Amino Acid Substitutions in Hexokinase II Among Patients With NIDDM

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Hexokinase (HK) II plays an important role in intracellular glucose metabolism by catalyzing the conversion of glucose to glucose-6-phosphate. HKII is considered to be a promising candidate gene for non-insulin-dependent diabetes mellitus (NIDDM) and insulin resistance. Therefore, we investigated the frequency of variants in the coding region of the HKII gene in patients with NIDDM. Initial screening included a population-based sample of 40 Finnish patients with typical NIDDM, and subsequent screening included an additional 72 patients with NIDDM. By applying single-strand conformation polymorphism analysis and direct sequencing, the following amino acid substitutions were found among the 112 NIDDM patients: $Ala^{314}Val$ in one patient (0.9%), $Arg^{353}Cys$ in three patients (2.7%), and $Arg^{775}Gln$ substitution in three patients (2.7%). We also screened 97 subjects with completely normal glucose tolerance and a negative family history of diabetes for these mutations. The $Ala^{314}Val$ and the $Arg^{353}Cys$ substitutions were not found in control subjects, but the $Arg^{775}Gln$ substitution was found in two (2.1%) control subjects. None of these mutations were located close to the glucose- and ATP-binding sites of HKII. We conclude that mutations of the HKII gene are not a major etiological factor for NIDDM in the Finnish population.
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Non-insulin-dependent diabetes mellitus (NIDDM) is a common disorder of glucose metabolism characterized by at least three major defects: insulin resistance, insulin deficiency, and elevated hepatic glucose production (1). Although it is still unclear in what sequence the defects in these processes occur, insulin resistance in skeletal muscle is the most typical finding in patients with NIDDM (2,3).

Heredity plays a major role in the etiology of NIDDM. However, the etiology of NIDDM is known only in a subset of well-defined families with maturity-onset diabetes of the young, in which mutations of the glucokinase (GK) gene lead to a mild form of insulin deficiency (4). In a small minority of NIDDM patients, mutations in the genes encoding insulin (5), the insulin receptor (6), and a mitochondrial tRNA (7) have been described.

The four mammalian hexokinases (HKs) (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) catalyze the conversion of glucose to glucose-6-phosphate, the first step of intracellular glucose metabolism (8). HKI–III consist of a single polypeptide chain of 100 kDa, have a high affinity for glucose, and are subject to allosteric inhibition by glucose-6-phosphate. In contrast, HKIV, also known as GK, has a molecular weight of 50 kDa, has a low affinity for glucose, and is not inhibited by glucose-6-phosphate (9). The striking sequence similarity between the NH_2-terminal and COOH-terminal halves of the HKI–III genes supports the notion that these isoenzymes evolved from HKIV and yeast HK-like enzymes by a process involving gene duplication and tandem ligation (10). HKI is prevalent in brain, kidney, erythrocytes, platelets, and placenta, and it has been cloned from rat brain (11), bovine heart muscle (12), and human kidney (13). HKII is abundant in skeletal muscle and adipose tissue (14) and is therefore a promising candidate gene for NIDDM. Like the rat gene, the human HKII gene consists of 18 exons (M.M., M.L., and S.S.D., unpublished observations) and localizes to chromosome 2 (14). Insulin induces HKII expression, which enhances glucose uptake in insulin-sensitive tissues. HKII cDNA has been cloned from rat (15,16) and human skeletal muscle libraries (17).

The following findings support the notion that HKII could be a promising candidate gene for NIDDM in humans. First, several metabolic studies on NIDDM patients have indicated that the rates of both glucose oxidation and nonoxidation are significantly reduced compared with normal subjects (18,19). Therefore, it is reasonable to assume that the defect in insulin action lies at a step proximal to the activation of the key enzymes involved in glucose oxidation and storage (for example, glucose transport or glucose phosphorylation). Because no defects in the GLUT4 gene have been found in NIDDM patients, HKII is the next logical candidate gene for NIDDM. Second, a decrease in skeletal muscle glucose-6-phosphate has been demonstrated recently in NIDDM using magnetic resonance imaging (20). Low glucose-6-phosphate levels are compatible with HKII deficiency. Third, in streptozocin-induced diabetic rats, the activity of HKII is markedly decreased in skeletal muscle and adipose tissue (8,21). Simultaneous reduction of glucose-6-phosphate in these animals rules out the possibility that low levels of HKII could be the result of allosteric inhibition of this enzyme by a high glucose-6-phosphate concentration.

