Minor Effect of GLUT1 Polymorphisms on Susceptibility to Diabetic Nephropathy in Type 1 Diabetes

Daniel P.K. Ng,[1,2] Luis Canani,[1,2] Shin-ichi Araki,[1,2] Adam Smiles,[1] Dariusz Moczulski,[1,2] James H. Warram,[1] and Andrzej S. Krolewski[1,2]

Elevation of intracellular glucose in mesangial cells as mediated by GLUT1 may be important in initiating cellular mechanisms that cause diabetic nephropathy. To determine whether DNA sequence differences in *GLUT1* confer susceptibility to this complication, single-nucleotide polymorphisms (SNPs) in this gene were examined using a large case-control study. SNPs examined included the known *Xba*I (intron 2) and *Hae*III SNPs (exon 2). Four novel SNPs located in three putative enhancers were also investigated. Homozygosity for the *Xba*I(-) allele was associated with diabetic nephropathy (odds ratio 1.83 [95% CI 1.01–3.33]). Furthermore, homozygosity for the A allele for a novel SNP (enhancer-2 SNP 1) located in a putative insulin-responsive enhancer-2 was associated with diabetic nephropathy (2.38 [1.16–4.90]). Patients who were homozygous for risk alleles at both *Xba*I SNP and enhancer-2 SNP 1 [i.e., homozygosity for *Xba*I(-)/A haplotype] also had an increased risk of diabetic nephropathy (2.40 [1.13–5.07]). Because enhancer-2 SNP 1 may directly control *GLUT1* expression, the strong linkage disequilibrium between the two SNPs likely accounts for *Xba*I SNP being associated with diabetic nephropathy. In conclusion, our study confirms that SNPs at the *GLUT1* locus are associated with susceptibility to diabetic nephropathy in type 1 diabetes. Although these SNPs confer a considerable personal risk for diabetic nephropathy, they account for a limited proportion of cases among type 1 diabetic patients. *Diabetes* 51: 2264–2269, 2002

The glucose transporter GLUT1 (OMIM 138140) is the major representative of the family of facilitative glucose transporters that are expressed in the glomerular mesangial cell (1,2). Elevated intracellular glucose levels in these cells as a result of diabetes are thought to affect a number of cellular pathways that may be involved in diabetic nephropathy. These pathogenic pathways include de novo synthesis of diacylglycerol, activation of protein kinase C, increased polyol pathway flux, and advanced glycation end product formation (3–5). In this regard, GLUT1 is likely to be pivotal in activating these pathways by raising intracellular glucose levels. Indeed, overexpression of GLUT1 in mesangial cells cultured under normoglycemic conditions mimics the diabetic phenotype, including the excessive production of extracellular matrix (6), a process likely to be of importance in bringing about diabetes-induced mesangial expansion and glomerulosclerosis. GLUT1 therefore appears to play a vital role in the pathogenesis of diabetic nephropathy (7,8).

Of late, several studies have examined *GLUT1* as a candidate gene that might confer susceptibility to diabetic nephropathy. These investigations have focused on testing whether the *Xba*I single-nucleotide polymorphism (SNP) located within intron 2 of *GLUT1* is associated with diabetic nephropathy by using case-control study designs (9–13). Inconsistent results have so far been reported. Although this *Xba*I SNP is not significantly associated with diabetic nephropathy in Spanish type 2 diabetic patients (9), heterozygosity for the *Xba*I(-) allele has been linked to susceptibility in Chinese patients (10). In contrast, homozygosity for the *Xba*I(+) allele was associated with increased risk in Caucasian type 2 patients in Poland (11). In studies conducted on type 1 diabetic patients, homozygosity for the *Xba*I(-) allele was associated with nephropathy in British Caucasians (12). However, this finding was not confirmed in a Danish study (13).

Although these discrepant findings might reflect the relative importance of *GLUT1* in different human populations, most of these studies have been conducted on a limited number of patients and, as such, may yield inconsistent findings. In addition, these studies suffer another potentially important limitation in that they have concentrated solely on the *Xba*I SNP. This polymorphism does not reside within a known regulatory region of the gene (i.e., promoter, enhancers, and silencer elements), and it is thus unclear whether it directly influences gene expression. Association of this SNP with diabetic nephropathy is likely attributable to linkage disequilibrium with the true functional polymorphism(s).

In view of these considerations, we have sought to find new polymorphisms in known and putative regulatory regions of *GLUT1*. Besides the promoter region, we searched for common polymorphisms in three other regions that exhibited sequence similarity to enhancer elements of the mouse and rat homologues (14,15). Together with the *Xba*I SNP and a common *Hae*III SNP located in

From the [1]Section on Genetics and Epidemiology, Joslin Diabetes Center, Boston, Massachusetts; and the [2]Department of Medicine, Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Andrzej S. Krolewski, MD, PHD, Section on Genetics and Epidemiology, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215. E-mail: andrzej.krolewski@joslin.harvard.edu.

Received for publication 6 December 2001 and accepted in revised form 3 April 2002.

Additional information can be found in an online appendix at http://diabetes.diabetesjournals.org.

