258X and *ABCG2* 126X. The present study was approved by the Ethics Committee of Nagoya University School of Medicine (approval number 288).

2. Materials and methods

2.1. Statistical power to detect a significant finding

Under the conditions that a genotype frequency in controls is 0.1, OR is 0.5, two-sided alpha error is 0.05, controls are 1000, and cases are 400, the statistical power is calculated to be 0.8137. As described below, the corresponding numbers of subjects were analyzed for males and females.

2.2. Subjects and data collection

Subjects were 5028 health checkup examinees in a health checkup center in Hamamatsu, Japan, who were the participants of Japan Multi-Institutional Collaborative Cohort Study (J-MICC Study) in 2007–2008 [15,16]. Written informed consent including genotyping was obtained from all participants. Excluding one participant without a blood sample, genotyping was successfully conducted for 5027 participants. Three participants with creatinine of 2.0 mg/dL or over were excluded from the analysis, and 5024 subjects remained.

The health checkup data including blood tests were used for this study. Peripheral blood was drawn in the morning from those with overnight fast. SUA was measured enzymatically (a uricase method) using an auto-analyzer.

2.3. Genotyping procedure

DNA was extracted from buffy coat conserved at −80 °C using a BioRobot® M48 (QIAGEN Group, Tokyo). *SLC2A9* rs11722228 polymorphism was genotyped by a polymerase chain reaction with confronting two-pair primers (PCR-CTPP) [17]. Each 25 µl reaction tube contained 50–80 ng DNA, 0.12 mM dNTP, 12.5 pmol of each primer, 0.5 U AmpliTaq Gold (Perkin-Elmer, Foster City, CA) and 2.5 µl of 10× PCR buffer including 15 mM MgCl$_2$. The PCR-CTPP was conducted with initial denaturation at 95 °C for 10 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The primers were F1: 5′-GGA GCT ATC ACT GCT CC-3′, R1: 5′-CAG AGG ACA GTA GAA ATG TTT GG-3′, F2: 5′-GAT CGA GCC CTG ATC ATC T-3′, and R2: 5′-TAG TGA TTC ATC CCT GGT G-3′. The amplified DNA fragments were 124 base pairs (bp) for the *C* allele, 172 bp for the *T* allele, and 255 bp for common band, as illustrated in Fig. 1. *ABCG2* Q126X and *SLC22A12* W258X were genotyped as described in the previous papers [2,18].

2.4. Statistical analysis

Hardy–Weinberg equilibrium was examined with a chi-square test. The percentages among different genotypes were also tested with a chi-square test. Means among three genotype groups were tested with one-way analysis of variance. Adjusted OR and 95% confidence interval (CI) were estimated using an unconditional logistic regression model. All statistical analyses were performed using STATA software version 11 (STATA, College Station, TX).

3. Results

The subjects were derived from 5024 health checkup examinees (3413 males and 1611 females) aged 35 to 69 years with creatinine <2.0 mg/dL. Table 1 shows the characteristics of the examinees. Those with SUA less than 3.0 mg/dL were 0.6% in males and 5.2% in females, while those with SUA of 7.0 mg/dL or over were 23.5% in males and
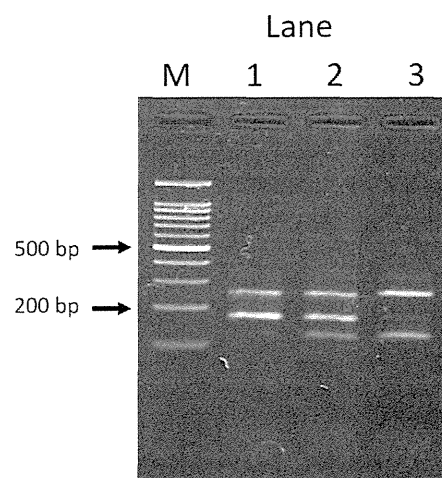

Fig. 1. Representative gel for *SLC2A9* rs11722228 polymorphism. Lane M, a 100-bp ladder; lane 1, a *TT* homozygote with fragments of 172 bp and 255 bp; lane 2, a *CT* heterozygote with fragments of 124 bp, 172 bp and 255 bp; and lane 3, a *CC* with fragments of 124 bp and 255 bp.

1.5% in females. The genotype frequency of *SLC2A9* rs11722228 was in Hardy–Weinberg equilibrium (p = 0.179 in males and p = 0.674 in females). The minor allele (*T* allele) frequency was 0.306 in males, 0.304 in females, and 0.305 in both sexes.

Among them, examinees with *SLC22A12* 258X (230 individuals), *ABCG2* 126X (250 individuals), and/or not genotyped (one for *SLC22A12* W258X and thirteen for *ABCG2* Q126X) were excluded from the analysis, and 4535 subjects (3082 males and 1453 females) with *SLC22A12* 258WW and *ABCG2* 126QQ remained. The genotype frequency was 2184 for *CC*, 1947 for *CT*, and 404 for *TT*, which was in Hardy–Weinberg equilibrium (p = 0.312). Table 2 shows the distribution of SUA levels according to the genotype. Although there was no marked difference in the distribution among three genotypes, males with SUA less than 5.0 mg/dL were 14.7% for *CC*, 10.6% for *CT*, and 7.8% for *TT* (p = 2.3E-4), and females with SUA less than 4.0 mg/dL were

Table 1
Characteristics of participants according to sex.

Characteristics	Males		Females	
	N	(%)	N	(%)
Total number	3413	(100)	1611	(100)
Age (years)				
35–39	265	(7.8)	192	(11.9)
40–49	983	(28.8)	496	(30.8)
50–59	1366	(40.0)	630	(39.1)
60–69	799	(23.4)	293	(18.2)
SUA (mg/dL)				
2.9 and below	21	(0.6)	84	(5.2)
3.0–4.9	522	(15.3)	1074	(66.7)
5.0–6.9	2069	(60.6)	429	(26.6)
7.0 and above	801	(23.5)	24	(1.5)
BMI (kg/m^2)				
18.4 and below	91	(2.7)	160	(9.9)
18.5–24.9	2423	(71.0)	1196	(74.2)
25.0 and above	899	(26.3)	255	(15.8)
Creatinine (mg/dL)				
0.0–0.4	0	(0.0)	33	(2.1)
0.5–0.9	2710	(79.4)	1575	(97.8)
1.0–1.4	701	(20.5)	3	(0.2)
1.5–1.9	2	(0.1)	0	(0.0)
SLC2A9 rs11722228				
CC	1627	(47.7)	785	(48.7)
CT	1483	(43.5)	674	(41.8)
TT	303	(8.9)	152	(9.4)

SUA; serum uric acid, BMI; body mass index, and BUN; blood urea nitrogen.

Table 2

Serum uric acid (SUA) distribution (%) according to *SLC2A9* rs11722228 genotype among Japanese health checkup examinees with *SLC22A12 258WW* and *ABCG 126QQ.*

Genotype	N	SUA (mg/dL)										Mean	95%CI[a]
		0.0–0.9	1.0–1.9	2.0–2.9	3.0–3.9	4.0–4.9	5.0–5.9	6.0–6.9	7.0–7.9	8.0–8.9	9.0 and above		
Males													
CC	1472	0.0	0.0	0.2	1.7	12.8	32.1	31.2	16.2	5.0	0.7	6.10	6.05–6.16
CT	1342	0.0	0.0	0.1	1.3	9.2	32.3	32.0	17.0	7.1	1.0	6.25	6.19–6.31
TT	268	0.0	0.0	0.0	2.2	5.6	24.3	34.7	25.4	6.7	1.1	6.45	6.32–6.59
Total	3082	0.0	0.0	0.1	1.6	10.6	31.5	31.9	17.3	6.1	0.9	6.20	6.16–6.24
Females													
CC	712	0.0	0.0	4.2	29.9	42.0	20.9	2.0	1.0	0.0	0.0	4.34	4.28–4.41
CT	605	0.0	0.2	2.8	22.5	43.5	22.5	6.8	1.3	0.5	0.0	4.59	4.51–4.66
TT	136	0.0	0.0	2.2	13.2	43.4	28.7	10.0	1.5	1.5	0.0	4.87	4.70–5.05
Total	1453	0.0	0.1	3.4	25.3	42.7	22.3	4.7	1.2	0.3	0.0	4.49	4.45–4.52

[a] 95% confidence interval for SUA mean.

34.1%, 25.5%, and 15.4% (p = 3.7E-6), respectively. Mean serum uric acid was 6.10 mg/dL for CC, 6.25 mg/dL for CT, and 6.45 mg/dL for TT among males (p = 1.5E-6), and 4.34 mg/dL, 4.59 mg/dL, and 4.87 mg/dL among females (p = 4.6E-11), respectively. The difference in the mean between CC genotype and TT genotype was 0.35 mg/dL in males and 0.53 mg/dL in females.

Table 3 shows the mean and standard deviation of SUA for each genotype according to sex and age group. The difference of mean SUA was 0.13 mg/dL for age 35–49 years, 0.45 mg/dL for age 50–59 years, and 0.55 mg/dL for age 60–69 years in males, while the difference in females was 0.54 mg/dL, 0.68 mg/dL, and 0.30 mg/dL, respectively.

The age-adjusted OR and 95% CI of low SUA (<5 mg/dL in males and <4 mg/dL in females) are shown in Table 4. Those with CT genotype or TT genotype had a significantly lower OR relative to those with CC genotype. From the view of hyperuricemia, those with TT genotype were found to be less resistant. The age-adjusted OR of SUA 7.0 mg/dL or over was 1.18 (95% CI, 1.00–1.40) for CT genotype and 1.62 (95% CI, 1.24–2.12) for TT genotype relative to CC genotype in males. In females, the OR was 1.99 (95%CI, 0.80–4.92) and 3.23 (95% CI, 0.95–11.1), respectively.

