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Polymorphic markers of the glycogen debranching enzyme gene allowing linkage analysis in families with glycogen storage disease type III

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Abstract

Glycogen storage disease type III (GSD-III), an autosomal recessive disease, is caused by deficient glycogen debranching enzyme (GDE) activity. We identified three polymorphic markers in the GDE gene using single strand conformation polymorphism (SSCP) analysis and DNA sequencing. They were −10G/A in the 5′ non-translated region of exon 3, 2001+8C/T in intron 16, and 3199C/T (P1067S) in exon 25. Two polymorphic markers (−10G/A and 2001+8C/T) were highly informative in both controls and GSD-III patients with heterozygosity values of 0.50 and 0.46, respectively. The third marker (3199C/T) had a heterozygosity value of 0.26. Restriction analysis of the PCR amplified genomic DNA products in two GSD-III families showed for the first time the potential use of these markers for carrier detection and prenatal diagnosis in this disease.

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Glycogen debranching enzyme (GDE), together with phosphorylase, is responsible for degradation of glycogen. The GDE is a 174 kDa monomeric protein, with both amylo-1,6-glucosidase (EC 3.2.1.33) and 1,4-α-D-glucan 4-α-D-glycosyltransferase (EC 2.4.1.25) activities at two independent catalytic sites.[1 2] Deficiency of GDE activity causes glycogen storage disease type III (GSD-III), an autosomal recessive disease, characterised by hepatomegaly, hypoglycaemia, short stature, and, in many patients, progressive myopathy and cardiomyopathy. Overt liver cirrhosis can occur and gross cardiac involvement may lead to death.[3] The variable phenotype is explained by differences in tissue expression of the defective enzyme. Most commonly the enzyme is deficient in both liver and muscle (GSD type IIIa). However, sometimes GDE is only deficient in liver and activity in muscle is normal (type IIIb). In rare cases, selective loss of only one of the two GDE activities (glucosidase (type IIIc) or transferase (type IIId)) has been documented.[4 5]

We and others have isolated the GDE gene and determined its cDNA sequences.[6 7] The GDE gene mRNA consists of a 4596 base pair coding region and a 2371 base pair 3′ non-translated region. The human gene is localised to chromosome 1p21.[8] The genomic structure of the human GDE gene has been determined and consists of 35 exons spanning at least 85 kb of DNA (Bao *et al*, manuscript submitted).[9]

We recently reported the first pathogenic mutation in GSD-IIIa; this mutation (4529insA) appears to be rare and has only been seen in one child with an unusually severe phenotype.[10] We also identified the striking association of exon 3 mutations (17delAG and Q6X) with GSD-IIIb patients.[11] However, these mutations account for less than 20% of the total mutant alleles in GSD-III. Because of the large size of the GDE gene, it is impractical to sequence the entire gene for routine diagnosis. Prenatal diagnosis using a qualitative assay or enzymatic assay requires a large number of viable cultured amniotic fluid cells or chorionic villi, which need a relative long period of cell culture, thus limiting its usefulness.[12–14] Furthermore, carrier detection using these methods has not been possible. In this study, we report three polymorphic markers in the GDE gene and show the potential use of these molecular markers in linkage analysis for carrier detection and prenatal diagnosis of GSD-III.

Materials and methods

PATIENTS

Our study group included 28 normal unrelated subjects and 18 patients with glycogen storage disease type III and their family members, the majority of whom were white. The patients with GSD-III were identified through our diagnostic facility for study of glycogen storage disease at Duke University Medical Center. Linkage analysis was studied in two GSD-III families. Family 1 had one affected child and a newborn baby. Family 2 had two affected children and the mother was pregnant. Blood (or cord blood from the newborn) was the source of genomic DNA.

SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP) ANALYSIS

The primers in table 1 were used to amplify the coding regions and intron-exon junctions of exon 3 (primers 1 and 2), exon 16 (primers 3 and 4), and exon 25 (primers 5 and 6) of the GDE gene. The DNA fragments were labelled

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Table 1 Primers used for genomic amplifications

Primer	*Sequence**	*Orientation*	*cDNA coordinates*
1	5'cgatattttaactccttttg3'	Sense	Derived from intron
2	5'agcacgcaaatgagcaaatc3'	Antisense	Derived from intron
3	5'gctatagaatagcactttgc3'	Sense	Derived from intron
4	5'aaagttgttggccatgtagg3'	Antisense	Derived from intron
5	5'cctacaagtaataaattcaa3'	Sense	Derived from intron
6	5'catattttaaccttgtca3'	Antisense	Derived from intron
7	5'GTGGAGTTCTTTTAATTCTT3'	Sense	−52 to −32
8	5'cttgcctactgaCCTTGTTC3'	Antisense	76 to 82 + intron sequence
9	5'GTGCTAGTGGAAGTACAAGA3'	Sense	1855 to 1874
10	5'AAATctggacaaaggtaaa3'	Antisense	Intron sequence +2002 to 2005
11	5'CCTGCCAATTCTTTCACC3'	Sense	2015 to 2032
12	5'GCTAGAGAAACACAACATT3'	Antisense	2064 to 2081

*Lower case letters represent the intron nucleotides

by addition of 0.25 µl α^{32}P dCTP (3000 Ci mmol^{-1}, 10 mCi ml^{-1}) into each 25 µl of polymerase chain reaction (PCR) mixture. The following PCR conditions were used: an initial denaturation at 95°C for five minutes, then 35 cycles of denaturation at 94°C for one minute, annealing at 55°C for 30 seconds for primers 1 and 2 (53°C for primers 3 and 4, and 52°C for primers 5 and 6), and extension at 72°C for 30 seconds, and followed by final extension at 72°C for 10 minutes. The PCR reactions were carried out with 1.5 mmol/l $MgCl_2$, 400 nmol/l of each primer, and about 150 ng genomic DNA as template. One microlitre of each PCR amplified sample was added to 10 µl of stop solution (95% formamide, 10 mmol/l NaOH, 0.25% bromophenol blue, and 0.25% Xylene cyanol), denatured for five minutes in a boiling water bath, and placed immediately on ice. Then 2.5 µl of each sample were loaded onto either one or both types of gels: (1) 0.75 × MDE gel (AT Biochemicals, Malvern, PA) containing 5% (V/V) glycerol; (2) 0.75 × MDE gel without glycerol. The gels were electrophoresed at 6–8 W either at room temperature for 18 hours or at 4°C for 36 hours. The gels were dried on 3 MM Whatman paper and exposed to Kodak Biomax MR film at −80°C.

DNA SEQUENCING

With the SSCP analysis, sequence polymorphisms were localised to specific regions within 150 to 360 bp. To identify the exact nucleotide changes, direct sequencing of PCR amplified genomic DNA fragments without subcloning was performed with the AmpliCycle™ Sequencing kit (Perkin Elmer, Foster City, CA) using γ^{32}P dATP (3000 Ci mmol^{-1}, 10 mCi ml^{-1}). In some cases the same PCR amplified genomic DNA fragments were also subcloned into pCR™TM II vector with the TA cloning kit (InVitrogen, San Diego, CA). Plasmid DNA was isolated, alkaline denatured, mixed with one original PCR primer, annealed, and sequenced with Sequenase 2.0 (US Biochemicals, Cleveland, OH) using [^{35}S] dATP. Either one or both strands were sequenced with the same sequencing primers as used in SSCP.

LINKAGE ANALYSIS BY RESTRICTION ENZYME DIGESTION OF PCR PRODUCTS

Restriction analysis was performed to confirm the polymorphisms and to provide a rapid screening for allele frequencies and linkage analysis in GSD-III families. Genomic DNA containing each of the suspected polymorphisms was amplified by PCR using a different set of primers. These primers are also listed in table 1. The amplified fragments were digested with their specific restriction enzymes: (1) fragments amplified from exon 3 (using primers 7 and 8) digested with *Xba*I for −10G/A; (2) fragments amplified from intron 16 (using primers 9 and 10) digested with *Nde*I for 2001+8C/T; and (3) fragments amplified from exon 25 (using primers 11 and 12) digested with *Rsa*I for 3199C/T (P1067S). The digested samples were then electrophoresed on a 2% agarose gel. All the restriction enzymes used were purchased from New England Biolabs, Inc (Beverly, MA).

