 1998). Contrariwise, the liability to macrovascular complications as hypertension and dyslipidemia is much lower than in DM2 patients (Isomaa et al. 1998).

The metabolic disease progress significantly and patients need treatment with insulin or with sulfonylurea derivatives (Hattersley 1998).

MODY3 is not absolutely penetrated, the phenotypic manifestation is involved by other genetic and environmental features – e.g. insulin resistance during pregnancy (Timsit et al. 2005).

Common variants in *HNF-1*α gene A98V and I27L are wide spread and were described in association with impaired GSIR, increased insulin sensitivity and with higher predisposition to DM2 (Chiu et al. 2003, Holmkvist et al. 2006). Therefore, some studies did not ascertain the association of these polymorphisms with DM2 (Wincler et al. 2005).

1.4 INSULIN PROMOTER FACTOR 1 (MODY4)

In 1997 was detected a homozygous mutation P63fsdelC→X in *IPF-1* gene in a child with pancreatic agenesis (Stoffers et al. 1997 [a) b)]). In 8 family members were identified other 3 missense mutations in *IPF-1* gene that strongly cosegregated with DM2 phenotype. *IPF-1* was recognised as a MODY4 gene lokus.

IPF-1 plays a key role in pancreatic embryogenesis and in maintenance of function of mature pancreas. It is expressed in β- and δ-cells. In β-cells IPF-1 directly regulates insulin expression and also expression of other genes: *GLUT2*, *Nkx6.1*, *Pax 4*, isled amyloid polypeptide (*IAPP*) and *GCK*; in δ-cells expression of somatostatine gene (Yamada et al. 1999).

Patients with MODY4 have higher susceptibility to microvascular diseases mostly to nephropathy. For the most part they are treated with insulin (Stoffers et al. 1997 [a) b)], Yamada et al. 1999).

1.5 HEPATOCYTE NUCLEAR FACTOR-1β (MODY5)

HNF-1β is a transcription factor involved in embryogenesis of pancreas and kidney and it acts in the regulatory network necessary to development and function of pancreatic cells. HNF-1β binds DNA as homodimer or heterodimer with HNF-1α (Beards et al. 1998).

Very few mutations in *HNF-1β* gene were detected. MODY5 is very rare.

1.6 NEUROGENIC DIFERENCIATION PROTEIN-1 (MODY6)

NeuroD1 comes under basic helix-loop-helix (bHLH) proteins participating in cell differentiation. It regulates the terminal differentiation of neurons in central nervous system, in the form of heterodimer with ubiquitous protein E47 binds to insulin promoter and affects the expression of other genes as *GCK*, *IAPP*, *Nkx2.2* and *Pax4* (Moates et al. 2003).

Mutations in *NeuroD1* were firstly described with DM2. In 2001 Fajans and his group published the association of *NeuroD1* with MODY.

Only few mutations have been identified. Phenotypic features of NeuroD1 mutations are very varied. Some patients were obese with high levels of insulin and C-peptide, the others were nonobese and their insulin secretion was very low (Malecki et al 1999).

One common polymorphism A45T in NeuroD1 gene was detected with impaired C-peptide secretion in relation to DM2 (Malecki et al 1999) and DM1 (Malecki et al. 2003).

2. AIMS

The aim of our study was to assess the variability of the *GCK* gene in the Czech diabetic and control populations and to find out the frequency of MODY2 in our set of MODY families.

For this reason two different screening methods were used: temperature gradient gel electrophoresis (TGGE) and single-strand conformation polymorphism (SSCP). The efficiency of these methods was compared and the positive findings were confirmed by direct sequencing.

The following aim was to assess the frequency of the polymorphism -30G>A in the B-promoter of the *GCK* gene in Czech diabetic and nondiabetic populations and to ascertain its possible association with diabetic phenotype.

The next aim was to assess the frequency of the polymorphism A98V (292C>T) in the *HNF-1*α gene in Czech diabetic and control populations and to establish its probable influence to impaired insulin secretion.

3. MATERIALS AND METHODS

3.1 SUBJECTS

We studied 12 index patients affected by hyperglycaemia (diabetes mellitus type 1 excluded) and with family history of higher glucose levels or diabetes in first degree relatives diagnosed before 25 years of age, according to the common criteria for the MODY diagnosis (Fajans et al. 2001), and their 10 family members.

