JIMD Reports
DOI 10.1007/8904_2015_447

RESEARCH REPORT

High Incidence of Biotinidase Deficiency from a Pilot Newborn Screening Study in Minas Gerais, Brazil

Marilis T. Lara • Juliana Gurgel-Giannetti • Marcos J.B. Aguiar • Roberto V.P. Ladeira • Nara O. Carvalho • Dora M. del Castillo • Marcos B. Viana • José N. Januario

Received: 06 March 2015 / Revised: 12 April 2015 / Accepted: 20 April 2015 / Published online: 13 May 2015

Abstract *Objective*: To assess the incidence of biotinidase deficiency among newborns and their clinical outcome up to one year of age in a large pilot screening study in Minas Gerais, Brazil.

Methods: A prospective cohort study was conducted from September 2007 to June 2008 with heel-prick blood samples collected on filter paper for the purpose of newborn screening. A qualitative colorimetric test was used as the primary screening method. Colorimetric-positive cases were further tested with a serum confirmatory assay. Gene sequencing was performed for eight children suspected with biotinidase deficiency and for some of their parents. Positive cases were daily supplemented with oral biotin and were followed up for approximately six years.

Results: Out of 182,891 newborns screened, 129 were suspected of having biotinidase deficiency. Partial deficiency was confirmed in seven children (one was homozygous for p.D543E) and profound deficiency in one child (homozygous p.H485Q). Thus the incidence was one in 22,861 live births (95% confidence interval 1:13,503 to 1:74,454) for profound and partial biotinidase deficiency combined. Two novel mutations were detected: p.A281V and p.E177K. In silico analysis and estimation of the enzyme activity in the children and their parents showed that p.A281V is pathogenic and p.E177K behaves like p.D444H.

Conclusion: The incidence of biotinidase deficiency in newborn screening in Minas Gerais was higher than several international studies. The sample size should be larger for final conclusions. Oral daily biotin apparently precluded clinical symptoms, but it may have been unnecessary in some newborns.

Communicated by: Georg Hoffmann

Competing interests: None declared

M.T. Lara
UFMG University Hospital, NUPAD – Center for Newborn Screening and Genetic Diagnostics, UFMG Federal University of Minas Gerais, Belo Horizonte, Brazil

J. Gurgel-Giannetti · M.J.B. Aguiar · M.B. Viana
Department of Pediatrics, NUPAD – Center for Newborn Screening and Genetic Diagnostics, UFMG Federal University of Minas Gerais, Belo Horizonte, Brazil

R.V.P. Ladeira · N.O. Carvalho · D.M. del Castillo
NUPAD – Center for Newborn Screening and Genetic Diagnostics, UFMG Federal University of Minas Gerais, Belo Horizonte, Brazil

J.N. Januario (✉)
Department of Medicine, NUPAD – Center for Newborn Screening and Genetic Diagnostics, UFMG Federal University of Minas Gerais, Av. Alfredo Balena 189, sala 1004, 30130-100, Belo Horizonte, Brazil
e-mail: nelio@nupad.medicina.ufmg.br; josenelio@gmail.com

Introduction

Biotinidase (E.C. 3.5.1.12) is the enzyme that recycles biotin. Biotinidase deficiency (BD; OMIM #253260 and *609019) is an autosomal recessive and inherited metabolic disorder with a varied phenotypic expression. The disorder is classified into two types according to the serum enzyme activity relative to a reference value: profound deficiency (up to 10%) and partial deficiency (from 10% to 30%) (Mcvoy et al. 1990; Wolf 2001). The biotinidase gene (*BTD*) has been mapped to the chromosome 3p25. Approximately 200 variants have been recognized (UTAH BTD Database 2013). The most prevalent mutation in the

world (allele frequency around 0.039) is c.1330G>C (p.D444H) that results in half of the activity of the enzyme produced by the wild-type gene (Swango et al. 1998). Most individuals with partial BD are compound heterozygotes, having the c.1330G>C mutation in combination with another mutation that causes severe deficiency of the biotinidase activity (Swango et al. 1998).

Profound BD usually manifests as neurological and skin disorders after the age of seven weeks. Patients who are not treated early enough often have hearing and visual impairments as well as motor and language delays. Simple and low-cost supplementation with oral biotin (5–20 mg daily for life) prevents clinical manifestations if provided early in life (Wolf 2001).

