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Molecular screening of the glucokinase gene in familial Type 2 (non-insulin-dependent) diabetes mellitus

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Summary The glucokinase locus has been implicated by linkage studies in several Caucasian pedigrees with early onset, autosomal dominant diabetes, and mutations have been identified in a large number of these pedigrees. Although mutations have been reported in some pedigrees with late onset Type 2 (non-insulin-dependent) diabetes mellitus, linkage studies of typical familial Type 2 diabetes did not suggest a major role for this locus. Nonetheless, linkage studies were consistent with the hypothesis that mutations of the glucokinase gene were responsible for the pathogenesis of Type 2 diabetes in a minority of pedigrees or one gene in a polygenic disorder. To systematically address this hypothesis, we examined 60 diabetic members of 18 pedigrees ascertained for two or more Type 2 diabetic siblings and eight unrelated diabetic spouses. Initially, the coding regions from each of the 11 glucokinase exons were examined by the sensitive technique of single strand conformation polymorphism analysis to screen for single nucleotide substitutions. Subsequently, we also sequenced each exon from an affected member of the single pedigree in which a glucokinase allele was most likely to segregate with diabetes. Single strand conformation polymorphism analysis detected only three variants, none of which altered the amino acid sequence. No coding or splice site mutations were detected. Likewise, no additional mutations were detected upon direct sequence analysis. However, additional screening of promoter and 3′ untranslated regions detected a variant pattern in the untranslated region of exon 10 which appeared to segregate with diabetes and impaired glucose tolerance in one pedigree. Sequence analysis demonstrated the deletion of a cytosine in exon 10 at position 906, but this deletion was not associated with Type 2 diabetes among unrelated spouses, was not linked to diabetes, and was not associated with significant elevations of fasting glucose or insulin among non-diabetic pedigree members. Similarly, two common variants in the islet promoter did not segregate with diabetes. We conclude that among typical familial Type 2 diabetes in a population representative of Northern European Caucasians, glucokinase mutations are an unlikely cause of diabetes. [Diabetologia (1994) 37: 182–187]

Key words Familial Type 2 (non-insulin-dependent) diabetes mellitus, molecular screening, single strand conformation polymorphisms, glucokinase gene.

The mode of transmission for typical Type 2 (non-insulin dependent) diabetes mellitus has not been established, but the genetic predisposition is generally accepted [1, 2]. Although the primary defect which accounts for this predisposition is uncertain, both impaired insulin action (insulin resistance) and defects of insulin secretion appear to characterize the diabetic state [3]. Glucokinase (ATP: D-glucose 6-phosphotransferase; EC 2.7.1.2) is among the few candidate genes which, if defective, might effect both pathways. Glucokinase (GCK) is expressed in pancreatic beta cells, where it may play a key role in the sensing and thus the coupling of insulin release to plasma glucose [4, 5]. GCK is also expressed in hepatocytes, where phosphorylation of glucose maintains a gradient for in-

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ward transport [4, 5]. Thus, GCK may be essential to hepatic glucose disposal. The GCK gene has recently been characterized [6, 7], and consists of 12 exons. Exons 2 to 10 are common to both liver and islet isoforms, but alternative first exons are expressed in the islet and liver forms of the enzyme.

A role for glucokinase in Type 2 diabetes has been strongly suggested by the observations of linkage in both French and English pedigrees with early onset autosomal dominant diabetes [8, 9]. Mutations have been identified in exons 2 to 10 in French pedigrees [6, 10, 11], exons 5 and 7 in Japanese pedigrees [12, 13], and exon 8 in English pedigrees [14]. In one pedigree, a mutation of exon 7 appeared to raise the set-point for glucose stimulated insulin secretion and reduce the beta-cell capactiy to respond to a continuous glucose infusion [15]. Although most of these mutations have been restricted to a single pedigree, in at least one instance a mutation detected in a pedigree with maturity onset diabetes of the young (MODY) was subsequently found in many diabetic members of a typical familial Type 2 diabetic pedigree [14].