4. Discussion

This study confirmed that mean SUA was significantly higher for *SLC2A9* rs11722228 *TT* genotype than for *CC* genotye among those with *SLC22A12 258WW* and *ABCG2 126QQ.* The difference was larger among

females than among males. The frequency of rs11722228 T allele was 0.305 among 5024 subjects, similar to that reported in ss44579198 HapMap-JST (0.289 of 2 N = 90), as well as HapMap-CEU European (0.333 of 2 N = 120), HapMap-HCB Asian (0.284 of 2 N = 88), and HapMap-YRI Sub-Saharan African (0.375 of 2 N = 120). The function of this polymorphism has not been reported, although GLUT9 is known to be an important molecule regulating SUA.

SUA is lower in females than in males. In addition, it is fully documented that age, menopause, meat consumption, alcohol intake, obesity, a sedentary lifestyle, dyslipidemia, insulin resistance, blood pressure, renal function, and drug use for hypertension were associated with SUA levels [19–24]. Accordingly, it is no doubt that SUA levels are determined by lifestyle and physical conditions. Meanwhile, recent studies elucidated that genotypes are also influential factors of SUA.

SLC22A12 258X allele and *ABCG2 126X* allele are actually very influential, but their alleles were relatively rare; 0.023 for *SLC22A12* 258X and 0.025 for *ABCG2* 126X in this dataset. The removal of those with either of the two alleles was thought to be essential to evaluate the impact of *SLC2A9* rs11722228. *ABCG2 141KK* genotype was also reported to elevate SUA levels, which was equivalent to *126WX*[2]. When 445 subjects with *141KK* genotype in the present subjects were excluded from the analysis, quite similar findings were observed. The mean SUA was 6.07 mg/dL for CC, 6.22 mg/dL for CT, and 6.42 mg/dL for TT among 2790 males, and 4.34 mg/dL, 4.58 mg/dL, and 4.76 mg/dL among 1324 females, respectively. To date, several polymorphisms

Table 3

Mean and standard deviation of serum uric acid (mg/dL) for *SLC2A9* rs11722228 genotype according to sex and age group among Japanese health checkup examinees with *SLC22A12 258WW* and *ABCG 126QQ.*

Genotype	Males (N = 3082)			Females (N = 1453)		
	35–49 years (N)	50–59 years (N)	60–69 years (N)	35–49 years (N)	50–59 years (N)	60–69 years (N)
CC	6.20 ± 1.15 (552)	6.02 ± 1.10 (580)	6.09 ± 1.04 (340)	4.12 ± 0.78 (292)	4.43 ± 0.84 (272)	4.62 ± 0.93 (148)
CT	6.32 ± 1.05 (485)	6.16 ± 1.19 (526)	6.28 ± 1.13 (331)	4.30 ± 0.82 (270)	4.70 ± 0.98 (240)	5.11 ± 1.07 (95)
TT	6.33 ± 1.07 (97)	6.47 ± 1.13 (119)	6.64 ± 1.18 (52)	4.66 ± 0.90 (62)	5.11 ± 1.13 (55)	4.92 ± 1.04 (19)
Whole	6.26 ± 1.10 (1134)	6.12 ± 1.15 (1225)	6.22 ± 1.10 (723)	4.25 ± 0.82 (624)	4.61 ± 0.95 (567)	4.82 ± 1.02 (262)

Table 4

Age-adjusted odds ratio (OR) and 95% confidence interval (95% CI) of low serum uric acid for *SLC2A9* rs11722228 genotype among Japanese health checkup examinees with *SLC22A12 258WW* and *ABCG 126QQ.*

Genotype	Males (N = 3082)						Females (N = 1453)					
	<5 mg/dL[a]		≥5 mg/dL[a]		OR	(95% CI)	<4 mg/dL[a]		≥4 mg/dL[a]		OR	(95% CI)
	N	(%)	N	(%)			N	(%)	N	(%)		
CC	217	(57.1)	1255	(46.4)	1	(Reference)	243	(58.1)	469	(45.3)	1	(Reference)
CT	142	(37.4)	1200	(44.4)	0.68	(0.54–0.85)	154	(36.8)	451	(43.6)	0.62	(0.49–0.80)
TT	21	(5.5)	247	(9.1)	0.49	(0.31–0.79)	21	(5.0)	115	(11.1)	0.32	(0.20–0.53)
Total	380	(100)	2702	(100)			418	(100)	1035	(100)		

[a] Serum uric acid levels.

including *MTHFR* C677T have been reported to have an association with SUA [25,26], but the impact was limited. This study indicated that *SLC2A9* rs11722228 had a substantial influence on SUA, though it was less marked than that of *SLC22A12* 258X and *ABCG2* 126X.

GLUT9 plays a role to reabsorb uric acid in kidney. The reduced activity is considered to lower SUA levels. The reported mutations (R380W and R198C in Japanese [9], L75R in an Israeli-Arab family, exon 7 deletion in Ashkenazi-Jewish [10], and Ile118HisfsX27 in a Czech family [11]) cause hypouricemia. Since the *CC* genotype of *SLC2A9* rs11722228 showed a lower mean SUA than the *TT* genotype, it indicated that the function of the *CC* genotype may be lower than that of the *TT* genotype, though there was no biological evidence. A recent study demonstrated that *SLC2A9* rs1014290 in intron 4 had a similar size of differences in the mean SUA among the genotypes, but the biological characteristics of the polymorphism were also not fully described [27].

The effect of *SLC2A9* rs11722228 on mean SUA was larger for females than for males in this study. The finding was consistent with the past studies [12,28–30]. Although it is known that estrogens reduce SUA levels possibly through more efficient renal clearance of SUA [31], the biological mechanism in connection with the GLUT9 function has not been clarified.

One study reported that the difference in mean SUA among the genotypes was larger for older groups in females, but not in males [29]. The present study found that the difference was larger in older groups in males, and not in females. The reason for the inconsistency was not clear.

One of the limitations in the present study was that the medication for hyperuricemia was not taken into account. Since many with hyperuricemia were treated to lower the SUA, it would tend to weaken the association of SUA with the genotype. Even under this situation, the highly significant moderate associations were observed in this study. Another limitation was no adjustment for the lifestyle factors associated with SUA. Generally speaking, genotypes are not associated with lifestyle, so that the confounding by lifestyle may be limited, if any, in this study. At least, sex, age, body mass index, and creatinine levels were not associated with the genotype.

5. Conclusions

SLC2A9 rs11722228 *TT* genotype was associated with higher SUA levels among those with *SLC22A12* 258*WW* and *ABCG2* 126*QQ*. The exclusion of those with *SLC22A12* 258X and *ABCG2* 126X made a clear comparison among the different genotypes of *SLC2A9* rs11722228. Since the T allele frequency of rs11722228 is similar among different ethnic groups, the impact remains to be examined in the other ethnic groups. In addition, the function of the polymorphism and linked polymorphisms needs to be examined.

Acknowledgments

References

[1] O. Woodward, A. Kottgen, J. Coresh, E. Boerwinkle, W. Guggino, M. Kottgen, Identification of a urate transporter, ABCG2, with a common functional polymorphism causing gout, Proc. Natl. Acad. Sci. USA 106 (2009) 10338–10342.

[2] H. Matsuo, T. Takada, K. Ichida, T. Nakamura, A. Nakayama, Y. Ikebuchi, K. Ito, Y. Kusanagi, T. Chiba, S. Tadokoro, Y. Takada, Y. Oikawa, H. Ioue, K. Suzuki, R. Okada, J. Nishiyama, H. Domoto, S. Watanabe, M. Fujita, Y. Morimoto, M. Naito, K. Nishio, A. Hishida, K. Wakai, Y. Asai, K. Niwa, K. Kamakura, S. Nonoyama, Y. Sakurai, T. Hosoya, Y. Kanai, H. Suzuki, N. Hamajima, N. Shinomiya, Common defects of

[3] K. Yamagishi, T. Tanigawa, A. Kitamura, A. Kottgen, A.R. Folsom, H. Iso, CIRCS Investigators, The rs2231142 variant of the ABCG2 gene is associated with uric acid levels and gout among Japanese people, Rheumatology 49 (2010) 1461–1465.

[4] A. Taniguchi, N. Kamatani, Control of renal uric acid excretion and gout, Curr. Opin. Rheumatol. 20 (2008) 192–197.

[5] A. Enomoto, H. Kimura, A. Chairoungdua, Y. Shigeta, P. Jutabha, S.H. Cha, M. Hosoyamada, M. Takeda, T. Sekine, T. Igarashi, H. Matsuo, Y. Kikuchi, T. Oda, K. Ichida, T. Hosoya, K. Shimokata, T. Niwa, Y. Kanai, H. Endou, Molecular identification of a renal urate–anion exchanger that regulates blood urate levels, Nature 417 (2002) 447–452.

[6] F. Komoda, T. Sekine, J. Inatomi, A. Enomoto, H. Endou, T. Ota, T. Matsuyama, T. Ogata, M. Ikeda, M. Awazu, K. Muroya, I. Kamimaki, T. Igarashi, The W258X mutation in SLC22A12 is the predominant cause of Japanese renal hypouricemia, Pediatr. Nephrol. 19 (2004) 728–733.

[7] K. Ichida, M. Hosoyamada, I. Hisatome, A. Enomoto, M. Hikita, H. Endou, T. Hosoya, Clinical and molecular analysis of patients with renal hypouricemia in Japan-influence of URAT1 gene on urinary urate excretion, J. Am. Soc. Nephrol. 15 (2004) 164–173.

