# Glucokinase Gene Variants in the Common Form of NIDDM

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**To determine whether a structural defect in glucokinase could be a primary cause of glucose intolerance in the common form of NIDDM, the prevalence of mutations in the gene in 60 American black NIDDM patients was investigated. First, by Southern blot analysis of DNA from a subset of randomly selected subjects ($n$ = 20), no gross deletions, insertions, or rearrangements of the gene were detected. Next, the 5′-untranslated and coding regions of the gene were amplified directly from genomic DNA by the polymerase chain reaction. PCR products were screened for mutations by using single-strand conformational polymorphism analysis. A total of nine variants were identified, with two in the 5′-UT regions of islet exon 1, two in the 5′-UT region of liver exon 1, and five in the coding regions. For islet exon 1, 5 of 60 NIDDM patients had both variants in the 5′-UT region; and for liver exon 1, two variants each occurred in 1 of 60 NIDDM patients. The coding region variants included a missense mutation in islet exon 1, substitution of Ala$^{11}$ (GCC) with Thr$^{11}$ (ACC), found in 2 patients. The biological consequences of this mutation and the mutations in the 5′-UT portion of the gene have yet to be determined. The rest of the variants were third base pair changes of codons, i.e., silent. A common polymorphism, which was in linkage equilibrium with microsatellite repeats GCK1 and GCK2, was found in intron 9, and a variant in intron 2 in both alleles of 1 patient. From these studies it was concluded that, although genetic variations in the coding sequence of the glucokinase gene are common, the amino acid sequence is highly conserved. The previous association of glucokinase alleles with NIDDM in American blacks has not been clarified by this analysis. Primary structural abnormalities of glucokinase do not play a significant role in the common form of NIDDM in this group.**
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Several lines of evidence suggest a strong genetic component of NIDDM (1). The enzymes that are involved in glucose metabolism are candidate genes for NIDDM. Glucokinase plays an important role in glucose metabolism by phosphorylation of glucose in the liver and pancreatic islet β-cells (2). Decreased hepatic glucokinase would alter glucose disposal by the liver, and decreased islet β-cell glucokinase might alter glucose-induced insulin secretion by β-cells.

A microsatellite repeat polymorphism at the 3′-end of the glucokinase gene, GCK1, was shown to be a genetic marker for NIDDM in American blacks (3) and Mauritian Creoles (4). Isolation of the human glucokinase gene (5) provided the sequence necessary to screen the gene at the single nucleotide level in NIDDM patients. DNA from American black NIDDM patients was amplified by the PCR and screened for nucleotide variants by SSCP (6). A total of nine different variants were identified within the 5′-UT and coding regions.

## RESEARCH DESIGN AND METHODS

The study population was described previously (3). A subset of NIDDM subjects ($n$ = 60) was randomly selected for this study. In this subset of subjects, the allelic frequency at GCK1 was essentially the same as described previously (3).

Southern blot analysis was performed as described

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NIDDM, non-insulin-dependent diabetes mellitus; 5′-UT, 5′-untranslated; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism; bp, base pair; GCK1, microsatellite at 3′-end of the glucokinase gene; GCK2, microsatellite at 5′-end of the glucokinase gene; TBE, Tris-borate/EDTA; 3′-UT, 3′-untranslated; MODY, maturity-onset diabetes of the young.

