Integration of PCR-Sequencing Analysis with Multiplex Ligation-Dependent Probe Amplification for Diagnosis of Hereditary Fructose

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RESEARCH REPORT

Integration of PCR-Sequencing Analysis with Multiplex Ligation-Dependent Probe Amplification for Diagnosis of Hereditary Fructose Intolerance

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Abstract Mutations in the *ALDOB* gene impair the activity of the hepatic aldolase B enzyme, causing hereditary fructose intolerance (HFI), an inherited autosomic recessive disease of carbohydrate metabolism, that can result in hypoglycemia, liver and kidney failure, coma, and death. Noninvasive diagnosis is possible by identifying mutant *ALDOB* alleles in suspected patients. We report the genetic characterization of a cohort of 18 HFI Caucasian patients, based on PCR-sequencing and Multiplex Ligation-dependent Probe Amplification (MLPA), with the identification of two novel genetic lesions: a small duplication c.940_941dupT (p.Trp314fsX22) and a large deletion encompassing the promoter region and exon 1. MLPA and long range-PCR (LR-PCR) also identified the recently reported g.7840_14288del6448 allele with a surprisingly high frequency (11%) within our patients' cohort. The most common p.Ala150Pro (44%), p.Ala175Asp (19%), p.Asn335Lys (8%), and/or the known c.360-363del4 (5%), p.Tyr204X (2.8%), IVS6 −2A>G (2.8%) mutant alleles were identified in 14 patients at a homozygous or compound-heterozygous level. The integration of PCR-sequencing analysis with exon-dosage tools [MLPA and quantitative fluorescent multiplex-PCR (QFM-PCR)] led to the full genotyping of patients within our cohort and to the identification of the new deletion encompassing the promoter region and exon 1.

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Introduction

Hereditary fructose intolerance (HFI) (EC 4.1.2.13; OMIM ID: 229600) is a rare (1 in 20,000 births, Steinman et al. 2001) recessive inherited disorder of carbohydrate metabolism, caused by catalytic deficiency of the aldolase B enzyme (ALDOB; EC 4.1.2.13). The ALDOB enzyme plays a key role in glycolysis and gluconeogenesis and, in mammals, is preferentially expressed in the liver. HFI patients manifest hypoglycemia, lactic acidosis, and gastrointestinal symptoms, such as severe abdominal pain and recurrent vomiting after consuming fructose-containing foods. HFI usually presents in infancy at the time of weaning, when fructose is added to the diet. Persistent ingestion of fructose and related sugars (such as sucrose and sorbitol) can lead to severe liver and kidney damage, seizures, coma, and risk of death (Alì et al. 1993). Patients who achieve adulthood develop a natural aversion to fruit and sweets (Alì et al. 1998; Steinman et al. 2001). Early diagnosis is essential, as patients can live a symptom-free life by avoiding sweet foods containing fructose, including

certain pharmacological preparations such as syrups, which contain problematic sugars.

Noninvasive diagnosis is possible using genomic DNA sequencing analysis by the detection of genetic lesions in the *ALDOB* gene (Alì et al. 1998), mapping on chromosome 9q. *ALDOB* consists of nine exons encoding a polypeptide containing 364 amino acids (Tolan and Penhoet 1986). At present, 56 *ALDOB* disease-causing mutations have been identified (Human Gene Mutation Database (HGMD), http://www.hgmd.cf.ac.uk/ac/index.php), but the severity of the disease appears not to be dependent upon the nature of the *ALDOB* mutation (Davit-Spraul et al. 2008). The mutated p.Ala150Pro allele (Cross et al. 1988) is the most frequent (Alì et al. 1998) and the frequency of heterozygous carriers has been estimated to be $1.32 \pm 0.49\%$ in the United Kingdom (James et al. 1996). p.Ala175Asp (Cross et al. 1990a) and p.Asn335Lys (Cross et al. 1990b) are also frequent *ALDOB* mutations. Together with the p.Ala150Pro, they account for about 72–76% of HFI mutated alleles worldwide (Santer et al. 2005; Tolan 1995; Cross et al. 1990a, b). Population screening has demonstrated that HFI patients are very frequently (94%) heterozygous for at least one of the three common mutations p.Ala150Pro, p.Ala175Asp, and p.Asn335Lys (Santer et al. 2005). The occurrence of these three genotypes in the same family with recurrent HFI has also been reported (Caciotti et al. 2008). Other population studies have estimated high frequencies of the p.Ala150Pro (50%) and p.Ala175Asp (30%) mutations in Italy (Santamaria et al. 1993; Sebastio et al. 1991), and it has been estimated that seven mutations account for 95% of HFI alleles in Italian patients (Esposito et al. 2004).

