

## RESEARCH ARTICLE

# Splicing Mutations, Mainly IVS6-1(G > T), Account for 70% of Fumarylacetoacetate Hydrolase (FAH) Gene Alterations, Including 7 Novel Mutations, in a Survey of 29 Tyrosinemia Type I Patients

J.A. Arranz,<sup>1\*</sup> E. Piñol,<sup>1</sup> L. Kozak,<sup>2</sup> C. Pérez-Cerdá,<sup>3</sup> B. Cormand,<sup>4</sup> M. Ugarte,<sup>3</sup> and E. Riudor<sup>1</sup>

<sup>1</sup>Unitat de Malalties Neurometabòliques, Hospital Materno-Infantil Vall d'Hebron, Barcelona, Spain; <sup>2</sup>Research Institute of Child Health, Department of Biochemical and Molecular Genetics, Brno, Czech Republic; <sup>3</sup>Centro de Diagnóstico de Enfermedades Moleculares, Universidad Autónoma de Madrid, Cantoblanco, Spain; <sup>4</sup>Departament de Genètica, Universitat de Barcelona, Barcelona, Spain

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Hereditary tyrosinemia type I (HTI) is an autosomal recessive disease characterized by a deficiency in fumarylacetoacetate hydrolase (FAH) activity. In this work, the FAH genotype was established in a group of 29 HTI patients, most of them from the Mediterranean area. We identified seven novel mutations—IVS8-1(G > A, IVS10-2(A > T), 938delC, E6/I6del26, W78X, Q328X, and G343W—and two previously described mutations—IVS6-1(G > T) and IVS12 + 5(G > A). Fully 92.8% of the patients were carriers of at least one splice site mutation, with IVS6-1(G > T) accounting for 58.9% of the total number of alleles. The splice mutation group of patients showed heterogeneous phenotypic patterns ranging from acute forms with severe liver malfunction to chronic forms with renal manifestations and slow progressive hepatic alterations. Qualitative FAH cDNA expression was the same in all IVS6-1(G > T) homozygous patients regardless of their clinical picture. One patient with a heterozygous combination of a nonsense (Q328X) and a frameshift (938delC) mutation showed an atypical clinical picture of hypotonia and repeated infections. Despite the high prevalence of IVS12+5(G > A) in the northwestern European population, we found only two patients with this mutation in our group. *Hum Mutat* 20:180–188, 2002. © 2002 Wiley-Liss, Inc.

KEY WORDS: FAH; fumarylacetoacetate hydrolase; SSCP; tyrosinemia, hereditary, type I; HTI; HTI; splice site; genotype–phenotype

DATABASES:

FAH – OMIM: 276700; GenBank: GI182392, NM\_000137 (cDNA)

## INTRODUCTION

Hereditary tyrosinemia type I (HTI or HTI, MIM# 276700) is an autosomal recessive disease characterized by a deficiency in fumarylacetoacetate hydrolase (FAH; E.C. 3.7.1.2) activity, catalyzing the last step in the tyrosine catabolic pathway [Lindblad et al., 1977]. It is a rare disorder, distributed worldwide, with an approximate incidence of 1:100,000. This figure may be higher in specific regions as a consequence of endogamic habits (carrier frequencies up to 1:20) or in isolated populations with a strong founder effect (e.g., the Sanguenay-Lac-St-Jean region in Quebec) [Grompe et al., 1994].

The phenotypic spectrum is heterogeneous [Mitchell et al., 2001], ranging from acute forms with early onset of some days to a few months and presenting

with severe liver malfunction proceeding frequently to death, to chronic forms mainly with renal manifestations and slow progressive hepatic alterations. Intermediate forms are frequent. Neurologic porphyria-like crises also appear in some patients. Patients with all forms have a high risk of developing hepatocarcinoma. Patients with the same genotype can present with very

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\*Correspondence to: J.A. Arranz, Unitat de Malalties Neurometabòliques, Hospital Materno-Infantil Vall d'Hebron, Ps. Vall d'Hebron 119-129, 08035 Barcelona, Spain.

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different clinical pictures, suggesting that epigenetic or environmental factors could play an important role in modulating the phenotype in HTI [Poudrier et al., 1998]. The relationship between phenotype and genotype is not clear and remains to be elucidated. A specific treatment (NTBC) has been developed and applied in most well-controlled patients, resulting in a dramatic improvement in the symptomatology [Holme and Lindstedt, 1998]. The long-term outcome provided by this treatment is still an open issue.

The human *FAH* gene has been localized to chromosome 15q23-q25; it spans approximately 35 Kb, contains 14 exons, and encodes a protein of 420 amino acids [Phaneuf et al., 1991]. *FAH* is mainly expressed in the liver and kidney although a basal level of expression has been detected in most other tissues.

Up to now, 33 different mutations resulting in HTI have been reported in the *FAH* gene [St-Louis and Tanguay, 1997; Bergman et al., 1998]. These include missense, nonsense, and splice-site mutations. Two different splice-site mutations are particularly prevalent in northwestern Europe and in the French origin population of Canada [IVS12+5(G>A)], and in the Mediterranean area [IVS6-1(G>T)]. Both mutations cause severe qualitative and quantitative mRNA expression alterations (deletions of regions or whole exons, misincorporation of intronic segments, etc.) that lead to defective *FAH* activity. Some authors have described different mRNA patterns produced by the same splice-site defect in different patients [Ploos van Amstel et al., 1996; Rootwelt et al., 1994].

In this work, a panel of 29 HTI patients, most of them from the Mediterranean area, were studied to identify the disease-causing mutations in the *FAH* gene. We investigated the cDNA patterns of several patients with different clinical phenotypes but the same genotype, particularly those with splice-site mutations, in an attempt to gain insight into the genotype-phenotype relationship.

