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Genetic epidemiology of the carnitine transporter *OCTN2* gene in a Japanese population and phenotypic characterization in Japanese pedigrees with primary systemic carnitine deficiency

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Serum free-carnitine levels were determined in 973 unrelated white collar workers in Akita, Japan. Fourteen of these participants consistently had serum free-carnitine levels below the fifth percentile (28 μM for females and 38 μM for males). The *OCTN2* (organic cation transporter) gene was sequenced for these 14 subjects, for 22 subjects whose carnitine levels were below the fifth percentile in the first screening but were normal in the second measurement and in 69 individuals with normal carnitine levels for two separate measurements. Polymorphic sequences defined three major haplotypes with equal frequency. Mutations were identified in nine subjects with low carnitine levels: Trp132X (three individuals), Ser467Cys (four), Trp283Cys (one) and Met179Leu (one). *In vitro* expression studies in HEK cells indicated that Ser467Cys and Trp283Cys, but not Met179Leu, significantly reduced L-carnitine uptake relative to the normal control. Trp132X and Ser467Cys were associated with specific haplotypes, suggesting a founder effect. A conservative estimate of the overall prevalence of heterozygotes was 1.01% in the Akita prefecture, Japan, giving an estimated incidence of primary systemic carnitine deficiency (MIM 212140) as 1 in 40 000 births. An echocardiographic study of the families of patients with primary carnitine deficiency revealed that the heterozygotes for *OCTN2* mutations were predisposed to late onset benign cardiac hypertrophy (odds ratio 15.1, 95% CI 1.39–164) compared with the wild-types. Sequencing of DNA isolated from three deceased siblings (1.5–8 years) in two families retrospectively confirmed that all three deceased subjects were homozygous for the *OCTN2* mutations.

INTRODUCTION

Primary systemic carnitine deficiency (SCD; MIM 212140) is a rare hereditary disease (1). This disease is treatable but is often overlooked (2,3). Although there is no direct evidence, as yet, several lines of circumstantial evidence imply that SCD may be one of the causes of sudden infant death syndrome (3–6).

The *OCTN2* gene (7) encodes for a Na-dependent carnitine transporter that was mapped at 5q31.1 where a genetic locus for SCD has been assigned by linkage analysis (8). It has been proven that mutations of *OCTN2* impair carnitine transport in SCD patients (9–12).

Due to the lack of a proper marker for diagnosis at the molecular level no genetic epidemiological studies have been done in the general population. The primary goal of the present study was, therefore, aimed at obtaining the prevalence of heterozygotes of *OCTN2* mutations in the general population.

Cardiac complications have been reported (13) in an SCD sibling who was judged to be heterozygous by serum free-carnitine level. Furthermore, in several families very young siblings of affected patients have been reported to die unexpectedly of unknown causes (3,14). There was, however, no direct retrospective molecular diagnosis of these deceased children to determine whether these children also suffered from SCD. These observations raise two questions: (i) whether the deceased children were homozygous for the *OCTN2* mutations;

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Table 1. Haplotypes of multiple site variants and mutations in a Japanese population

