

## Original Article

# Clinical, Biochemical and Genetic Analysis of Biotinidase Deficiency in Iranian Population

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## Abstract

**Background:** Biotinidase deficiency (BTD) is an autosomal recessive disorder of biotin metabolism. Biotin is a coenzyme that enhances the action of the four enzymes that play an important role in carbohydrates, amino acid, and fatty acid metabolism. Defects in these pathways cause severe metabolic disorder in the body. In general, biotinidase deficiency can be classified into two levels: partial and profound. The incidence of BTD is 1:40,000 to 1:60,000 births in the world, even though no convincing statistical data on the prevalence of this disorder exist in Iran. In this study, we aimed to set up a test for determining biotinidase activity among the Iranian population and report *BTD* mutations.

**Patients and Methods:** The quantitative method for the determination of biotinidase activity was set up in the National Biochemistry Reference Laboratory (NBRL) of Pasteur Institute of Iran in Tehran. To detect mutations in *BTD*, polymerase chain reaction (PCR) was performed followed by DNA sequencing.

**Results:** The biotinidase activity range values were 3.81 – 8.25 nmol/min/mL. We identified 8 BTD patients out of 47 cases with neurologic signs. We detected two mutations, c.98-104del7ins3 and p.Arg79Cys, in 5 patients with profound BTD, and one p.Asp444His mutation in 3 patients with partial BTD.

**Conclusion:** Infants suffering from BTD seem healthy during their first months of life. At present, the screening program for metabolic disorders such as BTD is in progress. The patients that are BTD deficient benefit from the availability of the tests, and consequently receive the Biotin supplements before being clinically affected.

**Keywords:** Biotinidase deficiency, BTD mutations, Iranian population

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## Introduction

Biotinidase deficiency (BTD) (OMIM 253260) is an autosomal recessive disorder associated with the absence of the biotin-recycling enzyme biotinidase.<sup>1,2</sup> Biotinidase is a mature protein consisting of 543 amino acids, and its encoding gene is located on chromosome 3p25 and consists of four exons and a cDNA with a total length of 1629 base pairs (bp).<sup>3-5</sup>

Biotin is an essential vitamin that serves as a coenzyme for four carboxylases (propionyl-COA carboxylase, 3-methylcrotonyl-COA carboxylase, pyruvate carboxylase, and acetyl-COA carboxylase) in humans. These enzymes play important roles in carbohydrate, amino acid, and fatty acid metabolism.<sup>6-9</sup> Defects in these pathways cause several disorders, including seizures, mental retardation, hypotonia, skin rash, alopecia, breathing abnormalities, ataxia, metabolic acidosis, hearing loss, eye

problems, and cellular immunological abnormalities that can lead to coma and death.<sup>10-12</sup> Biotin therapy can be an effective treatment to identify patients at birth, nowadays newborn screening for BTD is performed in many countries.<sup>13</sup>

If BTD remains untreated, young children with a profound BTD usually exhibit neurologic abnormalities, including seizures, hypotonia, ataxia, developmental delay, vision problems, hearing loss, and cutaneous abnormalities (e.g., alopecia, skin rash, and candidiasis). Older children and adolescents with profound biotinidase deficiency often exhibit motor limb weakness, spastic paresis, and decreased visual acuity. Once vision problems, hearing loss, and developmental delay occur, they are usually irreversible, even with biotin therapy. Individuals with partial biotinidase deficiency may have hypotonia, skin rash, and hair loss, particularly during times of stress.<sup>14</sup>

Based on the level of activity of the biotinidase enzyme in serum, patients could be classified into two levels: partial deficiency (10% – 30% enzyme activity) and profound deficiency (0% – 10% enzyme activity).<sup>15-17</sup>

According to previous studies, the incidence of BTD is 1:40,000 to 1:60,000 births in the world; however, in some countries, such as Saudi Arabia and Turkey, where there are high rates of consanguinity, this prevalence is higher.<sup>18-20</sup>

Up to now, no reliable statistical data exist on the BTD prevalence in Iran. Due to consanguineous marriages, it could be predicted that the prevalence of this disorder in Iran is higher than other

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countries. In this study, we aimed to set up tests for determining biotinidase activity and identify the disease-causing mutations in Iranian population for the first time.

