A 5' splice site mutation in fucosidosis

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Abstract

Fucosidosis is a rare, autosomal recessive, lysosomal storage disease, resulting from a deficiency of the enzyme α-L-fucosidase (EC 3.2.1.51). It is characterised clinically by progressive mental and motor deterioration, growth retardation, coarse facies, and frequent infections, but the course of the disease is variable. The gene encoding lysosomal α-L-fucosidase has been mapped to the short arm of chromosome 1 at position 1p34.1–36.1 and has been called FUCA1. Two mutations causing disease have been described previously, a C→T change in exon 8 giving rise to a premature, in frame TAA stop codon, and a deletion of at least two exons from the 3' end of the gene.

In this paper we present evidence that a homozygous G→A transition in the first position of the 5' splice site of intron 5 of FUCA1 is the disease causing mutation in a 9 year old child of distantly related parents. A new banding pattern was detected in the patient by Southern blotting of genomic DNA using TaqI restriction and a cDNA FUCA1 probe. The patient was homozygous for this pattern. Three sibs with α-fucosidase activity below the normal reference range and both parents were heterozygous. This pattern was not detected in 26 other fucosidosis patients and has not been found in any controls. The mutation was localised by a combination of restriction mapping using different cDNA probes, single stranded conformational polymorphism analysis of exons and flanking regions amplified by the polymerase chain reaction, and by direct sequencing of the amplified sequence. In view of the nature of the mutation, its cosegregation with the disease mutation and its absence in controls, it is probable that this 5' splice site mutation causes fucosidosis in this child.

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ASSAY OF LEUCOCYTE α-FUCOSIDASE
Leucocytes were isolated from heparinised blood as described previously.α-α-Fucosidase activity was measured in sonicated cell extracts by incubating 5 μl of the extract with 95 μl of H₂O and 100 μl of 1 M Tris/0.5 M NaCl solution to which 0.5 μl of 4-Methylumbelliferyl α-L-fucopyranoside in citrate-phosphate buffer, pH 4.5 (Melford Laboratories, Haverhill, Suffolk, UK), was added. The reaction was stopped by the addition of 2.5 ml of 0.25 mol/l glycine/NaOH, pH 10.4 and the fluorescence of the released 4-methylumbelliferone was read at 440 nm using an excitation wavelength of 365 nm. One unit of activity equals a rate of hydrolysis of 1 nmol per hour per mg of protein. Protein was measured using the method of Lowry et al. with human serum albumin as the standard.

α-FUCOSIDASE cDNA PROBES
Human fucosidase cDNA, encoding the major part of FUCAI, was digested with HindIII or EcoRI to generate fragments A, B, and C (fig 1). The fragments were separated by electrophoresis in agarose and the DNA extracted from the agarose using ‘GeneClean’ (BIO 101). The probes were generated by labelling the fragments by the random hexanucleotide procedure using [α-32P] dCTP (Amersham International).

SOUTHERN BLOT ANALYSIS
Genomic DNA was isolated from peripheral blood leucocytes by using the guanidinium chloride extraction procedure. DNA (10 μg) from members of the index family, 25 controls, and 26 other unrelated fucosidosis patients was digested with TaqI, BglII, or EcoRI restriction endonuclease according to the manufacturer’s instructions. The resulting fragments were separated on a 0.8 to 1% agarose gel and transferred to a nylon membrane by standard procedures. The blots were then hybridised with the radiolabelled α-fucosidase cDNA probes. Prehybridisation and hybridisations were carried out in 10× Denhardt’s solution, containing 4× SSC, 50 μg/ml denatured salmon sperm DNA, and 1% (w/v) sodium dodecyl sulphate (SDS) for 16 hours at 65°C. Filters were washed twice for 20 minutes at room temperature in 3× SSC containing 0.1% (w/v) SDS. This was followed by a higher stringency wash at 65°C for 30 minutes in 0.5× SSC containing 0.1% (w/v) SDS. The membrane was wrapped in ‘cling film’ and placed in an x ray cassette with Kodak Xomat film for 24 hours to a week.