[8] N. Iwai, Y. Mino, M. Hosoyamada, N. Tago, Y. Kokubo, H. Endou, A high prevalence of renal hypouricemia caused by inactive SLC22A12 in Japanese, Kidney Int. 66 (2004) 935–944.

[9] H. Matsuo, T. Chiba, S. Nagamori, A. Nakayama, H. Domoto, K. Phetdee, P. Wiriyasermkul, Y. Kikuchi, T. Oda, J. Nishiyama, T. Nakamura, Y. Morimoto, K. Kamakura, Y. Sakurai, S. Nonoyama, Y. Kanai, N. Shinomiya, Mutations in glucose transporter 9 gene SLC2A9 cause renal hypouricemia, Am. J. Hum. Genet. 83 (2008) 744–751.

[10] D. Dinour, N.K. Gray, S. Campbell, X. Shu, L. Sawyer, W. Richardson, G. Rechavi, N. Amariglio, L. Ganon, B.A. Sela, H. Bahat, M. Goldman, J. Weissgarten, M.R. Millar, A.F. Wright, E.J. Holtzman, Homozygous SLC2A9 mutations cause severe renal hypouricemia, J. Am. Soc. Nephrol. 21 (2010) 64–72.

[11] B. Stiburkova, K. Ichida, I. Sebesta, Novel homozygous insertion in SLC2A9 gene caused renal hypouricemia, Mol. Genet. Metab. 102 (2011) 430–435.

[12] M. Kolz, T. Johnson, S. Sanna, A. Teumer, V. Vitart, M. Perola, M. Mangino, E. Albrecht, C. Wallace, M. Farrall, A. Johansson, D.R. Nyholt, Y. Aulchenko, J.S. Beckmann, S. Bergmann, M. Bochud, M. Brown, H. Campbell, EUROSPAN Consortium, J. Connell, A. Dominiczak, G. Homuth, C. Lamina, M.I. McCarthy, ENGAGE Consortium, T. Meitinger, V. Mooser, P. Munroe, M. Nauck, J. Peden, H. Prokisch, P. Salo, V. Salomaa, N.J. Samani, D. Schlessinger, M. Uda, U. Völker, G. Waeber, D. Waterworth, R. Wang-Sattler, A.F. Wright, J. Adamski, J.B. Whitfield, U. Gyllensten, J.F. Wilson, I. Rudan, P. Pramstaller, H. Watkins, PROCARDIS Consortium, A. Doering, H.E. Wichmann, KORA Study, T.D. Spector, L. Peltonen, H. Völzke, R. Nagaraja, P. Vollenweider, M. Caulfield, WTCCC, T. Illig, C. Gieger, Meta-analysis of 28,141 individuals identifies common variants within five new loci that influence uric acid concentrations, PLoS Genet. 5 (2009) e1000504.

[13] B.A. Charles, D. Shriner, A. Doumatey, G. Chen, J. Zhou, H. Huang, A. Herbert, N.P. Gerry, M.F. Christman, A. Adeyemo, C.N. Rotimi, A genome-wide association study of serum uric acid in African Americans, BMC Med. Genomics 4 (2011) 17.

[14] Y. Kamatani, K. Matsuda, Y. Okada, M. Kubo, N. Hosono, Y. Daigo, Y. Nakamura, N. Kamatani, Genome-wide association study of hematological and biochemical traits in a Japanese population, Nat. Genet. 42 (2010) 210–215.

[15] The J-MICC Study Group, J-MICC Study Group, the Japan Multi-institutional Collaborative Cohort Study (J-MICC Study) to detect gene–environment interactions for cancer, Asian Pac. J. Cancer Prev. 8 (2007) 317–323.

[16] Y. Asai, M. Naito, M. Suzuki, A. Tomoda, M. Kuwabara, Y. Fukada, A. Okamoto, S. Oishi, K. Ikeda, T. Nakamura, Y. Misu, S. Katase, S. Tokumasu, K. Nishio, Y. Ishida, A. Hishida, E. Morita, S. Kawai, R. Okada, K. Wakai, A. Tamakoshi, N. Hamajima, Baseline data of Shizuoka area in the Japan Multi-institutional Collaborative Cohort Study (J-MICC Study), Nagoya J. Med. Sci. 71 (2009) 137–144.

[17] N. Hamajima, T. Saito, K. Matsuo, K. Kozaki, T. Takahashi, K. Tajima, Polymerase chain reaction with confronting two-pair primers for polymorphism genotyping, Jpn. J. Cancer Res. 91 (2000) 865–868.

[18] N. Hamajima, M. Naito, A. Hishida, R. Okada, Y. Asai, K. Wakai, Serum uric acid distribution according to SLC22A12 W258X genotype in a cross-sectional study of a general Japanese population, BMC Med. Genet. 12 (2011) 33.

[19] R.C. Burack, J.B. Keller, M.W. Higgins, Cardiovascular risk factors and obesity: are baseline levels of blood pressure, glucose, cholesterol and uric acid elevated prior to weight gain? J. Chronic Dis. 38 (1985) 865–872.

[20] T.G. Benedek, Correlations of serum uric acid and lipid concentrations in normal, gouty, and atherosclerotic men, Ann. Intern. Med. 66 (1967) 851–861.

[21] M. Alderman, Uric acid in hypertension and cardiovascular disease, Can. J. Cardiol. 15 (Suppl F) (1999) 20F–22F.

[22] C. Bengtsson, E. Tibblin, Serum uric acid levels in women: an epidemiological survey with special reference to women with high serum uric acid values, Acta Med. Scand. 196 (1974) 93–102.

[23] W. Rathmann, E. Funkhouser, A.R. Dyer, J.M. Roseman, Relations of hyperuricemia with the various components of the insulin resistance syndrome in young black and white adults: the CARDIA study. Coronary Artery Risk Development in Young Adults, Ann. Epidemiol. 8 (1998) 250–261.

[24] M. Nakashima, T. Uematsu, K. Kosuge, M. Kanamaru, Pilot study of the uricosuric effect of DuP-753, a new angiotensin II receptor antagonist, in healthy subjects, Eur. J. Clin. Pharmacol. 42 (1992) 333–335.

[25] J. Golbahar, M.A. Aminzadeh, Q.M. Al-Shboul, S. Kassab, G.R. Rezaian, Association of methylenetetrahydrofolate reductase (C677T) polymorphism with hyperuricemia, Nutr. Metab. Cardiovasc. 17 (2007) 462–467.

[26] S. Itou, Y. Goto, K. Suzuki, S. Kawai, M. Naito, Y. Ito, N. Hamajima, Significant association between methylentetrahydrofolate reductase 677T allele and hyper-uricemia among adult Japanese subjects, Nutr. Res. 29 (2009) 710–715.

[27] Y. Tobara, K. Kohara, R. Kawamoto, Y. Hiura, K. Nishimura, T. Morisaki, Y. Kokubo, T. Okamura, H. Tomoike, N. Iwai, T. Miki, Association of four genetic loci with uric acid levels and reduced renal function: the J-SHIPP Suita Study, Am. J. Nephrol. 32 (2010) 279–286.

[28] A. Brandstätter, S. Kiechl, B. Kollerits, S.C. Hunt, S. Coassin, J. Willeit, T.D. Adams, T. Illig, P.N. Hopkins, F. Kronenberg, Sex-specific association of the putative fructose transporter SLC2A9 variants with uric acid levels is modified by BMI, Diabetes Care 31 (2008) 1662–1667.

[29] A. Brandstätter, C. Lamina, S. Kiechl, S.C. Hunt, S. Coassin, B. Paulweber, F. Kramer, M. Summerer, J. Willeit, L. Kedenko, T.D. Adams, F. Kronenberg, Sex and age interaction with genetic association of atherogenic uric acid concentrations, Atherosclerosis 210 (2010) 474–478.

[30] A. Döring, C. Gieger, D. Mehta, H. Gohlke, H. Prokisch, S. Coassin, G. Fischer, K. Henke, N. Klopp, F. Kronenberg, B. Paulweber, A. Pfeufer, D. Rosskopf, H. Völzke, T. Illig, T. Meitinger, H.E. Wichmann, C. Meisinger, SLC2C9 influences uric acid con-centrations with pronounced sex-specific effects, Nat. Genet. 40 (2008) 430–436.

[31] A.E. Hak, H.K. Choi, Menopause, postmenopausal hormone use and serum uric acid levels in US women — the Third National Health and Nutrition Examination Survey, Arthritis Res. Ther. 10 (2008) R116.
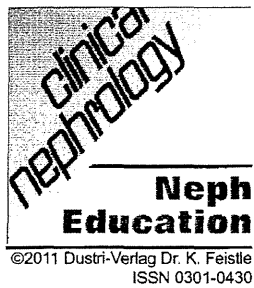
Two cases of nephrotic syndrome (NS)-induced acute kidney injury (AKI) associated with renal hypouricemia

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Abstract. Renal hypouricemia is a clinical disorder attributed to an increased renal urate excretion rate and is well known to involve a high risk of urolithiasis and exercise-induced acute kidney injury (AKI). This report concerns two interesting cases of nephrotic syndrome (NS)-induced AKI associated with renal hypouricemia. A 64-year-old female (Case 1) and a 37-year-old male (Case 2) were hospitalized because of AKI (serum creatinine: 2.07 mg/dl and 3.3 mg/dl, respectively), oliguria and NS. They were treated with prednisolone and temporary hemodialysis. Renal function improved, but hypouricemia persisted during hospitalization. Histological findings in both cases led to a diagnosis of minimal change nephrotic syndrome and identification of the diuretic phase of tubulointerstitial damage because of findings such as acute tubular necrosis. Furthermore, distal tubules of Case 2 showed an amorphous mass, possibly a uric acid crystal. Analysis of the two cases with the URAT1 gene, encoded by SLC22A12, found a homozygous mutation in exon 4 (W258stop) of each one. Our cases show that patients with renal hypouricemia may be susceptible to AKI without involvement of exercise if they possess some facilitators. Renal hypouricemic patients should therefore be carefully examined for all complications from renal hypouricemia because of high risk of AKI.

