JIMD Reports
DOI: 10.1007/8904_2011_10

Received: 4 October 2010; Revised: 28 October 2010;
Accepted: 15 December 2010; Published online: 22 June 2011
Communicated by: Verena Peters

Mutation Spectrum of Fumarylacetoacetase Gene and Clinical Aspects of Tyrosinemia Type I Disease

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Abstract Tyrosinemia type I (OMIM 276700) is a rare, autosomal recessive disorder caused by a deficiency in the fumarylacetoacetate hydrolase (FAH) enzyme. This study examined the spectrum of *FAH* gene mutation in 32 patients with tyrosinemia type I. In addition, clinical and biochemical findings were evaluated to establish a genotype–phenotype relationship in the patients. Mutation screening was performed using a 50K custom-designed resequencing microarray chip (TR_06_01r520489, Affymetrix) and sequencing analysis. Of the 12 different mutations found, 6 are categorized as novel. Three of the mutations-IVS6-1G>A, D233V, and IVS3-3C>G-are the most common in Turkish patients, comprising 25%, 17.1%, and 12.5% of mutant alleles, respectively.

Clinical evaluations suggest that the spectrum of symptoms observed in the patients with very early and early disease were of the more nonspecific form, whereas the patients with late-presenting disease had more of the distinctive form over the course of the disease. This study adds support to the notion that the D233V mutation is specific to the Turkish population.

Keywords FAH mutations · Genotype · Microarray · Phenotype · Resequencing chip · Tyrosinemia type I

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Introduction

Tyrosinemia type I (OMIM 276700) is an autosomal recessive disorder caused by a deficiency in the fumarylacetoacetate hydrolase (FAH) enzyme, which plays a role in the final step of tyrosine amino acid catabolism. The disease has panethnic distribution and varying frequencies worldwide. The disease primarily affects the liver and proximal renal tubular system. Liver disease can manifest as acute hepatic failure, mixed micro nodular cirrhosis, hepatocellular dysfunction, and hepatocellular carcinoma, and proximal tubular dysfunction causes such symptoms as hypophosphatemic rickets accompanying aminoaciduria, renal tubular acidosis, and glucosuria. Acute neurological crisis and cardiomyopathy may also develop in patients with the disease.

Three clinical phenotypes of the disease are described based on age at onset of symptoms: the acute form presents before 6 months of age with acute liver failure, the subacute form presents between 6 months and 1 year of age with liver disease, and the chronic form presents after 1 year of age with slowly progressive liver cirrhosis and hypophosphatemic rickets (Bergman et al. 1998; Mitchell et al. 2001; Chakrapani and Holme 2006). Van Spronsen et al. (1994, 1995) suggested another classification for tyrosinemia type I on the basis of survival rates and the age of symptom onset: very early form (onset of symptoms at <2 months of age), early form (onset of symptoms at 2–6 months of age), and late-presenting form (onset of symptoms at >6 months of age). Laboratory findings for tyrosinemia type I include

increased plasma levels of tyrosine (TYR), methionine (MET), and alpha-fetoprotein (AFP), and excessive urinary excretion of maleylacetoacetate (MAA), fumarylacetoacetate (FAA), and their derivatives-succinyl acetone (SA) and succinyl acetoacetate (SAA) (Chakrapani and Holme 2006).

The *FAH* gene has been mapped to chromosome 15q23-25 and contains 14 exons that code 420 amino acids (Awata et al. 1994). To date, more than 40 pathogenic mutations in the *FAH* gene have been reported in different populations. Some of the mutations appear to have accumulated in particular ethnic groups, i.e., IVS12+5G>A in French–Canadians (approximately 90% of alleles), IVS6-1G>T in the Mediterranean region (approximately 60% of alleles), W262X in Finns, D233V in Turks, and Q64H in Pakistanis (Rootwelt et al. 1996; St-Louis and Tanguay 1997; Bergman et al. 1998; Arranz et al. 2002; Elpeleg et al. 2002; Heath et al. 2002). Despite the fact that the spectrum of *FAH* gene mutation has been expanded, current knowledge is not adequate for establishing the disease's genotype–phenotype relationship.

