Clinical and Molecular Analysis of Patients with Renal Hypouricemia in Japan-Influence of URAT1 Gene on Urinary Urate Excretion

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Abstract. Renal hypouricemia is an inherited and heterogeneous disorder characterized by increased urate clearance (CUA). The authors recently established that urate was reabsorbed via URAT1 on the tubular apical membrane and that mutations in *SLC22A12* encoding URAT1 cause renal hypouricemia. This study was undertaken to elucidate and correlate clinical and genetic features of renal hypouricemia. The *SLC22A12* gene was sequenced in 32 unrelated idiopathic renal hypouricemia patients, and the relationships of serum urate levels, and CUA/creatinine clearance (Ccr) to *SLC22A12* genotype were examined. Uricosuric (probenecid and benzbromarone) and anti-uricosuric drug (pyrazinamide) loading tests were also performed in some patients. Three patients had exercise-induced acute renal failure (9.4%), and four patients had urolithiasis (12.5%). The authors identified eight new mutations and two previously reported mutations that result in loss of function. Thirty patients had *SLC22A12* mutations; 24 homozygotes and compound heterozygotes, and 6 heterozygotes. Mutation G774A dominated *SLC22A12* mutations (74.1% in 54 alleles). Serum urate levels were significantly lower and CUA/Ccr was significantly higher in heterozygotes compared with healthy subjects; these changes were even more significant in homozygotes and compound heterozygotes. These CUA/Ccr relations demonstrated a gene dosage effect that corresponds with the difference in serum urate levels. In contrast to healthy subjects, the CUA/Ccr of patients with homozygous and compound heterozygous *SLC22A12* mutations was unaffected by pyrazinamide, benzbromarone, and probenecid. The findings indicate that *SLC22A12* was responsible for most renal hypouricemia and that URAT1 is the primary reabsorptive urate transporter, targeted by pyrazinamide, benzbromarone, and probenecid *in vivo*.

Approximately 90% of all urate that is filtered through the glomerulus is eventually reabsorbed. A four-component hypothesis has been proposed to explain the renal urate transport mechanisms; it includes glomerular filtration, presecretory reabsorption, secretion, and postsecretory reabsorption (1,2). Renal hypouricemia is a common inherited and heterogeneous disorder characterized by impaired tubular urate transport (3). The incidence of renal hypouricemia has been reported to be 0.12 to 0.72% (4,5), and exercise-induced acute renal failure and nephrolithiasis have been reported as complications (6).

Renal hypouricemia has been classified into the following five types according to responses to the anti-uricosuric drug pyrazinamide, and the uricosuric drug, probenecid: (*a*) a presecretory reabsorptive defect with an attenuated response to both pyrazinamide and probenecid (3); (*b*) a post-secretory reabsorptive defect when pyrazinamide suppressible urate clearance (CUA) is not influenced by probenecid (7); (*c*) total inhibition of urate reabsorption when pyrazinamide induces elimination of CUA exceeding the rate of glomerular filtration (8); *(d)* enhanced secretion when the pyrazinamide suppressible CUA is increased by probenecid (9); and *(e)* subtotal defect in urate transport without any response to either pyrazinamide or probenecid (10).

The four-component hypothesis and the classification of renal hypouricemia are rather complicated and have presumed that pyrazinamide inhibits urate secretion. However, some reports using membrane vesicles have recently indicated that the anti-uricosuria induced by pyrazinamide was due to enhanced urate reabsorption through exchange of its active metabolite, pyrazine carboxylic acid (PZA), via the urate/anion exchanger at the brush-border membrane (11–13). Reconsideration of the four-component hypothesis and the classification of renal hypouricemia are crucial. Therefore, a classification based on gene mutations in *SLC22A12* and prospective urate transporter genes is needed.

Although membrane vesicles studies have suggested that a

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voltage-sensitive pathway and urate exchangers are located at both the apical and basolateral membranes of proximal tubule cells (12–14), no transporters have been identified that alter serum urate levels and urinary urate excretion *in vivo*. We recently cloned urate transporter 1 (URAT1 encoded by *SLC22A12*), located at the apical membrane in the proximal tubules. We also demonstrated that URAT1 regulates serum urate levels by showing that three patients with renal hypouricemia have defects in *SLC22A12* (15). The following questions remain: (*a*) What proportion of renal hypouricemia cases have *SLC22A12* defects? and (*b*) What variation of mutations exist in *SLC22A12*?

In this study, we elucidated clinical and genetic features of renal hypouricemia and the significance of URAT1 in maintaining serum urate levels *in vivo* using 32 unrelated patients. In addition, we performed loading tests with the anti-uricosuric drug pyrazinamide and the uricosuric drugs probenecid and benzbromarone in a number of the patients to establish URAT1 as the active site of pyrazinamide, probenecid, and benzbromarone for urate transport modification *in vivo*.

Materials and Methods

Materials

We examined 32 patients with idiopathic renal hypouricemia. These patients either came to Jikei University Hospital and Tottori University Hospital between January 1, 1998, and August 31, 2002, or their doctors consulted with Jikei University Hospital and Tottori University Hospital between January 1, 1998, and August 31, 2002. One patient was hospitalized for exercised-induced acute renal failure. These patients are unrelated and provided informed consent for various analysis procedures at the outset of the study. Pyrazinamide loading tests were performed in 15 patients, probenecid loading tests in 6 patients, and benzbromarone in 13 patients.

Methods

Pyrazinamide, probenecid, and benzbromarone loading tests were conducted according to the methods previously described (16–18). Serum urate, CUA, and creatinine clearance (Ccr) were measured before and after administration of 3g of pyrazinamide, 2 g of probenecid, or 100 mg of benzbromarone.

Genomic DNAs of the patients were isolated from peripheral blood cells using a QIAGEN blood and cell culture DNA kit (Qiagen, Hilden, Germany) for direct sequencing (19). Using primers designed from the sequences of the introns, the entire genomic DNA sequences equivalent to the open reading frame of URAT1 cDNA were determined (Table 1).

For the detection of the genotype of the G774A mutation in *SLC22A12*, the exon 4 of *SLC22A12* was amplified by nested PCR using the exonic primer RH-EX4f-*Msp*I and the intronic primer RH-r3. The nested PCR finally amplified a 135-bp fragment of genomic DNA, including nucleotide position 774. By setting C instead of T at the 3' end of primer RH-EX4f-*Msp*I, a new *Msp*I digestion site was created in the PCR product. Upon digestion of the PCR product with the restriction enzyme *Msp*I, only the PCR product that does not have a G to A base substitution at position 774 in *SLC22A12* is cleaved into 109-bp and 26-bp fragments.