The prevalence of BD in Minas Gerais, Brazil, is unknown. The state has approximately 20 million inhabitants, and 240,000 newborns are tested yearly. The state has been performing newborn screening tests since 1994 for phenylketonuria, congenital hypothyroidism, cystic fibrosis, and sickle cell disease. Only in 2014, BD was included in the mandatory panel of screened diseases.

This pilot study aimed to evaluate the incidence of BD in the Newborn Screening Program of Minas Gerais (NSP-MG). All of the suspected cases were supplemented with daily biotin and were referred for further laboratory and clinical follow-up evaluations.

Methods

A prospective cohort study was conducted on 182,942 newborns from September 2007 to June 2008. Possible partial or profound cases of BD were referred for biotin supplementation therapy (Wolf 2010) before the DNA analysis was available, and they have been maintained on biotin therapy to date. Mutation analyses were performed on DNA from the whole-blood samples of children suspected with BD and on DNA from the whole-blood samples of their parents in 2014–2015. The biochemical and molecular tests were performed at the laboratories of the Center for Newborn Screening and Genetic Diagnostics (Nupad-UFMG).

Dried blood samples (DBS) were collected on filter paper (Schleicher & Schuell 903) when the newborns were approximately five days old, which was in compliance with the NSP-MG protocol. Umtest Biotinidasa Test® (Tecnosuma) was used as the primary qualitative screening test (González et al. 2006). It was repeated on another DBS sample if positive. The quantitative serum test for the biochemical diagnosis was performed on the 120 newborns with positive results in screening tests, as described by Cowan et al. (2010). In 2014–2015, a second and third serum sample was drawn from five newborns and their parents (Table 1), and the biotinidase activity was determined again. The mean reference value at Nupad Laboratory is 7.2 nmol/min/mL. Accordingly, results below 0.8 nmol/min/mL (10% of the mean) were considered to be a profound deficiency; from 0.8 to 2.1 (10–30%), partial deficiency; from 2.2 to 5.1, suggestive of a heterozygous state; and results above 5.1 nmol/min/mL were considered "normal."

Mutation analyses were performed on the DNA of eight newborns with deficient biotinidase activity and on that of the parents of five newborns (see Table 1). Genomic DNA was extracted from peripheral blood lymphocytes with Chelex 100, Molecular Biology Grade Resin, Bio-Rad (Walsh et al. 1991).

DNA Sequencing

Exons 1–4 of the *BTD* gene as well as their flanking regions were amplified by polymerase chain reaction (PCR). Specific primers are available on request and were designed according to published papers (Pomponio et al. 1997; Thodi et al. 2011; Mühl et al. 2001).

Electrophoresis was performed on an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems). Analysis was based on the NCBI Reference Sequence NG_008019.1.

This study was approved by the UFMG institutional review board and the Minas Gerais State Health Administration.

Results

Of the 182,891 newborns who had satisfactory DBS (51 were excluded because the samples were unsatisfactory and the patients could not be found for another blood collection), 129 were suspected of BD based on the qualitative tests. BD was confirmed in ten of them by a quantitative serum test. Two newborns were excluded from the study because their families declined to participate during its molecular phase. Out of these eight cases, seven had partial and one a profound BD. Table 1 shows the quantitative tests and respective mutations.

Thus, the combined incidence of partial and profound BD based on these eight cases was 1:22,861 live births (95% confidence interval 1:13,503 to 1:74,454). The NSP-MG population coverage during the study period was 94.8% of all live births in the state.

Considering the initial group of ten newborns with BD, detected in the first quantitative test, their mean age for the first clinical appointment was 71 days (range, 30–285 days),

Table 1 Biochemical and molecular features of patients detected with biotinidase deficiency

