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Heterozygosity for the Common LCHAD Mutation (1528G>C) Is Not a Major Cause of HELLP Syndrome and the Prevalence of the Mutation in the Dutch Population Is Low

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ABSTRACT

Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency is an autosomal recessive disorder of mitochondrial fatty acid oxidation. Apart from life-threatening metabolic derangement with hypoketotic hypoglycemia, patients often show liver disease, cardiomyopathy, and neuropathy. A common mutation (1528G>C) in the gene coding for the α-subunit of the mitochondrial trifunctional protein harboring LCHAD activity is found in 87% of the alleles of patients. LCHAD is considered a rare disorder with only 63 patients reported in the literature. Whether this is due to a truly low prevalence of the disorder or because many patients remain unrecognized as a result of aspecific symptomatology is not clear. A remarkable association between LCHAD deficiency and the hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome, which is a severe complication of pregnancy, has been reported. Because of this, we studied the frequency of the common LCHAD mutation in the Dutch population by analyzing 2047 Guthrie cards and 113 women who had suffered from HELLP syndrome. To be able to perform this large-scale study in dried bloodspots, we developed a new sensitive PCR-restriction fragment length polymorphism method. The carrier frequency for the common LCHAD mutation in the Dutch population was found to be low (1:680), consistent with the observed low incidence of the disorder. In the group of women with a history of HELLP syndrome, the prevalence of the common LCHAD mutation was also low (1:113). We conclude that LCHAD deficiency is, indeed, a rare disorder and that heterozygosity for the common mutation is not a major cause of the HELLP syndrome. **(*Pediatr Res* 48: 151–154, 2000)**

Abbreviations

AFLP, acute fatty liver of pregnancy
HELLP, hemolysis, elevated liver enzymes, and low platelets
LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase
MCAD, medium-chain acyl-CoA dehydrogenase
PCR-RFLP, PCR-restriction fragment length polymorphism

LCHAD deficiency is one of the 13 inborn errors of mitochondrial fatty acid oxidation currently known. In patients with LCHAD deficiency, the oxidation of long-chain fatty acids is impaired due to mutations in the gene coding from the α-subunit of the mitochondrial trifunctional protein. The latter protein is an octamer of four α- and four β-subunits. The α-subunit harbors the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities, whereas the β-subunit carries the thiolase activity.

LCHAD deficiency is an autosomal recessive disorder. Patients usually present in infancy with recurrent attacks of hypoketotic hypoglycemia provoked by prolonged fasting, often during a minor intercurrent illness such as gastroenteritis (1). In addition, cardiomyopathy (1, 2) and hepatomegaly with cholestatic jaundice, which can sometimes progress to fulminant liver failure, are regularly observed (2, 3). Peripheral neuropathy and pigmentary retinopathy can occur during the course of the disease (2–5). LCHAD deficiency can also present as sudden infant death even in the neonatal period (2, 6).

The diagnosis of LCHAD deficiency is suggested by demonstrating the presence of large amounts of 3-hydroxy-dicarboxylic acids in the urine and by assessment of the acylcarnitine profile in plasma by tandem mass spectrometry (7). Definitive diagnosis requires enzymatic studies, which may be performed in liver, muscle, lymphocytes, and fibroblasts (8–10).

The gene for the α-subunit of the mitochondrial trifunctional protein carrying LCHAD activity is located on chromosome 2,

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and the genomic structure of the gene has been clarified (11). Most remarkable is the occurrence of a common point mutation (1528G>C) in LCHAD deficiency (12–14), accounting for 87% of the affected alleles in 70 patients investigated (12). This common mutation makes diagnosis at the molecular level feasible (12).