Herein we report two new *ALDOB* genetic lesions and the successful use of multiplex ligation-dependent probe amplification (MLPA) in the molecular diagnostic of HFI. We also report the allele frequencies of *ALDOB* mutations that we detected in 18 newly diagnosed HFI patients.

Materials and Methods

Patients

We examined a cohort of 18 Caucasian patients with phenotypic manifestations of the HFI clinical spectrum (Table 1). Their parents were also evaluated in order to confirm the heterozygous carrier status. Informed consent was obtained from all patients in accordance with local ethics committee recommendations.

Table 1 HFI patients' data and clinical manifestations

Patient	Pt1	Pt2	Pt3	Pt3's brother	Pt4	Pt5
Age at diagnosis	7 years	2 years	36 years	46 years	3 years and 9 months	6 months (weaning)
Age at present	11 years	30 years	37 years	47 years	6 years	1 year and 8 months
Origin	Albania	Italy	Italy	Italy	Italy	Italy
Growth	Normal	Normal	Lower limit of normal (tenth percentile)	Lower limit of normal (tenth percentile)	Normal	Normal
Age of presentation and symptoms[a]	5 months, aversion to fruit and sugar	6 months, aversion to fruit and sugar	Aversion to fruit and sugar	Aversion to fruit and sugar	Aversion to fruit and sugar, hypoglycemia	6 months, aversion to fruit and sugar, hypoglycemia
Gastrointestinal abnormalities	Diarrhea	Vomiting	Vomiting, diarrhea	Vomiting	–	Vomiting after fructose ingestion
Laboratory findings	Hyp-trans[b]	Hyp-trans[b]	Hyp-trans[b]	No	Hyp-trans[b]	Hyp-trans[b], slightly increased CK levels
Aversion to any source of fructose	Yes	Yes	Yes	Yes	Yes	Yes
Therapy	Diet	Diet	Diet	Diet	Diet	Diet
Other	Hepatic steatosis	Hepatic steatosis	Crohn's disease at 34 years, hepatic steatosis	Osteoporosis, hepatic steatosis	Hepatic steatosis	Hepatic steatosis

[a] When presenting, symptoms have followed sweets and/or fruit ingestion

[b] Hypertransaminasemia

Analysis of Genomic DNA

Genomic DNA was isolated using the EZ1 DNA Blood 350 µl Kit (QIAGEN, Hilden, Germany). The three *ALDOB* fragments which encompass exons 5, 8, and 9 including the p.Ala150Pro, p.Ala175Asp, and p.Asn335Lys frequent mutations were amplified and sequenced. In patients without common mutations or with only one mutant allele, molecular analysis was extended to the entire coding region. Oligonucleotides and PCR conditions are available on request.

Nucleotide positions were named on the basis of *ALDOB* reference sequence NG_012387.1 and HGVS standard nomenclature (http://www.hgvs.org/mutnomen). p.Ala150Pro, p.Ala175Asp, p.Asn335Lys, and p.Tyr204X correspond to mutations p.Ala149Pro, p.Ala174Asp, p.Asn334Lys, and p.Tyr203X, respectively, in HGMD nomenclature.

