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# HepG2/erythrocyte glucose transporter (GLUT1) gene in NIDDM: a population association study and molecular scanning in Japanese subjects

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**Summary** To evaluate the role of mutations in the glucose transporter (GLUT1) gene in Japanese patients with non-insulin-dependent diabetes mellitus (NIDDM), we first conducted a population association study using the XbaI polymorphism of the gene. A polymerase chain reaction (PCR)-based assay was developed and used for the analysis. When analysed in 91 diabetic patients and 87 non-diabetic control subjects, the distribution of the genotype frequency was significantly different between the two groups ($p = 0.0025$). The (–) allele was significantly associated with NIDDM (odds ratio 2.317, 95 % confidence interval 1.425–3.768). To identify possible mutation(s) in the GLUT1 gene, which was in linkage disequilibrium with the (–) allele, all ten exons of the gene were analysed by PCR single-strand conformation polymorphism (SSCP) analysis in 53 diabetic patients with at least one (–) allele. Variant SSCP patterns were detected in exons 2, 4, 5, 7, 9 and 10. Sequence analysis revealed that all the variants represented silent mutations. One of the variants in exon 2, GCT ($Ala^{15}$) → GCC(Ala), created a HaeIII restriction site. This polymorphism was common in Japanese subjects with heterozygosity of 0.36 and polymorphism information content 0.29. We conclude that the structural mutation of GLUT1 is rare and not likely to be a major genetic determinant of NIDDM in Japanese subjects. The XbaI (–) allele of the GLUT1 gene appeared to be a genetic marker of NIDDM in Japanese subjects. The possibility of the presence of mutation(s) in the regulatory region of the gene or in another locus nearby could not be excluded. [Diabetologia (1995) 38: 942–947]

**Key words** Non-insulin-dependent diabetes mellitus, genetics, GLUT1, single-strand conformation polymorphism, mutation.

Non-insulin-dependent diabetes mellitus (NIDDM) is a metabolic disorder characterised by chronic hyperglycaemia due to impaired fuel metabolism. Strong familial aggregation of the disease suggests that genetic components play a major role as determinants of susceptibility to the disease [1]. "Candidate genes", the mutation of which could determine the susceptibility to NIDDM, have been intensively studied. Recently, mutations in the glucokinase [2, 3] and mitochondrial genes [4–6] were found in a subset of NIDDM. The inherited defects in the greater part of the common form of NIDDM, however, remain to be identified.

HepG2/erythrocyte glucose transporter (GLUT1) is a member of the facilitative glucose transporter family. GLUT1 is the most widely expressed member of this family and transports glucose across the barrier between body tissues and blood supply [7]. It may be responsible for basal constitutive glucose uptake in most tissues; thus, defective GLUT1 could disturb basal glucose uptake and might result in chronic hyperglycaemia. The association between the

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*Abbreviations:* NIDDM, Non-insulin-dependent diabetes mellitus; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism; BMI, body mass index; RFLP, restriction fragment length polymorphism; TRE, TPA-responsive elements; GLUT1, glucose transporter 1.

**Table 1.** Clinical characteristics of subjects for the analysis of GLUT1 gene

| | *n* | Age (years) | Sex (% male) | Positive family history (%) | BMI (kg/m$^2$) |
|---|---|---|---|---|---|
| NIDDM subjects | 91 | 58.5 ± 11.3[a] | 41.8 | 38.5 | 22.7 ± 3.8 |
| Control subjects | 87 | 48.7 ± 8.4[a] | 40.2 | 0 | 22.3 ± 3.2 |

Values are mean ± SD; [a] $p < 0.01$

GLUT1 gene and NIDDM has been studied using restriction fragment length polymorphism (RFLP) of the gene in several racial groups including Japanese [8–13]. The results, however, are inconclusive. Some have reported positive associations [8, 13], but others reported negative [9, 10–12, 14], even in the same racial groups. Linkage was not found in familial NIDDM pedigrees [15, 16] or in Caucasian diabetic sib-sets [17]. These results suggested that mutations in the GLUT1 gene did not play a major role in the development of NIDDM. Linkage analysis, however, may not be definitive in the heterogeneous diseases with an unknown mode of inheritance; minor roles also could not be ruled out.

