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Hereditary Fructose Intolerance Caused by a Nonsense Mutation of the Aldolase B Gene

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Summary

The nucleotide sequence of a patient's aldolase B gene was determined and showed a substitution of a single nucleotide (C→A) at position 720 in the coding region, which resulted in the 240th amino acid, a cysteine, being changed to a stop codon (TGC→TGA). By an allele-specific oligonucleotide probe and polymerase chain reaction, the patient was shown to be homozygous for the mutation. To examine whether this mutation causes functional defect of the enzyme, the activity of the aldolase B from the patient, expressed in *Escherichia coli* by using expression plasmid, was measured. No activity was observed, and the predicted product was recovered from *E. coli* expression plasmid, indicating that this nonsense mutation was the cause of aldolase B deficiency.

Introduction

Aldolase is a glycolytic enzyme involved in the conversion of fructose-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone-1-phosphate. There are three isozymic forms for the enzyme: aldolase A, aldolase B, and aldolase C (Horecker et al. 1972). The structural genes for these isozymes have been cloned in man (Sakakibara et al. 1985*a*, 1985*b*; Tolan and Penhoet 1986; Maire et al. 1987; Mukai et al. 1987; Rottmann et al. 1987), as well as in the rat and the chicken Burgess and Penhoet 1985; Joh et al. 1986; Kukita et al. 1988). These isozymes are well regulated developmentally and tissue specifically at the transcriptional level. These findings now permit us to study the molecular basis of hereditary diseases caused by aldolase deficiency.

In inherited deficiency of aldolase in man there is red cell aldolase deficiency, which is associated with hereditary hemolytic anemia (Miwa et al. 1981) and later identified as aldolase A deficiency by us (Kishi et al. 1987). Another type of aldolase deficiency, hereditary fructose intolerance (HFI), was discovered 30 years ago as idiosyncrasy toward fructose (Chambers and Pratt 1956). The mutation affects aldolase B of the liver, intestine, and kidney and is transmitted as an autosomal recessive disorder. It is characterized by nausea, vomiting, and hypoglycemia following fructose ingestion which rapidly evolves to cirrhosis (Gitzelmann et al. 1983).

Here we describe a molecular analysis of HFI in Japan. We found that a C→A transversion occurred at amino acid position 240 in exon 7. By using expression plasmid in *Escherichia coli,* we proved that this mutation affected directly the function of the enzyme. Cross et al. (1988, 1990) also described the analysis of HFI at the nucleotide level. However, the mutation sites differed from that given in our results. Ours is a nonsense mutation, and theirs were missense mutations at different positions.

Subjects, Material, and Methods

Subjects

The patient, a 4-mo-old Japanese girl, had vomiting, appetite loss, and abdominal fullness from the time of her birth. Tyrosinemia was suspected on the basis

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of hypertyrosinemia, hypermethioninemia, and hepatic dysfunction. She died at the age of 4 mo, and her fructose-1-phosphate aldolase (aldolase B) activity in the liver was 9% of the normal level. Her disease was determined to be HFI. She was the second child, and her younger sister was also affected by this disease. Her parents are consanguineous and have no signs or symptoms of HFI (Nakamura et al. 1978).

Material

Restriction enzymes and other enzymes were from Takara Shuzo and Nippon Gene. Radioisotope-labeled compounds were from New England Nuclear. Reagents for measuring aldolase activity were from Boehringer Mannheim. A kit for the oligonucleotide-directed in vitro mutagenesis was from Amersham. Reagents for the synthesis of the oligonucleotides were from Applied Biosystem.

