MUTATION IN BRIEF

A Founder Mutation (R254X) of SLC22A5 (OCTN2) in Chinese Primary Carnitine Deficiency Patients

Nelson L.S. Tang[1], W.L. Hwu[2], Rachel T. Chan[3] , L.K. Law[1] , L.M. Fung[1], and W.M. Zhang[1]

[1] Department of Chemical Pathology, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong;
[2] National Taiwan University Hospital and National Taiwan University College of Medicine, Taiwan;
[3]Department of Pediatrics and Neonatology, Centro Hospitalar Conde S. Januario, Macau.

*Correspondence to: Nelson LS Tang, Associate Professor, Department of Chemical Pathology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong; Fax: 852-26322320 or 852-26365090; E-mail: nelsontang@cuhk.edu.hk

Communicated by Haig H. Kazazian, Jr.

Mutations in the SLC22A5 gene, which encodes for the plasma membrane carnitine transporter OCTN2, cause primary carnitine deficiency (PCD). After our first report of OCTN2 mutations in Chinese, three more Chinese PCD patients were identified. The parents of these families were non-consanguineous and these families were unrelated. Two novel truncating mutations were found: R254X, a single-base mutation at cDNA position 981 (c.981C>T); and Y387X (c.1382T>G). Two probands, one each from Taiwan and Macau, were homozygous for R254X. The other proband from Taiwan carried both R254X and Y387X. Two additional heterozygote carriers of R254X were also identified among 250 control samples, while none was detected for Y387X. The population carrier rate for R254X would be about 1 in 125. Haplotypes of R254X alleles were examined and patients homozygous for R254X were also homozygous for the same haplotype of intragenic and microsatellites markers. Analysis of population frequencies of haplotypes revealed that the chance of 4 chromosomes having arisen as independent events was 0.016. We conclude that R254X is probably a founder mutation in Chinese. Other previously reported mutations found in the Japanese population were also screening in 250 control samples but no carrier was identified, indicating that they were either very rare or not present in Southern Chinese.
© 2002 Wiley-Liss, Inc.

KEY WORDS: Primary carnitine deficiency; OCTN2; SLC22A5; founder mutation; Chinese.

INTRODUCTION

Primary carnitine deficiency (PCD, MIM# 212140) was first described in a case of lipid storage myopathy by Engel (Engel et al., 1973). The patient had very low carnitine concentrations in serum and various tissues, including skeletal and cardiac muscles. Therefore, this condition is also called systemic carnitine deficiency. Patients commonly present during infancy with acute metabolic decompensation and even as sudden death (Tang et al., 1998). Other PCD patients present with heart failure due to dilated cardiomyopathy in early childhood. Carnitine supplement is effective in preventing acute decompensation and reversing cardiomyopathy. Case reports with long term follow-up data suggested that patients on carnitine supplement grew up normally and thus, PCD was a treatable metabolic disease with potentially excellent clinical outcome (Cederbaum et al., 2000). A defect in carnitine transport across the cell membrane had been established as the etiology of this disease. Research from

Received 28 March 2002; accepted revised manuscript 14 June 2002.

© 2002 WILEY-LISS, INC.
DOI: 10.1002/humu.9053

our group and others showed that mutations in OCTN2 gene (SLC22A5; MIM# 603377), which encodes a plasma membrane carnitine transporter, were the molecular defect in PCD patients (Lamhonwah et al., 1998; Nezu et al., 1999; Tang et al., 1999; Wang et al., 1999).

After our first report demonstrating the OCTN2 was the disease-causative gene of PCD, other reports revealed additional mutations in OCTN2, and many of them were found in Caucasian patients (Burwinkel et al., 1999; Vaz et al., 1999; Wang et al., 2001). Therefore, we started to recruit more Chinese PCD patients for molecular genetic analysis with an objective of studying the spectrum of mutations in this ethnic group. Determination the carrier frequencies of OCTN2 mutations may provide an estimation of prevalence of this disease in Chinese. Therefore, those mutations found in this report were also screened in a sample of 250 anonymous umbilical cord blood specimens, which represented a sample of alleles from the local Chinese population.