To test the hypothesis that mutations in the coding region of the HKII gene are found in NIDDM, we determined the intron-exon structure of the gene (in preparation) and screened Finnish patients for amino acid variants. Three

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bp, base pair; GK, glucokinase; HK, hexokinase; NIDDM, non-insulin-dependent diabetes mellitus; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

TABLE 1
Clinical characteristics of the study groups

	Control group	NIDDM group
Sex (M/F)	84/13	56/56
Age (years)	55 ± 1	63 ± 1
Body mass index (kg/m^2)	26.6 ± 0.3	30.3 ± 0.5
Fasting glucose (mmol/l)	5.1 ± 0.1	9.2 ± 0.3
Duration of diabetes (years)	—	8.1 ± 0.8
Age at onset of diabetes (years)	—	54.0 ± 1.1
Treatment for diabetes (%)		
Diet	—	50
Oral drugs	—	28
Insulin	—	22

Data are means ± SE or percentages.

different amino acid substitutions of the HKII gene were found.

RESEARCH DESIGN AND METHODS

All subjects participating in this study were Finnish. The Finnish population is genetically quite homogeneous, descending mainly from a small number of founders of Baltic Finnish and German origin (22).

Initial screening. The subjects with NIDDM screened for HKII variants were selected from a previous population study (23). Altogether, 40 diabetic patients (18 men and 22 women) from this study were randomly selected for the initial analysis. Their mean age was 66 ± 1 years, body mass index 28.5 ± 0.8 kg/m^2, fasting blood glucose 10.0 ± 0.4 mmol/l, duration of diabetes 13.2 ± 1.5 years, and age at onset of diabetes 52.3 ± 1.8 years.

Additional screening. Screening for the amino acid substitutions observed in the initial screening was performed on an additional 49 patients with NIDDM selected randomly from the epidemiological study described above (23) and on 23 NIDDM patients subsequently recruited from the diabetes clinic of the Kuopio University Hospital. The 97 subjects with normal glucose tolerance were selected randomly from two previous population studies (24,25). None of the control subjects had any chronic disease, had been taking any drug treatment that could influence carbohydrate metabolism, had any abnormality in an oral glucose tolerance test (impaired glucose tolerance or diabetes according to the criteria of the World Health Organization [26]), or had hypertension (use of antihypertensive drugs or systolic/diastolic blood pressure >160/95 mmHg). Each control subject had a negative family history of diabetes. Every diabetic and control subject had normal liver, kidney, and thyroid function tests and no history of excessive alcohol intake. Diabetic patients fulfilled the World Health Organization criteria for diabetes and NIDDM (26). Table 1 shows clinical characteristics of all study subjects. The mean age of diabetic patients was 63 years. The diabetic patients who represented typical Finnish patients with NIDDM were obese and hyperglycemic, with a mean age at onset of diabetes of 54 years.

Study protocol. Every control subject participating in this study underwent an oral glucose tolerance test (75 g of glucose) to exclude NIDDM and impaired glucose tolerance. None of the insulin-treated patients had a history of ketoacidosis, and their fasting C-peptide level exceeded 0.20 nmol/l. Therefore, it is unlikely that our study population included a significant number of patients with insulin-dependent diabetes mellitus (27). Informed consent was obtained from all subjects after the purpose and potential risks of the study were explained to them. The protocol was approved by the ethics committee of the University of Kuopio and was in accordance with the Helsinki Declaration.

Analytical methods. Plasma glucose in an oral glucose tolerance test was measured by the glucose oxidase method (Glucose Auto & Stat HGA-1120 analyzer, Daiichi, Kyoto, Japan). Plasma C-peptide concentration was determined by radioimmunoassay (125J RIA kit, Incstar, Stillwater, MN).