POLYMERASE CHAIN REACTION (PCR) ANALYSIS
Exons 5 and 6 and their flanking regions were amplified by PCR using the primers in the table and fig 2. The primers were synthesised on an Applied Biosystems 381A DNA synthesiser, using DMT-biotin-C6-PA for primer 5 (Cambridge Research Biochemicals Ltd, Northwich, Cheshire, UK), according to the manufacturer’s instructions. Genomic DNA (500 ng) in a total volume of 100 μl was denatured at 94°C for 10 minutes, after which 2.5 units of Taq polymerase (Promega) were added and the mixture was held at 54°C for five minutes. This was followed by 30 cycles of one minute at 72°C, one minute at 94°C, and 30 seconds at 54°C. The amplified DNA was analysed by electrophoresis on a 1.5% agarose gel and visualised by staining with ethidium bromide; 20 μl of amplified product was digested with TaqI according to the manufacturer’s instructions (NBL, Framlington, Northumberland, UK) and analysed by electrophoresis on a 2% agarose gel followed by staining with ethidium bromide.

SINGLE STRANDED CONFORMATIONAL POLYMORPHISM (SSCP) ANALYSIS
Exons 5 and 6 and the exon 5/intron 5 boundary of the α-fucosidase gene were amplified by PCR using the primers and conditions already described. The reaction was carried out in a
volume of 50 μl using 500 ng of DNA, 25 pmol of each primer, 0.2 mmol/l dCTP, 2 mmol/l dATP, dGTP, dTTP, 0.1 μl of 40% dCTP. The amplified products were analysed by electrophoresis of 10 μl on a 1.5% agarose gel, followed by ethidium bromide staining. Five μl of the PCR product was diluted with 40 μl of 10 mmol/l EDTA/0.1% SDS and 5 μl of this was further diluted with 5 μl of gel loading buffer (80% formamide/89 mmol/l Tris/2 mmol/l EDTA/89 mmol/l boric acid, pH 8.0, with bromophenol blue and xylene cyanol added as indicator dyes). The DNA samples were denatured by heating at 95°C for three minutes and then placed on ice. Aliquots of 2.5 μl were loaded onto a non-denaturing 6% polyacrylamide gel (Protegol Lablogic) containing 5% glycerol. Electrophoresis was carried out at 4°C for 12 hours at 360 volts and then the gels were dried and exposed to x ray film without intensifying screen.

SEQEUNCING
The exon 5/intron 5 boundary was amplified by PCR using primers 5 and 6 (table, fig 2) under the conditions already described. The reaction was carried out in a volume of 100 μl containing 5 pmol of each primer, 2 mmol/l dNTPs, reaction buffer (Promega), and 500 ng of patient DNA. Fifty μl of the amplified product was mixed with 30 μl of magnetic Dynal M-280 streptavidin beads (Dynal, UK) and incubated at room temperature for five minutes and then placed in a magnetic rack. The beads with the bound double stranded amplified product were then incubated with 0.15 mol/l NaOH for five minutes to produce single stranded molecules. The biotinylated single strand attached to the beads and the unbiotinylated single strand in the supernatant were separated. Two volumes of absolute ethano
tol and 1/10 volume of 3 mol/l sodium acetate, pH 5.6, were added to the supernatant which was stored at −20°C for 12 hours and then centrifuged in a bench centrifuge for 15 minutes to recover the precipitated DNA. Water (7 μl) was added to the pellet and the DNA sequenced using the Sequenase kit (United States Biochemical Corporation) according to the manufacturer’s protocol and 5 pmol of the 5' biotinylated primer 5. The samples were heated to 85°C for two minutes before 2 μl was applied to a 6% denaturing polyacrylamide gel (Accugel, Lablogic) and electrophoresis was carried out at 55 W on a BRL (model 2) sequencing apparatus for two to two and a half hours. The gel was fixed in 10% methanol and 12% acetic acid for 30 minutes, then dried and exposed to x ray film at −70°C.

RESULTS
α-L-FUCOSIDASE ACTIVITY
The α-L-fucosidase activity in the leucocytes from members of the family is shown in fig 3. No activity could be detected in the patient (SB) consistent with the diagnosis of fucosidosis. Both parents had levels of activity at the lower end of the control reference range, and three of the five sibs had activity lower than normal. Another sib, SaB, had similar activity to her parents and one sib, DB, had normal activity.

SOUTHERN BLOT ANALYSIS
Genomic DNA was extracted from white blood cells of SB, digested with four different restriction endonucleases, and analysed by Southern blotting using probes A, A+B, B, and C. No abnormalities were seen after digestion with BglII, EcoRI, or PvuII. The normal pattern obtained with EcoRI excludes both the mutation obliterating an EcoRI site in exon 8* and the deletion at the 3' end, which is characterised by an extra junction fragment of 12 kb.2 SB was homozygous for the 12.0 kb allele of the BglII RFLP and the 7.0 kb allele of the PvuII RFLP (haplotype 1-1).16 Almost complete linkage disequilibrium has been found between these two polymorphisms in North American Caucasians but a significantly lower disequilibrium was found in Belgian Caucasians.2 The two sibs with a 3' deletion of FUCA12 also had haplotype 1-1.

