Identification of a New Point Mutation in the Human Molybdenum Cofactor Sulferase Gene That Is Responsible for Xanthinuria Type II

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A 43-year-old xanthinuric female was referred to our department because of hypouricemia. Routine laboratory data showed hypouricemia, a high level of plasma oxypurines, decreased urinary uric acid excretion, and increased urinary oxypurine excretion, with xanthine dehydrogenase activity in the duodenal mucosa below the limits of detection. In addition, allopurinol was not metabolized. From these findings, the patient was diagnosed with xanthinuria type II. To investigate the properties of xanthine dehydrogenase/xanthine oxidase (XDH/XO) deficiency, a cDNA sequence encoding XDH/XO, aldehyde oxidase (AO), and molybdenum cofactor sulferase (MCS), as well as immunoblotting analysis for XDH/XO protein, obtained from duodenal mucosa samples were performed. The XDH/XO cDNA and AO cDNA sequences of the xanthinuric patient were consistent with previously reported ones, whereas the MCS cDNA sequence revealed a point mutation of G to C in nucleotide 466, which changed codon 156 from GCC (Ala) to CCC (Pro). In addition, the MCS genomic DNA sequence including the site of the mutation revealed the same, suggesting that the xanthinuric patient was homozygous for this mutation. Such findings have not been previously reported for patients with xanthinuria type II.

XANTHINE dehydrogenase/xanthine oxidase (XDH/XO, EC 1.1.1.204) is a molybdenum iron-sulfur flavin hydroxylase that oxidizes a variety of purines and pterins. It contains a molybdenum cofactor, which is essential for the activity of xanthine dehydrogenase, and catalyzes hypoxanthine to uric acid via xanthine, as well as allopurinol (a xanthine oxidase inhibitor) to oxypurinol, and pyrazinamide (an antituberculous agent) to 5-hydroxy pyrazinamide.[1,2] A deficiency of XDH/XO is called xanthinuria, first reported by Dent and Philiport,[3] which is characterized by hypouricemia, hypouricosuria, and xanthinuria. Xanthinuria is classified into 2 groups, types I and II.[1,2,4] Patients with type I xanthinuria lack XDH/XO activity, but possesses aldehyde oxidase (AO) activity, while patients with type II lack both XDH/XO and AO activities,[1,3,4] suggesting a defect in the sulfuration of desulfo molybdenum. Patients with either type I or type II sometimes develop urinary tract xanthine stones or myositis, due to tissue deposition of xanthine. In previous studies,[5-7] the cloning of cDNA encoding XDH/XO was performed in rats, mice, and humans, and the chromosomal location of the *XDH/XO* gene (chromosome 2p22, 2p23) has been demonstrated in humans.[7,8] Further, mutations in the human *XDH/XO* gene in patients with xanthinuria type 1 have been found,[9,10] while a nonsense mutation in the human molybdenum cofactor sulferase (*MCS*) gene in 2 patients with xanthinuria type II has also been reported.[11] We recently observed a case of xanthinuria type II, which provided an opportunity to investigate the *XDH/XO*, *MCS*, and *AO* genes, as well as the XDH/XO protein.

MATERIALS AND METHODS

Patient

A 43-year-old xanthinuric female was referred to our department because of hypouricemia. There was no history of urinary calculi or myositis, and she had never received an antihyperuricemic agent. Her elder sister is one of our patients with xanthinuria type II, who we have previously reported.[1] A physical examination showed no abnormal findings besides obesity (body weight, 56 kg; height, 145 cm). Routine laboratory examination also showed no abnormal data for serum AST, serum ALT, serum alkaline phosphatase, serum creatinine, serum cholesterol, serum triglyceride, and compelte blood cell count, except for serum uric acid (5.3 μmol/L) and fasting plasma glucose (7.3 μmol/L). Duodenal mucosal tissue samples were obtained by endoscopic examination for isolation of total RNA, immunoblotting of XDH protein, and an XDH assay.

Allopurinol Loading Test

An allopurinol loading test was performed as described previously.[1,2] In brief, allopurinol (300 mg) was orally administered after an overnight fast, and urine was collected for 24 hours. Allopurinol and oxypurinol were measured by high-performance liquid chromatography (HPLC) as described previously.[10]

Determination of Hypoxanthine, Xanthine, and Uric Acid

The concentrations of hypoxanthine and xanthine were determined by HPLC as described previously,[1,2] and that of uric acid was determined by a uricase method, using a Wako Uric Acid Kit (Wako Pure Chemical Ltd, Osaka, Japan).