Synthesis of Oligonucleotides

The oligonucleotides (17-21 mers) for primers of polymerase chain reaction (PCR), sequencing, allele-specific oligonucleotide (ASO) hybridization, and site-directed mutagenesis were synthesized on a model 380A 3 Column DNA Synthesizer (Applied Biosystem) by using the methoxy phosphoramide method. The synthesized oligonucleotides were used directly, without purification, because these short oligonucleotides were of high purity. The primers for PCR and sequencing, which are located 30–225 bp upstream or downstream of each exon are as follows: exon 1, 5′GGTAGAAAGGGGAGGGC3′; exon 2, 5′GAGCCACCCATGGTTCTGTG3′ and 5′GTTGTTATATGATGAGACTG3′; exon 3, 5′TGGCTTGCTCCTTATGCTGC3′ and 5′GACAAAGCAGAAGTGAGGTG3′; exon 4, 5′ACTAATTCAACTCCTTGATCT3′ and 5′CCTTCATTTCTAGCTTACAC3′; exon 5, 5′CCATGGATCAGGTACAAAGG3′ and 5′GGTCCATTTGTAGTTATAGT3′; exon 6, 5′CTAGGTTCTGAGGCAGCTAG3′ and 5′TTATATGTTAAGTAACAGCTG3′; exon 7, 5′AATGTGCCAAGGTCAAGTGG3′ and 5′AAAGCTTGTGGCTCTCCAAA3′; exon 8, 5′TAAGAGGTGGCAGCATC3′; exon 9, 5′CCAGTCTCCTCTCTCAT3′, 5′GAGGGCTGAAAGGGAGC3′, and 5′GTGAAACCCGATAGCAG3′. The underlined sequences were used as sequencing primers, but all the sequences (underlined or not) of exons 2–7 were also used as PCR primers.

Cloning and Sequencing of a Mutant Aldolase B Gene

DNA obtained from the liver tissue of the HFI patient (Nakamura et al. 1978) was digested to completion with *Eco*RI and was size fractionated on an agarose gel. On the basis of the restriction map of the human aldolase B gene (Mukai et al. 1987), aldolase B gene–enriched fractions of 6 kb and 9 kb were recovered, ligated to λgtWES arms, packaged, and transfected *Escherichia coli* strain LE392. Two types of clones containing exons 1 or 7–9 and located on different 6-kb *Eco*RI fragments were isolated by using a probe derived from the human aldolase B cDNA clone pHABL120-3 (Sakakibara et al. 1985*a*). These clones were subcloned into pUC13 plasmid and were sequenced with internal primers by the modified method of dideoxy chain termination (Hattori and Sakaki 1986). However, cloning of the 9-kb fragment which includes exons 2–6 was not successful. Thus, except for exon 3, exons 2–6 were sequenced directly from genomic DNA after specific amplification of those exons by PCR (Gyllenstein and Erlich 1988) using Sequenase version 2. Exon 3 was amplified by PCR, and the double-stranded DNA was subcloned into pUC13 and was sequenced by the modified method of dideoxy chain termination (Hattori and Sakaki 1986).

DNA Amplification and Dot Blotting

The PCR of exon 7 genomic DNA including the mutation site was performed for 30 cycles by using a thermocycler (Techne BiGene PHC-1) according to a method described elsewhere (Saiki et al. 1988). A sample (4 μl) of each amplified DNA in 100 μl was denatured by 0.4 M NaOH and was blotted onto GeneScreen Plus nylon membranes according to a method described elsewhere (Mullis et al. 1986). The filters were then hybridized in a solution of 5 × SSPE (1 × SSPE = 0.18 M NaCl, 10 mM sodium phosphate [pH 7.7], 1 mM EDTA), 2 × Denhardt's solution, 0.5% SDS, and 100 μg herring sperm DNA/ml, with the following ASO probes either N 240 or M 240 labeled at the 5′end: N 240 (normal probe), 5′TATACTTCTTGGTGCAG3′; and M 240 (mutant probe), 5′TATACTTCTTGGTTCAG3′. After hybridization for 4 h at 43°C, filters were washed twice in 3 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at room temperature for 15 min and then in 6 × SSC at 45°C for 5 min. The filters were autoradiographed for 6 h at −70°C by using an intensifying screen.

