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Hawkinsinuria in two unrelated Greek newborns: identification of a novel variant, biochemical findings and treatment

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Abstract

Background: Hawkinsinuria is a rare inborn error of tyrosine metabolism.
Objectives: To study novel hawkinsinuria cases by monitoring their biochemical profile and conducting a mutation analysis.
Subjects and methods: Among 92,519 newborns that underwent expanded newborn screening, two unrelated cases with high tyrosine blood levels were further investigated by chromatographic techniques and via genetic testing for 4-hydroxyphenylpyruvate dioxygenase (HPD) gene.
Results: Elevated levels were monitored for blood/plasma tyrosine and for the specific diagnostic markers in urine. The two newborns were put on a special low tyrosine diet. Till completion of the 1st year of their life, liver function tests and brain MRI were normal. The mutation A33T was identified in both cases, while one neonate carried an additional novel mutation of *HPD* gene (V212M).
Conclusions: Two mutations of *HPD* gene, A33T, which are associated with hawkinsinuria and a novel one (V212M) were detected for the 1st time in Greek newborns.

Keywords: expanded screening; hawkinsinuria; mutational analysis; newborn; urine markers.

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Introduction

Tyrosinemia is an autosomal recessive disorder caused by the deficiency of enzymes involved in the amino acid tyrosine (Tyr) catabolism. The deficiency of these enzymes induces an elevation of Tyr levels, as well as its by products and their accumulation results in health problems. There are three Tyr enzyme deficiencies which are responsible for the clinical and laboratory findings of Tyr metabolic disorders (1, 2).

Tyrosinemia type III (OMIM 276710) is an autosomal recessive trait disease caused by the deficiency of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPD) (EC 1.13.11.27). This enzyme is the 2nd enzyme in the Tyr catabolism and its deficiency results in high Tyr blood levels and the increased excretion of phenolic metabolites in urine (3). Existing studies of the disease report mental retardation and/or neurological problems and/or liver problems of the affected patients if not treated (4). Mutation analysis of the *HPD* gene enables differential diagnosis among tyrosinemia I, II, and III and transient tyrosinemia (5). Administration of ascorbic acid, as well as low phenylalanine along with Tyr diet, constitute the most common treatment of the disease (1).

Additionally, it has been reported that hawkinsinuria, an autosomal dominant disorder, is also caused by a heterozygous defect in *HPD*. Hawkinsinuria is characterized by the excretion of hawkinsine in the patient's urine. Although symptoms of hawkinsinuria disappear during the 1st year of life, hawkinsine is still excreted (6). Previous (7) and recent (8) reports underline that besides hawkinsine, another specific marker, 4-hydroxycyclohexylacetic

acid, is excreted in the urine of these patients, too (Figure 1). Regarding mutational analysis, a homozygous patient for A268V mutation as well as two patients heterozygous for the missense A33T in a previous study (9) were reported. All the above patients suffer from hawkinsinuria. In general, very few cases of such patients have been reported in literature (10).

Expanded newborn screening using a tandem mass spectrometric method is the most common and useful tool to identify cases such as tyrosinemia or hawkinsinuria in the neonatal population, as such diseases are very rare and mostly symptomless during infant life (11). Particularly, hawkinsinuria seems to be very rare in Greek population, as no such patient has ever been reported. Liver needle biopsies have been currently replaced by gene mutation analysis and it is preferable by both doctors and parents. Therefore, differential diagnosis for hawkinsinuria, which is symptomless during the 1st days of life, can be obtained (10, 12).

In this study, we report two cases of Greek neonates with hawkinsinuria, detected via expanded newborn screening. Their potential clinical symptoms, the biochemical findings in blood, plasma, and urine were monitored during their 1st year of life. Mutational analysis of the *HPD* gene was conducted in order to reveal the presence of related mutations.

Materials and methods

Subjects

Dried blood spots (DBS, Whatman 903) were used (GE Healthcare, Buckinghamshire, UK) to screen 92519 newborns of Greek origin for metabolic disorders of amino acids and acylcarnitines. The blood was collected after 2–3 days of feeding with a heel prick. This procedure was approved by the Institutional Review Board of IASO hospital in agreement with the current revision of the Helsinki Declaration.

