GENETIC DISORDERS – DEVELOPMENT

A high prevalence of renal hypouricemia caused by inactive *SLC22A12* in Japanese

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A high prevalence of renal hypouricemia caused by inactive *SLC22A12* in Japanese.

Background. Recently, *SLC22A12* has been identified as a urate-anion exchanger in the human kidney.

Methods. We screened for polymorphisms of *SLC22A12* and conducted an association study between genetic polymorphisms and urate levels in an epidemiologic cohort representing the general population in Japan. Functional significance of mutations was assessed by oocyte expression analysis.

Results. We found five missense, one nonsense, and one deletion mutations [R90H, A226V, R228E, W258Stop, Q312L, D313A (deletion of 313D-333P), and R477H] in 24 subjects with hypouricemia recruited from an epidemiologic cohort (Suita Study) representing the general population in Japan ($N = 1875$). A statistical analysis indicated that the 90H ($N = 14$), 477H ($N = 5$), and 258Stop (hetero + homo $N = 82 + 3$) alleles were associated with hypouricemia. The alleles 228E and 313A (deletion of 313D-333P) were found just once in the total population. In vitro oocyte expression analysis indicated that 313A (deletion of 313D-333P) had no urate transport activity, indicating that this is a newly identified mutation for idiopathic renal hypouricemia. Intriguingly, the allele frequency of 258Stop was unexpectedly high (2.37%). However, this inactivating mutation does not seem to be harmful in the general population. The effects of common polymorphisms of *SLC22A12* were also investigated. Based on linkage disequilibrium, 16 common polymorphisms were categorized into six distinct groups, and six representative genotypes were determined. None of these six common polymorphisms affected the serum uric acid level. A haplotype analysis also suggested that these common genotypes/haplotypes were not important in determining the serum uric acid levels in the general population.

Conclusion. *SLC22A12* is a major gene for hypouricemia but not hyperuricemia in Japanese.

Urate is present in blood at higher levels in human blood than in other mammals, since humans have an effective renal urate reabsorption system despite the loss of hepatic uricase through evolutionary mutation [1]. Recently, *SLC22A12* has been identified as a urate-anion exchanger in the human kidney [2]. Inactivating mutations in *SLC22A12* have been shown to cause renal idiopathic hypouricemia [2]. It is possible that common genetic variants of *SLC22A12* may influence urate levels in the general population and may contribute to hyper- or hypouricemia. In the present study, we screened for polymorphisms of *SLC22A12* and conducted an association study between genetic polymorphisms and urate levels using an epidemiologic cohort representing the general population in Japan.

METHODS

Study population and DNA analysis

We screened for genetic polymorphisms in *SLC22A12* by sequencing the promoter regions (up to −2.0 kb) and all of the exons. We selected 93 healthy subjects (uric acid levels between 3 mg/dL and 8 mg/dL), 24 subjects with hypouricemia, and 36 subjects with hyperuricemia from among 1875 consecutive subjects recruited from the Suita Study. Since the uric acid level is influenced by many environmental factors, including gender, age, body mass index (BMI), plasma creatinine level, and alcohol consumption, we calculated residuals of uric acid levels by adjusting for the above-mentioned environmental factors. We selected the top 36 subjects with high residual values (above +2.1 mg/dL) as hyperuricemic subjects and the bottom 24 subjects with low residual values (below −2.1 mg/dL) as hypouricemic subjects. The selection criteria and design of the Suita Study have been described previously [3]. Briefly, the sample consisted of 14,200 men and women (30 to 79 years of age), stratified by gender and 10-year age groups who had been randomly selected from the municipal population registry. They were all invited by letter to attend regular cycles of follow-up examination (every 2 years). We routinely check 10 to 15 participants per day. DNA from leukocytes was collected from

Table 1. Primers for sequencing

Region	Primer sequence	
	Sense	Antisense
Promoter 1	5′-CCAGACTACCAGGAGCTCACTGG-3′	5′CAATGCCACCCTGAGATCTCTGTC-3′
Promoter 2	5′-CACACCCTGCATCAGCACACAC-3′	5′-GCTGAGAGGCTCGAGAGTGAGTG-3′
Exon 1	5′-GCTGGAGAAGCCACTGTGGGCAC-3′	5′-GCTGAGAGGCTCGAGAGTGAGTG-3′
Exon 2	5′-CTCACTGTTCCACAGGGTCTTGCTCT-3′	5′-GTTCCAGACCTCCCTACCTGGCAC-3′
Exon 3-Exon 4	5′-CTCAGCTCAGCGGGCAAGCATAGG-3′	5′-CAAGCCCTGGTTGAAGTGGGTGTC-3′
Exon 5-Exon 6	5′-GTTCCACAGAGTCATCCCTCCCTCC-3′	5′-CAGGGGATTTCTGCCTCCCTCTCTG-3′
Exon 7	5′-CTGGGAAGAAGGTCTCAGAGAGGAGGAG	5′-GAAGAGCTCGCTGCTGTAGATGGTGAT
Exon 8-Exon 9	5′-CACTCAGGACAGGATACCCAGATG-3′	5′-GTTGCCACTCTCCCAGAGATGTAC-3′
Exon 10	5′-CATCACATGCTCGGGAGCTCAGTTC-3′	5′-CAGGGTGATGGTGCACTGGGTCAC-3′
	5′-5′-CTCGTGGCTTCGGAGAGC-3′	5′-GTCAAGCAGAGGGTTGGCA-3-3′

The indicated regions were amplified by the primers listed in the Table.

participants who visited the National Cardiovascular Center between April 2002 and February 2003. All of the participants were Japanese, and only those who gave their written informed consent for genetic analyses of cardiovascular diseases were included. The ethics committee of the National Cardiovascular Center approved the study protocol.

Primer sequences are shown in Table 1. The genotype was determined by the TaqMan method (*http://www.appliedbiosystems.com/*) in 1875 consecutive subjects. Uric acid levels were determined using a kit (enzyme method, Uricolor-liquid; Ono Pharmaceutical Co., Ltd., Osaka, Japan).