Haplotype	Total no.	No. of subtype	5'-UTR	EX1	EX2	EX3	EX4	INT4	EX5	INT5	INT6	EX8	Description of mutation
A	69	64	CGC GCG	CTC	TGG	ATG	CTG	CCT	TGG	ACC	TAT	TCC	Wild-type
		1	CGC GCG	CTC	TGG	ATG	CTG	CCT	TGG	CCG	TAT	TCC	[17227A→C; 17229C→G]
		4	CGC GCG	CTC	TGG	ATG	CTG	CCT	TGG	ACC	TAT	*TGC*	22602C→G (Ser467Cys)
B	71	48	CGC GCG	CTT	TGG	ATG	CTA	CTT	TGG	ACC	TAT	TCC	[285C→T; 15524G→A; 15554C→T]
		3	CGC GCG	CTT	TGG	ATG	CTA	CTT	TGG	ACC	TAT	TCC	[285C→T; 15524G→A; 15554C→T] and 22402–22403insA[a]
		20	CGC GCG	CTT	TGG	ATG	CTA	CTT	TGG	CCG	TAT	TCC	[285C→T; 15524G→A; 15554C→T] and [17227A→C; 17229C→G]
C	68	43	CTC GTA	CTC	TGG	ATG	CTG	CCT	TGG	ACC	TGT	TCC	[–107G→T; –78C→T; –77G→A; 19159A→G]
		21	CTC GTA	CTC	TGG	ATG	CTG	CCT	TGG	CCG	TGT	TCC	[–107G→T; –78C→T; –77G→A; 19159A→G] and [17227A→C; 17229C→G]
		3	CTC GTA	CTC	*TGA*	ATG	CTG	CCT	TGG	ACC	TGT	TCC	[–107G→T; –78C→T; –77G→A; 19159A→G] and 8418G→A(Trp132X)
		1	CTC GTA	CTC	TGG	ATG	CTG	CCT	*TGT*	ACC	TGT	TCC	[–107G→T; –78C→T; –77G→A; 19159A→G] and 17086G→T(Trp283Cys)
Unclassified[b]		1				*TTG*							14226A→T(Met179Leu)

Letters in bold indicate mutated nucleotides. Italic letters indicate missense or nonsense mutations.
[a]Insertion of A in the 3'-UTR, resulting in an in-frame shift in this region.
[b]Unclassified, undetermined as to either A or B haplotype.

and (ii) whether heterozygotes for the *OCTN2* mutations develop cardiac complications.

RESULTS

Population-based study

A total of 557 males (mean age ± SD 32.4 ± 9.31 years, range 18–68, median 31) and 416 females (mean age ± SD 25.7 ± 6.13 years, range 18–62, median 24) participated in the first stage of screening. The serum free-carnitine concentrations were distributed normally with the mean serum free-carnitine ± SD for males being 50.4 ± 7.62 μM and for females 40.2 ± 7.19 μM. The mean was significantly higher in males than in females ($P < 0.01$). The screening cut-off levels of serum free-carnitine concentrations were set at 38 μM for males and 28 μM for females, which corresponded to the lowest 5% limit values (i.e. mean – 1.64 SD). Any individual with a serum free-carnitine level below the cut-off value was tentatively identified as a carrier candidate and those whose serum free-carnitine levels were above the cut-off values were assigned to the normal group.

All members of the carrier candidate group and randomly selected persons from the normal group (50 males and 30 females) were asked to return for further examination. Nineteen males (73% response rate) and 17 females (85% response rate) from the carrier candidate group, and 45 males (90% response rate) and 24 females (80% response rate) from the normal group, returned for the second phase.

Fourteen (seven males, seven females) of the carrier candidate group consistently had serum free-carnitine levels below the 5% lowest limit: mean ± SD was 35.2 ± 1.4 μM for males with a range of 33.8–37.2, and 25.2 ± 2.5 μM for females with a range of 21.0–27.1. In contrast, the remaining members of the carrier candidate group and the normal group had serum free-carnitine levels above the 5% lowest limit: mean ± SD was 52.4 ± 8.2 μM for males ($n = 57$) with a range of 38.4–71.3, and 39.0 ± 5.3 μM for females ($n = 34$) with a range of 30.6–48.2.

All 10 exons of the organic cation transporter (*OCTN2*) gene were sequenced in 105 subjects from members of the carrier candidates and normal groups. The structure pattern of the sequence polymorphisms allowed us to resolve the linkage and infer haplotypes. Haplotype A was found in 69 out of 210 chromosomes (32.9%), haplotype B was found in 71 out of 210 (33.8%) and haplotype C was found in 68 out of 210 (32.3%) (Table 1).

A truncated mutation, Trp132X (Fig. 1A), was found in three participants who had serum free-carnitine levels lower than the 5% lowest limit at two separate determinations. This mutation was also found in the AK family and was derived from maternal ancestors (10). The genotypes of these three carriers enabled us to infer multiple site linkage disequilibrium of this mutation with haplotype C because two were homozygous for haplotype C and the other had a genotype BC.