When two different mutations in *SLC22A12* were identified, we performed PCR in which the DNA products included the two mutations; the primers in Table 1 were used to identify whether one *SLC22A12* mutation and another *SLC22A12* mutation were located on the same allele or not. We direct-sequenced several clones after the amplified DNA fragment was subcloned using TOPO XL PCR Cloning Kit (Invitrogen, San Diego, CA).

For the construction of a missense mutant, we used the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutagenic oligonucleotide primers for generation of G269A, G412A, G490A, A1145T, T1298C, and deletion of 1639–1643 mutants were: 5'-GAGGCCCCATCAGTGCCGCC(A)CTTCCGCCAG CCACAGTGGCAGC-3', 5'-CGTGGCCAAGTGGAACCTC(A)T-GTGTGACTCTCACGCTCTGAAGCC-3', 5'-CTGGTGGGAGCT-GCTGCGTGC(A)GCCCTGCCTCAGACAGGTTTGGG-3', 5'-GGG CAGCAACATCTTCCTGCTCC(T)AATGTTCATTGGTGTCGTG G-3', 5'-CACGCTGGTGCCCCACGAAA(C)GGGGGCTCTGCGCT CAGCCTTG-3', and 5'-CATGGCACGCTGGGGAACTCT()A-AAATCCACACAGTTTTAGCCTCC-3', respectively (sense strands; mutated nucleotides are in parentheses). Proper construction of the mutated cDNA was confirmed by complete sequencing.

URAT1 mutant cRNA was synthesized, using an mMESSAGE mMACHINE kit (Ambion, Inc., Austin, TX), from each linearized URAT1 mutant cDNA by *Not*I. Poly $(A)^+$ tail was added using Poly (A) Tailing kit (Ambion). The injection of 50 ng of wild-type or mutant URAT1 cRNA into *Xenopus leavis* oocytes and maintenance of the oocytes and uptake studies were performed as described pre-

Table 1. The primers for genomic amplification of URAT1 coding region

Exon	Forward Primer		Reverse Primer	
1	RH-f1	cctcacgcggcctcagg gcccagtt	RH-r1	gggtccctcccaggactggaccttt
2	RH-f2	ccctcactgttccacagggtcttgctct	RH-r2	ccagcaagtagggcgctttctagacttg
3, 4	RH-f3	catagggtgggctctaggtgttccagag	RH-r3	ggagagtgggcaggatctcctctgagga
5	RH-f5	agcagtgggtacagggtagcagtctgag	RH-r5	agctgaggtccttggtgggtctcccagg
6	RH-f6	gctccctgggaagaaggtctcagagagg	RH-r6	tgtgccagcgagagccccgattccaggt
7	RH-f7-1	gccaaacccaaagggaagccatgctggcaagg	RH-r7-1	gccacacccacaatctgctccacgctcagacac
7	RH-f7-2	cctgagcccccaccgcccattgtt	RH-r7-2	cctgctctagtccagcacctccaa
8	RH-f8	gctgaagggagccctcatctgatcttgg	RH-r8	taggtctgggagaagccagtcctgcctg
9	RH-f9	gtgcaggatcaaggctgtgggcacacag	RH-r9	ctctgcttccgcctctgtcaagtgatgg
10	RH-f10	cttgggatggacacaggtcaagggtcag	RH-r10	cagagaacagaagaagtctcttcctctga
	RH-Ex4f/MspI	tgcagtggcctacggtgtgcgggacc		

viously (15,20). Uptake studies were performed at room temperature in ND96 (in mM: 96 NaCl, 2.0 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 5.0 HEPES, pH 7.4) containing 10 μM ^{14}C-urate (1.85 to 2.22 GBq/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO) and 100 μM cold urate. After uptakes were stopped and washed five times with ice-cold ND96, pH 7.4, each oocyte solubilized with 200 μl of 10% SDS was mixed with 2.5 ml of Aquasol-2 (Packard, Meriden, CT) for radioactivity determination using a scintillation counter (LC-3010; ALOKA, Tokyo, Japan).

The subcellular localization of URAT1 mutants was determined by immunocytochemical analysis. Anti-URAT1 N-terminal polyclonal antibody was obtained as 1.705 mg/ml of IgG fraction by immunization of rabbit with a keyhole limpet hemocyanin-conjugated synthetic peptide SELLDLVGGLGR, corresponding to URAT1 amino acid sequence 4–15, and by peptide affinity-purification. *Xenopus* oocytes, which were injected with 50 ng of wild-type or mutant URAT1 cRNA and cultured for 3 d at 18°C, were fixed in 4% paraformaldehyde in ND96 for 30 min at room temperature. Three-micrometer paraffin sections were stained with anti-URAT1 N-terminal antibody (1:100), followed by staining with Cy5-conjugated anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Images were visualized by a confocal laser microscope (FLUOVIEW FV500; Olympus Co., Tokyo, Japan).

Values for clinical data were expressed as mean ± SD. The experiments for urate uptake of URAT1 mutant *in vitro* were performed using three batches of oocytes, and results from the representative experiments were expressed as means ± standard errors, respectively. Statistical analyses were performed using the unpaired *t* test.

Results

Clinical Features of Patients with Renal Hypouricemia

Data related to urate handling of all the patients are shown in Table 2. Serum urate levels of all the patients were below 2 mg/dl and averaged 0.93 ± 0.49 mg/dl ($n = 32$). Both mean C_{UA} (68.3 ± 31.6 ml/min; $n = 30$) and mean fractional excretion of C_{UA} (C_{UA}/Ccr) (0.584 ± 0.264; $n = 32$) were significantly increased. These data are compatible with patterns observed in patients with renal hypouricemia. Three patients had a past history of exercise-induced acute renal failure (9.4%). Urolithiasis was identified in 4 of the 32 patients (12.5%). Two patients had chronic renal failure; one was due to hypertension, and another was due to unknown origin.

Mutation Analysis

Thirty of thirty-two patients had *SLC22A12* mutations (93.8%), whereas two patients had none in the URAT1-coding region. All the patients with serum urate levels of 1.0 mg/dl or below did have *SLC22A12* mutations. C_{UA}/Ccr over 1 was identified in only two patients. We detected *SLC22A12* mutations in 54 (84.4%) of 64 alleles. Eight new mutations in *SLC22A12* were identified and are summarized in Table 2, together with the two of three mutations we previously reported (C650T and G774A) (15). Mutations included two nonsense mutations, one splice-site mutation, a 5-bp deletion, and six missense mutations.

Two nonsense mutations, both G774A and C889T within exon 4 and 5 of *SLC22A12*, produce a truncated protein that lacks half of the mature protein. The G774A mutation was identified in 40 (74.1%) of 54 affected *SLC22A12* alleles, 11 homozygotes, 13 compound heterozygotes, and 5 heterozygotes (Table 2). The splice-site mutation (IVS2 + 1G→A) modifies the GT donor splice site of intron 2, suggesting aberrant splicing in this patient.