Remarkably, severe complications during pregnancy, including HELLP syndrome and AFLP, have been reported in mothers who are heterozygous carriers of the LCHAD mutation (14–16). HELLP syndrome is a serious complication of pregnancy occurring in approximately 4 to 20% of women with severe preeclampsia (17). AFLP is another complication of pregnancy characterized by severe progressive liver disease in approximately 50% of cases complicated by preeclampsia and is sometimes observed in combination with HELLP syndrome (15, 18). HELLP syndrome carries a high risk for serious morbidity and even mortality for the affected mother and her child (17). Although several mechanisms have been proposed, the exact pathophysiologic mechanism(s) causing HELLP syndrome and AFLP still remains unclear (17, 18). The similarities between the liver disease seen in LCHAD deficiency and in HELLP syndrome as well as in AFLP, with microvesicular or macrovesicular steatosis, in combination with the reported high incidence of these gestational complications in mothers heterozygous for the LCHAD mutation suggest a causal relationship between a compromised long-chain fatty acid oxidation and HELLP and AFLP (14–16, 19, 20). However, it is uncertain whether the risk of these gestational complications is limited to those pregnancies in which the fetus is homozygous for the LCHAD mutation. Nevertheless, in some centers, mutation screening is offered to all mothers who suffered from HELLP syndrome or AFLP to allow presymptomatic diagnosis of LCHAD-deficient newborns.

We decided to study the prevalence of the common LCHAD mutation (1528G>C) in women who suffered from HELLP syndrome during pregnancy and to compare this prevalence with the frequency of the 1528G>C mutation in the Dutch population to determine whether screening of mothers or their offspring for the LCHAD mutation is justified.

A PCR-RFLP method using a *Pst*I restriction site has previously been described, making detection of heterozygous individuals possible (12). However, an important drawback of this method is that the sensitivity is not high enough when samples with a relatively low DNA concentration are used, because of the large amplified fragment (640 bp). To be able to perform large-scale sensitive screening for the common LCHAD mutation in dried bloodspots, we developed a novel improved PCR-RFLP method and applied the method to establish the frequency of the 1528G>C mutation in bloodspots from control persons and women with a history of HELLP syndrome.

METHODS

DNA extraction from bloodspots. DNA was extracted from bloodspots by using Chelex (BioRad) essentially as described before (21) with some modifications. To this end, a sample (3-mm diameter) was taken from a dried bloodspot and washed with 1 mL of sterile water for 30 min at 50°C in a 1.5-mL Eppendorf tube. Thereafter, 200 μL of Chelex (50 g/L, pH 10.5) was added and incubated at 56°C for 30 min. Subsequently, the samples were mixed for 10 s and centrifuged (3 min, 10,000 $\times$ *g*), followed by an 8-min incubation in a boiling water bath. After cooling to room temperature, the samples were mixed for 10 s and centrifuged (3 min, 10,000 $\times$ *g*). Ten microliters of sample was used in a 25-μL PCR reaction.

PCR-RFLP for the common LCHAD (1528G>C) mutation. To increase sensitivity of the used PCR-RFLP method, we aimed to amplify a fragment smaller than the fragment of 640 bp used before (12). To prevent interference of the pseudogene as identified by Zang and Baldwin (11), a new primer had to be selected in intron 15. Because the sequence of this intron has not been published, we sequenced intron 15 completely (data not shown). Based on the obtained sequence, different primer sets were selected. Only with the primer set used here (see below), a specific PCR fragment with high yield was obtained. The product contains a predicted *Pst*I site that can serve as a convenient internal control.

Exon 15 and part of intron 15 were amplified in a 25-μL PCR reaction containing 10 mM Tris-HCl (pH 8.4 at 25°C), 1.2 mM $MgCl_2$, 50 mM KCl, 0.1 mg/mL BSA, dNTP (0.2 mM each), 2.5 U *Taq* polymerase (Promega), and the following primer set (12.5 pmol each): sense primer 5′-CCC TTG CCA GGT GAT TGG C-3′, antisense primer 5′-ACA AGC CTG GAG GTA AAA GG-3′. DNA amplification was performed in a PTC-100 thermocycler from M.J. Research, Inc., programmed as follows: 120 s at 96°C initial to cycling, five cycles of 30 s at 96°C, 30 s at 55°C, and 30 s at 72°C followed by 25 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C and the end of cycling 10 min at 72°C. The amplified fragment (224 bp) was directly digested after addition of 2.5 μL of buffer M and 5 U of *Pst*I (Boehringer Mannheim).

