

Screening of Mutations and Polymorphisms in the Glucokinase Gene in Czech Diabetic and Healthy Control Populations

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SHORT TITLE: GLUCOKINASE VARIANTS IN THE CZECH POPULATION

Summary

Glucokinase (GCK) plays a key role in glucose metabolism. *GCK* mutations are known as a pathogenic cause of maturity-onset diabetes of the young type 2 (MODY2). These mutations are also found in gestational diabetics. The aim of our study was to assess the variability of the *GCK* gene in the Czech diabetic and control populations. We screened all 10 exons specific for the pancreatic isoform of glucokinase (1a and 2-10) including the intron flanking regions in 722 subjects (in 12 patients with an unrecognised type of MODY and their 10 family members, 313 patients with diabetes mellitus type 2 (DM2), 141 gestational diabetics (GDM), 130 healthy offspring of diabetic parents, and 116 healthy controls without family history of DM2). In two MODY families we identified two mutations in exon 2 of the *GCK* gene: a novel mutation Val33Ala and the previously described mutation Glu40Lys. In other subgroups (excluding MODY families) we detected only intronic variants and previously described polymorphisms in exons 6 (Tyr215Tyr) and 7 (Ser263Ser), we did not find any known *GCK* pathogenic mutation. We observed no difference in the frequencies of *GCK* polymorphisms between Czech diabetic (DM2, GDM) and non-diabetic populations.

Keywords

glucokinase gene (*GCK*) - MODY2 - mutation - polymorphism - gestational diabetes - diabetes mellitus type 2

Introduction

Glucokinase comes under the hexokinase gene family and plays a key role in glucose homeostasis as a glucose sensor in pancreatic β -cells. 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). A reduction in β -cell GCK amount or activity increases the glucose threshold for insulin secretion, causing typical fasting hyperglycaemia (Byrne *et al.* 1994).

GCK is expressed in pancreatic β -cells, hepatocytes and variety of neural/neuroendocrine cells including pancreatic α -cells, L- and K- gut enterocytes and selected neurons (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 distinct splicing of the RNA transcript. The enzymes contain 465 amino acids and exon 1 varies in the diverse tissues due to the different promoter regions: the upstream promoter is functional in pancreas and brain, the downstream promoter is used only in liver (Stoffel *et al.* 1992, Gloyn 2003).

In view of its crucial role in the regulation of glucose-stimulated insulin secretion, it is possible that mutations in the *GCK* gene can cause both hyperglycaemia and hypoglycaemia. Genetic studies have shown that *GCK* mutations are responsible for three different disorders of glucose regulation. The most frequent mutations are heterozygous inactivating *GCK* mutations known as a pathogenetic cause of maturity-onset diabetes of young type 2 (MODY2) which is characterized by mild, persistent fasting hyperglycaemia, low glucose-stimulated insulin secretion, autosomal dominant inheritance and early onset (at least one affected family member with disease onset before 25 years of age) (Fajans *et al.* 2001). Missense mutations of *GCK* represent the most frequent cause of MODY2; to date more than

200 mutations with distinct enzymatic characteristics have been found. These mutations were also detected in 5-6 % women with gestational diabetes (Ellard *et al.* 2000).

Heterozygous activating missense *GCK* mutations causing persistent hyperinsulinemic hypoglycaemia of infancy (PHHI) and inactivating homozygous *GCK* mutations leading to permanent neonatal diabetes mellitus (PNDM) are much less frequent (Gloyn 2003).

The aim of our study was to assess the variability of the *GCK* gene in the Czech population and to identify possible differences of *GCK* variants distribution between diabetic and control populations.

Methods

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.

One hundred and forty one gestational diabetic subjects (GDM; examined 0.5-1 year after the delivery) from the Institute for Mother and Child Care in Prague, 313 patients with diagnosed diabetes mellitus type 2 (DM2) and 130 healthy offspring of diabetic parents unrelated to our DM2 patients were included in the analysis. The control group consisted of 116 unrelated healthy subjects without family history of DM2. The protocol of the study was approved by the local Ethic Committee and all subjects signed informed consent.