Single-strand conformation polymorphism (SSCP) analysis. DNA was prepared from peripheral blood leukocytes by the proteinase K-phenol-chloroform extraction method. All 18 exons and intron-exon junctions of the HKII gene were amplified with the polymerase chain reaction (PCR) using primers shown in Table 2, and the products were digested with the indicated restriction enzymes, if necessary, to obtain fragments of <230 base pairs (bp). SSCP analysis was performed essentially according to the method of Orita et al. (28). The reaction for PCR amplification contained a 10- to 20-μl volume, 100 ng genomic DNA, 5–10 pmol of each primer, 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 0.3–1 U Amplitaq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT), 1.5–2 μCi [α-^{32}P]dCTP, 62.5–200 μmol/l dNTP, and 1–1.5 mmol/l $MgCl_2$. For exon 10, 10% glycerol was added. PCR conditions were denaturation at 94°C for 2–4 min, followed by 35 cycles of denaturation at 92–94°C for 45–60 s, annealing at 62–66°C for 1 min, and extension at 72°C for 45–60 s with a final extension at 72°C for 4 min. The extension step was eliminated when the annealing temperature was >64°C. Before SSCP analysis, PCR fragments were digested with the restriction enzymes given in Table 2. After enzyme digestion, PCR products were first diluted 3- to 10-fold with 0.1% sodium dodecyl sulfate and 10 mmol/l EDTA and then mixed (1:1) with loading dye mix (95% formamide, 20 mmol/l EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol). After denaturation at 98°C for 3 min, samples were immediately placed on ice. Two microliters of each sample were loaded onto a 5% (PCR products ≥200 bp) or 6% (PCR products <200 bp) nondenaturing polyacrylamide gel (acrylamide/*N,N'*-methylene-bis-acrylamide ratio 49:1) containing 10% glycerol. Each sample was run at two different gel temperatures: *1*) at 40 W with fan cooling for ~6 h at a gel temperature of 27–28°C and *2*) at 45 W for ~4.5 h at a gel temperature of 38–40°C. The gel was dried and autoradiographed overnight at −70°C with intensifying screens.

Direct sequencing. Genomic DNA from individuals with variant single-strand conformers was used as a template in the amplification reaction as described above (total volume 100 μl containing 70 pmol of each primer and 5 U Amplitaq DNA polymerase). Amplified segments were purified by electrophoresis on a 1% low-melting-point agarose gel and directly sequenced using Sequenase (United States Biochemicals, Cleveland, OH) as previously described (29).

Statistical analysis. All calculations were performed using the SPSS/PC+ programs (SPSS, Chicago, IL). Data are presented as means ± SE.

RESULTS

Initial screening for sequence variants in the HKII gene. The nucleotide sequence variants were first screened among 40 NIDDM patients. All variants were detectable under both the low- and high-temperature conditions of electrophoresis of single-stranded DNA segments on polyacrylamide gels. Three amino acid substitutions were found: Ala^{314}Val (one patient), Arg^{353}Cys (one patient), and Arg^{775}Gln (one patient). The GCC 314 GTC substitution abolishes a *Hae* III restriction site, and the CGG 775 CAG substitution creates an *Alu* I restriction site. Therefore, the Ala^{314}Val and Arg^{775}Gln substitutions were verified by digestion of the PCR product with *Hae* III and *Alu* I, respectively, and by electrophoresis on an 8% nondenaturing polyacrylamide gel. We also found the following silent substitutions in 40 NIDDM patients: exon 4: CAA 142 CAT (AA in 22, AT in 16, and TT in 2 patients), exon 7: GAC 251 GAT (CC in 22, CT in 16, and TT in 2 patients), exon 15: AAC 692 AAT (CC in 12, CT in 23, and TT in 5 patients), exon 15: CCG 736 CCC (GG in 20, GC in 13, and CC in 7 patients), and exon 16: CTG 766 CTA (GG in 25, GA in 10, and AA in 5 patients).

