Introduction

Renal hypouricemia is a disorder characterized by the impaired urate handling in the renal tubules. Both inherited familial cases and sporadic cases are reported. The incidence of renal hypouricemia has been reported to be 0.12 to 0.72 %(1-3), and exercise-induced acute renal failure and nephrolithiasis are the common complications(4;5). Based largely on clearance and micropuncture data obtained in experimental animals, the major modes of renal urate handling have been described as 4-component theory that includes glomerular filtration, pre-secretory reabsorption, secretion, and post-secretory reabsorption(5-8). This 4-component theory has been applied to the classification of renal hypouricemia, and five types renal hypouricemia has been proposed according to the responses to the anti-uricosuric drug, pyrazinamide (PZA), and the uricosuric drug, probenecid: (a) a pre-secretory reabsorption defect(9), (b) a post-secretory reabsorption defect(10), (c) total inhibition of urate reabsorption(11), (d) enhanced secretion(12), and (e) subtotal defect in urate transport(13).

In 2002, Enomoto et al isolated human urate transporter 1 (hURAT1) and identified loss-of-function mutations in hURAT1 in 3 independent subjects with idiopathic renal hypouricemia, suggesting that hURAT1 is responsible for the regulation of serum urate levels in human(14). Recently Ichida et al examined the mutations in hURAT1 in 32 unrelated subjects with idiopathic renal hypouricemia and identified 8 new mutations(15). Komoda et al reported high prevalence of W258X mutation in hURAT1 in Japanese population(16).

In the present study, we investigate the clinical features and the mutations in hURAT1 gene in 7 families with pre-secretory reabsorption defect type familial renal hypouricemia and in one family with post-secretory reabsorption defect type familial renal hypouricemia. We identified a novel mutation as well as three previously reported mutations in the families with pre-secretory

reabsorption defect type, and *Xenopus* oocytes expressing these mutant hURAT1s showed substantially reduced urate transporting activity. However, no mutations in the coding region of hURAT1 could be detected in the family with post-secretory reabsorption defect type. These findings indicate the possibility that hURAT1 might be the responsible gene for the pre-secretory reabsorption defect type renal hypouricemia and that other transporter(s) might be involved in the post-secretory reabsorption defect type renal hypouricemia.

Materials and Methods

Patients

Twelve affected subjects and 26 family members belonging to eight unrelated Japanese families diagnosed with familial renal hypouricemia were the object of the present molecular study. Hypouricemia was defined as serum urate levels below 2.0 mg/dL. At least one member of each family had an episode of exercise-induced acute renal failure, and underwent both PZA and probenecid (or benzbromarone) loading tests to determine the type of disorder in urate handling in the kidney. Based on the results of these drug loading tests, 7 of 8 families (Family 1 to 7) were classified into the pre-secretory reabsorption defect type and the other family (Family 8) was the post-secretory reabsorption defect type. The pedigrees are depicted in Figure 1. The group of affected subjects was composed of 9 men and 3 women with a mean age of 23.3 years old. Clinical features of affected subjects in Family 2, 4, and 8 were previously reported(17;18). Written informed consent was obtained from each subject upon enrollment to this study. This study was approved by the Institutional Review Board of Kumamoto University.

Detection of mutations in URAT1 gene

All exons of the hURAT1 gene were screened for mutations in each affected subject and family members. Primers used for the hURAT1 sequence determination were described by Ichida et al(15). The technique used to detect mutations was the PCR-direct sequencing method. Briefly, genomic DNA from each subject was isolated from peripheral blood cells using GFX Genomic Blood DNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK). Each exon was amplified by PCR using the genomic DNA as a template. The PCR product was purified and subjected to sequencing reaction using the dideoxy chain termination method with fluorescent dye-labeled terminators on an automated sequencer (ABI model 310; Applied Biosystems, Foster City, CA). When two different mutations in hURAT1 gene were identified,

we performed PCR reaction in which the PCR product contains both of the mutations. The PCR product was then subcloned into pGEM easy T vector (Promega, Madison, WI, USA) and several clones were sequenced to determine if the two mutations are located on the same allele or not.