Although no universal common mutation had been found across ethnical groups, recurrent mutations in OCTN2 were reported in specific populations. The recurrent mutation S467C was found in PCD patients in the Akita Japanese population and was also believed to be an ancient mutation (Koizumi et al., 1999). For other examples, the W132X and W283C were found recurrent in East Asian populations (Koizumi et al., 1999; Nezu et al., 1999; Tang et al., 1999). In addition, R282X was common in Caucasian (Burwinkel et al., 1999; Vaz et al., 1999; Wang et al., 1999). Two other missense mutations were also found recurrent in specific geographic regions. R169W was found in Italian patients (Wang et al., 2000) and Y211C was found in patients from West African region (Vaz et al., 1999). These findings suggested that ethnic specific founder mutations existed in OCTN2. Therefore, mutations reported in other Asian population (mostly in Japanese) were also screened in our control sample to determine if they were present in Southern Chinese.

MATERIALS AND METHODS

Patients

Three unrelated Chinese patients were recruited for study. Two of them were living in Taiwan and one in Macau. The clinical history of the Macau family had been reported (Marques, 1998). The proband presented at 6 years of age with acute heart failure due to dilated cardiomyopathy. Diagnosis was confirmed by carnitine uptake assay on fibroblasts culture. She responded well to carnitine therapy.

The second case was a 4-year-old boy living in Taiwan (sample No. S85.). At presentation, he was admitted for acute heart failure and dilated cardiomyopathy. Serum free carnitine was 4 μmol/L (reference range: 25 – 61 μmol/L) and plasma ammonia concentration was elevated, up to 150 μmol/L. He had been followed up for 5 years after carnitine supplement and his developments were normal. His cardiac function was slightly subnormal, and had a borderline low ejection fraction.

The other Taiwan patient had been reported by Hwu et al (Hwu et al., 2000). The girl (sample No. S86) first presented at 7 months of age with impaired conscious state and hepatomegaly. Both plasma ammonia (up to 140 μmol/L) and liver enzymes were elevated during the acute metabolic crisis. She was treated with high dose carnitine supplement and showed a favorable response. There was no subsequent acute metabolic attack for a period of follow-up of four years. Her cardiac function remained normal. Diagnosis of PCD was confirmed in both Taiwan patients in cultured fibroblasts. For these two patients from Taiwan, only patient's samples were available and parents' samples were not available for study.

Mutation analysis

Mutation analysis was performed on genomic DNA extracted from peripheral blood samples. Individual exons of OCTN2 were amplified by PCR with previous reported primers (Tang et al., 1999). PCR products were then sequenced in both forward and reverse directions. PCR was carried out for 35 cycles with AmpliTaq Gold (Applied Biosystems, USA). PCR product was purified with Concert PCR product purification kit (Life Technology, USA) to remove unincorporated primers and nucleotides. DNA sequencing was performed by BigDye Cycle Sequencing kit (Applied Biosystems, USA) and analyzed with ABI-310 autosequencer.

Screening of mutations in population samples

In order to determine the frequencies of mutations in the general population of Southern Chinese, a consecutive collection of 250 anonymous umbilical cord blood samples was used as control to screen for such mutations.

Screening of mutations was performed by either PCR-restriction fragment length polymorphism or mismatched PCR- restriction fragment length polymorphism. The exact design of screening protocol was determined by whether the cutting site of a restriction enzyme was altered by the mutation. The protocols for screening of alleles in OCTN2 are shown in Table 1.

Table 1. Protocols for Detection of Alleles in OCTN2

Part A. Mutations causing primary carnitine deficiency

OCTN2 screening (reported in)	Codon	Exon	Primers	Restriction enzyme	Fragment sizes in wild type	Fragment sizes in mutant
-91-22del (Japanese)	1	1	OC21F2 GCAGGACCAAGGCGGCGGTGTCAG OC21R1 AGCTCGGGTTCAAGGACCGC	Nla III	170,459	516
W132X (Chinese)	132	2	SCD2F* SCD2R*	Nla IV	74	102
R254X (Chinese)	254	4	SCD4F* SCD4R*	FokI	149, 91	163, 91
W283C (Japanese)	283	5	SCD283misF CCTCTCACCCACACCCAC SCD283misR GTTCATCCCTGAGTCCACC	PflM I	140	155
Y387X (Chinese)	387	7	SCD7F* SCD7R*	Nde I	159, 149	308
S467C (Japanese)	467	8	SCD8F* SCD8R*	Fnu4HI	105, 90 , 97	105, 90 , 76
P478L (Chinese)	478	8	SCD478misF CCTTTTCCATGGTCTACGTG SCD478misR GGACTTACCAAGGTAAACGAAGTCG	Taq I	134	103
IVS8-1G>A (Japanese)		8	SCD9F* SCD9R*	Ban I	103,90	103,138

