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RESEARCH REPORT

Prevalence of Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase Deficiency in Estonia

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Abstract The aim of our study was to evaluate the prevalence of long chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) in the general Estonian population and among patients with symptoms suggestive of fatty acid oxidation (FAO) defects. We collected DNA from a cohort of 1,040 anonymous newborn blood spot samples. We screened these samples for the presence of the common c.1528G>C mutation in the *HADHA* gene. Based on the clinical suspicion of FAO defects, we screened suspected individuals since 2004 for the common c.1528G>C mutation in the *HADHA* gene and since 2008 in addition by tandem mass spectrometric analysis of plasma acylcarnitines. Our results showed that the carrier frequency of the c.1528G>C mutation in the Estonian population is high – 1:173. During the screening of symptomatic patients, we identified five LCHADD patients in four families. Three patients were retrospectively identified by molecular screening of the *HADHA* gene. One patient was homozygous for the c.1528G>C mutation in the *HADHA* gene, and two siblings were compound heterozygotes with *HADHA* genotype c.[1528G>C]+[1690-2A>G]. Among patients tested using acylcarnitine profiling, we identified two cases with an abnormal acylcarnitine profile typical to LCHADD. Molecular analysis showed homozygosity for c.1528G>C mutation. Based on a carrier frequency of 1:173 (95% Confidence Interval 1:76–1:454) and taking into account that the c.1528G>C mutation makes up 87.5% of disease alleles in Estonian LCHADD patients, the estimated prevalence of LCHADD in Estonia would be 1: 91,700.

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Abbreviations

LCHADD	Long chain 3-hydroxyacyl-CoA dehydrogenase deficiency
FAO	Fatty acid oxidation

Introduction

Fatty acids are the main source of metabolic energy in humans. Beta-oxidation, a major energy producing pathway in metabolically active tissues such as skeletal muscles and myocardium, also plays an essential role during periods of fasting and metabolic stress (Carpenter et al. 1992a; Tyni and Pihko 1999).

Mitochondrial inner membrane protein complex, the trifunctional protein (TFP) consists of 4 α-subunits harboring

long chain enoyl-CoA hydratase, long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) activity, and 4 β-subunits carrying long chain 3-ketothiolase activity (Kamijo et al. 1994; Carpenter et al. 1992a; Carpenter et al. 1992b). The TFP α-subunit is encoded by the *HADHA* gene (OMIM*600890) and the β-subunit by the *HADHB* gene (OMIM 143450), both of which lie on chromosome 2p23. In rare cases, all three functions of TFP are deficient, but isolated LCHAD deficiency in which hydratase activity is still normal and thiolase activity has decreased moderately is more common (Jlst et al. 1994).

LCHAD deficiency (LCHADD) is caused by mutations in the *HADHA* gene. More than 30 *HADHA* gene variations have been described to date (Gregersen and Olsen 2010), among them c.1528G>C (p.E510Q; NM_000182.4), which is by far the most common mutation, accounting for 87% of all LCHAD alleles. This missense mutation results in the substitution of glutamate to glutamine at amino acid position 510. As this change is located in the catalytically active region, it deteriorates the dehydrogenase activity of the α-subunit protein (Sims et al. 1995; Ijlst et al. 1996).

Isolated LCHADD is an autosomal recessive disease first described in 1989 by Wanders et al. Clinical manifestations of LCHADD can vary from early-onset cardiomyopathy, hypoglycemia, hepatopathy, coma, and sudden infant death syndrome (SIDS) to later onset myopathy, neuropathy, and pigmentary retinopathy (den Boer et al. 2002; Saudubray et al. 1999). Due to the variable clinical spectrum of the disease, it is not easy to differentiate isolated LCHADD from total TFP insufficiency or from other long chain fatty acid oxidation (FAO) disorders. The diagnosis of LCHAD or TFP deficiency is suggested by the finding of characteristic organic acid urine profiles or by an abnormal acylcarnitine profile (Matern 2008). There is generally high morbidity and mortality, and therefore prompt diagnosis at an early age is critical (den Boer et al. 2002). Early recognition and correct treatment are essential in order to lower the mortality rate and alleviate the metabolic crisis.