The restriction fragments were analyzed on a 2% (wt/vol) agarose gel with ethidium bromide staining. To validate this method, we performed PCR-RFLP in dried bloodspots from a control subject, a homozygous 1528G>C patient, and both parents.

Population screening for the common LCHAD mutation. In the Netherlands, approximately 99% of all newborns ($\pm$200,000 live births yearly) are tested for phenylketonuria and congenital hypothyroidism in a nationwide screening program by means of Guthrie cards. For this study, 2047 Guthrie cards were anonymously obtained from the screening laboratories representing the 12 Dutch provinces and the two largest cities (Amsterdam and Rotterdam) after approval by the Dutch health authorities. The total number of cards selected from each of the 14 screening areas and used in our population screening was proportional to the number of live births in each of these regions, which guarantees a demographic representation of the Dutch population.

Confidence intervals (CI) were calculated using the method for estimating the population carrier rate when some carriers are not detected as described by Parker and Philips (22), accounting for the 87% allele frequency of the common 1528G>C mutation in LCHAD-deficient patients.

Prevalence of the common LCHAD mutation in HELLP syndrome. HELLP syndrome was defined as hemolytic anemia (LDH > 600 U/L), elevated liver enzymes (ASAT > 70 U/L), and thrombocytopenia (thrombocytes $< 100 \times 10^9$/L) during pregnancy (23).

A total of 113 women who had suffered from HELLP syndrome during at least one of their pregnancies were included in this study. Inclusion was regardless of the outcome of the affected pregnancy. Bloodspots on Guthrie cards were collected from all 113 women. This study was approved by the Institutional Medical Ethical Committee.

RESULTS

To be able to do the studies described in this article, we had to set up a new sensitive method allowing unequivocal identification of the 1528G>C mutation in bloodspots. The result of this new procedure is shown in Figure 1.

After restriction of the amplified fragment from a control bloodspot, the predicted restriction fragment of 175 bp (and 49 bp) was obtained. In a patient known to be homozygous for the 1528G>C mutation, a smaller restriction fragment of 117 (58 and 49 bp) was found, indicating that *Pst*I has cut at both the control site and at the position of the mutation. Both fragments of 175 and 117 bp were visible using material from both parents, which is compatible with heterozygosity (Fig. 1, lower panel). The PCR product contains a predicted *Pst*I site that can serve as a convenient internal control (Fig. 1, upper panel).

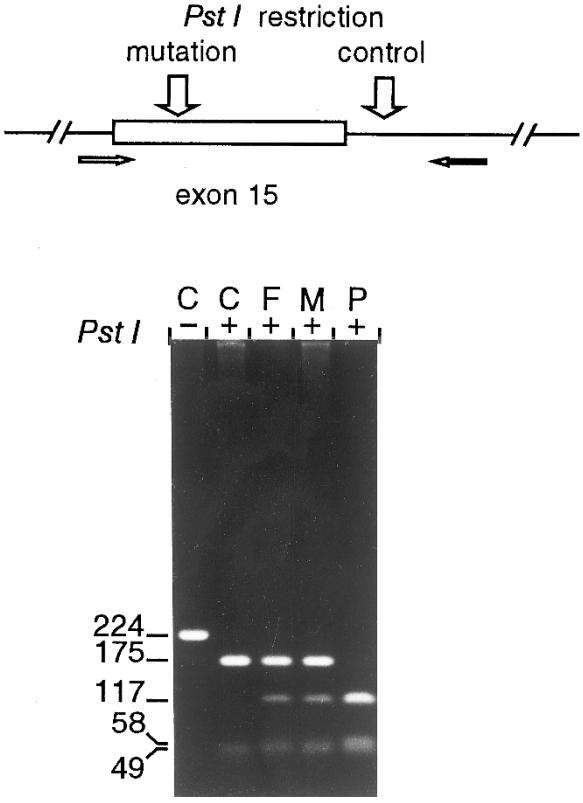

Figure 1. PCR-RFLP analysis of the 1528G>C mutation. *Upper part*, Schematic representation of a part of the gene. The primers are indicated by *horizontal arrows*; positions of the *Pst*I restriction sites are indicated by *vertical arrows*. *Lower part*, Ethidium bromide-stained agarose gel showing PCR-RFLP analysis using DNA extracted from dried bloodspots of a control subject (*C*), a LCHAD-deficient patient (*P*), and the father (*F*) and the mother (*M*) of the patient. PCR products were either directly loaded (−) or digested with *Pst*I (+) before electrophoresis.