	Sex	BTD activity (nmol/min/ml)	Allele 1 (protein change)	Allele 2 (protein change)	Deficiency classification
Child 1	M	0.7, 0.7	c.1455C>G (p.H485Q)	c.1455C>G (p.H485Q)	Profound
Mother	F	3.6, 3.3	c.1455C>G (p.H485Q)	Wild type	
Child 2	F	1.4, 1.7	c.842 C>T (p.A281V)[a]	c.1330G>C (p.D444H)	Partial
Mother	F	3.4	c.842 C>T (p.A281V)	Wild type	
Father	M	4.8	c.1330G>C (p.D444H)	Wild type	
Child 3	F	1.5, 1.4	c.1330G>C (p.D444H)	Wild (?) type	Partial
Mother	F	5.4	c.1330G>C (p.D444H)	Wild type	
Father	M	4.9	Wild type	Wild (?) type	
Child 4	F	2.0	c.529 G>A (p.E177K)[b]	c.1595C>T (p.T532M)	Partial
Mother	F	3.9	c.1595C>T (p.T532M)	Wild type	
Father	M	5.1	c.529 G>A (p.E177K)	Wild type	
Child 5	M	1.0, 1.2	c.1629C>A (p.D543E)	c.1629C>A (p.D543E)	Partial
Mother	F	3.1	c.1629C>A (p.D543E)	Wild type	
Father	M	4.6	c.1629C>A (p.D543E)	Wild type	
Child 6	F	1.8	c.511G>A;1330G>C (p.A171T; p.D444H)	c.1330G>C (p.D444H)	Partial
Child 7	F	1.8	c.511G>A;1330G>C (p.A171T; p.D444H)	c.1330G>C (p.D444H)	Partial
Child 8	M	1.8, 1.8	c.1489C>T (p.P497S)[c]	c.1330G>C (p.D444H)	Partial

Wild (?) = coding and splicing regions of *BTD* gene corresponded to the wild sequence, but promoter and intronic regions were not completely sequenced

[a] Newly described mutation predicted to affect protein function (see text)

[b] Newly described mutation predicted *not* to affect protein function (see text)

[c] A silent variant [c.1284C>T (Y428Y)] was also detected together with the pathogenic variant p.P497S, as reported by Wolf et al. (2005)

the mean age for diagnosis confirmation was 103 days (range, 38–258 days), and the mean age for starting biotin supplementation was 135 days (range, 60–300 days).

The mutation analysis (Table 1) detected two homozygous newborns: p.D543E/p.D543E with consanguineous parents and p.H485Q/p.H485Q without consanguineous parents. The first child was classified as partially deficient. His enzyme activity corresponded to 17% of the mean reference value. The second child was classified as profoundly deficient (9.7% of the mean reference value). Five double heterozygotes were detected: two patients with p.A171T;D444H/p.D444H, one with p.281V/p.D444H, one with p.P497S/p.D444H, and one with p.E177K/p.T532M. Child #3 (Table 1) was classified as having partial BD on the basis of biotinidase activity of 1.5 and 1.4 nmol/min/mL in the first and second serum samples, respectively. Only p.D444H was detected in the child and her mother. Her father's biotinidase activity was within the heterozygous range of values, although apparently having two wild alleles. Promoter and intronic regions of the *BTD* gene were not completely sequenced, and thus it is possible that he has a "hidden" pathogenic mutation.

Two novel mutations were identified, i.e., p.281V and p.E177K. The first one (child # 2, Table 1) was predicted to be pathogenic by SIFT (http://sift.bii.a-star.edu.sg/) and by estimation of the enzyme activity in the heterozygous mother and in the double heterozygous child (with D444H). The predicted value was zero. The second mutation (p.E177K) was predicted NOT pathogenic by SIFT. The estimated enzyme activity was 42%.

Up to one year of age, all newborns underwent normal clinical and neurological evaluations prior to and after oral biotin supplementation (10 mg daily).

Discussion

The combined incidence of BD in newborns in this pilot study was 1:22,861 live births. The frequency rates of BD reported in the literature are variable. Based on 36 pilot newborn screening programs conducted from January 1984 to December 1990 (Wolf and Heard 1990; Wolf 1991) in a sample of 8,532,617 newborns, the following figures were reported: profound BD, 1:112,271 live births; partial BD, 1:129,282 live births; and combined BD incidence, 1:60,089 live births. The combined incidence of BD was 1:47,486 out of 1,321,989 newborns in nine European countries (Loeber 2007). Thus, the incidence found in the current study was higher than that observed worldwide and in many countries. Nevertheless, pilot newborn screening

studies in isolated regions or selected groups have disclosed higher incidences (1:4,500 to 1:14,000) than that reported in the present research (Thodi et al. 2013; Dunkel et al. 1989; Lawler et al. 1992; Sarafoglou et al. 2009).

In Brazil, the available prevalence rates are divergent. A study in the State of Paraná (South) showed a combined prevalence of BD of 1:62,500 live births and 1:125,000 for partial BD (Pinto et al. 1998). The highest incidence (1:9,000) was reported by a private laboratory study (Neto et al. 2004) that reported the results from samples received from several Brazilian regions. The authors recognized several problems with the samples received for the serum quantitative tests. Although the epidemiological data are quite different, it is possible that regional variations in a large country like Brazil explain such divergent results.