## Patients and Methods

### Subjects

Patients who referred to the reference biochemistry laboratory of the pasture institute of Iran from April to December 2014 were included in this study. The consent forms were completed and signed by the children's parents. This study was designed and approved by the Ethics Committee of Pasture institute of Iran (No. 1754) and performed in accordance with its guideline.

To determine the reference range of biotinidase activity, serum specimens, and dried blood spots (DBS) were collected from 30 healthy adult volunteers. In addition, blood and DBS samples of forty-seven children with neurologic signs and their parents were collected. Family history, clinical symptoms, and informed consent were obtained from families.

### Chemicals and reagents

For the enzyme assay, Biotinyl-para aminobenzoic acid (B-PABA), Para aminobenzoic acid (PABA), Bovine Serum Albumin (BSA), N-(1-Naphthyl) ethylenediamine dihydrochloride monomethanolate (NEDD), Sodium Nitrite, and Ammonium Sulfamate were purchased from Sigma-Aldrich Co. Ltd (St. Louis, USA). Trichloroacetic acid, Dithiothreitol (DTT),  $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ , NaOH, EDTA- $\text{K}_2$  2H $_2\text{O}$  were obtained from Merck & Co. Inc (New York, USA). All reagents were of analytical grade, and all solutions were prepared with deionized-distilled water.

### Measurement of enzyme activity

Enzyme activity was measured by both qualitative and quantitative spectrophotometric methods.

### Quantitative method

A quantitative method was performed as previously described.<sup>21</sup> Biotinidase activity was measured in a final volume of 1 mL of a mixed reaction containing 50  $\mu\text{L}$  plasma, 0.4 M potassium phosphate buffer pH 6.0, 0.5 mM DTT, and either 0.15 mM or 1.5 mM B-PABA substrate (final concentrations in mixture reaction) that incubate at 30°C for 60 min.

The reaction was stopped by adding TCA (30%). After centrifugation (3000 rpm, 10 min), PABA was released into the supernatant and converted to a purple compound and quantified by measuring its absorbance at 546 nm. Biotinidase activity was

expressed as one nmol PABA released per minute in one milliliter of plasma (nmol/min/ml).

### Qualitative method

Qualitative method was performed as previously described.<sup>22</sup> Briefly, circles with 3 mm diameters from filter papers were added to cups then 30  $\mu\text{L}$  potassium phosphate buffer (50 mmol/L, pH 6.0) was added to each sample cups. The reaction cups were covered and incubated for 16 h in a humidified at 37°C. The reaction was terminated by the addition of 30- $\mu\text{L}$  trichloroacetic acid (1.84 mol/L) to each cup. For color development, 30  $\mu\text{L}$  sodium nitrite (14.5 mmol/L, freshly prepared) was added to the samples, followed by the addition of 30  $\mu\text{L}$  ammonium sulfamate (43.8 mmol/L) and N-1-naphthylethylenediamine dihydrochloride (3.86 mmol/L) at 3-min intervals. Color development was completed after 10 min. Samples with purple color were considered to have biotinidase activity, while straw-colored ones were considered to have little or no biotinidase activity.

### Polymerase chain reaction (PCR) and DNA sequencing

DNA was extracted from EDTA blood by using salting-out method, as previously described.<sup>23</sup> For PCR, 1  $\mu\text{L}$  genomic DNA was used as a template in a mixed reaction containing 10  $\times$  PCR Buffer (2.5  $\mu\text{L}$ ), 10 mM dNTPs (0.5  $\mu\text{L}$ ), 50 mM  $\text{MgCl}_2$  (0.75  $\mu\text{L}$ ), 100 pM each primer (1  $\mu\text{L}$ ), 1U AmpliTaq DNA Polymerase (0.2  $\mu\text{L}$ ), and distilled water up to 25  $\mu\text{L}$  final volume. The PCR primers used in this study are shown in Table 1. Exons 1, 2, and 3 were amplified as follows: an initial denaturation at 95°C for 5 min, followed by 32 cycles at 95°C for 45 s, 56°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. As exon 4 was long in length, two primer sets including 4a and 4b were designed. PCR amplification was conducted as follows: an initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 61.5°C for 30 s, and 72°C for 50 s, and a final extension at 72°C for 10 min. The PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN, Chatsworth, CA, USA), according to the manufacturer's instructions. DNA sequencing was performed as described previously<sup>24</sup> and results were evaluated using the Sequencing Analysis Software v 5.2 Patch 2 (Applied Biosystems, Foster City, CA, USA).

