

ORIGINAL ARTICLE

Mutational analysis for biotinidase deficiency of a Greek patients' cohort ascertained through expanded newborn screening

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Late-onset multiple carboxylase deficiency, also known as biotinidase (BTD) deficiency, is an autosomal recessively inherited disorder of biotin metabolism. Its early diagnosis and treatment seems that it can even fully prevent its various clinical manifestations. Mutations in the *BTD* gene scattered throughout its coding region have been detected in patients ascertained either through newborn screening or clinically. From March 2010 up to June 2011, 18 954 Greek neonates were subjected to biochemical determination of BTD activity through a semiquantitative fluoroimmunoassay. Subsequently, the first cohort of our 'suspected' samples was further tested for the presence of aberrations associated either with partial or profound BTD deficiency through sequencing of the coding region of the *BTD* gene, including splice-site junctions. On the basis of the molecular data derived from the study of our first cohort of 'suspected' samples, a panel of four mutations, most frequently encountered in the Greek population, was created, and a rapid, reliable and cost-effective real-time-based genotyping assay for the detection of these mutations was developed. This is the first report about the *BTD* mutational spectrum in Greece, and it could be a beneficial utility in the differential clinical diagnosis of BTD deficiency.

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INTRODUCTION

Multiple carboxylase deficiency (MCD) is a disorder of biotin metabolism, inherited with autosomal recessive mode. It is attributed either to mutations occurring in the *BTD* (biotinidase) gene resulting in the manifestation of the late-onset MCD or to mutations in the *HLCS* (holocarboxylase synthetase) gene giving rise to the early-onset MCD syndrome (OMIM number 253260). As the emergence of clinical symptoms, some of which are associated with severe impairment of the central nervous system, may be even fully prevented through early biotin supplementation in both types of MCD,¹ its early diagnosis is considered beneficial. For that reason, the determination of BTD activity has been incorporated in the routine newborn screening program of many countries.

Late-onset MCD, also known as BTD deficiency syndrome, is distinguished to partial and profound type on the basis of the mean enzyme–serum activity observed. Particularly, 10–30% of mean BTD serum activity is implicated to partial enzyme deficiency, whereas <10% activity to profound enzyme deficiency. The aforementioned biochemical data have been correlated with the presence of mutations in the *BTD* gene through genetic testing of positive samples.² Specific

mutations, such as the frequent p.Asp444His mutation, either in homozygosity or combined with other mutations, labeled as 'severe', are present in clinical cases of partial deficiency, whereas the 'severe' mutations either in homozygosity or in compound heterozygosity are implicated in the profound form of BTD deficiency.³ The carrier frequency of *BTD* mutations is approximately estimated to 1 in 120, whereas the combined incidence of profound and partial cases are about 1 in 60 000, worldwide.^{2,4}

Up to date, more than 100 mutations of the *BTD* gene have been recorded. These mutations are scattered throughout the coding region of the *BTD* gene and can be of any type.¹ Two distinct mutations in compound heterozygosity are detected in most clinical cases, except in populations characterized by significant consanguinity.⁵

The phenotype of the disorder includes a variety of clinical symptoms such as neurological (seizures, ataxia, hypotonia, developmental delay, hearing loss, problematic vision), gastroenterological (vomiting, feeding difficulties), dermatological (alopecia, skin rash) and even respiratory ones (apnea, tachypnea).^{2,6} The aforementioned heterogeneity regarding the clinical manifestations of the late-onset MCD syndrome could be explained by the diverse functions of the

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BTD protein. In fact, BTD has a critical role in the recycling of both free- and bound biotin, which is essential for the processing of amino acids, lipid and carbohydrate metabolic pathways. Furthermore, there is emerging evidence that BTD mediates both biotinylation and debiotinylation of histones, and consequently affecting processes such as DNA repair, replication and transcription.^{7–11} It is worth noting that the clinical manifestations of patients carrying the same mutations may vary even between members of the same family concerning the severity, as well as the age of onset. This might be due to the simultaneous presence of rare variants and/or epigenetic modifications, the action of modifier loci or even environmental effects.¹

The aim of this study was to successfully identify the 'suspected' samples under routine newborn screening procedures and to report the *BTD* mutational spectrum in the Greek population. More specifically, the frequency and the type of pathogenic alterations encountered in patients ascertained through newborn screening have been elucidated, and a rapid DNA-based confirmatory protocol for the 'suspected' samples has been developed.