We have previously reported on 18 pedigrees ascertained from the Utah population for two or more diabetic siblings with more typical age of onset [16, 17]. Although pedigree members were hyperinsulinaemic when compared with spouse control subjects, offspring of two affected parents were hypoinsulinaemic when compared with offspring of non-diabetic parents, thus suggesting possible defects in both beta-cell function and insulin action [16]. Using the two GCK microsatellite polymorphisms, we were able to reject linkage of GCK and diabetes for the pooled pedigrees under several models, including models with heterogeneity [18]. Lack of linkage was also reported among English Type 2 diabetic families by Cook et al. [19]. Nonetheless, nearly one-third of the pedigrees were uninformative (log of the odds [LOD] scores near 0), and one pedigree (pedigree 9) had an individual LOD score which approached 2 under an autosomal dominant model [18]. Linkage analysis has limited power to detect a mutation under models where the locus might account for fewer than 50% of pedigrees [20; SCE unpublished data] and where multiple genes may be involved. Thus, a role for GCK mutations as an important cause of typical familial diabetes in a minority of pedigrees or under a polygenic model of Type 2 diabetes could not be rejected.

To address the hypothesis that mutations in the coding region of the GCK gene contribute to the pathogenesis of common familial diabetes, we used single strand conformation polymorphism (SSCP) analysis to screen for mutations in the coding regions of the GCK gene for each GCK allele which appeared in at least one diabetic individual in each pedigree. Additionally, because the sensitivity of SSCP remains uncertain, we sequenced each exon for the pancreatic and liver isoforms from the single pedigree which appeared most likely to carry a defective GCK allele. Finally, we completed the SSCP screening for the promoter regions of both pancreatic and liver GCK isoforms and for the non-coding regions of exons 1 and 10 for the pedigrees in which linkage analysis of GCK and diabetes was most suggestive of GCK involvement.

Subjects, materials and methods

Study population

SSCP analysis [21, 22] was performed on DNA samples from 60 diabetic members drawn from 18 pedigrees and 8 unrelated spouses with Type 2 diabetes. All diabetic individuals were either under treatment for diabetes or met World Health Organization criteria for diabetes, and none were ketosis-prone. Sixteen pedigrees were selected for two or more diabetic siblings, as described in detail elsewhere [16, 17]. An additional two pedigrees were selected for a mixture of Type 1 and Type 2 diabetes, but included a Type 2 diabetic sibling pair. None of the pedigrees studied met the usual definitions for MODY. All pedigree members included in earlier analysis [17, 18] were studied. Within each pedigree, individuals were selected to ensure analysis of each independent GCK allele which appeared in a diabetic individual. GCK alleles were determined by assignment of haplotypes using the GCK-1 and GCK-2 polymorphisms [18]. The number of individuals required to examine each allele was dependent on whether parental alleles could be distinguished, and whether parents were alive. Although for a fully informative pedigree the four parental alleles might be examined with two individuals, many pedigrees were not fully informative and required analysis of all diabetic siblings.

Single strand conformation polymorphism analysis (SSCP)

DNA from members of each pedigree, as described above, was screened for mutations in alternative first exons for islet and liver isoforms and exons 2–10 according to the method of Orita et al. [21] and as we described previously [22]. Amplification was performed by polymerase chain reaction with Taq polymerase (Perkin-Elmer Cetus, Norwalk, Conn., USA) for 30 cycles, using the same primer sequences and similar conditions to those described elsewhere [23], except that exons 7–10 were amplified in the presence of 5% glycerol. For each exon, 0.2 μg of DNA was amplified in a 10 μl reaction volume in the presence of 0.3 μCi of α-^{32}P-dCTP (> 3000 Ci/mmol; Amersham, Arlington Heights, Ill., USA) and 5 pmol each primer, except exons 8 and 10 which were amplified with 1.25 mmol and 2.5 mmol of each primer, respectively. Amplified fragments were of size 193 bp to 387 bp. After amplification, samples were diluted to 60 μl in 0.1% SDS and 1 mmol/l EDTA. An aliquot of this sample was diluted 1:1 with loading buffer which contained 95% formamide. The diluted sample was denatured by boiling 5–10 min and loaded on 6.5% non-denaturing polyacrylamide gel on an IBI ST45 sequencing apparatus (35 cm × 41 cm; International Biotechnology, Inc., New Haven, Conn., USA). Each exon was examined under four conditions: 5.0% acrylamide at 22 °C; 5.0% acrylamide and 10% glycerol at 22 °C; 5.0% acrylamide at 4 °C; and 5.0% acrylamide with 10% glycerol at 4 °C. All gels were run at 30 watts with 1 × Tris-borate-EDTA (90 mmol/l Tris-borate, 2 mmol/l EDTA) for 6–16 h with a cooling fan. Gels were dried and exposed to