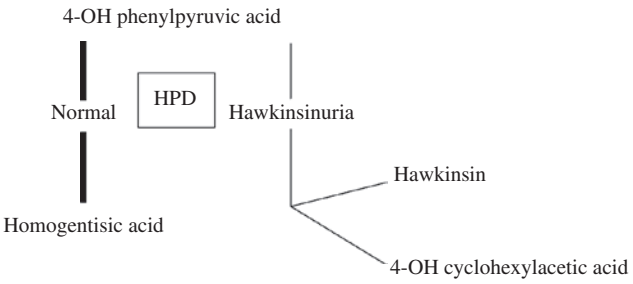

Figure 1: Schematic presentation of the reaction of 4-hydroxyphenylpyruvic acid resulting in the two characteristic hawkinsinuria markers in urine.

DBS screening via liquid chromatography tandem mass spectrometry

The protocol adopted for routine liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of DBS, via infusion, has been described in detail elsewhere (13). Our laboratory has been participating in a worldwide collaborative study in order to confirm the clinical validity of various cut-off values for newborn screening by LC-MS/MS (14). Regarding tyrosinemias, Tyr values of the aforementioned screening were considered as primal markers of the disease. If result exceeded the upper cut-off value (Tyr: 190 μM), the specimen was regarded as "positive", and a 2nd Guthrie card was requested immediately for repeat analysis along with a plasma and a urine sample.

LC-MS/MS method for plasma samples

The standard EZ:faast (Phenomenex, Torrance, CA, USA) LC-MS/MS protocol was utilized for the quantitative determination of Tyr among other amino acids, following elution from a C18 column. Amino acids were detected as derivatives using a procedure described previously in detail (15).

Gas chromatography-mass spectrometry (GC/MS) method for urine analysis

A previously described protocol (16) has been used for the derivatization of organic acids in urine samples using tropic acid as internal standard. Urine markers indicative for tyrosinemias and hawkinsinuria (7), such as 4-hydroxyphenyllactic acid, 4-hydroxyphenylpyruvic acid, and 4-hydroxyphenyllactic acid, can be identified and determined via this experimental procedure (Figure 2). The same protocol can be applied for the determination of 4-hydroxycyclohexylacetic acid, a specific diagnostic marker, besides hawkinsin, for Hawkinsinuria (7, 8).

DNA extraction from DBS

Genomic DNA was extracted from DBS following the standard chelex protocol (Chelex 100, Molecular Biology Grade Resin, Biorad, Athens, Greece). The quantity and quality of the DNA samples were determined with "NanoDrop" (Thermo Fisher Scientific, Wilmington, MA, USA).

DNA sequencing

Amplicons spanning the whole coding region of the *HPD* gene, including intron-exon boundaries, were amplified by polymerase chain reaction (PCR). The PCR products were purified with "Multiscreen PCRμ_{96} Filter Plate" (Millipore, MALVA, Athens, Greece), and then subjected to automated cycle sequencing with the Big Dye Terminator Cycle v3.1 sequencing kit (Life Technologies, Grand Island, NY, USA) and electrophoresis on an ABI 3130xl Sequencer (Life