ACR, albumin-to-creatinine ratio; ASO, allele-specific oligonucleotide; ESRD, end-stage renal disease; OR, odds ratio; PAR%, population attributable-risk percent; SNP, single-nucleotide polymorphism; USF, upstream stimulatory factor.

exon 2 (16), these novel polymorphisms were examined for association with diabetic nephropathy in a large case-control study.

RESEARCH DESIGN AND METHODS

Study groups. Individuals with type 1 diabetes were recruited for the study from among patients attending the Joslin Clinic in the decade 1991–2000. Diabetes was classified as type 1 if it was diagnosed before age 30 years and continuous treatment with insulin began within 1 year of diagnosis. During 1991–1993, we enrolled a 50% sample of Joslin Clinic attendees with type 1 diabetes in the age range 15–44 years (n = 1,598) in the Natural History of Microalbuminuria Study (17). On the basis of multiple measurements of the albumin-to-creatinine ratio (ACR) in random urine specimens during a 2-year observation period, patients were classified as having normoalbuminuria (n = 1,080), persistent microalbuminuria (n = 312), or advanced nephropathy (persistent proteinuria or end-stage renal disease [ESRD], n = 206) (17).

As unrelated control subjects for genetic studies on diabetic nephropathy, we selected Caucasian patients with normoalbuminuria and type 1 diabetes duration ≥15 years. Among the 1,080 normoalbuminuric participants in the Natural History of Microalbuminuria Study, 420 met these additional criteria, and we examined 307 (73%) of them.

As unrelated subjects for the genetic studies on diabetic nephropathy, we selected Caucasian patients with persistent proteinuria or ESRD. We were able to enroll and examine 152 of the 206 patients with advanced nephropathy identified in the Natural History Study described above. In addition to these subjects, we enrolled and examined 201 (86%) of 235 Caucasian patients with type 1 diabetes and proteinuria or ESRD attending the clinic between 1994 and 2000 who had not been included in the 50% random sample screened in 1991–1993 for the Natural History Study. The two groups of subjects did not differ with regard to sex, age at examination, duration of diabetes, or values of HbA_{1c}, total serum cholesterol, or serum triglycerides. Therefore, we pooled the two groups, bringing the total number of unrelated subjects with advanced diabetic nephropathy to 352.

Examination of study participants. After consenting to participate in the study, each subject had a standardized physical examination and provided a diabetes history regarding its diagnosis, treatment, and complications. Each individual provided a blood sample for biochemical measurements and DNA extraction. For the family-based study, parents provided blood samples for DNA extraction. The Committee on Human Subjects of the Joslin Diabetes Center approved the protocol and informed consent procedures.

Diagnosis of diabetic nephropathy. The diabetic nephropathy status of each patient was determined on the basis of questionnaires, medical records of the Joslin Clinic (supplemented with records of other physicians if necessary), and measurements of the ACR. Methods for measuring the ACR have been described previously (17). Patients were classified as control subjects (n = 307) if they had diabetes duration ≥15 years and the ACR (in mg/g) was <17 (men) or <25 (women) in at least two of the last three urine specimens. Patients with microalbuminuria or intermittent proteinuria were excluded from the study. Patients were considered case subjects (n = 352) if they had persistent proteinuria or if they had ESRD due to diabetic nephropathy. Persistent proteinuria was defined as two of three successive urinalyses positive by reagent strip (≥2+ on Multistix; Bayer Corporation, Diagnostics Division, Elkhart, IN) or by an ACR (in mg/g) ≥250 (men) or ≥ 355 (women). At the time of the first examination (1992–2000), case subjects were divided into those with persistent proteinuria (n = 260) and those with ESRD (n = 92). Those with proteinuria were followed until the end of July 2001, by which time ESRD had developed in 60 of them, i.e., renal dialysis had been initiated. These were considered incident cases of ESRD. The individuals who had begun dialysis or received a renal transplant before the first examination were considered prevalent cases of ESRD. For prevalent or incidence cases of ESRD, the year when dialysis began was used to calculate the duration of diabetes. In total, we had 152 case subjects with ESRD (92 prevalent and 60 incident case subjects). For the present study DNA was available for 230 control individuals with normoalbuminuria and 262 case subjects with persistent proteinuria (n = 151) or ESRD (n = 111).

In silico analysis. The DNA sequence of the human *GLUT1* has not yet been published but was obtained by querying GenBank (available online at http://www.ncbi.nlm.nih.gov/). A recent entry (GenBank accession no. NT_004852, 16 April 2001 release) containing human genomic sequence was found that contained the entire annotated *GLUT1* gene (located on human chromosome 1p).

Enhancer-1 and -2 have been described for mouse *GLUT1* (GenBank accession no. D10230 and D10231) (14). In addition, a separate enhancer element (denoted enhancer-3) has been described for the rat homolog (GenBank accession no. U82754) (15). To determine which part of the human gene exhibited sequence similarity to these three enhancers, we used the PipMaker program (available online at http://nog.cse.psu.edu/cgi-bin/pipmaker?basic), which identifies conserved segments between pairs of DNA sequence. The presence of transcription factor binding sites in DNA sequence was detected using the TESS program to query the TRANSFAC transcription factor database (available online at http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ = SEA-FR-Query).