The Japanese are one of the populations in which the association between the GLUT1 polymorphism and NIDDM has been suggested previously [8]. We first re-examined the association in our study population. Finding that the XbaI(−) allele of the gene was a genetic marker of NIDDM, we analysed the GLUT1 gene in the diabetic patients at single nucleotide level using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis to evaluate directly the role of the GLUT1 gene in the development of NIDDM.

## Subjects and methods

*Subjects.* NIDDM patients and non-diabetic control subjects were chosen randomly from the subjects we examined in a previous study [18]. All subjects were recruited from the outpatient clinics at Yamaguchi University Hospital and affiliated hospitals. They were unrelated Japanese over the age of 40 years, living in Yamaguchi Prefecture. Individuals with NIDDM met the diagnostic criteria of the World Health Organization. Non-diabetic subjects had a random plasma glucose of less than 6.7 mmol/l and claimed no personal or family history of diabetes. At the time of the recruitment, informed consent was obtained from each individual according to a protocol approved by the Human Studies Committee. Clinical characteristics of the subjects for the current study are summarized in Table 1. Control subjects were younger ($p < 0.01$) than diabetic patients. There was no difference between the two groups in gender and body mass index (BMI). None of the parameters were different from those of the subjects in the previous study in either NIDDM or control groups.

Genomic DNA was extracted from leukocytes as reported previously [9].

*Restriction fragment length polymorphism (RFLP) analysis.* XbaI RFLP of the human GLUT1 gene [19] was detected by Southern blot analysis of genomic DNA [9]. Because the classic RFLP analysis using Southern Blotting was laborious and time consuming, we established a PCR-based detection system. The polymorphic XbaI site was localized in the second intron of the gene by restriction analysis of a human GLUT1 genomic clone (kindly provided by Dr. M. Mueckler, Washington University, St. Louis, Mo., USA). The fragment containing the polymorphic XbaI site was subcloned and the flanking DNA sequences were determined. A 5′ primer (TGTGCAACCCATGAGCTAA) and a 3′ primer (CCTGGTCTCATCTGGATTCT) were synthesized on the DNA synthesizer (Model 392, Applied Biosystems Japan, Urayasu, Japan). A 1.1 kb DNA fragment including the polymorphic XbaI site was PCR amplified in 20 µl containing 250 ng of genomic DNA, 10 pmol each primer, 0.2 mmol/l each dNTP, 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.01 % gelatin and 0.25 unit AmpliTaq DNA polymerase (Perkin Elmer/Cetus, Norwalk, Conn., USA). After 30 cycles of amplification consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and 20 s, in a thermocycler (Program Temp Control System PC-700, ASTEC Co., Fukuoka, Japan), the products were purified using Prep-A-Gene DNA purification kit (Bio-Rad Laboratories, Richmond, Calif., USA), digested with 10-fold excess XbaI for 3–4 h and electrophoresed on a 1.2 % agarose gel. XbaI RFLP was detected by ethidium bromide staining.

A silent mutation in exon 2 created a HaeIII site. This polymorphism was also detected by PCR-RFLP assay. Exon 2 was amplified using the PCR primers for the SSCP analysis (see below). Amplified products were digested with HaeIII followed by the electrophoresis in 2.8 % agarose gel as for the XbaI polymorphism.

*SSCP analysis of the GLUT1 gene.* Ten sets of oligonucleotide primers were synthesized flanking the coding regions of each of 10 exons of the human GLUT1 gene (Table 2, [20]; a part of intronic sequences was kindly provided by Dr. G. I. Bell, University of Chicago, Chicago, Ill., USA).

PCR amplification was conducted in a 10-µl volume containing 100 ng genomic DNA, 2 pmol each primer, 0.1 mmol/l each dNTP, 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 2 mmol/l $MgCl_2$, 0.01 % gelatin, 0.25 unit AmpliTaq DNA polymerase and 0.67 µCi of α-[$^{32}$P]dCTP (3,000 Ci/mmol, Amersham, Tokyo, Japan). After an initial denaturation at 94 °C for 3 min, 30 cycles of amplification were performed with denaturation at 94 °C for 1 min, annealing for 1 min at the temperatures given in Table 2, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min.