A missense mutation, Ser467Cys, was found in four individuals (Fig. 1D). Three were homozygous for haplotype A and the other had a genotype AB. The other missense mutation, Trp283Cys (Fig. 1C), was found in one worker who was

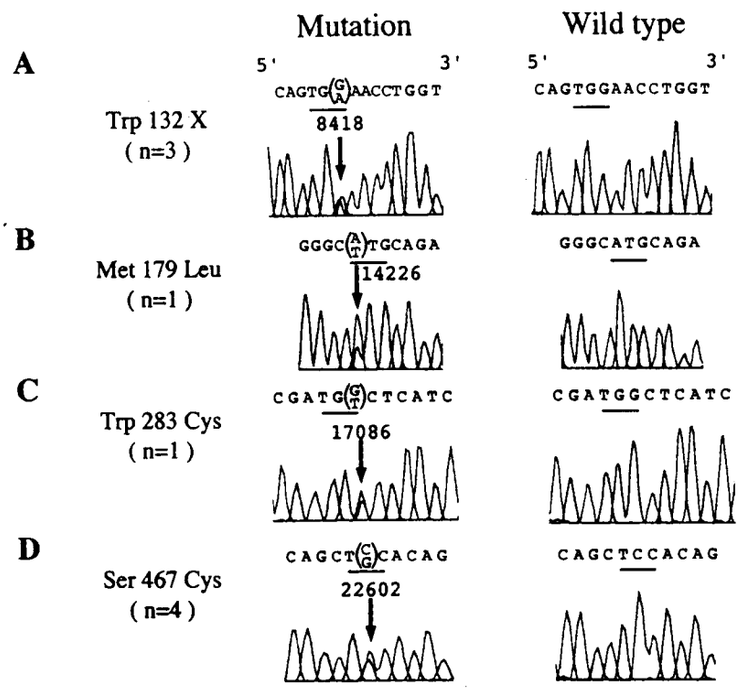

Figure 1. Direct sequences of four mutations detected in heterozygotes in the general population. (**A**) Trp132X [8418G→A] mutation in exon 2 was found in three unrelated individuals. (**B**) Met179Leu [14226A→T] mutation in exon 3 was found in one individual. (**C**) Trp283Cys [17086G→A] mutation in exon 5 was found in one individual. (**D**) Ser467Cys [22602C→G] mutation in exon 8 was found in four unrelated individuals. These mutations were confirmed by sequencing reverse strands (data not shown).

homozygous for haplotype C. These data enabled us to assign the Ser467Cys and Trp283Cys mutations to a chromosome carrying haplotypes A and C, respectively. The Met179Leu mutation (Fig. 1B) was found in a person who had a genotype AB, making determination of the haplotype of this mutation impossible. These carriers were shown to have serum free-carnitine levels lower than the 5% lowest limit at two separate determinations.

In vitro functional analysis for the missense mutations revealed that both Ser467Cys and Trp283Cys significantly reduced L-carnitine uptake to 11 and 2% of the normal control, respectively, while the Met179Leu mutation slightly reduced uptake to 74% (Fig. 2).

Four out of the five males who showed low serum carnitine phenotypes without mutations were vegetarians. The low carnitine phenotype of the remaining male, however, could not be elucidated by this study. The medical examinations did not reveal abnormalities in any member of the carrier candidate group, including those who did not attend the second phase, except one male (Ser467Cys) in his 30s who had diabetes and mild lipidemia (hypertriglyceridemia and hypercholesterolemia) and one female (Met179Leu) in her 30s, with a minor electrocardiogram (ECG) abnormality (abnormal Q wave) and mild hypercholesterolemia.

A lowest estimate of the prevalence of heterozygotes with impaired function mutations is calculated to be 0.82% (eight out of 973, CI 0.80–0.84%). The estimated population prevalence of impaired function mutations, when adjusted for the response rate at the second stage (0.73 for males and 0.85 for females), is calculated to be 1.01%, i.e. (2/0.73 + 6/0.85)/973, CI 0.99–1.03%, in the present population.

