Null alleles of the aldolase B gene in patients with hereditary fructose intolerance

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Abstract

We report three new mutations in the gene for aldolase B that are associated with hereditary fructose intolerance (HFI). Two nonsense mutations create opal termination codons: R3op (C→T, Arg³→ter, exon 2) was found in homozygous form in four affected members of a large consanguineous Turkish pedigree and R59op (C→T, Arg⁵⁹→ter, exon 3) was found on one allele in a woman of Austrian origin known to harbour one copy of the east European mutation, N334K (Asn³³⁴→Lys). The third mutation occurred in a French HFI patient known to be heterozygous for the widespread mutation, A174D (Ala¹⁷⁴→Asp): a single mutation, G→A, in the consensus acceptor site 3' of intron 6 was found on the remaining allele. These mutations are predicted to abrogate synthesis of functional protein and thus represent null alleles of aldolase B. The mutant alleles can be readily detected in the amplification refractory mutation system (ARMS) or (for R59op and 3' intron 6) by digestion of amplified genomic fragments with *Dde*I or *Alw*NI, respectively, to facilitate direct diagnosis of HFI by molecular analysis of aldolase B genes.

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Hereditary fructose intolerance (HFI) results from a deficiency of aldolase B (fructose-bisphosphate aldolase, EC 4.1.2.13) in the liver, intestine, and kidney.¹ The disorder is inherited as an autosomal recessive trait and studies from Switzerland estimate a frequency of 1 in 20 000 live births.²

HFI is characterised by abdominal symptoms, especially vomiting, that follow ingestion of fructose and related sugars. The symptoms are accompanied by hypoglycaemia, hypophosphataemia, and acidosis.³ Affected infants usually develop a protective aversion to sweet tasting foods and drinks but persistent exposure to harmful sugars leads to growth retardation, hepatic injury, and eventually death.⁴ However, the condition responds dramatically to a sugar exclusion diet and, once recognised, is compatible with a normal life expectancy. The mainstay of diagnosis is the intravenous fructose tolerance test but this procedure, and others based on enzymatic assay of tissue biopsy samples or ³¹P magnetic resonance spectroscopy,⁵ are too cumbersome or invasive for general use in the population.

The human gene encoding aldolase B has been fully characterised: it maps to chromosome 9q21.3→q22.2 and contains nine exons, the first of which is untranslated.^{6,7} Lately, we have identified mutations in this gene that are associated with HFI and have applied these findings to the diagnosis of the condition by direct analysis of genomic DNA.⁸⁻¹¹ Hitherto, three missense mutations in the human aldolase B gene have been found to be widely distributed: the mutations A149P, (Ala¹⁴⁹→Pro, G→C exon 5⁸⁻¹⁰), A174D, (Ala¹⁷⁴→Asp, C→A exon 5¹⁰), and N334K, (Asn³³⁴→Lys, C→G exon 9¹¹) are prevalent in European patients with HFI and are thus of diagnostic significance. However, until a more complete picture of the distribution of aldolase B mutations emerges, genetic diagnosis and screening for HFI will be limited to specific populations in which the allele frequencies can be predicted.⁹ Rare mutant alleles of aldolase B can be overlooked and result in failure to diagnose the condition by this means.

We report here three previously unknown mutations that inactivate aldolase B and have been identified in patients with HFI originating respectively from eastern Turkey, Austria, and France. We also describe simple methods for mutation analysis using the polymerase chain reaction (PCR). The procedures require only minute quantities of genomic DNA to establish the presence of these disease alleles in patients in whom HFI is suspected.

Figure 1 Pedigree of a Turkish family affected by hereditary fructose intolerance showing symptomatic and asymptomatic sibs of the proband marked by an arrow. The proband's mother had suffered one stillbirth and several spontaneous abortions, as depicted. In addition, severe intolerance of fructose containing foods had been reported by two forebears, who had died in adult life, and affected several living collateral relatives. Small circles next to symbols in this pedigree indicate persons from whom DNA was obtained for aldolase B gene analysis.