Although it is acknowledged that LCHADD is a rare but serious life-threatening disease, prevalence of LCHADD has been published for only a few countries, e.g., Sweden 1: 50,000 (Hagenfeldt et al. 1995) and Germany 1: 170,000 (Sander et al. 2005). The incidence of LCHADD by the combined data of the newborn screening programs of Australia, Germany, and USA is 1:250,000 (Lindner et al. 2010). The carrier frequency of the common LCHAD mutation, c.1528G>C, in the overall population is known in only a few populations, and the data show great differences, with 1:217 in Poland (Piekutowska-Abramczuk et al. 2010), 1:240 in Finland (Tyni and Pihko 1999) and zero in the Chinese population (of 1,200 individuals screened) (Zhu et al. 2005).

The main aim of this study was to evaluate the prevalence of LCHADD in the general Estonian population using readily available blood samples collected from newborns and among patients suspected of suffering from FAO defects. The clinical course and outcome in the first reported Estonian patients is described.

Material and Methods

Definition of Study Groups

Group of Anonymous Newborns (N = 1,040)

We collected DNA from a cohort of anonymous newborn samples. These neonates were consecutively born in Estonia in January 2005. We screened these samples for the presence of the c.1528G>C mutation in the *HADHA* gene. DNA was obtained from samples of dried blood spotted on Guthrie cards that had been collected by the Estonian newborn screening program for the detection of cases of phenylketonuria and congenital hypothyreosis. About 90% of newborns born during this month in the whole Estonia were covered.

Study Group of Symptomatic Patients with Suspicion of FAO Defects from the Period 2004–2007 (N = 102)

The blood samples from all individuals were sent to molecular laboratory for both LCHAD and MCAD deficiency from different hospitals in Estonia during 2004–2007. All individuals had clinical suspicion of FAO, but detailed clinical descriptions were not available for us. All were molecularly tested for the common c.1528G>C *HADHA* mutation that causes LCHADD. In some cases, in addition to the molecular analysis (including heterozygotes for c.1528G>C mutation found among general population), acylcarnitine profiling through tandem mass spectrometric (tandem MS) analysis was performed using dried blood spots collected on a Guthrie card in the Charité-Virchow Klinikum (Berlin, Germany).

Study Group of Symptomatic Patients with Clinical Suspicion of FAO Defects from the Period 2008–2010 (N = 425)

Since 2008, all patients with clinical symptoms of FAO defects were first investigated by tandem MS analysis of acylcarnitines from plasma. The indication list for studying plasma acylcarnitine profiles was developed and adapted by Duran (2003). If a child had at least one feature from

the indication list, plasma acylcarnitine analysis was performed. All investigated patients were selected from two regional hospitals (Tartu University Hospital and Tallinn Children's Hospital), which serve as reference centers for the whole of Estonia (which has a population of about 1.34 million). Patients with abnormal acylcarnitine profile were secondly molecularly tested for the common c.1528G>C *HADHA* mutation that causes LCHADD.

This study was approved by the Ethics Committee on Human Research of the University of Tartu.

Molecular Analysis

DNA Extraction

Patients' DNA was extracted from peripheral blood by the standard salting out method or from newborn screening test cards. DNA was extracted from 3 mm disc of the test card, which was soaked in 1 ml of distilled water for at least 2 h, with constant vigorous swirling of the tubes. Then the supernatant was discarded, 100 µl of methanol was added under a ventilation hood, and samples were incubated for 15 min at room temperature. In the next step, the methanol was discarded and 100 µl of freshly prepared 5 mM NaOH and 20–50 µl of mineral oil was added. Then samples were incubated at 100°C for 15 min and immediately placed on ice. The obtained DNA solution can be stored briefly at +4°C or at −20°C for years. This extraction method is cost-effective and also quite robust, as the DNA can easily be used for other PCR-based applications for at least 5 years (Teek et al. 2010; Laugesaar et al. 2010).