Micorsatellite genotyping

Genotyping of all family members belonging to Family 8 was performed by PCR with primer sets for D11S4191 and D11S4162 (Table 1). D11S4191 locus and D11S4162 locus are located upstream and downstream of hURAT1 gene respectively (Table 1). In brief, specific segments from genomic DNA were amplified with specific fluorescence-labeled primers for each marker locus on chromosome 11, the site of the hURAT1 gene. Genotypes were analyzed by an ABI373S autosequencer with Genescan software (Applied Biosystems) to measure the nucleotide length of amplified fragments from each allele. Heterozygosity of the markers used reported by Applied Biosystems was as follows: 0.87 for D11S4191 and 0.64 for D11S4162.

Functional analysis of mutant hURAT1

In order to introduce mutations that were found in the present study into wild type hURAT1 cDNA, we used the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instruction. Proper construction of the mutated cDNA was confirmed by complete sequencing. Mutant hURAT1 cRNA was synthesized using the mMESSAGE mMACHINE kit (Ambion, Inc., Austin, TX, USA) from each linearized mutant URAT1 cDNA. Poly $(A)^+$ tail was added by the Poly $(A)^+$ Tailing kit (Ambion) and synthesized cRNA was purified by the MEGAclear Purification kit (Ambion). Stage 5-6 *Xenopus* Oocytes were treated with 1 μg / mL of collagenase type I (Sigma, St. Louis, MO, USA) for 1 hour to remove follicle cell layers, and injected with 50 ng of cRNA for wild type or

mutant hURAT1 in 25 nL of water. Injected oocytes were kept at 19 °C in modified Barth's saline (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM HEPES, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$). Uptake studies were performed 72 hours after cRNA injection. Oocytes were incubated with ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) containing 10 μM ^{14}C-urate (1.85 to 2.22 GBq / mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) and 100 μM cold urate. Thirty minutes after incubation, oocytes were washed five times with ice-cold ND96, pH 7.4, and were solubilized with 500 μL of 10 % SDS. Each sample was then mixed with 2.5 ml of Emulsifier-safe (Packard Bioscience, Meriden, CT, USA) and the radioactivities were measured using a scintillation counter.

Statistical Analysis

Statistical significance was evaluated using ANOVA analysis followed by Newman-Keuls method. A value of $P < 0.05$ was regarded as statistically significant. Values are expressed as mean ± SE.

Results

Clinical features of affected subjects and related family members

Clinical features and genotypes of hURAT1 gene in the affected subjects and related family members of familial renal hypouricemia are described in Table 2. The affected subjects in Family 1 to 7 were classified into the pre-secretory reabsorption defect type according to the results from PZA and probenecid (or benzbromarone) loading tests, and the affected subjects in Family 8 were into the post-secretory reabsorption defect type. The results of PZA and probenecid loading tests were described in Table 3. Serum urate levels of all affected subjects were substantially decreased (0.60 ± 0.16 mg/dL, n = 12) and fractional excretion of urate (FEUA) levels were significantly increased (59.9 ± 12.6 %, n = 12). These data are compatible with the patterns observed in renal hypouricemia. Mutational analysis revealed that homozygous or compound heterozygous mutations in hURAT1 gene were identified in 9 of 12 affected subjects. Three subjects who had no mutations in hURAT1 gene were originated from the same family (Family 8). In 18 subjects who were identified to have heterozygous mutations in hURAT1 gene, serum urate levels were relatively lower (2.79 ± 0.54 mg/dL, n = 18) and FEUA levels were relatively higher (14.5 ± 3.7 %, n = 18) than the normal range (normal urate level: 2.5 – 7.0 mg/dL, normal FEUA level: less than 10.0 %). Except for the subjects belonging to Family 8, four subjects were shown to have no mutations in hURAT1 gene and they had normal urate and FEUA levels (urate: 5.78 ± 1.54 mg/dL, FEUA: 6.5 ± 1.8 %, n = 4).

Mutational analysis and microsatellite genotyping

Mutational analysis revealed that all of the affected subjects and 20 of 24 related family members in the pre-secretory reabsorption defect type familial renal hypouricemia (Family 1 – 7) had mutations in hURAT1 gene. We detected one nonsense mutation (G774A), two missense mutations (A1145T, T1253G) and one 5-bp deletion mutation (1639-1643 del-GTCCT) in these