Multiplex Ligation-Dependent Probe Amplification Analysis

MLPA was carried out using the SALSA MLPA KIT P255 ALDOB (MRC Holland, Amsterdam, The Netherlands), which contains 16 control probes, and 11 probes specific to the *ALDOB* gene, 1 probe for each exon and 2 probes that reveal the p.Ala150Pro and p.Ala174Asp mutations. Reactions were carried out as reported (www.mlpa.com) and confirmed at least in duplicate. Electrophoresis was performed with an ABI 3130XL sequencer (Applied Biosystems, Carlsbad, CA). For each sample, each peak area was normalized by dividing it by the combined area of all peaks in that sample. Normalized areas were then divided by the average normalized peak area from at least three normal controls. Values obtained indicate the allele copy numbers compared with normal controls (Hogervorst et al. 2003). Values of 0.7–1.3 were assumed as normal and <0.7 as deletions. False-positives were excluded by DNA sequence analysis of probe hybridization sites.

Multiplex-PCR Analysis to Confirm Homozygous Large Deletion

Combinations of PCR-fragments: exons 2 + 8; exons 3 + 7; exons 4 + 8; exons 5 + 8; and exons 6 + 8 were successfully assayed. Products were checked by agarose gel electrophoresis using 2% Agarose Low Melting (Eurobio, France) + 1% Standard Agarose (AB Analitica, Italy).

Breakpoint Determination

The breakpoint junctions of the large gene rearrangement encompassing exons 2–6 were amplified by long range-PCR (LR-PCR) using the Platinum Taq, DNA polymerase High Fidelity (Invitrogen, USA) and directly sequenced. PCR and sequencing conditions are available on request.

Quantitative Fluorescent Multiplex-PCR of Exons 1 and 2

ALDOB exons 1 and 2 were amplified in one multiplex reaction, including the internal quantitative control FECH8 (Dobrovolny et al. 2011). *ALDOB* exon 1 produces a fragment of 249 bp, *ALDOB* exon 2 of 319 bp, and FECH8 of 235 bp. PCR and sequencing conditions are available on request. PCR products were run on an ABI 3130XL sequencer (Applied Biosystems, Carlsbad, CA). For each sample, the peak areas of each *ALDOB* exon were normalized by dividing them by the peak area of the internal control FECH8. Normalized areas were then divided by the average normalized peak area from at least three normal controls. Values obtained were interpreted as described for MLPA. Validation of the assay was performed by analyzing 11 control DNAs and the genomic DNAs of the homozygous and heterozygous known *ALDOB* large deletion g.7840_14288del6448 that involves exon 2.

Results

ALDOB Mutated Alleles Identified in Our HFI Cohort and Their Distribution

The three most common mutations p.Ala150Pro, p.Ala175Asp, and p.Asn335Lys had a prevalence of 44, 19, and 8%, respectively, in agreement with the literature (Alì et al. 1998; Sebastio et al. 1991). Other known mutated alleles were found with minor frequencies, 5% for c. 360_363del4 (Dazzo and Tolan 1990), 2.8% for p.Tyr204X (Alì et al. 1993), and 2.8% for IVS6 −2A>G (Esposito et al. 2004). Five remarkable cases led to the identification of two new mutations (c.940_941dupT and a large deletion encompassing the promoter region and exon 1) and of the recently reported g.7840_14288del6448 large deletion (Esposito et al. 2010), which was found at a high frequency in our cohort (11%). Allele combinations identified in our cohort and their relative frequencies are reported in Table 2.

The New Mutated Allele c.940_941dupT

Molecular analysis of an Albanian proband (Pt1, Table 1) identified, at a heterozygous level, the new mutation c.940_941dupT in exon 8, combined with the recurrent mutation p.Ala175Asp. In silico analysis revealed that this new mutation causes a frameshift leading to the lesion

Table 2 *ALDOB* allele combinations identified in our cohort ($N = 18$)

