Carnitine Palmitoyltransferase II Deficiency Due to a Novel Gene Variant in a Patient With Rhabdomyolysis and ARF

Hidetoshi Kaneoka, MD, PhD, Noriko Uesugi, MD, PhD, Ayaka Moriguchi, PharB, Shinichi Hirose, MD, PhD, Masaki Takayanagi, MD, PhD, Seiji Yamaguchi, MD, PhD, Yosuke Shigematsu, MD, PhD, Tetsuhiko Yasuno, MD, Yoshie Sasatomi, MD, PhD, and Takao Saito, MD, PhD

• **Adult patients deficient in carnitine palmitoyltransferase II (CPT II) cannot generate sufficient amounts of energy, which results in rhabdomyolysis and acute renal failure (ARF). Its genetic basis has been recognized; but histopathologic changes, especially electron microscopic changes, have scarcely been described. The study subject is a patient with ARF caused by repetitive nontraumatic rhabdomyolysis. The acylcarnitine profile of serum and enzyme assay on skin fibroblasts confirmed the diagnosis of CPT II deficiency. Renal biopsy specimens were examined microscopically and immunohistochemically. The histological diagnosis was interstitial nephritis with acute tubular necrosis caused by rhabdomyolysis. Myoglobin in tubules was detected by means of immunohistochemistry and electron microscopy. The genetic structure of CPT II was analyzed in the patient and his family. Eight pairs of polymerase chain reaction (PCR) primers were designed to cover the coding region. Each PCR-amplified gene product was subjected to DNA sequencing, which unveiled heterozygosity at the CPT II locus consisting of a deletion of cytosine and thymine at codon 408, resulting in a stop signal at 420, as well as a mutation of arginine to cysteine at codon 631. The frame shift at 408 has never been described before. DNA sequencing of the family showed the deletion mutation from the mother and the point mutation from the father. We describe renopathological findings in a patient with CPT II deficiency associated with rhabdomyolysis, which suggested the pathological role of myoglobin casts in the development of tubular necrosis. Genetic analysis of the patient identified a novel variant of the CPT II gene. *Am J Kidney Dis* 45:596-602.**

INDEX WORDS: Rhabdomyolysis; acute renal failure (ARF); carnitine palmitoyltransferase II (CPT II) deficiency; frame shift; point mutation; electron microscopy.

CARNITINE palmitoyltransferase II (CPT II) is one of the key mitochondrial enzymes involved in β-oxidation of long-chain fatty acids. β-oxidation is activated when there is an immediate requirement for high levels of energy, for example, in exhausting sports, military exercises, and severe infection. Adult patients deficient in this enzyme cannot supply adequate amounts of energy, which results in acute rhabdomyolysis and, if not appropriately managed, acute renal failure (ARF).[1-3] There is sufficient evidence for a genetic basis of this disorder; however, the associated histopathologic findings, especially electron microscopic findings, have rarely been described. We report a case of ARF caused by repetitive and nontraumatic rhabdomyolysis resulting from CPT II deficiency. Renal biopsy specimens were examined by means of light microscopy, electron microscopy, and immunohistochemistry. Genetic structure and familial segregation also were determined.

CASE REPORT

A 24-year-old Japanese man was transferred to the emergency department of Fukuoka University Hospital and Clinic (Fukuoka, Japan) because of ARF. Three years before presentation, he developed an illness characterized by high fever, myalgia, respiratory symptoms, and dark-colored urine. After hospitalization, he was found to have markedly elevated

From the Departments of Internal Medicine, Pathology, and Pediatrics, Division of Nephrology and Rheumatology, Fukuoka University School of Medicine, Fukuoka; Chiba Children's Hospital, Chiba; Department of Pediatrics, Shimane University, Izumo, Japan; and Department of Health Science, University of Fukui, Fukui, Japan.

Received August 9, 2004; accepted in revised form December 1, 2004.

Originally published online as doi:10.1053/j.ajkd.2004.12.006 on January 27, 2005.

Supported in part by fund no. 026006 from the Central Research Institute of Fukuoka University and a grant for Progressive Renal Diseases from the Ministry of Health, Labor, and Welfare, Japan.

Presented in abstract form at the American Society of Nephrology Annual Meeting, St Louis, MO, October 31, 2004.

