RESEARCH NOTE

Importance of molecular diagnosis in the accurate diagnosis of systemic carnitine deficiency

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Introduction

Systemic carnitine deficiency (SCD: MIM212140) is a rare autosomal recessive disease resulting from defects in the *SLC22A5* gene. In this study, five Japanese SCD probands with low serum free carnitine levels were analysed of whom four were identified during newborn screening (NBS). Direct sequencing of *SLC22A5* revealed six different mutations: p.S467C ($n = 3$), p.R254X ($n = 2$), p.N32S ($n = 2$), p.W132X ($n = 1$), p.W283C ($n = 1$), and p.A451P ($n = 1$). Molecular diagnosis unmasked new SCD cases in two of five pedigrees. In another pedigree, a two-year and two-month-old child, who had not undergone NBS, was previously diagnosed with Reye's syndrome was found to be affected by SCD. Although serum free carnitine levels are informative, we suggest that confirmatory diagnostic sequencing procedures that include other family members are desirable to detect masked SCD subjects.

SCD is often fatal (Eriksson *et al*. 1988; Treem *et al*. 1988). In such cases its clinical profile is characterized by progressive cardiomyopathy, skeletal myopathy, hypoketotic hypoglycaemic encephalopathy, and hyperammonaemia (Chapoy *et al.* 1980; Magoulas and El-Hattab 2012). SCD is caused by mutations in the *SLC22A5* gene, and genetic epidemiology demonstrates that the incidence of SCD carriers is about 1% in Japan (Koizumi *et al.* 1999). Currently NBS is conducted in various countries for an early diagnosis of disorders of fatty-acid oxidation, including SCD.

Here we report the mutation analysis of five Japanese probands with low serum free carnitine levels, some of whom were identified in NBS trials. Direct sequencing of *SLC22A5* revealed six different mutations.

Materials and methods

Pedigrees and cases

This study was approved by the Ethics Committee of the Kyoto University Institutional Review Board and appropriate informed consent was obtained from all subjects. Molecular diagnoses were carried out at Kyoto University between 2008 and 2011.

Sequencing, restriction fragment length polymorphism (RFLP) analysis and bioinformatics analysis

Genomic DNA was isolated from 2 mL peripheral blood sample donated by patients and their blood relatives. We carried out PCR amplification of whole nine exons with 100 bp splicing donor and acceptor sites, 3′-UTR, and the potential promoter region of *SLC22A5* to detect functional variants. The PCR products were directly sequenced on an ABI Prism 3100 sequence analyser (Applied Biosystems, Foster City, USA). Presence of p.A451P RFLP was tested using the restriction enzyme *CviKI*-1 (New England Biolabs, Ipswich, USA). Primers are available on request. Two computer-based algorithms, PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/) were used to predict the pathogenicity of unpublished missense variants.

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Results and discussion

The mutational analysis of five unrelated pedigrees with SCD is reported in the current study (figure 1). In pedigree A, free carnitine levels in the dried blood spot (DBS) of the proband (A12) were measured as part of NBS, and found to be low at 5.6 μM (reference value >10 μM). Renal fractional excretion of free carnitine was elevated at 16.7% (reference value $<2.0\%$). Proband's elder sister (A11) donated blood samples for molecular diagnosis, and her serum free carnitine level was found to be 4.6 μM. Genomic DNA sequencing of A11 and A12 revealed that both sisters harboured two mutations: p.W132X and p.S467C (figure 2). Mutation p.W132X was transmitted from the mother and mutation p.S467C from the father.

The proband (B13) of pedigree B had low DBS free carnitine levels in NBS: 9.4 μM and 6.3 μM on days six and 14, respectively (figure 1). Renal fractional excretion of free carnitine was elevated at 17.6%. Carnitine administration (600 mg, twice a day) was therefore commenced on day 31. This was stopped at 10 months old for a period of two-weeks for differential diagnosis. Serum free carnitine levels were 56 μM immediately after the last administration; however, two weeks after cessation the level had decreased to 9 μM, confirming that the observed lowered serum free carnitine levels were consistent. Sequence analysis revealed the existence of two variants: p.R254X and p.A451P (figure 2). p.R254X was inherited from the mother and p.A451P from the father. The p.A451P allele was not found in 250 Japanese controls by RFLP using the restriction enzyme *CviKI*-1 (figure 2). Functional prediction by bioinformatics suggested that p.A451P is 'probably damaging' by Polyphen-2 and 'affecting protein function' by SIFT. Sequencing revealed no mutation of *SLC22A5* other than p.R254X and p.A451P in B13. His father (B1), who has an allele of p.A451P, had a low serum free carnitine level (34 μM) (reference value, >38 μM; Koizumi *et al.* 1999). These pieces of evidence consistently suggest that p.A451P is likely to be the causal mutation. Pathogenicity of p.A451P, however, needs to be confirmed experimentally in future.