All of the six missense mutations were localized within the putative transmembrane domains (G164S, T217M, and Q382L) or within the putative intracellular loops (R90H, V138M, and M430T) (Figure 1). None of the missense mutations lie within the extracellular loops of the URAT1 protein. Four of six single–amino acid residue mutations of *SLC22A12* (R90H, V138M, G164S, and Q382L) alter residues that are conserved in URAT1 and OAT4 encoded by *SLC22A11*. G269A and G412A were identified in 5 (9.3%) and 2 (3.7%) of 54 affected *SLC22A12* alleles, respectively, and G650T we previously reported in one (1.6%).

Functional and Immunocytochemical Analysis of URAT1 Mutants

The new missense mutations (G269A, G412A, G490A, A1145T, and T1289C) and the 5-bp deletion (1639-1643delGTCCT) were tested for urate transport activity by *in vitro* expression analysis in *Xenopus* oocytes. As shown in Figure 2, the urate transports of all URAT1 mutants were significantly decreased in comparison with that of wild-type and were similar to that of oocytes that were not injected with URAT1 cRNA. G490A, A1145T, and T1289C mutants partially conserved urate transport function.

To investigate the influence of URAT1 localization on urate transport activity, sections of mutant-expressing oocytes were stained with anti-URAT1 N-terminal polyclonal antibody. Staining at the plasma membrane was clearly observed in sections of wild-type URAT1-expressing oocytes (Figure 3). Oocytes expressing mutants G269A, G412A, G490A, or A1145T revealed significant immunopositive staining of the plasma membrane. In contrast, the sections of oocytes expressing T1289C or 1639-1643del mutants revealed only weak plasma membrane staining.

Genotype-Phenotype Correlation in Renal Hypouricemia

On the basis of the functional analysis experiment, we classified the 32 patients into three groups: (*a*) homozygotes and compound heterozygotes ($n = 24$); (*b*) heterozygotes ($n = 6$); and (*c*) patients with wild-type URAT1 ($n = 2$). Correlation of serum urate levels with C_{UA}/Ccr of the renal hypouricemia patients and the controls are shown in Figure 4. The curve plotted is similar to that previously reported using healthy volunteers (21). Although the areas on the curve representing the homozygotes and compound heterozygotes, the heterozygotes and patients with wild-type URAT1, and the controls overlapped, each group is distinctively clustered. Lower serum urate levels and higher C_{UA}/Ccr in the homozygotes and compound heterozygotes were statistically significant compared with the heterozygotes and the controls (Figure 5). Serum urate levels and C_{UA}/Ccr in the heterozygotes were significantly lower and higher than those of the controls, respectively. This

Table 2. Clinical data and SLC22A12 mutations of the patients with renal hypouricemia

No.	Gender	History	Complication	SLC22A12 Mutations		SUA (mg/dl)	$U_{UA}V$ (mg/d)	C_{UA} (ml/min)	C_{Cr} (ml/min)	C_{UA}/C_{Cr}
				Nucleotide	Amino Acid					
1	M			G774A/G774A	W258X	0.7	853.3	84.7	181.8	0.466
2	M			G774A/G774A	W258X	0.6	897.2	103.8	180.0	0.577
3	M			G774A/G774A	W258X	0.7	414.0	78.8	77.0	1.023
4	M			G774A/G774A	W258X	0.8	827.0	71.8	138.0	0.520
5	F			G774A/G774A	W258X	0.5	758.0	108.3	83.6	1.295
6	M	ARF, NS	IgAN	G774A/G774A	W258X	1.0	1144.5	79.5	93.4	0.851
7	M		CRF	G774A/G774A	W258X	1.8		30.6	33.0	0.927
8[b]	F		CRF	G774A/G774A	W258X	2.0		16.2	25.2	0.643
9	F	HSPN		G774A/G774A	W258X	0.5	514.0	66.0	96.8	0.682
10	M		IgAN	G774A/G774A	W258X	0.7	548.4	63.5	110.0	0.577
11	M			G774A/G774A	W258X	0.5	645.1	89.6	125.0	0.717
12	M			G774A/C650T	W258X/T217M	0.9	637.0	49.2	126.1	0.390
13	M	ARF		G774A/C889T	W258X/Q297X	0.7	511.2	50.7	105.8	0.479
14	M			G774A/IVS2+1G→A	W258X/Frameshift	0.8	527.2	42.1	84.6	0.498
15	M			G774A/G269A	W258X/R90H	0.7	1194.0	118.5	146.0	0.812
16	M			G774A/G269A	W258X/R90H	0.6	836.0	96.8	188.0	0.515
17	M		Proteinuria	G774A/G269A	W258X/R90H	0.7	649.4	64.4	107.0	0.602
18[b]	M			G774A/G269A	W258X/R90H	1.1	583.0	52.5	106.0	0.495
19	M			G774A/G269A	W258X/R90H	0.4	501.0	81.0	120.0	0.675
20	F		Urolithiasis	G774A/G412A	W258X/V138M	0.6	698.9	80.9	131.0	0.618
21	M		Urolithiasis	G774A/G412A	W258X/V138M	0.5	802.0	111.4	119.4	0.933
22	F			G774A/G490A	W258X/G164S	0.8	1229.8	106.8	191.0	0.559
23	F			G774A/1639–1643delGTCCT	W258X/Frameshift	0.6	423.2	49.0	90.8	0.540
24	F			G774A/A1145T	W258X/Q382L	0.8	564.0	49.0	109.0	0.450
25	F			G774A/+	W258X	0.9	595.2	45.9	118.8	0.386
26	F			G774A/+	W258X	1.7	468.0	19.1	145.0	0.132
27	M	ARF	Urolithiasis	G774A/+	W258X	0.6	1140.8	132.0	136.0	0.971
28[b]	F			G774A/+	W258X	2.0				0.262
29	F			G774A/+	W258X	0.6	554.0	64.1	126.0	0.509
30[b]	F			T1289C/+	M430T	1.5	703.6	16.6	85.0	0.195
31[b]	F		Urolithiasis	+/+		2.0	470.0	25.3	108.3	0.234
32[b]	F			+/+		1.5	891.6			0.167
Controls						5.03 ± 1.06	08.6 ± 51.7	7.3 ± 1.4	99.5 ± 4.5	0.073 ± 0.013

[a] UUAV, urinary urate excretion; ARF, Acute renal failure; NS, Nephrotic syndrome; IgAN, IgA nephropathy; CRF, chronic renal failure; HSPN, Henoch-Schonlein purpura nephritis. + denotes the wild-type allele. Controls were 8 healthy individuals (4 men and 4 women aged 28 to 45 yr).

[b] Patients in Tottori University.

