Brief Genetics Report

Molecular Analysis of *KCNJ10* on 1q as a Candidate Gene for Type 2 Diabetes in Pima Indians

Vidya S. Farook, Robert L. Hanson, Johanna K. Wolford, Clifton Bogardus, and Michal Prochazka

The *KCNJ10* gene is located within a region on chromosome 1q linked to type 2 diabetes in the Pima Indians and six other populations. We therefore investigated this gene as a potential type 2 diabetes candidate gene in Pima Indians. *KCNJ10* consists of two exons, spans ~33 kb, and we identified eight single-nucleotide polymorphisms (SNPs), including one (SNP2) in the coding region leading to a Glu359Lys substitution. Association studies were carried out in a case-control group composed of 149 affected and 150 unaffected Pimas, and the linkage analysis was performed in a linkage set of 1,338 Pimas. SNP1 in the promoter and SNP2 in the intron, which were in a complete linkage disequilibrium, and SNP5 in the 3′ untranslated region showed association with diabetes in the case-control group ($P = 0.02$ and $P = 0.01$, respectively). When genotyped in the linkage set, only the KCNJ10-SNP1 variant showed a modest association with type 2 diabetes ($P = 0.01$). KCNJ10-SNP1 is in a strong linkage disquilibrium with SNP14 of the adjacent *KCNJ9* locus, which we previously found to be associated with type 2 diabetes. After adjustment for *KCNJ10*-SNP1, the original linkage score at this locus was marginally reduced from 3.1 to 2.9. We conclude that these variants in *KCNJ10* are unlikely to be the cause of linkage of type 2 diabetes with 1q in Pima Indians. *Diabetes* 51:3342–3346, 2002

Type 2 diabetes is a heterogeneous disease that is caused by both genetic and environmental factors (1). We have recently performed a genome-wide linkage study of type 2 diabetes in the Pima Indians of Arizona and reported a suggestive evidence for linkage with young-onset diabetes within a 30-cM interval on 1q21-q23 (2). Subsequently, this region has been linked to type 2 diabetes in Caucasian families living in Utah (3), the Old Order Amish (4), Northern Europeans living in England (5), French Caucasians (6), northern China Han families (7), and community-based Caucasian pedigrees from the Framingham Offspring Study (8).

One possible candidate gene within the linked area is *KCNJ10* (*Kir4.1/KAB-2/Kir1.2/Bir1*), coding for a member of the inwardly rectifying potassium (K^+) channel family (Kir). Among members of the Kir channel family are proteins that participate in the inhibition of transmembrane K^+ transport throughout the nervous system, heart, and pancreas (9,10). Because insulin release depends on changes in pancreatic β-cell permeability for K^+ and a lower acute plasma insulin secretory response to intravenous glucose is a predictor of type 2 diabetes in Pima Indians (11), the Kir channel genes represent potential candidates for diabetes susceptibility. We have previously reported an association of allelic variants within the adjacent *KCNJ9* gene with diabetes ($P = 0.006$ in a case-control group), but the strength of the association was not sufficient to account for the 1q linkage in the Pimas (12).

Using BLAST searches of GenBank, we identified three bacterial artificial chromosomes (BACs) (201L5, 536C5, and 2338N5) containing portions of the *KCNJ10* locus. We determined that this gene spans ~33 kb and consists of two exons (Fig. 1). Exon 1 (226 bp) encodes the 5′ untranslated region (UTR), whereas exon 2 (5,478 bp) contains the entire coding and 3′UTR sequences. Furthermore, we determined that *KCNJ10* is located within 12 kb of *KCNJ9* and that the genes are arranged in a head-to-head transcriptional orientation. The predicted promoter region lacks the TATA and CAAT boxes but is GC rich (~70%), which is a common feature of many TATA-less promoters (13).

KCNJ10 mRNA has previously been detected in brain and kidney (14), and we expanded the expression analysis by PCR amplification of cDNAs prepared from 19 human tissues. In addition to brain and kidney, we also detected the 250-bp PCR fragment in spleen (Online Appendix 3).

We screened both *KCNJ10* exons, flanking intronic regions, 2 kb of the 5′ region and 5.4 kb of the 3′-flanking region using denaturing high-performance liquid chromatography (DHPLC) and identified eight variants designated *KCNJ10*-SNP1-*KCNJ10*-SNP8 (Fig. 1 and Online Appendix 2). These include a G→A variant in exon 2 (*KCNJ10*-SNP3) causing a Glu359Lys substitution. With the exception of the SNP1-SNP5 pair, most SNPs were in a strong linkage disequilibrium (LD), with D′ values (equal to an

From the Clinical Diabetes and Nutrition Section, Phoenix Epidemiology and Clinical Research Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Phoenix, Arizona.