Construction of a Mutant Aldolase B Expression Plasmid and Its Expression in Escherichia coli

pHAB141, the original *E. coli* expression plasmid of aldolase B, had been constructed previously (Sakakibara

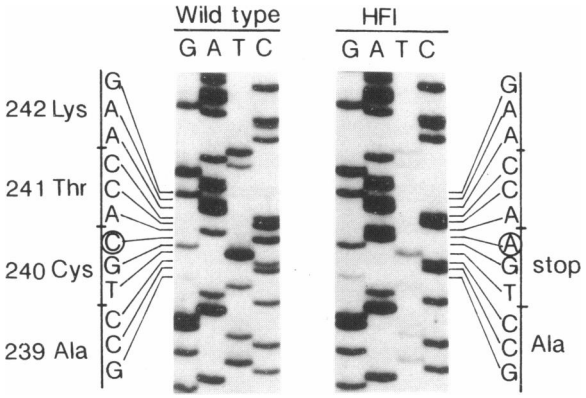

Figure 1 Comparison of the nucleotide sequence of the patient's and normal aldolase B. The nucleotide sequence was determined from a cloned lambda genomic DNA as described in Material and Methods. The amino acid sequence shown was deduced from the nucleotide sequence. *Wild Type,* Part of the nucleotide sequence from the normal aldolase B gene which was cloned previously (Mukai et al. 1987). *HFI,* Corresponding sequence of the patient's aldolase B cloned DNA. The substituted nucleotides are circled. The arrowhead indicates the substituted adenine.

et al. 1989). An *E. coli* plasmid pHAB240 carrying the same sequence as the patient's aldolase B cDNA was constructed by substituting a single nucleotide (C→A) at position 720 from the ATG initiation codon of pHAB141 by using the oligonucleotide-directed in vitro mutagenesis system (Nakamaye and Eckstein 1986) and was transfected into *E. coli* strain JM83. The substitution of the nucleotide was directly confirmed by DNA sequencing. To further ascertain that the mutagenesis occurred only in the 720th nucleotide position and not in other regions, the pHAB240 *Acc*I DNA fragment containing nucleotides from the 637th to the 729th position, a region which included the mutation site, was replaced with a same DNA piece from pHAB141. This plasmid was named pHAB141R. These transfectants were cultured overnight at 37°C and were collected. The cell pellets were suspended in buffer (25 mM Tris-HCl [pH7.5], 1 mM EDTA, 1 mM $PhMeSO_2F$) and were sonicated. Human aldolase activity in the lysates was then measured according to a method described elsewhere (Rajkumar et al. 1966). The enzyme assay was performed in the presence of 5 mM EDTA to inhibit the endogenous *E. coli* aldolase activity (Kishi et al. 1987).

Identification of the Mutant Protein by Maxicell Method

The mutant protein was identified by using the maxicell method. *Escherichia coli* strain CSR 603 (F^-, uvrA6 recA1 phr-1) carrying plasmid pHAB141, pHAB240, pHAB141R, or vector alone was grown and treated according to methods described elsewhere (Sancar et al. 1979).

Results

Nucleotide Sequence of a Mutant Aldolase B Gene

Human aldolase B gene has been cloned and sequenced (Mukai et al. 1987). It consists of nine exons interrupted by eight introns and spans 15 kb, as described previously. To know the nucleotide sequence of the patient's aldolase B gene, we have cloned the genomic DNA from the patient's lambda DNA library and have sequenced the coding exons of the aldolase B gene. When this sequence was compared with the normal counterpart, only a single base change (C→A) was found at position 720 of the ATG start codon in exon 7. This change caused the 240th amino acid, cysteine (TGC), to be the stop codon (TGA) (fig. 1). No other sequence changes were found in other exons as far as we sequenced.