SSCP was performed essentially using the method of Orita et al. [21]. Ten microlitres of the PCR products was mixed with 30 µl of 95 % formamide, 20 mmol/l EDTA, 0.05 % bromophenol blue and xylene cyanol. Immediately before electrophoresis, samples were heat-denatured at 94 °C for 3 min. Aliquots of the samples (1.5 µl) were electrophoresed under two different conditions: 1) 5 % polyacrylamide (49 : 1, acrylamide: N, N′-methylene-bis-acrylamide) gel in 1 × TBE [90 mmol/l Tris-borate (pH 8.3), 2 mmol/l EDTA] at 5–7 W

**Table 2.** PCR primers used for the SSCP analysis of GLUT1 gene

| Exon | Sense primer (bp) | Antisense primer (bp) | Product (bp) | Ta (°C) |
|---|---|---|---|---|
| 1 | AACAGCGAGCGTGCCGGTCGCTAGT | TAAGGCGGGCAGGAGTCTGCGCCTT (37) | 280 | 65 |
| 2 | CTCCCAGACACGCCTATAACAGT (20) | GGCTGGTGTCCATAAGCCAACG (12) | 173 | 66 |
| 3 | GCTTGCTCACCCAGGCTGCAT (12) | TCCAAGGGCAGTGCCAGGAC (13) | 227 | 66 |
| 4 | AGGAATGTGGGCACTGGCCT (21) | GTGCCAGGCAGGTAGATCCT (12) | 314 | 64 |
| 5 | AAAGGGGGTCAGGGCAGAGGCGCTCA (20) | GTGGGAAGAAGGCCAGGGCTCAGGGA (30) | 313 | 72 |
| 6 | ACTCTGAGCCACCCTCACCTTCCCT (13) | ACACTTGACCAGAGGGCTTGGCTGG (30) | 281 | 65 |
| 7 | CAGGTAGGCCCCAACAGTTTCTC (11) | TGGCTGGGGGGGCCAGTAAGC (10) | 170 | 70 |
| 8 | AGGGGTGGCACTGACATGTCTCT (8) | GCATCCCTCACTCTCCAGAACCT (10) | 166 | 68 |
| 9 | AACTTTTCCCCCTCTCCGTCATC (8) | GTGCGGGTGAGTATAGAGACAGT (12) | 270 | 66 |
| 10 | ATGACTCCAACCAAGTGTGTC (14) | TGTGCTCCTGAGAGATCCTTA | 297 | 58 |

Number of nucleotides between the end of each PCR primer and the corresponding exon-intron boundary in parentheses. The sense primer of exon 1 and the antisense primer of exon 10 are in the 5′ and 3′ untranslated regions, respectively. Ta, annealing temperatures

for 12 h or 30 W for 3–4 h; 2) 5 % polyacrylamide gel with 10 % glycerol in 1 × TBE at 5–7 W for 12 h, both at room temperature. After the electrophoresis, gels were dried and exposed to X-ray film with an intensifying screen for 16–24 h at – 80 °C.

*DNA sequencing and analysis.* Exons were PCR amplified from 200 ng of genomic DNA in a 20-μl volume. Products were electrophoresed on 1.2 % NuSieve GTG Agarose (FMC BioProducts, Rockland, Me., USA) to ensure a single specific product. The DNA-containing gel was cut out and digested by β-Agarase I (New England Biolabs, Beverly, Mass., USA) at 40 °C for 2 h. After the digestion, DNA was recovered by ethanol precipitation.

DNA sequencing was performed using Taq Dye Deoxy Terminator Cycle Sequencing Kit and model 373A DNA Sequencer (Applied Biosystems) according to the manufacturer's instructions.

Possible transcriptional regulatory elements near the polymorphic XbaI site in the second intron were searched by a computer program GENETYX-MAC version 6.01 (Software Development Co., Ltd., Tokyo, Japan).

### *Statistical analysis*

Statistical significance of difference between groups in quantitative variables was analysed by unpaired (two-tailed) *t*-tests. Associations between the polymorphic markers in the GLUT1 gene and NIDDM was assessed by Fisher's exact test or the chi-square test.