Pedigree-based study

Echocardiographic examinations of 11 pedigree members <20 years of age in the TH family, four heterozygotes and seven wild-types (Fig. 3) confirmed the absence of abnormalities in both the cardiac parameters and cardiac function in individuals as previously reported (8). Among the 31 members >20 years of age without confounding diagnosis, 10 heterozygotes were found to exceed the 95% upper limit of one of the echocardiographic parameters for Japanese, although ECGs and chest X-rays were normal. Individual II-4 in family TH, who was in her 70s, was diagnosed as having clinically apparent cardiac hypertrophy. The chest roentgenography and ECG revealed that she also had supraventricular premature contraction and cardiomegaly (63%).

The confounding diagnoses of hypertension in various cases, systemic lupus erythematosus with hypertension in III-13 in family TH and chronic renal failure with dialysis and hypertension in II-9 in family AK were found in the pedigrees (Fig. 3). Multiple logistic analysis showed that mutations, age and presence of the confounding factors were significant independent risk factors for echocardiographic abnormalities (Table 2). The

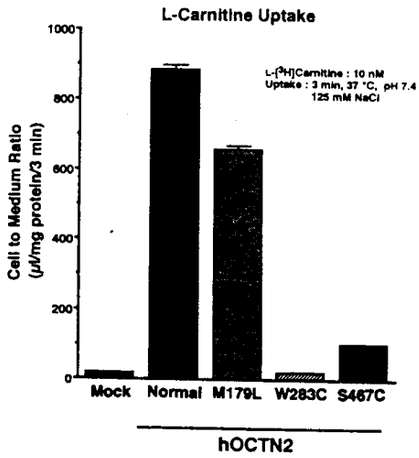

Figure 2. *In vitro* functional analysis of *hOCTN2* mutations. Uptake of L-[^{3}H]carnitine by normal *hOCTN2*- or mutant *hOCTN2*-transfected HEK293 cells. Uptake of [^{3}H]carnitine was measured in HEK293 cells transfected with wild-type *hOCTN2* (wild), mutant *hOCTN2*s found in workers [Met179Leu (M179L), Trp283Cys (W283C) and Ser467Cys (S467C)] or pcDNA3 vector alone (mock). Uptake was measured after incubation at 37°C for 3 min in L-[^{3}H]carnitine (10 nM) at pH 7.4 in 125 mM NaCl as previously reported (7), and data are shown as the means ± SE of three determinations. This experiment was repeated twice. All mutations reduced carnitine uptake significantly compared with the wild-type (ANOVA, $P < 0.01$).

mutations were found to be a larger risk factor (odds ratio 15.1, 95% CI 1.39–164) than other factors.

A retrospective diagnosis for IV-9 (Fig. 3) in family TH revealed homozygosity of the 23713G→A mutation in the *OCTN2* gene (data not shown), which is the acceptor site of intron 8, as previously reported (10). IV-9 died of unknown causes when he was 1.5 years of age after a 1 day episode of vomiting in 1989. In family KR, II-1 died suddenly when he was 8 years old in 1978 (Fig. 3). He was diagnosed as having cardiac hypertrophy when he entered elementary school at 6 years of age. His sibling, II-2, also died suddenly when he was 2 years of age after a 1 day episode of diarrhea and vomiting in 1974. Both subjects were retrospectively confirmed to be homozygous for the 113 bp deletion spanning the 5'-untranslated region (5'-UTR) to exon 1 (–91–22del) (10; data not shown).