194 gestational diabetic subjects (G; age = 32,5±4,63 years; BMI = 24,3±4,91 kg/m^2) from the Institute for Mother and Child Care in Prague who had persisting fasting hyperglycaemia in pregnancy and negative antibodies GADA (glutamic acid decarboxylase antibodies), ICA (islet cell antibodies) and IA2 (antibodies to protein tyrosine phosphatase), 339 patients with diagnosed diabetes mellitus type 2 (DM2; age = 60,0±8,38 years; BMI = 31,0±5,20 kg/m^2) and 141 healthy offspring of diabetic parents (PD; age = 38,8±11,61 years; BMI = 25,5±4,19 kg/m^2) not related with our group of DM2 were included in the analysis. 261 unrelated normoglycaemic subjects without family history of DM2 were collected as controls (K; age = 29,9±10,41 years; BMI = 23,3±3,76 kg/m^2). The study was approved by local Ethic Committee and signed informed consent was obtained from all subjects.

3.2 BIOCHEMICAL METHODS

After an overnight fast, a venous blood sample was obtained for the determination of a number of biochemical parameters, including a 75-g oral glucose tolerance test (OGTT), and anthropometric parameters were measured (body height, weight, waist and hip circumferences). Blood glucose level was evaluated by the glucose oxidase method (Beckman Glucose Analyzer 2). Immunoreactive insulin was assayed in probands not on insulin therapy using an immunoradiometric assay kit (Immunotech IRMA kit, Czech Rep). Serum level of C-peptide was evaluated by the immunoradiometric assay kit (Immunotech IRMA kit, Czech Rep). Glycosylated hemoglobin (HPLC BioRad, Czech Rep.) and glycosylated proteins (spectrophotometric redox reaction using nitroblue tetrazolium as a sensitive redox indicator for the specific quantification of fructosamine in alkaline solution) were determined.

3.3 MOLECULAR-GENETIC METHODS

Genomic DNA was isolated from peripheral blood lymphocytes using the QIAamp® DNA Blood Kit (QIAGEN, Germany). After measurement of the DNA concentration and DNA/protein ratio, the DNAs were used for the PCR amplification.

3.3.1 SCREENING FOR GCK SEQUENCE VARIANTS

Screening for sequence variants in all 10 exons and flanking intron regions of the *GCK* gene was performed by SSCP (ALFexpress II, Amersham Pharmacia Biotech) and/or by TGGE (TGGE MAXI, Biometra).

Genomic DNA was amplified by PCR (T-Gradient Cycler, Biometra). The reaction mix (12 µl) contained 1x reaction buffer (Perkin Elmer), 1-3 mM $MgCl_2$ (Tab. 2, 3), 160 µM deoxyribonucleoside triphosphates, 0.1 µM of each primer (Tab. 2, 3), 0.18 U Gold AmpliTaq polymerase (Perkin Elmer) and 20 ng DNA. The amplification ran under the following conditions: initial denaturation at 95 °C for 10 min; 35 amplification cycles - 95 °C for 30 s, specific annealing temperature T_a (Tab. 2, 3) for 30 s, and 72 °C for 1 min; final elongation at 72 °C for 10 min.

For exons 1a, 2-7, TGGE primers with GC-clamps (Tab. 2) were chosen by using the aided program Poland (http://www.changbioscience.com/primo/primomel. html) that allows prediction of the impact of mutations on the melting behaviour of the PCR products. Some of them were previously described (Stoffel et al. 1992), we linked the GC clamp and verified the melting domain.

PCR products for TGGE were denaturated adding 7M urea (1:1 by volume) at 95 °C for 5 minutes and than renaturated by slowly cooling (Δ 1 °C/30 sec) to room temperature. TGGE conditions: 8% acrylamide denaturating gel (with 7M urea) in 0.55x TBE at temperature gradient specific for each exon (Tab. 2), 300 V, 35 mA and 20 W for 4 hours. Gels were silver stained in a Hoefer automated gel stainer (Amersham Pharmacia Biotech) according to the standard DNA-staining protocol.

Tab. 2: TGGE primers, conditions of amplification and TGGE

Exon	Primer (5´→3´)	$MgCl_2$ (mM)	T_a (°C)	Temperature gradient (°C)	PCR product (bp)
1a	Forward: 5´-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GTC CAC TTC AGA AGC CTA CTG-3´* Reverse: 5´-TCA GAT TCT GAG GCT CAA AC-3´*	2	56	47-59	295
2	Forward: 5´-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GTG CAG ATG CCT GGT GAC AGC-3´* Reverse: 5´-CAC AGC TGC TTC TGG ATG AG-3´*	1,3	64	48-63	330
3	Forward: 5´-ACC CAG CCC AAG GCC AGC CTG TG-3´ Reverse: 5´-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GGT GGC ACC TCC CGT CAG GAC TAG C-3´	3	69	50-60	337
4	Forward: 5´-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CGG CCA GGT GTT GCA GTG TCC-3´ Reverse: 5´-TGA AGG CAG AGT TCC TCT GG-3´*	1,5	64	46-56	341
5	Forward: 5´-TCC AGA TAT GTT AGC CAC GAG-3´ Reverse: 5´-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GCC AAG GAG AAA GGC AGG CAG TG-3´	1	55	52-62	252
6	Forward: 5´-CCA GCA CTG CAG CTT CTG TG-3´* Reverse: 5´-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CGA GCC TCG GCA GTC TGG AAG-3´*	3	67	50-60	216
7	Forward: 5´-AGT GCA GCT CTC GCT GAC AG-3´* Reverse: 5´-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCA TCT GCC GCT GCA CCA GAG-3´*	2	65	54-64	325