## SUBJECTS AND METHODS

### Subjects

Mutation analysis was carried out in 29 HTI patients and 30 healthy individuals selected from laboratory staff volunteers and non-tyrosinemic patients. Diagnostic criteria included detection of succinyl-acetone and determination of porphobilinogen-synthase activity in erythrocytes and/or fumarylacetoacetate-hydrolase activity in fibroblasts. All patients were from the Mediterranean area (Spain: 25, Italy: one, and Morocco: one) except for two from central Europe (Brno, Czech Republic). Eight Spanish patients were from the Romany ethnic group (gypsies) whose genealogies showed a high degree of consanguinity. Additionally, 35 close relatives were screened to detect carrier status and to perform genetic counseling in some families.

None of the studied patients corresponded to the Mediterranean patients included in the study by Bergman et al.

[1998]. The two Czech patients had been previously screened for mutations IVS12+5(G>A), E357X, and E364X [Grompe et al., 1994].

Proband histories are summarized in Table 1.

### Mutation Screening Strategy

When available, RNA samples of the patients were used to screen for splicing abnormalities. These alterations were characterized by reverse transcription and polymerase chain reaction (RT-PCR) and subsequent sequencing of the resulting cDNA products. The splice site mutations were identified at the DNA level by PCR amplification of the suspected exon and its intronic flanks and direct sequencing of the purified product. The remaining exons were subsequently analyzed in patients with incomplete HTI genotypes, starting with exons 7, 8, and 12, where previous studies had reported a high proportion of changes [St-Louis and Tanguay, 1997; Bergman et al., 1998]. Restriction and SSCP analyses were performed to verify the mutations identified and for screening purposes in the remaining patients and relatives.

In addition, control DNA samples were sequenced to verify the wild-type sequence (Genebank accession numbers: L14646-L14670 for exons and intronic flanks, and M55150/GI182392 for cDNA) and possible polymorphic changes.

Informed consent was obtained from proband parents for the study. The study was approved by the local ethical committee.

### DNA Amplification and Sequencing

High-molecular-weight DNA was isolated from peripheral blood leukocytes, liver samples, and cultured fibroblasts of the probands according to standard commercial procedures (QIAamp mini/midi kit, Qiagen, Hilden, Germany). Exons 1–14 of the *FAH* gene and their flanking intronic sequences were amplified using the polymerase chain reaction. The amplification primer sequences used have been previously described [Ploos van Amstel et al., 1996]. The PCR mix consisted of 100 ng of genomic DNA, 20 pmol of each primer, 1.50 mM dNTPs, 5  $\mu$ L of 10 $\times$  amplification buffer (1.5 mM final MgCl<sub>2</sub> included), and 2 U of *Taq* DNA polymerase (all from Boehringer Mannheim, Mannheim, Germany) in a final volume of 50  $\mu$ L. The amplification protocol consisted of an initial step of 10 min at 95°C followed by 33 cycles of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C, and an additional extension step at 72°C for 15 min. The amplified products were visualized by electrophoresis on 1.5–2% agarose, then purified and directly sequenced by the dideoxy-chain termination method with a dye-terminator sequencing Kit (PE-Applied Biosystems, Foster City, CA).

### SSCP Analysis

We used the SSCP technique to screen some PCR-products of exons 6, 7, and 11 for the E6/I6del26, IVS6-1(G>T), and IVS10-2(A>T) mutations, respectively, and also to screen the other exons in some patients with unidentified mutations. It was performed according to standardized commercial protocols (Pharmacia, Uppsala, Sweden). Appropriate negative (wild-type) and positive (known mutation) controls were run in parallel with the samples under screening.

### Restriction Analysis

Several mutations were confirmed by restriction analysis of PCR products and agarose gel electrophoresis, following the supplier's instructions (Boehringer Mannheim, Mannheim,

TABLE 1. Origin, Clinical Findings, Biochemical Analysis, and Genotypes of the Patients