subjects. T1253G mutation was a novel mutation, and the other mutations were previously reported(14-16). None of these mutations were identified in 75 randomly chosen unrelated control Japanese individuals. Genotyping of hURAT1 gene in the related family members revealed the segregation of each mutated allele in the affected family, and elucidated the autosomal recessive inheritance of this disease. On the other hand, no mutations in hURAT1 gene were identified in 3 affected subjects and 2 related family members in the post-secretory reabsorption defect type familial renal hypouricemia (Family 8). We only determined the sequences of all exons and exon / intron boundaries of hURAT1 gene, but not entire hURAT1 genome. However, we could not detect any insertion, deletion, or substitution mutations in this family, suggesting the possibility that hURAT1 may not be a responsible gene for the post-secretory reabsorption defect type. In order to elucidate this possibility, we next performed linkage analysis of Family 8 using two microsatellite markers (D11S4191 and D11S4162) that are located upstream and downstream of hURAT1 gene (Table 1). As shown in Figure 2, segregation patterns of both D11S4191 and D11S4162 markers were totally different among the affected children although they showed the same phenotypes (hypouricemia). These findings strongly support the possibility that hURAT1 is not responsible for the post-secretory reabsorption defect type.

Functional analysis of hURAT1 mutants

In order to assess the functional significance of the new mutation (T1253G) as well as the three previously reported mutations (G774A, A1145T, and 1639-1643 del-GTCCT), we expressed these mutant or wild-type hURAT1 in *Xenopus* oocytes, and tested for the urate transporting activities. As shown in Figure 3, all hURAT1 mutants had significantly reduced urate transporting activities than wild-type hURAT1 (water: $0.03 \pm 0.005^{*}$ pmol / min / oocyte, wild type: 0.56 ± 0.021, G774A: $0.03 \pm 0.005^{*}$, A1145T: $0.05 \pm 0.009^{*}$, T1253G: $0.04 \pm 0.009^{*}$,

1639-1643 del-GTCCT: 0.06 ± 0.006*, *$P < 0.01$ vs. wild type, n = 12), and the transporting activities of hURAT1 mutant were almost similar to those of water-injected oocytes. These findings strongly support the fact that the mutations found in the current study account for the loss-of-function in hURAT1 and renal hypouricemia phenotype in the affected subjects.

Discussion

In the present study, we identified four mutations in hURAT1 gene in the subjects with the pre-secretory reabsorption defect type familial renal hypouricemia, one of which was a novel mutation. Functional analysis revealed that mutant hURAT1s showed substantially reduced urate transporting activities when they were expressed in *Xenopus* oocytes. We could not find any mutations in the sequence of all exons and exon/intron boundaries of hURAT1 gene in a family with post-secretory defect type. In addition, linkage analysis using microsatellite markers demonstrated no significant association of segregation pattern of hURAT1 locus in the affected subjects in this family, suggesting the possibility that another urate transporter(s) might also be involved in the pathogenesis of renal hypouricemia.

As mentioned in the introduction section, renal hypouricemia is traditionally classified into five types according to the responses to the PZA and the probenecid loading tests(5-8). Pre-secretory reabsorption defect type is characterized by the attenuated response to both PZA and probenecid. Post-secretory reabsorption defect type is accompanied with significant suppression of urate clearance by PZA and no influence on urate clearance by probenecid. FEUA of the affected subjects in Family 1 to 7 were approximately 51 to 88 % under basal condition. Since both PZA and probenecid loading tests had only modest effect on FEUA, they were diagnosed as pre-secretory reabsorption defect type(17). FEUA of the affected subjects in Family 8 were approximately 47 to 61 % under basal condition. PZA loading resulted in significant decrease in FEUA to 4 %, whereas probenecid had no effect on FEUA. Thus, this subject was diagnosed as post-secretory reabsorption defect type(18).

We identified four mutations in hURAT1 gene in subjects with pre-secretory reabsorption defect and functionally these mutations impaired urate transporting activity of hURAT1. G774A,

A1145T, and 1639-1643 del-GTCCT were previously reported(14-16) and T1253G was newly identified. G774A resulted in truncated immature hURAT1 protein, and 1639-1643 del-GTCCT is considered to reduce the routing of hURAT1 protein to the apical membrane by disrupting PDZ binding motif(15). Since A1145T and T1253G mutations were located in the 9th and 10th putative transmembrane domain respectively, these mutations might modulate the urate permeability of hURAT1. As previously reported by Ichida et al(15), our study also demonstrated a gene dosage effect of hURAT1 on FEUA and serum urate levels. There was a correlation between the number of wild type hURAT1 allele and FEUA or serum urate levels, suggesting the significance of hURAT1 in the regulation of serum urate levels.