TABLE 1
Primers, annealing temperature, and $MgCl_2$ concentration for PCR

| Region amplified | Forward primers | Reserve primers | Product | Annealing temperature | $MgCl_2$ |
|---|---|---|---|---|---|
| Islet exon 1–1 | CACTCCACACCTGGCTGGAGC | CATTGTTCTCCAACGAGTCGGC | 308 | 62 | 1.5 |
| Islet exon 1–2 | GAAGGGTCCAGAAGGGAATGC | CAGAATGCCCAATGGAGGGCG | 296 | 62 | 2.0 |
| Liver exon 1 | GCCCTGCCTCTGACCCCATGG | CAGTGCAAAGTCCCTAACTTTG | 310 | 70 | 2.0 |
| Liver exon 2A | GGACTGTCTCTGTACTGATGGCTC | CAGAAGGGCCTGGGAAGAAGAGG | 199 | 70 | 3.0 |
| Exon 2 | CCAGCCCGACTGCTCCCATCC | CTTCTGGATGAGGAGCCGGTTAC | 231 | 69 | 2.0 |
| Exon 3 | CCTGTGGGTGTCCCTGAGGC | GCATGGCCTTGGCCCCCTGC | 243 | 63 | 2.0 |
| Exon 4 | CATGCCAGATGGTCACCATGGC | CTCCCCTCATCTGCCTTCTGC | 193 | 63 | 2.0 |
| Exon 5 and 6 | GCCTCCAGATATGTTAGCAGCCACG | GCAGTCTGGAAGGGGCAGGGGTG | 387 | 71 | 2.0 |
| Exon 7 | GTGCAGCTCTCGCTGACAG | CCAGGGCCTGGGTTGTGG | 258 | 62 | 2.0 |
| Exon 8 | GCCTGCTGATGTAATGGACC | CTTTGCACCCACCCTCCTC | 220 | 60 | 1.5 |
| Exon 9 | TGGAGGGGGATGGACTGTCG | TTTGGGCCCCACTTTACCAGG | 338 | 64 | 2.0 |
| Exon 10–1 | GGGACGGCAGCCCTGCTTC | GCATCCTCCCTGCGCTTGCG | 206 | 62 | 2.5 |

earlier (5) on 20 diabetic subjects who were randomly selected from the current 60 subjects for SSCP analysis. Genomic DNA samples were digested with *Eco*RI or *Bam*HI. A full-length human islet cDNA (7) and a partial human liver cDNA containing exon 1 and 2A (8) were used as probes.

PCR amplification was conducted in a 10-μl volume containing 100 ng genomic DNA, 10 μM each primer (Table 1), 0.2 mM each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.25 U Amplitaq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT), and $MgCl_2$ as shown in Table 1. Either 1 μCi of α-[$^{32}$P]dCTP or 1.0 μM of [$^{32}$P]end-labeled primers (3) were added to the PCR mixture. The PCR conditions were initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at the temperature as shown in Table 1 for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 9 min. PCR conditions were optimized to ensure a single PCR product of the appropriate size on a 2% agarose gel. After being denatured at 90°C for 3 min in 42.5% formamide, 10 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol, samples were immediately placed on ice. Aliquots of each sample were electrophoresed under three different conditions to optimize detection of variants: *1*) 8% Long Ranger gel (Hydrolink, AT Biochem, Malvern, PA) with 10% glycerol in 1.2 × TBE at 20 watts for 14 h at ambient temperature; *2*) 0.5 × MDE gel (Hydrolink, AT Biochem) in 0.6 × TBE or 8% Long Ranger gel in 1.2 × TBE at 5 watts for 14 h at ambient temperature; and *3*) 8% Long Ranger gel in 1.2 × TBE at 30 watts for 8 h at 4°C.

Genomic DNA from individuals noted to have aberrant SSCP patterns was amplified as above except for a 100-μl volume containing 1 μg of genomic DNA and 2.5 U Amplitaq DNA polymerase. Amplified samples were resolved on 1% low-melting-point agarose to ensure a single specific product. The DNA was cut out of the gel and purified by PCR Magic Prep (Promega, Madison, WI). The purified products were sequenced by using a double-stranded DNA Cycle Sequencing System (BRL, Gaithersburg, MD) with either PCR primers or internal primers. All the variants were confirmed by sequencing in both directions.

## RESULTS AND DISCUSSION

Our previous study (3) showed that one allele (Z + 4) at the GCK1 locus was associated with NIDDM in American blacks. The hypothesis to be tested by the current analysis was that there was linkage disequilibrium between a variant of the glucokinase gene and the Z + 4 allele. Because 39% of NIDDM subjects have at least one Z + 4 allele (3), we elected to screen a random sample of 60 NIDDM subjects. By doing this, we were not only able to screen a relatively large number of Z + 4 allele, but also could detect the variants that were not linked to the Z + 4 allele.

The human glucokinase gene has recently been isolated and characterized (5). The gene spans a region of >50 kb on chromosome 7p (9) and is composed of 12 exons (islet exon 1, liver exon 1, liver exon 2A, which is expressed in a minor variant with an unclear physiological role, and exons 2–10, which are common to both islet and liver glucokinases). We designed 12 sets of primers (Table 1) to amplify all of the exons in fragments of <400 bp, including the 5′-UT and coding regions, along with their adjacent splice acceptor and donor sites. Islet exon 1 is 531 bp and was therefore amplified by two sets of primers. Exons 5 and 6 were amplified together with one set of primers because of a short intron (109 bp) between them. Exon 10, which contains a large 3′-UT region, was screened in the coding regions only.