Results

SCREENING POLYMORPHIC MARKERS WITH SSCP

Thirty-five pairs of PCR primers were designed so that they amplified the 5′ non-translated region as well as coding regions of each of the 35 exons, and all intron-exon junctions, of the GDE gene. SSCP analysis on MDE gels followed to detect mobility shift. The exact changes were then identified by DNA sequencing and confirmed by restriction analysis of PCR amplified genomic products using a different set of primers. The following three polymorphisms were detected.

Polymorphic marker 1

Fig 1A shows the SSCP pattern of a two allele polymorphism (allele 1: lanes 1, 2, 3, 4, 6, and 10; allele 2: lanes 5, 9, 11, 12, and 13; alleles 1 and 2: lanes 7 and 8) in exon 3 of 13 unrelated controls. DNA sequencing of the subclones showed that the polymorphism was the result of a G to A change in the 5′ non-translated region of exon 3 (−10G/A) (fig 1B). The same polymorphism was also observed in the GSD-III patients. The polymorphism created an *Xba*I restriction enzyme site. The allele frequencies of this polymorphism were: (1) normal controls: allele 1 (corresponding to G), 50%, allele 2 (corresponding to A), 50%, with a heterozygosity value of 0.50; (2) GSD-III patients: allele 1, 0.46%, allele 2, 0.54%, with a heterozygosity value of 0.50.

Polymorphic marker 2

Fig 2A shows a second SSCP pattern of a two allele polymorphism (allele A: lanes 1, 2, 4, and 7; allele B: lanes 5, 8, and 10; alleles A and B: lanes 3, 6, 9, and 11) in intron 16 of the unrelated controls. DNA sequencing (fig 2B) showed that this second polymorphism was the result of a C to T change (2001+8C/T). The same polymorphism was also observed in the GSD-III patients. The polymorphism created a *Nde*I restriction enzyme site. The