Part B. Silent polymorphisms and microsatellites for determination of haplotype

OCTN2 screening	Codon	Exon	Primers	Restriction enzyme	Fragment sizes in wild type	Fragment sizes in mutant
c.506C>T	95	1	OC21F2 GCAGGACCAAGGCGGCGGTGTCAG OC21R1 AGCTCGGGTTCAAGGACCGC	Bso BI	449, 180	629
c.1028G>A	269	4	SCD269misF TGCATTTGGCTACATGGTGC SCD269misR TCACCACCAGAGTGCCATGCA	Nsi I	112	92
Novel STR			octn2p1f GCAAAACAGTGAGACTCAAAATTT octn2p1r ATGGTTCTGGCAGTGCCTTT			

*These primers are those used in mutational analysis.

The two mutations in **bold** were novel mutations reported here.

Haplotype determination

Both intragenic single nucleotide polymorphisms and nearby microsatellite markers were studied to determine the haplotype of the recurrent R254X mutation. Haplotype frequencies of two intragenic polymorphisms,

c.506C>T in exon 1 and c.1028G>A in exon 4 were estimated from genotype data of 90 control subjects by the EH program (Terwilliger et al., 1994).

Two microsatellites with heterozygosity above 0.7, GATA51D11 (137.4 cM from p terminal on the Marshfield map) and AFM200ya9 (141.8 cM) were studied. The location of OCTN2 gene had not been precisely defined on genetic map but these two markers were mapped to flank the OCTN2 gene on the physical map and were 5 Mb and 1.2 Mb away from OCTN2 loci in chromosome 5, respectively. We searched for other novel repeat sequences in the contig containing OCTN2 (NT_007072). Three putative repeat elements were identified; one in accession AC008599 and two in accession AC003959. Both clones were located within 40 kbps from the OCTN2. The primers used to amplify the novel microsatellite were shown in Table 1.

RESULTS

Two truncating nonsense mutations accounted for the disease alleles found in these patients. Two probands (case from Macau and case S85 from Taiwan) were homozygote for R254X (c.981C>T) mutation, while the third patient (case S86 from Taiwan) was a compound heterozygote of R254X and Y387X (c.1382T>G). Both mutations were novel.

The R254X was a 981C>T transition, predicting a truncation at codon 254 (CGA to TGA) in exon 4 (sequence data reviewed but not shown). The other mutation Y387X was a 1382T>G transversion, changing codon 387 from TAT to TAG (data reviewed but not shown). Both mutations were confirmed on sequencing with reverse primers and PCR-RFLP (Table 1). Family members of the Macau case were genotyped for R254X mutation. It was found that mother and a younger sister were carriers of the mutation (Figure 1).

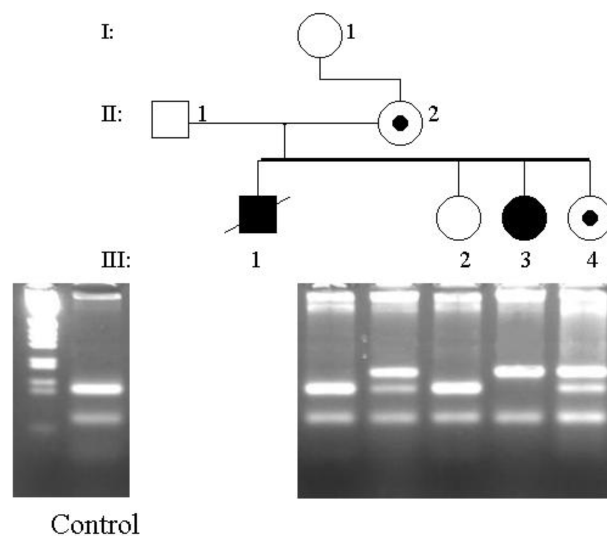

Figure 1. Genotype of family members of the Macau PCD family. The mother (II:2) and a younger sister (III:4) were carriers of the R254X mutation. The restriction digestion pattern of a control sample was shown in the low left corner (a 149 bp fragment present). III:3 was a PCD patient and homozygous for R254X and the restriction digestion showed only a 163 bp fragment. Carriers had both 149 bp and 163 bp fragments. The size marker was 1-kb size marker.