Statistical analysis

Values are expressed as mean ± SD. All statistical analyses were performed with the JMP statistical package and StatView 5.0 (SAS Institute, Inc., Cary, NC, USA). Multiple regression analyses indicated that the uric acid level was determined by gender, BMI, age, plasma creatinine, and alcohol consumption (ethanol g/day). The relationships between genotypes and uric acid levels were analyzed by one-way analysis of variance (ANOVA) and the Kruskal-Wallis test with the Bonferroni correction. A subject was categorized as having hyperuricemia when the subject was being treated with hypouricemic drugs or had a uric acid level higher than 7.0 mg/dL.

Linkage disequilibriums (R^2 values) between polymorphisms and estimation of haplotypes were calculated using the SNPAlyze statistical package (Dynacom, Kanagawa, Japan).

Functional analysis of *SLC22A12* mutants

Missense and deletion mutants were constructed with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Proper construction of the mutated cDNA was confirmed by sequencing. Mutant cRNA was synthesized using an mMESSAGE mMACHINE kit (Ambion, Inc., Austin, TX, USA). The injection of 50 ng

of cRNA into *Xenopus laevis* oocytes, and maintenance of the oocytes and uptake studies of ^{14}C-urate were performed as described previously [4].

RESULTS

Sequence analysis of *SLC22A12*

Of the total 1875 consecutive subjects, 50 had hypouricemia (<3.0 mg/dL) and 56 had hyperuricemia (>8.0 mg/dL). We selected 93 healthy subjects, 24 with hypouricemia (<3 mg/dL), and 36 with hyperuricemia (>8 mg/dL) for sequence analysis. Sequence analysis of *SLC22A12* in the 93 healthy subjects revealed eight polymorphisms in the promoter region and 20 polymorphisms in exonic regions (Fig. 1 and Table 2). Sequence analysis in 24 subjects with hypouricemia revealed seven additional missense mutations (Figs. 1 to 3, Table 2): R90H, A226V, R228E, W258Stop, Q312L, D313A (deletion of 313D-333P), and R477H. Intriguingly, we found 11 heterozygotes and two homozygotes for 258Stop among the 24 subjects with hypouricemia selected for sequence analyses (Table 2). Sequence analysis in the 36 subjects with hyperuricemia revealed no additional polymorphisms compared to those found in the 93 healthy subjects.

Association study with missense mutations

All of the missense and nonsense mutations were detected in the subjects with hypouricemia (Table 2). To confirm whether these mutations contribute to hypouricemia, the effects of these mutations on the uric acid level were first investigated by an association study. Table 3 shows the probes and primers for TaqMan.

The minor allele frequencies of A226V, R228E, and D313A (deletion of 313D-333P) were very low; only one allele in the total population (1875 subjects or 3750 alleles). Therefore, we cannot conclude whether these mutations actually contribute to hypouricemia based on a statistical analysis.

The 226V mutation was detected in a subject homozygous for the 258X allele. Thus, the hypouricemia in this
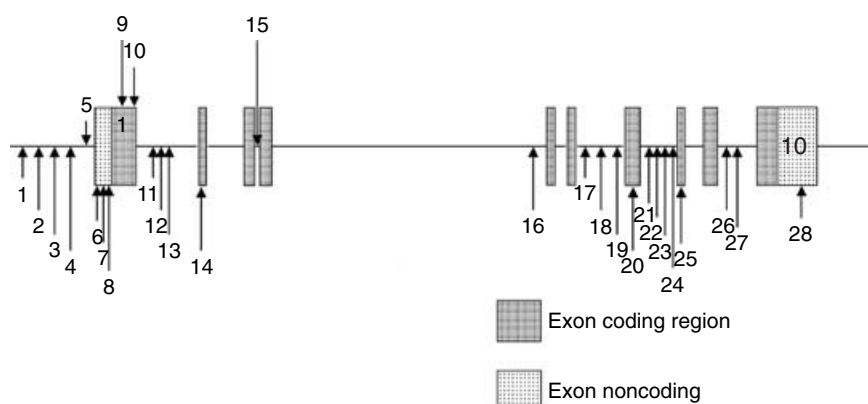
Fig. 1. Polymorphisms of SLC22A12. The sites for polymorphisms are shown on the SLC22A12 gene scheme. The arrows indicate the site of polymorphisms. The numbers of the polymorphic sites correspond to those in Table 2. Boxes indicate exons.

subject can be explained by this homozygosity of the 258X allele. Moreover, since alanine and valine are both hydrophobic amino acids, this homologous substitution would not seem to alter the function of URAT1.

The R228E missense mutation was tested with regard to urate transport activity by in vitro expression analysis in *Xenopus* oocytes. As shown in Figure 4, the urate transport activity of *SLC22A12* with 228E was not significantly less than that of the wild type. Although it is possible that this mutation might be in tight linkage disequilibrium with some other unidentified mutation that reduces the *SLC22A12* expression level, we could not confirm that this mutation was responsible for hypouricemia.

On the other hand, the urate transport activity of *SLC22A12* with 313A (deletion of 313D-333P) was significantly less than that of the wild type (Fig. 4). Thus, this is a newly identified mutation responsible for renal idiopathic hypouricemia in Japanese.

The Q312L mutation was detected in two subjects. One subject had one 312L+313A (deletion type) allele and had hypouricemia. The other had one 312L+313D (wild type) allele and a serum uric acid level of 5.5 mg/dL. Thus, the Q312L mutation seems to be neutral.

Subjects who were heterozygous for R90H (14 subjects) had significantly lower residuals of serum uric acid levels than those with the wild-type genotype ($P < 0.0004$, Kruskal-Wallis analysis followed by the Bonferroni correction). This R90H missense mutation has been previously reported in renal hypouricemic patients recruited from Jikei and Tottori University Hospitals who were different from our subjects with the 90H mutation [4]. The urate transport activity of *SLC22A12* with the 90H mutation has been reported to be significantly less than that of the wild type [4]. The allele frequency of 90H was 0.4%.