SNP discovery in GLUT1 regulatory regions. The 0.3-kb known human gene promoter (18) was amplified by PCR from genomic DNA of eight individuals. The putative human enhancer-1 (located ~3.6 kb upstream of exon 1), enhancer-3 (located ~6.6 kb upstream of exon 1), and enhancer-2 (located in intron 2) were likewise amplified. These PCR products were purified and then sequenced using an ABI 377 automated DNA sequencer machine in conjunction with dye terminator chemistry in accordance with the manufacturer's protocol (Perkin Elmer).

Genotyping of GLUT1 SNPs. The *Xba*I SNP represents a G-to-T transversion in intron 2 of *GLUT1* (Fig. 1). This was amplified from genomic DNA as a 1.1-kb PCR product using primers TGTGCAACCCATGAGCTAA (F′) and CCTGGTCTCATCTGGATTCT (R′) (16,19). PCR (25 μl reaction volume) was performed on 20 ng of genomic DNA using 0.6 units of *Taq* polymerase (PGC Scientific) with above primers in the presence of 1.5 mmol/l $MgCl_2$ for 30 cycles. Cycling parameters were: denaturation at 95°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 90 s, with final extension at 72°C for 10 min. PCR products were then digested with *Xba*I restriction endonuclease at 37°C for >3 h before fractionation on a 1.2% agarose gel. When the restriction site was present [denoted *Xba*I(+) allele], two fragments (0.9 and 0.2 kb) were visualized. In the absence of this site [denoted *Xba*I(-) allele], the 1.1-kb product remained intact.

The *Hae*III SNP is a common T-to-C transition (Ala^{15}→Ala^{15}) in exon 2 of the *GLUT1* gene (Fig. 1) (16). This was amplified from genomic DNA as a 173-bp PCR product using primers CTCCCAGACACGCCTATAACAGT (F′) and GGCTGGTGTCCATAAGCCAACG (R′). PCR was performed as above in the presence of 1.5 mmol/l $MgCl_2$ using *Taq* polymerase for 40 cycles with the following parameters: denaturation at 95°C for 45 s, annealing at 66°C for 45 s, and extension at 72°C for 60 s, with final extension at 72°C for 10 min. PCR products were digested with *Hae*III enzyme at 37°C for >3 h and then electrophoresed in a 2.8% agarose gel. The *Hae*III(+) allele was observed as two fragments of 138 and 35 bp after digestion, whereas the *Hae*III(-) allele was seen as the intact 173-bp product.

The SNP (denoted enhancer-1 SNP) found in putative enhancer-1 represents a C-to-T transition (Fig. 1). This SNP was amplified from genomic DNA as a 635-bp product using primers AAGATAAAGAAGGGATGCCG (F′) and CAGCATTCAGCTTGAACGAC (R′) by PCR as described above, with an annealing temperature of 61°C. The PCR product was then subjected to allele-specific oligonucleotide (ASO) hybridization (20) using the following probes: GGAACCCATCACTGTGT and GGAATCCATCACTGTGT.

Two SNPs (denoted enhancer-2 SNP 1 and enhancer-2 SNP 2) were found in close proximity (162 bp apart) within the putative enhancer-2 of *GLUT1* (Fig. 1). Both SNPs were amplified as a 403-bp product using the same pair of primers CGATGATGGAAATGCGAG (F′) and ACAAGGCACACGCAGTGA (R′), with an annealing temperature of 61°C. Detection of enhancer-2 SNP 1 was achieved using ASO hybridization with probes TCCCATGCATTGGAGGA and TCCCGTGCATTGGAGGA. Likewise, enhancer-2 SNP 2 was performed using ASO probes TTCTCGGGAGGAAGGCT and TTCTTGGGAGGAAGGCT.

An SNP (C to T transition) was located in putative enhancer-3 (Fig. 1). This polymorphism was amplified as a 525-bp fragment using primers AGACCTC CAGAAAACAATACG (F′) and GATCCAGCAACAGTACAAGC (R′), with annealing temperature of 58°C. Alleles were discriminated using ASO probes GATTCAGGTGAGGTAAC and GATTTAGGTGAGGTAAC.

Altogether, 93 and 89% of the 492 patients included in this case-control study were genotyped for the *Xba*I SNP and enhancer-1 SNP, respectively; the remainder could not be determined due to technical difficulties with PCR amplification. Of the patients, >98% were successfully genotyped for the remaining four SNPs studied.

Statistical analysis. Haplotype frequencies were estimated using the EH program (21). Categorical data were compared using χ^2 test (two-sided). For continuous variables, ANOVA was used in cases of multiple group comparisons, whereas Student's t test (two-sided) was used for pairwise comparisons. The Breslow-Day test was used to test for homogeneity of odds ratios (ORs). P values <0.05 were considered statistically significant.