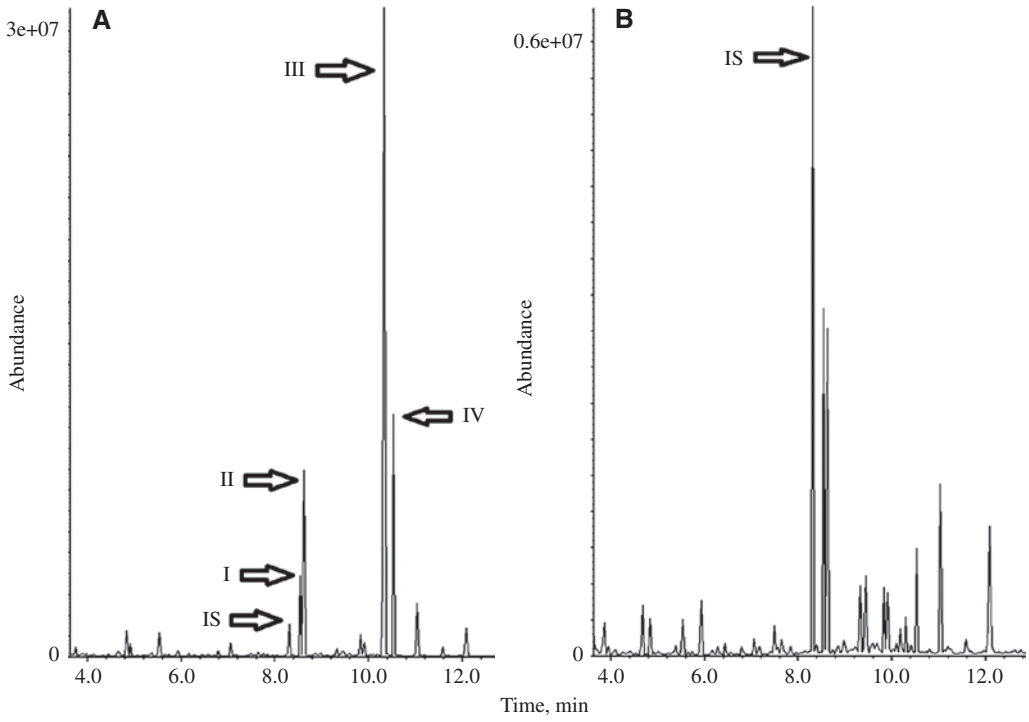

Figure 2: GC/MS chromatograms obtained from urine samples of (A) case 2 and (B) normal sample. Trimethylsilyl esters of the following organic acids were detected: (I) 4-hydroxycyclohexylacetic acid (tr=8.58 min), (II) 4-hydroxyphenylacetic acid (tr=8.62 min), (III) 4-hydroxyphenyllactic acid (tr=10.34 min), (IV) 4-hydroxyphenylpyruvic acid (tr=10.53 min), and tropic acid as internal standard (tr=8.40 min).

Technologies, Grand Island, NY, USA). Primer pairs were designed with NCBI/Primer Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are available upon request.

In silico analysis

The effect of the novel mutation on protein level was evaluated through SIFT and Provean tools (http://sift.jcvi.org/www/SIFT_seq_submit2.html), PolyPhen (http://genetics.bwh.harvard.edu/pph/) algorithms and Mutation Taster (http://www.mutationtaster.org/).

Results

Case 1

A full-term female infant was born from unrelated parents of Greek origin after an uneventful pregnancy and delivery with birth weight 2980 g. After a 3-day breastfeeding, blood spots were collected on a Guthrie card after a heel prick. The screening via LC-MS/MS showed elevated levels of Tyr (Table 1), whereas the other amino acids and succinylacetone were within normal ranges. After 24 h, a second blood sample on Guthrie card, as well as plasma and urine samples were requested. Tyr levels were also elevated in the second DBS sample, as well as in the plasma sample. In addition, increased concentration of 4-hydroxyphenylpyruvic acid was determined in urine sample, along with the pathognomonic marker 4-hydroxycyclohexylacetic acid. At this time, liver function tests, except gamma-glutamyl transferase (γ-Gt) (190 U/L, normal values 20–60 U/L), clinical and neurological examination were normal. Blood gases were evaluated and found normal.

Table 1: Tyrosine concentrations in dried blood spots (Guthrie cards) and plasma in the newborns at diagnosis.

Cases	Guthrie card (20–190 μM)[a]		Plasma (55–147 μM)[a]
	1st sample	2nd sample	
1	362.4	418.5	395.1
2	567.8	678.6	586.7

[a]Normal values in parentheses.

The natural protein intake was reduced from 2.5 to 0.8 g/kg/24 h. A Tyr-free product (Tyr-Anamix-Infant, SHS, UK) was utilized to replace a part of the natural protein intake, for example, the infant was on (0.8 g natural protein +1.8 g artificial protein)/kg/24 h. After 3 days on diet, Tyr blood levels were reduced to normal and Tyr metabolites in urine

disappeared. Now the baby is 12 months old, having and has excellent psychomotor development. DNA analysis revealed the presence of A33T mutation in heterozygosity. Unfortunately, parents were not willing to be subjected to mutational analysis.

Case 2, parents and sibling

A full-term female infant was born after a normal pregnancy and delivery with birth weight 3280 g. The parents were unrelated, both of Greek origin. After 2 days on formula feeding, blood drops were put on Guthrie card after a heel prick. Very high levels of Tyr, almost three times higher than the upper cut-off value, were measured in DBS and in plasma samples, as shown in Table 1. Additionally, four characteristic metabolites (Figure 2 and Table 2) including 4-hydroxycyclohexylacetic acid, the specific for hawkinsinuria marker, were detected in high levels in urine samples. At this time, liver function tests, except γ-Gt, were normal. The latter declined to normal after 1 week on Tyr-restricted diet. Blood gases were evaluated and found normal.