In addition, three polymorphisms were identified: silent mutations in codon 95 (c.506C>T) in exon 1, codon 269 (c.1028G>A) in exon 4, and a substitution in intron 4 (IVS4+13C>T). These polymorphisms served as intragenic markers to generate haplotype for further analysis of the novel recurrent mutation (R254X).

These intragenic polymorphisms were identical in the two probands who were homozygous for R254X. The third patient (S86) was a heterozygote of R254X and also the three polymorphisms. However, samples from the parents of the third patient were not available to establish the phase of the polymorphic markers.

Screening for recognized mutations to determinate the carrier frequencies in population

In addition, two heterozygote carriers of R254X were found in the 250 control samples by PCR-RFLP method. These two samples were sequenced for exon 4 and did confirm a carrier status of R254X. The results provided estimations of carrier rate and allelic frequency of R254X which were one in 125 and one in 250, respectively.

No carrier of Y387X or other recognized mutations (listed in Table 1) was identified in screening of control samples.

Haplotype analysis of chromosomes carrying R254X

As mentioned above, both patients homozygous for R254X were also homozygous for all the three intragenic polymorphisms and their shared haplotype was [c.506C>T; c.1028G>A; IVS4+13C>T]. Two of these polymorphisms (c.506C>T; c.1028G>A) were used to determine the haplotype frequencies in the general population. As polymorphisms of c.1028G>A and ; IVS4+13C>T are very close together, just 30 base pairs apart, and they are in complete linkage disequilibrium and not informative for further analysis.

The allele frequency of T at c.506 was 0.389 and that of A at c.1028 was 0.378 in the 90 control samples. And both alleles that were linked with R254X, were the minor alleles. The genotype data was then analyzed by the EH program and confirmed the presence of linkage disequilibrium (empirical chi square statistics, $p<0.05$). Table 2 shows the estimated haplotype distribution of the two polymorphisms. R254X was linked to the T-A haplotype which corresponded to haplotype group B in a previous study in Japanese (Koizumi et al., 1999).

Only one of the three novel microsatellites was polymorphic, which was located within accession AC008599, between position 38777 to 38887. It was a composite CA and TA repeats. Alleles of this microsatellite formed two clusters of fragments, 145 to 149 bps and 163 to 171 bps due to a 20bp-deletion in the shorter alleles and the heterozygosity of this microsatellite was 0.82. The two homozyogote R254X patients shared a common haplotype at all three microsatellites. The common haplotype was GATA51D11: 136 bp – novel STR: 149 bp – AFM200ya9: 190 bp. The other carriers (S85 and two healthy carriers) of R254X were also heterozygote for the above alleles supporting a common origin for this mutation.

DISCUSSION

The three Chinese patients studied here enriched the spectrum of clinical presentations of PCD found in Chinese. Clinical presentation of PCD can be divided into two major forms, (1) an early onset form, which is characterized by presentation in infancy with acute metabolic derangement, nonketotic hypoglycaemia or sudden infant death and (2) a cardiomyopathic form, which is characterized by dilated cardiomyopathy in early childhood. The phenotype in two homozygote patients (Macau case and S85 from Taiwan) were characterized by dilated cardiomyopathy, whereas the compound heterozygote patients (S86 from Taiwan) presented with metabolic derangement. This heterozygote patient carried another truncating mutation, therefore, it was unlikely that phenotype of R254X was confined to the cardiomyopathy form. Previous studies had also reported completely different phenotypes in probands from the same family (Wang et al., 2001). Therefore, phenotype of PCD is not related to the genotype of OCTN2.

Two novel mutations were found in Southern Chinese and one of them (R254X) accounted for 5 out of 6 (83%) mutant alleles. The mutant encodes for a truncated protein, which has only 5 of the 12 transmembranous domains, and it is certain that R254X is a loss-of-function mutant. This mutation has not been reported in other population and GC content of the region is not high. Both features indicate that this site is not particularly prone to spontaneous mutation. The identical intragenic and microsatellite haplotype found on the chromosomes carrying R254X further supports that it is a founder mutation in Southern Chinese. As blood samples from other family members were not available for study in the Taiwan families, the phase of polymorphisms and R254X could not be determined in the compound heterozygote Taiwan patient. Therefore, phase information was only derived from the two homozygote patients. An empirical determination of the probability that the 4 chromosomes carrying R254X had been arisen as independent sporadic mutations, was 0.016 (0.355^4). This calculation was based on the population frequency of their shared haplotype of intragenic markers, which was 0.355 (Table 2).