The population attributable-risk percent (PAR%) was calculated according to the following formula: $PAR\% = [(RR - 1)/(RR - 1 + 1/P_e)] \times 100$ (22). RR was estimated from the OR. The proportion of population who have exposure (P_e) to *Xba*I(-)*Xba*I(-) or enhancer-2 SNP 1 AA risk genotypes was based on the frequency of these genotypes in our control group (see RESULTS). This latter approximation is valid, considering that homozygosity for *Xba*I(-) occurred at

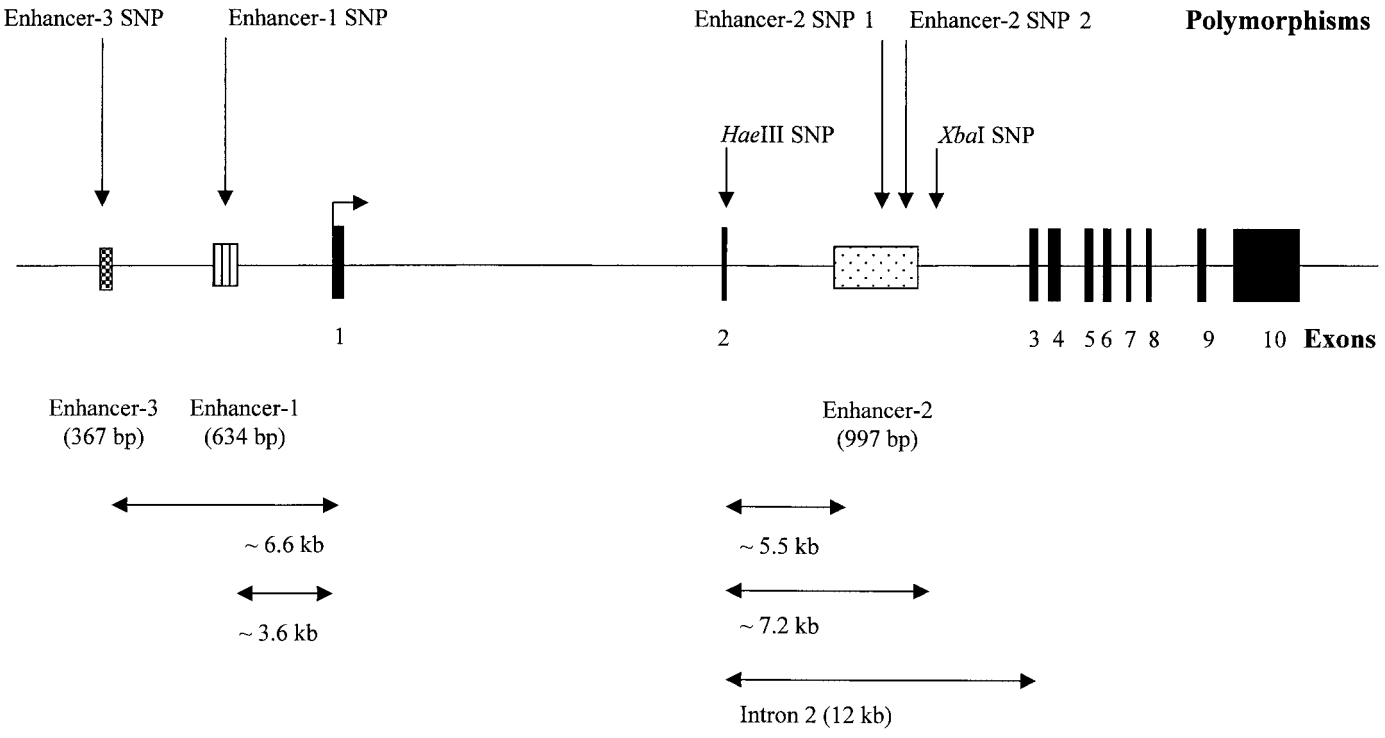

FIG. 1. Genomic structure of human *GLUT1* showing the location of the 10 exons and putative enhancers (1, 2, and 3). The six SNPs examined for association with diabetic nephropathy are also indicated.

a comparable frequency among control subjects in other independent studies conducted on type 1 diabetic Caucasians (12,13).

RESULTS

Identification of putative enhancer elements. Two regions of *GLUT1* were identified that showed substantial sequence similarity to enhancer-1 and enhancer-2 of the mouse homolog according to the PipMaker program (the sequence alignments are listed in the online appendix at http://diabetes.diabetesjournals.org). A 634-bp sequence (putative enhancer-1) found ~3.6 kb upstream of exon 1 was similar (65% identity over 645 bp) to the 610-bp enhancer-1, which is located 2.7 kb upstream of the mouse gene (Fig. 1) (14). Another sequence of 997-bp (putative enhancer-2) was likewise observed to share sequence similarity (46% identity over 1,159 bp) to the 1,342-bp mouse enhancer-2 (14). Like the mouse enhancer-2, this putative human enhancer is also located in intron 2 of *GLUT1* (Fig. 1). In addition, a 367-bp putative enhancer (denoted enhancer-3) located ~6.6 kb upstream of exon 1 was identified (Fig. 1). This *cis*-element exhibited significant sequence similarity (66% identity over 368 bp) to a 666-bp enhancer present at ~6 kb upstream of the rat *GLUT1* gene major transcription start site (15).