## Results

In this study, we measured the serum enzyme activity by qualitative and quantitative methods. Reference range values

**Table 1.** PCR primers used to sequence the *BTB* gene in patients affected by biotinidase deficiency

Exon	Primer	Product Size (bp)	Annealing Temperature (C°)
1	F: CGGTCTAAATTCGTCCACT R: GATTTAAGTAACGTGCGCT	553	53
2	F: CAGTACTACTGCGAGTGAGT R: AGGTAACTACCTGGATGCT	506	60
3	F: CAGAGTAACTTCCTGATGGT R: CCTTGTAACGTCAGACATTC	444	56
4a	F: GGTGGTCTCAATCTCCTGAC R: GTGGAGATAGCCTTCCTTTC	892	61
4b	F: AGTGGAACGTGAATGCTCCT R: CTGGGTGTCACTTGATCAAC	869	61

**Table 2.** Clinical signs and symptoms data of BTD patients, and biotinidase activity of their parents

Case	Sex	Age	Mother's Biotinidase Activity (nmol/min/ml)	Father's Biotinidase Activity (nmol/min/ml)	Clinical Signs and Symptoms	Neurological Signs	Consanguineous Parents	Ethnical Background
P1	F	12	2.46	3.42	Hearing loss, alopecia	Seizures	Yes	Fars
P2	F	3	5.01	5.17	Alopecia, optic abnormalities	Seizures	Yes	Torkaman
P3	F	6	3.71	3.66	- - -	- - -	No	Fars
P4	F	3	4.37	5.75	Skin rash, alopecia, immunological abnormalities	Seizures	No	Fars
P5	M	2.5	5.68	6.67	Optic abnormalities, immunological abnormalities	Seizures	Yes	Fars
P6	M	5	4.24	3.30	Hearing loss	Seizures	Yes	Fars
P7	F	3	3.48	4.19	Alopecia, optic abnormalities	Seizures	No	Lor
P8	M	3.5	6.10	5.58	Skin rash, alopecia	Seizures	No	Fars

**Table 3.** Biochemical and molecular characteristics of patients with biotinidase deficiency

Case	Biotinidase Activity (nmol/min/ml)	Mutation	Exon	Effect	Phenotype
P1	0.23	c.98-104del7ins3	2	p.Cys33PhefsX36	Profound
P2	0.05	c.98-104del7ins3	2	p.Cys33PhefsX36	Profound
P3	0.11	235C>T	2	p.Arg79Cys	Profound
P4	2.71	1330G>C	4	p.Asp444His	Partial
P5	2.53	1330G>C	4	p.Asp444His	Partial
P6	0.05	c.98-104del7ins3	2	p.Cys33PhefsX36	Profound
P7	0.05	c.98-104del7ins3	2	p.Cys33PhefsX36	Profound
P8	3.01	1330G>C	4	p.Asp444His	Partial

were 3.81 – 8.25 nmol/min/ml in the serum, with a mean value of  $6.03 \pm 1.11$  (1SD) nmol/min/ml in thirty healthy individuals. These data are in accordance with the reference range values declared in international resources.<sup>20</sup> After the standardization of experiment and method validation, we identified 8 BTD patients out of 47 that their plasma enzyme activity was in average  $1.09 \pm 1.37$  nmol/min/mL. The enzyme activity in the serum of patients' parents was in average  $4.54 \pm 1.19$  nmol/min/mL (Table 2). Clinical signs and symptoms of patients are illustrated in Table 2.