Additional screening for sequence variants in the HKII gene. Seventy-two additional NIDDM patients (a total of 112) as well as 97 subjects with completely normal glucose tolerance were screened for the amino acid substitutions at codons 314, 353, and 775 of the HKII gene (Table 3). The Ala^{314}Val substitution was found in one (0.9%) NIDDM patient, and the Arg^{353}Cys substitution was found in three (2.7%) NIDDM patients. The Arg^{775}Gln substitution was found in three (2.7%) NIDDM patients and in two (2.1%) control subjects. The presence of the Ala^{314}Val and the Arg^{775}Gln substitutions found by SSCP was verified also by digestion of the PCR product with *Hae* III and *Alu* I, respectively, and by electrophoresis as described above. The new Arg^{353}Cys substitutions were verified by direct sequencing. All patients with amino acid substitutions of the HKII

TABLE 2
Primers, size of the amplified fragment, enzyme digestion, and fragment size for SSCP of the HKII gene

No.	Sequence 5′→3′	Size of amplified fragment (bp)	Cleavage enzyme	Restriction fragments (bp)
1F	CTCTCGCGTCTCCGCCTCGG	178	None	None
1R	ACTGATGGAGGCCAGACCAC			
2F	TCCATGACGTACACCTATGCC	305	*Sau*3AI	120,185
2R	GGTCCCTGAGCAAAGCCAAC			
3F	GTCTCGGTTGGTTCCTGGAAG	246	None	None
3R	GTCACCAGGGGCTGCCCTG			
4F	TCGACCTCTCTGCTCACCAC	231	None	None
4R	GGCTCAGAAGTCCCTCTCAG			
5F	GTTTGAGGGGTGTGGTGTGAG	224	None	None
5R	AGTCTTCAGGGTGGACCCAG			
6F	CGCCCTCTGTGATGATGAAGG	194	None	None
6R	CCATCCCTTGGCCAGCACG			
7F	GCCGGAGCAGGCGTGTGC	258	None	None
7R	CCAAGCCCACTCGCACAGG			
8F	AGTACATGGGCAGTGGGGAC	255	None	None
8R	CAACTCGTGTGGTGATCCAGC			
9F	CACTGGCCACAGTGGGTCAG	335	*Pst* I	207,128
9R	AGGTACTCCAGGAACTCCGC			
10F	CCCTAACCATGGACACCTGTC	390	*Pst* I	214,176
10R	GGCCCACCATGTGAGCCAGG			
11F	TCCCCATGTTCTGCCCCAAC	250	None	None
11R	GGCAAGAGGTGACGTCCACC			
12F	GCAGCCTGCCCTGCCCAGG	206	None	None
12R	CCACAGGAGAGCCCTCCAG			
13F	GACCTGGGAGCTCTTCCCTG	209	None	None
13R	CCAGGCCAATCATTGAGGAGC			
14F	GGTTCACCTGTGAACTGGGC	478	*Eco*O109	145,67,224,42
15R	TCACTTGTTGCCATCCAGTCC			
16F	TGTCTCAACACATCCCTCCAC	284	*BST*NI	89,195
16R	CTTCAGTTTGTCTGCCAGTGAG			
17F	GGCTACCAGCCTTCTTGGTG	312	*Bgl* I	155,157
17R	CAGGGAGGGCTGGGATCAG			
18F	CCTGTGTCTTCCTCCCACC	265	None	None
18R	AAGGGGTCTGTCTCTGACAC			

gene had a positive family history of diabetes. Altogether, seven (6.1%) NIDDM patients and two (2.1%) control subjects had an amino acid substitution in the HKII gene (NS between the groups).

DISCUSSION

The low K_m human HKII is an important enzyme in the regulation of glycolysis in insulin-sensitive target cells, particularly in skeletal muscle and adipose tissue. Several lines of evidence (18–21) support the view that the HKII gene is a promising candidate gene for NIDDM and insulin resistance. In this study, we report previously unpublished amino acid variants of HKII that could potentially be associated with the risk of NIDDM.

Our study included typical middle-aged Finnish patients with NIDDM and corresponding control subjects who were healthy and did not have a positive family history of diabetes. Three amino acid substitutions in the HKII gene were found. The $Ala^{314}Val$ substitution was found in one NIDDM patient (0.9%), and the $Arg^{353}Cys$ substitution was found in three NIDDM patients (2.7%). These mutations were not found in control subjects. The $Arg^{775}Gln$ substitution was found in three NIDDM patients (2.7%) and in two control subjects (2.1%).