Introduction

Renal hypouricemia is a clinical disorder attributed to an increased renal excretion rate of urate. Although hypouricemia is mostly asymptomatic, it is well known to involve a high risk of urolithiasis and exercise-induced acute kidney injury (AKI) [1].

Some studies have dealt with the association of exercise-induced AKI with renal hypouricemia [2, 3], but no studies appear to have dealt with cases complicated with nephrotic syndrome (NS) and AKI associated with renal hypouricemia. Here we report two interesting cases of NS-induced AKI associated with renal hypouricemia.

Case reports

Case 1

A 64-year-old female experienced leg and facial edema in late May 2005. She had been treated with nonsteroidal antiinflammatory drugs (NSAIDs) because of lumbago since she was 30. When she visited a local clinic on June 1, her body weight had increased 3 kg. Laboratory findings showed a slight elevation of serum creatinine (s-Cr: 1.08 mg/dl). Two days later she visited the same clinic again and was diagnosed with AKI and NS because of elevated s-Cr (2.07 mg/dl), and diminished total protein (TP: 5.2 g/dl) and albumin (Alb: 2.3 g/dl), and proteinuria (3+). She was admitted to a general hospital on June 3.

After admission, she showed oliguria (urine volume: 186 ml/day), and general fatigue. Because her s-Cr level was elevated even more, she underwent hemodialysis (HD) for 3 days. Four days after admission, she was transferred to Kobe University Hospital on June 7.

On admission, her arterial blood pressure was 110/60 mmHg and pulse rate 72 beats/min.
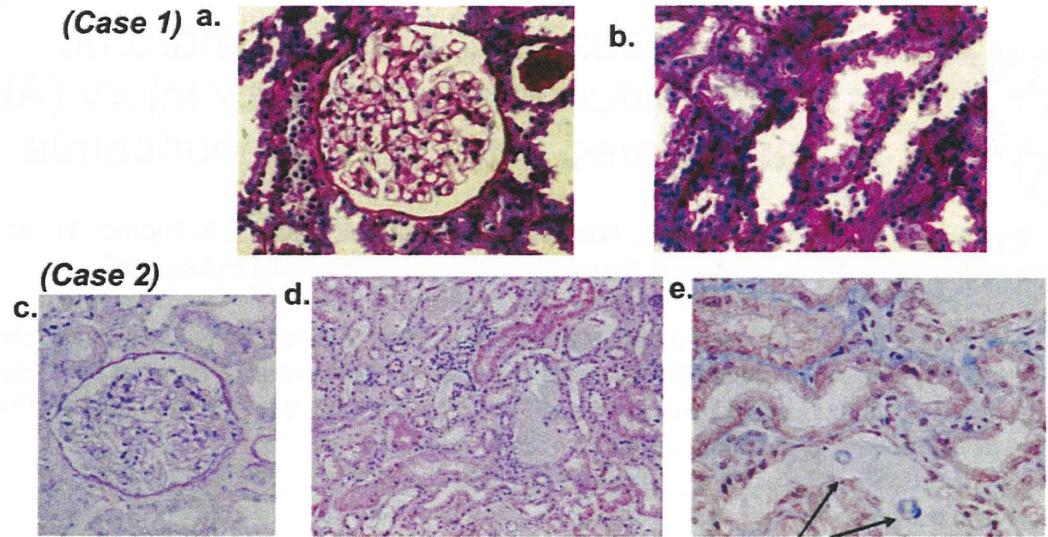
Figure 1. Renal pathology. a: Minor glomerular abnormalities (Periodic acid-Shiff (PAS) staining, × 400). b: Proximal and distal tubular cells had partial increase of nuclear density and swelling of nuclei. Tubular regenerative change of epithelial cells was focally observed. (PAS staining, × 400). c: Minor glomerular abnormalities (PAS staining, × 400). d: Vacuolization, desquamation, and regenerative changes of tubular epithelial cells was observed. Tubular regenerative changes in the proximal tubular epithelial cells, which indicate moderate patchy tubular injury was observed. (PAS staining, × 400). e: Crystalloid body (Urate salt?) was partly observed within hyaline cast(↑). (Masson Tricrome staining, × 400).

She weighed 51.3 kg and was 153 cm tall. She showed pitting edema in the pretibial area.

Laboratory evaluation showed hemoglobin (Hb) of 10.9 g/dl (109 g/l); platelet count (Plt): $230 \times 10^3/\mu l (230 \times 10^9/l)$; white blood cells (WBC): $6.5 \times 10^3/\mu l (6.5 \times 10^9/l)$; blood urea nitrogen (BUN): 21 mg/dl (7.5 mmol/l); S-Cr: 3.18 mg/dl (281.1 μmol/l); uric acid (UA): 4.0 mg/dl (normal: 3.8 ~ 5.1 mg/dl) (238 μmol/l); TP: 4.6 g/dl (46 g/l); Alb 1.7 g/dl (17 g/l); creatine kinase (CK): 132 U/l; total-cho 384 mg/dl. Urinalysis found protein 4+ (5.4 g/day), occult blood –, sediments; RBC 1 ~ 4/HPF, WBC 0 ~ 1/HPF, granular cast +, waxy cast +. Abdominal computed tomography (CT) scan, chest X-ray and electrocardiogram all appeared normal.

Renal pathology

Kidney biopsy was performed on July 20 (Day 41 of steroid therapy).

Light microscopy with Periodic Acid-Shiff (PAS) staining showed 23 glomeruli without any remarkable changes. Focal tubulointerstitial damage was observed. Proximal and distal tubular cells showed partial increase in nuclear density and swelling of nuclei, while tubular regenerative change of epithelial cells was focal (Figure 1).

Immunofluorescence demonstrated no staining for immunoglobulins complements.

Electron microscopy demonstrated mild foot process effacement. There were no electron dense deposits.

Our diagnosis of minimal change nephrotic syndrome (MCNS) was based on evidence of NS, minimal glomerular abnormalities, and a good response to steroid therapy. Renal biopsy was indicated for the diuretic phase of tubulo-interstitial damage such as acute tubular necrosis (ATN).

Clinical course

Because the clinical history indicated suspected NS-induced AKI, treatment with prednisolone (PSL) 50 mg/day was initiated after steroid pulse (methylprednisolone 1 g/day × 3 days). However, because oliguria and renal dysfunction continued (June 13: BUN 70 mg/dl; Cr 7.25 mg/dl; UA 6.8 mg/dl), the patient underwent HD 5 times in total.

Renal function improved to creatinine clearance (CCr) of 77.7 ml/min at hospital discharge, and proteinuria, which was 5.4 g/day, disappeared after steroid therapy.

Hypouricemia persisted during hospitalization (UA: 0.6 ~ 1.0 mg/dl), although a kid-

ney biopsy showed only slight tubular damage and the patient was already in recovery. Moreover, fractional excretion of uric acid (FEUA) ranged from 61.3 to 100% (normal: < 10%), suggesting an increase in the renal excretion rate of urate and renal hypouricemia. In consideration of the effect of AKI, probenecid and pyrazinamide loading tests were performed one year after discharge. Probenecid caused FEUA to increase from 61.3% to 66.4%, while pyrazinamide did not reduce FEUA to less than 53.0%. These test findings prompted us to classify this case as presecretory reabsorption defect of UA.

Figure 2. Mutation analysis of SLC22A12.

Case 2

A 37-year-old male experienced leg edema in late February 1999. After a checkup in April found proteinuria (4+), he presented with oliguria on May 10 and his body weight increased about 9 kg. When he visited the Jikei University Aoto hospital, laboratory findings showed elevation of s-Cr (3.3 mg/dl) and BUN (87.2 mg/dl). He was diagnosed with AKI and NS, and was hospitalized on May 15.

On admission, arterial blood pressure was 132/76 mmHg and pulse rate 76 beats/min. He weighed 91.2 kg and was 171.8 cm tall. He presented with general pitting edema.

Laboratory evaluation showed Hb of 15.3 g/dl (153 g/l); Plt 328 × 10^3/μl (328 × 10^9/l); WBC 9.1 × 10^3/μl (9.1 × 10^9/l); blood BUN 87.2 mg/dl (31.13 mmol/l); S-Cr 3.3 mg/dl (291.72 μmol/l); UA 3.3 mg/dl (196.3 μmol/l); TP 4.0 g/dl (40 g/l); Alb 2.1 g/dl (21 g/l); CK 98 U/l. Urinalysis found protein 4+ (17.38 g/day) and occult blood 2+, sediments; RBC 5 ~ 10/HPF, WBC 10 ~ 19/HPF, granular cast 3+, waxy cast +, fatty cast +. Abdominal CT scan, chest X-ray and electrocardiogram all appeared normal.

Renal pathology

Kidney biopsy was performed on July 1 (Day 47 of steroid therapy).