Mutation Detection in the HADHA Gene

For c.1528G>C detection, a slight modification of the PCR–RFLP method and primers described by Den Boer (den Boer et al. 2002) were used. PCR was carried out in 96-well plates with a total volume of 10 µl. We used a higher Mg_2Cl concentration (2.5 mM) than that described in the article by den Boer et al., i.e., 1.5 µl of DNA solution and 0.65U HOT FIREPol® DNA Polymerase (Solis BioDyne, Estonia) per reaction. In addition, for test card DNAs the number of PCR cycles was increased from 30 to 35 cycles. The amplified 224 bp PCR products were directly digested in the 96-well plate after adding 1.2 µl of Buffer O and 8U of restriction enzyme *Pst*I (Fermentas; Lithuania). The restriction fragments of 175 bp and 49 bp in wild-type alleles and 117 bp, 58 bp and 49 bp in mutant alleles were analyzed on a 2% (wt/vol) agarose gel with ethidium bromide staining.

HADHA Gene Sequencing

The 20 exons that make up the *HADHA* gene and nearby intronic elements were sequenced as described by Olpin et al. (2005).

Frequency of the HADHA c.1690-2A>G Mutant Allele

Genomic DNA isolated from 59 anonymized dried blood-spots collected from the general Danish population was used to determine the frequency of the *HADHA* c.1690-2A>G mutant allele by sequence analysis of a 355 bp PCR fragment covering exon 17 and nearby intronic elements. The study was performed with the approval of the Central Denmark Regional Committee on Biomedical Research Ethics.

Acylcarnitine Profiling of Plasma Samples

Acylcarnitines were analyzed as butylesters. Sample preparation was performed using standard methods (Matern 2008). About 20 µl of plasma was extracted, with 100 µl of methanol containing the deuterated internal standards and 300 µl acetonitrile was used to sediment plasma proteins. Samples were centrifuged at 4°C for 3 min, and the supernatants were removed. After evaporation, the extracts were butylated using 60 µl butanol/HCl at 65°C for 15 min. The samples were dried again on SpeedVac and finally dissolved in 100 µl acetonitrile/H_2O/formic acid (80:20:0,025, respectively). About 25 µl of the samples was injected into tandem MS (3200 Qtrap, Applied Biosystems MDS Sciex, Canada). Acylcarnitines were measured as positive precursor ion scan for the 85 Da fragment. Quantification was performed using labeled carnitine standards set B from Cambridge Isotopes Inc (MA, USA).

Clinical Data

Clinical data of diagnosed LCHADD cases were obtained from case histories.

Cases 1 and 2 (Family 1)

The first child in this family (a girl, Case 1) died in 1987 at the age of 3.5 months at the local hospital due to cardiomyopathy (no metabolic investigations or autopsy were performed).

The fourth child (a boy, Case 2) was born in 1995. He had his first acute attack of unconsciousness and muscular hypotonia at the age of 9 months. The child had delayed motor development and he needed frequent meals. He was

repeatedly hospitalized due to lethargy and hypoglycemia, additionally liver dysfunction and dilatative cardiomyopathy were observed. At the age of 2.5 years, he referred due to severe hypoglycemia (0.6 mmol/l), lactic acidemia (up to 5.0 mmol/l), and coma. Despite treatment, the child died 8 h after hospitalization. The autopsy showed cardiomyopathy and liver steatosis.

Case 3 (Family 2)

The boy was born in 2007. The mother's obstetric history was complicated: severe hepatic and renal dysfunction had been observed during the first pregnancy. The family's first child died during labor. The course of this pregnancy was also complicated: severe HELLP syndrome was diagnosed during the 28th week of pregnancy. Due to complications in the pregnancy, a caesarean section was performed on the 28th week of gestation. At the age of 6 months, the child was hospitalized. He was pale and weak, clinically presenting with symptoms characteristic of an upper respiratory illness. His blood glucose was slightly over the reference range of 6.3 mmol/l (reference 3.3–5.5 mmol/l) and the infusion with 0.9% NaCl solution was initiated. Six hours after hospitalization and hypoglycemia (0.2 mmol/l) was observed. The child died 10 h after hospitalization.