Allele combinations	Number of patients	Frequency (%)
p.Ala150Pro/p.Ala150Pro	5	28
p.Ala175Asp/p.Ala175Asp	2	11
c.360_363del4/p.Ala150Pro	2	11
p.Ala150Pro/p.Ala175Asp	1	5
p.Ala150Pro/p.Asn335Lys	1	5
p.Asn335Lys/p.Asn335Lys	1	5
p.Thr204X/IVS6 −2A>G	1	5
Remarkable cases		
c.940_941dupT/p.Ala175Asp	1	5
delEx1/p.Ala150Pro	1	5
g.7840_14288del6448/ g.7840_14288del6448	1	5
g.7840_14288del6448/p.Ala175Asp	1	5
g.7840_14288del6448/p.Ala150Pro	1	5

p.Trp314fsX22 at the protein level. Molecular analysis of the patient's relatives identified heterozygous c.940_941dupT in his father and heterozygous p.Ala175Asp in his mother and sister.

A New Large Deletion Encompassing the Promoter Region and Exon 1

The Italian patient Pt2 (Table 1) had previously received biochemical diagnosis of HFI after liver biopsy. DNA sequencing of the entire *ALDOB* gene coding sequence and of the fragment containing the promoter region and exon 1 (Coffee and Tolan 2010) only revealed the p.Ala150Pro mutation at a heterozygous status. MLPA analysis identified a deletion involving the region containing the promoter and exon 1 at the heterozygous status (Fig. 1b, lane 1). Direct sequencing and MLPA analysis of the patient's daughter led to the identification of the heterozygous p.Ala150Pro (Fig. 1b, lane 2), indicating that the two mutations were *in trans* in the genome of the proband. Since the p.Ala150Pro mutation is very frequent, we excluded the possibility that the carrier status of the daughter was inherited from the mother by also analyzing her mother at the *ALDOB* molecular level. Amplification of the *ALDOB* exon 1 and the promoter region with the specific primers (Coffee and Tolan 2010) did not identify genetic lesions in the proband, excluding an interference by an SNP within the annealing region of specific MLPA Probes. LR-PCR amplification with two different forward primers annealing one upstream and one in the promoter region and a reverse primer annealing in intron 2 from patient's DNA did not allow the determination of the exact breakpoint because the resulting amplicon was not different from the controls (data not shown). To confirm this new *ALDOB* gene rearrangement, we developed a specific quantitative fluorescent multiplex-PCR (QFM-PCR) assay for the dosage of *ALDOB* exons 1 and 2. Data obtained confirmed the MLPA data (Table 3).

Homozygous g.7840_14288del6448 Large Deletion

Molecular analysis of an Italian proband (Pt3, Table 1) revealed the large homozygous deletion g.7840_14288del6448 spanning exons 2–6, then confirmed by multiplex PCRs (data not shown). Molecular analysis of the patient's brother, who also shows classic HFI symptoms (Table 1), identified the same *ALDOB* genotype. MLPA analysis, performed on the DNAs of the proband, his brother and their parents, confirmed the homozygous deletion in the brother and showed the heterozygous level of the consanguineous parents (Fig. 1c). Since MLPA indicated that exon 1 was not involved, LR-PCR and sequencing analysis identified the g.7840_14288del6448 deletion. LR-PCR amplification produced an amplicon of 3,915 bp corresponding to the mutated allele, which is easily distinguishable from the WT of 10,364 bp, thus confirming the heterozygous status of the patient's parents.

Heterozygous g.7840_14288del6448 Large Deletion

Molecular analysis of a fourth Italian family (proband Pt4, Table 1) identified the p.Ala175Asp mutation that was seemingly at a homozygous level. This finding, however, was not confirmed by molecular analysis of the proband's parents' DNA. p.Ala175Asp was carried only by the father and was absent in the mother. MLPA and LR-PCR analysis revealed the g.7840_14288del6448 large deletion in the DNAs of the proband and her mother (Fig. 1d). An analogous condition was found in a fifth Italian family (Pt5, Table 1) where molecular analysis of the proband revealed the apparently homozygous p.Ala150Pro, which was not confirmed by analysis of parents' DNA. LR-PCR amplification and sequencing identified the heterozygous g.7840_14288del6448 in the DNAs of the proband and his father.