Subjects who were heterozygous for R477H (five subjects) had significantly lower residuals of serum uric acid levels that those with the wild-type genotype ($P < 0.0024$, Kruskal-Wallis analysis followed by the Bonfer-

roni correction). However, the urate transport activity of *SLC22A12* with 477H was not significantly less than that of the wild type (Fig. 4). This discrepancy might be explained by the hypothesis that some other unidentified mutations in tight linkage disequilibrium with the R477H mutation could reduce the expression level of *SLC22A12*. Another possibility is that the discrepancy between the statistical data and the functional analysis regarding the R477H mutation may reflect some limitation of the *Xenopus* oocyte expression system. This amino acid replacement might alter the processing of the transporter in mammalian cells. Additional studies will be needed to clarify this point. Further studies will be necessary to confirm these hypotheses. The allele frequency of 477H was 0.13%.

The W258Stop mutation has been reported previously, and the truncated transporter has been shown to have no transporter activity [2]. Subjects heterozygous or homozygous for this mutation had significantly lower residuals of serum uric acid levels than those with the wild-type genotype ($P < 0.0005$, respectively, Kruskal-Wallis analysis followed by the Bonferroni correction). We found one subject with possibly compound heterozygous mutations (258Stop and 90H) whose uric acid level was similar to that in subjects who were homozygous for the 258Stop allele (Table 4). One of the interesting findings in the present study is that this mutation occurs at a very high frequency among Japanese. We found 83 heterozygotes (including one compound heterozygote) and three homozygotes among 1875 subjects. The allele frequency of 258Stop was calculated to be 2.37%.

Other phenotypes and SLC22A12 mutations

Uric acid has been recognized to have several direct biologic functions. Thus, various phenotypes were compared in subjects with and without these mutations. As shown in Table 4, there were no significant differences in the incidence of hypertension, plasma creatinine level, age, or BMI between the two groups.

Table 2. Polymorphisms of *SLC22A12*

No.	SNP	dbSNP No.	Genotype frequency	Region	Amino acid variation	Sequence
1	C(-1957)T	505802	66/26/0	Promoter	-	GAACTGAGAAAGCATGTACAGGGCA[C/T]GTGGTGGAAGCAGTGGATGACTCAA
2	A(-1821)G	-	67/22/3	Promoter	-	GGAGGGGCATTTATGCCTCA[A/G]ATGGAGAGTTTTATGCCCAA
3	A(-1490)G	552307	66/26/0	Promoter	-	AGCTGGAATTACAGCTGTGCACCAC[A/G]ACCATGCTCAGCTAACTTTTTGTATT
4	G(-1193)A	-	92/1/0	Promoter	-	ACACTGCTGTCCACTCACCT[G/A]CCCTAGTAAGTCACCACTAG
5	C(-424)T	559946	88/27/0	Promoter	-	GGAGGCTGCACCTCCCTCGCGTCTG[C/T]GCCTGCCTCAACGCGGGTTAAACTT
6	G(-220)A	3825018	85/27/0	Exon 1/5'-UTR	-	GCTGGAGGTCTCGGAANTCACCTCAC[G/A]CGGCCTCAGGGCCCAGTTGGAGCCA
7	C(-75)T	-	92/1/0	Exon 1/5'-UTR	-	CACCGTGGGGAAACAGGCC[C/T]GTTGCCCTGGCCTCTTTGCC
8	G(-45)C	-	91/2/0	Exon 1/5'-UTR	-	CCTCTTTGCCCTGGGCCACA[G/A]CCTTTTGTGAAGTGGGCCCTC
9	C246T	3825017	46/38/7	Exon 1	N->N	TATTTCCATCCCGCCGGCCCCAA[C/T]CAGAGGCCCCA(C/T)CAGTGCCGCC(G/A)CT
10	C258T	3825016	66/25/0	Exon 1	H->H	GCCGGCCCCAA(C/T)CAGAGGCCCCA[C/T]CAGTGCCGCC(G/A)CTTCCGCCCAGCCA
11	C599T	-	66/26/0	Intron 1	-	GATCAGTCTACAAATGGGGCCAGCC[C/T]AGGCTCTTGGAGGTGCGGGGCAC
12	G616A	-	90/2/0	Intron 1	-	GCCCAGGCTCTTGGAGGTGC[G/A]GGGGCACCTGGGCGGGGCAC
13	C629T	-	91/1/0	Intron 1	-	AGGTGCGGGGCACCTGGG[C/T]GGGCACCTCTAAATGCTGGC
14	T1246C	-	65/26/0	Exon 2	H->H	AGTGGAACCTCGTGTGTGACTCTCA[T/C]GCTCTGAAGCCCATGGCCCAGTCCA
15	G2014A	-	91/2/0	Intron 3	-	GGCACTCTCCGTAGGTCTCT[G/A]ACCTGGCGCCATGCAGGGGG
16	T6092C	1529909	67/26/0	Intron 4	-	TCTGAGGCTGGCGCAGGCCAGGCAC[T/C]GGGGGCCACAGGCAATGACCCCTCCCACGC
17	CC6297-6298C	-	90/1/0	Intron 6	-	CTCAAACCCGACCCCTCCAGA[CC/C]CTCTCCCTGCCCCTGCATAG
18	C6610T	-	91/2/0	Intron 6	-	TCGGGGCTTCGCTGGCACCA[C/T]GGCCCCGGCCTCTGCTGGCT
19	G6948A	-	59/27/7	Intron 6	-	AGACCAAAACCGCCAAGGCCAAGGA[G/A]GGAAGGACTGTTCAGTGGCAGCTGG
20	C7421T	-	92/1/0	Exon 7	L->L	CCAAGATGGGGCGCCCTGCTGI[C/T]GCTGAGCACCACCCTCCTTGG
21	G7568A	-	92/1/0	Intron 7	-	CCTTGGCGGGGTCTCGGGCT[G/A]GGAAACAGCACCCTCCTTGG
22	G7752A	-	28/46/19	Intron 7	-	AGTTAAAATGAGAACTAAACT[G/A]ATGGATTGCAGGAGGAGGGA
23	GG7792-7793G	-	92/1/0	Intron 7	-	AAGAAGGACTCCTCGGGGCT[GG/G]CTCTGGGTGTCTGAGCGTGG
24	G7885A	-	26/46/20	Intron 7	-	AGATGGAGATGACTCCCAAACATTT[G/A]CAAAGAGGCCTGAAAGTCAGGGGACA
25	C8011T	-	26/45/20	Exon 8	L->L	AGAAATGGGGGCTCTGGCGCTCCAGCC[C/T]TGGCCGTGCTGGGGCTGGGCGGGGT
26	T8577C	-	27/45/19	Intron 9	-	CAGAACCAGTGAGTGGACCCAGCCT[T/C]GGGACCACCCCTCCTCCCACCAGA
27	G8711A	-	87/5/0	Intron 9	-	GTCACACAGTACATCTCTGG[G/A]AGAGTGGCAACCCAGGGCTC
28	G9463(-)	3832794	40/42/10	Exon 10/3'-UTF	-	TCGGAGCAGAGGGGTCAGGCCCA[G/-]GGGAACGAGCTGGCCTTGCCAACCC
29	C269T	-	21/3/0	Exon 1	R 90 H	GAGGCCCCACCAGTGCCGCGC[G/A]CTTCCGCCAGCCACAGTGGC
30	C2094T	-	23/1/0	Exon 4	A 226 V	TGCAGTGATGGAGTGGACGG[C/T]GGCACGGGCCGACCCTTGG
31	G2100A	-	23/1/0	Exon 4	R 228 E	GATGGAGTGGACGGCGGCACTG[G/A]GCCCAGACCCTTGGTGATGA
32	G2191A	-	12/11/1	Exon 4	W 258 Stop	GCCTACGGTGTGCGGGGACTG[G/A]ACACTGCTGCCAGCCTGGTGGT
33	A6231T	-	23/1/0	Exon 5	Q312L	CGGAAAGGGGGCAGTGC[A/T]GG[228base/-]CTCCTGCCAGCCTGGGCACC
34	6244-6471 del	-	23/1/0	Exon 5-Exon6	D 313 A	CGGAAAGGGGGCCAGTGCAGG[228base/-]CTCCTGCCAGCCTGGGCACC
35	G8391A	-	22/2/0	Exon 9	R 477 H	TGGGCCAGATGGCAGCCC[G/A]TGGAGGAGCCATCCTGGGGC