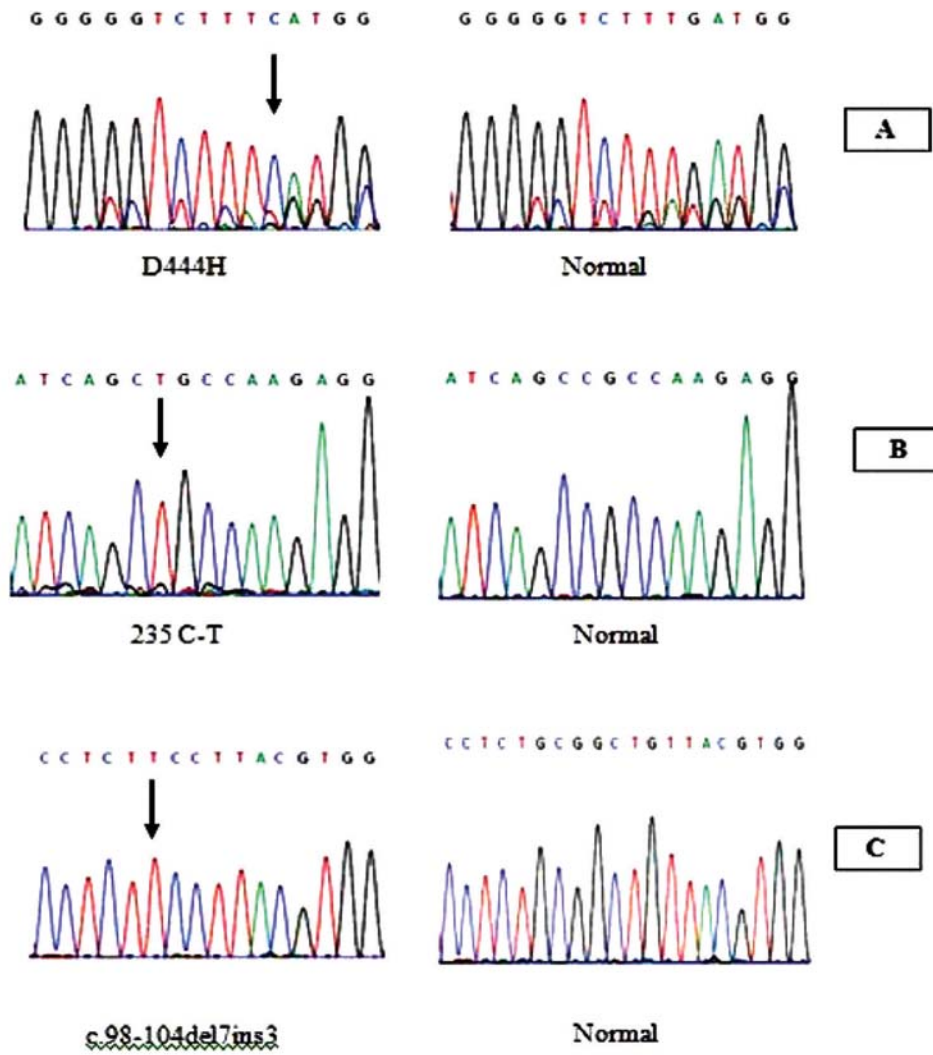
The presence of *BTD* mutations in the patients was assessed by PCR followed by DNA sequencing. The results are shown in Table 3. All patients were heterozygous for the mutations.

Four out of 8 BTD patients (P) possessed the c.98-104del7ins3 mutation, consisting in a deletion of 7 nucleotides (98 – 104) and an insertion of 3 nucleotides (TCC) in exon 2 that cause a frame shift in the enzyme sequence (Figure 1C). This group of patients had a biotinidase activity of about 0.05 nmol/min/mL, and profound BTD phenotype. In total, 3 patients (P4, P5, and P8) possessed the 1330G > C mutation, where the G to C substitution in exon 4b leads to Aspartic Acid to Histidine conversion (Figure 1A). These patients had a biotinidase activity of about 2.7 nmol/min/mL,

and a partial BTD phenotype. One patient possessed the 235C > T mutation, where the C to T substitution in exon 2 leads to Arginine to Cysteine conversion in a Biotinidase enzyme (Figure 1B). This patient had a biotinidase activity of about 0.11 nmol/min/mL, and profound BTD phenotype.

