研究成果の刊行に関する一覧表

書籍・総説

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
松尾洋孝	第7章 尿酸のトランスポーター	竹谷豊, 薩秀夫. 伊藤美紀子, 武田英二	栄養・食品機能とトランスポーター	建帛社	東京	2011	145-166
松尾洋孝	トランスポーターの分子機能を指標とした臨床遺伝学的解析による痛風の主要病因遺伝子ABCG2 の同定	金井好克	遺伝子医学MOOK	メディカル ドゥ	大阪	2011	116-125 (Vol. 19)
松尾洋孝	痛風の病因遺伝子	板倉光夫	痛風と核酸代謝	痛風・核酸代謝学会	東京	2010	159-169 (Vol. 34)
松尾洋孝, 高田龍平, 市田公美, 中村好宏, 鈴木洋史, 四ノ宮成祥	痛風の主要な病因遺伝子 ABCG2 の同定	池ノ内順一, 藤本豊士	実験医学	羊土社	東京	2010	1285-1289
中山昌喜, 松尾洋孝, 四ノ宮成祥	痛風の遺伝子		リウマチ科	科学評論社	東京	2010	689-694

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Stiburkova B, Ichida K, Sebesta I	Novel homozygous insertion in SLC2A9 gene caused renal hypouricemia	Mol Genet Metab	102(4)	430-435	2011
Hamajima N, Naito M, Hishida A, Okada R, Asai Y, Wakai K	Serum uric acid distribution according to SLC22A12 W258X genotype in a cross-sectional study of a general Japanese population	BMC Med Genet	12	in press	2011
Wakai K, Hamajima N, Okada R, Naito M, Morita E, Hishida A,	Profile of participants and genotype distributions of 108 polymorphisms in a cross-sectional study of associations	J Epidemiol	21(3)	223-235	2011

Kawai S, Nishio K, et al.	of genotypes with lifestyle and clinical factors: a project in the Japan multi-institutional collaborative cohort (J-MICC) study				
Hosoyamada M, Takiue Y, Morisaki H, Cheng J, Ikawa M, Okabe M, Morisaki T, Ichida K, Hosoya T, Shibasaki T	Establishment and analysis of SLC22A12 (URAT1) knockout mouse	Nucleosides Nucleotides Nucleic Acids	29(4-6)	314-320	2010
塚田愛，木村徹，Promsuk J, 安西尚彦, 市田公美, 櫻井裕之	尿酸トランスポーターURAT1 トランスジェニックマウスにおける尿酸の体内動態	痛風と核酸代謝	34	171-177	2010
Nakamura M, Anzai N, Jutabha P, Sato H, Sakurai H, Ichida K	Concentration-dependent inhibitory effect of irbesartan on renal uric acid transporters	J Pharmacol Sci	114(1)	115-118	2010
Shinohara Y, Hasegawa H, Kaneko T, Tamura Y, Hashimoto T, Ichida K	Analysis of [$^2H_7$]methionine, [$^2H_4$]methionine, methionine, [$^2H_4$]homocysteine and homocysteine in plasma by gas chromatography–mass spectrometry to follow the fate of administered [$^2H_7$]methionine	J Chromatogr B Analyt Technol Biomed Life Sci	878(3-4)	417-422	2010
Ikebuchi Y, Ito K, Takada T, Anzai N, Kanai Y, Suzuki H	Receptor for activated C-kinase 1 regulates the cell surface expression and function of ATP binding cassette G2	Drug Metab Dispos	38(12)	2320-8	2010
Hotta K, Nakamura M, Nakamura T, Matsuo T, Nakata Y, Kamohara S, Miyatake N, Kotani K, et al.	Polymorphisms in NRXN3, TFAP2B, MSRA, LYPLAL1, FTO and MC4R and their effect on visceral fat area in the Japanese population	J Hum Genet	55(11)	738-742	2010
Nakamura T	Commentary to 'a remark on rare variants'	J Hum Genet	55(5)	263-264	2010

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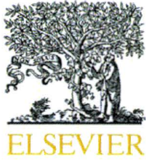

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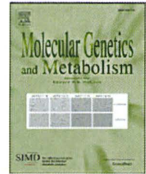

Novel homozygous insertion in *SLC2A9* gene caused renal hypouricemia

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Renal hypouricemia is a heterogeneous inherited disorder characterized by impaired uric acid handling in the renal tubules. Patients are usually asymptomatic; however, some may experience urolithiasis and/or acute kidney injury. Most of the described patients (compound heterozygous and/or homozygous) are Japanese with mutations in the *SLC22A12* gene (OMIM #220150). Four patients with renal hypouricemia caused by heterozygous defects and two families with homozygous mutations in the *SLC2A9* gene have been recently described (OMIM #612076).