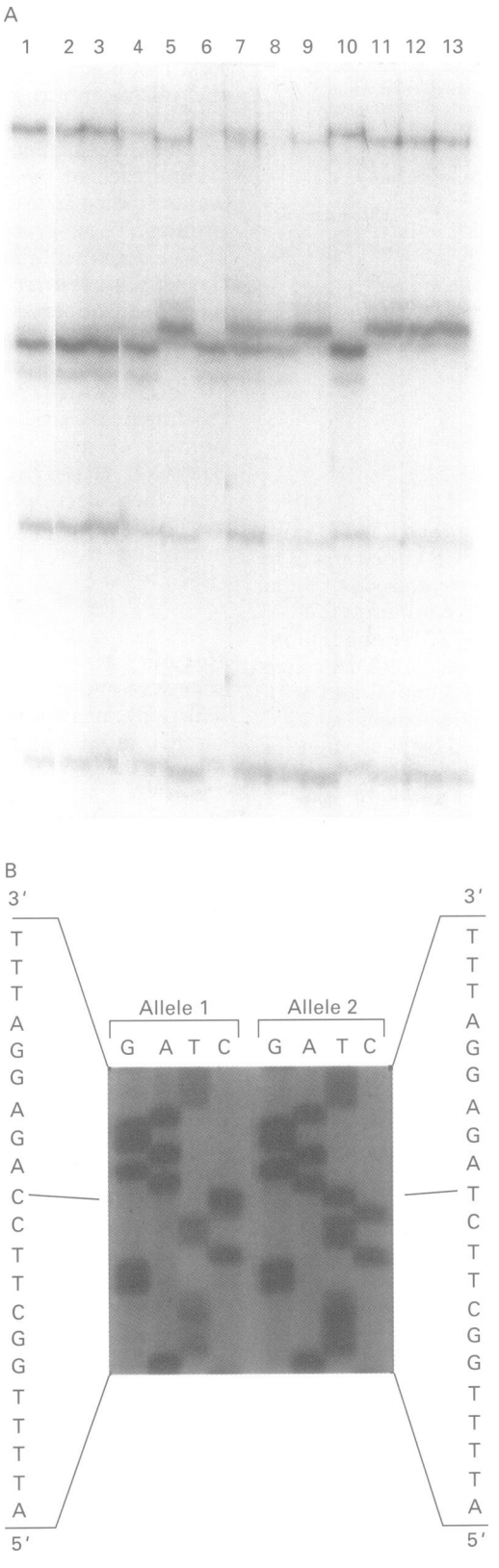


Figure 1 SSCP analysis and identification of the polymorphic marker (−10G/A) in exon 3. (A) SSCP analysis of PCR amplified genomic DNA of the unrelated controls. Allele 1: lanes 1, 2, 3, 4, 6, and 10; allele 2: lanes 5, 9, 11, 12, and 13; alleles 1 and 2: lanes 7 and 8. The gel contained 0.75 × MDE with 5% glycerol and was run at 4°C for 36 hours. (B) Partial DNA sequences of the GDE gene subclones of two unrelated controls with different SSCP patterns. The sequences shown represented an antisense strand. The polymorphism (−10G/A) resulting from G to A change is indicated.

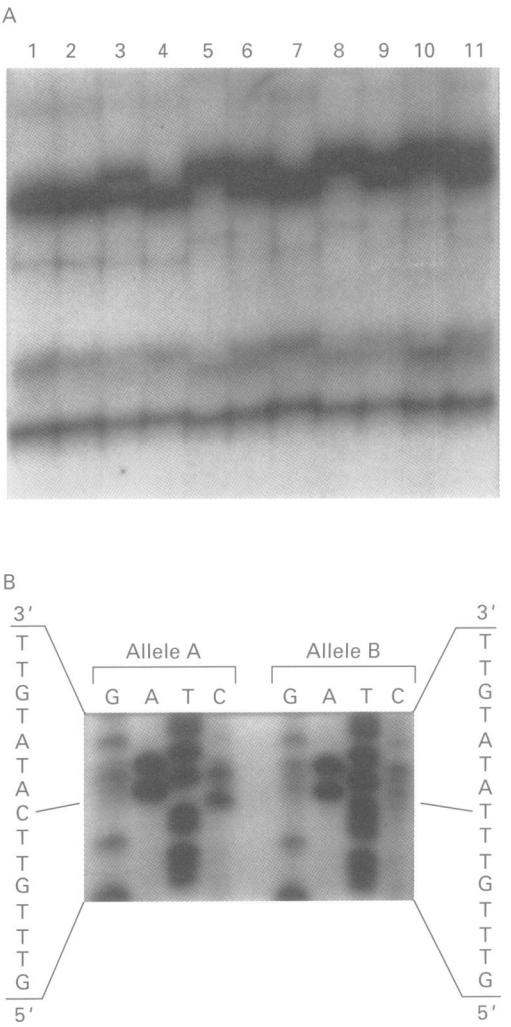


Figure 2 SSCP analysis and identification of the polymorphic marker (2001+8C/T) in intron 16. (A) SSCP analysis of PCR amplified genomic DNA of the unrelated controls. Allele A: lanes 1, 2, 4, and 7; allele B: lanes 5, 8, and 10; alleles A and B: lanes 3, 6, 9, and 11. (B) Partial DNA sequences of direct sequencing of PCR amplified genomic products of two unrelated controls with different SSCP patterns. The polymorphism (2001+8C/T) resulting from a C to T change is indicated.

allele frequencies of this polymorphism were: (1) normal controls: allele A, 64%, allele B, 36%, with a heterozygosity value of 0.46; (2) GSD-III patients: allele A, 54%, allele B, 46%, with a heterozygosity value of 0.50.