Screening for the common LCHAD mutation (1528G>C) by use of the new PCR-RFLP method in the 2047 Guthrie cards obtained form the neonatal screening centers detected three carriers for this mutation. No homozygous-deficient samples were found. The prevalence of the common (1528G>C) LCHAD mutation in the Dutch population is thus estimated to be 1:680 (95% CI of 1:325 to 1:1400).

Among the 113 women who suffered from HELLP syndrome during at least one of their pregnancies, one carrier for the 1528G>C mutation was identified (prevalence 1:113, 95% CI of 1:18 to 1:560).

The prevalences of the common LCHAD mutation among the general Dutch population and among women who suffered from the HELLP syndrome are not statistically different (2-tailed Fisher exact test, p value 0.19).

DISCUSSION

Inborn errors of mitochondria fatty acid oxidation are often diagnosed with considerable delay because of the aspecific symptomatology. For instance, 20% of the MCAD-deficient patients were diagnosed after death, and, in 25%, a sibling had suddenly died without a proper diagnosis (24) but probably due to MCAD deficiency. Although MCAD deficiency is a relatively common disorder among Caucasians with a prevalence of the common mutation (985G>A) ranging from 1:333 in Italy (25) to as high as 1:55 in the Netherlands (26), LCHAD deficiency seems to be a much rarer disorder. Whereas in the Netherlands every year approximately 14 patients are diagnosed with MCAD deficiency in accordance with the carrier frequency for the common mutation, only six patients with LCHAD deficiency have been identified in the last 10 y. However, it may well be that the diagnosis of LCHAD deficiency is even more frequently missed than MCAD deficiency because it is not generally known that cholestatic jaundice and cardiomyopathy can be the presenting signs and symptoms in fatty acid oxidation disorders such as LCHAD deficiency. For this reason, population screening for the common LCHAD mutation (1528G>C, allele frequency 87%) was performed.

Because the previously described PCR-RFLP method (12) has a low sensitivity for the heterozygous detection in samples with relatively low concentration of DNA such as bloodspots, a new and more sensitive method was developed. Recently Ding *et al.* (27) described a sensitive nested PCR-RFLP method. The origin of the PCR product was then confirmed by a gene (and not a pseudogene) specific restriction site for *Pvu*I. This 2-step amplification method works well but is too laborious for processing large numbers of samples. The method described here is a simple 1-step PCR-RFLP method that only amplifies the coding gene. Furthermore, the method uses a second *Pst*I site as internal control for the restriction, preventing false-negative results. Therefore, this PCR-RFLP method is superior to the previously described methods and allows heterozygous detection in dried bloodspots, making our carrier frequency studies possible.

The observed prevalence of the carrier frequency for the common LCHAD mutation of 1:680 (CI 1:325–1:1400) in the Dutch population is indeed much lower than that of the MCAD mutation (1:55) and, with approximately 200,000 live births yearly, corresponds well with the low number of patients diagnosed in the last decade. It is, therefore, unlikely that many patients are missed because of an aspecific presentation.

To study the relation between HELLP syndrome and AFLP on the one hand and LCHAD deficiency on the other hand, we compared the observed prevalence with the frequency of the 1528G>C mutation in a group of women who had suffered from HELLP syndrome.

It is important to keep in mind that our study only allows conclusions related to HELLP syndrome. Although AFLP is regarded as part of the spectrum of the group of gestational disorders that includes HELLP syndrome, further studies are necessary to see whether heterozygosity for the common LCHAD mutation is an important risk factor for the development of AFLP.

Our results clearly show that almost none of the women with a history of HELLP syndrome carries the 1528G>C mutation. These results are important especially because it has been suggested that mutation screening should be performed in women suffering from the HELLP syndrome (16). The results of our study provide no justification for this.

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