Address reprint requests to Hidetoshi Kaneoka, MD, PhD, Division of Nephrology and Rheumatology, Department of Internal Medicine, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Jonan-Ku, Fukuoka 814-0180, Japan. E-mail: kaneokah@fukuoka-u.ac.jp

0272-6386/05/4503-0018$30.00/0

doi:10.1053/j.ajkd.2004.12.006

Table 1. CPT I and II Activity in Cultured Skin Fibroblasts

	CPT I Activity		
	Without Malonyl Coenzyme A	With Malonyl Coenzyme A	CPT II Activity
Present case	0.90	0.20	0.02
CPT I deficiency	0.01	0.08	0.80
Healthy controls (n = 10)*	1.38 ± 0.54	0.30 ± 0.11	0.97 ± 0.25

NOTE. CPT activity expressed as nanomoles of palmitoyl-L-(methyl-^{14}C) carnitine per minute per milligrams of protein. CPT I activity is expressed as the difference in CPT I activity in the presence and absence of 50 μmol/L of malonyl-coenzyme A, which is a specific inhibitor of CPT I. CPT I and II activity was measured in cultured skin fibroblasts as described by Demaugre et al.[5] Briefly, activities were assayed in supernatant of fibroblast homogenates as palmitoyl-L-(methyl-^{14}C) carnitine formed from L-(methyl-^{14}C) carnitine and palmitoyl coenzyme A. Palmitoyl-L-(methyl-^{14}C) carnitine was extracted with isopropanol and measured for CPT I activity.

*Data expressed as mean ± SD.

levels of serum muscle enzymes and urine myoglobin, and a diagnosis of rhabdomyolysis, probably caused by a viral infection or drugs prescribed for a common cold, was made. He was treated by means of assisted ventilation through endotracheal intubation, hydration, and medical diuresis and discharged 2 weeks later without complications. During that admission, renal dysfunction was not recognized.

In the current illness, the patient reported high fever, general malaise, myalgia, flulike symptoms, dyspnea, and dark-colored urine. He was seen by the family physician and referred to a consultant at a local medical center, where he was found to have rhabdomyolysis and ARF, and he was transferred to our unit. He was born to a nonconsanguinous couple. He smoked 40 cigarettes per day and was an occasional drinker. Physical examination on admission was not remarkable, with alert consciousness, normal neurological signs and reflexes, and arterial blood pressure of 150/96 mm Hg. Initial laboratory investigations showed positive test results for inflammation (circulating leukocytes, 17.4 × $10^3/\mu$L [× 10^9/L]; C-reactive protein, 21.5 mg/dL), high serum levels of myogenic enzymes (aspartate aminotransferase, 1,645 IU/L; alanine aminotransferase, 571 IU/L; lactic dehydrogenase, 4,340 IU/L; creatine kinase, 127,600 IU/L; aldolase, 132.8 U/L [normal, 0.5 to 3.1 U/L]; and myoglobin, 63,000 ng/mL [normal, <60]), and evidence of renal damage (proteinuria, 2^+ by means of test strips; myoglobinuria with myoglobin of 370,000 ng/mL; blood urea nitrogen, 27 mg/dL [9.6 mmol/L]; serum creatinine, 3.1 mg/dL [274 μmol/L]; β_2-microglobulin, 21.5 mg/L [normal, 0.8 to 1.8 mg/L]; and 24-hour creatinine clearance, 15 mL/min [0.25 mL/s]). Urine sediments showed 1 to 4 red blood cells and 10 to 20 white blood cells per high-power field, as well as a few granular and epithelial casts per low-power field. Serological test results for toxoplasma, mycoplasma, and antinuclear autoantibodies were negative. Lymphocyte stimulation tests for drugs administered for the flulike symptoms were negative. Diagnoses of rhabdomyolysis and ARF were made. The patient recovered successfully from ARF with hydration and diuresis and continuous hemofiltration and hemodialysis for 4 consecutive days, with resultant normalization of 24-hour urine volumes.

The repetitive rhabdomyolysis and complete recovery from ARF prompted us to investigate genetic and metabolic disorders in this patient after obtaining a signed consent form. Skin fibroblasts were surgically harvested from the forearm, cultured, and used for additional metabolic and genetic analyses. The acylcarnitine profile in a serum sample obtained during the acute phase by using tandem mass spectrometry showed markedly elevated levels of long-chain acylcarnitines, suggesting the diagnosis of CPT II deficiency (data not shown).[4] Enzyme assay using cultured skin fibroblasts showed low enzymatic activity and confirmed the diagnosis of CPT II deficiency[5] (Table 1).