Light microscopy with PAS staining showed global sclerosis in 2 of 18 glomeruli with minimal abnormalities.
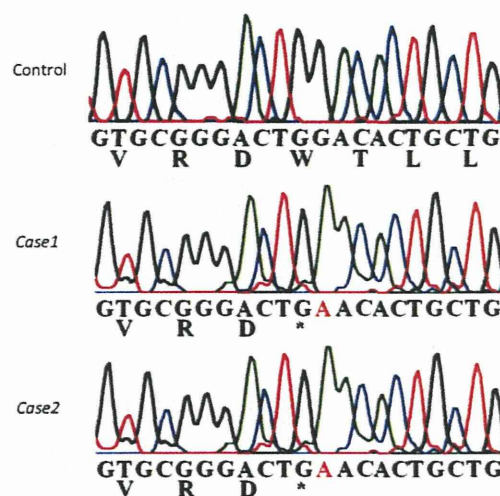
Tubular regenerative changes were also observed in the proximal tubular cells, indicating patchy tubular injury. Furthermore, distal tubules were filled with hyaline casts which had in part escaped to the interstitium. Crystalloid bodies (Urate salt?) were found in part of the hyaline casts, suggesting that obstructive nephropathy might be involved (Figure 1).

Immunofluorescence demonstrated mild insudative deposits of IgM and C1q in glomerular mesangial areas.

The diagnosis of MCNS was evident. Renal biopsy was indicated for the recovery phase of tubulo-interstitial damage such as acute tubular necrosis.

Clinical course

Because the clinical history indicated suspected NS-induced AKI, treatment with PSL 50 mg/day was initiated. But because oliguria and renal dysfunction continued (May 27: BUN 131 mg/dl; Cr 4.7 mg/dl; UA 5.3 mg/dl), HD was administered 10 times in total.

Renal function improved to CCr 64.5 ml/min, and proteinuria decreased from 17.38 g/day (urinary quantity: 500 ml/day) to 2.86 g/day at discharge.

Hypouricemia persisted during hospitalization (UA: 0.8 ~ 1.1 mg/dl) and FEUA was 64%, suggesting an increase in the renal excretion rate of urate. Probenecid and pyrazinamide loading were not administered.

Two cases sequence analysis of the URAT1 Gene

Mutation in URAT1 is known as a cause of renal hypouricemia [4]. We analyzed two cases with the URAT1, encoded by SLC22A12 and reported by Ichida et al [1], and found a homozygous mutation in exon 4 (W258stop) in each one (Figure 2).

Discussion

Renal hypouricemia is a common hereditary disease in Japanese and possibly in the non-Ashkenazi Jewish ethnic group [4]. It is accompanied by urolithiasis and exercise-induced AKI in 10% of patients [1, 2]. In 2002, Ishikawa reviewed exercise-induced AKI including 49 reported cases of exercise-induced AKI with renal hypouricemia and clarified the characteristics [5]. In the same year, Enomoto et al. identified a urate transporter, URAT1, responsible for renal hypouricemia [6].

Renal hypouricemia leads to low antioxidant capability because of less UA, a scavenger of free radicals. Exercise-induced AKI with renal hypouricemia is suggested to result from increased free radicals caused by exercise, which induces renal reperfusion injury due to renal vasoconstriction.

Interestingly, our cases had no exercise before the manifestation of AKI. We do not know whether renal hypouricemia plays a causal role in the development of NS (MCNS)-induced AKI, because there have been no reports on cases like ours.

Recent reports note about the importance of reactive oxygen species (ROS) in kidney disease [7, 8, 9]. ROS have been considered to have roles in the pathogenesis of glomerular disease such as NS, postischemic or toxic AKI. Cellular defense mechanisms against ROS include enzyme systems that directly remove some species (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic scavengers that are either endogenous molecules (albumin, glutathione, and UA) or derived from the diet (vitamin C, vitamin E, carotenoids, selenium and zinc) [10].

This accounts for the increase in oxidative stress in renal hypouricemia patients with NS because of hypoalbuminemia and low intake of certain antioxidant components due to appetite loss resulting from NS. These results indicate that renal hypouricemia with NS is likely to lead to kidney injury under increased oxidative stress even without exercise that exceeds the antioxidant capacity of the human body. It is therefore a matter of concern that our cases with NS and renal hypouricemia also had high oxidative stress, since this can lead to AKI just as exercise can.

As for the frequency of cases needing dialysis due to AKI with MCNS, Shibasaki et al. in 1990 [11] reported that 12 patients showed AKI followed by primary NS among 420 cases, and 3 cases (2 cases of MCNS, 1 case of FGS) needed continuous and transient dialysis. In addition, Kawai et al. in 1994 [12] reported a case of renal hypouricemia with NS and idiopatic hypercalcemia, but their case did not lead to AKI. Our cases can be considered rare in the sense that the incidence of AKI due to MCNS and renal hypouricemia needed transient HD.

Furthermore, Case 1 had been taking NSAIDs. According to the review by Ishikawa [5], about 37.9% of patients with exercise-induced AKI had taken analgesics including NSAIDs. Therefore, NSAIDs might facilitate exercise-induced AKI.

The renal biopsy of Case 2 showed an amorphous mass, possibly a UA crystal. Renal hypouricemia is well known to lead to urolithiasis, and renal tubular obstruction with UA crystals resulting from explosive UA loading to tubules is another causative candidate.

In our cases AKI was induced not only by the reduction in effective circulatory volume related to MCNS and administration of NSAIDs but also by renal hypouricemia, which features obstruction with UA and antioxidant fragility, and also oxidative stress related to NS.

When a patient suffering from AKI with renal hypouricemia is first seen, it is generally difficult to diagnose renal hypouricemia because of the increasing level of UA. Since Case 1 was admitted after two dialysis sessions, it is difficult to determine whether the level of UA was low or high. The UA level of Case 2 was within normal range, but could be low in view of AKI. For this reason, careful attention should be paid to any signs of hypouricemia during the recovery phase of AKI.

Renal involvement with renal hypouricemia is focused on exercised-induced AKI

only. But our cases show that patients with renal hypouricemia may be susceptible to AKI without involvement of exercise if they possess some facilitators. Renal hypouricemic patients with NS as in our cases should therefore be carefully examined in consideration of the possibility of causing AKI depending on antioxidant stress.

References

[1] Ichida K, Hosoyamada M, Hisatome I et al. Clinical and molecular analysis of patients with renal hypouricemia in Japan-influence of URAT1 gene on urinary urate excretion. J Am Soc Nephrol. 2004; 15: 164-173.

[2] Erley ChMM, Hirschberg RR, Hoefer W, Schaefer K. Acute renal failure due to uric acid nephropathy in a patient with renal hypouricemia. Klin Wochenschr. 1989; 67: 308-312.

[3] Ohta T, Sakano T, Igarashi T, Itami N, Ogawa T, and members of the ARF Associated with Renal Hypouricemia Research Group. Exercise-induced acute renal failure associated with renal hypouricaemia: results of a questionnaire-based survey in Japan. Nephrol Dial Transplant. 2004; 19: 1447-1453.

[4] Ichida K, Hosoyamada M, Kamitsuji S et al. Age and origin of the G774A mutation in SLC22A12 causing renal hypouricemia in Japanese. Clin Genet. 2008; 74: 243-251.

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

[6] Enomoto A, Kimura H, Chairoungdua A et al. Molecular identification of a renal urate anion exchanger that regulates blood urate levels. Nature. 2002; 417: 447-452.

[7] Clemens MR, Burza-Zanetti Z. Lipid abnormalities and peroxidation of erythrocytes in nephritic syndrome. Nephron. 1989; 53: 325-329.

[8] Ginevri F, Ghiggeri GM, Candiano G, Oleggini R, Bertelli R. Peroxidative damage of erythrocyte membrane in children with nephrotic syndrome. Pediatr Nephrol. 1989; 3: 25-32.

[9] Warwick G, Waller H, Ferns G. Antioxidant vitamin concentrations and LDL oxidation in nephrotic syndrome. Ann Clin Biochem. 2000; 37: 488-491.

[10] Bakr A, Abul Hassan S, Shoker M, Zaki M, Hassan R. Oxidant stress in primary nephritic syndrome: dose it modulate the response to corticosteroids? Pediatr Nephrol. 2009; 24: 2375-2380.

[11] Shibasaki T, Nakano H, Kanai T et al. Characterization of acute renal failure due to primary nephrotic syndrome. Japanese J Nephrol. 1991; 33: 91-95.

[12] Kawai S, Yamada M, Osawa G. Renal hypouricemia: Incomplete combined defect. Japanese J Nephrol. 1994; 36: 955-961.