This study examined the spectrum of *FAH* gene mutation in 32 patients with tyrosinemia type I. Of the 12 different mutations detected, 6 are categorized herein as novel. Based on the clinical and laboratory findings at the time of diagnosis, an attempt was made to establish a genotype–phenotype relationship.

Materials and Methods

In all, 32 Turkish patients with tyrosinemia type I that, to the best of our knowledge, came from unrelated families were examined for *FAH* gene mutations. Clinical diagnosis was based on increased levels of tyrosine in serum and the presence of SA in urine. No enzymatic studies were performed. The patients were categorized as very early (onset of symptoms at <2 months of age), early (onset of symptoms at 2–6 months of age), and late-presenting (onset of symptoms at >6 months of age), according to van Spronsen classification. Genotype and phenotype profiles, together with biochemical findings, are summarized in Table 1.

Mutation screening was performed using a 50K resequencing microarray chip (TR_06_01r520489/Affymetrix) custom designed by our research group to sequence all exonic sequences and their flanking intronic sequences for the following 14 genes responsible for 10 different types of inborn errors of metabolic diseases: the *ALDOB* gene for hereditary fructose intolerance, the *ATP7B* gene for Wilson disease, *BCKDHA*, *BCKDHB*, *DBT*, and *DLD* genes for maple syrup urine disease, the *FAH* gene for tyrosinemia type I, the *FBP1* gene for fructose 1–6 diphosphatase deficiency, the *GALT* gene for galactosemia, the *GCDH* gene for glutaric aciduria type I, the *MUT* gene for methylmalonic acidemia, *PCCA* and *PCCB* genes for propionic acidemia, and the *PAH* gene for phenylketonuria. Genomic DNA was extracted from blood samples using the salting-out technique. Fifty healthy individuals were selected as a control group to test the identified nucleotide changes and their frequencies in normal people. The study protocol was approved by the Hacettepe University Ethics Committee.

In brief, the *FAH* gene was amplified from genomic DNA via long-range polymerase chain reaction (PCR). The primer sets used for long-range PCR, and the related exons and amplicon sizes are given in Table 2. After purification (Qiagen kit), all PCR products were quantified (NanoDrop Technologies) and equimolar quantities were pooled. After the fragmentation step, fragmented PCR products were end-labeled using a biotin-labeling reagent and hybridized with DNA arrays (GeneChip Resequencing Assay Kit, Affymetrix). Arrays were processed via washing and staining on a fluidics station. Scanned arrays were analyzed using Affymetrix GeneChip resequencing analysis software.

Direct DNA sequencing was performed using primers specific for each exon to confirm all of the nucleotide changes detected by the microarray resequencing chip. Sequences of the exonic primers will be supplied upon request. For the sequencing reaction, BigDye Terminator Cycle Sequencing v.3.1 (Applied Biosystems, Foster City, CA) and sense or anti-sense primers were used in both the forward and reverse directions. An ABI 3130 capillary electrophoresis system was used for automated sequencing (Applied Biosystems) with the POP7 polymer. Sequencing chromogram files were analyzed using sequencing analysis software.

Results and Discussion

In total, two patients had very early disease, 15 had early disease, and eight had late-presenting disease. Six patients who were diagnosed via selective newborn screening because of a positive family history were excluded from clinical classification, and one patient, whose clinical and biochemical findings were not available, was excluded from classification. While two patients with the very early form presented with gastrointestinal hemorrhagia and acute liver failure that mimicked other acute neonatal problems, 15 patients with the early form had broader clinical symptomatology, including acute hepatic insufficiency, sepsis, hepatomegaly, chronic diarrhea, and rickets. On the contrary, hepatomegaly and rickets were primarily observed in patients with the late-presenting form.