Immunohistochemical and Electron Microscopy

One month after the onset, serum creatinine and 24-hour creatinine clearance values were 1.4 mg/dL (124 μmol/L) and 27 mL/min (0.45 mL/s), respectively. Urinalysis still showed 1^+ proteinuria with a few granular and epithelial casts. To determine the significance and extent of renal involvement, rather than its existence, a renal biopsy was performed. The obtained tissue sample was examined by means of light microscopy, immunohistochemistry, and electron microscopy, using methods described previously.[6] For light microscopy, paraffin-embedded sections were stained with hematoxylin-eosin, periodic acid–Schiff, periodic acid–methenamine silver, and Masson trichrome. For immunohistochemistry, sections were stained with polyclonal antisera to human immunoglobulin G (IgG), IgA, IgM, complements 1q and 3, and fibrinogen. Immunoperoxidase staining also was performed using rabbit antihuman myoglobin antibody (Dako, Glostrup, Denmark).

Light microscopic examination showed no remarkable changes in glomeruli; however, patchy and dense cellular infiltration was identified in the interstitium, together with acute necrosis of the proximal tubules. Distal tubules contained coarse granular light-brown pigmented casts (Fig 1A) and other protein-rich casts. Necrosis of tubular epithelia, disruption of the tubular basement membrane, and inflammatory cells were seen in several distal tubules, especially those containing pigmented casts. Dense inflammatory infiltrates, composed mainly of lymphocytes, histiocytes, neutrophils,