** primers by Stoffel et al. 1992*

For PCR/SSCP and PCR/sequencing Cy-5 labeled primers were used (Tab. 3), some of them were previously described (Stoffel et al. 1992), some of them were designed by primer analysis software OLIGO® (MedProbe) (Tab. 3).

PCR products for SSCP were denaturated adding 95% formamide (1:2.5 by volume) at 95 °C for 4 minutes. SSCP conditions: 6% nondenaturating acrylamide gel in 1x TBE at two different temperatures: 10 °C and 25 °C, 1200 V, 40 mA and 35 W for 5 hours (ALFexpress II, Amersham Pharmacia Biotech).

For TGGE and SSCP we used the positive controls for each exon, which were kindly offered by Prof. J. Gräßler (Department of Internal Medicine III, University Clinic Carl Gustav Carus of the Technical University Dresden, Germany) and by Prof. J. A. Maassen (Department of Molecular Cell Biology LUMC, Leiden University Medical Centre, The Netherlands) (Tab. 4).

Tab. 3: SSCP primers and conditions of amplification

Exon	Primer (5´→3´)	$MgCl_2$ (mM)	T_a (°C)	PCR product (bp)
1a	Forward: Cy5 5´-TCA AAA GCT GTC CCC AGG TCA-3´ Reverse: Cy5 5´-ATG GAA TGT TGG GGA CAG GCA-3´	1,5	61	491
2	Forward: Cy5 5´-GTG AGG CCC TCG GTG TGC AGA-3´ Reverse: Cy5 5´-TCG GGC TGG CTG TGA GTC TGG-3´	1,5	60	362
3	Forward: Cy5 5´-TAA TAT CCG GCT TCA GTC ACC-3´* Reverse: Cy5 5´-CTG AGA TCC TGC ATG CCT TG-3´*	2	62	295
4	Forward: Cy5 5´-TAG CTT GGC TTG AGG CCG TG-3´* Reverse: Cy5 5´-TGA AGG CAG AGT TCC TCT GG-3´*	2	62	272
5	Forward: Cy5 5´-GCA GCC ACG AGG CCT ATC TC-3´* Reverse: Cy5 5´-GAG AAA GGC AGG CAG TGC TG- 3´*	1,5	61	195
6	Forward: Cy5 5´-CAC CCA CCC CAG CAC TGC CTG-3´ Reverse: Cy5 5´-AGG GAG CCT CGG CAG TCT GGA-3´	1,5	62	216
7	Forward: Cy5 5´-CGG GGC AGT GCA GCT CTC GCT-3´ Reverse: Cy5 5´-CTC CCA TCT GCC GCT GCA CCA-3´	1,5	65	285
8	Forward: Cy5 5´-GCC CTC CCT CGT GCC TGC TGA- 3´ Reverse: Cy5 5´-TCG CCC TGA GAC CAA GTC TGC- 3´	1	65	278
9	Forward: Cy5 5´-CCG CCG CTG GAG GGG GAT GGA- 3´ Reverse: Cy5 5´-GCG CGC TTT TTG GGC CCC ACT- 3´	2	60	352
10	Forward: Cy5 5´-GTC GAC TGC GTG CAG GGC GC-3´* Reverse: Cy5 5´-TGT GGC ATC CTC CCT GCG CT- 3´*	1,5	68	263

** primers by Stoffel et al. 1992*

Tab. 4: Positive controls used in SSCP and TGGE

Exon	Positive controls
1a	A11T (31 G>A)
2	E40fsins20 R43fsinsC
3	Y108H (322T>C)
4	F150S (449T>C) V154fsdelTG IVS4+2del15
5	R186X (556C>T)
6	Y215Y (645C>T; homozygot) Y215Y (645C>T; heterozygot)
7	A259T (775G>A) G261R (781G>A)
8	C382Y (1145G>A) G299R (895G>C)
9	A384T (1150G>A) R392C (1174C>T) R403fsdelC IVS9+8T>C
10	V455E (1364T>A)

DNA polymorphisms (bands with abnormal mobility on acrylamide gel detected by SSCP or by TGGE) were confirmed by direct sequencing in both directions. PCR products for sequencig were purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham, Biosciences) and amplified using Thermo Sequenase Cycle Sequencing Kit (USB), than denaturated adding 95% formamide (3:1 by volume) at 80 °C for 3 minutes. Sequencing conditions: 5.5% Long Ranger® denaturating gel (with 6M urea) (Cambrex Bio Science Rockland) in 0.5x TBE at 55 °C, 1500 V, 60 mA and 25 W for 6-8 hours (ALFexpress II,

Amersham Pharmacia Biotech). Using fluorescent PCR primers these mutations were subsequently detected by an automated DNA sequencer (ALFexpress II, Amersham Pharmacia Biotech).