Neurologic crisis and restrictive cardiomyopathy, which disappeared after the initiation of treatment, were noted during the follow-up of two patients with the late-presenting form. All but one patient excreted SA in their urine at

Table 1 Clinical and biochemical findings and genotypes of the patients

Patient No	Age of Diag. (mo)	Hepatic and tubular function								Mutation			
		Presenting symptoms	Tyr levels (mg/dl)	Synthetic function (PT/PTT)	ALT/ AST	Total Bil. levels (mg/dl)	a-FP IU/ml[a] (N 0–5.8)	SA	Tubulopathy	Type	Exon	Nucleotide	Protein	Outcome
1	2 day	GIS bleeding	25.1	Abnormal	40/102	3	855.000	+	NA	VE	2	c.191delA (Hom)		Well
2	3	HM	10.6	Abnormal	49/83	1.1	>308	+	–	E	3	IVS3-3C>G (Hom)		Liver Ca,Tx
3	2	Sepsis, HM	8.7	Abnormal	23/56	5.4	71.028	+	NA	E	3	IVS3-3C>G (Hom)		Exitus
4	18	HM	4.4	Normal	36/57	0.3	550.6	+	+	L	3	IVS3-3C>G (Hom)		Well
5	45	Sibling hist.	7.5	NA	20/45	1.3	231.715	+	–	–[b]	3	IVS3-3C>G (Hom)		Well
6	2	Hepatomegaly	20	NA	36/114	3.19	78.273	+	NA	E	5	c.(440–441) del 8 nt		Non follow up
7	9	Ascites,HM	9.5	Abnormal	35/93	3.1	50.884	+	+	L	6	IVS6-1G>T (Hom)		Non follow up
8	4 day	Sibling hist.	12.8	Abnormal	29/103	20.1	152.652	+	NA	–[b]	6	IVS6-1G>T (Hom)		Non follow up
9	12	Cr. diarrhea HM,CMP	24	Abnormal	31/75	2.9	37.615	+	+	L	6	IVS6-1G>T (Hom)		Non follow up
10	4	HM	NA	Abnormal	16/22	0.7	NA	+	+	E	6	IVS6-1G>T (Hom)		Liver Ca
11	9 y	Rickets	6.8	Normal	27/37	0.63	37.25	+	+	E	6	IVS6-1G>T (Hom)		Well
12	36	HM	10.9	Abnormal	35/62	0.6	5135	+	+	L	6	IVS6-1G>T (Hom)		Well
13	3.5 y	Fanconi syn.	9.2	Abnormal	38/49	NA	6987	+	+	L	6	IVS6-1G>T/?		Non follow up
14	6	HSM	6.0	Abnormal	20/84	1.43	35.000	+	+	E	6	IVS6-1G>T (Hom)		Well
15	1.5	Sibling hist.	9.9	NA	23/76	3.18	5031	–	–	–[b]	6/8	IVS6-1G>T/c.698A>T	D233V (CH)	Well
16	26 day	Sibling hist.	9.4	Abnormal	23/145	3.67	369.643	+	–	–[b]	6	c.497T>G	V166G (Hom)	Well
17	1.5	Sibling hist.	15.6	NA	18/49	3.44	686.200	+	NA	–[b]	6	c.497T>G	V166G (Hom)	Well
18	19 m	Cr. diarrhea	9.8	Abnormal	28/80	0.59	34.479	+	NA	L	6	c.520C>T	R174X (Hom)	Nor. crisis
19	3	Rickets	17	Abnormal	27/77	NA	NA	+	+	E	8	c.696C>A	N232K (Hom)	Liver Ca-Tx-Ex
20	3	Diarrhea-HM	9.6	Abnormal	27/90	3.2	97.625	+	–	E	8	c.698A>T	D233V (Hom)	Well
21	3.5	Diarrhea-HM	10.6	Abnormal	30/84	0.64	761.5	+	–	E	8	c.698A>T	D233V (Hom)	Well
22	4	HM, rickets	10.3	NA	44/60	NA	>533	+	+	E	8	c.698A>T	D233V (Hom)	Liver Ca,Tx,Well
23	4	Hip.rickets	9	NA	14/13	0.8	NA	+	+	E	8	c.698A>T	D233V (Hom)	Liver Ca,Ex
24	20 day	Liver failure	6.3	Abnormal	88/312	6.5	52.830	+	+	VE	8	c.698A>T	D233V (Hom)	Well
25	7.5	HM	6.7	Abnormal	44/101	1.25	109.761	+	+	E	8/12	c.1107delG/c.698A>T	N344Tfsx/D233V (CH)	Non follow up
26	6	HM	8.6	Abnormal	30/53	2.3	36.570	+	–	E	9	c.709C>T	R237X (Hom)	Well
27	13	HM	9.5	Abnormal	98/245	5.08	1200	+	+	L	9	c.709C>T	R237X (Hom)	Exitus
28	4	Rickets	7.7	NA	27/64	0.36	18.7	+	+	E	9	c.776T>A	V259D (Hom)	Liver Ca,Tx,Well
29	12	HM	15.4	Normal	26/42	1.1	13.688	+	+	L	9	IVS9+2T>C (Hom)		Liver Ca,Tx,Well
30	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	9	IVS9+2T>C (Hom)		Non follow up
31	4	Sibling hist.	2.6	NA	32/87	1.5	>29.340	+	–	–[b]	12	IVS12+5G>A (Hom)		Well
32	5	Hepatomegaly	9.1	Abnormal	52/102	–	435	+	NA	E	12	IVS12+5G>A (Hom)		Well