Direct Sequencing of cDNA

Total RNA was obtained from the duodenal mucosa using a chloroform-phenol extraction method, after which cDNA was reverse-transcribed from the total RNA of the obtained materials. The polymerase chain reaction (PCR) primers and conditions for the XDH/XO cDNA and AO cDNA amplifications were previously reported by Sakamoto et al[10] and Ichida et al,[11] respectively. The PCR primers for the MCS cDNA amplification are shown in Table 1 and were used under the following conditions. cDNA was denatured at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension step of 7 minutes. PCR amplifications were performed in a volume of 25 μL containing 50 mmol/L KCl, 10 mmiol/L Tris-HCl (pH 8.8), 1.5 mmol/L $MgCl_2$, 0.1 % Triton X-100,

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Table 1. Primers for cDNA of MCS

Primer	Sequence (5′ to 3′)	Position	Amplified Product
HMCS1-F	GCCGGCTTCGCGCACTT	−54-−38	240
HMCS1-R	CTGGGAGAACAAGGTGGCAC	167-186	
HMCS2-F	CGCGGCTAGCCTACGGCTAC	71-90	470
HMCS2-R	TGCAGACCACAGGTCCTCTG	521-540	
HMCS3-F	GTCGCTTCTGTTACCTCACC	428-447	348
HMCS3-R	GGACAAAGTCGGCCTGGTGA	756-775	
HMCS4-F	CGCCTGGGAAGTGGTTTGTG	686-705	371
HMCS4-R	CAAGGTGAAGGTGTGCTGCT	1037-1056	
HMCS5-F	GAGGCAGTCGGTAGCTCAGA	921-940	361
HMCS5-R	CCAGTGTTACAGAAGCAGCCAGT	1258-1281	
HMCS6-F	GAGGTTCAGGGCCCGATCAT	1150-1169	402
HMCS6-R	AGCCTGGCTGTCTGCTGATG	1532-1551	
HMCS7-F	GGATGATGTCCAGGCCTTTC	1425-1444	382
HMCS7-R	CCTGGTCACCTCAAATGCAG	1787-1806	
HMCS8-F	CAGCCGACTCCTTCAGAGAA	1690-1709	342
HMCS8-R	ACAGACCCTGCTTTGGCGAA	2012-2031	
HMCS9-F	CTTGCGGCAAAGGATCATGG	1926-1945	391
HMCS9-R	GCTGAGATCCTTCAGTGAGA	2297-2316	
HMCS10-F	TGGCCACCCTTTCTCTGGTG	2183-2202	361
HMCS10-R	GCATGCATCAGGTACATGCC	2524-2543	
HMCS11-F	CCAGATGATTTGCATCGACC	2433-2452	277
HMCS11-R	CACTGTAAAAGAGAAACT	2691-2709	

200 μmol/L each of dATP, dCTP, dGTP, dTTP, 0.1 μg of cDNA, 10 pmol of each primer, and 1.5 U of Taq polymerase in a DNA thermal cycler (Gene AMP PCR System 9400; Perkin Elmer, Norwalk, CT). Amplified DNA products were sequenced by the dye-termination method using a DNA sequencing kit (Perkin-Elmer, Foster, CA) with the same primers as for the PCR assay, and analyzed using an ABI PRISM 310 (Applied Biosystems, Foster, CA).

Direct Sequencing of Genomic DNA Including the Point Mutation Site

Genomic DNA was extracted from blood using a DNA Quick II kit (Dainipponn Pharmaceuticals, Osaka, Japan). PCR was performed as described above with the forward primer (TGGCCTGATGTTACGTCTGC) designed from intron 1, and the reverse primer (CCTGGCCTATTAATCCAGCG) designed from intron 2.