After an overnight fast, a venous blood sample was obtained for the determination of biochemical parameters (Table 1), followed by a 75-g oral glucose tolerance test (OGTT) in non-diabetics and GDM subjects. Blood glucose level was evaluated by the glucose oxidase method (Beckman Glucose Analyzer 2). Immunoreactive insulin was assayed in probands

which were 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 also determined. OGTT derived parameters of insulin secretion (HOMA F, insulinogenic index) and insulin sensitivity (1/HOMA R, Matsuda index) were calculated (Matthews *et al.* 1985, Matsuda and De Fronzo 1999).

Genomic DNA was isolated from peripheral blood lymphocytes using the QIAamp® DNA Blood Kit (QIAGEN, Germany). Screening for sequence variants in all 10 exons and flanking intron regions of the *GCK* gene was performed by SSCP (Single-stranded Conformation Polymorphism; ALFexpress II, Amersham Pharmacia Biotech, Sweden) and/or by TGGE (Temperature Gradient Gel Electrophoresis; TGGE MAXI, Biometra, Germany). Genomic DNA was amplified by PCR (T-Gradient Cycler, Biometra, Germany). The reaction mix (30 µl) contained 1x reaction buffer (Perkin Elmer), 1-3 mM MgCl₂ (Table 2), 160 µM deoxyribonucleoside triphosphates, 0.1 µM of each primer (Table 2), 0.45 U Gold AmpliTaq polymerase (Perkin Elmer) and 30 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 T_a (Table 2) 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 (Table 2) were chosen using the aided program Poland (<http://www.changbioscience.com/primo/primomel.html>), which allows prediction of the impact of mutations on the melting behaviour of the PCR products. Some of these primers were previously described (Stoffel *et al.* 1992), we linked the GC clamp and verified the melting domain. For PCR/SSCP and PCR/sequencing Cy-5 labeled

primers were used (Table 2), some of them were previously described (Stoffel *et al.* 1992), some of them were designed by primer analysis software OLIGO[®] (MedProbe) (Table 2). Because of the melting profiles of exons 8-10 the TGGE method for these DNA fragments was not applicable.

PCR products for TGGE were denatured by the addition of 7M urea (1:1 by volume) at 95 °C for 5 minutes and then renatured by slow cooling ($\Delta 1$ °C/30 sec) to room temperature. TGGE conditions: 8% acrylamide denaturing gel (with 7M urea) in 0.55x TBE at temperature gradient specific for each exon (Table 2), 300 V, 35 mA and 20 W for 4 hours. The gels were silver stained in a Hoefer automated gel stainer (Amersham Pharmacia Biotech, Sweden) according to the standard DNA-staining protocol.

PCR products for SSCP were denatured by addition of 95% formamide (1:2.5 by volume) at 95 °C for 4 minutes. SSCP conditions: 6% non-denaturing 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, Sweden).

We used the positive controls for each exon, which were kindly provided by Dr. Thomas Selisko and Dr. Peter Schwarz (University Clinic Carl Gustav Carus of the Technical University Dresden, Germany) and by Prof. J. A. Maassen (Leiden University Medical Centre, The Netherlands) (Table 3).

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 sequencing were purified using GFX[™] PCR DNA and the Gel Band Purification Kit (Amersham, Biosciences) and amplified using the Thermo Sequenase Cycle Sequencing Kit (USB), then denatured by the addition of 95% formamide (3:1 by volume) at 80 °C for 3 minutes. Sequencing conditions: 5.5% Long Ranger[®] denaturing 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, Sweden). Using fluorescent PCR primers these mutations were subsequently detected by an automated DNA sequencer (ALFexpress II, Amersham Pharmacia Biotech, Sweden).