3.3.2 SCREENING FOR -30G>A POLYMORPHISM IN B-PROMOTER OF GCK GENE

At first we used the RFLP (restriction fragments length polymorphism) method to identify all genotypes — GG, GA and AA.

Genomic DNA was amplified by PCR (T-Gradient Cycler, Biometra). The reaction mix (12 µl) contained 1x reaction buffer (Perkin Elmer), 1.5 mM $MgCl_2$, 160 µM deoxyribonucleoside triphosphates, 0.1 µM of each primer, 0.18 U Gold AmpliTaq polymerase (Perkin Elmer) and 20 ng DNA. The amplification ran under the following conditions: initial denaturation at 95 °C for 10 min; 35 amplification cycles - 95 °C for 30 s, annealing temperature at 57 °C for 30 s, and 72 °C for 1 min; final elongation at 72 °C for 10 min.

Previously described primers were used: Forward 5'-TGC ATG GCA GCT CTA ATG AC-3' and Reverse: 5'-ATT CTC CTG CCA GGG CTT AC-3' (März et al. 2004).

Amplification products were digested with restriction endonuclease MwoI and analyzed by 3% agarose gel electrophoresis. The G allele yielded fragments of 82 bp and 25 bp, the A allele was not digested — 107 bp.

For the reason of the costs of MwoI enzyme we used for follow-on analysis the SSCP method.

Genomic DNA was amplified by PCR (T-Gradient Cycler, Biometra) under the above described conditions for PCR-RFLP method.

Cy-5 labeled primers were used: Forward 5'-Cy5-CAA GGC GAT TGA GTG GTC ACC ATG-3´, Reverse: 5'-Cy5-GAC TGT GTC TCT CAC ATC CTA GCC-3´ (Rissanen et al. 1998).

PCR products for SSCP were denaturated adding 95% formamide (1:2.5 by volume) at 95 °C for 4 minutes. SSCP conditions: 6% nondenaturating acrylamide gel in 1x TBE at 22 °C, 1200 V, 40 mA and 35 W for 5 hours (ALFexpress II, Amersham Pharmacia Biotech).

Genotypes GG, GA and AA were used as positive controls in every run.

3.3.3 SCREENING FOR A98V POLYMORPHISM IN HNF-1α GENE

The RFLP analysis was used.

Genomic DNA was amplified by PCR (T-Gradient Cycler, Biometra). The reaction mix (12 µl) contained 1x reaction buffer (Perkin Elmer), 1.5 mM $MgCl_2$, 160 µM deoxyribonucleoside triphosphates, 0.1 µM of each primer, 0.18 U Gold AmpliTaq polymerase (Perkin Elmer) and 20 ng DNA. The amplification ran under the following conditions: initial denaturation at 95 °C for 10 min; 35 amplification cycles - 95 °C for 30 s, annealing temperature at 65 °C for 30 s, and 72 °C for 1 min; final elongation at 72 °C for 10 min.

Primers were designed by primer analysis software OLIGO® (MedProbe): Forward 5'-GGG AGG CGG CTA GCG TGG TGG AC-3' a Reverse: 5'-GAA GAC TCA ACT CAG GGG TAC T-3'.

Amplification products were digested with restriction endonuclease AvaII and analyzed by 1.5% agarose gel electrophoresis. The T allele (valin) yielded fragments of 354 bp and 129 bp, the C allele (alanin) was not digested — 474 bp.

3.4 STATISTICAL METHODS

The statistical analysis was performed by program NCSS 2004 (Statistical Solutions, Saugus, MA, USA).

Biochemical and anthropometrical parameters in DM2 patients were described by descriptive statistics. Biochemical and anthropometrical parameters between other groups (G and PD) and control population were compared using ANCOVA with adjustment for BMI and age.

The genotypic distribution of polymorphism was proved by Hardy-Weinberg equilibrium (HWE). Than the possible differences of polymorphism frequencies between groups were examined by chi-square test (χ^2-test).

Association between genotypes and biochemical and anthropometrical parameters in each group was evaluated by ANCOVA with adjustment for BMI and age.