## Discussion

BTD is an autosomal recessive disorder due to the deficiency of biotinidase enzyme. Clinical signs such as vomiting, hypotonia, and seizures accompanied by metabolic keto-lactic acidosis or mild hyperammonemia are often observed in inherited metabolic diseases. Individuals with BTD may exhibit clinical features that are misdiagnosed as other disorders, such as isolated carboxylase deficiency, before they are correctly identified.<sup>25,26</sup> Other symptoms, more characteristic of biotinidase deficiency, (e.g., skin rash and alopecia), can also occur in children with a nutritional biotin deficiency, holocarboxylase synthetase deficiency, or essential fatty acid deficiency. Therefore, it is essential to carry out a differential diagnosis by determining enzyme activity and genetic testing.<sup>25</sup>



**Figure 1.** Definition of *BTBD9* mutations; **A)** The p.Asp444His mutation consists of the G to T substitution in exon 4b; **B)** The 235 C>T mutation consists of the C to T substitution in exon 2; **C)** The c.98-104del7ins3 mutation consists of the deletion of 7 nucleotides (98–104) and insertion of 3 nucleotides (TCC) in exon 2. Arrows indicate the site of mutations.

Patients affected by BTBD9 should be tested for biotinidase deficiency even if they do not exhibit any symptom.<sup>14</sup> The incidence of BTBD9 in Iran is high due to a high prevalence consanguineous marriages. In this study, we surveyed patients both biochemically and genetically and provided the information concerning enzyme activity and mutation analysis in addition to clinical manifestations. These profiles allow analyzing the effect of each mutation on the clinical symptoms and enzyme activity in each case. Similar to previous reports, in the current study the enzyme activity deficiency was classified in two manners, including partial or profound deficiency that are useful to analyze the pathogenicity level of each mutation.

In the early stages of our study, 47 patients with neurological symptoms were examined for enzyme activity. We found that 8 out of 47 patients were biotinidase deficient, and their biotinidase activity was in average  $1.09 \pm 1.37$  nmol/min/mL. After molecular analyses, we identified three types of mutations that already observed in previous studies.<sup>27</sup>

The c.98-104del7ins3 and p.Arg79Cys mutations were detected in 5 patients with profound BTBD9, while the p.Asp444His mutation

was found in 3 patients with partial BTBD9. All patients enrolled in our study were homozygous for BTBD9, maybe due to the high rate of consanguineous marriages in Iran. Previous Iranian report by Khalilian, et al. detected the c.98-104del7ins3 mutation in 3 months years old girl with the BTBD9 profound deficiency. This mutation is a deletion/insertion mutation in exon 2 causing to a frameshift. Four out of eight patients had the c.98-104del7ins3 mutation with the profound enzyme deficiency.<sup>28</sup>

In contrast, in U.S. patients, the most common cause of profound BTBD9 is the double mutation p.Ala171Thr and p.Asp444His.<sup>29</sup> In another study conducted in Turkey, the most common BTBD9 mutation was the c.98-104 del7ins3, which is consistent with our results.<sup>30</sup>

In our study, we observed the p.Asp444His mutation in patients with partial BTBD9 phenotypes (P4, P5, and P8). This result is consistent with studies conducted in other parts of the world.<sup>27,30,31</sup>

All patients except one case (P3) showed the clinical symptoms, including seizures, and cutaneous abnormalities (skin rash, alopecia) that are common in biotinidase deficiency. Although, patient No. 3 had no symptom; she suffered from profound

biotinidase deficiency. It has been mentioned that the type of her mutation differed from the rest of the patients. It inherited the mutation, 235C > T at exon 2. In addition, she was diagnosed at birth and subjected to early biotin treatments. Furthermore, the immunological abnormality was only among children with partial deficiency, due to the p.Asp444His mutation.

According to our knowledge, except to one case study, there are no reports from the type and frequency of mutations leading BTM from Iran and ours is the first findings. However, there are some limitations in the current study. The small sample size and single center study is the most momentous. Moreover, the sequencing data from the parents are not available.

In conclusion, screening the biotinidase deficiency by both enzyme activity measurement and sequencing is momentous for early diagnosis and determination of carriers. Furthermore, the follow-up after confirmation of diagnosis is also very important. We need international guidelines to set up a relevant and accurate process (i.e., diagnosis, treatment, and follow up). For determination of genetic risk, clarification of carrier status, and discussion of the availability of prenatal testing, biotin deficiency tests should be carried out before pregnancy. In the future, it would be appropriate to offer to young adult carriers of BTM mutations, or at risk of being carriers of BTM mutations, the possibility to participate to newborn screening programs.

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