Address correspondence and reprint requests to Vidya S. Farook, Clinical Diabetes and Nutrition Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 4212 North 16th St., Phoenix, AZ 85016. E-mail: vfarook@mail.nih.gov.

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BAC, bacterial artificial chromosome; DHPLC, denaturing high-performance liquid chromatography; LD, linkage disequilibrium; LOD, logarithm of odds; RFLP, restriction fragment length polymorphism; SNP, single-nucleotide polymorphism; UTR, untranslated region.

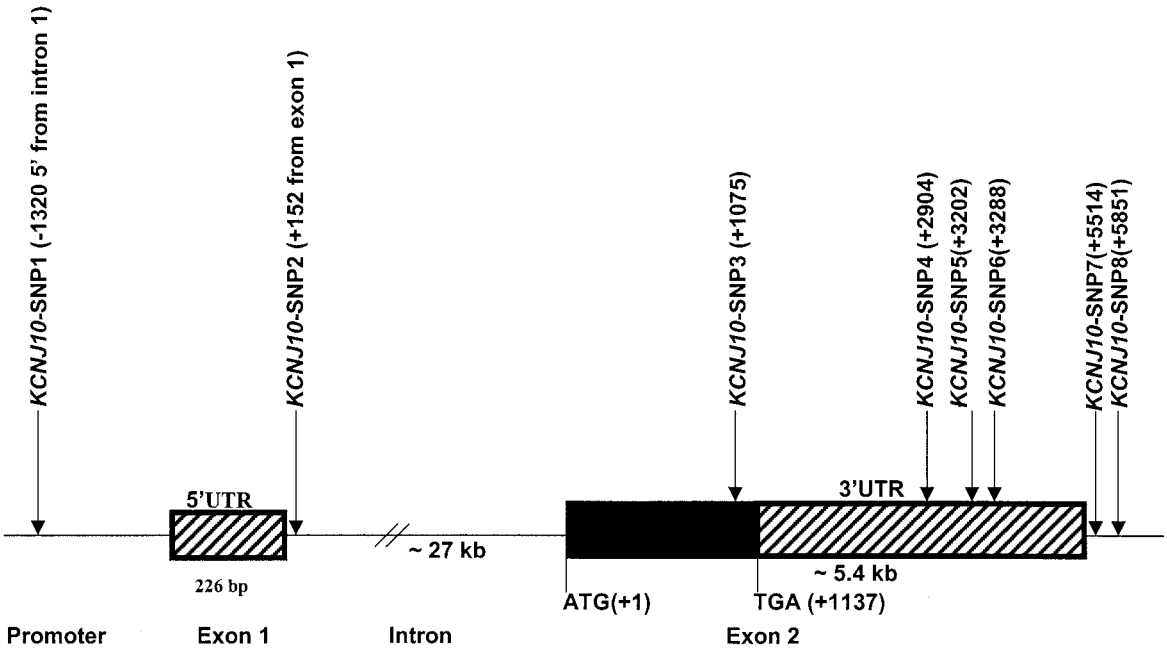

FIG. 1. Genomic structure of the *KCNJ10* gene. ■, coding region of the *KCNJ10* gene; ▨, noncoding region of the *KCNJ10* gene. The positions of SNPs 3–8 are numbered considering the adenosine in the translation initiation order of ATG as +1.

estimate of LD quantitation; see RESEARCH DESIGN AND METHODS) equal to or approaching 1 (Table 1).

All detected polymorphisms, allelic frequencies, and results of the association analyses are summarized in Table 2 and in Online Appendix 2. In the case-control groups none of these SNPs showed a statistically significant association with diabetes. There was a tendency for carriers of the less common T-allele at SNP1 and more common T-allele at SNP5 to have a higher prevalence of diabetes. These relations were stronger among the relatively smaller case-control groups (50 individuals in each) selected from the original linkage study population, and in this group the association with both SNP1 and SNP5 were statistically significant ($P = 0.02$ and $P = 0.01$, respectively).

When analyzed in the full original linkage set of 1,338 individuals who participated in the genomic scan, we found a modest association of type 2 diabetes with SNP1 ($P = 0.01$). Nevertheless, there was no substantial effect of this variant on the logarithm of odds (LOD) score (original LOD score 3.1; after adjustment 2.9). No significant association for young-onset diabetes (the trait that originally indicated linkage to this region) or effect on the LOD was seen with SNP5 (Table 2).