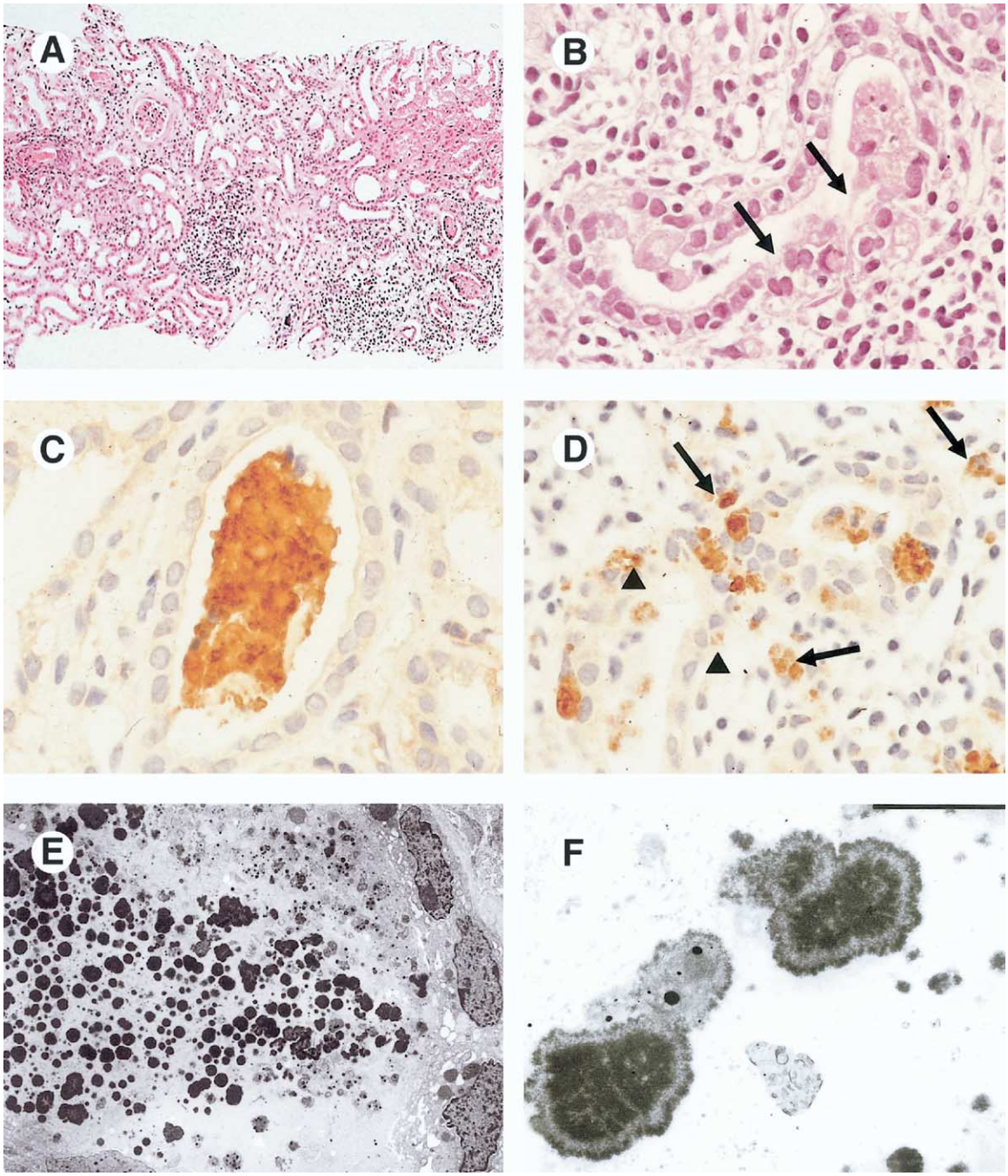

Fig 1. Microscopic examination. (A) Note severe inflammatory cell infiltration around tubular myoglobin casts and damaged tubules. (Hematoxylin and eosin stain; original magnification ×100.) (B) The other distal tubule containing a similar cast shows necrosis of epithelial cells (arrow) with infiltration of inflammatory cells. (Hematoxylin and eosin stain; original magnification ×400.) Myoglobin (brown) can be identified on (C) pigmented casts, (D) tubular epithelial cells (arrowhead), and (D) macrophages (arrow) by means of immunohistochemical study. Electron microscopy shows (E) numerous osmiophilic casts (original magnification ×2,900) composed of an electron-dense core surrounded by an electron-lucent rim, as seen (F) under higher magnification. (Original magnification ×10,000.)

Table 2. Primers Used for PCR Amplification and Direct DNA Sequencing

PCR	Exon		Sequence	Fragment Length (bp)	Reference
1	1	Sense	5′-CGGCCTTGTGTTTAGACTCC-3′	360	This report
		Antisense	5′-CTTCCAGATTAGGGGCTGTG-3′		This report
2	2	Sense	5′-GCCTTACACTGACCCTGCTT-3′	290	This report
		Antisense	5′-AGGTTCTGGGTTCCTGGAGA-3′		19
3	3	Sense	5′-TTCCAGGTTTTAGGGCTATG-3′	360	19
		Antisense	5′-GGAGGATGAGACGTTACTTC-3′		19
4	4	Sense	5′-TAGGGACAGCATTAACATTT-3′	430	19
		Antisense	5′-TGGCCTTGTCATCAGTGAAG-3′		19
5	4	Sense	5′-GTCCCAGTATTTTCGGCTTT-3′	380	19
		Antisense	5′-TGTGGGACAAGTGGACAAGG-3′		19
6	4	Sense	5′-GAGTTTCCCCTGGCATACCT-3′	510	This report
		Antisense	5′-GCCTCCTCTCTGAAACTGGA-3′		This report
7	4	Sense	5′-ACAGCTGCTAAGGAAAAGTT-3′	410	19
		Antisense	5′-CAAGACCCAAGGGCATGCTC-3′		19
8	5	Sense	5′-CTGAGACGCTGGTTTTCCA-3′	404	19
		Antisense	5′-GGTAGCTTTTCATCTGCCCA-3′		19

NOTE. PCR primers also were used as sequencing primers.

and plasma cells, were noted around the affected tubules (Fig 1B).

Immunofluorescent study showed no glomerular staining for IgG, IgA, IgM, C3, C1q, or fibrinogen (data not shown). Immunoperoxidase staining using rabbit antihuman myoglobin antibody showed strong positivity for brown-colored pigmented casts (Fig 1C), tubular epithelial cells (arrowhead), and inflammatory cells (arrow; Fig 1D). Electron microscopy showed numerous osmiophilic granular casts in the distal tubules (Fig 1E). At greater magnification (Fig 1F), casts were composed of a dense core surrounded by an electron-lucent rim, indicating they were myoglobin casts. The diagnosis based on biopsy examination was interstitial nephritis with acute tubular necrosis caused by rhabdomyolysis.