Mutation Analysis of the Affected Pedigree

To determine whether the patient is homozygous for this mutation, the sequence of the exon 7 was amplified by PCR and was examined by dot blot hybridization

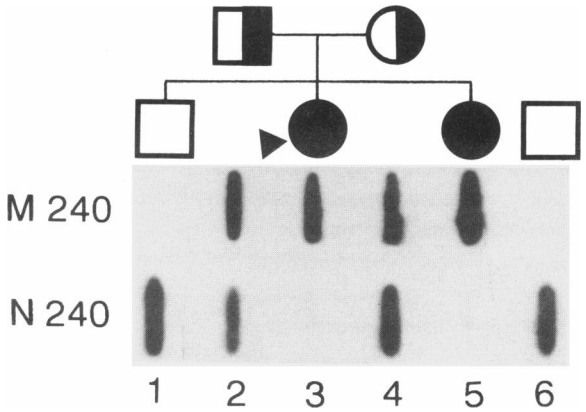

Figure 2 Genotype analysis of an affected pedigree. Exon 7 DNA of the individual was specially amplified by the PCR and hybridized with the following probes: M 240—mutant probe, 5′TATACTTCTTGGTTCAG3′; N 240—normal probe, 5′TATACTTCTTGGTGCAG3′ (lane 1, unaffected brother; lane 2, father; lane 3, propositus [arrowhead]; lane 4, mother; lane 5, affected sister; lane 6, normal unrelated individual). The parents are distant cousins. Open and solid symbols represent the normal haplotype and the mutant haplotype, respectively.

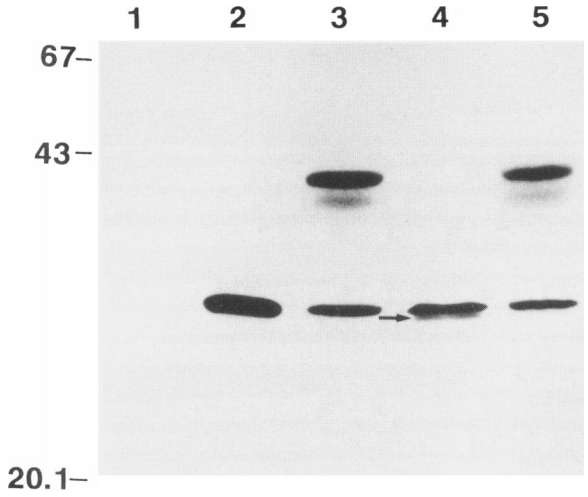

Figure 3 Identification of proteins produced by a mutant cDNA. The polypeptides encoded by plasmids were labeled with [^{35}S] methionine by using a maxicell system (Sancar et al. 1979) and were analyzed by electrophoresis on 12.5% SDS/polyacrylamide gels, followed by fluorography. The positions of BSA (67 kD), eggwhite ovalbumin (43 kD), and trypsin inhibitor (20.1 kD) are indicated. Each lane represents the product obtained from CSR603 cells harboring one of the plasmids as follows: lane 1, no plasmid; lane 2, vector plasmid alone; lane 3, pHAB141 (normal form); lane 4, pHAB240 (mutant form); lane 5, pHAB141R (resubstituted form). Mutant aldolase B protein is marked by an arrow.

with the ASO probe, N 240 (normal probe) or M 240 (mutant probe). Her family members were also examined. The N 240 probe hybridized with the genomic DNAs of her parents, her brother, and a normal unrelated individual, whereas the M 240 probe hybridized with the DNAs of the proband, her sister, and, again, her parents (fig. 2). These findings indicate that the patient and her sister are homozygous for this mutation and that the disease is transmitted in the manner characteristic for an autosomal recessive inheritance.

A Single Base Mutation Causes the Loss of Aldolase B Activity

To examine whether the nucleotide substitution in exon 7 affects the aldolase B activity, aldolase B cDNA was prepared from a normal control and from the mutant (patient) by oligonucleotide-directed in vitro mutagenesis. The DNAs were inserted into an *Escherichia coli* expression vector and were expressed in *E. coli,* as has been described elsewhere (Kishi et al. 1987; Sakakibara et al. 1989). The results indicated that the normal control, pHAB141, exhibited the class 1 aldolase activity as has been described elsewhere (Sakakibara et al. 1989), whereas no such aldolase activity was detected in the mutant, pHAB240, or in the cells carrying vector alone, unless EDTA was omitted from the assay system. The resubstituted sample, pHAB141R, showed restored enzyme activity (data not shown).