MATERIALS AND METHODS

Patients

Blood samples were collected from neonates on Whatman filter papers following the standard procedure, to be screened for the presence of a series of metabolic disorders, as well as cystic fibrosis, congenital adrenal hyperplasia and BTD deficiency. The newborn screening procedure, including confirmatory genetic testing was approved by the Institutional Review Board of IASO hospital, in agreement with the 1975 Helsinki statement, revised in 1983. A coded designation was utilized to protect patient anonymity.

Determination of enzyme activity

A semiquantitative fluoroimmunoassay (Delfia Biotinidase kit; Perkin Elmer, Boston, MA, USA) was adopted to determine the BTD activity on dried blood spots. According to the manufacturer's instructions, values $< 19 \text{ nmol min}^{-1} \text{ dl}^{-1}$ are implicated to profound BTD deficiency, whereas values $> 49 \text{ nmol min}^{-1} \text{ dl}^{-1}$ are considered as normal. The obtained values, which fell in the range between $19 \text{ nmol min}^{-1} \text{ dl}^{-1}$ and $49 \text{ nmol min}^{-1} \text{ dl}^{-1}$, indicate possible partial enzyme deficiency.

DNA extraction from dried blood spots

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

DNA sequencing

Exons 1–4 of the *BTD* gene as well as their flanking regions were amplified by PCR. The PCR products were purified with 'Multiscreen PCR_μ96 Filter Plate' (Millipore, Malva, Athens, Greece), and then subjected to an automated cycle sequencing with the Big Dye Terminator Cycle v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) and electrophoresis on an ABI Prism 310 Sequencer (Perkin Elmer, Applied Biosystems). Primer pairs were designed with NCBI/Primer Blast Tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and are available upon request.

Real-time PCR-based genotyping assay

Four distinct real-time PCR assays were developed for the rapid screening of 'suspected' samples concerning the presence of p.Asp444His, p.Gln456His, p.Thr532Met and c.98_104del7ins3 mutations. Primers and probes for these assays were synthesized by VBC Genomics Bioscience Research GmbH (Vienna, Austria). The total volume of the PCR reaction was $10 \mu\text{l}$ and consisted of $1 \times$ KAPA PROBE FAST Universal $2 \times$ qPCR Master Mix (Kappa Biosystems, Boston, MA, USA), $0.75 \mu\text{M}$ forward/reverse primers mix, $0.5 \mu\text{M}$ HEX/6FAM probes MIX and $3.75 \text{ ng} \mu\text{l}^{-1}$ of DNA. All PCR reactions were performed in a

Rotor-Gene 6000 (Corbett Research, Bioanalytica S.A., Athens, Greece). The cycling conditions were as follows: 60°C for 30 s (1 cycle), 95°C for 10 min (1 cycle), 95°C for 15 s or 60°C for 1 min (45 cycles), 60°C for 30 s (1 cycle). Positive controls, either a heterozygote or a homozygote sample for each mutation, as well as non-template controls were included in each PCR run, so as to check reaction efficiency and to exclude any contaminants' presence.

RESULTS

Screening for BTD deficiency among neonates

From March 2010 up to June 2011, 18 954 neonates born in Athens were subjected to massive biochemical determination of BTD activity through a semiquantitative fluoroimmunoassay. Values between $25 \text{ nmol min}^{-1} \text{ dl}^{-1}$ and $50 \text{ nmol min}^{-1} \text{ dl}^{-1}$ were obtained in 69 samples, which were further tested through molecular techniques to confirm the primary biochemical result.