DNA from NIDDM patients was screened by molecular scanning with the SSCP technique. This technique is very sensitive but not without limitations. For example, if an individual had a deletion in one allele, the DNA from the normal allele would still be amplified and result in a normal SSCP pattern. To exclude this possibility, genomic DNA from 20 randomly selected patients was examined by Southern blot analysis with labeled islet and liver cDNA probes. No deletions, insertions, or gross rearrangements were found (data not shown).

The sensitivity for detecting single-base changes by SSCP analysis has not been well defined. Our previous experience showed this method was capable of detecting 7 of 7 known variants at insulin locus (10). To optimize detection, the PCR products were examined by three electrophoretic conditions (6). DNA samples from 60

TABLE 2
Nucleotide variations of glucokinase in 60 American black NIDDM subjects

| Exon | Nucleotide* | Common | Variant | *n* (%) |
|---|---|---|---|---|
| Exon 1 (islet) | 220 | **G** | **T** | 5 (8.33) |
| | 403 | **C** | **G** | 5 (8.33) |
| | 517 | **G**CC (Ala$^{11}$) | **A**CC (Thr$^{11}$) | 2 (3.33) |
| Exon 1 (liver) | 99 | **A** | **G** | 1 (1.66) |
| | 125 | **C** | **T** | 1 (1.66) |
| Exon 3 | 629 | GA**C** (Asp$^{112}$) | GA**T** (Asp$^{112}$) | 1 (1.66) |
| Exon 6 | 908 | AC**G** (Thr$^{205}$) | AC**C** (Thr$^{205}$) | 1 (1.66) |
| | 950 | TG**C** (Cys$^{219}$) | TG**T** (Cys$^{219}$) | 1 (1.66) |
| Exon 8 | 1244 | GG**G** (Gly$^{317}$) | GG**C** (Gly$^{317}$) | 1 (1.66) |
| Intron | | | Genotype | n (%) |
| Intron 1B† | | | C/C | 59 (98.34) |
| | | | C/T | 0 |
| | | | T/T | 1 (1.66) |
| Intron 9‡ | | | C/C | 38 (63.33) |
| | | | C/T | 18 (30.00) |
| | | | T/T | 4 (6.67) |

*For the amino acid and nucleotide numbering, see ref. 5 for islet and liver exon 1, and see ref. 8 for the common exons 2–10.
†Partial sequence of intron 1B: 5′-tcccc**c**tccctgtgcagGTAGAGCAGATCCT-3′ (Intron 1B is in the lower case, exon 2 is in the upper case, and the bold **c** is the mutation site).
‡Partial sequence of intron 9: 5′-TGCACCCAGgtgagcc**c**gcccgc-3′ (Exon 9 is in the upper case, intron 9 is in the lower case, and the bold c is the polymorphic site).

NIDDM patients were examined by PCR and SSCP method (12 exons, including islet exon 1, liver exon 1, liver exon 2A, and the common exons 2–10). A total of nine exon variants were identified within the gene (Table 2).

Two variants found in islet exon 1 were in the 5′-UT region of the gene. The islet glucokinase has a relatively long 5′-UT region 487 (7) vs. 168 (8) bp for the liver form and because it has been shown in experimental animals (11) that glucokinase activity changes under certain conditions in the absence of changes in mRNA, translational control could be important in glucokinase gene expression. The effects of the relatively common 5′-UT changes noted are unknown, although in a sampling of 43 nondiabetic individuals, 4 and 3 were found to have 5′-UT variants, respectively. In the 5′-UT of liver exon 1, two variants were identified, each in 1 NIDDM subject.

Five variants occurred within the coding region of the gene in a single allele for each patient. These variants are without obvious biological consequence, except the substitution of Ala$^{11}$ (**G**CC) with Thr$^{11}$ (**A**CC) in islet exon 1 in 2 patients. Interestingly, islet exon 1 in human and rat glucokinases are identical in 14 of 15 amino acids (7,13). The only difference is Thr$^{11}$ in the rat (12). The biological activities of the human and rat islet glucokinase enzymes have not been compared, so the effects of this mutation are unknown. The Thr$^{11}$ to Ala$^{11}$ mutation, however, was as common in a small sampling of nondiabetic individuals (4 of 43). The other four variants encoded third bp (i.e., silent) changes of the codons. A potentially useful intron polymorphism (allele frequency 22%) was found in intron 9 (Table 2), which may serve as a marker in future genetic studies.