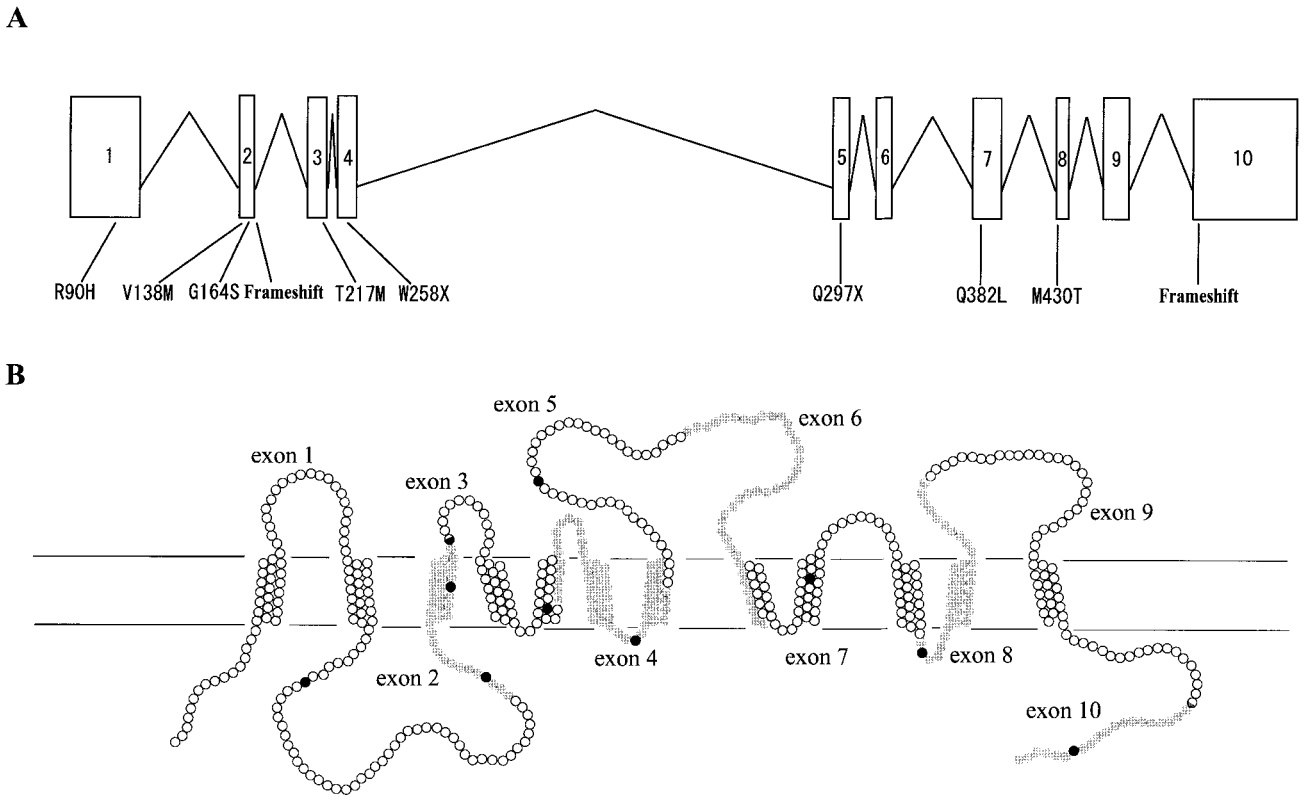

Figure 1. The predicted membrane-spanning secondary structure of URAT1 and positions of the mutations. (A) Boxes indicate exons of *SLC22A12*. The mutations are pointed by line. (B) Predicted secondary structure of URAT1 is shown. Each circle represents one amino acid. A chain of open or gray circles indicates that corresponding amino acids were derived from the same exon. Mutations are indicated by closed circles.

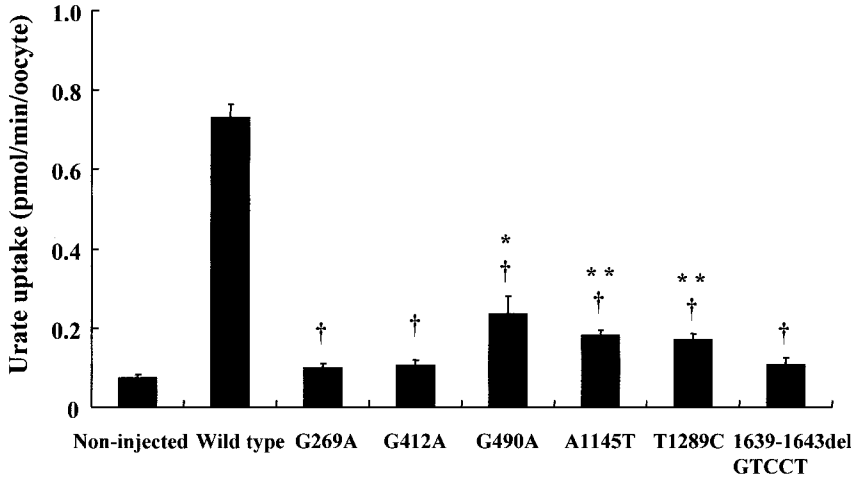

Figure 2. Urate transport ability of the URAT1 mutants found in the patients. Urate uptakes by all the oocyte-expressed mutants were significantly decreased compared with oocytes injected with wild-type URAT1 cRNA († $P < 0.001$). Urate uptakes of G490A, A1145T, and T1289C mutants–expressed oocytes significantly increased compared with the non-injected oocytes. * $P < 0.01$; ** $P < 0.001$.

relation of CUA/Ccr among the homozygotes and compound heterozygotes, the heterozygotes, and the controls demonstrated a gene dosage effect, correlating with the difference in serum urate levels. The two patients with wild-type URAT1 had levels intermediate between the heterozygotes and the controls.