Results

The basic characteristics of the study subjects are given in Table 1. Impaired fasting glucose (≥ 5.6 mmol/l) was found in 5 offspring of DM2, in 3 GDM. The impaired glucose tolerance (glucose in 120th min of OGTT ≥ 7.8 and < 11.1 mmol/l) was discovered in 4 offspring of DM2, 4 GDM and 1 control woman. All other tested subjects had normal glucose tolerance according to WHO criteria. Even if the women with diagnosed gestational diabetes improved their glucose tolerance after delivery, significantly higher fasting (ANCOVA, adjustment for age and BMI; $p < 0.03$) and OGTT stimulated glucose levels ($p < 0.001$, but still in normal range) in comparison with control women were detected. In GDM decreased insulin sensitivity ($1/\text{HOMA R}$ $p < 0.05$) but sustained β -cell function was ascertained. Also in offspring of DM2 increased fasting ($p < 0.03$) and stimulated glucose levels ($p < 0.03$), significantly higher fasting insulin ($p < 0.006$) and lower insulin sensitivity ($1/\text{HOMA R}$, $p < 0.002$) in comparison with control subjects was detected. However, insulin secretion (HOMA F, insulinogenic index) did not significantly differ between these groups.

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

Exons 1a–7 were screened by both methods: TGGE and SSCP. As we did not find suitable primers with GC-clamps for TGGE to allocate a fragment with an applicable melting domain for exons 8–10, these exons were analyzed by SSCP only. All positive controls (Table 3) were analyzed by SSCP and/or TGGE in every run.

Detected gene variants are given in Table 4.

Among 12 MODY families, two *GCK* mutations and one intronic variant in index patients as well as in their family members were found. The first one is a novel mutation of Val33Ala (98T>C) in exon 2 of the *GCK* gene in a patient with MODY (Fig. 1). This is a 12-year-old boy whose hyperglycaemia was incidentally diagnosed in 10 months of age. He has mild fasting hyperglycaemia and slightly increased glycosylated haemoglobin. Other biochemical parameters are in the physiological ranges. In OGTT he had fasting hyperglycaemia, and the glycaemia in 2 hours is on the lower border of the impaired glucose tolerance (IGT) range. But glucose tolerance is still normal. Negative antibodies against islet cell antigens and sufficient insulin secretion disagree with DM1, MODY1 and MODY3. The genetic analysis revealed the same mutation in the proband's mother, who had exhibited gestational diabetes, later was diagnosed with DM2, which was being treated with diet so far. His maternal grandfather suffers from DM2.

The second mutation observed in our MODY families was the previously described mismatch Glu40Lys (118G>A) primarily identified in the Czech population (Průhová *et al.* 2003). We detected this mutation in a 30-year-old man whose impaired glucose tolerance was diagnosed in 7 years of age. His mother and grandparents from the mother's side also have impaired glucose tolerance. They refused the biochemical and genetic testing.

Intronic variant IVS4+87C>A was detected in a MODY 42-year-old index patient with impaired glucose tolerance and in his two offspring, in one of them in homozygous form.

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

Discussion

For *GCK* mutational screening of the Czech diabetic and nondiabetic subjects two different methods were used: TGGE and SSCP. These methods can be used to screen a large number of exons or other DNA fragments when only samples with abnormal electrophoretic mobility are then subjected to DNA sequencing. Although both screening methods are based on various physical properties of DNA, we could confirm the high sensitivity of both methods and 100% concordance of TGGE and SSCP screening results consequently confirmed by direct sequencing in both directions. However, due to the plenitude of mutations in the *GCK* gene and the limited number of positive controls, it is not completely sure that all *GCK* gene variants were revealed in our sets of subjects.

Even though MODY is a relatively rare form of diabetes mellitus, some studies suggest that it may not be so uncommon as hypothesized and that 2-5 % of patients with DM2 may in fact have MODY (Ledermann 1995). Recent data evidence supports that MODY is prevalent in approximately 1-2 % of diabetic patients in Europe (Owen and Hattersley 2001). MODY2 and MODY3 represent the most common forms of MODY in Europe. The relative prevalence of MODY2 among all MODY patients varies greatly in studies from different populations: from 46-56 % in France (Froguel *et 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 (Průhová *et al.* 2003) to 11-20 % in the UK (Thomson *et al.* 2003), 10 % in Denmark (Johansen *et al.* 2005), 8 % in Germany (Lindner *et al.* 1999) and 3.5 % in Scandinavia (Lehto *et al.* 1999). This data indicates that *GCK* mutations are more common in southern European populations than in northern Europe, where HNF-1 α (MODY3) mutations play the major role in MODY causes.