XDH/XO Protein Immunoblotting

Using postmitochondrial supernatants from the samples, immunoblotting was performed as described previously.[10] In brief, XDH/XO was transferred electrophoretically from a 10 % dodium dodecyl sulfate (SDS)-polyacrylamide gel to a PVDF membrane at 1 mA/cm^2 for 90 minutes, using Trans Blot SD (Bio-Rad Laboratories, Hercules, CA). After incubation with Block Ace (Dainippon Pharmaceuticals) overnight at 4°C, the PVDF membrane was washed with 50 mmol/L Tris-buffered saline (pH7.4) (TBS) and then incubated for 3 hours at room temperature with a 1:200 dilution of anti-human xanthine oxidase rabbit serum in TBS containing 10 % (vol/vol) Block Ace. The PVDF membrane was washed 3 times, for 10 minutes per wash, with TBS and then incubated with a 1:500 dilution of a secondary antibody (biotinylated anti-rabbit IgG; Vector Laboratories, Burlingame, CA) for 3 hours at room temperature. Next, the PVDF membrane was washed 4 times with TBS and incubated with avidin-biotin complex (Vectastain ABC Elite kit; Vector Laboratories). Finally, the XDH/XO peptide was visualized by incubation with 0.01% H_2O_2 in TBS containing 0.05% diaminobenzidine.

Enzyme Activity

The activity of XDH/XO was determined as described previously.[10] In brief, the reaction mixtures contained 50 mmol/L phosphate buffer (pH 8.5), 20% cell or tissue homogenate, and 1 mmol/L NAD in a final volume of 200 μL. After preincubation for 5 minutes at 37°C, the reaction was initiated by the addition of 100 μL of 1 mmol/L xanthine. After either 5 or 20 minutes, the reaction was stopped by the addition of 25 μL of 20% $HClO_4$, followed by neutralization with 25 μL of 1 mol/L K_2CO_3. The solution was then passed through a 4A Chromatodisc (Kurabo, Osaka, Japan) for HPLC. The HPLC apparatus consisted of an LC-6A chromatograph (Shimadzu, Kyoto, Japan), an SPD-6AV UV-VIS Spectrophotometric Detector (Shimadzu), a C-R6A Chromatopac (Shimadzu), and a Wakosil 18C-200 column (Wako, Osaka, Japan). The mobile phase consisted of 20 mmol/L potassium phosphate buffer (pH 2.2), with a flow rate of 1 mL/min and absorbance at 254 nm. XDH/XO activities were expressed as nmol/min/mg protein.

RESULTS

Laboratory Data

Plasma hypoxanthine was 8.2 μmol/l (reference range, 1.1 to 3.0 μmol/L), plasma xanthine was 12.2 μmol/L (reference range, 0.7-1.2 μmol/L), urinary hypoxanthine excretion was 56.1 μmol/h (reference range, 4 to 5.7 μmol/h), urinary xanthine excretion was 75.2μmol/h (reference range, 3.5 to 4.6 μmol/h), and urinary uric acid excretion could not detected.

XDH/XO activity in duodenal mucosal samples from the patient was below the limits of detection. After administration of 300 mg (2.2 mmol) allopurinol, the 24-hour urinary excretion of allopurinol and oxypurinol was 1521 μmol (reference range, 223 to 335 μmol) and below the limits of detection (reference range, 1,118 to 1,597 μmol), respectively. From these findings, the patient was diagnosed as having xanthinuria type II.

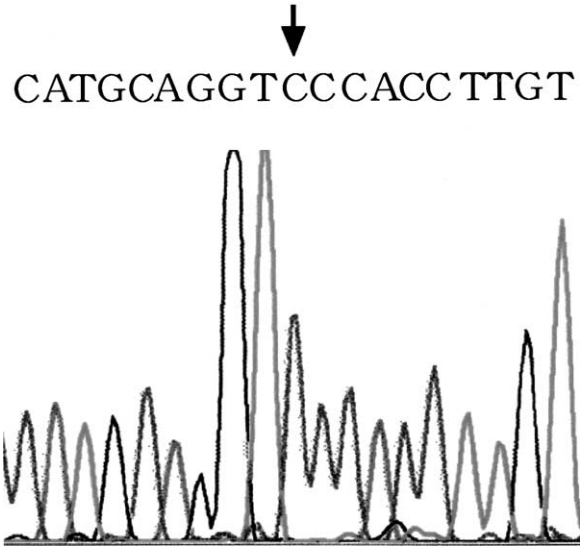

Fig 1. Direct sequence of genomic DNA including the site of the point mutation (arrow, point mutation of G to C in exon 2 of MCS genomic DNA in patient with xanthinuria type II).

Sequence of XDH cDNA , AO cDNA, and MCS cDNA From Xanthinuric Duodenal Mucosa

XDH cDNA from the xanthinuric duodenal mucosa was the same as that from the normal duodenal mucosa,[12] and AO cDNA was the same as that of a normal liver.[11] However, DNA sequence analysis of MCS cDNA from the xanthinuric duodenal mucosa identified a point mutation of G to C in nucleotide 466, which changed codon 156 from GCC (Ala) to CCC (Pro).