The base number was from ATG. We analyzed bold SNPs by TaqMan system. The upper table was obtained from normal 93 subjects, and the lower table was from 24 subjects with hypouricemis.
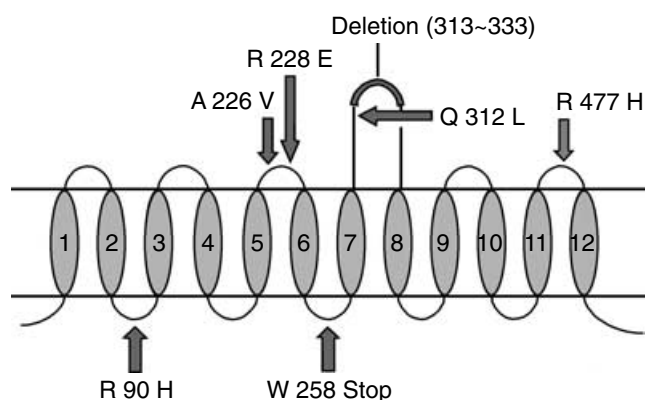
Fig. 2. Mutations in *SLC22A12*. The sites for missense or nonsense mutations are shown on the predicted protein structure of the uric acid-anion transporter. Numbers 1–12 indicate transmembrane domains.

Association study with common polymorphisms

The effects of common polymorphisms of *SLC22A12* were also investigated. None of these polymorphisms affected the amino acid sequence of *SLC22A12*. Based on linkage disequilibrium, common polymorphisms were categorized into six distinct groups (Table 5), and representative genotypes in each of the six groups were determined. Since the R90H, D313A, R477H, and W258Stop mutations all strongly affected uric acid levels, subjects with these mutations were excluded from the following association studies ($N = 1708$).

Table 6 shows the effects of common polymorphisms on residuals of the uric acid level. None of these six polymorphisms affected serum uric acid levels. We corrected *P* values using the Bonferroni method (Pc) by multiplying by 6 (number of polymorphisms analyzed).

The standard deviation of the residuals of the serum uric acid level was 1.0 (Table 6). The total sample size was about 1700. If a subgroup comprised 10% of the total population, we should be able to detect a difference between mean values of 0.3 mg/dL with a statistical power of 0.87 (alfa = 0.010, two-tailed). Thus, we can conclude that common polymorphisms of *SLC22A12* do not influence residuals of the uric acid level by at least 0.3 mg/dL.

We constructed haplotypes defined by the C(–1957)T, W258Stop, and G9463 genotypes, and compared the frequencies of the haplotypes between subjects with normal serum uric acid levels (<7.0 mg/dL, $N = 1597$) and subjects with hyperuricemia (≥ 7.0 mg/dL and/or treatment with hypouricemic drugs, $N = 256$). We selected the C(–1957)T and G9453 genotypes because these genotype had marginal effects on uric acid levels by a simple ANOVA analysis (Table 6). In this haplotype analysis, we excluded subjects with R90H, D313A, or R477H, since the allele frequencies of these genotype were very low. As shown in Table 7, the frequency of the haplotype (111) was significantly higher in subjects with normal serum uric acid levels than in those with hyperuricemia. This haplotype (111) corresponded to the allele 258Stop. However, no significant differences were observed in the frequencies of other haplotypes between the two groups, which suggests that common polymorphisms did not influence uric acid levels.