The linkage relationships between the SSCP variants and the microsatellite repeats at either GCK1 or GCK2, another microsatellite at the 5′-end of the glucokinase gene (5), were evaluated. Interestingly, 5 NIDDM subjects had both variants in islet 5′-UT region, and all 5 subjects had a least one Z allele (Table 3). This observation suggested the possibility of linkage disequilibrium among these two variants and GCK1. However, in the absence of family data, haplotype analysis could not be done for these relatively uncommon variants. Haplotypes were used to define the linkage relationship between intron 9 polymorphism and GCK1 (Table 4), revealing that this polymorphism was randomly associated with GCK1. A similar result was found between the intron 9 polymorphism and GCK2 (data not shown).

Amplification of liver exon 1 was not consistent for 26 patients. Among these 26 patients, Southern blot analysis on 6 patients revealed no gross deletions, insertions, or rearrangements. They were amplified with another set of primers (CATGGGCCTACCCTCCCTTTC and GGTAATC TGCAAAACCAAGGGCC) that were further distal to the previous set (Table 1). No difference was found in the flanking regior of the previous set of primers (Table 1) in 4 patients by direct sequencing. Variable amplification may be attributable to polymorphism in these introns, although we have no proof of this.

TABLE 3
Linkage relationships between SSCP variants and GCK1

| Islet Exon 1 | Genotype | Patient ID | GCK1 |
|---|---|---|---|
| Nucleotide 220 | G/T | 123 | Z/Z+2 |
| Nucleotide 403 | C/G | 111 | Z/Z |
| | | 99 | Z/Z |
| | | 85 | Z/Z+2 |
| | | 42 | Z/Z |
| Nucleotide 517 | **G**CC(Ala)/**A**CC(Thr) | 103 | Z/Z+2 |
| | | 60 | Z/Z+2 |

TABLE 4
Linkage relationship between intron 9 polymorphism and GCK1

| Haplotype | | Observed | | Expected |
|---|---|---|---|---|
| I–9 | GCK1 | *n* | Frequency (%) | frequency (%) |
| C | Z | 34 | 38.20 | 37.86 |
| C | Z+2 | 18 | 20.22 | 17.63 |
| C | Z+4 | 12 | 13.48 | 15.67 |
| C | Z+10 | 10 | 11.24 | 7.18 |
| T | Z | 10 | 11.24 | 10.46 |
| T | Z+2 | 3 | 3.37 | 4.88 |
| T | Z+4 | 2 | 2.25 | 4.33 |
| T | Z+10 | — | | 1.99 |
| Total | | 89 | 100.00 | 100.00 |

The frequencies of variations in the intron, 5′-UT, and coding regions are very similar. A total of two variants (1 of 225 bp, 0.45%) among 449 bp of intron, which is adjacent to exon, five variants among 1433 bp (1 of 287 bp, 0.34%) of coding sequence, and four variants (1 of 164, 0.61%) among 654 bp of 5′-UT, were identified from 60 NIDDM patients. As compared with the previous study of insulin gene variants in the same population (10), more coding variants and less intron variants were found in the glucokinase gene.

While this work was in progress, linkage between the glucokinase gene and MODY, a variant of NIDDM, was found (13,14). Subsequently, a nonsense mutation in the glucokinase gene was identified in one of the MODY families (15). We tested our SSCP system with the above published mutant, and four other unpublished mutants (provided by Philippe Froguel), and the present system was capable of detecting all of the variants. These results suggest that although mutations of the glucokinase gene may occur in as many as 50% of Caucasian MODY patients, they are very rare in American black NIDDM patients. Glucokinase defects in MODY families may result in mild β-cell dysfunction, which may not be a major feature of NIDDM in general (14).

This study did not find a mutation in the coding sequence that could explain the previous positive association in the American blacks (3). These results indicate that the glucokinase gene is highly conserved in the common form of NIDDM, and that structural changes in glucokinase do not contribute to this disease in the great majority of NIDDM patients. The previous association noted in American blacks is therefore either spurious, or attributable to mutations in adjacent regulatory regions of the gene that have yet to be defined.

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