We describe the clinical history, biochemical and molecular genetics findings of a Czech family with renal hypouricemia. The concentration of serum uric acid in the proband (16-year-old Czech girl with unrelated parents) was 0.17 ± 0.05 mg/dl and expressed as an increase in the fractional excretion of uric acid ($194 \pm 99\%$). The sequencing analysis of the coding region of uric acid transporters *SLC22A12, SLC2A9, SLC17A3, ABCC4* and *ABCG2*, was performed. Analysis of genomic DNA revealed novel one nucleotide homozygote insertion in exon 3 in the *SLC2A9* gene in proband and her brother resulting in a truncated protein (p. Ile118HisfsX27). No sequence variants in other candidate uric acid transporter were found. Homozygous loss-of-function mutations cause massive renal hypouricemia *via* total loss of uric acid absorption; however, they do not necessarily lead to nephrolithiasis and acute kidney injury. In contrast to previously reported heterozygous patients with renal hypouricemia type 2, we did not find even slight hypouricemia and found no decrease in the FE-UA of the heterozygous parents of the reported siblings.

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1. Introduction

Uric acid (UA) is the end product of purine metabolism in humans. The absence of the hepatic enzyme uricase and efficient renal UA reabsorption contribute to tenfold higher blood uric acid levels in humans compared to other mammals, and it has been suggested that higher UA levels have evolutionary advantages: (i) UA is a potent antioxidant, (ii) maintains blood pressure under low salt conditions [1] and (iii) may help arrest inflammatory demyelinating diseases such as optic neuritis linked to multiple sclerosis, through scavenging of peroxynitrite in the central nervous system [2].

Serum uric acid levels are determined by the balance between production and excretion. Transport mechanisms are localized in the proximal tubule, where UA is secreted and extensively reabsorbed by specific transporter proteins that reside in the apical and basolateral membranes. As a result, the percentage of excreted UA is only around 10% of the uric acid filtered through the glomerular membranes.

The most common genes that influence the level of serum UA, *via* renal UA excretion, are *SLC22A12* [3] and *SLC2A9* [4,5]. The *SLC22A12* gene maps to chromosome 11q13 and codes two transcript variants of the URAT1 transporter, which are specifically expressed on the apical membrane of proximal tubules in the kidneys (10 exons, 553 and 332 amino acids) and also in human vascular smooth muscle cells. URAT1 was identified in 2002 as the main transporter involved in uric acid reabsorption, where it regulates blood UA levels and plays a central role in reabsorption of UA from the glomerular filtrate. In 2008, the main role of *SLC2A9* in UA reabsorption was identified [4]. The *SLC2A9* gene maps to chromosome 4p15.3-p16 and exists in two variants: GLUT9a (12 exons, 540 amino acids) and GLUT9b (13 exons, 512 amino acids). GLUT9a is transcribed primarily in the kidneys, liver, placenta and leukocytes. Expression of GLUT9b has been observed only in the kidneys and placenta [6]. Recently results from a series of genome-wide association scans revealed a significant correlation between genetic variants in *SLC2A9* and serum UA levels, excretion fraction of uric acid, gout [4,5], blood pressure [7] and body mass index [8]. The genetic variants of *SLC2A9* are associated with reduced urinary UA clearance, which fits with common variations of *SLC2A9* which lead to changes in serum UA concentrations [7].

The consequential transporters have been shown to transport UA: OAT1, OAT3, OAT4 and OAT10 from the organic anion transporter

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(OAT) family [9,10], voltage-driven organic anion transporter OATv1 [11], and the multidrug-resistance-associated protein 4 MRP4, also known as the ATP-binding cassette transporter subfamily C 4 *ABCC4* [12]. Recently, two new loci associated with serum UA concentration were identified on the apical membrane of proximal tubule cells: *ABCG2*, the transporter of the ATP-binding cassette ABC family [13] and *SLC17A3*, a sodium phosphate transporter [12,14]. Additionally, a binding partner for URAT1, the multivalent PDZ domain-containing protein PDZK1, which regulates transport activity, was identified [15].

Despite much research in renal uric acid transport, the mechanism that determines serum UA concentration has not been fully revealed. Altered renal excretion of UA accounts for most hyperuricemia and gout [16]. It has been postulated that defects in tubular UA transport causes hypouricemia and that decreased renal UA clearance leads to hyperuricemia that induces gout, hypertension, and cardiovascular diseases [17].