4. RESULTS

4.1 CHARACTERIZATION OF GROUPS

Gestational diabetics had higher fasting and stimulating levels of glycaemia during OGTT (but still in physiological ranges) compared with control population. They had also increased insulin resistance, but the function of β-cells was well reserved.

In the group of offspring of DM2 we found out higher fasting and stimulating levels of glycaemia and insulin during OGTT (but still in physiological ranges) compared with control population. Offspring had also increased insulin resistance, but the function of β-cells was well reserved.

4.2 SCREENING FOR *GCK* SEQUENCING VARIANTS

All 10 exons specific for pancreatic glucokinase (1a and 2-10) including intron/exon boundaries were examined by SSCP and/or TGGE in total 722 subjects.

Exons 1a – 7 were screened by both methods: TGGE and SSCP. For exons 8 – 10 we did not find suitable primers with GC-clamps for TGGE to allocate a fragment with applicable melting domain. All positive controls (Tab. 4) were detected by both screening methods in every run.

Detected gene variants are given in table 5.

Among 12 MODY families there were found 2 *GCK* mutations and one intronic variant in index patients as well as in their family members. The first one is a novel mutation V33A (transition of thymine to cytosine at the nucleotide position 98) in exon 2 of *GCK* gene in a patient with MODY. This is a 12 years old boy whose hyperglycaemia was incidentally diagnosed in 10 months of age. He has low fasting hyperglycaemia and low increased glycosylated haemoglobin. Other parameters are in the physiological ranges. During OGTT he had fasting hyperglycaemia, and the glycaemia in 2 hours is on the lower boundary of impaired glucose tolerance (IGT). But glucose tolerance is still normal. Negative antibodies (GADA, ICA and IA2) and sufficient insulin secretion in the first phase of ivGTT disagree with DM1 or with MODY1 and MODY3.

Tab. 5: GCK *gene variants in the Czech diabetic and nondiabetic populations*

	GCK gene varians / (number of subjects)				
GCK gene	MODY (n=12 basic patients + 10 offspring)	DM2 (n=313)	G (n=141)	PD (n=130)	K (n=116)
exon 1a		IVS1+4T>A / (n=1)	IVS1+4T>A / (n=1)	IVS1+4T>A / (n=1)	
exon 2	Val33Ala (98T>C) / (1+1) Glu40Lys (118G>A) / (1+1)	IVS2-12C>T / (n=3)	IVS2+1G>A / (n=1)	IVS2-12C>T / (n=1)	IVS2-12C>T / (n=2)
exon 3			IVS3+9C>T / (n=1)		IVS3-23C>T / (n=2)
exon 4	IVS4+87C>A (n=2) IVS4+87C>A hom. (n=1)				IVS4+26C>A (n=1)
exon 5					
exon 6		Tyr215Tyr (645C>T) / (n=2)	Tyr215Tyr (645C>T) / (n=2)	Tyr215Tyr (645C>T) / (n=1)	Tyr215Tyr (645C>T) / (n=1)
exon 7		Ser263Ser (789C>T) / (n=1) IVS7-15C>G/ (n=1)			
exon 8		IVS8+18G>A / (n=2)	IVS8+18G>A / (n=2)		
exon 9	IVS9+8T>C (33,3%)	IVS9+8T>C / (31%); IVS9+49G>A / (7,3%); IVS9+8T>C + IVS9+49G>A / (1,7%)	IVS9+8T>C / (24,5%); IVS9+49G>A / (9,2%); IVS9+8T>C + IVS9+49G>A / (5,1%)	IVS9+8T>C / (33,3%); IVS9+49G>A / (11,9%); IVS9+8T>C + IVS9+49G>A / (2,4%)	IVS9+8T>C / (35,5%); IVS9+49G>A / (13,1%); IVS9+8T>C + IVS9+49G>A / (0,9%)
exon 10					

We made the genetic analysis of the DNAs of proband´s mother and his younger brother. It revealed the same mutation in his mother, which had gestational diabetes, and now she suffers from DM2 treated with diet so far. His grandfather suffers from DM2.

The second mutation is the previously described mismatch E40K (transition of guanine to adenin at the nucleotide position 118), primary identified in the Czech population (Pruhova et al. 2003).

Intronic variant IVS4+87C>A was detected in a MODY index patient and in his two offspring, in one of them in homozygous form.

In a gestational diabetic we detected a novel intronic mutation IVS2+1G>A, that affects splice donor site.

In our sets of studied subject we found eleven different intronic variants and the previously described polymorphisms in the exons 6 and 7. With regard to relatively low frequency of *GCK* gene variants we can not find the phenotypic differences between the probands with the wild type of *GCK* gene and the probands with a *GCK* gene variant.