Mutational analysis

The confirmatory molecular testing procedure of our study was conducted in two stages. During the first stage, 44 infants with values indicative of partial BTD deficiency were screened for the presence of point mutations or small insertions/deletions through sequencing of the coding region of the *BTD* gene, including splice site junctions. In the second stage, 25 'suspected' samples were screened for the four mutations most frequently encountered in the cohort of the 'suspected' samples of the first stage, through a rapid real-time based PCR genotyping assay developed in our laboratory.

Regarding the first stage of our molecular study, 19 of the 44 neonates found to carry a mutation in one allele associated either with profound or partial BTD deficiency. Specifically, nine were carriers of the rather common p.Asp444His mutation, which is associated with 50% decreased enzyme activity. The p.Asp444His mutation, in combination with a 'severe' aberration, results in partial BTD deficiency, as it seems to be the case in 3 out of the 44 samples (Table 1). Moreover, one sample of our primary cohort carried the aforementioned mutation in both alleles. The particular genotype has been designated to partial loss of enzyme activity ($\sim 25\%$ of mean serum activity) on the basis of the literature.^{12,13} The most common 'severe' alterations worldwide, p.Gln456His and p.Cys33PhefsTer36, were detected in 5 out of the 19 samples (p.Gln456His: BTD 2, 3 and 42; p.Cys33PhefsTer36: BTD 4 and 35).

Furthermore, a novel amino-acid change in codon 211, which resulted from the c.632G>A nucleotide change, was identified. Its effect on protein level has been evaluated through SIFT (http://sift.jcvi.org/www/SIFT_seq_submit2.html) and PolyPhen (<http://genetics.bwh.harvard.edu/pph/>) algorithms. According to the results of the above predictor tools, the p.Arg211His alteration is benign (Table 2). The Human Splice Finder (v.2.4) web tool (<http://www.umd.be/HSF/>) was also used to find out any potential effect of the particular nucleotide change (c.632G>A) on the splicing process. It is really interesting that the Human Splice Finder main algorithm implicates this nucleotide change in the abrogation of the splice donor site, as well as the fact that the other algorithms used for the prediction of enhancer/silencer motifs incorporated in the Human Splice Finder main page indicate that the c.632G>A not only induces the abrogation of enhancer-binding sites, but also the creation of a silencer motif (Table 2).

Finally, one nonsense, p.Gln88Ter, and two missense mutations, p.Val199Met and p.Thr532Met, have been identified in 4 out of the 19 carriers. Except from mutations, three silent polymorphisms were recorded, too (Table 3).

During the second stage of our study, 25 'suspected' samples were subjected to mutational analysis. Five newborns carried in heterozygosity and one in homozygosity of the p.Asp444His mutation, whereas two others were carriers of the p.Thr532Met and p.Cys33PhefsTer36, respectively (Table 4). The seven carriers detected by our rapid molecular screening method were further subjected to sequencing of the whole coding region of the *BTD* gene, to exclude the possible presence of a second aberration. However, no other mutation was detected in any of these carriers. All the above data are summarized in Tables 1 and 4.

Table 1 Germline mutations detected in the *BTD* gene in the first cohort of 'suspected' samples