Drug Loading Tests for Renal Hypouricemia Patients

We conducted pyrazinamide, probenecid, and benzbromarone loading tests in a number of the patients. CUA/Ccr of the homozygotes and compound heterozygotes and the heterozygotes did not change significantly in response to the pyrazinamide and benzbromarone loading tests, although those of

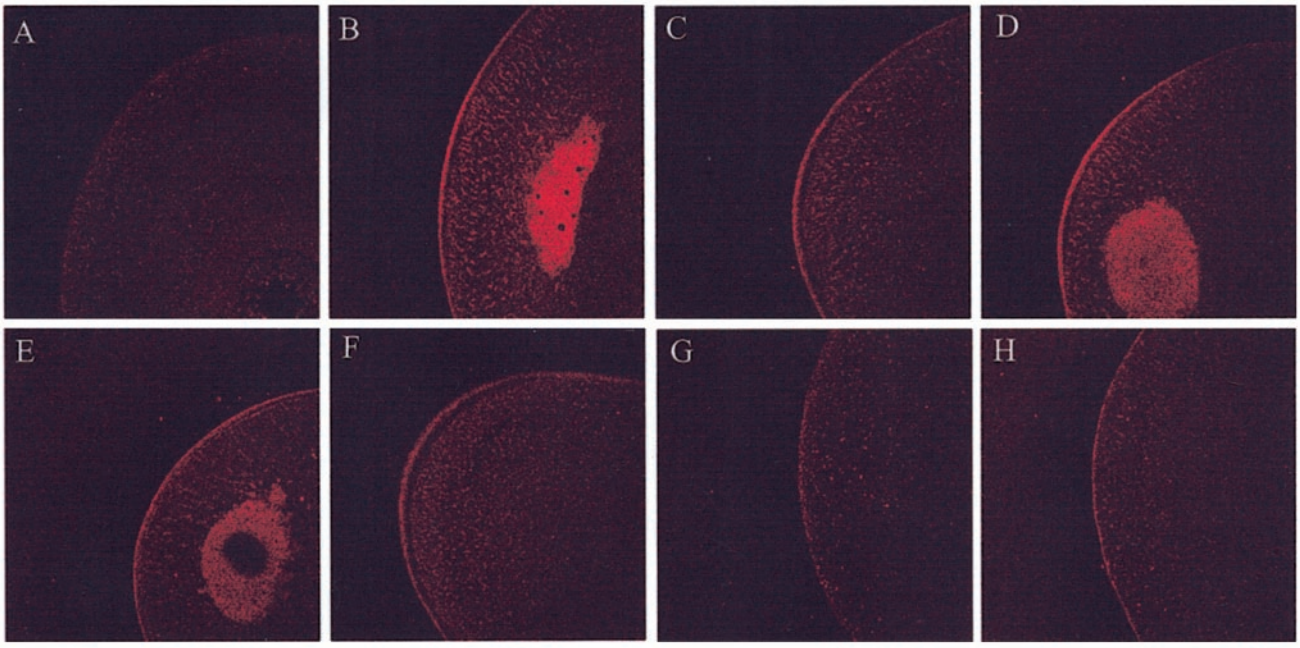

Figure 3. Immunocytochemical analysis of oocytes injected with 50 ng of cRNA encoding the wild-type or mutant URAT1. (A and B) Water-injected oocyte (A) and oocyte injected with the wild-type cRNA (B). (C through H) Oocytes injected with cRNA of G269A (C), G412A (D), G490A (E), A1145T (F), T1289C (G), or 1639–1643del (H). The mutants G269A, G412A, G490A, and A1145T exhibited similar staining to wild-type URAT1 at the plasma membrane. Staining of mutant T1289C and 1639–1643del was faint. Typical results of representative experiments are shown.

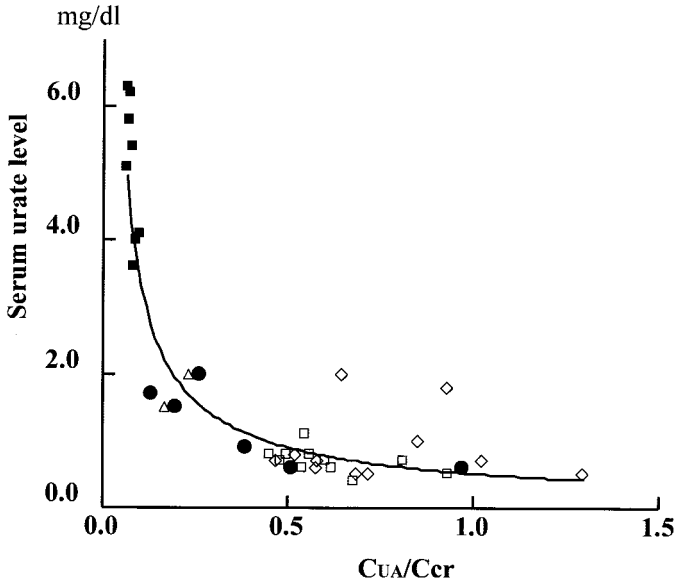

Figure 4. Correlation of serum urate levels with CUA/Ccr of the patients with renal hypouricemia and the controls. ◇: homozygotes; □: compound heterozygotes; ●: heterozygotes; △: patients with wild type URAT1; ■: controls. The regression curve is plotted. R: correlation coefficient, $y = 0.5068x^{-0.8227}$, $R^2 = 0.8056$.

healthy subjects markedly decreased and increased, respectively (Figure 6). On the other hand, CUA/Ccr in the homozygotes and compound heterozygotes increased significantly in response to the probenecid loading test ($P < 0.01$). In addition, a patient with wild-type URAT1 had responses similar to healthy subjects for three loading tests.

Discussion

This study examined 32 unrelated patients with renal hypouricemia to correlate clinical and genetic features. We described eight new *SLC22A12* renal hypouricemia mutations, which , together with those reported previously, increases the number of known mutations in *SLC22A12* to 11. Mutation G774A is revealed as the major *SLC22A12* renal hypouricemia allele (74.1%).

The incidence rates of exercise-induced acute renal failure and urolithiasis in patients with renal hypouricemia have not been adequately determined to date, despite the fact that most renal hypouricemic patients have been identified from the onset of these two conditions (18,22,23). The reported incidence rate of renal hypouricemia is 0.12% (5), and fewer than 70 patients with renal hypouricemia have been reported to develop exercise-induced acute renal failure in Japan. Accordingly, the perceived incidence rate of exercise-induced acute renal failure in patients with renal hypouricemia has been extremely lower than our frequency of 9.4%. A number of patients with renal hypouricemia who developed exercise-induced acute renal failure might have been overlooked due to the fact that the serum urate levels in these patients increase to the normal to high-normal range, during an exercise-induced acute renal failure episode. How renal hypouricemia accounts for exercise-induced acute renal failure remains an open question. Two mechanisms have been proposed: either urate nephropathy results from an increase in urate production during exercise (6,18), or renal reperfusion injury due to vasoconstriction results from an exercise-induced increase in oxygen free radicals and a lack of urate, free radical scavengers (24). The