Case	Origin ethnic group	Phenotype/form	Treatment/outcome	FAH activity <sup>a</sup> $\mu\text{mol}/\text{min/g}$ protein	Mutation
HT-1	Eastern Spain/ Gypsy	Chronic/renal	Diet up to 7 years; Diet + NTBC from 7 years/GOOD	n.a.	IVS6-1 (G>T)/IVS6-1 (G>T)
HT-2	Eastern Spain/ Gypsy	Acute/hepatorenal	Diet + NTBC/ GOOD	n.a.	IVS6-1 (G>T)/IVS6-1 (G>T)
HT-3	Eastern Spain/ Gypsy (Brother of HT-2)	Asymptomatic (neonatal diagnosis and treatment)	Diet + NTBC/ GOOD	n.a.	IVS6-1 (G>T)/IVS6-1 (G>T)
HT-4	Eastern Spain/ Gypsy	Acute/hepatorenal	Diet/EXITUS	0.03 <sup>b</sup>	IVS6-1 (G>T)/IVS6-1 (G>T)
HT-5	Eastern Spain/ Gypsy	Acute/hepatorenal	Diet + NTBC/ GOOD	n.a.	IVS6-1 (G>T)/IVS6-1 (G>T)
HT-8	Eastern Spain/ Gypsy	Intermediate/hepatic	Diet + NTBC/ GOOD	n.a.	IVS6-1 (G>T)/IVS6-1 (G>T)
HT-23	Eastern Spain/ Gypsy	Acute/hepatorenal	Diet + NTBC/ GOOD	n.a.	IVS6-1 (G>T)/IVS6-1 (G>T)
HT-6	Eastern Spain/ Moroccan	Acute/hepatorenal	Diet + NTBC/ GOOD	n.a.	IVS6-1 (G>T)/IVS6-1 (G>T)
HT-7	Northern Spain	Acute/hepatorenal	Diet + Transplant/ GOOD	n.a.	IVS6-1 (G>T)/IVS6-1 (G>T)
HT-14	Central Spain	Acute/hepatic	Diet + transplant/ EXITUS	0.31 <sup>c</sup>	IVS6-1 (G>T)/IVS6-1 (G>T)
HT-19	Northern Spain	Acute/hepatorenal	Diet + transplant/ GOOD	0.26 <sup>c</sup>	IVS6-1 (G>T)/IVS6-1 (G>T)
HT-28	Northern Spain	Acute/hepatorenal	Diet + transplant	n.a.	IVS6-1 (G>T)/IVS6-1 (G>T)
HT-29	Northern Spain/ Gypsy	Acute/hepatorenal	-/EXITUS	n.a.	IVS6-1 (G>T)/IVS6-1 (G>T)
HT-15	Central Spain	Acute/hepatorenal	-/EXIUS	0.14 <sup>c</sup>	IVS6-1 (G>T)/233G>A (W78X)
HT-25	Central Spain	Acute/hepatorenal	Diet/EXITUS	n.a.	IVS6-1 (G>T)/233G>A (W78X) <sup>d</sup>
HT-16	Central/Spain	Acute/hepatorenal	Diet + transplant/ GOOD	0.25 <sup>c</sup>	IVS6-1 (G>T)/1027G>T (G343W)
HT-17	Northern Spain	Chronic/Renal	Diet + transplant/ GOOD	0.21 <sup>c</sup>	IVS6-1 (G>T)/1027G>T (G343W)
HT-18	Central Spain	Acute/hepatorenal	-/EXITUS	0.17 <sup>c</sup>	IVS6-1 (G>T)/982C>T (Q328X)
HT-9	Brno-(Czech Rep.)	Chronic/hepatorenal	Diet/GOOD	0.08	IVS6-1 (G>T)/E6/16del26 <sup>d</sup>
HT-10	Brno-(Czech Rep.)	Chronic/hepatic	Diet/GOOD	0.09	IVS6-1 (G>T) <sup>f</sup>
HT-11	Italian	Acute/hepatorenal	Diet + NT BC/ <sup>e</sup>	n.a.	IVS6-1 (G>T) <sup>f</sup>
HT-26	Central Spain	Acute/hepatorenal	Diet/EXITUS	0.21 <sup>c</sup>	IVS6-1 (G>T) <sup>f</sup>
HT-27	Southern Spain	Acute/hepatorenal	Diet + transplant	n.a.	233G>A (W78X)/233G>A (W78X)
HT-12	Southern Spain	Hypotonia, repeated infections	Diet + NTBC/GOOD	n.a.	982C>T/938delC <sup>d</sup>
HT-22	Northwestern Spain	Acute/hepatic	Diet + retransplant/ GOOD	0.17 <sup>c</sup>	IVS12 + 5(G>A)/IVS12 + 5(G>A)
HT-24	Central Spain	Acute/hepatorenal	-/EXITUS	n.a.	IVS8-1(G>A)/IVS8-1(G>A)
HT-21	Eastern Spain	Acute/hepatorenal	Diet + NTBC/ GOOD	0.14 <sup>c</sup>	IVS8-1(G>A) <sup>f</sup>
HT-13	Southern Spain	Acute/hepatic	Diet + transplant/ GOOD	0.5 <sup>b</sup>	IVS10-2(A>T) <sup>d,f</sup>
HT-20	Eastern Spain	Intermediate/ hepatorenal	Diet + NTBC/ GOOD	0.19 <sup>c</sup>	IVS12 + 5(G>A) <sup>f</sup>

<sup>a</sup>Normal range: 1.3–3.1.

<sup>b</sup>Dr. E Holme, Sahlgren's Hospital, Gotheborg, Sweden.

<sup>c</sup>Dr. R. Berger, Wilhelmina Children's Hospital, Utrecht, The Netherlands.

<sup>d</sup>Mutation segregation in parents were verified.

<sup>e</sup>After diagnosis and starting treatment with NTBC, the patient developed hepatocarcinoma. Chemotherapy was applied with remission of the hepatocarcinoma and metastasis.

<sup>f</sup>No mutation identified so far.

n.a., not available.

Germany). The length of the fragments resulting from the digestion of the normal and the mutated PCR products for each mutation are indicated in Table 2.

### Reverse Transcription/PCR

Blood or tissue lysates were prepared from 5–10 mL of whole blood, small liver biopsy samples, or cultured fibroblast pellets using a protective solution (RNA/DNA Stabilization Kit for Blood/Bone Marrow, Boehringer Mannheim, Mannheim, Germany). The mRNA fraction was isolated with the help of the mRNA Isolation Kit for Blood/Bone Marrow

(Boehringer Mannheim, Mannheim, Germany). Reverse transcription PCR was performed using primers FAHm1f (5'-CTCTCCGCACGCCACCTTAG-3') and FAHm14r (5'-AGGGAGGACCCCAGTCACAG-3') in a single tube using the Titan one-tube RT/PCR kit (Boehringer Mannheim, Mannheim, Germany). A second PCR was performed by using nested primers FAHm1fi (5'-CAGCCGTGCCGGGTGCTCTT-3') and FAHm14ri (5'-GTGCTTGTGCCCCTTTGTTTC-3') over 33 cycles, using one-twentieth of the first RT-PCR reaction.

The PCR products were visualized, purified, and sequenced as described above. The calculation of splicing scores and the

TABLE 2. Distribution and Frequency of Mutations Found in the FAH Gene in 29 HTI Patients

Mutation	Effect	Region	Number of alleles/ frequency (%)	Restriction enzyme detection (normal/ mutant, bp)	First description
IVS6-1 (G>T)	Splice	I6/E7	33/58.9%	- <i>Cel</i> II (102 + 73/175)	Timmers and Grompe [1996]
IVS8-1 (G>A)	Splice	I8/E9	3/5.4%	- <i>Pst</i> I (42 + 84 + 150/ 42 + 234)	This report
IVS12 + 5 (G>A)	Splice	E12/I12	3/5.4%		Kvittingen et al. [1993]
IVS10-2 (A>T)	Splice	I10/E11	1/1.8%		This report
938delC	Frameshift + stop	E11	1/1.8%		This report
E6/I6del26	Splice	E6/I6	1/1.8%	+ <i>Mva</i> I / + <i>Eco</i> RII (64 + 161/62 + 137)	This report
del(548-553) + (553 + 20)				- <i>Mva</i> I / - <i>Eco</i> RII (14 + 22 + 51 + 71 + 94/ 22 + 65 + 71 + 94)	
233G>A (W78X)	Stop	E3	4/7.1%	- <i>Pst</i> I (27 + 33 + 24 + 129/ 27 + 33 + 153)	This report
982C>T (Q328X)	Stop	E12	2/3.6%	- <i>Msp</i> I (4 + 36 + 52 + 121/ 4 + 52 + 157)	This report
1027G>T (G343W)	Gly → Trp	E12	2/3.6%		This report
Not identified			6/10.7%		

Nucleotide numbering starts at the A of the first ATG (GenBank G1182392).

search for consensus sites were aided by Alex's Splice Site Finder program, version 0.4 ([www.genet.sickkids.on.ca](http://www.genet.sickkids.on.ca)).