We were unable to detect any mutation in hURAT1 gene in the subject with post-secretory reabsorption defect. Sequence analysis was performed only for all exons and exon/intron boundaries and we could not detect any mutation so far. Then we performed linkage analysis using microsatellite markers that are located around the hURAT1 locus. The linkage analysis revealed that segregation patterns of hURAT1 gene are not identical in the affected subjects in the family with post-secretory reabsorption defect type although they had similar hypouricemia. These findings strongly suggest the possibility that another urate transporter(s) might be involved in the post-secretory reabsorption defect type. Also, the fact that the response to PZA loading test in this subject is completely different from subjects with pre-secretory defect type supports our hypothesis.

In summary, our study revealed a novel mutation in hURAT1 gene in the pre-secretory reabsorption defect type of familial renal hypouricemia. In addition, microsatellite linkage analysis indicated that hURAT1 locus is not associated with the hypouricemic phenotype in the post-secretory reabsorption defect type. Our findings would provide a new insight into the

understandings of structure-function relationship of hURAT1. Future studies are required to identify a responsible gene for the post-secretory reabsorption defect type renal hypouricemia.

Acknowledgement

The authors thank Dr. Hideyuki Saya, Dr. Hideo Nakamura, Dr. Keishi Makino, Ms. Masayo Obata (Kumamoto University), and Dr. Motowo Nakajima (Novartis Pharma) for helpful suggestions and technical assistances for the microsatellite analysis. This work was supported by the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan (13877166 and 14370321 to K. T., 13770602 and 15790432 to K. K.), the Salt Science Research Foundation (0330 to K. K.), the Japan Heart Foundation Research Grant (to K. K.).

Reprint requests to Kenichiro Kitamura, M.D., Ph.D., Assistant Professor, Department of Nephrology, Kumamoto University Graduate School of Medical Sciences, 1-1-1 Honjo, Kumamoto, Kumamoto 860-8556, Japan.

E-mail: ken@gpo.kumamoto-u.ac.jp

Table 1

Primers used for the microsatellite genotyping

Locus	Position		Forward primer	Reverse primer	Size range
	Genetic (cM)	Physical (Kb)			
D11S4191	60.09	60251	GCAAGATGGCCAATTAGAAG	TTTTGGTTGGAATGTAGTTGTTTAT	111-135
URAT1		64620			
D11S4162	72.82	71198	GTTCTCCAGAGAGACAGCAC	GAGAGCAACACTATTGCCC	263-269