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DIAGNOSTIC TESTS FOR PRIMARY RENAL HYPOURICEMIA

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□   *Primary renal hypouricemia is a genetic disorder characterized by defective renal uric acid (UA) reabsorption with complications such as nephrolithiasis and exercise-induced acute renal failure. The known causes are: defects in the SLC22A12 gene, encoding the human urate transporter 1 (hURAT1), and also impairment of voltage urate transporter (URATv1), encoded by SLC2A9 (GLUT9) gene. Diagnosis is based on hypouricemia (<119 μmol/L) and increased fractional excretion of UA (>10%). To date, the cases with mutations in hURAT1 gene have been reported in East Asia only. More than 100 Japanese patients have been described. Hypouricemia is sometimes overlooked; therefore, we have set up the flowchart for this disorder. The patients were selected for molecular analysis from 620 Czech hypouricemic patients. Secondary causes of hyperuricosuric hypouricemia were excluded. The estimations of (1) serum UA, (2) excretion fraction of UA, and (3) analysis of hURAT1 and URATv1 genes follow. Three transitions and one deletion (four times) in SLC22A12 gene and one nucleotide insertion in SLC2A9 gene in seven Czech patients were found. Three patients had acute renal failure and urate nephrolithiasis. In addition, five nonsynonymous sequence variants and three nonsynonymous sequence variants in SLC2A9 gene were found in two UK patients suffering from acute renal failure. Our finding of the defects in SLC22A12 and SLC2A9 genes gives further evidence of the causative genes of primary renal hypouricemia and supports their important role in regulation of serum urate levels in humans.*

INTRODUCTION

Primary hereditary renal hypouricemia (OMIM: 220150) is an inborn error of renal membrane transport of uric acid, with an autosomal recessive mode of inheritance.[1] This genetic disorder is characterized by defective renal uric acid reabsorption. The known major causes are defects in the *SLC22A12* gene,[2] encoding the human urate transporter 1 (hURAT1), and also impairment of the voltage urate transporter (URATv1), encoded by *SLC2A9* (GLUT9) gene.[3] The hURAT1 mainly acts as an influx transporter for urate at apical membrane at the proximal renal tubule.[2] URATv1 (GLUT9) is an efflux transporter of intracellular urate from the tubular cell to the interstitium/blood space.[3] The majority of patients are asymptomatic, but some may experience urolithiasis and/or be predisposed to exercise-induced acute renal failure. Some patients developed hematuria only. Diagnosis is based on hypouricemia (<119 μmol/L) and an increased fractional excretion of UA (>10%). Confirmation of the diagnosis is accomplished by molecular analysis of the *SCL22A12* and *SLC2A9* genes. Therapy is based on alkalization of urine, drinking of plenty of water, and avoidance of strenuous exercise.[2–4] To date, the cases with mutations in *hURAT1* gene have been reported only in East Asia, with more than 100 Japanese patients described.[5,6]

Although the measurement of uric acid in serum and urine is a useful diagnostic tool for many inborn errors of purine metabolism, asymptomatic hypouricemia is sometimes overlooked. We therefore suggest a flowchart for the diagnosis of primary hereditary renal hypouricemia.

MATERIALS AND METHODS

The patients (with hypouricemia and increased fractional excretion of uric acid) were selected for molecular analysis of *SLC22A12* and *SLC2A9* genes from 620 Czech hypouricemic patients. Those hypouricemic individuals were found from 3600 blood and urine samples. Serum and urinary uric acid and creatinine were determined. Uric acid in serum and urine was measured by a specific enzymatic method. Creatinine in plasma and urine was measured by the Jaffé reaction adapted for an autoanalyzer. The fraction of uric acid excreted was evaluated as uric acid clearance factored by creatinine clearance × 100. Sequence analysis by automated DNA sequencer (Applied Biosystems 3100) of the coding region of *SLC22A12* and *SLC2A9* genes was performed after informed consent.
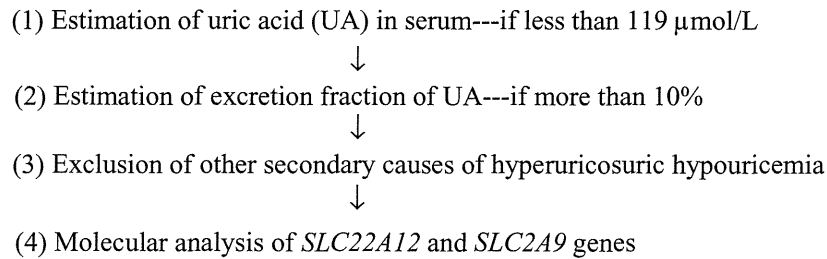
(1) Estimation of uric acid (UA) in serum—if less than 119 µmol/L

↓

(2) Estimation of excretion fraction of UA—if more than 10%

↓

(3) Exclusion of other secondary causes of hyperuricosuric hypouricemia

↓

(4) Molecular analysis of *SLC22A12* and *SLC2A9* genes

FIGURE 1 Protocol for the diagnosis of hereditary renal hypouricemia.

RESULTS

Figure 1 shows our established protocol for the diagnosis of primary hereditary renal hypouricemia. The first step is measurement of uric acid in serum. When a patient with hypouricemia is identified, estimation of the fraction excretion of uric acid follows. The exclusion of secondary causes of hyperuricosuric hypouricemia (such as Wilson disease and Fanconi syndrome, etc.) is important before molecular analysis of the *SLC22A12* and *SLC2A9* genes.

Using this flowchart, we were able to diagnose eight new patients with hypouricemia and an increased fractional excretion of uric acid due to primary hereditary renal hypouricemia (Table 1). All the patients fulfilled the criteria for idiopathic renal hypouricemia, which consist of (1) hypouricemia (serum uric acid concentration of less than 119 µmol/L), (2) fractional excretion of uric acid more than 10%, and (3) no other secondary causes of hyperuricosuric hypouricemia such as Fanconi syndrome, Wilson disease, or drug-induced tubulopathy were found.

We have found three transitions and deletion in the *SLC22A12* gene. One nucleotide insertion in the *SLC2A9* gene was found.[7] Three patients

TABLE 1 Clinical features and mutations (patients 1–6 in *SLC22A12* gene and patients 7–8 in *SLC2A9* gene)

Case	Sex	Age (yrs)	UA (µmol/L)	FEUA (%)	ARF	Uro-lithiasis	Mutation
1.	F	73	124	52.4	+	−	g. 82948302del
2.	F	39	58	53.4	+	−	g. 82948302del/g.9184C/T
3.	F	53	78	60.3	−	−	g. 82948302del/g.9184C/T
4.	M	35	63	43.0	−	−	g. 8145G/Cg.9214G/A
5.	F	15	35	55.2	−	−	g. 8294-8302del g.9184C/T
6.	M	5	95	52.6	−	+	1242-1250delGCTGGCAGG
7.	F	18	11	240.0	−	−	g. 43412_43413insC
8.	M	23	10	220.0	−	−	g. 43412_43413insC

UA—serum uric acid; FEUA—fractional excretion of uric acid; ARF—exercise-induced acute renal failure.

had acute renal failure and urate nephrolithiasis. In addition, five nonsynonymous sequence variants and three nonsynonymous sequence variants in *SLC2A9* gene were found in two UK patients suffering from acute renal failure.

DISCUSSION

The molecular basis of urate transport in kidney was uncertain until 2002, when Enomoto et al. discovered hURAT1, which is encoded by *SLC22A12* gene and regulates blood urate levels.[2] Since that time, more than 100 cases with hereditary renal hypouricemia due to mutations in the *SCL22A12* gene have been identified in Japan,[4–6] and this number is unique worldwide. It was found that W258X nonsense mutation is the major cause of renal hypouricemia in Japanese patients.[6]

We have performed mutational analysis of the *SLC22A12* and *SLC2A9* genes in non-Asian patients. Hypouricemia with increased fractional excretion of uric acid is consistent with the findings previously reported[4–6] and this finding confirms the causative role of these genes on primary hereditary renal hypouricemia. There is a growing interest in understanding the genetic determinants of urate homeostasis in view of the fact that recent clinical and epidemiological studies have found that soluble uric acid has an important biological role.[8] Hyperuricemia may be a primary risk factor for several common disorders, including metabolic syndrome, cardiovascular disease, hypertension, and kidney disease.[9,10] Our study suggests that primary hereditary renal hypouricemia is not restricted to East Asian populations, as previously thought. As hypouricemia itself does not induce any symptoms, hereditary renal hypouricemia is sometimes overlooked. Our experience in detection of other inborn errors of metabolism with hypouricemia shows that every finding of persistent hypouricemia needs further detailed purine metabolic investigations in specialized biochemical-genetic laboratories in order to identify different types of hereditary xanthinuria, other causes of primary hypouricemia (such as purine nucleosidase deficiency), and to exclude secondary causes of hypouricemia. These include conditions with increased renal uric acid excretion in conjunction with isolated or generalized tubular defects (Fanconi syndrome, Wilson disease, cystinosis, heavy metal poisoning) or conditions with decreased uric acid synthesis (severe liver disease) or medication with uricosuric agents (salicylates >2 g/day).

In conclusion, our finding of genetic defects in *SCL22A12* and *SLC2A9* provides further evidence of the causative genes of primary renal hypouricemia and supports their important role in the regulation of serum urate levels in humans. Hereditary renal hypouricemia is still an unrecognized condition with a significant incidence in European populations.