In 1,338 individuals who participated in the genome-wide linkage study, there was also modest LD between SNP1 and SNP5, such that the more frequent allele at one locus was associated with the less frequent allele at the other ($D' = -0.40$). When haplotypes at both SNP1 and SNP5 were analyzed (Table 3), there was an indication that the relatively small number of individuals ($n = 101$) who unambiguously carried the haplotype with the rare allele at both loci had a higher prevalence of diabetes (72%) than the remaining individuals (58%). However, adjustment for the effect at both SNP1 and SNP5 also did not attenuate the linkage signal (not shown).

Although the Glu359Lys variant was not associated with type 2 diabetes, the Glu359 residue is conserved in mouse, rat, and human, and any potential effect of this amino acid substitution on the function of the protein therefore remains to be elucidated. Alleles at *KCNJ10*-SNP1 are in a strong LD ($D' = 0.97$) with SNP14 at the adjacent *KCNJ9*, which we have previously found to be associated with diabetes in this population (12). Because the associated alleles have nearly identical frequencies, it is difficult to determine which of the two SNPs is more strongly associated with diabetes. However, re-analysis of the linkage of diabetes after accounting for the effect of either of these SNPs does not attenuate the LOD score signal, and we conclude that the observed associations are not of sufficient magnitude to account for the linkage of 1q21-q23 with diabetes in this population (2). It is possible, however, that both *KCNJ10*-SNP1 and *KCNJ9*-SNP14 are in a modest LD with a diabetes susceptibility locus elsewhere

TABLE 1
LD estimates (D′ above the diagonal) and associated *P* values (below the diagonal) between pairs of SNPs in KCNJ10 in the case-control study

	SNP1	SNP3	SNP4	SNP5	SNP8
SNP1 (0.67)	—	1.00	−0.91	−0.19	−0.65
SNP3 (0.95)	<0.01	—	−1.00	1.00	−1.00
SNP4 (0.86)	<0.01	0.04	—	−0.87	−0.81
SNP5 (0.61)	0.07	<0.01	<0.01	—	−1.00
SNP8 (0.75)	<0.01	<0.01	<0.01	<0.01	—

D′ represents the extent of LD between alleles at each locus, expressed as a proportion of the maximum possible given the direction of association. A positive D′ number represents an association between the more frequent alleles at each locus, whereas a negative number represents an association between the more frequent allele at one locus and the less frequent allele at the other locus. The *P* value for the null hypothesis of no LD is also shown. SNP1 and SNP2 (not listed here) are in complete LD; SNP6 and SNP7 were rare (0.03) and their D′ could not be reliably assessed in relation to the other markers. The numbers in parenthesis represent the frequency of the most common allele of that particular SNP.

TABLE 2
Genotypic distribution for *KCNJ10* polymorphism and odds ratio (OR) with 95% CI for diabetes in participants in the case-control study and genome-wide linkage studies