Identification of polymorphisms in putative enhancers and the promoter region. Sequencing of putative enhancer regions of *GLUT1* amplified from genomic DNA revealed four common SNPs. Of these, one is located in enhancer-1 and consists of a C-to-T transition (termed enhancer-1 SNP) (Fig. 1). Two SNPs were found in putative enhancer-2 and comprised an A-to-G transition (enhancer-2 SNP 1) and a C-to-T transition (enhancer-2 SNP 2) (Fig. 1). The fourth SNP (termed enhancer-3 SNP) is a C-to-T transition and resides in enhancer-3 (Fig. 1). No polymorphisms were found in the *GLUT1* promoter region.

Of the many potential transcription sites detected in the vicinity of the enhancer SNPs, some were predicted with greater certainty than others (i.e., above default secondary threshold in the TESS program). Of these better predictions, a site for the transcription factor CytR was predicted with both C and T alleles of enhancer-1 SNP. On the other hand, sites for GATA-1 and HSTF appeared to be specific for the C allele. The CytR and GATA-1 sites were detected in the mouse but not the rat homolog. An upstream stimulatory factor (USF) binding site was detected with both A and G alleles of enhancer-2 SNP 1, but it was absent in the mouse homolog. Likewise, sites for MGF and LyF-1 were detected with both C and T alleles of enhancer-2 SNP 2 but were not predicted in the mouse homolog. In the case of enhancer-3 SNP, a deltaEF site was predicted with both C and T alleles. However, predicted sites for NF-X3, tal-1, E12, and TFE3-S were specific for the C allele, whereas sites for Cdx-1 and MBF-1 were confined to the T allele. None of these enhancer-3 SNP–associated sites were conserved in the rat homolog.

Patient characteristics. Selected clinical characteristics of control and case subjects are presented in Table 1. Diabetes duration and sex ratio were similar between the two groups. Values for HbA_{1c}, serum total cholesterol, and triglycerides were significantly elevated in case subjects compared with control subjects at the time of examination ($P < 0.01$ for each parameter). Case subjects were slightly younger than control subjects when diabetes was diagnosed ($P = 0.03$).

Allele and genotype distribution of GLUT1 SNPs among case and control subjects. All six SNPs were

TABLE 1
Selected clinical characteristics of type 1 diabetic subjects according to nephropathy status

Clinical characteristics at time of examination	Control subjects	Case subjects
Number (*n*)	230	262
Sex (M/F)	112/118	134/128
Age at diabetes diagnosis (years)*	13 ± 7	11 ± 6
Duration of diabetes (years)	26 ± 7	25 ± 8
HbA_{1c} (%)†	8.1 ± 1.2	9.1 ± 1.7
Total cholesterol (mg/dl)†	194.0 ± 40.2	240.3 ± 76.4
Triglycerides (mg/dl)†	110.1 ± 57.4	184.7 ± 132.9
Systolic blood pressure (mmHg)†	118.3 ± 16.5	133.4 ± 22.0
Diastolic blood pressure (mmHg)†	71.1 ± 9.7	79.6 ± 11.4
Patients with ESRD (%)	-	42.4%

Data are means ± SD. *$P < 0.05$ for control vs. case subjects; †$P < 0.01$ for control vs. case subjects.

examined for association with diabetic nephropathy using a case-control study design. Genotype frequencies for all polymorphisms did not deviate significantly from Hardy-Weinberg equilibrium when case and control subjects were analyzed together or as separate groups.

The overall genotype distribution of the *Xba*I SNP was not significantly different between case and control subjects (Table 2). However, homozygosity for the *Xba*I(-) allele was associated with a slightly increased risk of diabetic nephropathy compared with the other genotypes combined (OR 1.83 [95%CI 1.01–3.33]). A significant difference in overall genotype distribution between case and control subjects was observed for enhancer-2 SNP 1 ($P = 0.036$) (Table 2). Specifically, there was an excess of the AA genotype among case subjects (10.7%) compared with control subjects (4.8%). These homozygotes were at a significantly higher risk of diabetic nephropathy compared with the AG and GG genotypes combined (OR 2.38 [95% CI 1.16–4.90]). The genotype distribution of both *Xba*I SNP and enhancer-2 SNP 1 were similar among case subjects when examined as subcategories based on whether these patients were proteinuric or had developed ESRD.

The effect of glycemic control and duration of diabetes on the association between these SNPs and diabetic nephropathy was examined. Stratifying according to the median HbA_{1c} value of 8.5%, the association was seen in both resulting strata (i.e., with $HbA_{1c} \leq 8.5\%$ or with $HbA_{1c} > 8.5\%$). Similarly, stratifying by the median duration of diabetes (25 years) revealed that the observed associations were also independent of this parameter. In addition, ANOVA revealed that *Xba*I SNP and enhancer-2 SNP 1 genotypes did not affect HbA_{1c} values and systolic and diastolic blood pressure values in either control or case subjects.