## RESULTS

### Mutation Analysis of Genomic DNA

The results of the mutation analysis are depicted in Tables 1 and 2. In the 29 HTI patients, we found nine different alterations, seven of which were new. The most prevalent mutation in our series was IVS6-1(G>T), which alters a consensus acceptor splice site in intron 6. This change was detected in 21 of 28 patients (33/56 alleles), either at homozygosity or heterozygosity (Patient HT3, brother of HT2, was not included in the statistics). In addition, six novel mutations were found: two splice-site mutations (IVS8-1(G>A) and IVS10-2(A>T)); two nonsense transitions (982C>T (Q328X) and 233G>A (W78X)); one missense transversion (1027G>T) resulting in a glycine to tryptophan substitution at codon 343 (G343W); one point deletion (938delC) causing a frameshift and introducing a premature stop codon 60 codons downstream; and a 26-nt deletion spanning the last six nt of exon 6 and the first 20 nt of intron 6 (E6/I6del26). The mutation IVS12+5 (G>A), prevalent in other populations, was found only twice in our series of patients. After extensive mutation analysis, six alleles remained with unknown alterations. Figure 1 shows the restriction analysis of the newly described mutations. Figure 2 shows the sequence analysis of mutation F6/I6del26.

The segregation of the disease alleles within the families was verified in several cases, IVS10-2(A>T) (Patient HT13), IVS8-1(G>A) (Patient HT21),

938delC (Patient HT12), E6/I6del26 (Patient HT9), 233G>A (Patient HT25; no parents samples available for Patients HT15 and HT27), and 982C>T (Patient HT12; no parents samples available for Patient HT18), excluding hemizyosity in patients apparently homozygous for a given mutation (data not shown except for Patient HT9 in Fig. 1E and 1F, Patient HT13 in Fig. 1G, and Patient HT21 in Fig. 3B).

The presence of the identified changes in healthy individuals was ruled out by sequence analysis of more than 70 wild-type chromosomes from 30 control volunteers and from several healthy relatives.

### RNA Analysis by RT-PCR

To determine the effect of the splice-site mutations at the RNA level, reverse transcription and PCR was performed on mRNA samples from peripheral lymphocytes, fibroblasts, and liver and kidney (depending on availability in each patient) using FAH-specific primers. Control mRNA was isolated from several tissues: lymphocytes, fibroblasts, liver, and chorionic villi. Owing to the low expression level of FAH in lymphocytes and fibroblasts, a second nested amplification was required. The control samples yielded the same cDNA pattern in every tissue assayed.

Those patients bearing only missense mutations or point deletions showed the same 1323-nt cDNA band as found in the control samples. Sequencing of four overlapping RT-PCR fragments spanning the entire coding region of the FAH gene