Genetic Structure of CPT II From the Patient and His Family

Written informed consents for genetic investigation were obtained from the patient and family members. Direct DNA sequencing of polymerase chain reaction (PCR)–amplified gene products was performed to determine the CPT II gene of the patient.[7-9] A new approach was used. Eight pairs of PCR primers were designed by overlapping to cover the complete stretch of the coding sequence (Table 2; GenBank accession no. M58581). Genomic DNA was extracted from cultured fibroblasts using a standard method.[7] The PCR protocol was as follows: 30 cycles of 1 minute at 94°C for denaturation, 1 minute at 60°C for annealing, and 1 minute at 72°C for extension and 1 cycle of 10 minutes at 60°C for completion. Each PCR-amplified gene product was then subjected to DNA sequencing on an automated DNA sequencer (ABI 3100 Genetic Analyzer; Applied Biosystems-Hitachi, Tokyo, Japan) using the PCR primer as the sequencing primer and the BigDye Terminator v3.1 cycle-sequencing kit (Applied Biosystems, Foster City, CA). Sequences from the 5′ end were confirmed by those from the 3′ end.

The 8 DNA sequences obtained from 8 PCR products were assembled (data not shown), and the resulting coding sequence was analyzed. Sequencing from the 5′ end and that from the 3′ end showed a deletion of CT from TCT at codon Y408 (1223delCT), resulting in a stop signal at codon 420 (Fig 2), and a sense mutation of arginine to cysteine at codon 631 (1891C→T; R631C). Because our sequencing strategy determines 2 haploids at once, a family study was deemed necessary to confirm the genotypes. The family study showed that the frame-shift mutation came from the mother (M) and the full-length point mutation came from the father (P). The younger brother (S2) shared the frame shift, but still had an intact genotype from the father. The youngest brother (S3) shared the 2 genotypes with the patient (Fig 3), but, to date, he has never reported symptoms suggestive of CPT II deficiency. All family members shared the same single-nucleotide polymorphisms at the 3 reported positions in the CPT II region.

DISCUSSION

Nontraumatic or non–drug-induced rhabdomyolysis is seen after exhaustive exercise and after severe infection, evidenced by dark-colored urine, recognized as one of the frequent causes of ARF, and, in rare cases, presents repeatedly and among family members.[9-13] Familial rhabdomyolysis often is attributed to deficiency of enzymes related to the metabolism of long-chain fatty acids. CPT II mutation is the most common disorder affecting mitochondrial β-oxidation. Genetic analyses have been carried out extensively.[2,3,8,14-20] In marked contrast to ordinary autosomal recessive traits, which usually are genotyped by restriction enzyme fragment length polymorphism, sequence-specific oligonucleotide hybridization, se-

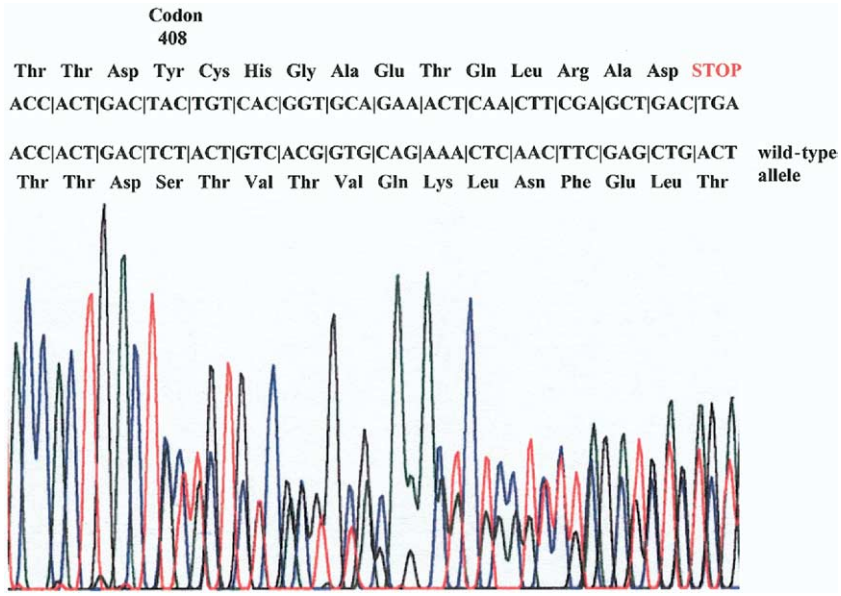

Fig 2. Direct DNA sequencing of CPT II–specific PCR product. Two nucleotides CT were deleted from codon 408 (1223delCT; Y408), resulting in a mixture of sequences consisting of the wild-type sequence and frame-shift sequence. Stop codon was identified at codon 420 on the frame-shift sequence.

