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IDENTIFICATION OF A COMMON MUTATION IN PATIENTS WITH MEDIUM-CHAIN ACYL-CoA DEHYDROGENASE DEFICIENCY

Yoichi Matsubara[1*], Kuniaki Narisawa[1], Shigeaki Miyabayashi[2], Keiya Tada[2], Paul M. Coates[3], Claude Bachmann[4], Louis J. Elsas II[5], Rodney J. Pollitt[6], William J. Rhead[7] and Charles R. Roe[8]

Departments of [1]Biochemical Genetics and [2]Pediatrics, Tohoku University School of Medicine, 1-1 Seiryomachi, Aobaku, Sendai 980, JAPAN

[3]Department of Pediatrics, The Children's Hospital of Philadelphia, Philadelphia, PA 19104

[4]Laboratorie Central de Chimie Clinique, CHUV, CH 1101 Lausanne,SWITZERLAND

[5]Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322

[6]The Children's Hospital, Sheffield S10 2TH, U.K.

[7]Department of Pediatrics, University of Iowa Hospitals and Clinics, Iowa City, IA 52242

[8]Department of Pediatrics, Duke University Medical Center, Durham, NC 27710

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SUMMARY: Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is one of the most common recessively inherited metabolic diseases in man. We have studied fibroblast cultures obtained from three patients with MCAD deficiency by sequencing the entire coding region of MCAD mRNA. A single A to G nucleotide replacement which resulted in lysine^{329}-to-glutamic acid^{329} substitution of the MCAD protein was identified in all cultures. Furthermore, this point mutation was present in 91 % (31 of 34) of mutant MCAD alleles, indicating that the majority of cases with MCAD deficiency are caused by this type of mutation.

Medium-chain acyl-CoA dehydrogenase (MCAD; EC 1.3.99.3) deficiency is an autosomal recessive disorder which has been known to cause sudden infant death and Reye-like syndrome among

*To whom all correspondence should be addressed.

The abbreviations used are: MCAD, medium-chain acyl-CoA dehydrogenase; PCR, polymerase chain reaction.

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children (1). The incidence of the disease is not known with certainty, but may be as high as 1 in 10,000 births in Caucasians, making it among the most common recessively inherited metabolic diseases (2).

The MCAD gene is located on human chromosome 1 and the nucleotide sequence of MCAD cDNA has been reported (3,4,5). Recently, Strauss et al. identified molecular defects in the MCAD gene in a patient with this disorder (6). The mutations included aberrant splicing of MCAD mRNA and partial deletion of the mitochondrial transit peptide of the MCAD precursor. However, their studies of fibroblasts from other patients with MCAD deficiency suggested that these types of mutations were not likely to be the cause of MCAD deficiency in most patients (6). This latter observation is in line with an earlier study on 13 human MCAD-deficient fibroblast cultures using anti-MCAD antibody by Ikeda et al.(7). They demonstrated that the MCAD precursor in these 13 patients was synthesized and processed to its mature form with molecular sizes identical to those in normal cultures. The data suggested that MCAD-deficiency in these patients was most likely due to point mutation(s) in the MCAD gene.

We report here a point mutation in the MCAD gene, which may be responsible for the majority of cases with MCAD deficiency. A preliminary communication of this study has been published elsewhere (8).

MATERIALS AND METHODS

Fibroblast cultures were obtained from three Caucasian patients (A, B and C) with MCAD deficiency. These cultures were among the 13 previously described (assigned as C, D and F in ref. 7) to have immunoreactive MCAD precursor and mature proteins of normal subunit sizes; their MCAD activities ranged from 7 to 12% of the mean control value. Dried blood spots on filter papers were collected from other 14 unrelated patients with proven diagnosis of MCAD deficiency (13 Caucasians and one Pakistani) and their parents.

mRNA was isolated from these fibroblast cultures according to a described method (9) followed by purification with oligo(dT)

column. First strand cDNA was synthesized by reverse-transcription of 1 µg of mRNA and subjected to polymerase chain reaction (PCR)(10) in order to amplify the entire coding region of MCAD cDNA. PCR was performed in a total volume of 100 µl containing 50 mM KCl, 10 mM Tris-HCl(pH 8.3), 1.5 mM $MgCl_2$, 0.01 %(w/v) gelatin, 200 µM of each dNTP, 1 µM of each oligonucleotide primer, 1/20 (v/v)of synthesized first strand cDNA, and 2.5 units of Taq DNA polymerase (Perkin-Elmer Cetus). The oligonucleotides used for PCR were: dGAACGGAGAGCCAACATGGC and dCAGTGGCTTGTGTTCTAGT T. The first amplification cycle consisted of 5 min of denaturation at 97 °C, 5 min of annealing at 55 °C, and 30 min of polymerization at 72 °C. The subsequent 30 cycles were carried out at 94 °C for 1 min, followed by 55 °C for 1 min, and 72 °C for 1 min. The amplified DNA fragments (1326 bp) were cloned into pGEM-Blue (Promega) and sequenced by the dideoxy-sequencing method (11).