R254X was screened in a sample of 250 unselected Hong Kong Chinese samples, which represented the population gene pool. The carrier rate for R254X was 1 in 125. The estimated incidence of PCD due to R254X mutation is 1 in 62, 500, i.e. 1 / (4 x 125 x 125). This estimation would be lower than the disease incidence of PCD

as only cases caused by this single mutation was counted and there were at least three other recognized mutations in Chinese patients (Tang et al., 2000).

Table 2. Estimated Haplotypes of c.506C>T and c.1028G>A in 90 Unrelated Samples from the General Population by EH Algorithm*

c.506	**c.1028**	**Estimated Haplotype Frequency**
C	G	0.588
C	A	0.023
T	G	0.034
T	**A**	**0.355**

*The haplotype [c.506C>T; c.1028G>A] in linkage with R254X is shown in Bold.

Hyperammonaemia has not been considered as a feature in PCD until recently. Both probands from Taiwan experienced significance hyperammonaemia during acute illness when plasma ammonia level was up to 150 µmol/L (reference range: less than 50 µmol/L). As hyperammonaemia is a diagnostic feature for urea cycle defects and during acute illness, it may lead to confusion in the diagnostic workup. The exact cause for secondary hyperammonaemia is uncertain in PCD patients, as only few hyperammonaemic cases were reported (Hwu et al., 2000). It is not certain if hyperammonaemia only occurs in a minority of PCD patients or it is a common phenomenon but not being elicited. Interestingly, hyperammonaemia had been associated with other fatty acid oxidation defects, such as carnitine-acylcarnitine translocase deficiency (Roschinger et al., 2000) and medium chain acyl-CoA dehydrogenase deficiency (Catzeflis et al., 1990; Touma et al., 1992). These reports suggested that secondary hyperammonaemia might occur in patients with various defects in the fatty acid oxidation pathway and accumulation of fatty acid acyl-CoA might lead to secondary urea cycle blockage.

In the mouse model of PCD, juvenile visceral steatosis (jvs) mice, hyperammonaemia was a constant feature during the weaning period (Horiuchi et al., 1994). The jvs mice carried a homozygote mutation in the mouse octn2 gene (Nezu et al., 1999). Activities of all urea cycle enzymes were suppressed in these animals, for example, the mean carbamoyl phosphate synthetase (CPS) activity was only 11% of normal (Imamura et al., 1990). Subsequent studies from the same group reported that oleic acid suppressed expression of CPS gene through a direct interaction on an AP-1 element which acted as an enhancer of CPS gene (Saheki et al., 2000). Therefore, secondary hyperammonaemia in human may be caused by a similar mechanism. The reason why hyperammonaemia was not found in all patients may be related to different degrees of protein catabolism during the acute crisis. From a practical point of view, it is necessary to search for secondary causes other than urea cycle defects (like PCD and fatty acids oxidation defects) in infants presenting with hyperammonaemia.

In summary, a founder mutation of OCTN2 was found in Chinese and the population allelic frequency of this mutation suggested that PCD might be a common metabolic disease in Southern Chinese.

REFERENCES

Burwinkel B, Kreuder J, Schweitzer S, Vorgerd M, Gempel K, Gerbitz KD, Kilimann MW. 1999. Carnitine transporter OCTN2 mutations in systemic primary carnitine deficiency: a novel Arg169Gln mutation and a recurrent Arg282ter mutation associated with an unconventional splicing abnormality. Biochem Biophys Res Commun 261: 484-7.

Catzeflis C, Bachmann C, Hale DE, Coates PM, Wiesmann U, Colombo JP, Joris F, Deleze G. 1990. Early diagnosis and treatment of neonatal medium-chain acyl-CoA dehydrogenase deficiency: report of two siblings. Eur J Pediatr 149: 577-81.

Cederbaum S, Dipple K, Vilain E, Miller M, Koo-McCoy S, Hsu BY, Ganguly A, Stanley C. 2000. Clinical follow-up and molecular etiology of the original case of carnitine transporter deficiency. J Inherit Metab Dis 23: 119.

Engel AG, Angelini C. 1973. Carnitine deficiency of human skeletal muscle with associated lipid storage myopathy: a new syndrome. Science 179: 899-902.

Horiuchi M, Kobayashi K, Yamaguchi S, Shimizu N, Koizumi T, Nikaido H, Hayakawa J, Kuwajima M, Saheki T. 1994. Primary defect of juvenile visceral steatosis (jvs) mouse with systemic carnitine deficiency is probably in renal carnitine transport system. Biochim Biophys Acta 1226: 25-30.