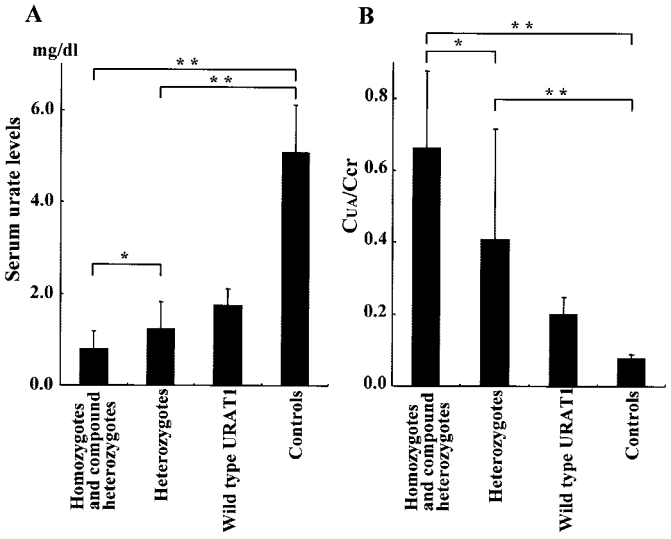

Figure 5. (A) Serum urate levels of the patients with homozygous and compound heterozygous *SLC22A12* mutations, the patients with heterozygous *SLC22A12* mutations, the patients with wild-type URAT1, and controls. (B) CUA/Ccr of the patients with homozygous and compound heterozygous *SLC22A12* mutations, the patients with heterozygous *SLC22A12* mutations, the patients with wild-type URAT1, and controls. Serum urate levels and CUA/Ccr in the patients with wild-type URAT1 were not statistically analyzed with those of other groups because only two patients were classified for the patients with wild-type URAT1. * $P < 0.05$; ** $P < 0.001$.

first hypothesis raises an interesting question; why has tubular urate precipitation been identified in renal biopsy specimens of only one (6) of these renal hypouricemic patients with exercise-induced acute renal failure (25–28)? Conversely, with regards to the second hypothesis, why has exercise-induced acute renal failure not been reported in xanthinuric patients who lack xanthine dehydrogenase and whose serum urate levels are almost 1 mg/dl or below, similar to renal hypouricemic patients? As *SLC22A12* renal hypouricemia is proven to be primarily responsible for the renal hypouricemia documented in this study, we need to reconsider the mechanism from the viewpoint of URAT1 functional loss. Although it is difficult to obtain evidence for these hypotheses, understanding this mechanism may provide insights into other etiologies of acute renal failure.

The prevalence of urolithiasis is approximately 2 to 3% in the general population; however, 12.5% of the patients in this study had a history of urolithiasis. This high prevalence is probably related to the increased amount of urinary urate in these patients compared with healthy subjects, because the correlation of urinary urate with the prevalence of urolithiasis is well known (29,30). Renal hypouricemia should be recognized as one of the diseases to consider that leads to the formation of kidney stones.

This study demonstrated that URAT1 was responsible for most of the renal hypouricemia, especially severe renal hypouricemia with serum urate levels of 1.0 mg/dl or less. We also demonstrated a gene dosage effect of *SLC22A12* on CUA/Ccr, correlating with the difference in serum urate levels, despite the possibility that some renal hypouricemia alleles lacking mutations may be explained by large deletions or insertions, mutations in the promoter region, or mutations in intron sequences that were not screened. These facts indicate that URAT1 acts as the most significant renal tubular urate transporter for reabsorption and fluctuations of serum urate levels *in vivo*. Additionally, the fact that 24 of 30 patients had homozygous or compound heterozygous *SLC22A12* mutations indicates that *SLC22A12* renal hypouricemia is autosomal recessive in principle, which is compatible with previous reports (22,31). In contrast, the present study showed individual variability in the *SLC22A12* renal hypouricemia phenotype, as illustrated by the different CUA/Ccr and serum urate levels. For example, the homozygous G774A mutations resulted in variable CUA/Ccr values from 0.466 to 1.295. We preliminarily found some healthy subjects with the heterozygous G774A mutation whose serum urate level and CUA/Ccr were in the lower normal range (data not shown). This variability and both phenotypes, normouricemia and hypouricemia, in subjects with heterozygous *SLC22A12* mutations suggest that other environmental and/or genetic factors affect CUA/Ccr and serum urate levels.

Functional analysis showed that five new missense mutations and the 5-bp deletion out of eight new mutations resulted in loss of urate transport activity. T1289C and 1639-1643del mutant proteins were present only at the plasma membrane, as demonstrated by immunocytochemical analysis. These finding indicate that impaired routing of URAT1 to the cell surface might be related with the loss of function in T1289C and 1639-1643del mutants. URAT1 has PDZ binding motifs at the C terminus, suggesting that URAT1 interacts with a multiple PDZ protein. PDZ domains are often involved in scaffolding protein complexes at plasma membranes, maintaining cell polarity, and signal transduction (32). Further investigation is needed, including identification of the PDZ protein that interacts with the PDZ domain of URAT1, and the interaction of URAT1 with the PDZ protein.

The new splice-site mutation (IVS2 + 1G→A) should not induce urate transport activity by modification of the GT donor splice site of intron 2. Accordingly, all of the mutations in this study, including the new nonsense C889T mutation and missense C650T mutation previously reported (15), should lack urate transport activity. These analyses correspond to the phenotype of our patients with *SLC22A12* renal hypouricemia. Interestingly, the G774A mutation accounts for 74.1% of *SLC22A12* mutations in our patients. The G774A mutation could have occurred in ancient history and spread over Japan such as the APRT *J mutation of the adenine phosphoribosyltransferase gene (33). Out of 32 patients, two patients without any *SLC22A12* mutation were natives of the Chuugoku region in the western part of Japan. Accordingly, the variation of *SLC22A12* mutations and the frequency of *SLC22A12* renal hypouricemia would differ somewhat in the area.

Guggino *et al.* (11) and Roch-Ramel *et al.* (13) suggested that the anti-uricosuric effect of PZA was due to enhanced urate reabsorption through exchange of PZA via the urate/