Normal values of liver transaminase: ALT (6–50 IU/L for 0–5 days, 35–140 for 1–19 years), AST (5-45IU/ml for 0–5 days, 15–55 IU/ml for 1–19 years)

NA Not available, *HM* Hepatomegaly, *CMP* Restrictive cardiomyapathy, *GIS* Gastro intestinal system, Tx. Liver transplantation, *VE* Very early form, *E* Early form, *L* Late form, *Hom* Homozygous, *CH* Compound heterozygous

[a]It is accepted as 1 ng of AFP is approximately equal to 1 mIU (James et al. 1981; Wu et al. 1981; Blohm et al. 1998)

[b]Marker showed the patients with sibling history and diagnosed by screening

Table 2 Amplification of *FAH* gene fragments with long PCR using four primer sets

Amplicon	Exon	Amplicon size (bp)	Sequence of the primers
PCR1	1	5069	F-primer: TGCCAAACAGGTTGAATACAGAGGTTGTAACT
			R-primer: ACTCTTCTAGACCAAAGCACTTACCGCAGAC
PCR2	2,3,4,5	9672	F-primer: AAAGAGGGCAGGGAAACAAACTTTGACTAAG
			R-primer: TCATCACTGCCAAGGACACTCATATAGACACT
PCR3	6,7,8,9,10	11635	F-primer: TTAATCCAATAAAGGAACCAAGGTGGGTAA
			R-primer: ACCCTGGAACTGACAGTGAGACAGACAT
PCR4	11,12,13,14	12901	F-primer: CTGAGTGTCTTGCAGACAGACCCGAGAT
			R-primer: TAGAGAGCAGGGGTTTTAGCTGAGTCAACA

the time of diagnosis. Patient 15, who was screened for tyrosinemia type I at the age of 1.5 months because of a positive history (an affected older sibling), did not excrete SA in his urine (according to gas chromatography-mass spectrometry (GC-MS) results) despite the fact that plasma tyrosine and alpha-fetoprotein levels were elevated. The diagnosis of this patient was made via mutation analysis. During follow-up, GC-MS analysis detected SA in the urine of patient 14 at age 4 months. Haagen and Duran (1987) reported a case with tyrosinemia type I in which SA results were negative at 7 months of age. They proved that this finding was due to the low sensitivity of the test method used, which implies that there is a need for more sensitive tests.

Blood tyrosine levels of the patients in this study ranged from 4.4 to 25.1 mg dL^{-1}. A high-peak tyrosine level (25.1 mg dL^{-1}) was observed in one patient carrying the homozygous c.191delA mutation. AFP levels were higher than the normal range in all the patients at the time of diagnosis; however, based on the variation in the age of the patients at the time of diagnosis, AFP levels seemed to decrease considerably in terms of newborn period as time passes even if any treatment was given. A meaningful correlation was not observed between AFP level, and the severity of symptoms and disease type.

Altered coagulation parameters (PT and PTT) indicative of hepatic synthesis dysfunction were observed in all but except three of the patients in this study. In addition, two patients with a sibling history did not have distinctive clinical findings, but did have abnormal coagulation parameters at the time of diagnosis. Interestingly, although AST levels were elevated in 25 patients, the level of ALT (a specific enzyme for liver tissue) was significantly elevated in only five patients. Bilirubin levels were near the normal range at the time of diagnosis in all the patients. In summary, altered levels of tyrosine, AFP, coagulation parameters (PT/PTT), and AST were considered sensitive biomarkers for the diagnosis of tyrosinemia type I.