Methods

PATIENTS

The diagnosis of hereditary fructose intolerance was suspected on the basis of a characteristic history of sugar intolerance and rejection of sweet tasting foodstuffs.³ These were the sole diagnostic features of disease in four members of a large kindred indigenous to the province of Mersin in southern Turkey. Several consanguineous unions were identified on investigation of this pedigree (fig 1). The mother of six surviving children (one infant had been stillborn and there had been several spontaneous abortions) had clearly identified foods which provoked vomiting with failure to thrive in four of her infants after weaning and

had adjusted their diets accordingly. Neither parent nor the two other sibs were intolerant of sweet tasting foods or fruit. A history of sugar intolerance had been obtained in several forebears and surviving cousins. In two sisters from Austria, the diagnosis of HFI was suspected on the basis of characteristic symptoms and confirmed by enzymatic assay of fructaldolases in liver biopsy samples.³ The suspected diagnosis of HFI was confirmed similarly in a female infant of French origin.

MOLECULAR ANALYSIS OF ALDOLASE B GENE

Peripheral blood leucocytes were used for isolation of DNA and the PCR was used to amplify aldolase B gene fragments containing exons and splice junctions, as described previously.¹⁰ To screen for common mutations responsible for HFI, PCR products and respective plasmid controls were blotted onto nylon membranes and probed by hybridisation to ³²P end labelled allele specific oligonucleotides. Patients with unidentified aldolase B alleles were further investigated by DNA sequencing: exon-containing amplified aldolase B fragments were separated by electrophoresis in agarose gels and, after staining with ethidium bromide and illumination under ultraviolet light,⁸ were excised for purification and double stranded DNA sequencing¹² using T₇ DNA polymerase (Sequenase^R, version 2.0, US Biochemical Corporation), according to recommendations. The primers used for sequencing were as previously described.¹⁰ In the Turkish pedigree, molecular analysis of aldolase B genes from the proband involved amplification of splice junctions and exons 4, 5, and 9 before decisive sequence information was obtained from exon 2. In the molecular analysis of the Austrian and French patients with HFI, all exons and splice junctions were amplified and sequenced.

Analysis of DNA by the ARMS¹³ for detection of the R3op nonsense mutation involved the use of the antisense primer, E2+ (GTTGTT-ATATGATGAGACTG)¹⁰ and the mutant, R3op (ACCTGTCACCATGGCCAACT) or wild type, R3+ primer (ACCTGTCACCATGGCCAACT), respectively. The expected 156 base pair product was amplified using 30 cycles in the PCR, where each cycle consisted of 94°C for one minute, 55°C for one minute, and 72°C for one minute. For restriction analysis of amplified genomic fragments containing exon 3, and intron 6 with exon 7 sequences, PCR products were digested with the endonucleases *Dde*I (Northumbria Biologicals Ltd) and *Alw*NI (New England Biolabs), respectively, according to the manufacturer's recommendations. The final products were analysed by staining after agarose gel electrophoresis.⁸

Results

The patients were investigated for the presence of the mutant alleles A149P, A174D, and N334K that are frequently associated with hereditary fructose intolerance in Europe. It was found that the proband of the Turkish

Figure 2 Direct DNA sequencing of amplified human aldolase B genes. (A) Sequence of wild type (obtained from a healthy control subject) and mutant alleles of exons 2 from the proband of the Turkish pedigree affected by fructose intolerance. In the patient, homozygosity for a C→T transition in the first base of the genomic sequence corresponding to codon 3 (R3op) was found, as shown by the G→A mutation here depicted in the antisense strand. (B) Sequence of wild type and mutant alleles of exons 3 in the proband of the Austrian pedigree affected by fructose intolerance. The patient is heterozygous for a C→T transition in the first base of the genomic sequence corresponding to codon 59 (R59op). In the figure the complementary G→A mutation is depicted in the antisense strand of DNA. (C) Sequence of wild type and mutant alleles of the intron 6/exon 7 junction site from the French patient with fructose intolerance. The patient is heterozygous for a G→A transition in the last base at the 3' end of intron 6. The sequence depicted is of the sense strand.

family had only wild type alleles at these loci, whereas the two Austrian patients and the infant from France carried one copy of the N334K and A174D alleles, respectively.

To identify aldolase B mutations in the uncharacterised alleles, individual exons and splice junction sites were amplified in the PCR and sequenced. In the proband of the Turkish family, a C→T transition in the first base of the arginine codon at position 3 in exon 2 generated the opal termination codon T(U)GA, designated R3op. Inspection of the double stranded sequencing ladder indicated that the patient was homozygous for this nonsense mutation (fig 2A).