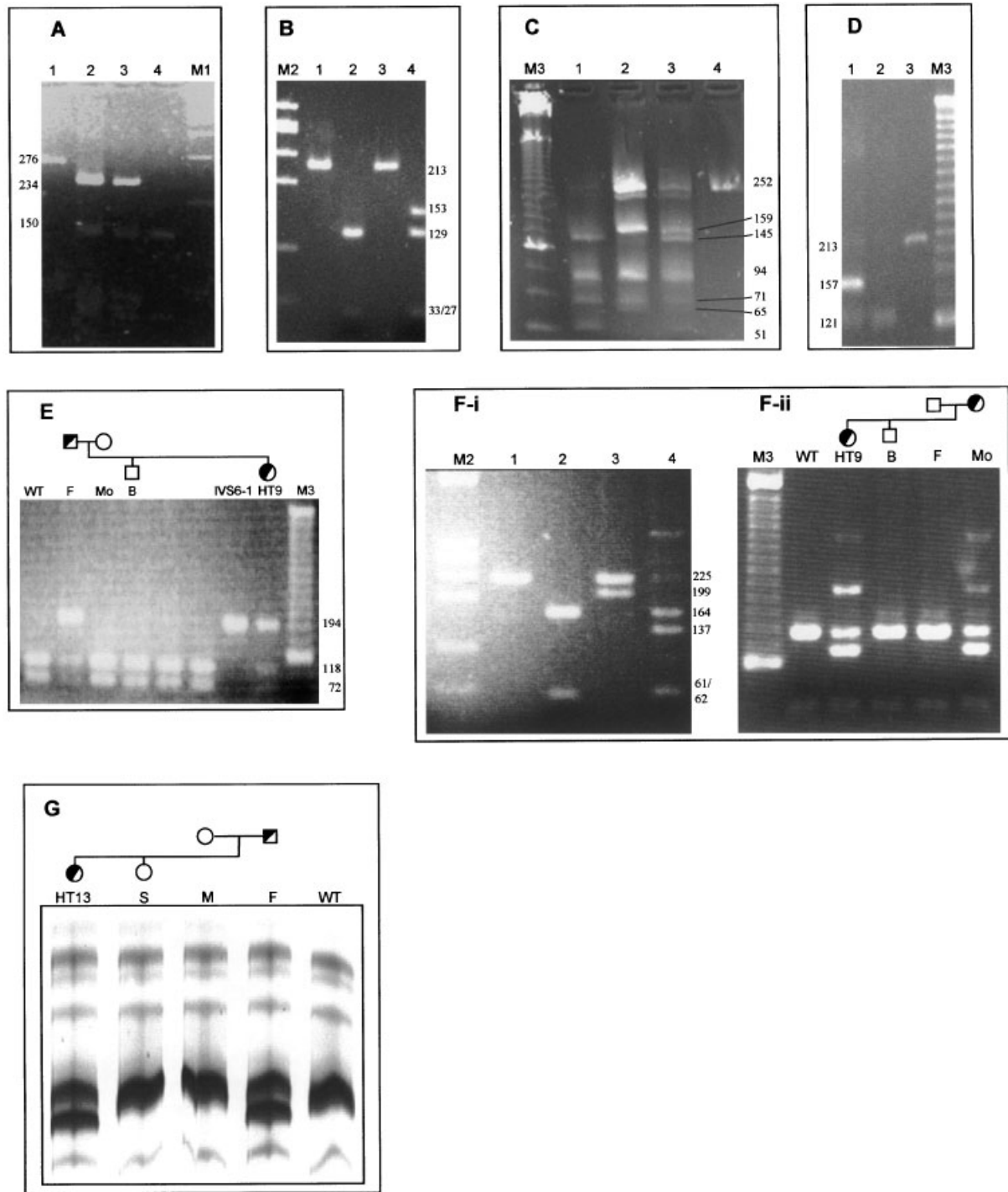


FIGURE 1. Detection of new mutations by PCR amplification of genomic DNA and restriction analysis. M1: ladder 100 bp. M2:  $\phi$ X174-*Hae*III. M3: ladder 25 bp. Side numbers indicate sizes of DNA fragments in bp. A: IVS8-1(g>a) mutation analyzed by *Pst*I digestion of exon 9; lane 1: undigested PCR product; lanes 2–3: heterozygous subjects, HT21 and his mother; lane 4: healthy control. B: Q328X mutation detected by *Pst*I digestion of exon 12; lanes 1 and 3: undigested PCR products from a healthy control and a heterozygous patient, respectively; lanes 2 and 4: the same samples after digestion. C: Analysis of W78X mutation by *Eco*RII digestion of exon 3; lane 1: healthy control; lane 2: homozygous patient HT27; lane 3: heterozygous patient HT15; lane 4: undigested product. D: Mutation G343W detected by *Msp*I digestion of exon 12; lane 1: heterozygous patient; lane 2: healthy control; lane 3: undigested product. E: Segregation of mutation IVS6-1(g>t) in the family of Patient HT9, by *Cel*II digestion; lane 1: wild-type (WT) control; lane 2: father (F); lane 3: mother (M); lane 4: brother (B); lane 7: homozygous control patient for IVS6-1(g>t); lane 8: Patient HT9; lanes 5–6: two other patients who do not bear this mutation. F-I: Detection of E6/I6del26 by *Mva*I digestion; lane 1: undigested healthy control; lane 2: the same sample after digestion; lane 3: undigested patient HT9; lane 4: Patient HT9 after digestion. F-ii: Familial study of mutation E6/I6del26 by *Mva*I digestion: lane 2: healthy control (WT); lane 3: Patient HT9; lane 4: brother (B); lane 5: father (F); lane 6: mother (M). G: Familial study of the IVS10-2(a>t) mutation by SSCP analysis; lane 1: Patient HT13; lane 2: sister (S); lane 3: mother (M); lane 4: father (F); lane 5: healthy control (WT).

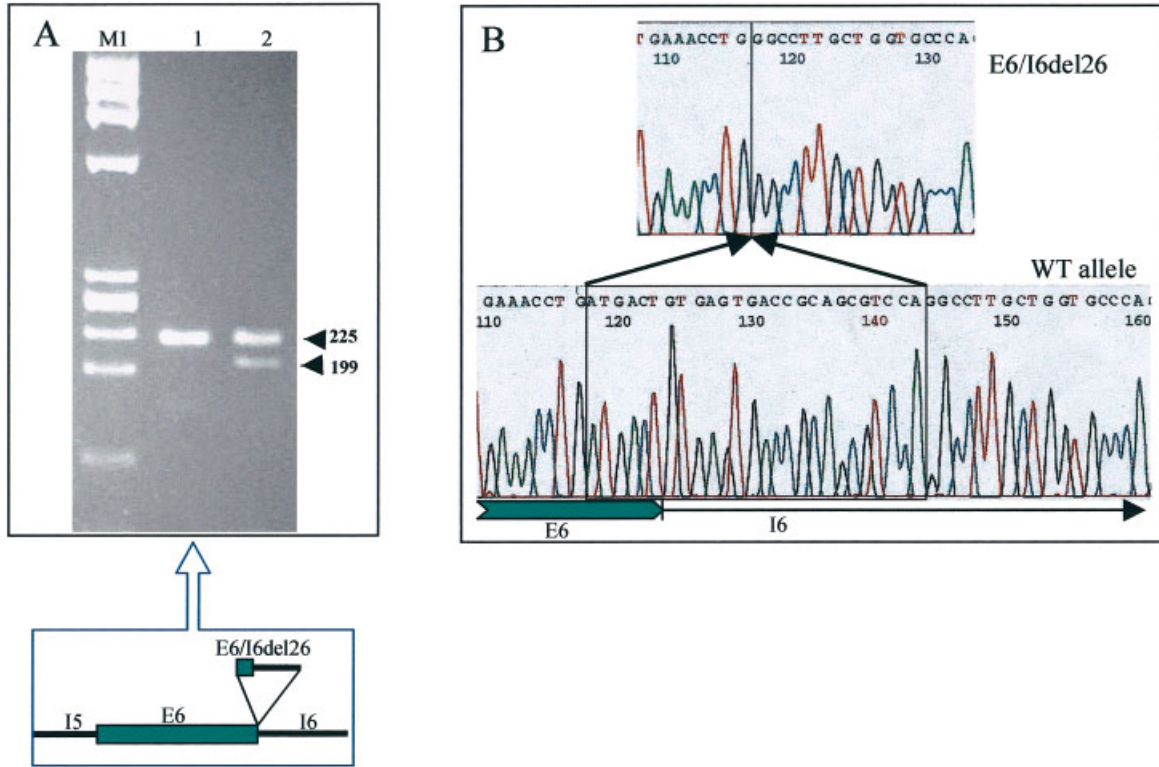


FIGURE 2. Genomic DNA analysis of mutation E6/I6del26 in Patient HT9. **A:** PCR amplification of exon 6 and flanking intronic sequences; lane 1: healthy control; lane 2: Patient HT9; M1:  $\phi$ X174 DNA-*Hae*III. Below, schematic representation of the deleted region spanning the last six nt of exon 6 and the first 20 nt of intron 6. **B:** Sequence analysis of the exon 6/intron 6 PCR product in the patient E6/I6del26 allele and in a WT allele.

allowed confirmation of the mutations (data not shown).