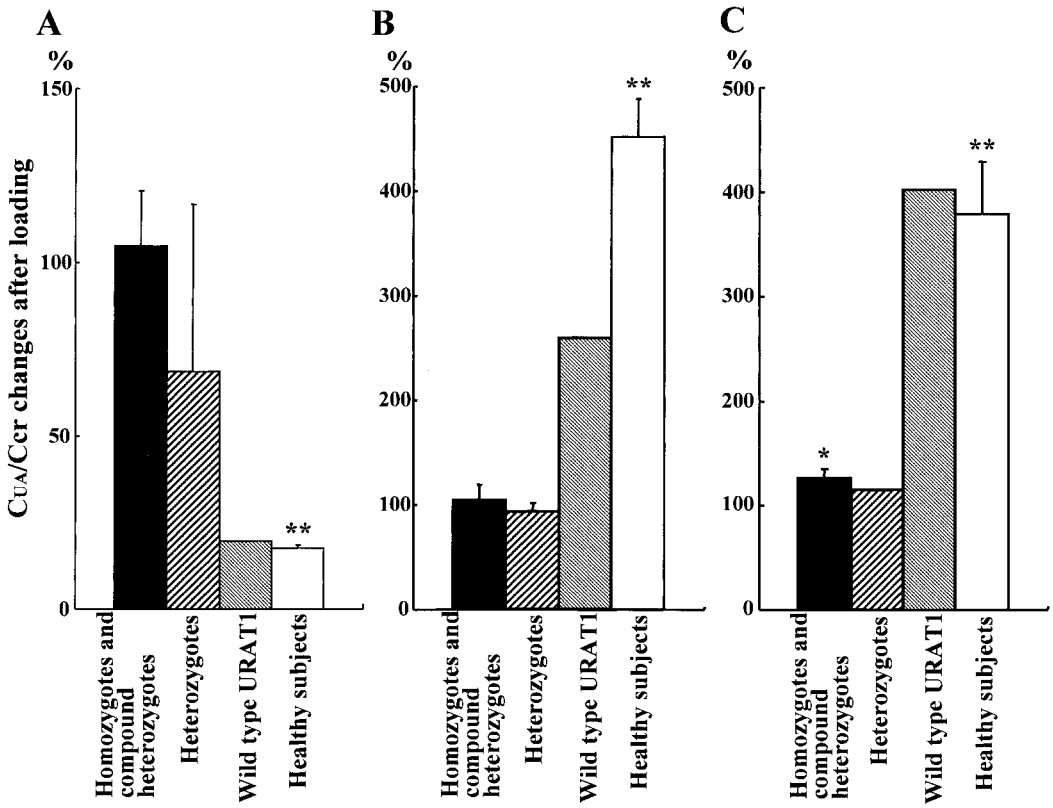

Figure 6. Changes of CUA/Ccr ratio of the patients with homozygous and compound heterozygous *SLC22A12* mutations, the patients with heterozygous *SLC22A12* mutations, and the patients with wild-type URAT1 by the loading tests. The rate of change is shown by % of the CUA/Ccr value before loading. Data of healthy subjects were previously described (16,17). * $P < 0.05$; ** $P < 0.001$ *versus* the CUA/Ccr value before loading. (A) Pyrazinamide loading test. This test was performed for ten patients with homozygous and compound heterozygous *SLC22A12* mutations, four patients with heterozygous *SLC22A12* mutations, and one patient with wild type URAT1. (B) Benzbromarone loading test. This test was performed for eight patients with homozygous and compound heterozygous *SLC22A12* mutations, four patients with heterozygous *SLC22A12* mutations, and one patient with wild-type URAT1. (C) Probenecid loading test. This test was performed for four patients with homozygous and compound heterozygous *SLC22A12* mutations, one patient with heterozygous *SLC22A12* mutations, and one patient with wild-type URAT1. We did not conduct statistical analysis of the reaction in the patient with wild-type URAT1 for the three loading tests, nor in the patient with heterozygous *SLC22A12* mutations for the probenecid loading test.

anion exchanger at the brush-border membrane after PZA moved into proximal cells by sodium-cotransport. In contrast, benzbromarone was suggested to inhibit urate reabsorption from the lumen side via the same urate/anion exchanger used by PZA (13, 34). Using *Xenopus* oocytes injected with URAT1 complementary RNA, we demonstrated that urate is reabsorbed via URAT1 in exchange for organic anions such as lactate at the apical membrane, that URAT1 has affinity for both uricosuric and anti-uricosuric drugs such as benzbromarone, probenecid, and PZA, and that urate uptake was transstimulated by PZA (15). The typical responses for pyrazinamide, benzbromarone, and probenecid loading tests were not observed in the homozygotes and compound heterozygotes. From the absence of these drugs' effects in these patients, we concluded that pyrazinamide, benzbromarone, and probenecid act on URAT1 as the target for their anti-uricosuric and uricosuric effects *in vivo*. These results correspond with previous studies *in vitro* (11,13,15,34). In addition, a slight but significant increase in CUA/Ccr of all the homozygotes and compound heterozygotes was observed in the probenecid loading test, suggesting that probenecid might also act on another urate transporter. It has been reported that a population of renal hypouricemic patients with a post-secretory reabsorptive defect have a pyrazinamide suppressible CUA that is not influenced by probenecid (5,7,35,36). This renal hypouricemia might be due to another urate transporter defect.

We identified two hypouricemic patients without *SLC22A12* mutations in whom serum urate levels were > 1.0 mg/dl and CUA/Ccr was approximately 0.2. It is possible that they have mutations in other *SLC22A12* regions such as the promoter region or the intron that affects URAT1 function. The responses for the loading tests in one of the two patients were similar to those of healthy subjects, rather than those of patients with URAT1 deficiency. Furthermore, most CUA/Ccr in the patients with *SLC22A12* mutation did not exceed 1, despite the urate secretion mechanism in the tubules. These facts also suggest that another urate transporter acts on the tubules for urate reabsorption. If the unknown urate reabsorptive transporter plays a role in maintaining serum urate levels, we should recognize the transporter deficit as renal hypouricemia. Since

URAT1 is responsible for most renal hypouricemia, the ability of the unknown urate reabsorptive transporter to regulate serum urate levels should not be greater than that of URAT1. We might be able to identify subjects with the unknown urate reabsorptive transporter disorder in the groups who have moderately lower serum urate levels.

In summary, this study correlated clinical and genetic features of renal hypouricemia. Clinically, the frequency of exercise-induced acute renal failure in patients with renal hypouricemia was higher than initially perceived. Genetically, this study illustrated that URAT1 is the main reabsorptive urate transporter because *SLC22A12* was responsible for most renal hypouricemia, and the G774A mutation dominated *SLC22A12* mutations in Japan. Furthermore, we established that pyrazinamide, benzbromarone, and probenecid exert anti-uricosuric and uricosuric effects by acting on URAT1 *in vivo*.