Table 2

Clinical features and URAT1 mutations in the families with renal hypouricemia

Family		Age	Gender	S-UA (mg/dL)	FEUA (%)	S-Cr (mg/dL)	Type	hURAT1 genotype Nucleotide	Amino acid
1	I : 1	76	M	5.80	5.9	0.81		Wild tvoe	Wild tvoe
	I : 2	70	F	2.70	15.9	0.60		A1145T (heterozygous)	Q382L (heterozygous)
	II : 1	48	M	3.80	11.5	0.86		G774A (heterozygous)	W258X (heterozygous)
	II : 2	47	F	2.20	11.7	0.48		A1145T (heterozygous)	Q382L (heterozygous)
	III : 1	18	M	0.90	53.8	0.75	Pre	G774A / A1145T	W258X / Q382L
	III : 2	15	M	4.10	4.3	0.78		A1145 (heterozygous)	Q382L (heterozygous)
2	I : 1	78	M	3.00	15.5	0.98		G774A (heterozygous)	W258X (heterozygous)
	I : 2	77	F	2.60	10.7	0.68		1639-1643delGTCCT (heterozygous)	V547fsX602 (heterozygous)
	II : 1	54	M	4.30	9.0	0.59		Wild type	Wild type
	II : 2	53	F	0.50	50.6	0.70	Pre	G774A / 1639-1643delGTCCT	W258X / V547fsX602
	III : 1	12	F	2.30	10.3	0.43		1639-1643delGTCCT (heterozygous)	V547fsX602 (heterozygous)
	III : 2	10	M	3.00	10.9	0.55		1639-1643delGTCCT (heterozygous)	V547fsX602 (heterozygous)
3	I : 1	72	M	7.90	4.7	1.4		Wild type	Wild type
	I : 2	68	F	2.60	18.4	0.7		G774A (heterozygous)	W258X (heterozygous)
	II : 1		M	N.D.	N.D.	N.D.		G774A (heterozygous)	W258X (heterozygous)
	II : 2	41	F	2.10	14.2	0.6		G774A (heterozygous)	W258X (heterozygous)
	III : 1	17	M	0.50	61.9	0.9	Pre	G774A / G774A	W258X / W258X
	III : 2	19	M	5.10	6.4	1.1		Wild type	Wild type
4	I : 1	73	F	5.50	9.1	0.94		G774A (heterozygous)	W258X (heterozygous)
	II : 1	42	M	0.50	66.2	0.77	Pre	G774A / 1639-1643delGTCCT	W258X / V547fsX602
	II : 2	42	F	1.40	20.8	0.54		G774A (heterozygous)	W258X (heterozygous)
	III : 1	14	F	0.50	57.3	0.49		G774A / G774A	W258X / W258X
	III : 2	11	F	0.60	52.7	0.55		G774A / G774A	W258X / W258X
5	I : 1	54	M	2.70	17.6	0.9		G774A (heterozygous)	W258X (heterozygous)
	I : 2	49	F	3.00	10.5	0.4		G774A (heterozygous)	W258X (heterozygous)
	II : 1	18	M	0.50	80.0	0.7	Pre	G774A / G774A	W258X / W258X
6	I : 1	70	M	3.30	16.3	0.87		G774A (heterozygous)	W258X (heterozygous)
	I : 2	68	F	2.50	22.9	0.59		T1253G (heterozygous)	L418R (hetero)
	II : 1	43	M	0.35	88.0	0.76	Pre	G774A / T1253G	W258X / L418R
7	I : 1	44	M	3.70	14.3	1.0		G774A (heterozygous)	W258X (heterozygous)
	I : 2	42	F	2.10	14.4	0.6		G774A (heterozygous)	W258X (heterozygous)
	II : 1	16	M	0.80	58.7	0.8	Pre	G774A / G774A	W258X / W258X
	II : 2	13	M	N.D.	N.D.	N.D.		G774A (heterozygous)	W258X (heterozygous)
8	I : 1		M	3.60	7.8	0.8		Wild type	Wild type
	I : 2		F	2.80	11.0	0.5		Wild type	Wild type
	II : 1	17	M	0.80	47.8	0.5	Post	Wild type	Wild type
	II : 2	16	M	0.60	55.0	0.4		Wild type	Wild type
	II : 3	14	M	0.60	47.0	0.4		Wild type	Wild type

Pre: pre-secretory reabsorption defect type, Post: post-secretory reabsorption defect type, N.D.: not determined

Table 3

Results of pyrazinamide and probenecid loading tests

		FEUA (%)			
		Pyrazinamide loading test		Probenecid loading test	
Family		Before	After	Before	After
1	III : 1	41.1	36.3	45.7	63.3
2	II : 2	81.0	67.0	89.0	94.0
3	III : 1	61.0	57.5	55.0	57.8
4	II : 1	62.0	62.0	55.0	59.0
5	II : 1	90.0	78.0	80.0	85.0
6	II : 1	109.0	105.0	84.0	108.0
7	II : 1	45.0	40.0	55.0	50.0
8	II : 1	61.0	4.0	47.0	47.0

Figure Legend

Figure 1

Pedigrees of 8 Japanese families with renal hypouricemia.

Affected subjects are indicated by the filled symbols, and unaffected subjects by unfilled symbols. Deceased subject (diagonal line) is of unknown phenotype. Below the symbol for each subject, genotypes of URAT1 gene are shown. Mutations identified in each family are indicated below the family number, and superscribed on the genotypes. +: wild type allele, -: mutant allele.

Figure 2

Genotypings of family members in Family 8.

The electropherogram of the amplified fragments for D11S4191 (left panel) and D11S4162 (right panel) were shown. Segregation patterns of the parents' alleles were completely different among the children.

Figure 3

Urate transporting activities of mutant hURAT1.

Twenty-five nanogram of mutant hURAT1 cRNAs were injected into *Xenopus* oocytes and ^{14}C-urate uptake was measured 72 hours after injection. All mutant hURAT1s showed significantly reduced urate transporting activities compared with wild type ($^{*}P < 0.01$).