The natural protein intake was reduced from 2.5 to 0.8 g/kg/24 h, whereas 1.8 g/kg/24 h protein was added from a Tyr-free product (Tyr-Anamix-Infant SHS, UK). After 3 days on this diet, Tyr levels were decreased to normal and pathological urine metabolites disappeared. Neurologic clinical examination, as well as MRI of the brain, revealed no abnormal signs. The baby is now 13 months old, she is well developed, following the special therapeutic diet. The infant proved to be compound heterozygote for A33T and V212M (Figure 3) after mutational analysis of the *HPD* gene (Table 3). V212M is reported for the 1st time and therefore in silico analysis tools were used in order to infer its potential pathogenicity. According to Polyphen2, this mutation is predicted to be probably damaging with a score of 0.984 (sensitivity: 0.55; specificity: 0.94). SIFT tool indicated that this mutation is probably damaging with a score of 0.01 (cut-off score: 0.05) and Provean characterized this mutation as deleterious with a score of –2.88

Table 2: Abnormal values of tyrosinemias-related metabolites in urine of cases 1 and 2 at diagnosis.

Cases	Metabolites	Concentration, μmol/mmol creatinine	Normal values, μmol/mmol creatinine
1	4-hydroxycyclohexylacetic acid	5.6	0–2.2
	4-hydroxyphenylpyruvic acid	102.8	0–3.4
2	4-hydroxycyclohexylacetic acid	57.3	0–2.2
	4-hydroxyphenyllactic acid	1410.3	0.1–5.8
	4-hydroxyphenylpyruvic acid	537.4	0–3.4
	4-hydroxyphenylacetic acid	268.7	0.1–11.5

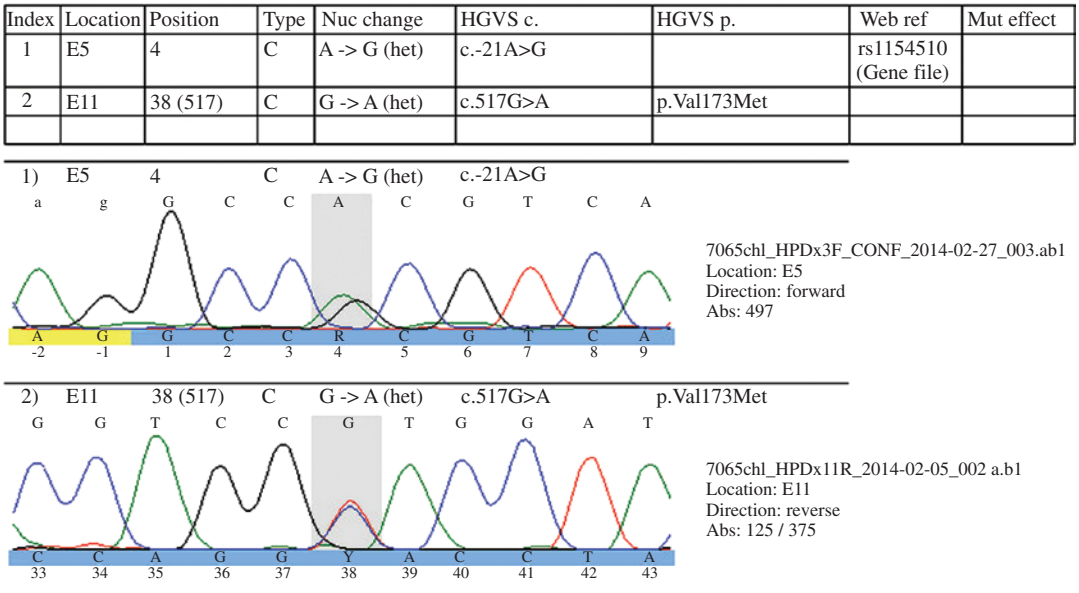

Figure 3: Graphic representation generated from SeqNext tool (JSI) of *HPD* exons 4 (top) and 10 (bottom), respectively, corresponding to case 2.

Table 3: Mutations identified in the two cases of 4-hydroxyphenylpyruvic-acid dioxygenase (HPD) deficiency and in parents and sibling (brother) of case 2.

Cases	Mutations
1	A33T/WT
2	A33T/V212M
Father	A33T/A33T, V212M
Mother	A33T/WT
Sibling	A33T/A33T, V212M

(cut-off score: –2.50). As for Mutation Taster, it predicted that this mutation is "disease causing".

The frequency of the novel mutation was determined in a group of 96 control samples which were randomly selected from neonates' cohort that had been proved to be negative for all the "metabolic markers" tested in the context of expanded neonatal screening. Particularly, the samples were subjected to sequencing of the coding region of the *HPD* gene which includes the novel mutation. None of the samples carried the p.V212M.