DISCUSSION

SCD is a treatable hereditary disease inherited in an autosomal recessive mode and, at present, is considered to be a rare disease with only 30 cases reported in the world since 1988 (6,15,16). The absence of mutations in the coding regions of 91 individuals with normal serum free-carnitine levels at least once in the two separate determinations suggests that a false negative rate for carriers is negligible, thus validating the estimation of the prevalence of *OCTN2* mutations in the general population of the Tohoku district of Japan. In this study an estimate of the prevalence of mutations associated with impaired carnitine transport was at least 0.822%, implying 1 SCD patient per 60 000 births. If adjusted by the attendance at the second screening, the rate is 1.01%, giving an estimated incidence of primary systemic carnitine deficiency as 1 in 40 000 births.

It is of note that two mutations, Trp132X and Ser467Cys, accounted for seven of eight deleterious mutations found in the present study. The former mutation was found in three unrelated participants with the same *OCTN2* haplotype who are from various locations in Akita prefecture. This mutation was also found recently in a Chinese patient (12), although of unknown haplotype, which may indicate that this mutation may represent a type of SCD among Asian patients. The other mutation, Ser467Cys, was found in four unrelated workers with the same *OCTN2* haplotype who are from Akita and Iwate.

Akita prefecture is located in the northern part of Japan and has a population size of 1 200 000. The demographic characteristics of the Edo era (17–19C), when both social and geographical isolation were enforced, are still well preserved in rural areas of Akita due to low migration rates from other parts of Japan. Historically, large-scale population migrations from the western part of Japan to Tohoku district occurred several times before the Edo era. Taken together, such a high prevalence of these two mutations may, therefore, be indicative of a strong founder effect in the Tohoku district.

As previously reported (8), heterozygotes that carry missense or truncated mutations had consistently low serum free-carnitine phenotypes. Both Ser467Cys and Trp283Cys mutations impaired *in vitro* carnitine uptake as predicted from the *in vivo* low serum free-carnitine phenotype. In contrast, the Met179Leu mutation increased carnitine uptake *in vitro* in HEK cells, although a carrier of this mutation had a low carnitine phenotype. This case implies that there may be other mechanisms for reducing carnitine transporter activity and several possibilities should be considered, including: (i) a dominant-negative effect of cell type-specific mechanisms as reported in somatotrophs (17); (ii) an instability of mutated *OCTN2* mRNA; and (iii) mutations outside the region examined in the present study.

The clinical investigation using direct sequence analysis retrospectively diagnosed all three deceased children of affected families as SCD. In addition, we confirmed that heterozygotes <20 years of age did not have any echocardiographic abnormalities. These and our previous findings (8) may indicate that *OCTN2* mutations are not a likely risk factor for life-threatening cardiac complications in juvenile heterozygotes.

Among middle-aged members of the pedigrees, however, echocardiography revealed that mutation of the carnitine transporter might be a risk factor for a marginal, yet detectable, left ventricular hypertrophy. This phenotypic profile shows a clear contrast with homozygotes that develop cardiomegaly in childhood (6,18). Such clinical changes were so subtle that ordinary health check-ups, using ECG and chest roentgenography without echocardiography, would not detect them. These changes are generally late onset and of a benign type with a low penetrance, suggesting a synergistic involvement of non-genetic factors such as aging and/or environmental factors in addition to *OCTN2* mutations. This finding appears to be in accord with reported evidence that a low carnitine phenotype and aging may act synergistically to lower the efficiency of ATP production in humans (19).