Strong linkage disequilibrium exists between enhancer-2 SNP 1 and the *Xba*I SNP. Specifically, 100% of enhancer-2 SNP 1 AA homozygotes were also homozygous for the *Xba*I(-) allele. Because the *Xba*I SNP is more polymorphic

TABLE 2
Genotype distribution of GLUT1 SNPs among control and case subjects

Polymorphism and genotypes	Control subjects	Case subjects	*P* (two-sided χ^2 test)
Enhancer-3 SNP (in distal promoter region)			
CT	118 (51.8)	133 (52.0)	NS
TT	65 (28.5)	57 (22.3)	
CC	45 (19.7)	66 (25.8)	
Enhancer-1 SNP (in proximal promoter region)			
TT	135 (64.3)	139 (61.0)	NS
CT	67 (31.9)	80 (35.1)	
CC	8 (3.8)	9 (4.0)	
HaeIII SNP (in exon 2)			
HaeIII(+)HaeIII(+)	144 (63.4)	154 (59.0)	NS
HaeIII(+)HaeIII(−)	70 (30.8)	97 (37.2)	
HaeIII(−)HaeIII(−)	13 (5.7)	10 (3.8)	
Enhancer-2 SNP 1 (in intron 2)			
GG	122 (53.0)	140 (53.4)	0.036
AG	97 (42.2)	94 (35.9)	
AA	11 (4.8)	28 (10.7)	
AG + GG	219 (95.2)	234 (89.3)	0.016
AA "risk genotype"	11 (4.8)	28 (10.7)	
Enhancer-2 SNP 2 (in intron 2)			
CC	144 (63.2)	162 (62.6)	NS
CT	78 (34.2)	91 (35.1)	
TT	6 (2.6)	6 (2.3)	
Xbal SNP (in intron 2)			
Xbal(+)Xbal(+)	99 (47.8)	106 (42.6)	NS
Xbal(+)Xbal(−)	90 (43.5)	106 (42.6)	
Xbal(−)Xbal(−)	18 (8.7)	37 (14.9)	
Xbal(+)Xbal(+) + Xbal(+)Xbal(−)	189 (91.3)	212 (85.1)	0.044
Xbal(−)Xbal(−) "risk genotype"	18 (8.7)	37 (14.9)	

Data are *n* (%).

than enhancer-2 SNP 1, the enhancer-2 SNP 1 AA genotype was detected in 67% of *Xba*I(-) homozygotes (data not shown). Frequency of haplotypes formed from these two markers was estimated using the EH program. As with allele frequencies of the individual markers, overall haplotype distribution did not differ between case and control subjects (P = NS). However, patients who were homozygous for risk alleles at both *Xba*I SNP and enhancer-2 SNP 1 [i.e., homozygosity for *Xba*I(-)/A haplotype] were at an increased risk of diabetic nephropathy compared with those without this genotype (OR 2.40 [95% CI 1.13–5.07]) (data not shown). This OR is similar to that found when either SNP was considered alone. This suggested an absence of a combined effect between these two SNPs on the development of diabetic nephropathy.

A significant association (as reflected by OR) thus exists between either *Xba*I SNP or enhancer-2 SNP 1 and diabetic nephropathy in Caucasians with type 1 diabetes. To gauge the magnitude of the effect of these *GLUT1* polymorphisms in conferring risk of this complication in our population, the PAR% was calculated. Using the procedure described in RESEARCH DESIGN AND METHODS, we estimated PAR% for *Xba*I(-)*Xba*I(-) homozygosity to be 6.73%, whereas that for enhancer-2 SNP 1 AA homozygosity was very similar at 6.21%.

The four remaining SNPs in *GLUT1* were also investigated in the case-control study (Fig. 1). No association was observed between these markers and the development of diabetic nephropathy (Table 2).

DISCUSSION

The present study was carried out to comprehensively investigate the role of *GLUT1* in conferring genetic susceptibility to nephropathy in type 1 diabetic patients. Previous studies have shown that polymorphisms located in exons of this gene do not alter the amino acid sequence of the protein (16,23). Thus, if *GLUT1* does contribute to genetic susceptibility, the causative polymorphism(s) is likely to be located in a regulatory portion of the gene.

Aside from the basal promoter (18), the regulatory regions of human *GLUT1* gene have not been well characterized. To circumvent this knowledge deficit, we identified putative enhancer regions by sequence similarity to known enhancer regions of the mouse (14) and rat genes (15). Common polymorphisms identified in these enhancer regions were then examined for association with diabetic nephropathy using a case-control study design. In addition, we took advantage of our large patient collection to test whether the *GLUT1 Xba*I SNP was associated with the development of this microvascular complication because conflicting results have so far been reported for this genetic marker (9–13).

Our study demonstrated that among Caucasian type 1 diabetic patients, *Xba*I(-)*Xba*I(-) homozygotes were at a slightly increased risk of diabetic nephropathy compared with patients with other genotypes. This result is in apparent agreement with the findings of a small British study by Hodgkinson et al. (12), which found homozygosity for *Xba*I(-) to be associated with diabetic nephropathy (OR 3.74 [95%CI 1.39–10.05], our calculation). This OR, though approximately twofold higher than that observed in our study, was not statistically different (Breslow-Day test for homogeneity of ORs, P = NS). On the other hand, this British study may have provided a less accurate estimate of the strength of the association because it consisted of only 44 control and 70 case subjects. In comparison, a total of 207 control and 249 case subjects were genotyped for the *Xba*I SNP in our study. It is reasonable that our four-times larger data set should yield a more reliable estimate of the genotype distribution. This should also make our study less prone to detecting spurious associations that may inadvertently occur in smaller studies, especially when the gene effect is minor.