Polymorphic marker 3

There was a third SSCP pattern of a two allele polymorphism (data not shown) in the coding region of exon 25 in the unrelated controls. DNA sequencing (fig 3) showed that this polymorphism was the result of a C to T change at nucleotide 3199, which changed amino acid codon proline to serine. This same polymorphism was also observed in the GSD-III patients. The polymorphism abolished an *Rsa*I restriction enzyme site. The allele frequencies of this polymorphism were: (1) normal controls: allele I 15%, allele II 85%, with a heterozygosity value of 0.26; (2) GSD-III patients: allele I 18%, allele II 82%, with a heterozygosity value of 0.30.

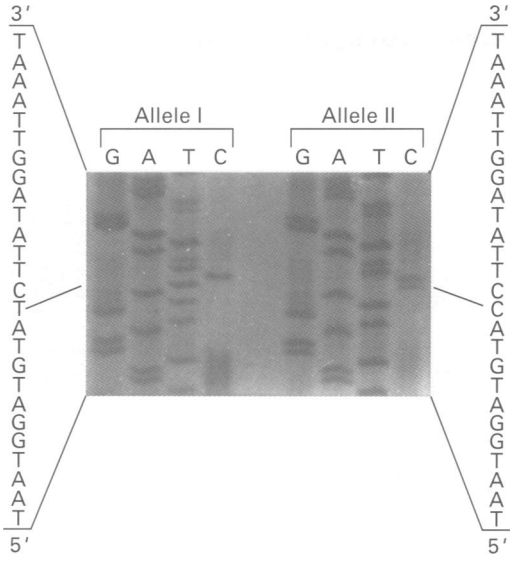
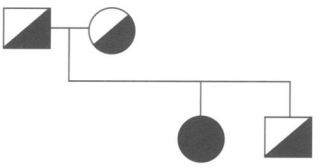


Figure 3 Identification of the polymorphic marker (3199C/T) in exon 25. Partial DNA sequences of direct sequencing of PCR amplified genomic products of two unrelated controls with two different SSCP patterns. The polymorphism (3199C/T) resulting from a C to T change is indicated.