XAR-5 film (Kodak, Rochester, NY, USA) at −70 °C without an intensifier screen for 24–48 h. Non-denatured samples were included on each gel to determine the location of any residual non-denatured DNA.

Liver and islet promoter regions were initially examined in eight pedigrees which were considered most likely to have GCK mutations based on earlier linkage analysis, using conditions as described above. The islet promoter was subsequently examined by SSCP in the remaining population under conditions which detected the variants identified in the initial eight pedigrees. The 3′ untranslated region of exon 10 was examined under all four SSCP conditions for individuals from the two pedigrees which had the highest LOD scores in linkage studies (autosomal dominant model). The region of exon 10 (3′ untranslated) which contained the variant was initially amplified with primers GCTTTGAATACCCCCCAGAGAC and CACAGTCCTAATGCACAGAAGTC as described above, but with 2.5 pmol/l of each primer in the presence of 2.0 mmol/l magnesium and 5% glycerol, with an annealing temperature of 64 °C.

DNA sequence analysis

All sequencing experiments were performed after subcloning of the amplified exon into M13mp18 or M13mp19. Exons 3, 7 and 9 were amplified with primers which were modified to include sequences for an EcoR1 site on the 5′ primer and the Hind III recognition sequence on the 3′ primer. After amplification, samples were treated with 100 μg/ml proteinase K for 1 h at 37 °C [24] followed by standard phenol/chloroform extraction. Redissolved samples were simultaneously digested with Hind III and EcoRI and cloned into appropriately digested M13mp18 and M13mp19 vector. For all other exons kinase-treated primers were used for amplification. After treatment with proteinase K, DNA was purified on low melting temperature agarose or by direct phenol/chloroform extraction [25]. Redissolved samples were treated with DNA polymerase, large fragment and purified by phenol/chloroform extraction prior to cloning into M13mp18 or M13mp19 prepared with SmaI [25]. Templates were screened for inserts of the correct size by PCR with the original amplification primers [26]. All subcloned exons were sequenced by dideoxy sequencing with ^{35}S dCTP and Sequenase (United States Biochemical, Cleveland, OH, USA) using the M13 universal primer, according to manufacturer's protocols and as described elsewhere [22]. At least eight clones were sequenced for each exon examined. Sequencing products were separated on an 6.5% or 8% polyacrylamide sequencing gel. Sequencing data were compared with cDNA sequence [27], sequence of control samples, and genomic sequence [7].

Screening for deletion in exon 10 untranslated region

The 69 bp region containing the single nucleotide deletion was amplified with primers (5′) AGCGCTGGCTCAGGAAGAAAC and (3′)TGTCATATGATGGGGTTGTCC for 30 cycles (1 min at 94 °C, 1 min at 51 °C, 1 min at 72 °C) as described above in the presence of 1.5 mmol/l MgCl and with 5% glycerol. The 5′ primer was labelled with γ-^{32}P ATP (> 5000 Ci/mmol; Amersham). Following amplification the samples were reheated to 94 °C and cooled to room temperature over 30 min to promote heteroduplex formation. The deletion was detected by heteroduplex analysis of non-denatured samples separated for 12–14 h at 800 volts on Hydrolink-MDE gels (J. T. Baker, Phillipsburg, NJ, USA) according to manufacturer's protocols. Following electrophoresis the gel was dried and exposed to XAR-5 film for 12–36 h. Additionally, the deletion was detected under denaturing conditions by mixing 7 μl of amplification mix with 3.5 μl of 95% formamide/10 mmol/l NaOH tracking dye. After heating to 95 °C for 5 min, samples were separated at 60 watts for 3–3.5 h on 7% acrylamide gel containing 32% formamide and 5.7 mol/l urea.