4.3 SCREENING FOR -30G>A POLYMORPHISM IN B-PROMOTER OF *GCK* GENE

Frequencies of genotypes GG, GA and AA in studied groups are given in table 6. HWE was confirmed. The frequencies did not differ among groups.

Tab. 6: Frequencies of genotypes of the -30G>A polymorphism

	Genotype		
	GG	**GA**	**AA**
DM2	240 (70,8 %)	88 (26,0 %)	11 (3,2 %)
G	108 (65,5 %)	55 (33,3 %)	2 (1,2 %)
PD	91 (64,5 %)	44 (31,2 %)	6 (4,3 %)
K	194 (74,3 %)	62 (23,8 %)	5 (1,9 %)

In control population the minor allele A in homozygous form was associated with increased fasting glucose (p = 0,025) and with stimulated levels of glycaemia during OGTT (p = 0,001). The homozygotes AA had also increased fasting (NS) and stimulated secretion of C-peptide (p = 0,048) and of insulin (NS). Insulin resistance was higher in AA subjects (HOMA R, p = 0,010).

The heterozygotes GA did not differ in biochemical and anthropometrical parameters from wild type homozygotes GG. In our study the minor allele A shows a dose effect (recessive model of inheritance).

In groups of DM2, gestational diabetics and offspring no association with biochemical and anthropometrical parameters was found.

4.4 SCREENING FOR A98V POLYMORPHISM IN *HNF-1α* GENE

The genotypic frequencies of the codon 98 polymorphism in *HNF-1α* gene among groups are given in table 7. All the observed frequencies were in HWE. The allele T (valin) was not more common in diabetic than in nondiabetic groups.

Tab. 7: Frequencies of genotypes of the A98V polymorphism

	Genotype		
	CC	**CT**	**TT**
DM2	224 (95,3 %)	11 (4,7 %)	0
G	182 (93,8 %)	11 (5,7 %)	1 (0,5 %)
K	99 (95,2 %)	5 (4,8 %)	0

In control population we identified the association of T allele with the increased plasma levels of C-peptide and insulin in the second phase of OGTT. The T allele carriers had higher insulin resistance (NS), increased insulinogenic index (marginally NS) and improved function of β-cells (HOMA F, p = 0,027).

In diabetic populations (DM2 and G) no effect of the T allele on any anthropometrical and biochemical parameters was observed.

5. DISCUSSION

Even though MODY is a relatively rare form of diabetes mellitus, some studies suggest that it may not be so uncommon and 2-5 % of patients with diabetes mellitus type 2 may in fact have MODY (Ledermann 1995). Recent data evidence that MODY assumes approximately 1-2 % of diabetic patients in Europe (Owen and Hattersley 2001). The most common forms of MODY in Europe are MODY2 and MODY3. The relative prevalence of MODY2 of all MODY carriers varies greatly in studies from different populations from 46-56 % in France (Froguel at al. 1993, Velho et al. 1997), 41-61 % in Italy (Massa et al. 2001, Mantovani et al. 2003), 25-41 % in Spain (Costa et al. 2000, Barrio et al. 2002) and 31 % in the Czech Republic (Pruhova et al. 2003) to 11-20 % in the UK (Zhang et al. 1995, Thomson et al. 2003), 10 % in Denmark (Johansen et al. 2005), 8 % in Germany (Lindner at al. 1999) and 3.5 % in Scandinavia (Lehto et al. 1999). These data indicate that *GCK* mutations are more common in the southern European populations than in the northern Europe, where HNF-1α mutations play the major role in MODY causes.

The clinical features of MODY2 are very moderate and the diagnostics is very often accidental (Hattersley et al. 1992, Hattersley 2005). The clinical diagnosis depends on the age of the patient and it happens frequently that slim children are thought to be in the beginning stages of diabetes type 1, pregnant women are diagnosed as gestational diabetics and older patients are classified to suffer from diabetes mellitus type 2 (Hattersley 2005). It was the reason why we decided to screen *GCK* gene variants in Czech population not only in MODY patients (additional MODY families than published in Pruhova et al. 2003), but also in groups with common forms of diabetes – in patients with diabetes mellitus type 2 and gestational diabetics; and in nondiabetic groups – in healthy probands with family history of DM2 (offspring) and in control population without family history of DM2.

5.1 TGGE AND SSCP METHODS

For *GCK* mutational screening of the Czech diabetic and nondiabetic subjects two different methods were used: TGGE and SSCP.