		Genotype			Dominant model		Recessive model		Additive model		Overall
	Status	11	12	22	OR (95 % CI)	*P*	OR (95 % CI)	*P*	OR (95 % CI)	*P*	*P*
All participants in case-control study											
SNP1	Nondiabetic	71 (53)	51 (38)	13 (10)							
	Diabetic	65 (44)	60 (41)	22 (15)	0.61 (0.29–1.26)	0.207	0.72 (0.45–1.14)	0.190	0.75 (0.53–1.05)	0.097	0.263
SNP3	Nondiabetic	134 (93)	10 (7)	0 (0)	—	—					
	Diabetic	135 (91)	13 (9)	0 (0)	—	—	0.78 (0.33–1.82)	0.666	0.77 (0.38–1.83)	0.561	0.666
SNP4	Nondiabetic	113 (79)	26 (18)	4 (3)	—	—					
	Diabetic	115 (79)	27 (18)	3 (2)	1.36 (0.30–6.21)	0.722	1.02 (0.58–1.80)	1.000	1.05 (0.64–1.70)	0.856	0.967
SNP5	Nondiabetic	58 (40)	59 (41)	27 (19)							
	Diabetic	60 (40)	73 (49)	17 (11)	1.81 (0.94–3.48)	0.101	0.99 (0.62–1.57)	1.000	1.16 (0.83–1.61)	0.381	0.163
SNP8	Nondiabetic	74 (52)	56 (39)	12 (8)							
	Diabetic	83 (56)	57 (39)	8 (5)	1.62 (0.64–4.08)	0.358	1.17 (0.74–1.86)	0.556	1.20 (0.83–1.74)	0.338	0.563
Participants in case-control study and in genome-wide linkage study											
SNP1	Nondiabetic	23 (55)	17 (41)	2 (5)							
	Diabetic	17 (35)	24 (50)	8 (16)	0.26 (0.05–1.28)	0.100	0.44 (0.19–1.02)	0.061	0.47 (0.24–0.92)	0.027	0.088
SNP3	Nondiabetic	39 (89)	5 (11)	0 (0)	—	—					
	Diabetic	45 (90)	5 (10)	0 (0)	—	—	1.15 (0.31–4.29)	1.000	1.15 (0.31–4.28)	0.831	1.000
SNP4	Nondiabetic	35 (80)	9 (20)	0 (0)	—	—					
	Diabetic	35 (71)	12 (25)	2 (4)	—	—	0.64 (0.25–1.68)	0.472	0.59 (0.25–1.41)	0.236	0.500
SNP5	Nondiabetic	15 (34)	18 (41)	11 (25)							
	Diabetic	20 (40)	27 (54)	3 (6)	5.22 (1.35–20.2)	0.018	1.29 (0.56–2.99)	0.670	1.71 (0.98–3.15)	0.083	0.035
SNP8	Nondiabetic	21 (48)	19 (43)	4 (9)							
	Diabetic	29 (60)	18 (38)	1 (2)	4.70 (0.5–43.8)	0.189	1.67 (0.73–3.82)	0.295	1.75 (0.87–3.53)	0.119	0.242
All participants in genome-wide linkage study											
SNP1	Nondiabetic	216 (50)	182 (42)	35 (8)							
	Diabetic	250 (40)	296 (48)	76 (12)	0.62 (0.39–0.99)	0.043	0.75 (0.55–1.02)	0.071	0.76 (0.64–0.95)	0.018	0.048
SNP5	Nondiabetic	169 (40)	195 (47)	55 (13)							
	Diabetic	226 (37)	296 (49)	86 (14)	1.05 (0.66–1.67)	0.842	0.88 (0.65–1.20)	0.436	0.95 (0.75–1.20)	0.669	0.663

Data are *n* (%) unless otherwise indicated. For all SNPs the more common allele is coded as "1" and the less common allele is coded as "2." For the "dominant" model ORs are calculated comparing those homozygous or heterozygous for the common allele (11 or 12) with the odds for those homozygous for the less common allele (22). Similarly, the "recessive" model compares those homozygous for the common allele (11) with the others (12 or 22). In the additive model, the OR is calculated for each copy of the common allele. In the case-control study, *P* values are calculated by Fisher's exact test, except for the additive model, for which the *P* value is calculated by the χ^2 test. For the subjects in the genome-wide linkage study, the ORs and *P* values are calculated by generalized estimating equations that adjust for age, sex, birth year, and ethnicity and that account for family membership (sibship). The dominant model is not analyzed in instances where there are too few individuals homozygous for the rare allele to obtain reliable estimates. The genotypes are presented as total numbers and their percent in parenthesis.

TABLE 3
Associations of combinations of genotypes in *KCN9* and *KCNJ10* SNPs with diabetes

Genotype		Diabetes *n*	Diabetes (%)	OR (95% CI)
KCNJ9-SNP1	*KCNJ10-SNP1*			
11	11	435	54	0.57 (0.32–1.01)
11	12	62	50	0.43 (0.19–0.99)
12	11	15	40	0.22 (0.05–0.91)
12	12	393	64	0.77 (0.43–1.38)
12	22	22	77	1.07 (0.36–3.00)
11	22	4	75	
22	11	1	0	1.01 (0.37–2.76)
22	12	4	40	
22	22	84	65	1.00 (referent)
KCNJ10-SNP1	*KCNJ10-SNP5*			
11	11	136	49	0.26 (0.12–0.59)
11	12	232	56	0.30 (0.14–0.66)
11	22	86	58	0.37 (0.14–0.93)
12	11	205	63	0.38 (0.17–0.84)
12	12	210	61	0.39 (0.18–0.84)
12	22	47	60	0.22 (0.08–0.63)
22	11	51	59	0.38 (0.16–0.98)
22	12	46	78	
22	22	8	100	1.00 (referent)
KCNJ9-SNP14	*KCNJ10-SNP5*			
11	11	160	46	0.20 (0.09–0.47)
11	12	251	56	0.28 (0.13–0.64)
11	22	82	59	0.35 (0.12–0.97)
12	11	181	67	0.40 (0.17–0.95)
12	12	189	62	0.34 (0.15–0.79)
12	22	46	59	0.21 (0.07–0.60)
22	11	40	50	0.33 (0.12–0.86)
22	12	36	78	
22	22	8	100	1.00 (referent)