When TaqI digested genomic DNA from SB was analysed using probe B, the 5.0 kb band which had been observed in all controls disappeared and an extra 1.8 kb band was observed (fig 4). SB appears to be homozygous for a new TaqI restriction site, which has not been detected in any control samples or other patients by us or others.2 The parents showed both the normal 5.0 kb and the aberrant 1.8 kb band. The new TaqI restriction site cosegregated with the fucosidosis allele in the family of SB (fig 3). The abnormal TaqI restriction pattern was not present in 26 additional unrelated fucosidosis patients, nor in the control population (n = 25). The parents and three of

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Figure 2 Primers used in the location of the new TaqI mutation.

Figure 3 Family pedigree with α-fucosidase enzyme activity as compared to TaqI Southern blot pattern of FUCA1. Normal reference range of α-fucosidase in leucocytes 30–189 nmol/h/mg.
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The 5-4 site mutation in...from the family of the patient hybridised to α-fucosidase cDNA probe B. F. father; M, mother; SB, patient; AB, DB, HB, NB, and SaB, sibs of patient; C, control.

the patient's sibs were heterozygous for this TaqI restriction pattern. The sib DB, who had the highest activity, and the sib SaB, whose activity was similar to that of her parents, were not heterozygous for the TaqI polymorphism.

LOCALISATION OF THE NEW TaqI RESTRICTION SITE
The sequence change producing the new TaqI site appears to be associated with an allele causing a deficiency of α-fucosidase. The new TaqI site results in the loss of a 5·0 kb band and the generation of a new 1·8 kb band. It was not possible to localise the new TaqI site by direct restriction mapping because the sizes of some of the intronic sequences are not known. However, it was possible to deduce which section of the gene corresponded to the 5 kb fragment by the use of probes that cover different regions of the cDNA sequence and knowledge of the sequence. The new TaqI site was not observed after hybridisation with probe A which covers exons 1, 2, and part of 3, indicating that it is not in this region of the gene. The 5·4 kb TaqI band is much less intense than other bands when probe B is used for hybridisation but of comparable intensity when probe A + B is used. The HincII site separating probes A and B is in exon 3, 5 bp upstream from the 5' end of exon 3 (fig 1). This suggests that the 5·4 kb fragment spans exon 3. The 5·4 kb fragment is unaffected in patient SB, thereby excluding exon 3 as the location of the new TaqI site. Probe C, which covers the 3' end of exon 8 and the 3' non-coding region, only hybridises to the 2·4 kb fragment. As no TaqI sites are present in exon 7, intron 7, and exon 8, according to the published sequence the 2·4 kb fragment must span exons 7 and 8. As the 2·4 kb fragment is intact in patient SB, the new TaqI site cannot be in exons 7 and 8 or intron 7. The absence of the 2·4 kb TaqI fragment in patients with the 3' deletion that includes exons 7 and 8 supports this conclusion. Exon 4 can also be excluded for the following reason. Exon 4 is only 106 bp long and there is a TaqI site 158 bp upstream. As the new TaqI site has to be 1·8 or 3·2 kb from an existing TaqI site, it cannot be in or flanking exon 4. For these reasons the 5 kb TaqI fragment in normal DNA, which is split by the new TaqI site, must span exon 5 or 6. This was investigated by amplifying exons 5 and 6 and their flanking regions from the patient and controls by PCR (fig 2) and analysing the amplified molecules for sequence differences.

ANALYSIS OF EXON 6
Primer complementary to 5' and 3' intronic sequences flanking exon 6 (primers 1 and 2 in the table and fig 2) were used to prepare amplified DNA from SB and a control. Both amplification products failed to cut with TaqI, indicating that the new TaqI site in SB is not in exon 6. Further evidence that a new TaqI site or another sequence change was not present in exon 6 of SB was obtained by SSCP analysis of the amplified products from SB and controls (fig 5A). Confirmation of the identity of the sequences of exon 6 and its flanking regions in SB and controls was obtained by direct sequencing of the amplified products and comparison with the published sequence.

ANALYSIS OF EXON 5
DNA from the patient and controls was amplified using primers specific for the 5' and 3' ends of exon 5 (primers 3 and 4 in the table and fig 2). There are no TaqI sites in the 5' and 3' ends of exon 5 covered by the primers, according to the published sequence. The amplified material from both sources consistently failed to be cut by TaqI, suggesting that the new TaqI site was not in exon 5. SSCP analysis of amplified exon 5 from SB and controls also indicated that the sequence of exon 5 was the same in the patient and controls. This was confirmed by direct sequencing of the amplified products.