Genotype data for the *Xba*I SNP in our patient sample was also compared with a recent Danish study that was smaller but still comparable in size (192 control and 175 case subjects). This study, conducted on type 1 diabetic Caucasians, did not detect any association between this SNP and diabetic nephropathy (13). Because our study found the association between this SNP and diabetic nephropathy to be small, failure of the Danish study to observe this association might have occurred by chance. Alternatively, it might be contemplated that *GLUT1* confers variable genetic risk to diabetic nephropathy in different Caucasian populations.

Aside from investigating *Xba*I SNP, a novel finding in our study is the identification of enhancer-2 SNP 1. Like the *Xba*I SNP, this new SNP is also located in intron 2, at a distance <2 kb from the former polymorphism. Results of our case-control study revealed that homozygosity for the A allele of enhancer-2 SNP 1 was significantly associated with diabetic nephropathy. Strong linkage disequilibrium was observed between this marker and *Xba*I SNP, and this likely accounted for the latter being associated with diabetic nephropathy.

However, in contrast to *Xba*I SNP, enhancer-2 SNP 1 could be the causative polymorphism directly influencing genetic susceptibility to diabetic nephropathy because it resides in a putative enhancer region that has potential to modulate gene expression. In this regard, the murine homologues of human enhancer-2 (and enhancer-1) have been previously demonstrated to confer insulin-responsiveness to reporter gene constructs when stably transfected into NIH/3T3 HIR3.5 cells (24). Also, enhancer-2 SNP 1 is located within a predicted binding site for USF, which is a transcription factor that confers responsiveness to stimulation by insulin and glucose (25,26). Thus, it might be hypothesized that in diabetic patients with the enhancer-2 SNP 1 AA risk genotype, high intracellular glucose levels may accumulate in mesangial cells in response to insulin and hyperglycemia (27). Excessive intracellular glucose levels may then activate various pathogenetic mechanisms that exacerbate mesangial expansion and glomerulosclerosis in diabetic nephropathy (6,28,29). Because these enhancer elements have so far been identified based on similarity between human and rodent genomic sequences, the effect of these putative enhancers on gene transcription could be examined in future studies. In addition, the effect of allelic variants of enhancer-2 SNP 1 on enhancer function may be tested to provide insight into the regulation of *GLUT1*.

In conclusion, our study confirms that SNPs at the *GLUT1* locus are associated with susceptibility to diabetic nephropathy in type 1 diabetes. Although these SNPs

confer a considerable personal risk to diabetic nephropathy, they account for a limited proportion of cases among type 1 diabetic patients.

ACKNOWLEDGMENTS

This research was supported by National Institutes of Health Grant DK41526. D.N. is a recipient of the National Science & Technology Board/Singapore Economic Development Board Biomedical Sciences Fellowship. L.C. is a recipient of a fellowship from Juvenile Diabetes Foundation International (3-2001-343).