in this region or that an additional separate susceptibility gene(s) is located in the region. It is also possible that the association of *KCNJ10*-SNP1 (and of *KCNJ9*-SNP14) with diabetes arose simply by chance and that the susceptibility polymorphism(s) that explains the linkage result is located some distance away. In either case, we conclude that effects of the *KCNJ9* and *KCNJ10* polymorphisms by themselves do not account for the diabetes linkage on chromosome 1q. However, elucidation of the *KCNJ10* genomic structure and identification of eight novel variants will be informative for association studies of this candidate gene with type 2 diabetes in other populations.

RESEARCH DESIGN AND METHODS

Subjects. The present data are derived from participants in a longitudinal study of the development of type 2 diabetes (2) diagnosed according to World Health Organization criteria (2). Association analyses were conducted using a case-control study design including 149 affected individuals with the youngest age at onset of diabetes <25 years and 150 unaffected individuals ≥45 years of age who remained nondiabetic based on the oral glucose tolerance test. None of the individuals were first-degree relatives. This sample size has 75–95% power to detect a difference of at least 15% in the prevalence of diabetes between groups with and without the high-risk genotype at $P < 0.05$ (15). A number of the individuals in the case-control study were not included in the original linkage study. To account for the possibility of heterogeneity in the genetic causes of diabetes between those included in the linkage study and other individuals in the population, statistical analyses were done both in the full case-control sample as well as in a subset (50 affected and 50 unaffected) who had participated in the genomic scan. Any SNP associated with diabetes was subsequently analyzed in the entire population sample of 1,338 Pima Indians used in the original linkage study (2).

DNA amplification and expression analysis. BACs 201L5 and 536C5 were purchased from Research Genetics (Huntsville, AL) and used to determine the genomic structure of *KCNJ10* by long-distance PCR with the Expand Long Template PCR system (Roche Molecular Biochemicals, Indianapolis, IN) combined with BLAST searches of human genomic sequences available in the nonredundant and high throughput genomic sequences databases (http:/www.ncbi.nlm.nih.gov/BLAST). Sequences were determined using the BigDye Terminator cycle sequencing kit and analyzed on an ABI 377 Sequence Detection System (Applied Biosystems, Foster City, CA).

The tissue distribution of the *KCNJ10* transcript was determined by PCR using Quick-Clone cDNAs from 19 human tissues and Advantage Klentaq polymerase (Clontech, Palo Alto, CA). The β-2-Microglobulin transcript was utilized as an internal standard to roughly estimate quantitative differences in *KCNJ10* expression between tissues. A duplex PCR was performed using a *KCNJ10* sense primer (5′-GGACAAGCCCTTATCTGATC-3′) designed from exon 1 combined with an antisense primer (5′-TCTTTTGTCAGGACTCTCCG-3′) from exon 2 of *KCNJ10*, and the sense primer 5′-TGTCTTTCAGCAAGGACTGGTC-3′ plus antisense primer 5′-TGATGCTGCTTACATGTCTCGAT-3′ for the β-2-Microglobulin control.

Variant detection and genotyping. The primers used for *KCNJ10* structure determination, variant (SNP) detection, and sequencing are available in Online Appendix 1. For SNP screening, we used two separate DNA pools, each pool comprised of five diabetic and five nondiabetic samples. All samples were from different subjects, thus representing a total of 20 individuals. Potential sequence variants were detected by DHPLC using the WAVE DNA fragment analysis system (Transgenomic, Omaha, NE) and confirmed by sequencing as previously described (16).

All SNPs were initially genotyped by either PCR-restriction fragment length polymorphism (RFLP) using the appropriate primers and restriction enzymes, pyrosequencing, or by capillary sequencing (Online Appendix 2). The *KCNJ10*-SNP2 did not alter a restriction site and was therefore typed by mismatch PCR-RFLP (17). The mismatch consisted of a C→G substitution at the second base preceding the 3′ end of this primer (5′-CAGGAGGCTCCATTTGGGA**g**T-3′; the mismatched nucleotide shown in lower, bold case). In combination with this primer, the presence of a '***T***' at the variant position results in a unique artificial *Rsa*I site (G*T*Ac; in this orientation the mismatched '**g**' introduced by the reverse primer corresponds to a '**c**'), whereas the recognition site is absent in the context of the alternative 'C' allele (G*C*Ac). The digested products were resolved on 3–4% agarose gels. All restriction enzymes were purchased from New England Biolabs (Boston, MA) or Gibco BRL (Gaithersburg, MD) and used according to the manufacturer's instructions.