Discussion

Molecular analysis of the *ALDOB* gene sequence is the least invasive diagnostic tool available for rapid confirmation of HFI in suspected patients. From a large cohort of patients suspected of HFI, we found 18 to be carrying different genotypes. Only 2 of 18 patients didn't carry any common allele. However, only 10/18 (55%) carried combinations of the three common mutations detectable

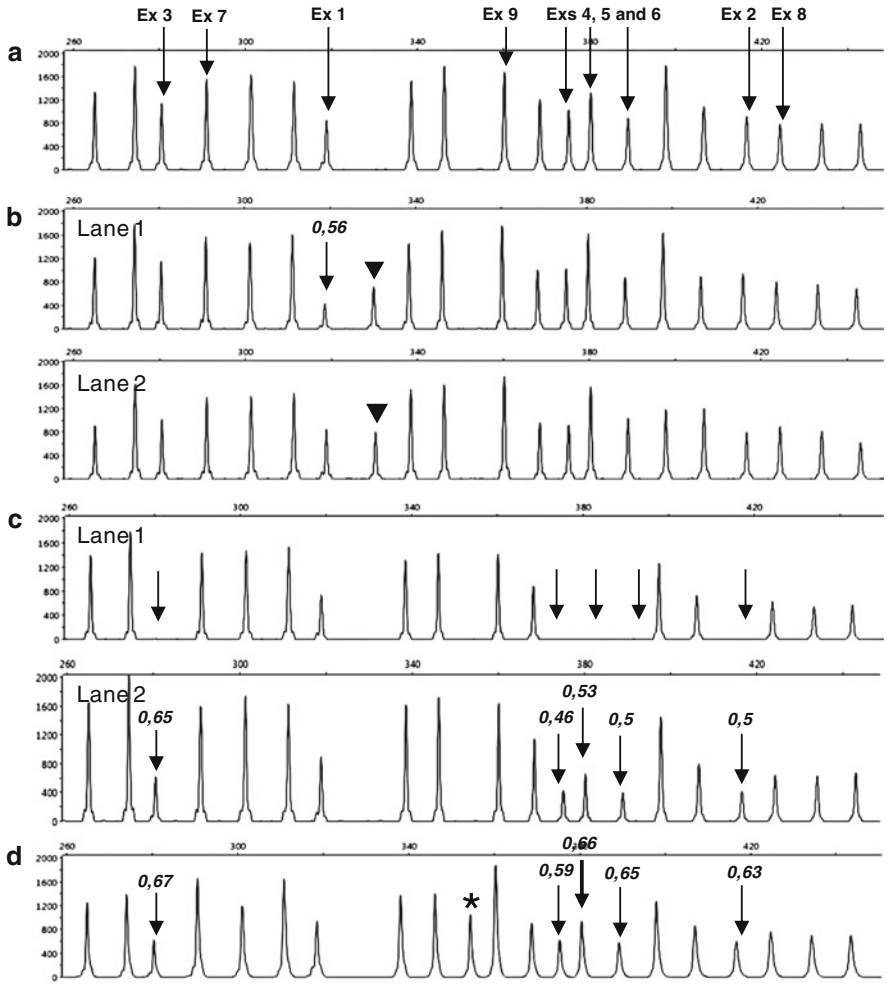

Fig. 1 Detection of *ALDOB* alterations by MLPA. *x*-axis: fragment size; *y*-axis: fluorescence intensity. Probe mix contains 22 probes and 9 of them recognize the *ALDOB* exons. Two probes are specific to the common mutations p.Ala150Pro (indicated by the *triangle*) and p.Ala175Asp (indicated by the *star*). These probes only generate a signal for samples containing such mutations. (**a**) Control sample. (**b**) *Lane 1* represents Pt2 profile. The *arrow* indicates a decreased peak area, corresponding to the heterozygous deletion of exon 1. Pt2's daughter (*lane 2*) inherited the p.Ala150Pro allele. (**c**) *Lane 1* illustrates the Pt3 homozygous deletion of exons 2–6 as the corresponding peaks are absent. (**c**) *Lane 2* and (**d**) represent the profiles of Pt3's father and Pt4 that show heterozygous deletion of exons 2–6. Normalized relative peak area ratios that indicate deletions are shown