quence-specific PCR, and so on, mutations of CPT II deficiency are located along the entire stretch of the coding region. Gene sequencing is indispensable for determining the mutation in affected patients, especially in our case. We used direct DNA sequencing of PCR-amplified gene products to avoid the time-consuming cloning and sequencing. To our knowledge, 41 sites of mutation in the 5 exons and 2 sites in introns have been reported.[3,8] Four of the 41 sites were frame-shift mutations based on nucleotide insertions or deletions. However, the deletion at codon Y408 (1223delCT), seen in our case, has never been described before.

The association between types and sites of mutations and clinical features has been a focus of interest. Unfortunately, the crystallography of CPT II has not been achieved to date; however, several functional analyses related to the genetic variances have been reported. Mutation of serine at codon 113 to leucine (338C→T; S113L), which is the most frequent, 60% in European unrelated alleles,[18] was reported to be closely related to adult-type rhabdomyolysis. The pathological function of R631C (1891C→T), also detected in the present family, is still controversial. This phenotype was recognized as important for the stability of the enzyme and sometimes assigned as a "severe" infantile systemic type, but sometimes as a "mild" adult muscular type.[19] The CPT II molecule resides on the mitochondrial inner membrane as a homotetramer[21] and anchors to the membrane with codon 464 through 496. Because our patient has a shorter peptide, which lacked the anchor sequence, it is conceivable that enzyme activity was very low.

The genotypes of CPT II of the youngest brother were identical to those of the patient. Recently, peroxisome proliferator-activated receptor α was shown to have important roles in the regulation of CPT II gene transcription.[22] Additional analyses are required.

Intraluminal myoglobin casts are attributed to myoglobinuric ARF through the induction of tubular obstruction and luminal urinary stasis, followed by reduced glomerular infiltration and increased toxin uptake by proximal tubules.[23] However, direct histological evidence for tubular damage by myoglobin casts has not yet been provided. Our immunohistochemical study shows that myoglobin was evident not only in tubular casts, but also in tubular epithelial cells and interstitial phagocytotic cells. The cast-containing distal tubules showed severe injury with epithelial cell necrosis, disruption of the basement membrane, and invasion of inflammatory cells. Dense cellular infiltration in the interstitium was recognized around the affected tubules. Solez et al[24] analyzed 57 renal biopsy specimens from patients with potentially reversible ARF and emphasized that necrosis of individual tubular epithelial cells appeared to be a continuing