Figure 1

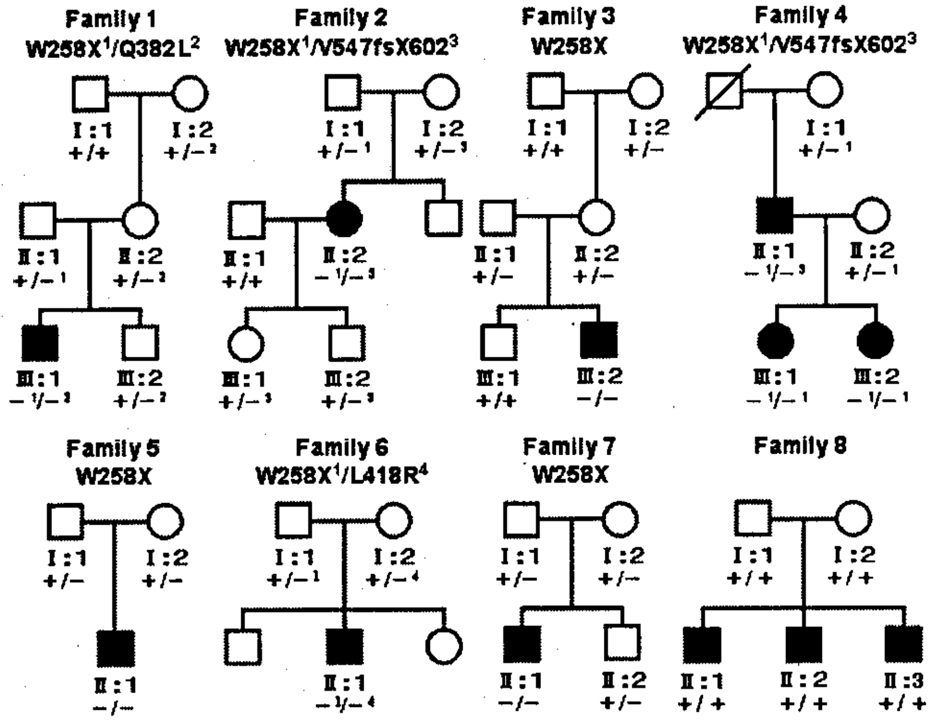


Figure 2

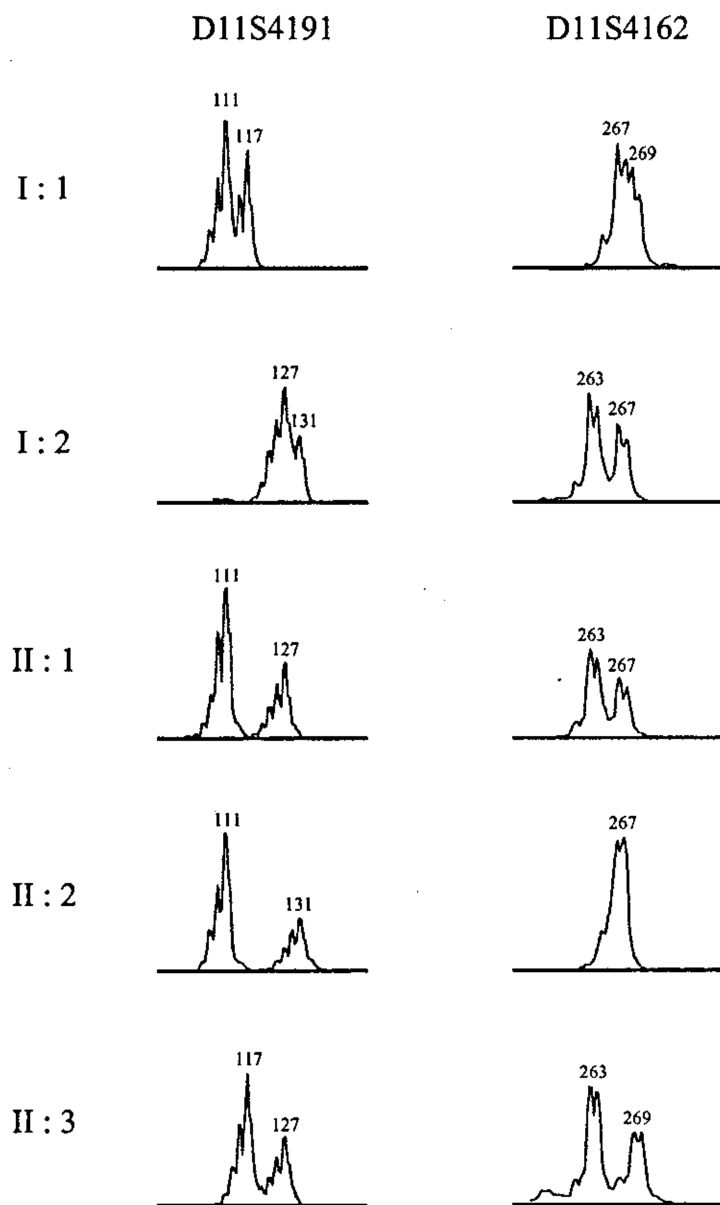
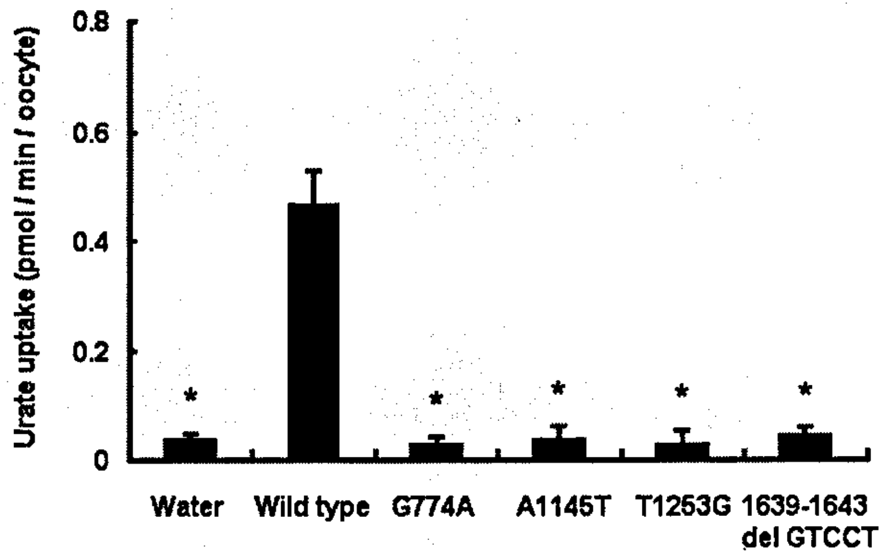