The clinical features of MODY2 are usually mild, and diagnosis is often accidental (Hattersley 2005). The disorder is frequently misdiagnosed and clinical diagnosis depends on the age of the patient: slim children are considered to be in the initial stages of diabetes type 1, pregnant women are diagnosed as gestational diabetics, and older patients are classified to suffer from diabetes mellitus type 2. This is the reason why we decided to screen *GCK* gene variants in Czech population not only in MODY patients (including additional MODY families to those published in (Průhová *et al.* 2003)), but also in groups with common forms of diabetes – in type 2 and gestational diabetics in comparison with non-diabetic groups, in healthy offspring of DM2 and healthy subjects without family history of diabetes mellitus.

Among our 12 MODY families the *GCK* gene mutations were detected in two of them, which represents 16.7 % of MODY2 genotype. In other tested groups of subjects we have not detected any *GCK* mutations, only several polymorphisms were found.

The screening for *GCK* mutations and polymorphisms in patients with DM2 and in gestational diabetics in European populations was not widely performed and their prevalence indicated in a few studies was relatively low. Very few polymorphisms of the *GCK* gene have been reported in literature. Sequence variants in the coding and flanking regions of *GCK* were detected in about 5 % of the 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 carried out in patients with DM2 and with IGT. The following 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 non-diabetic populations (Johansen *et al.* 2005,

Zouali *et al.* 1993) and IVS8+18G>A in about 1 % of diabetic population but not in healthy offspring or in the control population (Johansen *et al.* 2005, Lehto *et al.* 1999).

The detection rate of the above mentioned sequence variants, except of IVS9+8T>C, was much lower in our cohort of Czech diabetics type 2 and gestational diabetics in comparison with rates reported in 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 non-diabetic subjects (Thomson *et al.* 2003, Johansen *et al.* 2005, Lehto *et al.* 1999, Zouali *et al.* 1993, Laakso *et al.* 1995). Ser263Ser (789C>T) located in exon 7 was detected in one of our DM2 patients. Interestingly, the rare polymorphisms Ser263Ser and Tyr215Tyr were also identified in infants who died suddenly and unexpectedly (Forsyth *et al.* 2005).

The most common polymorphism is IVS9+8T>C. We detected its minor allele in about 30 % of both diabetic and non-diabetic populations. In other European populations, the frequency of this polymorphism was reported as being 36 % in DM2 subjects and 27.5 % in patients with IGT from Finland (Laakso *et al.* 1995), 15 % in the MODY patients in UK (Thomson *et al.* 2003) and 14 % in Italian families with early-onset DM2 (Gagnoli *et al.* 2001) or else the frequency was not stated: in French MODY and DM2 patients (Froguel *et al.* 1993), 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 also detected the polymorphism IVS9+49G>A in frequencies varying from ~7 to 13 % among groups. In the Italian subjects the frequency of the minor allele was about 3 % (Gagnoli *et al.* 2001).

Six of the ten identified intronic variants in our study 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, we did not detect some of the intronic variants of *GCK* that were identified in other European populations. The most often mentioned polymorphisms that we did not detect are: IVS2+11G>A (Průhová *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).

Conclusions

We found a novel heterozygous missense mutation Val33Ala in exon 2 of the *GCK* gene in a Czech MODY family. However, our study did not provide evidence of the *GCK* gene as a risk gene in the pathogenesis of diabetes mellitus type 2 or of gestational diabetes in the Czech population because we did not find any known *GCK* pathogenic mutations in these subjects or any differences in the frequencies of *GCK* polymorphisms between Czech diabetic and non-diabetic populations.

Acknowledgements

This study was supported by the grants IGA MH CR NR/7809-5.

Authors are not aware of any potential conflict of interest.