ANALYSIS OF INTRON 5
To amplify across the 5' splice site of intron 5, primers were synthesised specific for the middle of exon 5 and a sequence in intron 5 (primers 5 and 6 in the table and fig 2), which should result in a 269 bp product. The amplified product from controls did not cut with TaqI, whereas the product from SB was cut into two unequal fragments (fig 6). As the exonic portion of the PCR product was 201 bp...
in length and this was not cleaved by TaqI, the new TaqI site must be in the first 68 bases of intron 5. SSCP confirmed that there was a difference in sequences of the amplified products obtained with these primers from SB and controls (fig 5B). Sequencing showed a single base transition from G to A in SB at the first base in intron 5 (fig 7). This creates a new TaqI site of TCGA at the exon 5/intron 5 boundary and is consistent with the approximate sizes of the digestion fragments obtained with TaqI from the amplified material.

Discussion

Our patient with fucosidosis does not have either of the two previously described disease causing mutations in the α-fucosidase gene, the 3′ deletion or the premature termination signal in exon 8. However, she is homozygous for a new TaqI polymorphism owing to a g→a transition at position +1 of the exon 5/intron 5′ splice site. This caused the loss of a 5-0 kb band and the appearance of a new 1-8 kb band. It is assumed that a 3-2 kb band is also generated but that it is undetectable on the blot, either because it comigrates with the 3-3 kb band, or more likely, because it is an intronic sequence and therefore not detectable with the cDNA probe. The absence of this sequence change in controls and its cosegregation with the enzyme deficiency in the family strongly suggest that it is the disease causing mutation in this child. The sequence gt is highly conserved at the 5′ splice site of mammalian introns.17-19 A mutation of the g at the +1 position of a 5′ splicing site consistently leads to abnormal processing of messenger RNA, either exon skipping or activation of a cryptic splice site.20 5′ splice site mutations have been reported in genes for several other human lysosomal enzymes. These mutations lead to an almost complete deficiency of the enzyme when they occur homozygously in patients. A g→a transition in the 5′ splice site of intron 2 of the α chain of β-hexosaminidase has been found in a compound heterozygote with Tay-Sachs disease.21 Truncated mRNA lacking exon 2 is produced along with normal length mRNA from the normal allele. One of the two major mutations responsible for Tay-Sachs disease in Ashkenazi Jews is a g→c transversion in the 5′ splice site of intron 12 of the same gene, which leads to no detectable mRNA.22-24 In metachromatic leucodystrophy, g→a transitions in the +1 5′ splice site of introns 25 and 39 are present in mutant arylsulphatase A alleles associated with the more severe, early onset, late infantile form of the disease. A g→t transversion in the +1 position of the 5′ splice site of intron 6 of α-galactosidase resulted exclusively in skipping of exon 6 in a patient with Fabry disease.25 A patient with Gaucher disease was also found to have a 5′ splice site mutation in intron 2 of the β-glucocerebrosidase gene.27

The possible consequence of a mutation in an intron 5′ splice site can be assessed semi-quantitatively by comparing the statistical estimates for the probable use of the normal and mutant splice sites.19 Based on the -2 to +6 consensus sequence for a 5′ splice site, the g→a transition in the exon 5 donor splice site of α-fucosidase would cause a decrease in the likelihood score from 86 to 68. All the other 5′ splice site mutations in lysosomal enzyme genes led to a very similar drop in the calculated likelihood score for the use of the splice site. The
strongly supports our suggestion that the mutation at the +1 position of the 5' splice site of intron 5 is the disease causing mutation in our patient. It would be expected to lead to incorrect processing of mRNA and a deficiency of functional enzyme. Unfortunately it has not been possible to study the mRNA or enzymic protein in this family.

To date only three disease causing mutations have been fully characterised in cases of fucosidosis. Therefore it is not yet possible to correlate the genotype with clinical phenotype. Like the patients with the 3' deletion or prematurity stop codon in exon 8, our patient has negligible α-fucosidase activity in her white blood cells and clinical symptoms were detected between 1 and 2 years of age. Historically fucosidosis was classified into type 1, which was characterised by a rapidly progressive neurological deterioration and death before the age of 5 years, and type 2, which had a slower progression. However, patients homozygous for the mutation in exon 8 have been classified as both type 1 and type 2 on clinical criteria. Therefore it is possible that other factors contribute to the course of the disease.

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