Table 3 QFM-PCR results

Patient	Fluorescence ratios[a]	
	Exon 1	Exon 2
Pt2	0.54	1.12
Pt2's daughter	1.19	1.16
Pt3	1.11	0
Pt3's father	1.14	0.55
Pt3's mother	1.12	0.53

[a] Fluorescence ratios are calculated as indicated in the "Materials and Methods" section and are the average of two experiments

with the routinely sequence analysis, while 8/18 (45%) carried, at least, one not common allele. Concerning the last eight patients the sequencing analysis was extended to the entire coding region of ALDOB gene of all of them. Such approach led to the diagnosis in 4 of them (4/18; 22%), while in the other 4 (22%) the diagnosis was made by integration of molecular analysis with MLPA and/or QF-PCR and/or LR-PCR. These methods have also confirmed their parents' carrier status.

We identified a new mutation consisting of a duplication of a T in exon 8 (c.940_941dupT) that alters the *ALDOB* coding region leading to the p.Trp314fsX22 protein alteration. It was combined with the p.Ala175Asp in an

Albanian family in which recessive inheritance was confirmed by molecular analysis of the patient's parents.

Molecular analysis also revealed a high prevalence, within our cohort, of the recently reported g.7840_14288del6448 *ALDOB* allele, which carries a large deletion spanning exons 2–6. The incidence of this new emerging allele in our patient cohort (11%) was higher than that of the common p.Asn335Lys mutation (8%). As previously reported (Esposito et al. 2010), we also found such deletion in patients with Italian origin, thus a founder effect cannot be excluded. However, it is important to screen for this allele in patients with apparently homozygous *ALDOB* mutations in exons 2–6, when one parent does not carry the expected mutation, in cases where parents' DNAs are not available for analysis and in heterozygous HFI patients for whom the second mutation has not been identified.

We used MLPA to investigate *ALDOB* gene rearrangements and it was proved here to be very efficient in detecting the g.7840_14288del6448, also at a heterozygous level.

MLPA analysis allowed us to complete the genetic analysis in Patient 2 in whom PCR-sequencing analysis had revealed only the p.Ala150Pro mutation at a heterozygous level. The second mutated allele consists of a new *ALDOB* deletion that involves the promoter region and exon 1. The deletion was confirmed by specific QFM-PCR assay for the dosage of *ALDOB* exons 1 and 2 that we developed and validated successfully on the DNAs of the homozygous and heterozygous large deletion, g.7840_14288del6448. *ALDOB* exon 1 is a noncoding exon that is supposed to be involved in the regulation of *ALDOB* mRNA translation (Coffee and Tolan 2010) and mutations in this region are reported as causing HFI (Coffee and Tolan 2010). Thus, *ALDOB* gene expression in our patient could be altered by this mutation both at the transcription-regulation level and at the protein level.

Our data indicate the importance of extending sequence analysis to the entire coding sequence and intron/exon boundaries of the *ALDOB* gene of suspected HFI patients, in accordance with recent reports (Esposito et al. 2010, Coffee and Tolan 2010). The heterozygous status or the mutation negative status for the most common mutations do not exclude the affected status as such patients should be further investigated at the molecular level. To this purpose, PCR-sequencing analysis should be extended to the entire coding sequence. Moreover, the *ALDOB* MLPA test improves detection rates.

This report demonstrates the importance of screening the *ALDOB* gene of suspected patients, especially infants, since a fructose (and related sugars)-free diet can prevent the potentially serious manifestations of the disease.

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Synopsis

The here combined exon-dosage MLPA and QFM-PCR tools ensured that two new *ALDOB* mutations were detected and that a known deletion emerged with a surprisingly high frequency.

Conflict of Interest

Nothing to declare.

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