There is no conflict of interest.

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Figure 1. The novel *GCK* mutation Val33Ala.

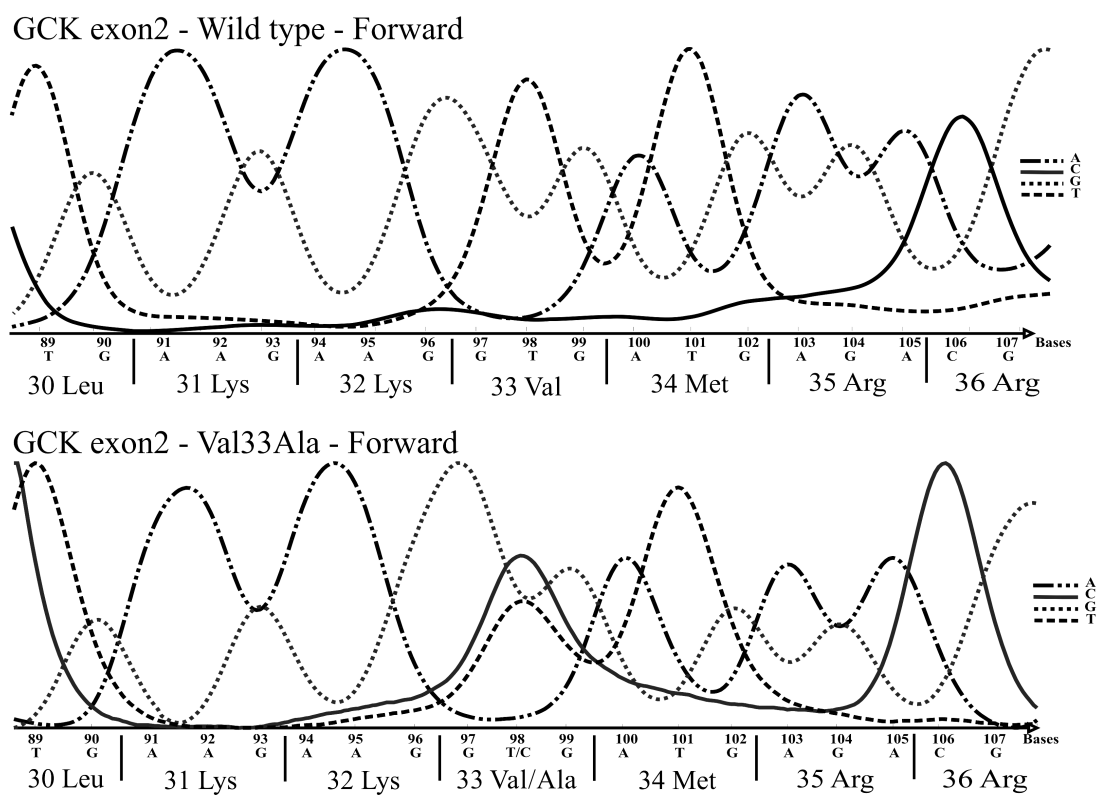


Table 1. Characterization of studied diabetic and non-diabetic groups.

	Type 2 diabetics	Gestational diabetics	Healthy offspring	Control population
Number (male/female)	313 (122/191)	141 (0/141)	130 (46/84)	116 (42/74)
Age [years]	58.86 ± 7.88	33.14 ± 6.44	39.12 ± 11.16	31.10 ± 10.26
BMI [kg/m ²]	30.53 ± 5.53	26.41 ± 5.03	25.51 ± 4.16	23.13 ± 3.38
Fasting glucose [mmol/l]	8.96 ± 3.34	5.3 ± 1.85	4.83 ± 0.55	4.69 ± 0.46
Fasting insulin [mIU/l]	15.77 ± 10.44	10.21 ± 11.03	8.88 ± 6.01	6.98 ± 4.15
Fasting C-peptide [pmol/l]	0.89 ± 0.53	0.73 ± 0.32	0.71 ± 0.35	0.58 ± 0.23
HbA_{1c} [%]	9.08 ± 2.36	5.20 ± 1.77	5.60 ± 1.05	5.16 ± 1.16
Glycosylated proteins [%]	1.61 ± 0.32	1.17 ± 0.22	1.10 ± 0.13	1.09 ± 0.12

Values are presented as means ± SD

Table 2. TGGE primer sequences with GC-clamp. Cy5 labeled primers for SSCP and sequencing.
Annealing temperature (T_a) of amplification and temperature gradient of TGGE (Tgradient).