Since the mutation neither creates nor destroys a restriction enzyme recognition site, the amplification refractory mutation system (ARMS)¹³ was used to confirm the segregation of R3op. The ARMS, which is a modification of the PCR, was carried out in the presence of an exon 2 antisense oligonucleotide primer (E2⁺) and sense primers for the wild type (R3⁺) or mutant alleles (R3op), respectively. The sense primers differ only at their 3' end by a single base, so that discriminatory amplification will occur according to the presence of the wild type or mutant sequences in the template DNA. We observed that DNA obtained from a healthy subject gave the predicted 156 base pair product with the R3⁺ primer only, indi-

cating the presence of wild type alleles at this locus (fig 3A). However, for the proband, only the R3op or mutant primer gave rise to the amplified product, confirming that this patient was homozygous for R3op. This genotype was assigned to the patient's three symptomatic sibs (fig 3A, lanes 3, 7, and 8) who gave rise to the same 156 base pair product only when the R3op primer was used in the PCR. Similar analysis of DNA from both parents and two remaining asymptomatic sibs (fig 3A, lanes 1, 2, 5, and 6) where products were obtained in the ARMS using wild type and mutant primers, indicated heterozygosity for R3op. The results showed that symptomatic disease was only associated with homozygosity for the R3op mutation and that consanguinity had led to transmission of two copies of a mutant allele of aldolase B that were identical by descent from a common ancestor.

Direct sequencing of exon 3 of aldolase B after amplification of DNA obtained from the proband of the Austrian family with HFI showed a C→T transition of the first base of arginine codon at position 59, also generating an opal termination signal, R59op (fig 2B). This nonsense mutation creates a new recognition site for the restriction endonuclease *DdeI*, thus allowing ready confirmation of its presence in genomic DNA (fig 3B). The 440 base pair fragment containing exon 3 normally encompasses a single *DdeI* site (C↓TNAG) and this serves as an internal control for the action of the endonuclease, since complete digestion of DNA that is wild type at this locus would give rise to two fragments of 328 and 112 base pairs. However, digestion of DNA from the patient produced two additional fragments of 189 and 139 base pairs generated from further cleavage of the 328 base pair fragment derived from a mutant allele containing the predicted new *DdeI* site. It had been determined earlier that the patient and her symptomatic sister had inherited one copy of the mutant allele N334K from their mother. Thus, compound heterozygosity for a new, paternally derived, R59op allele, that is, genotype N334K/R59op, was associated with symptomatic disease.

Sequence analysis of DNA encompassing intron 6 and exon 7 of the French patient with hereditary fructose intolerance showed a single base transition, G→A in the last base 3' in intron 6 (fig 2C). The 3' intronic mutation abolishes the normal recognition sequence for the endonuclease *AlwNI* (CAG↓NNNCTG) thus allowing confirmatory analysis in the PCR. Digestion of the 414 base pair fragment containing intron 6/exon 7 sequences derived from wild type alleles with *AlwNI* gives rise to two fragments of 332 and 82 base pairs (fig 3C). However, simultaneous digestion of DNA obtained from the patient with HFI showed the presence of a residual 414 bp fragment, corresponding to the undigested mutant allele. This patient was also known to carry the mutation A174D inherited from her father, and hence is heterozygous for the new G→A splice junction site lesion, with the genotype A174D/3' intron 6, G→A.

Figure 3 (A) Agarose gel electrophoresis of products of the ARMS used to detect the mutation R3op in the Turkish pedigree. Lanes 1–8, first degree family members. Lane 4, proband. Control lane shows DNA from a healthy control subject. (i) R3⁺ oligonucleotide primer. (ii) R3op oligonucleotide primer. (B) Agarose gel of *DdeI* digests of amplified exon 3 fragments of aldolase B used to detect the nonsense mutation, R59op. Lane 1, DNA obtained from a healthy control subject. Lane 2, Austrian patient with hereditary fructose intolerance. U shows undigested 440 bp exon 3 sequence after amplification in the PCR. (C) Agarose gel of *AlwNI* digests of amplified fragments of intron 6 and exon 7 of aldolase B gene used to detect the splice site mutation G→A in 3' intron 6. Lane 1, DNA obtained from a healthy control subject. Lane 2, DNA from a French patient with hereditary fructose intolerance. U shows an undigested 414 bp product after amplification in the PCR.