Results

Screening of 60 diabetic members of 18 pedigrees and 8 diabetic spouses resulted in the evaluation of approximately 88 independent GCK alleles which appeared in at least one diabetic individual. Liver and islet exon 1, exons 2–9, and the coding region of exon 10 were examined by SSCP analysis, as described above and elsewhere [21, 22]. Variant SSCP patterns were detected in islet exon 1, exon 3 and exon 9. No variations were detected in any other exon under any gel condition. The variant pattern in islet exon 1 was noted in six pedigrees with gels run at 22 °C either with or without glycerol. A variant pattern in the exon 3 amplification product was noted only under conditions of 22 °C with glycerol, and was detected in only two pedigrees. The variant in the exon 9 amplification product was easily detected only under conditions of 22 °C with 10% glycerol, and was very common with a minor allele frequency of 0.2. This variant was present in 13 of 18 pedigrees. None of these variants appeared to segregate with diabetes. Sequence analysis of islet exon 1, exon 3, and exon 9 PCR products showed a guanine for cytosine substitution 84 bp upstream from the first methionine (nucleotide 403 in [7]) in the 5′ untranslated region of islet exon 1, an adenine for guanine substitution 8 bp upstream from the 5′ splice site of exon 3, and a thymidine for cytosine substitution 8 nucleotides downstream from the 3′ splice site in intron 9, respectively. Thus, all variants were in non-coding or intron regions, and none altered the amino acid sequence.

Although SSCP appears to have high sensitivity when amplified samples are examined under four conditions [21, 22], uncertainty about the actual sensitivity and our previous findings suggestive of linkage with Type 2 diabetes in pedigree 9 [18] suggested a need to determine the actual sequence of the GCK coding region for the candidate allele from this pedigree. Translated regions of liver and islet exons 1 and exon 10 and exons 2–9 were amplified, subcloned and sequenced from an affected member of this pedigree. Only the common intron 9 variant was detected, and we found no mutation which accounted for the observed segregation [18].

In the absence of a coding mutation which could explain our linkage findings, we extended our SSCP analysis to the promoter region for both islet and liver isoforms and the 3′ untranslated region of exon 10 for 7 individuals drawn from the two pedigrees with the most suggestive LOD scores. Variants were detected in the islet promoter region at positions -194 (G→A) and -30

Table 1. Characteristics of pedigree members with and without the exon 10 deletion

Del	*n*	BMI	Age	Fast Gluc	1-h Gluc	2-h Gluc	Fast Insulin	1-h insulin
WT	77	26.7	38	4.98 ± 0.62	7.56 ± 3.48	5.28 ± 2.19	79.1 ± 59.3	481.2 ± 416.8
Del	58	26.7	35	5.42 ± 2.48	7.82 ± 3.65	4.82 ± 2.27	89.5 ± 62.5	574.7 ± 592.9

Characteristics of pedigree members who carry at least one copy of the deletion (Del) and those who are homozygous for the non-deleted allele (WT). *n*, Number of individuals in each group; BMI, mean body mass index; Age, mean age; Fast Gluc, mean fasting glucose (mmol/l); 1-h Gluc, mean glucose at 1 h during a 75-g glucose tolerance test (mmol/l); 2-h Gluc, mean glucose at 2 h during a 75-g glucose tolerance test (mmol/l); Fast Insulin, fasting insulin, pmol/l; 1-h insulin, mean insulin at 1 h during a 75-g glucose tolerance test (pmol/l). None of the differences approach significance

(A→G) in separate pedigrees, but neither appeared to segregate with diabetes. Subsequent screening demonstrated the -194 variant in 6 of 18 pedigrees (allele frequency 7%) and the -30 variant in 10 of 18 pedigrees (allele frequency 10%).