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References

1. Diamond HS, Paolino JS: Evidence for a postsecretory reabsorptive site for uric acid in man. *J Clin Invest* 52: 1491–1499, 1973
2. Levinson DJ, Sorensen LB: Renal handling of uric acid in normal and gouty subject: Evidence for a 4-component system. *Ann Rheum Dis* 39: 173–179, 1980
3. Greene ML, Marcus R, Aurbach GD, Kazam ES, Seegmiller JE: Hypouricemia due to isolated renal tubular defect: Dalmation dog mutation in man. *Am J Med* 53: 361–367, 1972
4. Van Peenen HJ: Causes of hypouricemia. *Ann Intern Med* 78: 977–978, 1973
5. Hisatome I, Ogino K, Kotake H, Ishiko R, Saito M, Hasegawa J, Mashiba H, Nakamoto S: Cause of persistent hypouricemia in outpatients. *Nephron* 51: 13–16, 1989
6. Erley CM, Hirschberg RR, Hoefer W, Schaefer K: Acute renal failure due to uric acid nephropathy in a patient with renal hypouricemia. *Klin Wochenschr* 67: 308–312, 1989
7. Barrientos A, Perez-Diaz V, Diaz-Gonzalez R, Rodicio JL: Hypouricemia by defect in the tubular reabsorption. *Arch Intern Med* 139: 787–789, 1979
8. Simkin PA, Skeith MD, Healey LA: Suppression of uric acid secretion in a patient with renal hypouricemia. *Adv Exp Med Biol* 41: 723–728, 1974
9. Shichiri M, Matsuda O, Shiigai T, Takeuchi J, Kanayama M: Hypouricemia due to an increment in renal tubular urate secretion. *Arch Intern Med* 142: 1855–1857, 1982
10. Shichiri M, Itoh H, Iwamoto H, Hirata Y, Marumo F: Renal tubular hypouricemia: Evidence for defect of both secretion and reabsorption. *Nephron* 56: 421–426, 1990
11. Guggino SE, Aronson PS: Paradoxical effects of pyrazinoate and nicotinate on urate transport in dog renal microvillus membranes. *J Clin Invest* 76: 543–547, 1985
12. Roch-Ramel F, Guisan B, Schild L: Indirect coupling of urate and p-aminohippurate transport to sodium in human brush-border membrane vesicles. *Am J Physiol* 270: F61–F68, 1996
13. Roch-Ramel F, Guisan B, Diezi J: Effects of uricosuric and antiuricosuric agents on urate transport in human brush-border membrane vesicles. *J Pharmacol Exp Ther* 280: 839–845, 1997
14. Kahn AM, Shelat H, Weinman EJ: Urate and p-aminohippurate transport in rat renal basolateral vesicles. *Am J Physiol* 249: F654–F661, 1985
15. Enomoto A, Kimura H, Chairoungdua A, Shigeta Y, Jutabha P, Ho Cha S, Hosoyamada M, Takeda M, Sekine T, Igarashi T, Matsuo H, Kikuchi Y, Oda T, Ichida K, Hosoya T, Shimokata K, Niwa T, Kanai Y, Endou H: Molecular identification of a renal urate anion exchanger that regulates blood urate levels. *Nature* 417: 447–452, 2002
16. Hisatome I, Ogino K, Saito M, Miyamoto J, Hasegawa J, Kotake H, Mashiba H, Nakamoto S: Renal hypouricemia due to an isolated renal defect of urate transport. *Nephron* 49: 81–83, 1988
17. Hisatome I, Sasaki N, Yamakawa M, Kobayashi M, Tanaka Y, Kosaka H, Yoshida A, Kotake H, Mashiba H, Takeda A, et al: Two cases of persistent hypouricemia associated with diabetes mellitus. *Nephron* 61: 196–199, 1992
18. Yeun JY, Hasbargen JA: Renal hypouricemia: Prevention of exercise-induced acute renal failure and a review of the literature. *Am J Kidney Dis* 25: 937–946, 1995
19. Ichida K, Amaya Y, Kamatani N, Nishino T, Hosoya T, Sakai O: Identification of two mutations in human xanthine dehydrogenase gene responsible for classical type I xanthinuria. *J Clin Invest* 99: 2391–2397, 1997
20. Ichida K, Hosoyamada M, Kimura H, Takeda M, Utsunomiya Y, Hosoya T, Endou H: Urate transport via the human PAH transporter hOAT1 and its gene structure. *Kidney Int* 63: 143–155, 2003
21. Lang F, Greger R, Oberleithner H, Griss E, Lang K, Pastner D, Dittrich P, Deetjen P: Renal handling of urate in healthy man in hyperuricaemia and renal insufficiency: circadian fluctuation, effect of water diuresis and of uricosuric agents. *Eur J Clin Invest* 10: 285–292, 1980
22. de Vries A, Sperling O: Inborn hypouricemia due to isolated renal tubular defect. *Biomedicine* 30: 75–80, 1979
23. Kotake T, Miura N, Ito H: Renal tubular hypouricemia and calcium urolithiasis. *Scanning Microsc* 7: 417–421, 1993
24. Murakami T, Kawakami H, Fukuda M, Shiigi H: Recurrence of acute renal failure and renal hypouricemia. *Pediatr Nephrol* 7: 772–773, 1993
25. Ishikawa I, Sakurai Y, Masuzaki S, Sugishita N, Shinoda A, Shikura N: Exercise-induced acute renal failure in 3 patients with renal hypouricemia. *Nippon Jinzo Gakkai Shi* 32: 923–928, 1990
26. Numabe A, Tsukada H, Sugimoto T, Ono H, Hirao S, Abe M, Yagi S: A case of acute renal failure in a patient with idiopathic hypouricemia [In Japanese]. *Nippon Jinzo Gakkai Shi* 34: 841–845, 1992
27. Igarashi T, Sekine T, Sugimura H, Hayakawa H, Arayama T: Acute renal failure after exercise in a child with renal hypouricemia. *Pediatr Nephrol* 7: 292–293, 1993
28. Ninomiya M, Ito Y, Nishi A, Matsumoto T, Koga A, Hori Y, Nishida H, Nomura G, Kato H: Recurrent exercise-induced acute renal failure in renal hypouricemia. *Acta Paediatr* 85: 1009–1011, 1996
29. Yu T, Gutman AB: Uric acid nephrolithiasis in gout. Predisposing factors. *Ann Intern Med* 67: 1133–1148, 1967

30. Grover PK, Ryall RL, Marshall VR: Effect of urate on calcium oxalate crystallization in human urine: evidence for a promotory role of hyperuricosuria in urolithiasis. *Clin Sci (Lond)* 79: 9–15, 1990
31. Gafter U, Zuta A, Frydman M, Lewinski UH, Levi J: Hypouricemia due to familial isolated renal tubular uricosuria. Evaluation with the combined pyrazinamide-probenecid test. *Miner Electrolyte Metab* 15: 309–314, 1989
32. Hung AY, Sheng M: PDZ domains: Structural modules for protein complex assembly. *J Biol Chem* 277: 5699–5702, 2002
33. Kamatani N, Terai C, Kim SY, Chen CL, Yamanaka H, Hakoda M, Totokawa S, Kashiwazaki S: The origin of the most common mutation of adenine phosphoribosyltransferase among Japanese goes back to a prehistoric era. *Hum Genet* 98: 596–600, 1996
34. Dan T, Koga H: Uricosurics inhibit urate transporter in rat renal brush border membrane vesicles. *Eur J Pharmacol* 187: 303–312, 1990
35. Sorensen LB, Levinson DJ: Isolated defect in postsecretory reabsorption of uric acid. *Ann Rheum Dis* 39: 180–183, 1980
36. Tofuku Y, Kuroda M, Takeda R: Hypouricemia due to renal urate wasting. Two types of tubular transport defect. *Nephron* 30: 39–44, 1982