Case 4 (Family 3)

At the age of 13 months, a girl (born in 2009) became lethargic during an acute viral infection. Hypoglycemia (glucose 2.4 mmol/l), hepatic dysfunction, and increased serum lactic acid (5.8 mmol/l; ref. <2.2) were observed. A diagnosis of LCHADD was later confirmed. A low fat and high carbohydrate treatment was initiated.

Case 5 (Family 4)

The boy (born in 2010) had muscular hypotonia and frequent vomiting since newborn age. At the age of 5 months, he was hospitalized due to somnolence, mild diarrhea, and vomiting. In the hospital generalized muscular hypotonia, hepatomegaly and liver dysfunction was observed. Diagnosis of LCHADD was later confirmed. Dietary treatment with carnitine supplementation (dose 50 mg/kg/die) was initiated, and the child's condition rapidly improved.

Results

Frequency of the c.1528G>C *HADHA* Mutation in the General Estonian Population

We tested 1,040 anonymous Estonian neonates from the general population who were consecutively born in 2005 for the presence of the c.1528G>C mutation in the *HADHA* gene. We detected six heterozygotes for the c.1528G>C mutation, and no c.1528G>C homozygotes. The acylcarnitine profile of all heterozygous individuals was measured from a newborn Guthrie card in the Charité-Virchow Klinikum (Berlin, Germany). None had biochemical abnormalities suggestive of LCHADD. This indicates a carrier frequency of 1 in 173 (95% Confidence Interval 1:76–1:454; Table 1).

Results of Selective Screening of Symptomatic Patients with the Suspicion of FAO Defects

During the screening of symptomatic patients, we identified four patients in three families with LCHADD. Among 102 individuals who were molecularly tested for the c.1528G>C *HADHA* mutation between 2005 and 2007, we found one patient to be homozygous for the c.1528G>C mutation (case 3) and one father who was heterozygous for the same mutation (family 1). His two children had died at an early age (Case 1 and 2). *HADHA* gene sequencing revealed that his wife was heterozygous for a c.1690-2A>G mutation in intron 16 of the *HADHA* gene. The c.1690-2A>G mutation was not identified in 59 DNA samples collected from the general population. The

Table 1 The carrier frequency of the c.1528G>C mutation in different countries

Population	No. of investigated individuals/carriers	Carrier frequency of c.1528G>C mutation	References
Finland	1200/5	1:240	Tyni and Pihko (1999)
The Netherlands	2047/3	1:680	den Boer et al. (2000)
Poland (whole country)	4137/22	1:189	Piekutowska-Abramczuk et al. (2010)
Poland (Pomerania)	2976/41	1:73	Piekutowska-Abramczuk et al. (2010)
China	1200/0	0	Zhu et al. (2005)
Estonia	1040/6	1:173	This study

c.1690-2A>G mutation most likely causes abnormal *HADHA* splicing. According to the splice site prediction program (http://www.fruitfly.org/seq_tools/splice.html), the c.1690-2A>G mutation changes the splice score of the IVS16 3' splice site from 0.97 to 0.0 (maximum splice score is 1.0). Unfortunately the in vivo consequences of the splice site mutation could not be investigated, as no cDNA was available from the mother and the two deceased children. In addition, no DNA was available from those children.

Of the 425 patients who were tested using acylcarnitine profiling in 2008–2010, we identified two patients (Cases 4 and 5) who had an abnormal acylcarnitine profile typical to LCHADD. Molecular analysis showed that both patients were homozygous for the c.1528G>C mutation in the *HADHA* gene.

In our relatively small patient group, we identified eight independent LCHADD alleles from four families. Seven of them (87.5%) carried the c.1528G>C mutation, and in one allele the c.1690-2A>G mutation was found after *HADHA* gene sequencing. Based on a c.1528G>C heterozygote frequency of 1:173 among the general Estonian population and taking into account that this mutation is present on 87.5% of disease alleles in our patients, we calculated that the estimated prevalence of LCHADD in Estonia is 1:91,700.