KCNJ10-SNP1 was genotyped in 1,338 Pima Indians using the pyrosequencing technology (Pyrosequencing, Uppsala, Sweden). Briefly, templates were amplified from genomic DNA using the biotinylated forward primer 5′-TGACCACAAATCAGCACAACT-3′ and unmodified reverse primer 5′-CCAGTGTTACCATATTGAATTG-3′. The pyrosequencing was performed with primer 5′-GAATGTAGCTTAGTTTAAC-3′. *KCNJ10*-SNP5 and *KCNJ10*-SNP8 were also genotyped by pyrosequencing using the following forward primers and biotinylated reverse primers: 5′-GTGTGTGTCTATACCTTCCTAT-3′ plus 5′-GAATCCTAAGACATATGAGACA-3′ for SNP5 and 5′-AGAAGATGCCAGATCGCATTA-3′ plus 5′-GTTCCTGTCCCTGTGAAATGT-3′ for SNP8. SNPs were scored with sequencing primers 5′-GTTAACCTCCATCTGGTT-3′ (SNP5) and 5′-AAGCAATAGCAGGTACCA-3′ (SNP8), respectively. Pyrosequencing was carried out as instructed by the manufacturer with slight modifications. The PCR was carried out in a final volume of 25 μl with an initial denaturing at 96°C for 3 min followed by 40 cycles of 96°C for 20 s, 57°C for 20 s, 72°C for 30s, and a final extension at 72°C for 5 min.

KCNJ10-SNP5 was genotyped in 1,338 Pima Indians by sequencing with the BigDye Terminator chemistry and the ABI 3700 Sequence Detection System (Applied Biosystems, Foster City, CA).

Statistical analyses. A χ^2 test and Fisher's exact test were (SAS Institute, Cary, NC) used for analysis of the case and control subjects to assess statistical evidence for association. P values <0.05 were considered statistically significant, and the odds ratio was used to determine the strength of the association between prevalence of the at-risk genotype and affection status. To maximize power, associations were calculated under three different models, one assuming the common allele is dominant to the less common allele, one assuming it is recessive, and one assuming an additive relation between number of common alleles and prevalence of diabetes. In addition, an overall P value was calculated for the null hypothesis of no difference in diabetes prevalence among the three different genotypes (2 df). None of the genotype frequencies differed significantly from that predicted by Hardy-Weinberg equilibrium. LD between pairs of SNPs within *KCNJ10* was estimated among the 299 unrelated individuals in the case-control group with the Estimating Haplotypes program (18). The degree of LD was quantified by D′, which represents the degree to which observed haplotype frequencies differ

from that expected under linkage equilibrium, expressed as a proportion of the maximum possible disequilibrium given the observed allele frequencies (19). Haplotype frequencies for pairs of SNP1, SNP5, and SNP6 within *KCNJ10* and *KCNJ9*-SNP14 were calculated using the ILINK program, which accounts for the relations among individuals (20). Associations with combination *KCNJ10*-SNP1 and *KCNJ9*-SNP14 polymorphisms were assessed using the same procedures used for single SNPs.

For assessment of the association of the *KCNJ10*-SNP1 genotypes with diabetes among the participants in linkage study, logistic regression was used to control for age, sex, and year of birth. To account for nonindependence introduced by inclusion of multiple siblings in a sibship, the logistic models were fit using binomial generalized estimating equations (21).

Linkage analyses were performed as in the previously published genomic scan using the Haseman-Elston method (2). The extent to which *KCNJ10*-SNP1 and *KCNJ10*-SNP5 contributed to the observed linkage results was investigated by determining the association of genotypes with type 2 diabetes and "adjusting" the diabetes score for the SNP effect by subtracting the prevalence of diabetes in the individual's genotypic category from an indicator variable for affection status. The Haseman-Elston regression with this "adjusted" diabetes score in the 1q21-q23 region was compared with the original unadjusted linkage result.

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Sequences reported in this article were deposited with the GenBank database under accession nos. AF482709, AF482710, and AH011548.

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