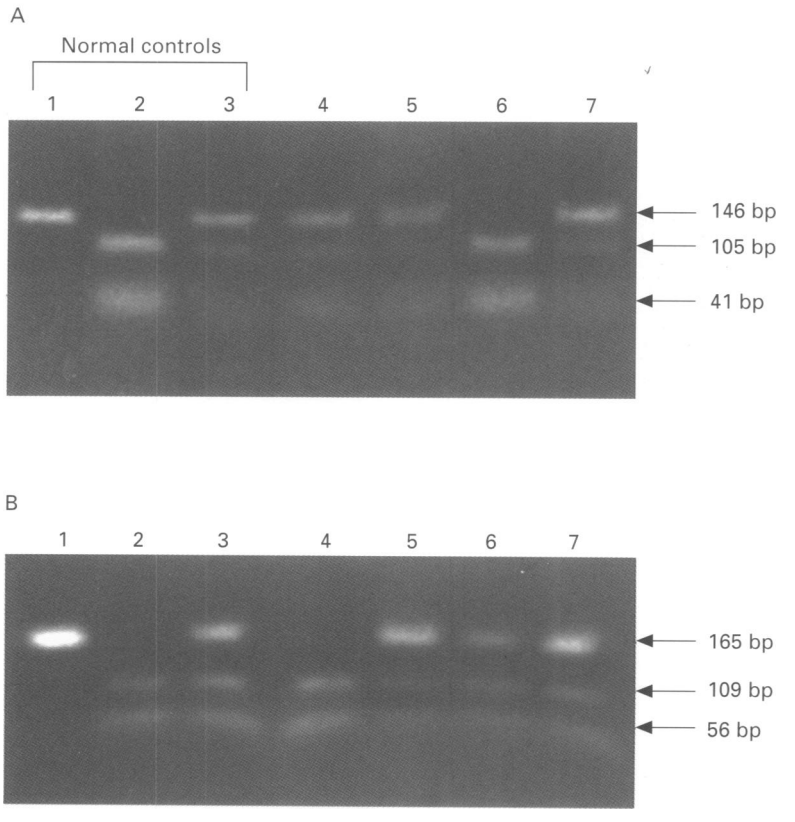


Figure 4 Linkage analysis of family 1 using the polymorphic markers (−10G/A; 2001+8C/T) by restriction digestion. (A) XbaI digestion of PCR amplified genomic DNA fragments of family 1 in exon 3. Digestion of the DNA of three unrelated controls yielded three patterns. Lane 1: completely undigested as allele 1; lane 2: completely digested as allele 2; lane 3: partially digested as alleles 1 and 2. Lanes 4, 5, and 7: father, mother, and the newborn baby, respectively, partially digested; lane 6, the affected child, completely digested. (B) NdeI digestion of PCR amplified genomic DNA fragments of family 1 in intron 16. Lane 1: completely undigested as allele A; lane 2: completely digested as allele B; lane 3: partially digested as alleles A and B. Lane 4: father, completely digested; lanes 5 and 6: mother, the affected child, and the newborn baby, respectively, partially digested.

LINKAGE ANALYSIS BY RESTRICTION ENZYME DIGESTION OF PCR PRODUCTS

Family 1

The proband was a 14 year old female with GSD-III diagnosed by liver biopsy. The parents asked about the possibility of diagnosing GSD-III without liver biopsy in their newborn baby. Linkage analysis using cord blood DNA of the newborn was performed. Restriction analysis of polymorphic marker 1 showed that the proband had inherited allele 2 from both parents and both parents were heterozygotes for alleles 1 and 2 (fig 4A). The newborn had the same restriction pattern as the parents, indicating carrier status. Further study of polymorphic marker 2 indicated that the baby had inherited the mutant allele from his mother and the normal allele from his father (fig 4B). The infant now aged 6 months is healthy and well, without evidence of GSD-III.

Family 2

This family requested prenatal diagnosis of GSD-III at 10 weeks of gestation after two children were born with the disease. The family was completely informative for polymorphic marker 2 (data not shown). Prenatal diagnosis using linkage analysis was therefore possible. The pregnancy, however, ended in spontaneous abortion before the scheduled amniocentesis.

Discussion

RFLPs of the GDE gene in Jewish populations have been reported.[15] All Jewish GSD-III patients of north African extraction have the same RFLP pattern suggesting the presence of a single mutation in that population. We report in this study three polymorphic markers in the GDE gene in the American population, the majority of whom were white, as well as a rapid PCR screening method for these markers. They were −10G/A in the 5′ non-translated region of exon 3, 2001+8C/T in intron 16, and 3199C/T (P1067S) in exon 25 with heterozygosity values of 0.50, 0.46, and 0.26, respectively. The heterozygosity values were similar in GSD-III patients and unrelated normal controls, indicating that there was no linkage disequilibrium between these markers and the disease.

Carrier detection for GSD-III by measuring debranching enzyme activity has been proven to be technically difficult because the assay uses limit dextrin as the substrate and is an indirect method.[12–14] On the other hand, prenatal diagnosis of GSD-III by a qualitative analysis for GDE enzyme activity or direct measurement of the enzyme activity or an immunoblot assay is possible.[13] However, the immunoblot assay cannot be offered to a family in which the proband has cross reactive material to the GDE protein. The enzyme activity assay has low sensitivity as described earlier, and the qualitative assay for GDE activity requires a large number of viable cultured amniotic fluid cells or chorionic villi with and without glucose, which takes a relatively long time for cell culture, thus affecting its usefulness. Mutation analysis

in the GDE gene is most useful for prenatal diagnosis and for carrier detection within affected families. However, the mutations we have reported so far only account for less than 30% of the total mutant alleles in GSD-III.[10 11] The polymorphic markers reported here have the advantage that they can be used for carrier detection requiring only a small number of uncultured cells for prenatal diagnosis. Three of the five GSD-III affected families studied were either completely informative or partially informative for these markers.

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