Temperature gradient gel electrophoresis (TGGE) is a rapid and very sensitive screening method for detection of point mutations. The fragment size suited ranges from 100 bp to 1000 bp, the optimum length is ~ 600 bp with the critical base pair(s) in the centre of the fragment. Its ability to separate homologous sequences is attributed to two

physical properties of DNA: the effect of base pair sequence on the helix-coil or 'melting' transition, and the electrophoretic mobility of duplex DNA with single stranded regions. DNA varying in the primary structure differs in the stability of its first melting domain and unwinds at different positions in the gel. Close to 100% of point mutations can be detected, when the displacement of heteroduplices and wild-type bands is higher than the resolution of the gel (Steger 1994).

Single-strand conformation polymorphism (SSCP) is the simplest and most frequently used method for mutation screening. A strand of single-stranded DNA folds differently from another if it differs by a single base, and mutation-induced changes of tertiary structure of the DNA results in different mobilities for the two strands. Mutation detection for PCR-SSCP is generally high, under optimal conditions ~ 80 % - 90 % in a single run for fragments shorter than 300 bp (150 – 250 bp being the optimum). The sensitivity of PCR-SSCP decreases with increasing fragment length (Hayashi and Yandell 1993). Seeing that the sensitivity of SSCP depends on temperature and ionic environment we established two different SSCP temperatures (10 °C and 25 °C) to resolve the wild-type and all possible variants in a particular electrophoretic run.

These methods may be used to screen a large number of exons or other DNA fragments and only samples with abnormal electrophoretic mobility are then subjected to DNA sequencing.

Due to the plenitude of mutations in *GCK* gene and to the limited number of positive controls, that we used by screening, it is not 100% sure that all the *GCK* gene variants were revealed in our sets of subjects. However, although both screening methods are based on various physical properties of DNA, we could confirm the high sensitivity of both methods and the 100% concordance of TGGE and SSCP screening results consequently confirmed by direct sequencing in both directions.

5.2 SCREENING FOR *GCK* SEQUENCING VARIANTS

Among our 12 MODY families we detected the *GCK* gene mutations in two of them and an intronic variant in one of them (Tab. 5). MODY2 amounted 16.7 % of our set of MODY families. This fact that it represents approximately half detection rate than in previously described Czech population study (Pruhova et al. 2003) could be the cause of the relatively small amount of our studied MODY families.

In the other tested groups of subjects we have detected only one mutation in intron/exon boundary in a gestational diabetic, several polymorphisms were found.

The screening of *GCK* mutations and polymorphisms in patients with late-onset DM2 and in gestational diabetics in European populations was not widely performed and the

indicated prevalence in the rare studies was relatively low. Studies of GCK mutations in gestational diabetics suggested the highest prevalence of 6 % in UK (Saker et al. 1996) and 5.6 % in France (Zouali et al. 1993).

Very few polymorphisms of the *GCK* gene have been reported in the literature. Sequence variants in the coding and flanking regions of *GCK* were detected in about 5 % in French DM2 subjects. In the index probands with exon variants Tyr215Tyr and Trp257Arg diabetes had been diagnosed during pregnancy (Zouali et al. 1993). In Finland the population study of *GCK* variants was achieved in patients with DM2 and with IGT. These polymorphisms were found: Tyr215Tyr in 2.8 % DM2 and 5 % IGT; 403C>G (5´untranslated region of exon 1a) in 16.7 % DM2 and 17.5 % IGT and IVS9+8T>C in 36 % DM2 and 27.5 % IGT (Laakso et al. 1995). Intronic variant IVS2-12C>T was identified in about 1 % of diabetic as well as nondiabetic populations (Johansen et al. 2005, Zouali et al. 1993) and IVS8+18G>A in about 1 % of diabetic population but not in healthy offspring and control population (Johansen et al. 2005, Lehto et al. 1999).

The detection rate of the above mentioned sequence variants exept of IVS9+8T>C was much lower in Czech diabetics type 2 and gestational diabetics in comparison with French, Finnish and UK populations even after phenotypic selection for early-onset and family history of diabetes.

The silent substitution Tyr215Tyr (645C>T) in exon 6 was detected across European populations and we confirmed its frequency about 1 % in diabetic as well as nondiabetic subjects (Thomson et al. 2003, Johansen et al. 2005, Lehto et al. 1999, Zouali et al. 1993, Laakso et al. 1995). The Ser263Ser (789C>T) located in exon 7 was detected in one of our DM2 patients. Interestingly, these rare polymorphisms Ser263Ser and Tyr215Tyr were also identified in infants who died suddenly and unexpectedly (Forsyth et al. 2005).