Sequence of Genomic DNA Including the Mutation Site

In the patient with xanthinuria type II, a point mutation of G to C was found in exon 2 of MCS genomic DNA (Fig 1), indicating that they were homozygous for this mutation. This mutation was also found in her elder sister with xanthinuria type II.

Immunoreactive XDH/XO Protein

An immunoblot analysis was performed after electrophoresis, using postmitochondrial supernatants from the xanthinuric and normal duodenal mucosa specimens. A 150-kd band was found in both, indicating that the immunoreactive XDH/XO protein is produced in this patient (Fig 2).

DISCUSSION

The common feature of XDH/XO is that molybdenum is bound by 1 oxygen and 1 sulfur atom in their active forms. The removal of the sulfur ligand of molybdenum in XDH/XO and AO by cyanide treatment forms inactive desulfo forms,[13] suggesting that the active forms require the sulfur ligand. The molybdenum cofactor sulferase sulfrates desulfo molybdenum cofactor to change inactive desufo XDH/XO and AO forms to their active sulfo forms.[14] Therefore, a deficiency of this enzyme causes XO and AO deficiencies, which is known as xanthinuria type II.

In *Drosophila melanogaster*, some mutations at the maroo-lile locus (ma-l) have been shown to cause inactivation of molybdenum hydroxylase, XDH and AO.[14] Thus, it has been suggested that the ma-l locus includes the gene encoding the putative enzyme that sulfrates desulfo molybdenum cofactor. Recently, the *ma-l*[15]and human *MCS* genes[11] were sequenced. Since the *MCS* gene contains a pyridoxal phosphate binding site motif,[11] its protein has been proposed to supply sulfur to the molybdenum center.

In a recent study,[11] the same nonsense substitution of human MCS was identified in 2 independent xanthinuria type II patients. This mutation is a C to T base change at nucleotide position 1255, which results in a nonsense substitution from CGA (Arg) to TGA (Ter) at codon 419. In the present xanthinuria type II patient, XDH cDNA and AO cDNA were identical with those of normal subjects. In addition, a 150-kd XDH protein was detected, which is also found in normal subjects. However, a cDNA sequence analysis of MCS cDNA identified a point mutation of G to C in nucleotide 466, which changed codon 156 from GCC (Ala) to CCC (Pro). Further, a point mutation of G to C was found in exon 2 of the MCS genomic DNA of the patient (Fig 1), as well as in her elder sister with xanthinuria. These findings strongly suggest that a new point mutation in the human *MCS* gene is responsible for xanthinuria type II.

Since the mutation involves the *XDH/XO* gene in patients with xanthinuria type I, the XDH/XO protein may or may not be present. On the other hand, since the mutation does not involve the *XDH/XO* gene but rather the *MCS* gene, the XDH/XO protein may be present in type II patients. In fact, the XDH/XO protein was identified in a xanthinuria type II patient using a double diffusion method in a previous study,[16] as well as by an immunoblot method in the present study.

In conclusion, we report a new mutation in the human *MCS* gene in a patient with xanthinuria type II. This mutation is suggested to be responsible for the absence of MCS activity, which leads to an absence of XDH/XO activity. However, to determine whether it is the cause of MCS activity loss, further investigation, including possible changes in the tertiary structure of the MCS protein caused by this mutant gene, is required.

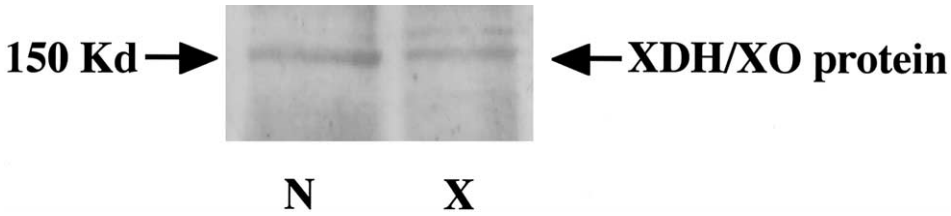

Fig 2. Immunoblots of XDH/XO. Postmitochondrial supernatant (50 μg protein) obtained from the duodenal mucosa was applied. N, normal duodenal mucosa; X, xanthinuric duodenal mucosa.

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