DISCUSSION

In the present study, we identified six missense mutations and one nonsense mutation in *SLC22A12* in 24 subjects with hypouricemia selected from the total 1875 subjects recruited from the Suita Study. The allele frequencies of 90H and 258Stop, which have been shown to contribute to renal hypouricemia in other hospital-based study populations-[2, 4], were 0.40% and 2.37%, respectively. The present epidemiologic study showed that the prevalence of these inactive *SLC22A12* mutations was unexpectedly high in Japanese. We also demonstrated, based on an in vitro expression analysis with *Xenopus* oocytes, that the newly identified D313A (deletion of 313D-333P) mutation caused renal hypouricemia in Japanese. We then investigated the effects of common polymorphisms of *SLC22A12* on serum uric acid levels. None of the common polymorphisms seemed to affect serum uric acid levels.

Many studies have found that uric acid is not an independent risk factor for cardiovascular diseases after controlling for other risk factors [5–8]. The uric acid level is higher in men and postmenopausal women because estrogen is uricosuric [9]. Obesity often accompanies hyperuricemia because insulin stimulates sodium and urate reabsorption in the proximal tubule [9]. Uric acid is elevated in subjects with renal diseases as a result of reduced glomerular filtration rate and urate excretion. Alcohol intake results in elevated uric acid levels due to increased generation and decreased excretion [10]. Since the uric acid level is influenced by the above-mentioned cardiovascular risk factors, it has been considered to be simply a marker for cardiovascular diseases. However, uric acid has also been recognized to be more than simply an inert bystander and indeed to have several important biologic functions that could be either beneficial or harmful to humans.

Ames et al [11] hypothesized that uric acid provides an antioxidant defense in humans against oxidant- and radical-causing aging and cancer. Uric acid seems to be one of the most important antioxidants in human plasma [11–13]. A positive correlation has been reported between uric acid levels and life span in primates [14]. However, in the present study, the mean age of subjects with inactive *SLC22A12* alleles was not significantly different from that of subjects without inactive *SLC22A12* alleles. The allele frequency of 258Stop in subjects
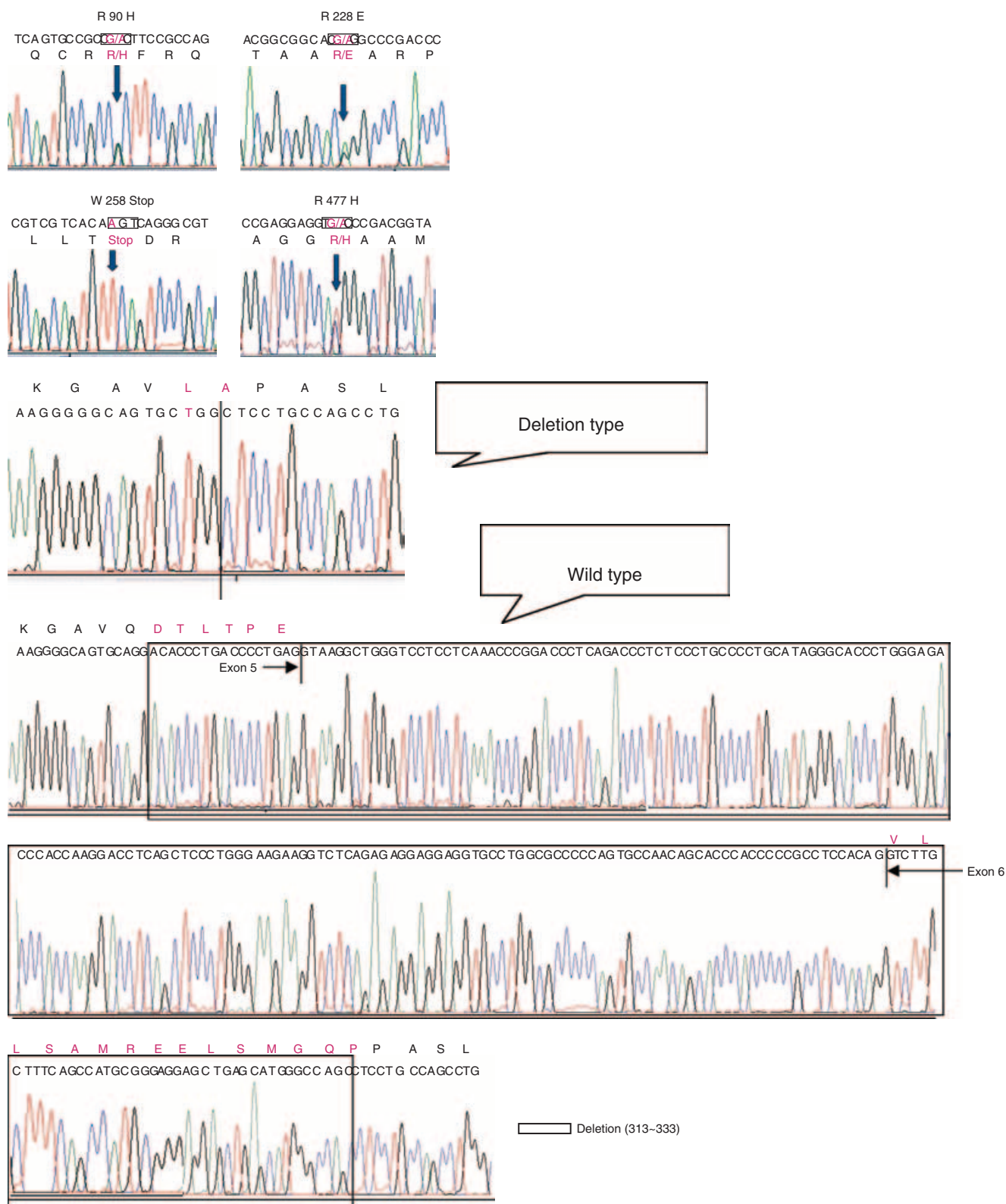
Fig. 3. Sequences for missense mutations. The sequences around the missense mutations (R90H, R228E, D313A, and R477H) and the nonsense mutation (W258Stop) are shown. The arrows indicate the sites of mutations.