## Results

*XbaI RFLP analysis at the GLUT1 locus.* Southern blotting revealed a two-allele polymorphism of 5.9 kb ( + allele) and 6.2 kb ( – allele) as reported previously [9, 19]. To establish a PCR-based detection system, we localized the polymorphic XbaI site in the second intron and determined the flanking sequence (Fig. 1). PCR-RFLP produced three bands of different sizes (Fig. 2). A 1.1 kb band corresponds to the (–) allele, and a set of 0.9 kb and 0.2 kb bands correspond to (+) allele. The results from the two methods were identical.

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tctaga1atcc taagatcaag gttcgagagg ccacatccag ccagcagcct
gtttgtgcaa cccatgagct aa2caatggtt ttacatttta caaggttgag
aaaaaagaac aatgagtgac agaggccata tgtggctcac agaccctacc
taatggattc tagctagcct tttacataaa atgtttaccc cagtccggct
ctggagcact ttgacttgca tgctccctac ttacagggaa ctcaccacct
gtaaggtggg cagcctcata ccctgaagtc taGa3aagctc ttccttcccc
tgagctggag tctggccctg gcacctctgc cctctggggc tacagagtgc
tgggttcctc tgccaggagc agccgagcag ggtctggaga ctagctttat
tccctgaggc aggcccgtag ttccttcagg ttcctcctta ctcttacatt
cttagggtaa tgatttctgg caatttcaaa atggaaaacc ttgtccctaa
ggctcctccg aaagaggtga cttcttttct tgagagagca acagtgacag
cagtaacagc ttacccatgt gccaggcagt gttcgccgcc tcatgccccc
```

**Fig. 1.** Nucleotide sequence flanking the polymorphic XbaI site in the second intron of the human GLUT1 gene. XbaI sites (1, 3) and the site of the 5′ primer for the PCR-RFLP analysis (2) are underlined. G to T transversion in the second XbaI recognition site (3, capitalized) abolishes the recognition site. The 3′ PCR primer locates about 1.1 kb downstream of the 5 primer

Observed genotypic frequency for the XbaI polymorphism did not differ from that predicted from Hardy-Weinberg equilibrium in the control or the diabetic group. Heterozygosity of the polymorphism in the control subjects was 0.303, and the polymorphism information content was 0.257.

Distribution of the genotype frequency was significantly different between the diabetic patients and the non-diabetic control subjects (Table 3, chi-square was 11.974, $df = 2$, $p = 0.0025$). When the allele fre-

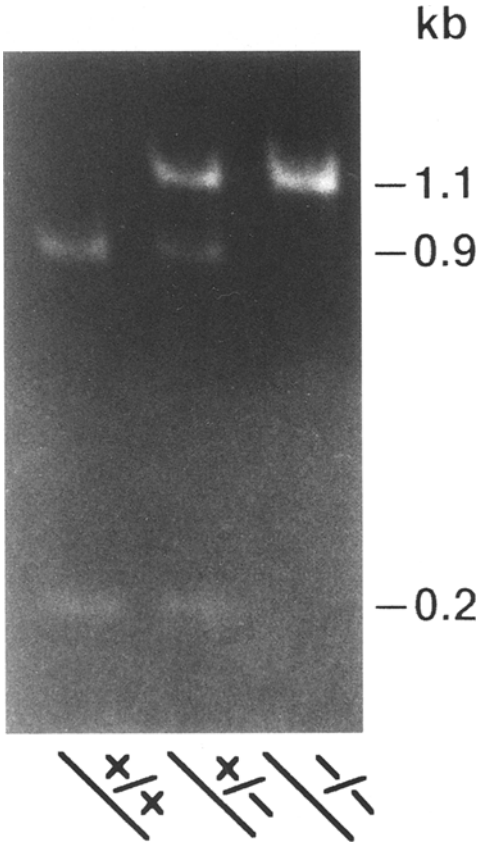


**Fig. 2.** XbaI RFLP of human GLUT1 gene detected by PCR-RFLP analysis. Sizes of the fragments are shown in kb on the right. Genotypes assigned for each individual are shown below the lanes

quency was compared, the (–) allele was significantly associated with NIDDM (odds ratio 2.317, 95 % confidence interval 1.425–3.768, Table 3).

Clinical characteristics were compared among groups with each genotype in the diabetic patients. The average BMI did not differ (data not shown). $HbA_{1c}$ in (+/–) group was marginally lower than in (+/+) group ($7.9 \pm 1.7$ % vs $8.7 \pm 2.0$ %, mean ± SD, $p < 0.05$).

*Variation of the GLUT1 gene.* From the population association study, the (–) allele of the XbaI RFLP appeared to be a risk factor for NIDDM in Japanese subjects. Since Murakami et al. [22] suggested that the TPA-responsive elements (TRE) in the second intron of the mouse GLUT1 gene regulate gene transcription, we examined the possibility that the XbaI polymorphism itself might be involved in the transcriptional gene regulation. The polymorphism did not create or abolish any known regulatory elements including TRE (Fig. 1 and data not shown).

We then assessed the possibility that the increased risk was due to the mutation(s) in the coding region of the GLUT1 gene by direct analysis of the gene. All of the ten exons of the GLUT1 gene were