REFERENCES

1. Sperling, O. Hereditary renal hypouricemia. *Mol. Genet. Metab.* **2006**, 89, 14–18.
2. Enomoto, A.; Kimura, H.; Chairoungdua, A.; Shigeta, Y.; Jutabha, P.; Cha, S.H.; 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* **2002**, 417, 447–452.
3. Vitart, V.; Rudan, I.; Hayward, C.; Gray, N.K.; Floyd, J.; Palmer, C.N.; Knott, S.A.; Kolcic, I.; Polasek, O.; Graessler, J.; Wilson, J.F.; Marinaki, A.; Riches, P.L.; Shu, X.; Janicijevic, B.; Smolej-Narancic, N.; Gorgoni, B.; Morgan, J.; Campbell, S.; Biloglav, Z.; Barac-Lauc, L.; Pericic, M.; Klaric, I.M.; Zgaga, L.; Skaric-Juric, T.; Wild, S.H.; Richardson, W.A.; Hohenstein, P.; Kimber, C.H.; Tenesa, A.; Donnelly, L.A.; Fairbanks, L.D.; Aringer, M.; McKeigue, P.M.; Ralson, S.H.; Morris, A.D.; Rudan, P.; Hastie, N. D.; Campbell, H.; Wright, A.F. SLC2A9 is a newly identified urate transporter influencing serum concentration, urate excretion and gout. *Nat. Genet.* **2008**, 40, 437–442.
4. Ichida, K.; Hosoyamada, M.; Hisatome, I.; Enomoto, A.; Hikita, M.; Endou, H.; Hosoya, T. Clinical and molecular analysis of patients with renal hypouricemia in Japan–influence of URAT1 gene on urinary urate excretion. *J. Am. Soc. Nephrol.* **2004**, 15, 164–173.
5. Komatsuda, A.; Iwamoto, K.; Wakui, H.; Sawada, K.; Yamaguchi, A. Analysis of mutation in the urate transporter 1 (URAT1) gene of Japanese patients with hypouricemia in northern Japan and review of the literature. *Ren. Fail.* **2006**, 28, 223–227.
6. Komoda, F.; Sekine, T.; Inatomi, J.; Enomoto, A.; Endou, H.; Ota, T.; Matsuyama, T., Ogata, T.; Ikeda, M.; Awazu, M.; Muroya, K.; Kamimaki, I.; Igarashi, T. The W258X mutation in SLC22A12 is the predominant cause of Japanese renal hypouricemia. *Pediatr. Nephrol.* **2004**, 19, 728–733.
7. Stiburkova, B.; Ichida, K.; Sebesta, I. Novel homozygous insertion in SLC2A9 gene caused renal hypouricemia. *Mol. Genet. Metab.* **2011**, 102, 430–435.
8. Ichida, K. What lies behind serum urate concentration? Insights from genetic and genomic studies. *Genome Med.* **2009**, 1, 118.1–118.10.
9. Johnson, R.J.; Titte, S.; Cade, J.R.; Rideout, B.A.; Oliver, W.J. Uric acid, evolution and primitive cultures. *Semin. Nephrol.* **2005**, 25, 3–8.
10. Bos, M.J.; Koudstaal, P.J.; Hofman, A.; Witteman, J.C.; Breteler, M.M. Uric acid is a risk factor for myocardial infarction and stroke: the Rotterdam study. *Stroke* **2006**, 37, 1503–1507.

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STABLE ISOTOPE DILUTION MASS SPECTROMETRIC ASSAY FOR PRPP USING ENZYMATIC PROCEDURES

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☐ *5-Phosphoribosyl-1-pyrophosphate (PRPP) is an important regulator of de novo purine synthesis. A method for the measurement of PRPP in erythrocytes was designed, which is based on the determination of [$^{13}C_5$]glutamate derived from [$^{13}C_5$]glutamine following the utilization of PRPP by the action of amidophosphoribosyltransferase. The present study describes a gas chromatographic-mass spectrometric method for determination of [$^{13}C_5$]glutamate using [$^{13}C_2$]glutamate as an internal standard. The methods involved purification by anion-exchange chromatography using a BondElut SAX and derivatization with isobutyl chlorocarbonate in water-methanol-pyridine. Quantitation was performed by selected ion monitoring of the protonated molecular ions in the chemical ionization mode. The intra-day reproducibility in the amounts of [$^{13}C_5$]glutamate determined was in good agreement with the actual amounts added in erythrocytes. A linear relationship was found between the amount of PRPP added and the amount of [$^{13}C_5$]glutamate formed from [$^{13}C_5$]glutamine using amidophosphoribosyltransferase.*

Keywords PRPP; amidophosphoribosyltransferase; GC-MS; glutamate; stable isotope

INTRODUCTION

5-Phosphoribosyl-1-pyrophosphate (PRPP) is a substrate common to several metabolic pathways including the biosynthesis of purine and pyrimidine nucleotides and the salvage pathway for purines. PRPP also serves as both a substrate and an activator of amidophosphoribosyltransferase (ATase), the first and presumed rate-determining enzyme of de novo purine synthesis. Uric acid is the end product of purine metabolism in humans. Hyperuricemia is a risk factor for gout and results from either overproduction or renal underexcretion of uric acid. Elevated intracellular PRPP is the driving

force for acceleration of uric acid synthesis in two rare inherited enzyme defects resulting in overproduction hyperuricemia, hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency, and PRPP synthetase (PRS) overactivity.[1,2] Elevation of PRPP is a basis for idiopathic overproduction hyperuricemia, but there is no study about the intracellular concentration of PRPP in the patients with idiopathic overproduction hyperuricemia. Erythrocyte PRPP concentrations are markedly increased in two rare inherited enzyme abnormalities, HPRT deficiency[3] and PRS overactivity,[4] resulting in overproduction hyperuricemia. The measurement of erythrocyte PRPP concentrations is, therefore, an important screening procedure in the investigation of patients with idiopathic hyperuricemia.

A number of radioenzymatic assays have been described for the measurement of PRPP in biological samples.[5–8] The basic approach utilizes the conversion of PRPP to a radioactive derivative through its reaction with a ^3H or ^{14}C-labeled base under catalysis by the appropriate phosphoribosyltransferase. The methods have involved trapping of evolved $^{14}CO_2$ or separation of radioactive products by various chromatographic techniques. The present study was carried out to develop a gas chromatography-mass spectrometry (GC-MS) method for determination of PRPP in erythrocytes using ATase and stable-isotopically labeled substrate. We used $[^{13}C_5]$glutamine ($[^{13}C_5]$Gln) as the substrate and measured the enzyme product $[^{13}C_5]$glutamate ($[^{13}C_5]$Glu) by GC-MS using $[^{13}C_2]$glutamate ($[^{13}C_2]$Glu) as the internal standard.

$$[^{13}C_5]Gln + PRPP \xrightarrow[Mg^+]{ATase} [^{13}C_5]Glu + phosphoribosylamine + PPi$$
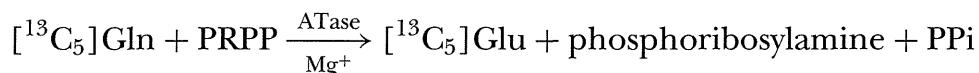
MATERIALS AND METHODS

Chemical and Reagent

Glutamine (Gln), glutamate (Glu), and isobutyl chloroformate were purchased from Wako (Osaka, Japan). $[^{13}C_5]$Gln (98 atom%^{13}C) and $[^{13}C_5]$Glu (98 atom%^{13}C) were purchased from Isotec (Miamisburg, OH, USA). $[^{13}C_2]$Glu (99 atom%^{13}C) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). PRPP sodium salt was purchased from Sigma (St. Louis, MO, USA). A strong anion-exchange solid-phase extraction column BondElut SAX (1 mL/100 mg) was purchased from Varian (Harbor City, OH, USA), and 10% hydrogen chloride in methanol (10% HCl/methanol) was purchased from the Tokyo Chemical Industry (Tokyo, Japan). ATase was prepared as described previously.[9] All other chemicals and solvents were of an analytical grade.

Gas Chromatography-Mass Spectrometry With Selected Ion Monitoring (GC-MS-SIM)

GC-MS-SIM measurements were made with a Shimadzu (Kyoto, Japan) QP-2000 quadrupole gas chromatograph-mass spectrometer. A methylsilicone bonded-phase fused-silica capillary column SPB-1 (10 m × 0.25 mm I.D.) with 0.25 μm film thickness (Supelco, Bellefonte, PA, USA) was connected directly to the ion source. Helium was used as the carrier gas. A split-splitless injection system was used with a septum purge flow-rate of 3.0 mL/minute. The initial column temperature was set at 120°C. After the sample injection, it was maintained for 2 minutes and was increased at 20°C/minute to 220°C and maintained there for 1 minute. The mass spectrometer was operated in a chemical ionization (CI) mode with isobutene as the reagent gas. The ion source temperature was 280°C. SIM was performed on the protonated molecular ions at m/z 276, 278, 281 for the N(O)-isobutoxycarbonyl methyl ester (iBCME) derivatives of Glu, [$^{13}C_2$]Glu, and [$^{13}C_5$]Glu, respectively.

Sample Preparation for GC-MS-SIM

Venous blood samples were obtained in heparinized tubes from a healthy volunteer. Erythrocytes were isolated by centrifuging at 900 g for 15 minutes at 4°C and washed twice with two volumes of ice-cold saline and then packed by centrifuging at 6000 g for 10 minutes at 4°C. Lysates were obtained by freezing and thawing the red blood cells twice. The lysates were stored at −80°C until analysis. The frozen lysates were defrosted in a 4°C water bath. To a polypropylene tube (75 × 15 mm ID) were added 0.05 mL of lysate and 0.2 mL of 50 mM Tris-HCl buffer (pH 7.4). After heating for 45 seconds in boiling water, the sample was immediately chilled on ice. Following the addition of [$^{13}C_2$]Glu (5.10 nmol/0.05 mL) as the internal standard, the sample was deproteinized with 2 mL of methanol. After centrifugation at 900 g for 5 minutes, the supernatant was applied to a BondElut SAX cartridge, which was prewashed and activated with 1 mL of methanol, 1 mL of water, 2 mL of 1M NaOH, 4 mL of water, 2 mL of acetic acid, and 4 mL of water. The cartridge was washed with 1 mL of 80% methanol/water, and then the glutamate species were eluted with 0.5 mL of 10% HCl/methanol. After removal of the solvent under a stream of nitrogen at 40°C, the residue was dissolved in 0.5 mL of a mixture of water-methanol-pyridine (30:16:4, v/v), and 0.02 mL of isobutyl chloroformate was added. The mixture was shaken for 10 seconds on a vortex mixer. The sample was extracted with 1 mL of chloroform. After evaporating to dryness under a stream of nitrogen, the residue was dissolved in 0.05 mL of ethyl acetate. A volume of 0.2–1.0 μL of the solution was subjected to GC-MS-SIM.