Discussion

In this paper we report the identification of three hitherto unknown mutations in the aldolase B gene that have been detected in patients with intolerance of fructose. The mutations, designated R3op, R59op, and 3' intron 6 [G→A], create null alleles of aldolase B, since they are predicted to prevent synthesis of the 363 amino acid polypeptide which, as a homotetramer, gives rise to catalytically active enzyme.¹⁴

R3op would allow the synthesis of a tripeptide only and R59op would give rise to a markedly shortened translation product lacking essential substrate binding residues as well as the active site lysine at position 229.¹⁵ The effects of the G→A transition in the last base of the consensus acceptor splice site in intron 6 are less predictable but the effects of a base substitution in this invariant nucleotide are likely to be drastic. Processing of heteronuclear RNA transcribed from this allele is likely to lead to the formation of aldolase B mRNA lacking exon 7 or to the activation of a cryptic 3' splice site with elongation or truncation of the exon 7 sequence that contains the catalytically essential lysine 229.¹⁶ Activation of cryptic splice sites as a result of lesions in consensus sites is usually inefficient and the abundance of any abnormal mRNA species is likely to be reduced, either because of low rates of processing or because of instability.

Other null mutations of aldolase B have been reported in patients with hereditary fructose intolerance. These include intragenic deletions of 1 bp to 1.65 kb,¹⁷ nonsense mutations,^{18,19} a splice site variant,²⁰ and a translation initiation codon mutation.¹⁹ Homozygosity for the R3op nonsense mutation, which completely prevents synthesis of aldolase B, provides evidence that aldolase B activity is not required for gluconeogenesis in patients with hereditary fructose intolerance. Characteristically, in the absence of recent fructose ingestion, these patients withstand starvation normally.³ Several patients have now been reported to have inherited two putative null alleles of aldolase B¹⁷⁻¹⁹ and, as in the patients described here, no differences in their clinical presentation or phenotype ascribable to the complete absence of enzymatic function are apparent. There is thus no evidence of adverse selection against these null mutations in the human aldolase B gene nor evidence for any evolutionary advantage that favours spread of mutations which result in synthesis of a variant enzyme with residual catalytic activity. It is interesting that, including the present findings, none of these mutations has been convincingly detected in more than one pedigree. They may thus represent private mutations confined to single pedigrees and in this sense reflect the background of spontaneous mutations that occur at this locus. A possible exception to this is the frameshift mutation L288ΔC, a single base deletion that was identified in patients from Sicily; however, its apparent restriction to three subjects from an island community raises the possibility that the lesion occurs only in one extended pedi-

gree.¹⁰ As in the case of a patient previously described with homozygosity for a 6 bp deletion¹⁷ who was a member of a large consanguineous Swiss family with HFI,²¹ local inbreeding in the Turkish pedigree carrying the mutation R3op increased the probability of inheriting two copies of a rare allele by descent from an ancestor common to both parents.

All three mutations identified here represent transitions, the most frequently observed point mutation. This is attributable to the chemical instability of cytosine, which constitutes a mutational "hot spot" in eukaryotic genomes. Unmethylated cytosines deaminate spontaneously to form uracil but this can be repaired after excision. However, in eukaryotic DNA, cytosines in CpG dinucleotides are prone to modification by DNA methylases; 5-methyl cytosines cannot be excised from the DNA strand and after deamination form thymine.²² Thymine derived from cytosines on either strand of DNA thus mispair during replication and lead to C→T or G→A transitions, as in the aldolase B mutations described in this paper.

Finally, it is noteworthy that we report here the first mutant allele of aldolase B responsible for hereditary fructose intolerance in patients originating from a community in eastern Turkey. It is our aim to improve methods for the direct diagnosis of HFI by molecular analysis of the aldolase B gene, and to date about 85% of disease alleles have been identified in patients of west European ancestry.²³ To enhance the diagnostic yield of mutation analysis in HFI, it is clearly desirable to ascertain whether other widespread alleles, such as the null alleles described here that cause aldolase B deficiency, are present. Studies of the phenylalanine hydroxylase gene in Turkish patients with phenylketonuria indicate that about 40% of the mutant alleles in this population are in linkage with a rare haplotype in northern Europe²⁴ and other mutations have yet to be defined in Turkey. Given that the rate of consanguineous marriage exceeds 20% in Turkey,²⁵ a relatively increased frequency of recessively transmitted metabolic errors such as HFI might be expected, as has been confirmed for phenylketonuria.²⁶ At present, we do not have evidence that any of the null alleles of aldolase B we have identified occur outside single pedigrees affected by HFI but the means by which they may be detected are described to facilitate further investigation of this question.

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