The RT-PCR results of the IVS6-1(G>T) mutation in homozygous patients showed a complex pattern with three distinct bands. Sequence analysis revealed that all three lacked the first five nucleotides of exon 7; additionally, the first 13 nucleotides of exon 8 were lost in one band and all of exon 8 was skipped in another, in agreement with previous reports [Bergman et al., 1998]. This pattern was the same in all the patients homozygous for this mutation regardless of the tissue of origin of the mRNA sample (lymphocytes, fibroblasts, or liver) (see Fig. 3 for the cDNA patterns of the splicing mutations).

In Patient HT21, a compound heterozygote for mutation IVS8-1(G>A) and a second, still unidentified alteration, the cDNA pattern consisted of a highly predominant 1,100-bp band lacking exons 8 and 9, and a very faint 1,200-bp band (Fig. 3A), both smaller than the expected 1,323-nt normal band. The RT-PCR analysis of the patient's parents showed that each of them bore one of these two bands (Fig. 3B), indicating that the second mutation in this patient may also involve a splice site. The results in Patient HT22, homozygous for the IVS12+5(G>A) mutation, showed a complex pattern of several bands,

currently under study, compatible with the results previously described by other authors [Rootwelt et al., 1994]: an upper band, longer than the control, corresponding to the insertion of 105 nt of intron 11, and two lower bands, smaller than the control, one lacking E12 and the other lacking E12 + E13. We have encountered a third band of approximately 900 bp that needs further characterization. Patient HT20 bears the IVS12+5(G>A) in one allele (the other mutation has not been identified so far) and her abnormal RT-PCR pattern (Fig. 3A), under study, could be interpreted as another variant of the patterns observed in that mutation: it shows a main band of ~1,200 bp compatible with the skipping of exon 12 [Rootwelt et al., 1994], and a very slight band of ~1,100 bp compatible with the skipping of both exon 12 and 13. There are also a lower band of ~1,000 bp and a diffuse smear area between 1,200 and 1,400 bp that remain unexplained. We do not know if the other undetected mutation may play some influence on this pattern.

**Genotype-Phenotype Correlation**

A heterogeneous spectrum of clinical manifestations was observed in the group of 13 patients homozygous for the IVS6-1(G>T) mutation, ranging from a chronic renal phenotype without important

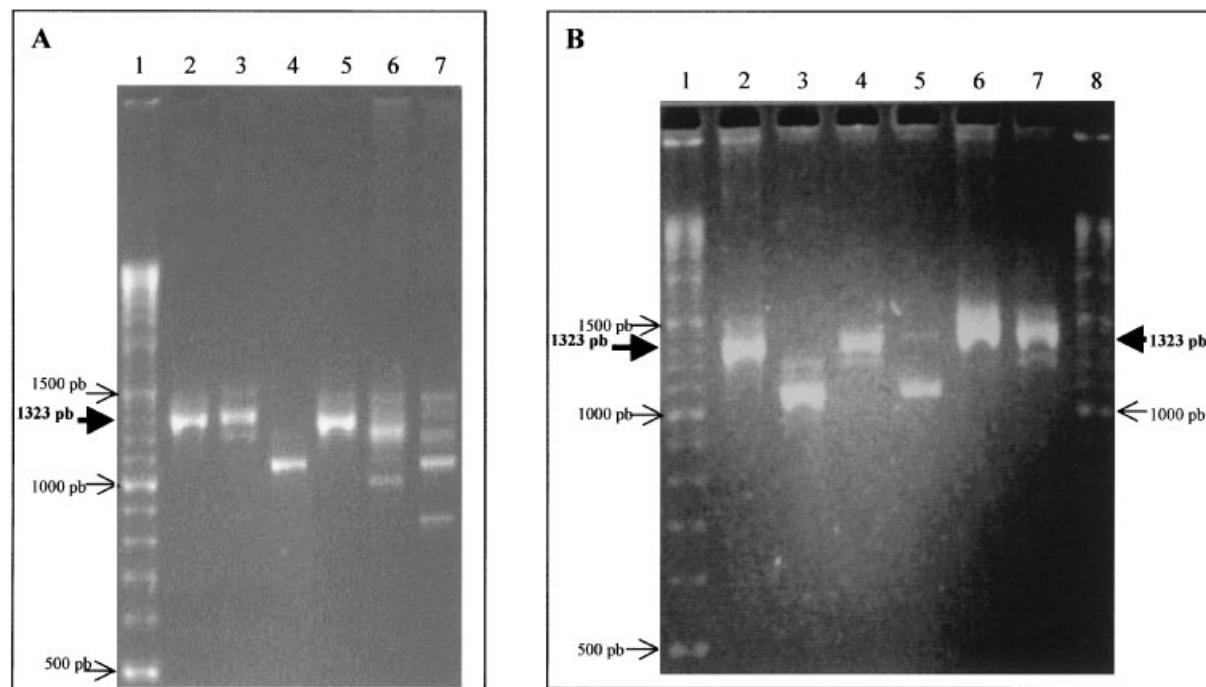


FIGURE 3. Electrophoregrams showing different cDNA patterns in several tyrosinemic patients and some relatives carrying splicing mutations and other alterations, obtained by RT-PCR of the entire coding region. **A:** Lane 1: Molecular weight marker (ladder 100 bp); lane 2: healthy control; lane 3: Patient HT14, homozygous for the IVS6-1(G>T) mutation; lane 4: Patient HT21, heterozygous for the IVS8-1(G>A) mutation; lane 5: Patient HT12, 982C>T (G343W)/938delC; lane 6: Patient HT20, heterozygous for IVS12+5G>A / unknown alteration; lane 7: Patient HT22, homozygous for the IVS12+5(G>A) mutation. **B:** Segregation of mutation IVS8-1(G>A) in the parents of Patient HT21; lanes 1 and 8: molecular weight marker (ladder 100 bp); lanes 2 and 6: cDNA from wild-type controls; lane 7: cDNA from an homozygous IVS6-1(G>T) (used here for comparison); lane 3: cDNA from Patient HT21; lane 4: cDNA from his father; lane 5: cDNA from his mother.