**Table 3.** Genotypic and allelic frequencies of XbaI RFLP

| | NIDDM subjects | Control subjects |
|---|---|---|
| Genotype[a] | | |
| +/+ | 38 (0.42) | 56 (0.64) |
| +/– | 42 (0.46) | 29 (0.33) |
| –/– | 11 (0.12) | 2 (0.02) |
| Total | 91 | 87 |
| Allele[b] | | |
| + | 118 (0.65) | 141 (0.81) |
| – | 64 (0.35) | 33 (0.19) |
| Total | 182 | 174 |

[a] $p = 0.0025$ ($\chi^2 = 11.974$, $df = 2$) between NIDDM and control subjects;
[b] $p = 0.0008$ (Fisher's exact test) between NIDDM and control subjects

screened by PCR-SSCP in 53 NIDDM patients with at least one XbaI (–) allele. Variant SSCP patterns were detected in exons 2, 4, 5, 7, 9 and 10. All samples showing variant patterns were subjected to direct sequencing and nucleotide substitutions were identified (Table 4). All the variants were silent mutations without amino acid substitution. Although the nucleotide substitutions in exons 4, 5, 7 and 10 created GT or AG dinucleotides, the flanking sequences did not match well with the known splicing consensus. Therefore it was unlikely that these changes altered GLUT1 mRNA processing by activating cryptic splicing.

One of the silent mutations in exon 2, T to C transition at nucleotide 45 in the codon for $Ala^{15}$, is relatively common in the Japanese population. The C allele, a variant from the reported sequence [23], has been found to be more common in the Japanese population. This transition created an HaeIII restriction site and the polymorphism was detectable by the PCR-RFLP method. When exon 2 was PCR amplified and digested with HaeIII, the (–) allele (with T at nucleotide 45) gave a single 138 bp band on agarose gel electrophoresis, whereas the (+) allele (with C at 45) gave a set of 103 bp and 35 bp bands. When analysed in NIDDM patients and control subjects, the allelic and genotypic frequencies did not differ between these two groups (Table 5, $p = 0.3805$, Fisher's exact test). The heterozygosity of the polymorphism in the non-diabetic subjects was 0.359 and the polymorphism information content 0.291.

## Discussion

Among the members of facilitative glucose transporters, liver/islet glucose transporter (GLUT2) and insulin-regulatable glucose transporter (GLUT4) were extensively studied as candidates for genetic determinants of susceptibility to NIDDM [10, 15, 24–29]. Al-

**Table 4.** SSCP analysis of GLUT1 gene in NIDDM patients

| Exon | Nucleotide | Consensus | Variant | $n^a$ |
|---|---|---|---|---|
| 2 | 45 | GCT ($Ala^{15}$) | GCC ($Ala^{15}$) | 44 |
| 4 | 399 | TGC ($Cys^{133}$) | TGT ($Cys^{133}$) | 5 |
| 5 | 588 | CCG ($Pro^{196}$) | CCA ($Pro^{196}$) | 5 |
| 7 | 906 | GGG ($Gly^{302}$) | GGT ($Gly^{302}$) | 2 |
| 9 | 1170 | ATC ($Ile^{390}$) | ATT ($Ile^{390}$) | 3 |
| 10 | 1372 | CGG ($Arg^{458}$) | AGG ($Agr^{458}$) | 6 |

Nucleotide, amino acid numbers and consensus sequences published previously [23].
[a] Number of patients with at least one variant allele. Genotypic and allelic frequencies of the common polymorphism in exon 2 (T/C) are shown in Table 5. Other rare variants in exons 4, 5, 7, 9, 10 were all observed in heterozygotes with the consensus sequence

**Table 5.** Genotypic and allelic frequencies of HaeIII RFLP in exon 2 of the GLUT1 gene

| | NIDDM patients | Control subjects |
|---|---|---|
| Genotype[a] | | |
| +/+ | 46 (0.54) | 46 (0.57) |
| +/– | 30 (0.35) | 32 (0.40) |
| –/– | 9 (0.11) | 3 (0.04) |
| Total | 85 | 81 |
| Allele[b] | | |
| + | 122 (0.72) | 124 (0.77) |
| – | 48 (0.28) | 38 (0.23) |
| Total | 170 | 162 |

(+) allele corresponds to GCC ($Ala^{15}$) and (–) allele to GCT ($Ala^{15}$) in Table 4.