The most common polymorphism is the IVS9+8T>C. We detected the frequency of its minor allele in about 30 % of Czech diabetic and nondiabetic populations. The frequency in other European populations was about 36 % in the Finnish DM2 population and 27.5 % in patients with IGT (Laakso et al. 1995), 15 % in the MODY patients in UK (Thomson et al. 2003) and 14 % in Italian families with early-onset DM2 (Gragnoli et al. 2001) or the frequency was not adduced: in the French MODY and DM2 (27), in Scandinavian subjects with early-onset DM2 and control population (Lehto et al. 1999) and in Danish MODY populations Johansen et al. 2005.

In intron 9 we detected also the polymorphism IVS9+49G>A in frequencies varying from ~7 to 13 % among groups. In Italian population the frequency of the minor allele was about 3 % (Gragnoli et al. 2001).

Six of ten by us identified intronic variants have not been previously described: IVS1+4T>A, IVS3+9C>T, IVS3-23C>T, IVS4+26C>A, IVS4+87C>A and IVS7-15C>G.

On the other hand, some intronic variants of *GCK* that we did not detected were identified in European populations. The mostly mentioned are: IVS2+11G>A (Pruhova et al. 2003, Johansen et al. 2005, Lehto et al. 1999, Zouali et al. 1993) and IVS7-7T>A (Johansen et al. 2005, Lehto et al. 1999).

5.3 SCREENING FOR -30G>A POLYMORPHISM IN B-PROMOTER OF *GCK* GENE

The minor allele A of the B-promoter polymorphism was in several studies associated with higher risk of impaired glucose tolerance, DM2 (Rose et al. 2005) and gestational diabetes (Zouali et al. 1993, Shaat et al. 2006). Nevertheless, there are studies that did not confirm the A allele as a risk of these metabolic disorders (Rissanen et al. 1998). In our study we did not find as well any differences in genotype frequencies among diabetic and nondiabetic populations.

However, in control population in homozygous allele A carriers we confirmed the previously observed increase of fasting and stimulated glucose levels (Rose et al. 2005, Shaat et al. 2006) but conversely to previous studies fasting and stimulated levels of C-peptide and insulin were in these subjects also increased. The impaired insulin sensitivity in AA homozygotes was observed.

The occurrence of minor allele A demonstrated the recessive model of inheritance as in other studies (Zaidi et al. 1997, März et al. 2004), heterozygotes GA pronounced no effect to any anthropometrical and biochemical parameters.

5.4 SCREENING FOR A98V POLYMORPHISM IN *HNF-1α* GENE

The A98V (292C>T) variant in *HNF-1α* gene is one of the most envisaged polymorphism between common forms of MODY genes.

The allele T was described in relation to impaired insulin secretion. In healthy subjects (Urhammer et al. 1997) and gestational diabetics (Lauenborg et al. 2004) was observed the decrease of C-peptide and insulin secretion in TC homozygotes. In T allele carriers from control population we did not note impaired insulin secretion, contrariwise, in the second phase of OGTT we measured higher levels of C-peptide and insulin. T allele was also associated with worse insulin sensitivity, increased β-cell secretion and higher insulinogenic index in opposite to previously published data (Homkvist et al. 2006).

In diabetic population (DM2 and G) no effect of T allele was observed.

6. CONCLUSIONS

The aims of our study were performed.

Twelve families with unrecognized type of MODY were collected.

Quite large cohorts of DM2 patients, direct offspring of DM2 patients, gestational diabetics and sufficiently large group of control subjects were completed. All the probands underwent a detailed anthropometric and biochemical characterisation. Data were filled in an electronic database.

The DNA bank was established and completed.

For *GCK* gene we adopted screening methods SSCP for all exons specific for β-cells (1a-10) and TGGE for exons 1a-7 and we confirmed their high sensitivity and the 100% concordance of both methods. Results were consequently confirmed by direct sequencing in both directions.

We found a novel heterozygous missense mutation V33A and a previously published mutation E40K in exon 2 of *GCK* gene in two differnent Czech MODY families. However, our study did not provide the evidence of *GC*K gene as a risk gene in the pathogenesis of diabetes mellitus type 2 or of the gestational diabetes in Czech population because we identified only one intronic mutation in a gestational diabetic and no differences in the frequencies of *GCK* polymorphisms between Czech diabetic and nondiabetic populations.

We assessed the frequency of common variant -30G>A in B-promoter of *GCK* gene. Although we did not detect the higher frequency of minor allele A in diabetic in comparison to nondiabetic population, we found out its influence in homozygote form to increased fasting and stimulated levels of glucose and to higher insulin resistance in healthy subjects without family history of DM2. We confirmed the dosage effect of the A allele.

We assessed the frequency of A98V polymorphism in *HNF-1α* gene that did not differ among diabetic and nondiabetic populations. We did not confirm the association of minor T allele (valin) with impaired insulin secretion. In opposite, in control population we identified increases of C-peptide and insulin secretions during the second phase of OGTT and the higher secretion of the β-cells.

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