hepatic symptoms to acute liver failure in the first months. Qualitative mRNA expression did not clarify the discrepancies, as all these patients showed the same pattern. In addition, no phenotype differences were observed when these patients were compared with the group showing compound heterozygosity for the IVS6-1(G>T) and a stop mutation in the other allele (Patients HT15, HT18, and HT25). Interestingly, Patient HT9, compound heterozygous for mutations IVS6-1(G>T) and E6/I6del26, presented with a relatively mild clinical phenotype. She is now 20 years old, has suffered from renal manifestations without hepatic or neurological crises, has never been treated with NTBC, and is only under dietary treatment.

Only one patient (HT12), compound heterozygous for a stop and a frameshift mutation (982C>T/938delC), presented with a clearly different clinical picture. He showed mostly neurological manifestations (hypotonia and some pain crises), which correlated well with his very high levels (720 mmol/mol creat. Normal: 0–3) of 5-aminolevulinic acid (5-ALA) at diagnosis. This metabolite never normalized (but substantially decreased to 15–30 mmol/mol creat) five years after the start of NTBC treatment and specific diet. Other toxic metabolites did reach

the normal range, particularly succinyl-acetone (SA) from 450 to <1 mmol/mol creat a few months after the start of NTBC. Clinical symptoms also disappeared soon.

Patient HT13, with a severe clinical picture, conserved a relatively high residual FAH activity (23% of mean control) and only trace levels of succinyl-acetone in urine (see Table 1). The absence of correlation between this metabolite and liver damage raised doubts about the pathogenic mechanisms in this patient [Riudor et al., 1991]. The patient presented the IVS10-2(A>T) mutation, which alters the consensus acceptor site for intron 10, while the other allele mutation remains unknown. RNA material was not available for study.

## DISCUSSION

### Mutation Analysis and Genotype-Phenotype Correlation

The results of mutation analysis in 29 HTI patients mainly from the Mediterranean area show a high prevalence (59%) of the IVS6-1(G>T) allele. These data concur with the analysis of Bergman et al. [1998] in 25 patients of several ethnic origins. Our data may be influenced to some degree by the fact that several

patients homozygous for the IVS6-1(G>T) mutation were Romanians, with demonstrated consanguinity in their genealogies. Nevertheless, this mutation continues to be the most widely represented in our area (45.2% of the alleles and 66.7% of the patients) when Romanians are not included. This result allows the design of screening strategies in the Mediterranean area based on direct mutation analysis. Interestingly, the two Czech patients studied here were heterozygous for the IVS6-1(G>T) mutation. Several Dutch patients reported by Ploos van Amstel et al. [1996] were also carriers of this splice-site mutation. In contrast, we found only two patients with the IVS12+5(G>A) allele, which is prevalent in north-western European populations. The patient HT22, homozygous for the mutation, comes from north-western Spain, raising the possibility of a Celtic connection with other northwestern European patients. The Dutch studies [Ploos van Amstel et al., 1996; Bergman et al., 1998] also failed to detect this mutation in non-northwestern European patients. On the basis of these data, molecular screening protocols based on the analysis of mutations IVS6-1(G>T) and IVS12+5(G>A) may allow the identification of the majority of alleles in HTI patients of European origin. The rest of the mutations identified in this study (missense or nonsense) were present in only one or two families.

Our studies did not allow the establishment of clear genotype-phenotype correlations. One mechanism that has been proposed to contribute to phenotype variability in HTI patients is the self-induced reversion of mutations [Kvittingen et al., 1994]. This would generate a mosaic pattern in the liver, with immunoreactive nodules to FAH antibodies having a selective growth advantage over non-reacting nodules [Overturf et al., 1996]. It has been suggested that the clinical variation observed in patients is caused by the magnitude of the reversal events and/or by the timing at which reversion occurred during fetal liver development [Poudrier et al., 1998]. These findings have been described in some patients with a very mild clinical phenotype [Kim et al., 2000] but continued presence of the biochemical hallmarks the disease.

### Molecular Characterization of the Mutations

The cDNA pattern associated with the IVS6-1(G>T) mutation, the most prevalent in our panel of patients, can be explained in part by the presence of two cryptic splice acceptor sites 5 nt downstream of exon 7 and 13 nt downstream of exon 8. The mutation increases the informative value of the exon 7 cryptic site from 73.2 to 79.0 and decreases the value of the natural site from 82.2 to 66.2. Surprisingly, the value of the cryptic acceptor site in exon 8 is higher (87.6) than that of the natural site (77.1). Thus, the mutation, located in the intron 6-exon 7

junction, also alters the recognition systems of the exon 8 boundaries (e.g., skipping or activation of a cryptic site).