[a] $p = 0.2265$ ($\chi^2 = 2.97$, $df = 2$) between NIDDM and control subjects;
[b] $p = 0.3805$ (Fisher's exact test) between NIDDM and control subjects

though a defective GLUT2 was identified in one single patient with NIDDM [27, 30], the mutations in these glucose transporters appeared to be rare and do not play a major role in the development of NIDDM. The role of HepG2/erythrocyte (GLUT1) glucose transporter, which is ubiquitously expressed and facilitates basal glucose transport into the cells, is less clear. The gene has not been studied at a single nucleotide level.

Li et al. [8] reported an association between NIDDM and the XbaI polymorphism of the GLUT1 gene in Japanese subjects. To further assess the role of GLUT1 mutation in NIDDM, we first tested whether the association could be replicated in our population. Our data also showed that the XbaI (–) allele was significantly associated with NIDDM (odds ratio 2.317, 95% confidence interval 1.425–3.768).

To examine whether the increased risk of NIDDM with the XbaI (–) allele was due to mutation(s) in the GLUT1 gene, we analysed all ten exons of the gene by PCR-SSCP in the diabetic patients with at least one (–) allele. We found only several silent mutations without functional significance. Although gross rearrangements of the gene, including insertion or deletion, could not be detected by PCR-SSCP analysis, we did not see aberrant bands other than those expected for the RFLPs during the course of XbaI and other RFLP analyses of the gene by Southern blot analysis. We chose 53 patients with at least one XbaI (–) allele for the SSCP analysis; 42 of whom were heterozygous at the locus, however, having one XbaI (+) allele. Thus it is unlikely that we have missed a common mutation in linkage disequilibrium with the XbaI (+) allele in Japanese subjects. These results indicate that structural changes in GLUT1 are not a major determinant of the susceptibility to NIDDM in Japanese subjects, although we cannot exclude a small possibility that we have missed the mutation because of the limitation of the sensitivity of the analysis.

We observed an association between the XbaI polymorphism of the GLUT1 gene and NIDDM in Japanese subjects, but were unable to identify an attributable mutation in the coding region. Although a population association study may be useful in the genetic analysis of complex diseases like NIDDM, it suffers from methodological deficiencies which could lead to a spurious observation [14, 31]. Thus, our conflicting results may be explained by the spurious association. The genotypic and allelic frequency distributions of the polymorphism in our samples however were very similar to those observed by Li et al. [8] in a smaller sample. The possibility that the association could be attributable to a mutation in the undefined regulatory region of the gene or in an adjacent gene must not be discarded. In this regard the mouse diabetic gene (db) and rat obesity gene (fa) were localized in a region including GLUT1, Cjun and Lck genes, all of which also made a cluster on chromosome 1 (1p 31–36) in human (32, 33).

We conclude that the structural mutation of the GLUT1 glucose transporter is rare, and is unlikely to be a major determinant of genetic susceptibility to NIDDM in Japanese subjects. The XbaI (–) allele of the GLUT1 gene appeared to be a genetic marker of NIDDM in Japanese subjects. The possibility of the presence of mutation(s) in the regulatory region of the gene or in the loci nearby could not be excluded.

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