REFERENCES

1. Wakisaka M, He Q, Spiro MJ, Spiro RG: Glucose entry into rat mesangial cells is mediated by both Na+-coupled and facilitative transporters. *Diabetologia* 38:291–297, 1995
2. Heilig CW, Liu Y, England RL, Freytag SO, Gilbert JD, Heilig KO, Zhu M, Concepcion LA, Brosius FC III: D-glucose stimulates mesangial cell GLUT1 expression and basal and IGF-1–sensitive glucose uptake in rat mesangial cells: implications for diabetic nephropathy. *Diabetes* 46:1030–1039, 1997
3. Larkins RG, Dunlop ME: The link between hyperglycemia and diabetic nephropathy. *Diabetologia* 35:499–504, 1992
4. Vlassara H: Recent progress in advanced glycation end products and diabetic complications. *Diabetes* 46 (Suppl. 2):S19–S25, 1997
5. Koya D, King GL: Protein kinase C activation and the development of diabetic complications (Review). *Diabetes* 47:859–866, 1998
6. Heilig CW, Concepcion LA, Riser BL, Freytag SO, Zhu M, Cortes P: Overexpression of glucose transporters in rat mesangial cells cultured in a normal glucose milieu mimics the diabetic phenotype. *J Clin Invest* 96:1802–1814, 1995
7. Heilig CW, Brosius FC III, Henry DN: Glucose transporters of the glomerulus and the implications for diabetic nephropathy. *Kidney Int* 60:S91–S99, 1997
8. Mogyorosi A, Ziyadeh FN: GLUT1 and TGF-β: the link between hyperglycaemia and diabetic nephropathy. *Nephrol Dial Transplant* 14:2827–2829, 1999
9. Gutierrez C, Vendrell J, Pastor R, Broch M, Aguilar C, Llor C, Simon I, Richart C: GLUT1 gene polymorphism in non-insulin-dependent diabetes mellitus: genetic susceptibility relationship with cardiovascular risk factors and microangiopathic complications in a Mediterranean population. *Diabetes Res Clin Pract* 41:113–120, 1998
10. Liu ZH, Guan TJ, Chen ZH, Li LS: Glucose transporter (GLUT1) allele (XbaI-) associated with nephropathy in non-insulin-dependent diabetes mellitus. *Kidney Int* 55:1843–1848, 1999
11. Grzeszczak W, Moczulski DK, Zychma M, Zukowska-Szczechowska E, Trautsolt W, Szydlowska I: Role of GLUT1 gene in susceptibility to diabetic nephropathy in type 2 diabetes. *Kidney Int* 59:631–636, 2001
12. Hodgkinson AD, Millward BA, Demaine AG: Polymorphisms of the glucose transporter (GLUT1) gene are associated with diabetic nephropathy. *Kidney Int* 59:985–989, 2001
13. Tarnow L, Grarup N, Hansen T, Parving HH, Pedersen O: Diabetic microvascular complications are not associated with two polymorphisms in the GLUT-1 and PC-1 genes regulating glucose metabolism in Caucasian type 1 diabetic patients. *Nephrol Dial Transplant* 16:1653–1656, 2001
14. Murakami T, Nishiyama T, Shirotani T, Shinohara Y, Kan M, Ishii K, Kanai F, Nakazuru S, Ebina Y: Identification of two enhancer elements in the gene encoding the type 1 glucose transporter from the mouse which are responsive to serum, growth factor and oncogenes. *J Biol Chem* 267:9300–9306, 1992
15. Behrooz A, Ismail-Beigi F: Dual control of Glut1 glucose transporter gene expression by hypoxia and by inhibition of oxidative phosphorylation. *J Biol Chem* 272:5555–5562, 1997
16. Tao T, Tanizawa Y, Matsutani A, Matsubara A, Kaneko T, Kaku K: HepG2/erythrocyte glucose transporter (GLUT1) gene in NIDDM: a population association study and molecular scanning in Japanese subjects. *Diabetologia* 38:942–947, 1995
17. Warram JH, Gearin G, Laffel L, Krolewski AS: Effect of duration of type 1 diabetes on the prevalence of stages of diabetic nephropathy defined by urinary albumin/creatinine ratio. *J Am Soc Nephrol* 7:930–937, 1996
18. Fukumoto H, Seino S, Imura H, Seino Y, Bell GI: Characterization and expression of human HepG2/erythrocyte glucose-transporter gene. *Diabetes* 37:657–661, 1988
19. Shows TB, Eddy RL, Byers MG, Fukushima Y, Dehaven CR, Murray JC, Bell GI: Polymorphic human glucose transporter gene (*GLUT*) is on chromosome 1p31.3→p35. *Diabetes* 36:546–549, 1987
20. Araki S, Moczulski DK, Hanna L, Scott LJ, Warram JH, Krolewski AS: APOE polymorphisms and the development of diabetic nephropathy in Type 1 diabetes: results of case-control and family based studies. *Diabetes* 49:2190–2195, 2000
21. Terwillinger J, Ott J: *Handbook of Human Genetic Linkage*. Baltimore, MD, John Hopkins University, 1994, p. 188–198
22. Hennekens CH, Buring JE: *Epidemiology in Medicine*. Mayrent SL, Ed. Boston, Little, Brown and Company, p. 54–98
23. Baroni MG, D'Andrea MP, Capici F, Buzzetti R, Cavallo MG, Fallucca F, Giovannini C, Pozzilli P: High frequency of polymorphism but no mutations found in the GLUT1 glucose transporter gene in NIDDM and familial obesity by SSCP analysis. *Hum Genet* 102:479–482, 1998
24. Todaka M, Nishiyama T, Murakami T, Saito S, Ito K, Kanai F, Kan M, Ishii K, Hayashi H, Shichiri M, Ebina Y: The role of insulin in activation of two enhancers in the mouse GLUT1 gene. *J Biol Chem* 269:29265–29270, 1994
25. LeFrancois-Martinez AM, Martinez A, Antoine B, Raymondjean M, Kahn A: Upstream stimulatory factor proteins are major components of the glucose response complex of the L-type pyruvate kinase gene promoter. *J Biol Chem* 270:2640–2643, 1995
26. Travers MT, Vallance AJ, Gourlay HT, Gill CA, Klein I, Bottema CB, Barber MC: Promoter I of the ovine acteyl-CoA carboxylase-á gene: an E-box motif at −114 in the proximal promoter binds upstream stimulatory factor (USF)-1 and USF-2 and acts as an insulin-response sequence in differentiating adipocytes. *Biochem J* 359:273–284, 2001
27. Arnqvist HJ, Ballermann BJ, King GL: Receptors for and effects of insulin and IGF-I in rat glomerular mesangial cells. *Am J Physiol* 254:C411–C416, 1988
28. Henry DN, Busik JV, Brosius FC III, Heilig CW: Glucose transporters control gene expression of aldose reductase, PKCα, and GLUT1 in mesangial cells in vitro. *Am J Physiol* 277: F97–F104, 1999
29. Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M: Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci U S A* 97:12222–12226, 2000