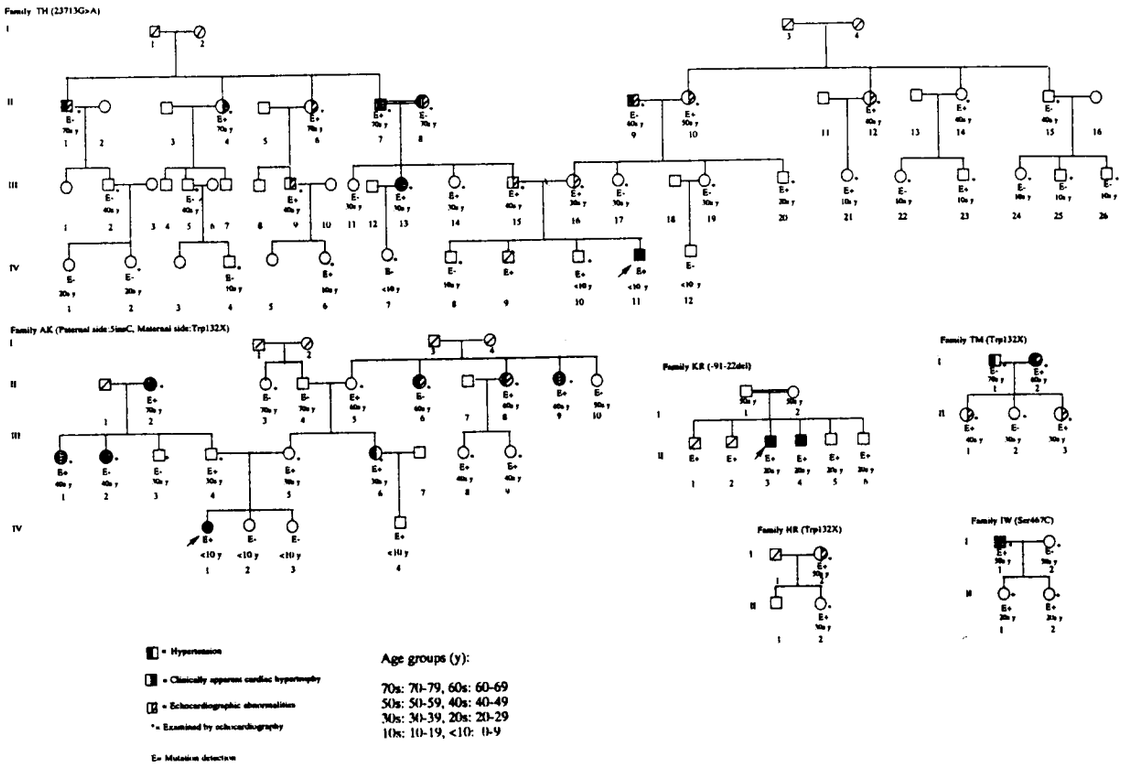

Figure 3. Pedigrees of the six families with *OCTN2* mutations. Clinically apparent cardiac hypertrophy was as defined in the text. Those with echocardiographic abnormalities had at least one of the parameters above the 95% limit of the normal range for Japanese. To protect privacy ages are shown in age groups when information of age was available.

A careful scrutiny of the TH pedigree reveals cardiac abnormalities without *OCTN2* mutations on both sides of the pedigree, although each side is unrelated, seeming to confound our tentative conclusion. However, it should be pointed out that non-carrier cases with cardiac abnormalities always have hypertension, which is one of the well-established risk factors for cardiac hypertrophy. In contrast, 10 heterozygotes had cardiac complications without hypertension or other confounding diagnoses in these pedigrees. Therefore, it is very unlikely that masked genetic factors other than deleterious *OCTN2* mutations are associated with cardiac abnormalities in these families. To resolve this uncertainty we are currently undertaking a population-based epidemiological study in Akita.

Finally, it is worthwhile mentioning that the present observation confirms earlier reports which showed that serum free-carnitine levels were low in vegetarians (20,21). Low carnitine levels, however, do not suggest a nutritionally deficient status of carnitine in vegetarians under ordinary conditions (20,21). The present study seems to support these earlier findings because the annual health check-up did not find any discernible clinical problems in vegetarians with a low carnitine phenotype.

MATERIALS AND METHODS

Population-based study

Members of the study population were white collar workers working mostly as salespeople in Akita prefecture. About 4 out of 5 of the males and 9 out of 10 of the females were originally from Akita prefecture while the remaining workers were from other prefectures in Tohoku and Hokkaido districts. These workers were all members of a local mutual aid union, an organization for medical health coverage, for salespersons. The study population consisted of members of this union who lived in the city of Akita and its urban areas. Once a year a medical check-up program is offered to all union members. This program includes ECG, chest roentgenography, a battery of biochemical tests [asparatate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase and γ-GTP] and lipid values [total cholesterol, free fatty acid and high density lipoprotein (HDL) cholesterol]. Ninety-five percent of the eligible workers received medical check-ups during this study in the Akita area.