Hwu WL, Chiang SC, Chang MH, Wang TR. 2000. Carnitine transporter defect presenting with hyperammonemia: report of one case. Acta Pediatr Taiwan 41: 36-8.

Imamura Y, Saheki T, Arakawa H, Noda T, Koizumi T, Nikaido H, Hayakawa J. 1990. Urea cycle disorder in C3H-H-2 degree mice with juvenile steatosis of viscera. Febs Lett 260: 119-21.

Koizumi A, Nozaki J, Ohura T, Kayo T, Wada Y, Nezu J, Ohashi R, Tamai I, Shoji Y, Takada G, Kibira S, Matsuishi T, Tsuji A. 1999. Genetic epidemiology of the carnitine transporter OCTN2 gene in a Japanese population and phenotypic characterization in Japanese pedigrees with primary systemic carnitine deficiency. Hum Mol Genet 8: 2247-54.

Lamhonwah AM, Tein I. 1998. Carnitine uptake defect: frameshift mutations in the human plasmalemmal carnitine transporter gene. Biochem Biophys Res Commun 252: 396-401.

Marques JS. 1998. Dilated cardiomyopathy caused by plasma membrane carnitine transport defect. J Inherit Metab Dis 21: 428-9.

Nezu J, Tamai I, Oku A, Ohashi R, Yabuuchi H, Hashimoto N, Nikaido H, Sai Y, Koizumi A, Shoji Y, Takada G, Matsuishi T, Yoshino M, Kato H, Ohura T, Tsujimoto G, Hayakawa J, Shimane M, Tsuji A. 1999. Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter. Nat Genet 21: 91-4.

Roschinger W, Muntau AC, Duran M, Dorland L, L IJ, Wanders RJ, Roscher AA. 2000. Carnitine-acylcarnitine translocase deficiency: metabolic consequences of an impaired mitochondrial carnitine cycle. Clin Chim Acta 298: 55-68.

Saheki T, Li MX, Kobayashi K. 2000. Antagonizing effect of AP-1 on glucocorticoid induction of urea cycle enzymes: a study of hyperammonemia in carnitine-deficient, juvenile visceral steatosis mice. Mol Genet Metab 71: 545-51.

Tang NL, Ganapathy V, Wu X, Hui J, Seth P, Yuen PM, Fok TF, Hjelm NM. 1999. Mutations of OCTN2, an organic cation/carnitine transporter, lead to deficient cellular carnitine uptake in primary carnitine deficiency. Hum Mol Genet 8: 655-60.

Tang NL, Hui J, Law LK, To KF, Ruiter JP, L IJ, Wanders RJ, Ho CS, Fok TF, Yuen PM, Hjelm NM. 1998. Primary plasmalemmal carnitine transporter defect manifested with dicarboxylic aciduria and impaired fatty acid oxidation. J Inherit Metab Dis 21: 423-5.

Tang NLS, Hui J, Law LK, To KF, Cheung KL, Hjelm NM, Yuen PK, Fok TF. 2000. Primary carnitine deficiency in Chinese. Chinese Med J-peking 113: 376-380.

Terwilliger J, Ott J. 1994. Handbook for human genetic linkage. Baltimore, John Hopkins University Press.

Touma EH, Charpentier C. 1992. Medium chain acyl-CoA dehydrogenase deficiency. Arch Dis Child 67: 142-5.

Vaz FM, Scholte HR, Ruiter J, Hussaarts-Odijk LM, Pereira RR, Schweitzer S, de Klerk JB, Waterham HR and Wanders RJ. 1999. Identification of two novel mutations in OCTN2 of three patients with systemic carnitine deficiency. Hum Genet 105: 157-61.

Wang Y, Korman SH, Ye J, Gargus JJ, Gutman A, Taroni F, Garavaglia B, Longo N. 2001. Phenotype and genotype variation in primary carnitine deficiency. Genet Med 3 : 387-92.

Wang Y, Taroni F, Garavaglia B, Longo N. 2000. Functional analysis of mutations in the OCTN2 transporter causing primary carnitine deficiency: lack of genotype-phenotype correlation. Hum Mutat 16: 401-7.

Wang Y, Ye J, Ganapathy V, Longo N. 1999. Mutations in the organic cation/carnitine transporter OCTN2 in primary carnitine deficiency. Proc Natl Acad Sci U S A 96: 2356-60.