Calibration Curves and Quantitation

To each of a series of standards containing known amounts of Glu (0.51–51.02 nmol) and [$^{13}C_5$]Glu (0.05–5.10 nmol), 5.10 nmol of [$^{13}C_2$]Glu was added as the internal standard. Each sample was prepared in triplicate. The samples were derivatized and analyzed as described above. After correcting the peak-area values using the values of relative contributions by the equations described previously,[10] the peak-area ratios (*m/z* 276 versus *m/z* 278 for Glu and *m/z* 281 versus *m/z* 278 for [$^{13}C_5$]Glu) were determined. The curves were obtained by an unweighted least-squares linear fitting of the peak-area ratios versus the amounts added to each sample. Erythrocyte concentrations were calculated by comparing the peak-area ratios obtained from the unknown samples with those obtained from standard mixtures.

Accuracy and Precision

Accuracy and precision were determined by assaying four preparations of 0.05 mL portions of lysate spiked with [$^{13}C_5$]Glu (0.51, 1.02, 2.55, and 5.10 nmol). Each sample was prepared in triplicate. Following the addition of [$^{13}C_2$]Glu (5.10 nmol) as the internal standard, the samples were derivatized and measured as described above.

Enzymatic Procedure

A reaction mixture consisting of [$^{13}C_5$]Gln (250 nmol), $MgCl_2$ (5 μmol) and standard PRPP (0.49-47.2 nmol) in 0.45 mL of 50 mM Tris-HCl buffer (pH 7.4) was added in a polypropylene tube (75 × 15 mm ID). The reaction was initiated by the addition of 0.05 mL of ATase solution (0.2 mg protein/mL). After incubation at 37°C for 10 minutes, the reaction was stopped by the addition of 2 mL of ice-cold methanol. Following the addition of [$^{13}C_2$]Glu (5.10 nmol/0.05 mL) as the internal standard, the sample was centrifuged at 900 *g* for 5 minutes. The supernatant was purified, derivatized, and analyzed as described above.

RESULTS AND DISCUSSION

Several derivatization methods have been used to measure glutamate by GC-MS.[11–13] Acid-catalyzed esterification of the carboxylic moiety of amino acids is commonly employed, but the derivatization causes deamidation of glutamine to glutamate.[14] We have previously used the N(O,S)-alkoxycarbonyl alkyl ester derivatives for a simultaneous quantitation of [$^2H_7$]methionine, [$^2H_4$]methionine, methionine, [$^2H_4$]homocysteine,

and homocysteine in plasma by GC-MS using [^{13}C]methionine and [^{13}C$_2$]homocystine as analytical standards.[15] A one-pot derivatization of Glu species to the isobutyloxycarbonyl methyl ester (iBCME) was achieved by the reaction with isobutyl chloroformate in a solution of water-methanol-pyridine. The reaction was completed in seconds at room temperature. The iBCME derivative of Glu showed good chromatographic behavior and eluted at 5.1 minutes. Since iBCME derivatives of [^{13}C$_5$]Glu, [^{13}C$_2$]Glu, and Glu gave strong protonated molecular ions [M+H]$^+$ at m/z 281, 278, 276 in the chemical ionization mass spectra, we have chosen these ions for selected ion monitoring. Reference Gln with a certified purity of 99% was derivatized and subjected to GC-MS-SIM. The Glu detected was 0.88 ± 0.09% ($n = 3$), indicating almost no transformation of Gln to Glu.

Ion-exchange chromatography provides a simple method for extracting amino acids from biological fluids. In the present method, we used an anion-exchange cartridge column BondElut SAX to extract Glu from the reaction mixture prior to GC-MS analysis. The recovery of [^{13}C$_5$]Glu was higher than 90%. In contrast, [^{13}C$_5$]Gln was not retained on the column and eluted almost immediately. The use of the iBCME derivatization and the anion-exchange column made it possible to measure Glu in samples containing Gln without degrading the compound of interest.

Calibration curves were prepared from a series of samples containing varying amounts of [^{13}C$_5$]Glu in the range of 0.05–5.10 nmol and Glu in the range of 5.10–51.0 nmol. When the peak-area ratios were plotted against the molar ratios, good correlations were found ($R = 1.000$). The accuracy and precision of the assays were determined by spiking 50 μL of red blood cell lysates with 0.51–5.10 nmol of [^{13}C$_5$]Glu. The boiling step served to inactivate enzymes, which catalyze Glu generation and utilization in erythrocytes. There was no interference in the vicinity of the peaks analyzed in the SIM. The variance of intra-day precisions of the assay of [^{13}C$_5$]Glu was less than 4%, while the accuracy of the relative errors was −0.92% to 1.27%. The erythrocyte concentration of Glu was 254 ± 7 nmol/mL in a healthy male. The data correspond to previous results in healthy subjects.[16]

The GC-MS method was applied for determination of PRPP. [^{13}C$_5$]Gln was incubated with different concentrations of PRPP in the presence of ATase and the [^{13}C$_5$]Glu formed was determined by GC-MS-SIM. There was a linear relationship between the amount of [^{13}C$_5$]Glu formed and the amount of PRPP added from 0.49 to 47.2 nmol (Figure 1).

In conclusion, a novel GC-MS method has been developed for the assay of PRPP by the determination of [^{13}C$_5$]Glu derived from [^{13}C$_5$]Gln catalyzed by ATase.

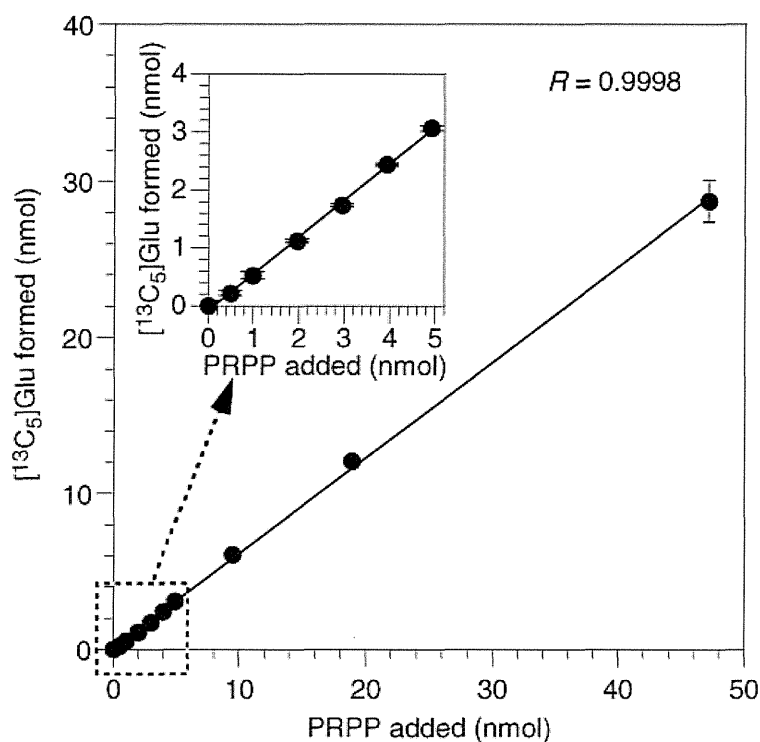**FIGURE 1** Relation between PRPP added and [$^{13}C_5$]Glu formed. All values represent the mean ± SD of three independent experiments.

REFERENCES

1. Becker, M.A. Patterns of phosphoribosylpyrophosphate and ribose-5-phosphate concentration and generation in fibroblasts from patients with gout and purine overproduction. *J. Clin. Invest.* **1976**, 57, 308–318.

2. Becker, M.A. Hyperuricemia and gout, in *The Metabolic & Molecular Bases of Inherited Disease*, 8th ed., ed. C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, McGraw-Hill, New York, 2001, pp. 2513–2535.

3. Fox, I.H.; Kelley, W.N. Phosphoribosylpyrophosphate in men: biochemical and clinical significance. *Ann. Int. Med.* **1971**, 74, 424–433.

4. Becker, M.A.; Meyer, L.J.; Wood, A.W.; Seegmiller, J.E. Purine overproduction in man associated with increased phosphoribosylpyrophosphate synthetase activity. *Science* **1973**, 179, 1123–1126.

5. Becker, M.A.; Meyer, L.J.; Seegmiller, J.E. Gout with purine overproduction due to increased phosphoribosylpyrophosphate synthetase activity. *Am. J. Med.* **1973**, 55, 232–242.

6. Tax, W.J.M.; Veerkamp, J.H. A simple and sensitive method for estimating the concentration and synthesis of 5-phosphoribosyl 1-pyrophosphate in red blood cells. *Clin. Chim. Acta* **1977**, 78, 209–216.

7. Ipata, P.L.; Mura, U.; Camici, M.; Giovannitti, M.P. A coupled optical assay for adenine phosphoribosyltransferase and its extension for the spectrometric and radioenzymatic determination of 5-phosphoribosyl-1-pyrophosphate in mixtures and in tissue extracts. *Anal. Biochem.* **1987**, 164, 411–417.

8. King, M.T.; Passonneau, J.V.; Veech, R.L. Radiometric measurement of phosphoribosylpyrophosphate and ribose 5-phosphate by enzymatic procedures. *Anal. Biochem.* **1990**, 187, 179–186.

9. Mantsala, P.; Zalkin, H. Glutamine nucleotide sequence of *Saccharomyces cerevisiae ADE4* encoding phosphoribosylpyrophosphate amidotransferase. *J. Biol. Chem.* **1984**, 259, 8478–8484.

10. Hasegawa, H.; Shinohara, Y.; Hashimoto, T.; Matsuda, R.; Hayashi, Y. Prediction of measurement uncertainty isotope dilution gas chromatography/mass spectrometry. *J. Chromatogr. A* **2006**, 1136, 226–230.

11. Darmaun, D.; Manary, M.J.; Matthews, D.E. A method for measuring both glutamine and glutamate levels and stable isotopic enrichments. *Anal. Biochem.* **1985**, 147, 92–102.