In pedigree C (figure 1), NBS revealed a low DBS free carnitine level in the fourth child (C14; 8.3 μM) and levels were shown to be very low in the mother (4.1 μM). Molecular diagnosis revealed that the mother unexpectedly carried two known variants: p.R254X and p.S467C. Her renal fractional excretion of free carnitine was elevated at 17.7%. All four children including the proband (C14) (figure 2) were found to be heterozygous, while the father had two wild-type alleles. This is similar to reports of other maternal cases in which SCD was identified in mothers following the NBS of neonates (El-Hattab *et al.* 2010; Lee *et al.* 2010).

The proband of pedigree D (figure 1) was a two-year and two-month-old child (D13). His medical history had been uneventful until the age of 2 years 2 months when he was admitted to hospital with symptoms of hypoglycaemia, elevated liver enzymes and hyperammonaemia; Reye's syndrome was suspected. His nasal discharge was positive for respiratory syncytial virus. After admission, he developed encephalopathy and became comatose with a flat electromyography and no spontaneous respiration. Because

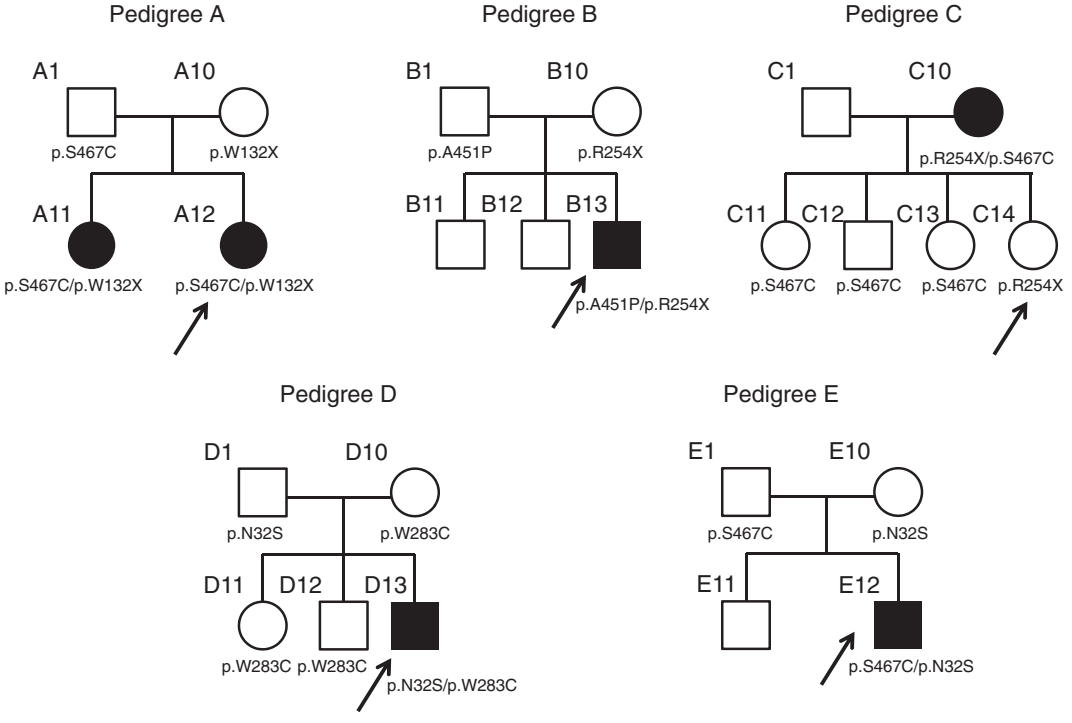

Figure 1. Pedigree and genetic diagnosis of five Japanese families with SCD. SCD patients are shown as black circles or squares. Arrows indicate probands.