Sample ID	Allele 1	Allele 2
BTD 2 ^a	Wild type	p.Gln456His
BTD 3 ^a	Wild type	p.Gln456His
BTD 4	Wild type	p.Cys33PhefsTer36
BTD 5	p.Cys186Tyr	p.Asp444His
BTD 7	Wild type	p.Thr532Meth
BTD 8	Wild type	p.Arg211His
BTD 9	Wild type	p.Val199Meth
BTD 11	Wild type	p.Val199Meth
BTD 13	Wild type	p.Asp444His
BTD 17	Wild type	p.Asp444His
BTD 18	Wild type	p.Asp444His
BTD 19	Wild type	p.Asp444His
BTD 20	p.Thr532Meth	p.Asp444His
BTD 21	Wild type	p.Asp444His
BTD 24	Wild type	p.Asp444His
BTD 25	Wild type	p.Gln88Ter
BTD 27	p.Arg157His	p.Asp444His
BTD 32	Wild type	p.Asp444His
BTD 33	Wild type	p.Asp444His
BTD 34	p.Asp444His	p.Asp444His
BTD 35	Wild type	p.Cys33PhefsTer36
BTD 41	Wild type	p.Asp444His
BTD 42	Wild type	p.Gln456His

Abbreviation: BTD, biotinidase.
Sequencing of exons 1–4.
Novel alterations written in bold.
^aTwins.

DISCUSSION

BTD deficiency is a metabolic disorder with variable phenotypic expression, as it seems to affect multiple organic systems. False-positive results might occur in the biochemical assay (screening procedure) for various reasons, such as infant's prematurity or mishandling of the sample before its arrival in the laboratory. However, mutational analysis seems to be able to readily discriminate between patients heterozygote for profound BTD deficiency and patients suffering from partial enzyme deficiency.^{2,4,16} The aforementioned facts motivated our laboratory to incorporate genetic testing as a second-tier screening step in the cohort of the 'suspected' samples.

In total, eight distinct germ line mutations, p.Asp444His, p.Gln456His, p.Cys33PhefsTer36, p.Thr532Met, p.Arg157His, p.Val199Met, p.Cys186Tyr and p.Arg211His, were identified in our cohorts of 'suspected' samples. The p.Arg211His has never been described before to the best of our knowledge. On the basis of the results of the *in-silico*

Table 3 Silent polymorphisms detected in the *BTD* gene

Sample ID	Polymorphisms
BTD 16	p.Leu215Leu
BTD 30	p.Cys471Cys
BTD 31	p.Cys471Cys, p.Ser181Ser

Abbreviation: BTD, biotinidase.

Table 4 Germline mutations detected in the *BTD* gene in the second cohort of our 'suspected' samples^a

Sample ID	Allele 1	Allele 2
BTD 48	Wild type	p.Asp444His
BTD 52	Wild type	p.Asp444His
BTD 53	Wild type	p.Asp444His
BTD 57	p.Asp444His	p.Asp444His
BTD 59	Wild type	p.Asp444His
BTD 62	Wild type	p.Cys33PhefsTer36
BTD 63	Wild type	p.Thr532Met
BTD 67	Wild type	p.Asp444His

Abbreviation: BTD, biotinidase.

^aChecking for the presence of p.Cys33PhefsTer36, p.Asp444His, p.Gln456His, p.Thr532Met.

Table 2 *In-silico* analysis of a novel mutation *BTD*, p.Arg211His

Methods of in-silico analysis	Result	Comments
SIFT score	0.43 → tolerated	If SIFT score < 0.05, then the aa substitution is predicted to affect protein function
PolyPhen	Benign (1.299)	If PolyPhen score > 2, then the aa substitution is predicted to affect protein function
Human Splice Finder v2.4 (HSF) (**)	'wt' SD broken (68.94/42.11)	
HSF Matrices		
ESE Finder	c.625 SC35 → variation (79.29/76.52) c.627 SF2/ASF (IgM-BRCA1) → site broken (76.0/0.0)	Scores predicted for the wt seq/score predicted for the mut seq (%)
EIEs from Wang <i>et al.</i> ¹⁴	c.628 → new site	
ESE motifs from HSF	c.632 (9G8) → site broken	
Silencer motifs from Sironi <i>et al.</i> ¹⁵	c.626 (motif 3) → variation (60.17/73.79)	

Abbreviation: HSF, Human Splice Finder.

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