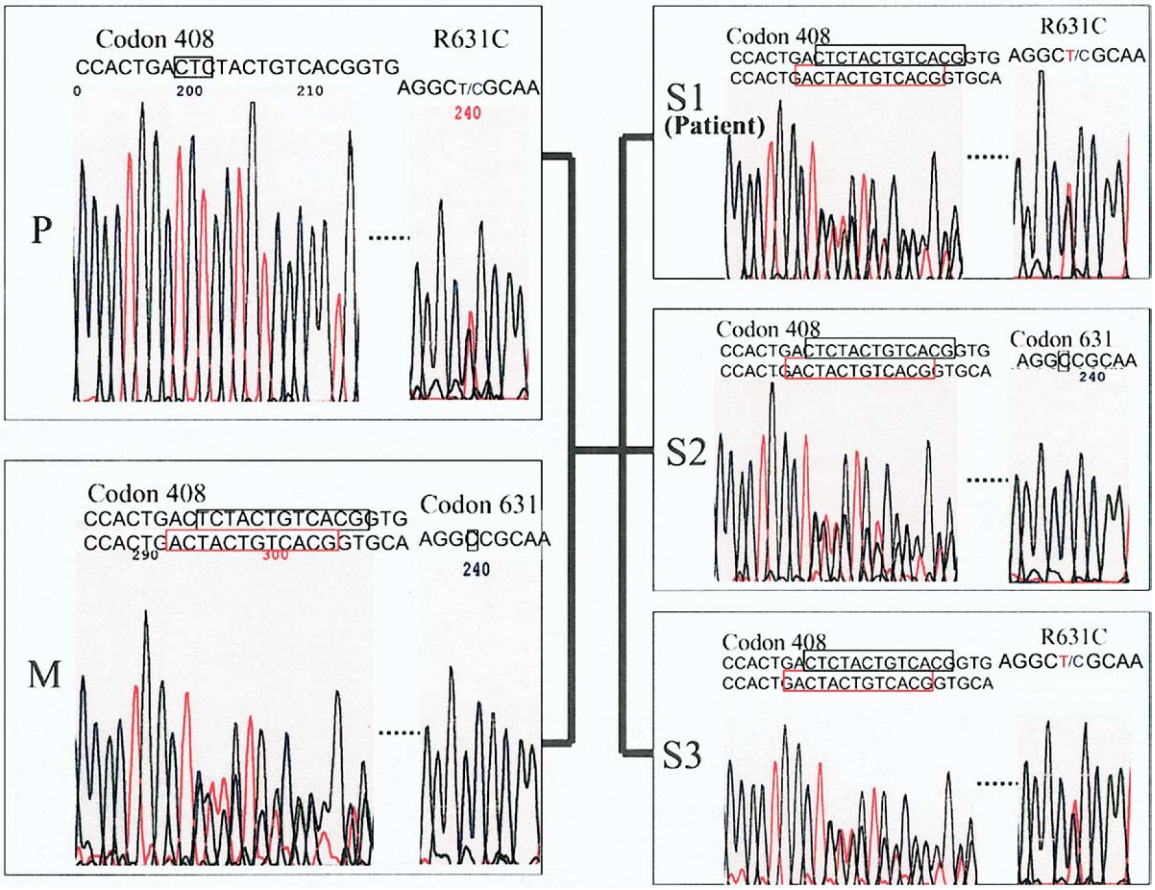

Fig 3. Sequence analyses of mutations of exons 4 and 5 in the family. The frame-shift mutation at codon Y408 (1223delCT) arose from the mother (M), and the point mutation R631C (1891C →T), from the father (P).

process in ARF. The histopathologic findings in our case strongly suggest tubulotoxicity of the myoglobin casts and resultant ARF. We identified intraluminal myoglobin casts in the electron microscopic study, which has rarely been described in patients with myoglobinuric ARF. The major feature of the cast was the electron-lucent peripheral rim relative to the dense core, similar to the myoglobin casts described previously by Nadasdy and Racusen.[25]

In conclusion, we describe renopathological findings in a patient with CPT II deficiency associated with rhabdomyolysis. These findings emphasize the pathological role of myoglobin casts in the development of tubular necrosis. Genetic analysis of the patient and family members identified a novel variant of the CPT II coding gene.

ACKNOWLEDGMENT

The authors thank Kaoru Kojima for excellent technical and secretarial assistance.

REFERENCES

1. van der Leij FR, Huijkman NCA, Boomsma C, Kuipers JRG, Bartelds B: Genomics of the human carnitine acyltransferase genes. Mol Genet Metab 71:139-153, 2000

2. Thuillier L, Rostane H, Droin V, et al: Correlation between genotype, metabolic data, and clinical presentation in carnitine palmitoyltransferase 2 (CPT2) deficiency. Hum Mutat 21:493-501, 2003

3. Sigauke E, Rakheja D, Kitson K, Bennett MJ: Carnitine palmitoyltransferase II deficiency: A clinical, biochemical, and molecular review. Lab Invest 83:1543-1554, 2003

4. Shigematsu Y, Hirano S, Hata I, et al: Selective screening for fatty acid oxidation disorders by tandem mass spectrometry: Difficulties in practical discrimination. J Chromatogr B Analyt Technol Biomed Life Sci 792:63-72, 2003

5. Demaugre F, Bonnefont J-P, Colonna M, Cepanec C, Leroux J-P, Saudubray J-M: Infantile form of carnitine

palmitoyltransferase II deficiency with hepatomuscular symptoms and sudden death. Physiopathological approach to carnitine palmitoyltransferase II deficiencies. J Clin Invest 87:859-864, 1991

6. Saito T, Sato H, Kudo K, et al: Lipoprotein glomerulopathy: Glomerular lipoprotein thrombi in a patient with hyperlipoproteinemia. Am J Kidney Dis 13:148-153, 1989