Table 3. Probes and primers for TaqMan

SNP	Region	Sequence primers/probes
C(-1957)T		GAGAAAGCATGTACAGGGCA[T/C]GTGGTGGAAGCAGTGGATGA 5'-GGGTGGGAACTGAGAAAGCA-3' (Sense) 5'-VIC-TCCACCACATGCCCTGT-MGB-3' 5'-GCTCTGCACCTTGAGTCATCC-3' (Antisense) 5'-FAM-CCACCACGTGCCCTGT-MGB-3'
A(-1821)G		GGAGGGGCATTTATGCCTCA[A/G]ATGGAGAGTTTTATGCCAA 5'-TTGGCTTGGTAAGCCATGTG-3' (Sense) 5'-VIC-ACTCTCCATTTGAGGCAT-MGB-3' 5'-TGATGGCAACCCAGGTGTT-3' (Antisense) 5'-FAM-CTCTCCATCTGAGGCAT-MGB-3'
C246T		TCCATCCCGCCGGGCCCCCAA[C/T]CAGAGGCCCCACCAGTGCCG 5'-GCCCTCCTGGCTATTTCCA-3' (Sense) 5'-VIC-CGGGCCCCAACCAGAGGC-TAMRA-3' 5'-TGGCATTGGGGTCCAAGA-3' (Antisense) 5'-FAM-CCCCAATCAGAGGCCCCACC-TAMRA-3'
C269T	R 90 H	GAGGCCCCACCAGTGCCGCC[G/A]CTTCCGCCAGCCACAGTGGC 5'-GCCCTCCTGCGCTATTTCCAT-3' (Sense) 5'-VIC-TGCCGCCGCTTCCGCC-TAMRA-3' 5'-CCAAGAGCTGCCACTGTGG-3' (Antisense) 5'-FAM-AGTGCCGCCAGCCACCAG-TAMRA-3'
C599T		GTCTACAAAATGGGGCCAGCC[C/T]AGGCTCTTGGAGGTGCGGGG 5'-CGGCAGGGATCAGTCTACAAAT-3' (Sense) 5'-VIC-AGAGCCTGGGCTGG-MGB-3' 5'-TCCAGGTGCCAGCATTTAGAG-3' (Antisense) 5'-FAM-AGCCTAGGCTGGC-MGB-3'
C2094T	A 226 V	TGCAGTGATGGAGTGGACGG[C/T]GGCACGGGCCGACCCTGG 5'-AGAGAGTTCAAGGTCATCACCAA-3' (Sense) 5'-VIC-CCCGTGCCGCCGTCCACT-TAMRA-3' 5'-AACCACTCGGTCTCCTTGCA-3' (Antisense) 5'-FAM-CCCGTGCCACCGTCCACTCC-TAMRA-3'
G2100A	R 228 E	GATGGAGTGGACGGCGGGCAC[G/A]GGCCCGACCCTTGGTGATGA 5'-TCGGTCTCCTTGCAGTGATG-3' (Sense) 5'-VIC-CGGCACGGGCCCGACC-TAMRA-3' 5'-GCTGAAGCCCAGAGAGTTCAA-3' (Antisense) 5'-FAM-CGGCACAGGGCCGACCTTG-TAMRA-3'
G2191A	W 258 Stop	GCCTACGGTGTGCGGGACTG[G/A]ACACTGCTGCAGCTGGTGGT 5'-AGGAGTACAAAAAGCAGAGGAAGAAG-3' (Sense) 5'-VIC-TGCAGCAGTGTCCAGTCCCGC-TAMRA-3' 5'-CCCTTGGTGATGACCTTGAACT-3' (Antisense) 5'-FAM-TGCAGCAGTGTTCAGTCCCGCA-TAMRA-3'
6244-6471 del	D 313 A (313D-333P del)	CGGAAAGGGGGCAGTGCAGG[*/-]CTCCCTGCCAGCCTGGCACC 5'-GGCTGCCATCAACGGAA-3' (Sense) 5'-VIC-ACCTCAGCTCCCTGG-MGB-3' 5'-GTGGAGATACAGGTCCGGAAG-3' (Antisense) 5'-FAM-CAGGAGCCTGCACTG-MGB-3'
G6948A		AAAACCGCCAAGGCCAAGGA[G/A]GGAAGGACTGTTCAGTGCA 5'-CATCCAGCAAAGACCAAAACC-3' (Sense) 5'-VIC-AAGGAGGGAAGGAC-MGB-3' 5'-GCTCCAGCTGCCACTGAAC-3' (Antisense) 5'-FAM-CAAGGAAGGAAGGAC-MGB-3'
G8391A	R 477 H	TGGGCCAGATGGCAGCCC[G/A]TGGAGGAGCCATCCTGGGGC 5'-CGCTTATAGGTGCATCTGCATT-3' (Sense) 5'-VIC-CTCCTCCACGGGCTGCCATCT-TAMRA-3' 5'-ACTGGCACCGTCCCATACA-3' (Antisense) 5'-FAM-CTCCTCCATGGGCTGCCATCTG-TAMRA-3'
C8577T		CCAGTGAGTGGACCCAGCCT[C/T]GGGACCACCCTCCTCCCA 5'-CCAAGATGTGCAGAACCAGTGA-3' (Sense) 5'-VIC-CAGCCTTGGGACCA-MGB-3' 5'-TGCCCTGTGTCTAGGGTTCTCT-3' (Antisense) 5'-FAM-CAGCCTCGGGACC-MGB-3'
G9463(-)		GAGCAGAGGGGTCAGGCCCA[G/-]GGGAACGAGCTGGCCTTGCC 5'-CTCGTGGCTTCGGGGAGC-3' (Sense) 5'-VIC-AGCTCGTTCCCTGGGCCTGA-MGB-3' 5'-GTCAAGCAGAGGGTTGGCA-3' (Antisense) 5'-FAM-CTCGTTCCCCTGGGCCTGAC-MGB-3'