Serum free-carnitine levels were determined for all participants during the annual medical check-up. All participants in the second phase donated 3 ml of blood for molecular analysis.

Table 2. Multiple logistic analysis for echocardiographic abnormalities

Factor	Odds ratio	95% CI	*P*-value
Gender (male versus female)	1.36	0.22–8.25	>0.05
Age (every 20 years)	4.16	1.27–13.6	0.02
Mutation (wild-type versus heterozygotes)	15.1	1.39–164	0.03
Hypertension and other confounding factors (no versus yes)	12.4	1.00–156	0.05

Multiple logistic analyses were conducted by incorporating the above four factors simultaneously.

Table 3. Primers for amplification, sequencing and mutation detection

Exon	Forward primer	Sequence	Reverse primer	Sequence	Product siz (bp)
1	*OC21F2*	5'-GCA GGA CCA AGG CGG CGG TGT CAG-3'	*OC21R1*	5'-AG CTC GGG TTC AAG GAC CGC-3'	629
1	*OC21F5*	5'-TGT CCT CCG TGT TCC TGA TA-3'	*OC21R1*	5'-AG CTC GGG TTC AAG GAC CGC-3'	354
2	*OC22F3*	5'-CCT GAC TAA GTG AGT TCA CA-3'	*OC22R1*	5'-TCA AGG GCC AGG CAC ACG CT-3'	305
3	OC234F2	5'-TTC ACA CCC ACT TAC TGG ATG GAT-3'	*OC23R3*	5'-GAA AGG TAG GTG ATG GGA TG-3'	325
4	*OC24F3*	5'-GGA ACC CAA ATT AAA CTG C-3'	OC24R1	5'-CTG CCC TCT AGT GAA GGC CA-3'	288
5	*OC25F3*	5'-GGT TCT GCA ACC TTA TTC-3'	*OC25R1*	5'-AGG GCT GGG TGC TGC TGC TC-3'	297
6	OC26F2	5'-TCT GAC CAC CTC TTC TTC CCA TAC-3'	*OC26R1*	5'-TAA ACA AGA GGC CCA ATG GC-3'	217
7	*OC27F3*	5'-TAC AGG TTG GGA AAG ATG-3'	*OC27R1*	5'-ATT GAG ACA GCC TGG TAG AC-3'	344
8	OC28F2	5'-TAT GTT TGT TTT GCT CTC AAT AGC-3'	*OC28R1*	5'-CAG CTC ACA TTC AAG CCA GT-3'	329
9	*OC29F1*	5'-ATA AAG GGG TAG ATG AGA GA-3'	OC28R2	5'-TCT GTG AGA GGG AGT TTG CGA GTA-3'	308
10	*OC210F3*	5'-CTT GTT TGT TTG GAG ACT G-3'	*OC210R1*	5'-CTG CAC AAG CTG GCC ATT TC-3	227
Pedigree-specific primers					
Detection of 113 bp deletion in family KR					
1	*OC21F2*	5'-GCA GGA CCA AGG CGG CGG TGT CAG-3'	*OC21R2*	5'-ACG GAG GAC AGG CCG GTG AAG CCA-3'	285
Mutation of splicing acceptor site in intron 8 in family TH					
9	*OC29F3*	5'-AGA GTC CTG GGA GCA TAA-3'	*OCTN29R*	5'-CCT TTG ACT CTT AGC ATC-3'	220

Primers not in italic were obtained as in our previous publication (10). Those in italic were newly designed for optimization to obtain better sequence or PC results.