Figure 3



Reference List

1. **Hisatome I, Ogino K, Kotake H, Ishiko R, Saito M, Hasegawa J, Mashiba H, Nakamoto S** 1989 Cause of persistent hypouricemia in outpatients. Nephron 51:13-16

2. **Igarashi T** 1993 Normal serum uric acid concentrations for age and sex and incidence of renal hypouricaemia in Japanese school children. Pediatr Nephrol 7:239-240

3. **Van Peenen HJ** 1973 Causes of hypouricemia. Ann Intern Med 78:977-978

4. **Ishikawa I** 2002 Acute renal failure with severe loin pain and patchy renal ischemia after anaerobic exercise in patients with or without renal hypouricemia. Nephron 91:559-570

5. **Sperling O** 1992 Renal hypouricemia: classification, tubular defect and clinical consequences. Contrib Nephrol 100:1-14

6. **Halabe A, Sperling O** 1994 Uric acid nephrolithiasis. Miner Electrolyte Metab 20:424-431

7. **Maesaka JK, Fishbane S** 1998 Regulation of renal urate excretion: a critical review. Am J Kidney Dis 32:917-933

8. **Pritchard JB, Miller DS** 1993 Mechanisms mediating renal secretion of organic anions and cations. Physiol Rev 73:765-796

9. **Greene ML, Marcus R, Aurbach GD, Kazam ES, Seegmiller JE** 1972 Hypouricemia due to isolated renal tubular defect. Dalmatian dog mutation in man. Am J Med 53:361-367

10. **Barrientos A, Perez-Diaz V, Diaz-Gonzalez R, Rodicio JL** 1979 Hypouricemia by defect in the tubular reabsorption. Arch Intern Med 139:787-789

11. **Simkin PA, Skeith MD, Healey LA** 1974 Suppression of uric acid secretion in a patient with renal hypouricemia. Adv Exp Med Biol 41:723-728

12. **Shichiri M, Matsuda O, Shiigai T, Takeuchi J, Kanayama M** 1982 Hypouricemia due to an increment in renal tubular urate secretion. Arch Intern Med 142:1855-1857

13. **Shichiri M, Itoh H, Iwamoto H, Hirata Y, Marumo F** 1990 Renal tubular hypouricemia: evidence for defect of both secretion and reabsorption. Nephron 56:421-426