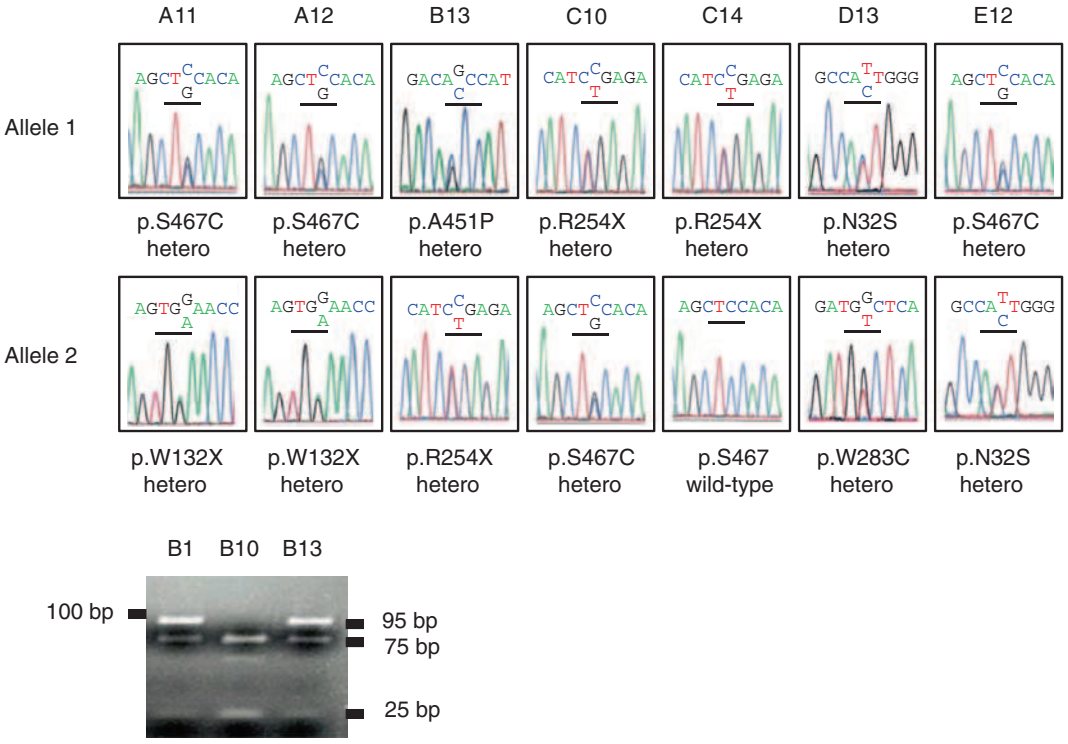

Figure 2. Sequencing analysis of *SLC22A5* mutations in SCD patients and NBS neonates (upper) and RFLP analysis for p.A451P in pedigree B (lower). The p.A451P mutation abolishes the *CviKI*-1 restriction site thus the 95bp PCR product remains undigested (4% metaphor gel).

the Reye's syndrome was suspected, his serum free carnitine level was determined and found to be very low (2.2 μM). Sequencing revealed that he is a compound heterozygote of p.N32S and p.W283C, while his two other siblings are carriers of p.W283C (figure 2). p.N32S was inherited from his father and p.W283C from his mother. If NBS had been conducted in this case, low serum free carnitine levels might have been detected during the early neonatal period and encephalopathy would have been prevented.

In pedigree E (figure 1), NBS revealed low DBS free carnitine levels (9.1 μM) in proband E12. Renal fractional excretion of free carnitine was elevated at 12.6%. Direct sequencing revealed that E12 is a compound heterozygote of p.S467C and p.N32S variants (figure 2). p.S467C was inherited from his father and p.N32S from his mother.

In the present study, molecular diagnosis unveiled two new masked cases (A11 and C10) and corrected a diagnosis of SCD with a carrier for one proband (C14) screened in NBS (figure 1). The new masked cases represent asymptomatic individuals. We also confirmed that a serious case (D13) who was suspected to have Reye's syndrome was instead affected by SCD. It has previously been reported that mild viral infection precipitates as Reye's-like syndrome in SCD subjects (Chapoy *et al.* 1980).

Most of the variants detected in this study are well known, including p.S467C, p.W132X, p.W283C, p.R254X and p.N32S. These have previously been reported in SCD cases in various countries, including Japan, suggesting the existence of founder mutations (Koizumi *et al*. 1999; Lamhonwah *et al*. 2002; Lamhonwah *et al*. 2004; Nezu *et al*. 1999; Tang *et al*. 1999). The only novel variant in the present study is p.A451P. We therefore suggest that known founder mutations should be searched initially to ensure that mutation screening is cost-effective and not time-consuming.

In conclusion, albeit high clinical specificity of measuring serum carnitine levels, confirmatory diagnostic sequencing procedures including other family members are desirable for suspected SCD cases following NBS.

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