Several missense mutations of the GK gene have been described that considerably lower V_{max} or increase K_m (30,31). Mutations located close to the glucose-binding site of GK have measured V_{max} values of 0.5% of the wild type (31). The reduction in catalytic activity caused by these mutations is consistent with the notion that these residues maintain the conformation of the substrate-binding site. HKI–III have two glucose-binding sites that are similar to those in yeast HK and human GK. Four residues implicated in binding of glucose by yeast HK are conserved in both the regulatory (residues 155, 209, 260, and 294) and catalytic (603, 657, 708, and 742) domains of human HK (13). The sequence of the putative ATP-binding site (residues 448–453 and 896–901) is also conserved (13). Arora et al. (32) used site-directed mutagenesis to evaluate the role of amino acid residues predicted to interact with either glucose or ATP-binding sites in the COOH-terminal half of tumor HK. Their results demonstrated that mutant enzymes (Ser^{603}, Asp^{657}, Glu^{708}, and Glu^{742} were mutated to either Ala or Gly) had HK activity (V_{max}) <10% that of the normal enzyme. Similarly,

TABLE 3
Variants of the HKII gene in control subjects and in patients with NIDDM

Exon	Codon	Change	Control subjects (n = 97)	NIDDM patients (n = 112)
8	314	GCC→GTC (Ala→Val)	0	1 (0.9)
9	353	CGT→TGT (Arg→Cys)	0	3 (2.7)
16	775	CGG→CAG (Arg→Gln)	2 (2.1)	3 (2.7)

Data are n (%).

mutations affecting the ATP-binding domain significantly reduced HK activity. Thus, mutations in the glucose- and ATP-binding sites of the human skeletal muscle HKII gene could potentially reduce HKII activity and lead to disturbances in glucose metabolism and insulin resistance.

None of the amino acid substitutions observed in this study corresponds to known mutations in either human GK or the yeast HK, and none is located in the glucose- or ATP-binding sites. With respect to the $Ala^{314}Val$ substitution, Ala is conserved in the rat HKI and HKII enzymes but not in the rat HKIII (Ser) or in the equivalent positions of rat and human GK (Val at position 310) or the yeast HK (Asp or Tyr at position 322). Therefore, it seems unlikely that Ala→Val substitution at residue 314 could significantly influence enzymatic activity of HKII. The presence of a positively charged residue at position 353 is conserved in the rat and human HKI but not in the rat HKII (Tyr) or the human GK (Tyr at 349). The $Arg^{353}Cys$ substitution creates a new disulfide bond-forming residue and potentially could influence enzymatic activity of HKII. The $Arg^{775}Gln$ substitution is unlikely to influence enzyme activity since Arg at the equivalent position is not well conserved among the HK (conserved only in the rat HKII; the human and rat GK enzymes have Gln at the equivalent position [residue 335]). The influence of the $Ala^{314}Val$, $Arg^{353}Cys$, and $Arg^{776}Gln$ substitutions on the enzymatic activity of HKII is possible to evaluate definitely only by expressing the variant alleles in cultured cells. This work is in progress. Furthermore, the association of HKII mutations with NIDDM will be tested by linkage analysis in affected families.

SSCP has been shown to be a powerful method to detect sequence variants in a variety of genes. To increase the probability of finding sequence variants, we screened all exons at two different temperatures. Furthermore, all the PCR fragments were ≤230 bp in length to maximize the probability of finding mutations of the HKII gene. The method we used has been shown to detect all sequence variants of the lipoprotein lipase gene (33,34), and it has been successfully applied also in the screening of variants of the green and red opsin genes (35,36) and the insulin receptor substrate-1 gene (37).

In conclusion, although three amino acid substitutions in HKII were found in the Finnish NIDDM patients, their combined frequency in this patient population is only 6.1%. Assuming that we have not missed a significant number of substitutions in the HKII gene, our findings indicate that mutations of the HKII gene are not a major etiological factor for NIDDM in the Finnish population. Because the Finnish population descends mainly from a small number of founders of Baltic Finnish and German origin, the possibility that variants of the HKII gene could play a more significant role in the etiology of NIDDM in other populations is not excluded.

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