Pedigree-based study

Six unrelated families participated in this study (Fig. 3). The members of families AK and TH currently suffering from systemic carnitine deficiency disease were briefly discussed in previous papers (8,10). Additional members of family TH were asked to participate in this study. Two siblings in family KR (14) and a sibling of the proband in family TH died of unknown causes. DNA was isolated from peripheral blood samples from the participants to detect pedigree-specific mutations. Retrospective diagnoses were conducted using DNA isolated from umbilical cord stumps of the three deceased children. Preservation of the umbilical cord as a remembrance of a child's birth is a traditional custom common in Japan. Pieces of the umbilical cord stumps (5 × 5 mm) were excised, pulverized in liquid nitrogen and DNA was then extracted from the powdered samples.

To expand the pedigrees we asked heterozygous individuals in the worker population to join a pedigree-based study. Four of the seven families chosen for the study declined to join the study because their parents did not live near the city of Akita. Blood relatives of three families (families TM, HR and IW) participated in the study.

Members of families AK, TH, TM, HR and IW participati in the study were given medical examinations that includ echocardiogram, blood pressure determination, ECG, che roentgenography, a biochemical panel (ALT, AST, LDH, alk line phosphatase and γ-GTP) and lipid values (total chole terol, free fatty acid and HDL cholesterol). Hypertension w defined by blood pressure: systolic ≥160 mmHg and/ diastolic ≥95 mmHg. Medical histories were obtained from a the participants during interviews.

We evaluated three echocardiography parameters: intrave tricular septum wall thickness, the left ventricular posteri wall thickness and left ventricular weight. The first two paran eters were compared with the 95% upper limit among Japane (>13 years) stratified by sex, age, height and body mass inde (BMI): range 10–11 mm for females and 11–12 mm for mal (22). The left ventricular muscle weight was estimated by th published proposed equation (23). These values we compared with the 95% upper limit among Japanese (>1 years) stratified by sex, age, height and BMI: range 174–205 for females and 227–273 g for males (22). The echocardic graphic parameters were standardized by body surface area for children ≤13 years of age and compared with standar values (24). A participant was judged to have an echocardic

graphic abnormality if one of the 95% upper limits was exceeded. A clinical diagnosis of apparent cardiac hypertrophy was given when the ventricular wall thickness was >13 mm.

Molecular analyses

Thirteen primer pairs were synthesized (Table 3) in order to PCR amplify all exons of human *OCTN2* for direct sequencing. The primers were designed based on the genomic sequence of *OCTN2* reported in GenBank (accession no. AB016625). Primers were ~80 bp upstream and downstream of each exon to cover sequences of splice donor and acceptor sites and the branch site. Direct sequencing was conducted as previously reported (10). Both forward and reverse strands were directly sequenced to confirm mutations. Mutations were described based on a nomenclature recommendation (25).

The expression constructs for the three missense mutations found in the population study were made using the GeneEditor *In Vitro* Site-Directed Mutagenesis System (Promega, Madison, WI) and the original construct (PCDNA2/OCTN2) (10) as template. Both wild-type and mutant constructs were transfected into HEK293 cells, which do not have carnitine transport activity, and transport activities for L-[^{3}H]carnitine were measured as described previously (7). Uptake was measured in triplicate for each sample after incubation at 37°C for 3 min and the experiment was repeated twice.

Pedigree-specific mutations were detected by either direct sequencing using the primers in Table 3 or by PCR restriction fragment length polymorphism analysis as previously reported (10).

Other methods

Serum free-carnitine levels were determined by an enzymatic cycling method (26). These samples were collected as previously reported (8).

Ethical issues

The Institutional Review Board for Akita University School of Medicine approved the present study. Written informed consents for carnitine determinations and DNA sequence analyses of the *OCTN2* gene were obtained from all participants in both population and pedigree studies.

Statistical analysis

Values are presented as means ± SD or means ± SE. The increase in risk of echocardiographic abnormalities, including cardiac hypertrophy, in heterozygous carriers was determined by multiple logistic analysis. To adjust for confounding effects (i.e. gender, age, confounding diseases such as hypertension and others) these factors were simultaneously incorporated in a multiple logistic analysis. All statistical analyses were conducted with SAS (SAS Institute, Cary, NC). A P-value <5% was considered significant in this study.

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