*ACACCCTGACCCCTGAGGGTAAGGCTGGGTCTCCTCAAAACCGGACCCTGCCCCTGCCCTCCCTGCCGTCTGGGTCTGGGTGCCTGGCCTCTCCCTGAGGTTAAGGCTGGGTCTCCTCAAAACCGGACCCTGCCCCTGCCCTCCCTGCTGCTAGGGCGCACCCTGGGAGACCTCCCTGGGAAGAA
GGTCTCAGAGAGGAGGAGGGTGCCTGGCGCCCTGGCGCCGCCCCAGTGCCAACAGCACCCCACCCCCGCCTCCACAGGTTCGTTTGCTTTCAGCCATGCGGGAGGAGCTGAGCATGGGCCCAGC

Table 4. Subjects' characteristics according to genotypes

	Wild	R90H/RH	H/RH+W258X	W258X/WX	W258X/XX	R477H/RH	R228E/RE	D313A/DA	Mutation(+)
N(M)	1768 (814)	14 (7)	1 (0)	82 (37)	3 (2)	5 (4)	1 (0)	1 (1)	107 (51)
UA	5.3 (1.3)	4.1 (1.2)[a]	1.2	3.7 (1.1)[c]	0.9 (0.3)[c]	3.3 (1.5)[b]	3.0	2.7	3.65 (1.2)
Res-UA	0.1 (1.0)	−1.1 (1.5)[c]	−4.1	−1.4 (0.8)[c]	−4.1 (0.6)[c]	−2.7 (1.9)[c]	−2.2	−2.7	−1.5 (1.1)
HUD	60	0	0	0	0	0	0	0	0
HU	255	0	0	1	0	0	0	0	1
Age	64.6 (11.2)	66.5 (10.3)	80	65.6 (10.9)	70.0 (4.4)	70.6 (3.7)	70	42	66.1 (10.7)
BMI	22.7 (0.2)	22.7 (0.2)	20.6	22.7 (2.7)	20.6 (2.7)	24.8 (4.5)	21.9	22.2	22.7 (2.8)
Cre	0.7 (0.2)	0.7 (0.2)	1	0.7 (0.2)	0.6 (0.3)	0.8 (0.2)	0.8	0.7	0.7 (0.2)
HTN (%)	722 (40.8)	9	1	36 (43.9)	2	2	0	0	50 (46.7)

Abbreviations are: N(M), number of subjects (number of male subjects); UA, uric acid (mg/dL); Res-UA, residulas of uric acid levels adjusting for sex, age, BMI, creatinine, and alcohol; HUD, number of subjects with hypouricemic treatment; HU, number of subjects with hyperuricemia; BMI, body mass index (kg/m^2); Cre; creatinine (mg/dL); HTN, number of subjects with hypertension. Values are mean(SD).

P values were calculated by Kruskal-Wallis test followed by the Bonferroni correction.

[a] $P < 0.05$, [b] $P < 0.005$, [c] $P < 0.0005$ compared with the wild type.

Table 5. Linkage disequilibrium among polymorphisms

	C(-1957)T	A(-1821)G	T(-1490)C	C(-924)T	G(-220)A	C(246)T	C(258)T	C(599)T	T(1246)C	T(6092)C	G(6948)A	G(7752)A	G(7885)T	C(8011)T	T(8577)C	G(9453)-
C(-1957)T		0.02954	0.95541	0.955687	0.95679	0.06552	0.95385	0.95541	0.93533	0.91154	0.05645	0.12891	0.14713	0.14945	0.14843	0.08611
A(-1821)G			0.02895	0.03087	0.03034	0.06859	0.02597	0.02846	0.02774	0.02692	0.06158	0.19854	0.19879	0.19295	0.2109	0.35191
T(-1490)C				0.91682	0.91667	0.06377	0.90867	0.91154	0.91171	0.86744	0.05645	0.12476	0.14277	0.14498	0.14396	0.08611
C(-924)T					1	0.06968	0.91413	0.91667	0.91682	0.87632	0.05992	0.14191	0.16084	0.15516	0.16291	0.09121
G(-220)A						0.06977	0.91998	0.91651	0.91667	0.87601	0.05983	0.14409	0.16338	0.15763	0.16561	0.09166
C(246)T							0.06369	0.06383	0.06369	0.06076	0.61916	0.20924	0.29214	0.29623	0.2847	0.18754
C(258)T								1	1	0.95592	0.05544	0.12948	0.14843	0.1509	0.15298	0.07865
C(599)T									1	0.95549	0.05645	0.13311	0.15156	0.15399	0.15298	0.08198
T(1246)C										0.95541	0.05314	0.12664	0.14498	0.14727	0.14631	0.08654
T(6092)C											0.0525	0.1198	0.13715	0.13927	0.13787	0.0799
G(6948)A												0.18099	0.25975	0.26021	0.24736	0.16452
G(7752)A													0.93596	0.93527	0.91444	0.5385
G7885A														1	0.97787	0.56275
C(8011)T															1	0.55882
T(8577)C																0.58434