Discussion

No proven LCHADD cases were reported in Estonia until 2005. We decided to establish whether this was due to the poor clinical recognition of the affected patients or to the low incidence of genetic changes causing LCHADD. In the first step, we investigated the carrier frequency of the common c.1528G>C *HADHA* mutation in the general Estonian population, in order to obtain an estimated prevalence of LCHADD in Estonia. We assumed that the c.1528G>C mutation is the most common LCHADD mutation in Estonia, as is the case in other European (neighboring) countries (Finland, Poland, The Netherlands) (Piekutowska-Abramczuk et al. 2010; Tyni and Pihko 1999; den Boer et al. 2000). Our study results showed that the frequency of heterozygotes for the c.1528G>C mutation in Estonia is high – 1:173 similar to neighboring counties – Finland and Poland (Tyni and Pihko 1999; Piekutowska-Abramczuk et al. 2010) (Table 1). Based on the heterozygote frequency and taking into account that the common mutation is present on 87.5% of disease alleles in our small cohort of patients, we calculated that the prevalence of LCHADD in Estonia is 1:91,700. As the annual birth rate in Estonia in recent years is about 16,000, we can expect one LCHADD patient every 5–6 years. During the last 3 years, however, we have diagnosed one LCHADD case every year. Therefore, the final prevalence may be even higher after the commencement of the nationwide mass screening program for FAO in Estonia. Our results show that the prevalence of LCHADD is comparable to Poland, where the prevalence is 1:118,336 (Piekutowska-Abramczuk et al. 2010), and higher than 1:250,000 observed in Australia, Germany, and the USA (Lindner et al. 2010).

In all of our patients, clinical symptoms appeared at around the age of 1 year (5.5–14 months). At least three patients had hypoglycemia in initial presentation, and in at least two cases hypoglycemic episodes prior to diagnosis were fatal (cases 2 and 3). Retrospective analysis of the disease histories of those two cases revealed that both patients had symptoms indicating LCHADD prior the fatal episode of the disease. These children might not have died if early specific diagnostic possibilities and treatment had been available. Acylcarnitine profiling by tandem MS is considered to be the "cornerstone" in the diagnostics of FAO defects. This method has been available in Estonia since 2008 for symptomatic patients only. Although mutational screening was available a few years earlier, the introduction of tandem MS-based acylcarnitine analysis allowed much wider recruitment of patients with symptoms suggestive of FAO disorders. According to our experience, the indications given by Duran (2003) are very practical and suitable for the selective screening of FAO defects.

In summary, our main goal for the future is to introduce acylcarnitine analysis to all newborns as a part of our national newborn screening program.

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Concise One-Sentence Take-Home Message

The carrier frequency of the c.1528G>C mutation in the *HADHA* gene in the Estonian population is 1:173.

Abbreviated Title

LCHADD in Estonia

References to Electronic Databases

OMIM #609016

Details of the Contributions Made by Individual Authors

K. Joost – development of method and performance of tandem MS analysis of acylcarnitines of all investigated individuals, diagnosis of suspected and confirmed cases, compilation of manuscript, treatment of a patient with LCHAD deficiency.

K. Õunap – planning of study, selection of suspected cases, moderation of cooperation between different centers, diagnosis of suspected patients, compilation of manuscript

R. Žordania – selection of suspected cases, diagnosis and evaluation of a family with LCHAD deficiency

M.-L. Uudelepp – evaluation and treatment of a patient with LCHAD deficiency

R.K. Olsen – sequencing of *HADHA* gene

K. Kall – performance of organic acid GC/MS analyses during the confirmation/exclusion of suspected LCHADD cases

K. Kilk – development of tandem MS analysis of acylcarnitines

U. Soomets – planning of strategy for biochemical diagnosis using tandem MS

T. Kahre – performance of molecular analysis for main mutation in *HADHA* gene in all investigated individuals, compilation of manuscript

Guarantor of Article

Kairit Joost

Statement of Competing Interest

All of the authors confirm that they have no competing interests to declare. There is no financial or nonfinancial interest in publishing this article.

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Details of the Ethics Approval

The Ethics Review Committee on Human Research of the University of Tartu approved the study.

Patient Consent Statement

Informed consent was obtained from the parents of the children involved in the research.

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