Genomic DNA from fibroblast cultures was prepared as described (12). Extraction of DNA from dried blood spots (13) was performed as follows. Thirty mm^2 of filter paper with dried blood was soaked in 10 µl of methanol for 5 min, dried, and boiled in 60 µl of H_2O for 15 min. After centrifugation at 12,000 x g for 10 min, 20 µl of the supernatant was directly used for PCR amplification.

RESULTS

Sequencing of MCAD cDNAs derived from fibroblast cultures from patients with MCAD deficiency identified an A to G replacement at nucleotide 985 in all three cultures. This mutation resulted in the substitution of lysine (codon AAA) by glutamic acid (codon GAA) at amino acid residue 329 of the MCAD precursor subunit (Figure 1)(4). Other nucleotide replacements observed were either a silent mutation (T→G at 363) not altering

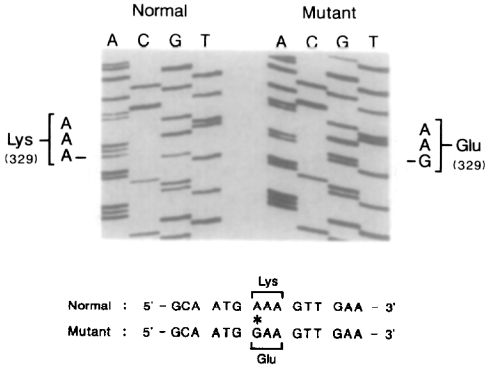

Figure 1. Sequence analysis of a point mutation in the MCAD gene. The A-to-G transition results in the substitution of Lys^{329} by Glu^{329}.

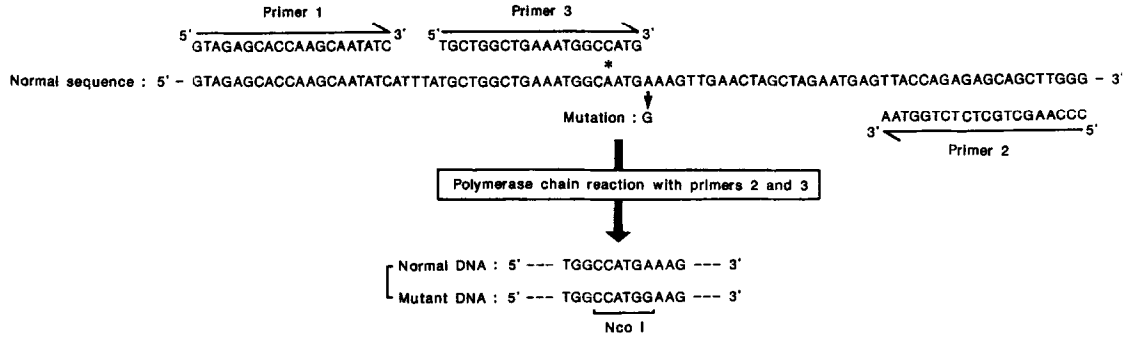

Figure 2. Amplification of genomic DNA fragments containing the mutation site by PCR. Oligonucleotide primers 1 and 2 were used to amplify DNA for dot-blot hybridization analysis. Primers 2 and 3 were employed to carry out 'modified' PCR (see text). The asterisk indicates a mismatched C residue in primer 3. The conditions for both PCR are the same as those described in MATERIALS AND METHODS, except that polymerization time in the first cycle was 2 min.

an amino acid (threonine) in patient C or an error caused by Taq DNA polymerase (C→T at 1211) in patient B. The latter was verified by direct sequencing (14) of the PCR product.

The presence of the Lys^{329}-to-Glu^{329} mutation was further confirmed by the analysis of genomic DNA extracted from these cultures. A DNA fragment containing the mutation site was PCR-amplified with two oligonucleotide primers (Figure 2, primers 1 and 2) and analyzed by dot-blot hybridization using allele specific oligonucleotide probes (Figure 3(a)). Positive hybridization with only the mutant probe indicated that all three patients are homozygous for the mutation (Figure 3(a), A, B and C). In contrast, the mutation was not observed in 29 normal individuals (20 Caucasians, 3 Pakistani, and 6 Japanese; data not shown).

In order to carry out further studies, a novel PCR method for detecting this mutation was developed. A 63 bp DNA fragment containing the mutation site in the MCAD gene was amplified using oligonucleotide primers 2 and 3 as shown in Figure 2. Primer 3 is adjacent to the point mutation and has a