The most common mutation was D444H, which was present in seven of the 16 alleles studied, as has been reported by almost all investigators, including a recent Brazilian genetic study on BD (Borsatto et al. 2014). Two novel mutations were detected. The pA281V mutation was observed in compound heterozygosity with a D444H mutation in a girl. The mutation segregated with the parents. Biotinidase activity of the mutant enzyme was estimated to be zero, according to the activity data from the child and her parents. Furthermore, SIFT analysis also suggested a pathogenic variant. The E177K mutation was described in compound heterozygosity with p.T532M. Allele-independent segregation was also demonstrated in this case. The enzyme activity of T532M, considered to be pathogenic in a homozygous child derived from a newborn screening program (Norrgard et al. 1999; patient No. 415), was reported to be 0.6 nmol/min/mL (8.4% of the mean reference value for the laboratory). The estimated activity for this mutant in the present study was also 0.6 nmol/min/mL (8.3% of the mean reference value in our laboratory). The estimated activity of biotinidase for the E177K was quite similar to that usually estimated for the D444H enzyme. The pathogenic nature of the p.H485Q mutation has been considered to be uncertain (UTAH BTD Database 2013) because no enzyme activity and clinical information were available in a patient with a compound H485Q/A171T_D444 mutation. The homozygous p.H485Q in the present report was associated with a profound BD; thus, it should be considered pathogenic.

The D543E mutation has been previously assigned as pathogenic when compounded with p.Q456H or c.587delC in two Hispanic children (Cowan et al. 2012), both with profound BD. At the UTAH BTD Database (2013), it was compounded with p.D444H and resulted in a biotinidase activity level of 2.4 nmol/min/mL. A Brazilian study also reported a child with the same double heterozygosity and activity level of 2.6 nmol/min/mL (Borsatto et al. 2014; patient No. 18). If a mean reference value of 7.2 nmol/min/mL is adopted and p.D444H is considered to have half the activity of the wild enzyme, the activity of p.D543E would be 16.7% and 22.2%, respectively. The estimated value in the homozygous child in the present report was 16.7%. Thus, the present report confirmed the pathogenicity of p.D543E which should be considered a mutant that leads, when homozygous, to a severe partial biotinidase deficiency.

In conclusion, this study describes a pilot newborn screening study that estimated an incidence rate of 1:22,861 live births for partial and profound BD combined. This incidence rate was higher than that reported in several large studies worldwide. The cases were asymptomatic prior to the start of biotin supplementation and remained so up to one year of follow-up examinations. Two novel mutations were detected: one pathogenic (p.A281V) and the other (p.E177K) with biotinidase activity similar to D444H. Two already registered mutations were confirmed as pathogenic (p.H485Q and p.D543E).

Acknowledgments The authors gratefully acknowledge the technical and scientific staff of the Center for Newborn Screening and Genetic Diagnostics (Nupad/UFMG) for their involvement and logistical support. The contribution of the technologist Daniela Magalhães Nolasco was essential for the standardization of the biotinidase serum analysis. Marcos Antunes Lopes, Lívia Uliana, and their teams collaborated for the successful recall of the families for confirmatory tests. The authors also wish to thank Nupad and Fapemig for their financial support. Marcos Borato Viana received a researcher grant from CNPq (Brazilian National Council for Research).

Synopsis

This extensive pilot study showed a high incidence (1:22,861) of biotinidase deficiency in Brazilian newborns through a three-phase laboratory procedure: a colorimetric screening test, confirmatory determination of serum biotinidase activity, and gene sequencing (two novel mutants were detected).

Compliance with Ethics Guidelines

Conflict of Interest

The authors declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent

was obtained from all patients for being included in the study.

Details of the Contributions of Individual Authors

Study concept and design: Januario, Gurgel-Giannetti, Lara; Acquisition of data: Lara, Ladeira; Analysis and interpretation of data: Lara, Januario, Viana, del Castillo; Drafting of the manuscript: Lara, Gurgel-Giannetti; Critical revision of the manuscript for important intellectual content: Januario, Viana, Gurgel-Giannetti, Aguiar; Molecular and Biochemical analysis: Ladeira, Carvalho, del Castillo; Final proofreading: Januario, Viana; Paper Guarantor: Januario

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