In total, 12 different disease-causing mutations were observed in 32 Turkish patients with tyrosinemia type I (Table 1): 4 missense mutations, 1 nonsense mutation, 4 splicing mutations, and 3 deletion-type mutations. Of the 12 mutations detected, 6 (V166G, R174X, D233V, R237X, IVS6-1G>A, and IVS12+5G>A) have been previously reported, the others (N232K, V259D, c.191delA, IVS3-3C>G, c.(440–441)del8 nt, and IVS9+2T>C) are designated as novel. Each novel mutation was screened in 50 control individuals and all were negative. In this study, IVS6-1G>A, D233V, and IVS3-3C>G were the most common mutations, comprising 25%, 17.1%, and 12.5% of the mutant alleles, respectively.

It is well known that the IVS6-1G>A mutation is the most frequently seen nucleotide change in the Mediterranean region, and accounts for approximately 60% of the deleterious alleles in the *FAH* gene (Bergman et al. 1998; Arranz et al. 2002). D233V is known to be specific to the Turkish population, as it has not been reported in other ethnic groups thus far, which is in agreement with the present results (Rootwelt et al. 1994, 1996). Surprisingly, IVS12+5G>A, which is reportedly the most prevalent mutation worldwide (about 25% of alleles), was detected homozygously in only 2 patients in this study. It was suggested that D233V mutation causes *FAH* dysfunction by directly affecting the Ca^{2+} ligand at the active site, and that V166G mutation occurring near the active site may cause misfolding by directly affecting the active site geometry (Timm et al. 1999). Similarly, it could be predicted that N232K located in strand 8 of the β-roll at the active site may impair Ca^{2+} ligands, as does the D233V mutation. On the contrary, it could be suggested that another novel mutation-V259D-located in strand 9 of the β roll at the active site may cause dysfunction via misfolding, as does V166G.

Large cohorts are necessary to establish genotype–phenotype relationships for infrequent mutations; however, genotype–phenotype relationships were evaluated for the three most common mutations (IVS6-1G>A, D233V, and IVS3-3C>G) observed in this study. On the basis of age of diagnosis, presenting symptoms, tyrosine levels, and biochemical markers, a meaningful genotype–phenotype relationship for these three mutations was not observed. These findings are consistent with the consensus that appears in the literature.

Hepatocellular carcinoma developed in 7 of our patients. All of these patients were treated irregularly and inadequately with NTBC. In addition, there was not any information

available concerning NTBC plasma levels or urinary succinylacetone excretion at the time these patients received NTBC treatment. Among these 7 patients, 5 who carried N232K, D233V, V259D, and IVS3-3C>G mutations developed liver cancer between 10 and 12 years of age. The patient carrying the IVS9+2T>C mutation developed liver cancer at 6 years of age. The patient with the IVS6-1G>T mutation was 24 years old when diagnosed with liver cancer. The AFP level in this patient was normal (4.16 IU mL^{-1}), and AFP was not immunohistochemically detected in liver tissue, whereas histological test results of liver tissue were compatible with neoplasm. On the contrary, noticeable increases in the AFP levels were not observed in these patients at the time liver cancer was diagnosed.

As a consequence, the present findings suggest that AFP has limited value for the early detection of liver cancer in tyrosinemia type I patients. Five patients in this study with the D233V mutation-3 of whom were previously reported by Rootwelt et al. (1994) developed liver carcinoma during follow-up at our clinic. Although 5 patients are not sufficient to conclude that the D233V mutation is a risk factor for liver cancer, patients carrying this nucleotide change should be followed up closely for liver neoplasm.

In conclusion, this study presents 6 novel mutations that were observed in tyrosinemia type I patients. The microarray resequencing method used to prescreen nucleotide changes was confirmed to be a rapid and cost-effective method for screening a large number of samples. Moreover, results of this study indicate that some mutations might be associated with a high risk of developing liver neoplasm.

Acknowledgement This study was supported by the State Planning Organization of Turkey (project No: DPT 2006K120640).

Synopsis

The article describes different type of FAH mutations and their clinical outcomes in tyrosinemia type 1 patients.

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