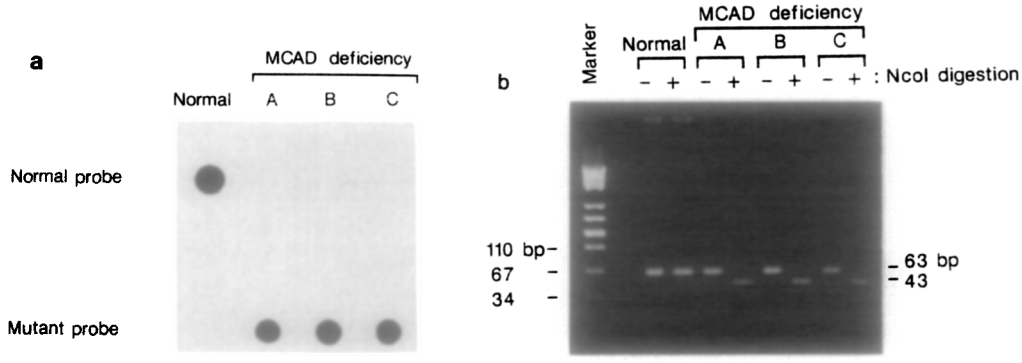

Figure 3. Detection of the point mutation in DNA from three patients with MCAD deficiency by (a) dot-blot hybridization using allele specific oligonucleotide probes and (b) 'modified' PCR with mismatched primer. (a) Genomic DNA fragments (88 bp) from MCAD-deficient patients (A, B, and C) were PCR-amplified with primers 1 and 2 (see Figure 2), denatured in 0.3 M NaOH, and immobilized on GeneScreen Plus membranes (NEN Research Products). The membranes were hybridized overnight at 40 °C with ^{32}P-labeled oligonucleotide probes, complementary to either the normal sequence (dTGGCAATGAAAGTTGAA) or the mutant sequence (dTTCAACTTCC ATTGCCA), in a hybridization solution containing 50 mM Tris-HCl(pH 7.5), 1M NaCl, 1% sodium dodecyl sulfate(SDS), 10% dextran sulfate and 100 µg/ml denatured salmon sperm DNA. The filters were then washed twice in 6x SSC, 0.1% SDS at 40 °C for 10 min, once at 48 °C for 10 min and finally once at 52 °C for 3 min in the same buffer followed by autoradiography at -70 °C. (b) DNA fragments (63 bp) were PCR amplified with primers 2 and 3 (see Figure 2), incubated with(+) or without(-) Nco I, and electrophoresed on 3 % agarose gel.

mismatched C residue 4 bases from the 3' end as indicated by an asterisk. Theoretically, when mutant DNA is amplified using these primers, the mismatched nucleotide creates a new Nco I restriction site (C'CATGG) at the mutation site, which is not present in the original sequence (Figure 2). On the other hand, when normal DNA is amplified, it does not yield an Nco I recognition site. This 'modified' PCR method was applied to DNA from patients A, B, and C (Figure 3(b)). Amplified DNA from each patient was completely cleaved to a 43 bp fragment by Nco I, indicating that these patients are apparently homozygous for the mutation. This observation was consistent with the data obtained by dot-blot hybridization (Figure 3(a)).

Fourteen additional cases of MCAD deficiency have been analyzed by the 'modified' PCR method employing dried blood spots

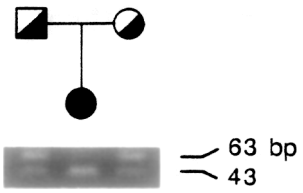

Figure 4. Transmission of the point mutation in a family with an MCAD-deficient child as analyzed by the 'modified' PCR method followed by Nco I digestion and agarose gel electrophoresis.

on Guthrie cards. Twelve of them exhibited the same mutation in homozygous form, whereas one patient was heterozygous for the mutation and one patient did not have this mutation, indicating the remarkably high incidence (31 out of 34 mutant alleles) of the Lys^{329}-to-Glu^{329} mutation (data not shown). Mendelian transmission of the mutation was demonstrated in a family with an affected child (Figure 4). In this family the parents are heterozygous for the mutation.

DISCUSSION

The Lys^{329}-to-Glu^{329} substitution was the only mutation identified in the three fibroblast cultures, which have immunoreactive precursor and mature MCAD proteins of normal subunit sizes (7). Therefore we concluded that this mutation is responsible for abolishing the catalytic activity of MCAD. The data obtained from 29 normal individuals and 14 patients, as well as their parents, were consistent with the conclusion.

Our study demonstrated a remarkably high incidence of the Lys^{329}-to-Glu^{329} mutation among patients, indicating that this is the most common mutation causing MCAD deficiency, at least in the Caucasian population. Of interest is the Pakistani patient who is also homozygous for the mutation. Although a detailed family pedigree of this patient was not available, the observation suggests that the mutation might have arisen during evolution before racial divergence occurred.

Early diagnosis or even presymptomatic diagnosis of MCAD deficiency is important, since the occurrence of life-threatening symptoms can often be prevented by simple dietary management(1). Currently, only a handful of laboratories can offer the confirmatory diagnosis of MCAD deficiency, because of the cumbersome nature of enzymatic assay methods (1) or the technologically demanding nature of mass spectrometric analysis of abnormal metabolites (1,15). However, our 'modified' PCR method can be easily performed in any molecular diagnostic laboratory. Unlike conventional dot-blot analysis, the method does not require radioisotopes or a cumbersome hybridization procedure. It utilizes dried blood spots on filter paper, allowing easy transportation of specimens. Retrospective studies of deceased patients are also possible, because many newborn screening programs store the used cards for considerable lengths of time. These advantages, plus the notably high frequency of Lys^{329}-to-Glu^{329} mutation, imply that our method may be used as a molecular diagnostic method for MCAD deficiency as well as for the detection of carriers.

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