In this case, parents and sibling were subjected to mutational analysis. The results, as depicted in Table 3, indicate that the mutations A33T and V212M are in cis based on parents' genotypes. Particularly, it seems that case 2 inherited the A33T-V212M allele from her father and a wild-type allele from her mother, while her brother inherited the A33T-V212M allele from his father and the A33T allele from his mother. At this point, it should be noted that both parents had no abnormal findings in urine analysis and their medical history is free from symptoms related to any metabolic disorder. However, this outcome was not so unexpected, as symptoms abate as the individual passes out of infancy (17). Regarding the sibling, he had been subjected to expanded newborn screening with a normal amino acid profile, whereas in a GC analysis following his sister's findings, no abnormal levels of related metabolites were observed.

Discussion

HPD enzyme plays a critical role in Tyr catabolism, catalyzing the reaction of 4-hydroxyphenylpyruvic acid to homogentisic acid. HPD deficiency may lead to hawkinsinuria, which is characterized by elevated Tyr blood levels and the excretion of Tyr metabolic products in urine (4), including hawkinsin and 4-hydroxycyclohexylacetic acid.

In both cases, neonates presented high Tyr blood (DBS) and plasma levels. The lower Tyr concentration measured in case 1 (Table 1), as compared to that of case 2, can be attributed to breastfeeding (case 1) which contains less natural protein than that of a common formula, as well as to the presence of the novel mutation in case 2. Furthermore, the profiles obtained from the urine samples were also different. In particular, in case 1 only 4-hydroxyphenylpyruvic acid and 4-hydroxycyclohexylacetic acid were detected, whereas in case 2 all potential metabolites (4-hydroxyphenylpyruvic acid, 4-hydroxyphenyllactic acid, 4-hydroxyphenylacetic acid and 4-hydroxycyclohexylacetic acid) were monitored. This difference may be also attributed to the genotypes of the two cases, as well as to the higher protein content of the formula with which the infant was fed. Specifically, case 1 is heterozygote for A33T, a mutation associated with hawkinsinuria (5, 18). The characteristic finding in urine, hawkinsine, a sulfur amino acid, could not be detected via our GC/MS protocol; however, the 2nd specific marker, 4-hydroxycyclohexylacetic acid was determined in both cases. Therefore, the genotype, the biochemical findings, and the absence of clinical manifestation of case 1 provide strong support for the establishment of the diagnosis for this disorder.

In case 2, two mutations were identified (A33T and V212M) in cis indicating a dominant inheritance pattern. With regards to the novel mutation, in silico analysis showed that it is probably damaging and this can be an explanation of the increased concentrations of Tyr metabolites in urine that were detected in case 2. However, in vitro and in vivo analysis is required to support assumptions about the potential effect of the combination A33T and V212M on enzyme activity. Regarding the latter, the residue 212 is located in the HPPD_C_like domain (C-terminal domain of HPD) as shown in UniProt database (http://www.uniprot.org/).

Based on the combined data of phenotypes and genotypes of family of case 2, one could suggest that the clinical entity of hawkinsinuria is attributed to the potential synergistic effect of more than one genetic factors along with environmental ones. This is evident for father and sibling in case 2, who carry the same A33T–V212M allele as the proband, but no clinical manifestations were observed.

Both cases were on special Tyr-restricted diet until the end of the 1st year of their life. This decision was made up for prevention reasons as symptoms of the disease are reported to be present early in life (first weeks). At this point, it should be stated that in both cases, ascorbic acid drops were initially given, but with poor results, as only 10% of Tyr blood levels were reduced. At the end of the 1st year of life, both babies were put on free diet and subjected to testing of Tyr in plasma and for associated metabolites in urine. In both cases no aberrant findings were monitored. These laboratory results are in accordance with their normal psychomotor development.

Conclusion

Two cases of hawkinsinuria were reported for the 1st time in two newborns of unrelated Greek parents. Expanded newborn screening resulted in early detection of the patients with the above-mentioned metabolic disease, enabling prevention of the related symptoms. The initial findings in DBS were confirmed by measurements in plasma and the detection of characteristic urine organic acid profiles associated to Tyr catabolic disorder, including the pathognomonic 4-hydroxycyclohexylacetic acid. Mutational analysis of the *HPD* gene revealed the presence of a well-known mutation A33T in both neonates in heterozygosity, whereas a novel one, V212M was identified (in cis) in case 2.

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Conflict of interests statement: All the authors declare no conflict of interest.

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