Screening of the untranslated region of exon 10 detected a variant in pedigree 9, the pedigree which previously demonstrated the highest LOD score under autosomal dominant models [18]. Unlike other variants, this one was detected in the non-denatured region of the SSCP gel, and appeared to segregate with diabetes and impaired glucose tolerance. Sequence analysis demonstrated a deletion of a cytosine at nucleotide + 906 from the beginning of exon 10. Further screening identified the pattern in three additional pedigrees and the mutation was confirmed by sequence analysis in one pedigree, where the pattern appeared to segregate with diabetes. For unclear reasons the deletion was not completely reproducible, however, and we devised new primers to amplify a smaller product. With the smaller product we reliably and reproducibly detected the single nucleotide deletion by both heteroduplex methods [28] and by size separation on denaturing gels.

The deletion was present in at least one diabetic member in 10 of 18 families, but was present in all diabetic family members in only two pedigrees. The deletion was present in 16 of 66 unrelated non-diabetic individuals (one homozygote) for an allele frequency of 12.8%. Using this frequency and repeating our earlier linkage analysis with the addition of this marker under an autosomal dominant model in which GCK mutations would contribute to only a fraction of Type 2 diabetes, we again found no evidence for linkage. Furthermore, comparison of fasting glucose and insulin levels and 1-h glucose and insulin levels from a 75-g glucose tolerance test demonstrated no significant differences among either unrelated individuals or among 77 pedigree non-diabetic members without the deletion and 58 members with the deletion (Table 1).

Discussion

Although other candidate genes for diabetes may play a role in occasional pedigrees, GCK is the first defined gene to have a clear role in multiple pedigrees with the Type 2 diabetic phenotype. Among those pedigrees in which this locus has been implicated in diabetes by linkage studies or by definition of the mutation, beta-cell function appears to be reduced. However, those individuals described with GCK mutations have had mild diabetes which stands in contrast to the more typical presentation of Type 2 diabetes in our pedigrees. Mutations of exon 8 (codon 299; [14]) and exon 5 (termination codon; [12]) have been associated with the late-onset diabetic phenotype, and neither mutation appeared to be present upon screening additional typical Type 2 diabetic individuals. We [18] and others [11, 19] have not found linkage of GCK and diabetes among typical familial Type 2 diabetic pedigrees. In screening of a single diabetic individual from 21 families with transmission of diabetes consistent with autosomal dominant transmission, Froguel et al. [11] also failed to identify any mutations. Finally, although case control studies have detected an association of certain alleles (as defined by the 3′ flanking microsatellite polymorphism) with Type 2 diabetes in both American Black [29] and Mauritian Creole [30] populations, Chiu et al. [23] failed to identify mutations which were likely to alter GCK function among American Blacks.

The mild glucose intolerance which typifies all of the reported GCK mutations suggested to us that for these mutations to account for the severity of typical Type 2 diabetes, they would have to be present with a second defect. This model is consistent with current models of the pathogenesis of Type 2 diabetes which suggest defects of both insulin secretion and insulin action. In this setting linkage studies might have limited power. Additionally, linkage studies in our pedigrees could not exclude a role for GCK mutations in over one-third of families which were uniformative for the known markers. Finally, even among typical Type 2 diabetic pedigrees in which GCK mutations have been identified, the mutation was not present in all diabetic individuals [14]. Thus, reported linkage studies do not fully address the role of GCK in typical Type 2 diabetes.

We are aware of three other reported studies which utilized SSCP to examine all glucokinase exons. We are not aware of any published studies which included promoter and untranslated regions. Chiu et al. [23] identified nine variants among a population of unrelated American Blacks, including the exon 1 and intron 9

variants which were also identified in the present study. These individuals were not drawn from families with a strong familial aggregation of diabetes, however. Sakura et al. [13] examined 100 Japanese individuals with one affected family member and onset before age 40, but the criteria used to diagnose diabetes were unclear. They identified a single mutation in a 10-year-old with mild diabetes. Clearly these investigations do not address the frequency of GCK mutations among Caucasian populations. Froguel et al. [11] examined 21 individuals drawn from 21 French (Caucasian) families with diabetes in consecutive generations. However, in contrast to our study affected individuals apparently included those with mild fasting hyperglycaemia. While the strategy of Froguel et al. [11] was appropriate to detect GCK mutations segregating in an autosomal dominant fashion, this strategy might not detect such mutations if they were part of a polygenic disorder.