To identify the mutant aldolase B gene product, *E. coli* CSR 603 carrying pHAB240 was labeled with [^{35}S] methionine by the maxicell method, and the extract was analyzed by SDS/PAGE, as shown in figure 3. In CSR603 cells carrying pHAB141 (lane 3) or pHAB141R (lane 5), there was a distinct band with molecular weight of 40 kD, in addition to the 28-kD *amp* gene product. The 40-kD band corresponds to normal aldolase B (I. Takahashi, Y. Takasaki, T. Mukai, and K. Hori, unpublished data). In CSR603 cells carrying the mutant pHAB240 (lane 4), one specific extra band of 27 kD, instead of normal aldolase B protein, was always observed just below the 28-kD *amp* gene product. This 27-kD product was never observed either in cells having the vector alone (lane 2) or in a recombinant plasmid carrying a normal aldolase B (lanes 3 and 5). Furthermore, this molecular weight was consistent with the size estimated from the amino acid sequence of the mutant molecule. Thus, in size, this 27-kD product should correspond to the mutant aldolase B protein (fig. 3).

Discussion

We found a homozygous mutation affecting the aldolase B gene in a Japanese family having HFI. The mutations were caused by a single nucleotide substitution within the coding region of the gene. This substitution changed cysteine to a stop codon. To confirm that this mutation causes a functional defect of the aldolase B, a recombinant plasmid was constructed by expressing, in *Escherichia coli,* human aldolase B from a cDNA. No aldolase activity was observed in the mutant cDNA. However, the product of this mutant molecule was detected by using a maxicell system, as shown in figure 3. Although the minor band migrating faster than the *amp* gene product could be a breakdown product or a processed form of beta-lactamase, instead of a mutant aldolase B, we excluded these possibilities because no product was observed in the normal control which gives the same mobility as the minor band seen in the mutant (see fig. 3, lanes 3 and 4) and because the actual molecular weight of the mutant aldolase B gave a slightly smaller size (26.3 kD, as calculated from the amino acid sequence) compared with that of the beta-lactamase.

Vertebrate aldolase has three isozymic forms, A, B, and C. An X-ray crystallographic analysis revealed that rabbit muscle aldolase has a single compact domain structure (Sygusch et al. 1987). The amino acid sequences of aldolase are highly conserved through the A, B, and C forms (Kukita et al. 1988), suggesting that the three isozymic forms would have a basically similar compact molecular form. Our results in the present paper reveal that the C-terminal fragment, as large as one-third of the total length (amino acid residues 241–363), was missing in the patient's enzyme, causing a complete loss of the enzymatic activity. The inactivation would be directly attributable to the deletion of this C-terminal end of the molecule, although the defective enzyme still carries the active site Lys-229 and the C-1 phosphate-binding site arg-148 (Horecker et al. 1972) on the residual N-terminal fragment, because the region defective in the patient's enzyme is capable of forming a repetitive α-helices/β-strand structure (Tsutsumi et al. 1984) and is an essential part in the formation of the β-barrel structure which is required for catalysis (Sygusch et al. 1987).

HFI is uncommon in Japan. Only five cases from three pedigrees have been described (Nakamura et al. 1978; Shiokawa et al. 1981; Maeda et al. 1985). The majority of the cases have been reported in Europe and North America. A survey in Switzerland estimated to be the frequency of 1/20,000 and the carrier frequency to be 1% (Gitzelmann et al. 1983). In addition, the diagnosis is often mistaken as hypertyrosinemia. Thus both the detection of heterozygotes and the neonatal diagnosis of HFI patients would be important. HFI has been analyzed at the nucleotide level, and several HFI patients and heterozygotes have been detected by DNA analysis of their blood (Cross et al. 1988, 1990). By such analyses two different missense mutations and a frameshift mutation were found in more than 95% of 50 patients with HFI. It appears that the mutation reported here is another new type. The prevalence of this mutation in Japan, relative to that of the common missense mutation described in Europe and the United States, is not clear at present, because the other two pedigrees in Japan have not been examined. Although we analyzed only one pedigree, the ASO probe described here could be used to determine the genotypic frequency and might help in diagnosis and heterozygote detection.

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