Exon	Method	Primer Sequences (5'→3')	MgCl ₂ [mM]	T _a [°C]	Tgradient [°C]	Size [bp]
1a	SSCP and sequencing	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
1a	TGGE	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' Stoffel et al. 1992, forward primer with GC clamp	2	56	47-59	295
2	SSCP and sequencing	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
2	TGGE	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' Stoffel et al. 1992, forward primer with GC clamp	1.3	64	48-63	330
3	SSCP and sequencing	Forward: Cy5 5'-TAA TAT CCG GCT TCA GTC ACC Reverse: Cy5 5'-CTG AGA TCC TGC ATG CCT TG-3' Stoffel et al. 1992, labeled Cy5	2	62		295
3	TGGE	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	SSCP and sequencing	Forward: Cy5 5'-TAG CTT GGC TTG AGG CCG TG-3' Reverse: Cy5 5'-TGA AGG CAG AGT TCC TCT GG-3' Stoffel et al. 1992, labeled Cy5	2	62		272
4	TGGE	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' Reverse primer: Stoffel et al. 1992	1.5	64	46-56	341
5	SSCP and sequencing	Forward: Cy5 5'-GCA GCC ACG AGG CCT ATC TC-3' Reverse: Cy5 5'-GAG AAA GGC AGG CAG TGC TG-3' Stoffel et al. 1992, labeled Cy5	1.5	61		195
5	TGGE	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	SSCP and sequencing	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
6	TGGE	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' Stoffel et al. 1992, reverse primer with GC clamp	3	67	50-60	216
7	SSCP and sequencing	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
7	TGGE	Forward: 5'-AGT GCA GCT CTC GCT GAC AG-3' Reverse: 5'-GCC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCA TCT GCC GCT GCA CCA GAG-3' Stoffel et al. 1992, reverse primer with GC clamp	2	65	54-64	325
8	SSCP and sequencing	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	SSCP and sequencing	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	SSCP and sequencing	Forward: Cy5 5'-GTC GAC TGC GTG CAG GGC GC-3' Reverse: Cy5 5'-TGT GGC ATC CTC CCT GCG CT-3' Stoffel et al. 1992, labeled Cy5	1.5	68		263

Table 3. Positive controls that were used for screening methods.

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

Table 4. *GCK* gene variants in Czech diabetic and non-diabetic populations.

Type of polymorphisms / (number of carriers)					
MODY (n=12 index patients n=10 family members)		Type 2 diabetics (n=313)	Gestational diabetics (n=141)	Healthy offspring (n=130)	Control population (n=116)
exon 1a		IVS1+4T>A (n=1)	IVS1+4T>A (n=1)	IVS1+4T>A (n=1)	
exon 2	Val33Ala (98T>C) (n=1+1) Glu40Lys (118G>A) (n=1+1)	IVS2-12 C>T (n=3)	IVS2+1 G>A (n=1)	IVS2-12 C>T (n=1)	IVS2-12 C>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 (31%);	IVS9+8T>C (24.5%);	IVS9+8T>C (33.3%);	IVS9+8T>C (35.5%);
	IVS9+8T>C (33.3%)	IVS9+49G>A (7.3%);	IVS9+49G>A (9.2%);	IVS9+49G>A (11.9%);	IVS9+49G>A (13.1%);
		IVS9+8T>C + IVS9+49G>A (1.7%)	IVS9+8T>C + IVS9+49G>A (5.1%)	IVS9+8T>C + IVS9+49G>A (2.4%)	IVS9+8T>C + IVS9+49G>A (0.9%)
exon 10					