7. Kaneoka H, Hsu K-C, Takeda Y, Sharp GC, Hoffman RW: Molecular genetic analysis of HLA-DR and HLA-DQ genes among anti-U1-70-kd autoantibody positive connective tissue disease patients. Arthritis Rheum 35:83-94, 1992

8. Wataya K, Akanuma J, Cavadini P, et al: Two CPT2 mutations in three Japanese patients with carnitine palmitoyltransferase II deficiency: Functional analysis and association with polymorphic haplotypes and two clinical phenotypes. Hum Mutat 11:377-386, 1998

9. Yang B-Z, Ding J-H, Roe D, Dewese T, Day DW, Roe CR: A novel mutation identified in carnitine palmitoyltransferase II deficiency. Mol Genet Metab 63:110-115, 1998

10. Berkman N, Meirow D, Katzir Z, Bar-On H: Acute renal failure due to carnitine palmitoyltransferase deficiency. J Intern Med 233:295-297, 1993

11. Faigel HC: Carnitine palmitoyltransferase deficiency in a college athlete: A case report and literature review. College Health 44:51-54, 1995

12. Kelly KJ, Garland JS, Tang TT, Shug AL, Chusid MJ: Fatal rhabdomyolysis following influenza infection in a girl with familial carnitine palmityl transferase deficiency. Pediatrics 84:312-316, 1989

13. Keverline JP: Recurrent rhabdomyolysis associated with influenza-like illness in a weight-lifter. J Sports Med Phys Fitness 38:177-179, 1998

14. Bonnefont J-P, Demaugre F, Prip-Buus C, et al: Carnitine palmitoyltransferase deficiencies. Mol Genet Metab 68:424-440, 1999

15. Minoletti F, Colombo I, Martin AL, et al: Localization of the human gene for carnitine palmitoyltransferase to 1p13-p11 by nonradioactive *in situ* hybridization. Genomics 13:1372-1374, 1992

16. Finocchiaro G, Taroni F, Rocchi M, et al: cDNA cloning, sequence analysis, and chromosomal localization of the gene for human carnitine palmitoyltransferase. Proc Natl Acad Sci U S A 88:661-665, 1991

17. Taroni F, Verderio E, Fiorucci S, et al: Molecular characterization of inherited carnitine palmitoyltransferase II deficiency. Proc Natl Acad Sci U S A 89:8429-8433, 1992

18. Taroni F, Verderio E, Dworzak F, Willems PJ, Cavadini P, DiDonato S: Identification of a common mutation in the carnitine palmitoyltransferase II gene in familial recurrent myoglobinuria patients. Nat Genet 4:314-319, 1993

19. Thuillier L, Sevin C, Demaugre F, et al: Genotype/phenotype correlation in carnitine palmitoyl transferase II deficiency: Lessons from a compound heterozygous patient. Neuromusc Dis 10:200-205, 2000

20. Wieser T, Deschauer M, Olek K, Hermann T, Zierz S: Carnitine palmitoyltransferase II deficiency. Molecular and biochemical analysis of 32 patients. Neurology 60:1351-1353, 2003

21. Finocchiaro G, Colombo I, DiDonato S: Purification, characterization and partial amino acid sequences of carnitine palmitoyl-transferase from human liver. FEBS Lett 274:163-166, 1990

22. Barrero MJ, Camarero N, Marrero PF, Haro D: Control of human carnitine palmitoyltransferase II gene transcription by peroxisome proliferator-activated receptor through a partially conserved peroxisome proliferator-responsive element. Biochem J 369:721-729, 2003

23. Zager RA: Rhabdomyolysis and myohemoglobinuric acute renal failure. Kidney Int 49:314-326, 1996

24. Solez K, Morel-Maroger L, Sraer J-D: The morphology of "acute tubular necrosis" in man: Analysis of 57 renal biopsies and a comparison with the glycerol model. Medicine 58:362-376, 1979

25. Nadasdy T, Racusen LC: Renal injury caused by therapeutic and diagnostic agents and abuse of analgesics and narcotics, in Jennette JC, Olson JL, Schwartz MM, Silva FG (eds): Heptinstall's Pathology of the Kidney (ed 5). Philadelphia, PA, Lippincott-Raven, 1998, pp 811-862