Seven novel mutations were identified (see Table 2), as described above. Owing to their intrinsic characteristics, all are probably disease-causing mutations; two mutations, 233G>A(W78X) and 982C>T(Q328X), generate stop codons that may lead either to truncated protein products or, more likely, to the degradation of the corresponding mRNA transcript by nonsense-mediated mRNA decay (NMD) surveillance mechanisms [see Culbertson, 1999 for a review]. Mutation IVS10-2(A>T) alters the consensus AG-dinucleotide of the splice acceptor site (from score 96.4 to 80.4) of intron 10 and probably (not studied yet) modifies the correct splicing of exon 11. Mutation IVS8-1(G>A) alters the splice acceptor site of intron 8, causing skipping of exons 8 and 9 in the mature mRNA. The reading frame is conserved but the lost regions (amino acids 203–279) include essential regions within the active site of the enzyme where other previously described pathogenic mutations are situated [Timm et al., 1999]. A similar mutation, IVS8-1(G>C), was found to be disease-causing by producing the skipping of exon 9 [Bergman et al., 1998]. A missense mutation, 1027G>T(G343W), produces a change of glycine 343 (hydrophilic) to tryptophan (hydrophobic). This glycine is conserved in all the studied FAH proteins (*Emeritella nidulans*, *Arabidopsis thaliana*, *Mus musculus*, *Rattus norvegicus*, and *Homo sapiens*) and is situated in a hinge between an  $\alpha$ -helix segment in sheet B and a  $\beta$ -segment in sheet A, near the active site of the enzyme [Timm et al., 1999]. The single-nucleotide deletion 938delC produces a frameshift from threonine 313 and introduces a new stop codon 60 amino acids downstream. Finally, a deletion spanning the last six nucleotides of exon 6 and the first 20 nucleotides of the adjacent intron 6 was detected in Patient HT9 (E6/I6del26). The other allele carries the IVS6-1(G>T) mutation. The presence of the two mutations in trans in the patient was confirmed by restriction analysis (Fig. 1E and 1F) and SSCP analysis (data not shown) of parental genomic DNA. The effect of these mutations on the mature transcripts of the patients' lymphocytes is currently under study, but we hypothesize the presence of abnormally spliced RNA product(s) owing to the lack of the 5' splice site of intron 6 in the E6/I6del26 allele, in addition to the proved splicing alterations due to the IVS6-1(G>T) mutation.

In conclusion, this study demonstrates a relatively high homogeneity of the HTI mutational spectrum in southern Europe, which has diagnostic and methodological consequences. In addition, the mutational spectrum is widened with seven new pathogenic alterations. And finally, the difficulties in establishing genotype-phenotype correlations are highlighted.

Clearly, further studies are necessary to investigate additional factors that may contribute to the clinical phenotype.

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### REFERENCES

- Bergman AJIW, van den Berg IET, Brink W, Poll-The BT, Ploos van Amstel JK, Berger R. 1998. Spectrum of mutations in the fumarylacetoacetate hydrolase gene of tyrosinemia type 1 patients in northwestern Europe and Mediterranean countries. *Hum Mutat* 12:19–26.
- Culbertson MR. 1999. RNA surveillance. Unforeseen consequences for gene expression, inherited genetic disorders and cancer. *Trends Genet* 15:74–80.
- Grompe M, St-Louis M, Demers SI, Al-Dhalimy M, Leclerc B, Tanguay RM. 1994. A single mutation of the fumarylacetoacetate hydrolase gene in French Canadians with hereditary tyrosinemia type I. *N Engl J Med* 331:353–357.
- Holme E, Lindstedt S. 1998. Tyrosinaemia type I and NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione). *J Inherit Metab Dis* 21:507–517.
- Kim SZ, Kupke KG, Lerardi-Curto L, Holme E, Greter J, Tanguay RM, Poudrier J, D'Astous M, Lettre F, Hahn SH, Levy HL. 2000. Hepatocellular carcinoma despite long-term survival in chronic tyrosinaemia I. *J Inherit Metab Dis* 23:791–804.
- Kvittingen EA, Rootwelt H, Brandtzaeg P, Bergman A, Berger R. 1993. Hereditary tyrosinemia type I. *J Clin Invest* 91:1816–1821.
- Kvittingen EA, Rootwelt H, Berger R, Brandtzaeg P. 1994. Self-induced correction of the genetic defect in tyrosinemia type I. *J Clin Invest* 94:1657–1661.
- Lindblad B, Lindstedt S, Steen G. 1977. On the enzymatic defects in hereditary tyrosinemia. *Proc Natl Acad Sci USA* 74:4641–4645.
- Mitchell GA, Grompe M, Lambert M, Tanguay RM. 2001. Hypertyrosinemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B, editors. *The metabolic and molecular bases of inherited disease*, 8th ed. New York: McGraw-Hill. p 1777–1805.
- Overturf K, Al-Dhalimy M, Tanguay R, Brantly M, Ou CN, Finegold M, Grompe M. 1996. Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tyrosinemia type I. *Nat Genet* 12:266–273.
- Phaneuf D, Labelle Y, Berube D, Arden K, Cavenee W, Gagne R, Tanguay RM. 1991. Cloning and expression of the cDNA encoding human fumarylacetoacetate hydrolase, the enzyme deficient in hereditary tyrosinemia: assignment of the gene to chromosome 15. *Am J Hum Genet* 48:525–535.
- Ploos van Amstel JK, Bergman AJIW, van Beurden EACM, Roijers JFM, Peelen T, van den Berg IET, Poll-The BT, Kvittingen EA, Berger R. 1996. Hereditary tyrosinemia type 1: novel missense, nonsense and splice consensus mutations in the human fumarylacetoacetate hydrolase gene; variability of the genotype–phenotype relationship. *Hum Genet* 97:51–59.
- Poudrier J, Lettre F, Scriver CR, Larochelle J, Tanguay RM. 1998. Different clinical forms of hereditary tyrosinemia (type I) in patients with identical genotypes. *Mol Genet Metab* 64:119–125.
- Riudor E, Ribes A, Lloret J, Friden J, Holme E, Jakobs C, Martínez-Ibáñez V. 1991. Liver transplantation in two children with tyrosinaemia type I: biochemical aspects. *J Inherit Metab Dis* 14:281–284.
- Rootwelt H, Kristensen T, Berger R, Høie K, Kvittingen A. 1994. Tyrosinemia type 1—complex splicing defects and a missense mutation in the fumarylacetoacetase gene. *Hum Genet* 94:235–239.
- St-Louis M, Tanguay RM. 1997. Mutations in the fumarylacetoacetate hydrolase gene causing hereditary tyrosinemia type I: overview. *Hum Mutat* 9:291–299.
- Timm DE, Mueller HA, Bhanumoorthy P, Harp JM, Bunick GJ. 1999. Crystal structure and mechanism of a carbon-carbon bond hydrolase. *Structure* 7:1023–1033.
- Timmers C, Grompe M. 1996. Six novel mutations in the fumarylacetoacetate hydrolase gene of patients with hereditary tyrosinemia type I. *Hum Mutat* 7:367–369.