Linkage disequilibrium (LD) was assessed by calculating R-square values by SNPAlyze. SNPs with low allele frequency (<0.1) were excluded from the analysis.
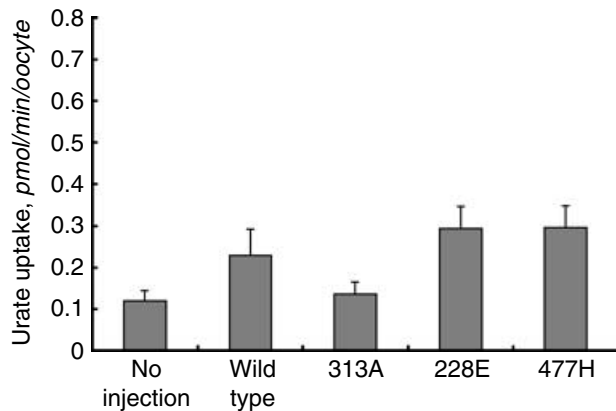
Fig. 4. Urate transport ability of the *SLC22A12* mutants. Urate uptake by the oocyte injected with the 313A (deletion of 313D-333P) mutant cRNA was significantly less than that with the wild-type cRNA (*P* < 0.01 vs. wild-type). Urate uptake by the oocyte injected with the 228E or 477H cRNA was not significantly different from that with the wild-type cRNA. Statistical analysis was performed with Tukey-Kramer's HSD test. Vertical bar indicates standard deviation (*N* = 8).

Table 6. Effects of common polymorphisms of *SLC22A12* on the residuals of uric acid levels

	AA	Aa	aa	*P* value	Pc
C(-1957)T	0.0 (1.0) (1188)	0.0 (1.0) (465)	0.3 (0.9) (55)	0.0823	0.4938
A(-1821)G	0.0 (1.0) (1169)	0.0 (0.9) (493)	−0.1 (1.0) (46)	0.7545	1.0000
C246T	0.0 (1.0) (929)	0.0 (1.0) (657)	0.0 (1.1) (122)	0.9965	1.0000
G6948A	0.0 (1.0) (920)	0.0 (1.0) (672)	0.0 (1.0) (387)	0.5437	1.0000
G7752A	−0.1 (1.0) (442)	0.0 (1.0) (879)	0.1 (1.0) (387)	0.1560	0.936
G9463-	0.1 (1.0) (578)	0.0 (1.0) (736)	−1.0 (1.0) (394)	0.0502	0.3012

Values are mean(SD). The number of subjects is indicated in parentheses. AA, Aa, and aa indicate major homozygotes, heterozygotes, and minor homozygotes. *P* values are corrected by the Bonferroni method (Pc).

Table 7. Haplotype analysis

Haplotype	Fr	Fr in N (*N* = 1597)	Fr in HU (*N* = 256)	*P* value	Pc
000	43.01	43.14	42.19	0.6849	1.0000
001	37.88	37.60	39.65	0.3766	1.0000
101	16.65	16.44	17.99	0.3918	1.0000
111	2.37	2.72	0.2	<0.0001	<0.0005
100	0.08	0.09	0.00	0.3448	1.0000

Haplotype is constructed with C(-1957)T, W258Stop, and G9463-genotypes. Major allele is indicated as "0" and minor allele is indicated as "1." The differences in frequencies (Fr) of haplotypes are compared between subjects with normal serum uric acid levels (N) and those with hyperuricemia (HU). The definition of HU is described in the text. *P* values are calculated by chi-square analysis, and are corrected by the Bonferroni method (multiplying the number of comparison = 5). The frequencies of the haplotypes (110, 011, and 010) have been calculated to be very low (<0.1%) in Japanese and are not included in Table 7.

incidence of hypertension. Our preliminary analysis indicated that the frequency of the 258Stop allele in 516 subjects with myocardial infarction (1.84%) was not significantly different from that in the general population (2.46%). Again, inactive *SLC22A12* alleles seem to be neutral.

The unexpectedly high prevalence (~3%) of inactive *SLC22A12* alleles suggests that at least these mutations may not be harmful to humans. Idiopathic renal hypouricemia has been reported to be associated with exercise-induced acute renal failure [17], and this nephropathy may be a possible major adverse influence of inactive *SLC22A12* mutations. However, subjects with inactive *SLC22A12* alleles had normal creatinine levels. We found four homozygotes with inactive *SLC22A12* alleles (the expected number was 1), and these four subjects also had normal creatinine levels. Since renal function, except for urine-concentrating ability, recovered after exercise-induced acute renal failure in hypouricemic subjects, more thorough characterization of subjects with inactive *SLC22A12* will be necessary to assess the incidence of this nephropathy. Since the Suita study principally involves general health checkups, it might be difficult to conduct more specific research-oriented laboratory and physiologic tests. This drawback of the present epidemiologic study could perhaps be offset by patient-oriented clinical studies. However, an unexpectedly high prevalence of *SLC22A12* inactive mutations in Japanese could only be clarified by epidemiologic studies.

Eight of our 1875 subjects had uric acid levels below 2.0 mg/dL. Of these eight subjects, four were homozygous [three homozygotes (258Stop) and one compound heterozygote (258Stop+90H)], and two were heterozygous (258Stop) for the inactive mutations reported here. The cause of hypouricemia in the remaining two subjects could not be explained by the mutations reported here. Whether this implies the existence of another urate transporter or mutations in the regulatory region of *SLC22A12* remains to be clarified.

<75 years (2.30%) was not significantly different from that in subjects ≥75 years (2.67%). The genotype frequencies did not deviate from Hardy-Weinberg equilibrium. Since subjects with inactive *SLC22A12* alleles have a low uric acid level throughout their life span, low uric acid levels may not be associated with reduced longevity.

Higher uric acid levels may contribute to endothelial dysfunction. Serum uric acid and nitric oxide levels have been reported to vary during the day in a reciprocal manner [15]. Waring et al [16] reported that uric acid infusion resulted in impaired acetylcholine-induced vasodilation in the forearm. Thus, hyperuricemia itself may lead to cardiovascular diseases, including hypertension and ischemic heart diseases. If so, inactive *SLC22A12* alleles might protect against hypertension and ischemic heart diseases. However, as shown in the present study, inactive *SLC22A12* alleles were not associated with a reduced

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