The current study differs from those previously published in several ways. First, our families were ascertained for a minimum of a sibling pair with typical late-onset diabetes, generally requiring therapy. Although we have included individuals with impaired glucose tolerance for linkage studies under some models, SSCP analysis was restricted to individuals with Type 2 diabetes by World Health Organization criteria. Because these families have a strong aggregation of diabetes (in most cases three or more diabetic siblings), we would expect that such pedigrees are enriched for genes which predispose to diabetes. Secondly, rather than testing a single member of each family, our goal was to examine every GCK allele which segregated in a diabetic individual. We believe that this strategy is appropriate when molecular screening is used to examine genes which are not linked to diabetes, since under polygenic or multifactorial models individuals within a pedigree may have diabetes from another cause and not inherit the candidate gene mutation. Finally, unlike other reported studies, our population is representative of Northern European extraction, and thus may be more similar to the population in which GCK mutations have been identified in late-onset diabetes [14]. Arguably the testing of 60 unrelated diabetic individuals from our population would give a better estimate of the frequency of glucokinase mutations in Type 2 diabetes, but we believe that screening families with evidence for a genetic defect predisposing to diabetes is more efficient. If genetic defects are not detected in the extensive screening of members of families with multiple affected members, they are unlikely to be a significant cause for the familial aggregation of Type 2 diabetes.

Although we detected no mutations which altered the protein sequence, we have identified two islet promoter variants and a single nucleotide deletion in the untranslated region of exon 10. The promoter variants were also detected in American Blacks [KC, YT, MAP, unpublished data] and do not segregate with diabetes. Thus, these variants are probably silent. Although the exon 10 deletion was present on a haplotype which segregated with diabetes and impaired glucose tolerance in an autosomal dominant fashion in the pedigree with the highest LOD score on our initial analysis, repeat linkage analysis with the inclusion of this marker remained strongly negative. The variant, which to our knowledge has not been previously reported, was not associated with Type 2 diabetes in the small population of unrelated individuals examined, and did not appear to alter fasting glucose among pedigree members who carried the variant. Furthermore, the deletion is in the untranslated region of exon 10 where it has no known biological function. Thus, we suspect that the presence of this deletion in a large number of affected individuals results from the high frequency, and that this deletion also represents a silent polymorphism. Large case control studies would be required to determine whether this deletion or the promoter variants have an effect not detectable in our study, however.

The sensitivity of our screening technique is critical to our conclusion that GCK mutations do not play an important role in typical Type 2 diabetes among Caucasians. The SSCP technique has been used extensively to identify mutations in our laboratories and by our colleagues [31], and has been used to detect most mutations reported in the GCK gene to date [6, 10]. In our laboratory (SCE, HQ, MH), the technique has detected all mutations which were initially detected by DNA sequence analysis, although we have had several instances where the mutation was only detected under one of four conditions and we have found no consistent condition which could be eliminated ([22] and SCE, unpublished data). Limited validation of the technique by other investigators has also suggested high sensitivity [32], and sensitivity may exceed 90% when all conditions originally described are included and fragment size is kept below 400 bp. Although a recent study suggested limitations in the ability of SSCP to detect mutations in regions which were predicted not to alter secondary structure [33], these investigators examined only the two SSCP conditions which in our experience are least often informative (22 °C without glycerol and 4 °C with glycerol). Furthermore, as our identification of the exon 10 deletion demonstrated, alterations from heteroduplex mismatch are often detected on SSCP gels. Thus, we believe that we would be unlikely to miss an important mutation under the conditions of the present study. Our sequence analysis supports this conclusion.

In conclusion, the present study confirms the findings of our earlier linkage study and expands the results of Froguel et al. [11] to suggest that GCK exon mutations are not a significant cause of diabetes among typical Caucasian Type 2 diabetic families. Early onset (MODY) diabetes is extremely rare in our population, and these mutations may be more common in populations with a higher prevalence of MODY. We also can-

not exclude a small role for GCK regulatory mutations in regions other than the promoters and untranslated regions.

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