Hypouricemia, defined as a serum UA levels<2 mg/dl, can result from decreased UA production, but it is more commonly due to decreased renal tubular UA reabsorption. Renal hypouricemia is a heterogeneous inherited disorder with an unknown pathogenesis characterized by impaired tubular UA transport, reabsorption insufficiency and/or acceleration of secretion with severe complications, such as acute kidney injury (AKI) and nephrolithiasis. Diagnosis is based on biochemical markers: hypouricemia and increased fractional excretion of uric acid (FE-UA). Therapy is not available. To date, homozygous or compound heterozygous patients with loss-of-function mutations in *SLC22A12* (renal hypouricemia type 1 RHUC1) have been reported [18,19]. Recently, heterozygous missense mutations in *SLC2A9* in Japanese patients [20,21], and homozygous loss-of-function mutations in Israeli-Arab and Ashkenazi-Jewish patients, causing severe hypouricemia, complicated by nephrolithiasis and AKI were described [22]. These cases demonstrate that *SLC2A9* is a novel causative gene linked to renal hypouricemia type 2 (RHUC2).

We report on a Czech family with severe hypouricemia caused by an insertion in the *SLC2A9* gene, but no sequence variants in other uric acid transporters *SLC22A12, SLC17A3, ABCC4* and *ABCG2*.

2. Materials and methods

2.1. Clinical and biochemical findings

All patients received information about the study, agreed and signed an informed consent. All tests were performed in accordance with the standards of the institutional ethics committee, which approved this project (MSM 0021620806). The proband was a 16-year-old Czech girl with unrelated parents. She was born after a normal pregnancy and delivery. Except for her brother, who had urolithiasis, she had no other family history of AKI or renal disease. At the age of 13 she was admitted to hospital complaining of abdominal pain. Physical examination showed marked tenderness in the right lower quadrant of the abdomen. There was no vomiting and stools were normal. Uric acid in serum and urine was measured using specific enzyme methods. Creatinine in plasma and urine was measured using the Jaffé reaction, adapted to an auto analyzer (Hitachi Automatic Analyzer 902, Roche). FE-UA was evaluated as [uric acid clearance factored by creatinine clearance × 100]. HPLC determination of hypoxanthine, xanthine, allopurinol and oxipurinol in urine and plasma were performed on an Alliance 2695 and Photodiode Array Detector 2998 (Waters, USA). Before HPLC analyses, the urine samples were diluted with water to give a creatinine concentration of 1 mmol/L. Serum aliquots were deproteinized with an equal volume of 2 M perchloric acid. Samples were centrifuged for 5 min at 7000×*g* and supernatants were collected and neutralized with 2.5 M KHCO3. After a subsequent centrifugation step (7000×*g*, 5 min.), the potassium perchlorate precipitate was removed and the supernatants were injected onto the chromatograph. A 20 µl aliquot of the diluted urine or plasma extract was injected. The determination was carried out using a reverse-phase Prontosil C18 AQ column 200 mm×4.0 mm i.d., 3 µm particle size (Bischoff Chromatography, Leonberg, Germany) and eluted using a linear 15 min. gradient from 0 to 30% of acetonitrile in a 40 mM ammonium acetate buffer (pH 5), a flow rate of 0.7 ml/min and peaks were detected using UV absorbance scanned over the range of 230–305 nm.

Serum cystatin C was measured using Imunoturbidimetrie (particle-enhanced), immunoparticles LX002 (DakoCytomation).

2.2. Uric acid transporters genes analysis

2.2.1. PCR amplification of coding exons and sequence analysis

The sequencing analysis of the coding region of uric acid transporters *SLC22A12, SLC2A9, SLC17A3, ABCC4* and *ABCG2*, was performed after informed consent. The genomic DNA for PCR analysis was isolated from blood samples (Qiagen columns). All exons were amplified using PCR and sequenced directly. Fifty nanograms of genomic DNA was amplified in 50 µl containing 2.5 U Taq-Purple DNA polymerase, 200 µM dNTPs and 0.15 µM primers. Amplification products were gel purified (1% agarose with 1 × TAE buffer) and subsequently purified using Wizard SV gel and PCR Clean-up system (Promega, Madison, WI, USA). DNA sequencing was performed using an automated DNA sequencer (Applied Biosystems 3100-Avant Genetic Analyzer; Applied Biosystems, USA).

2.2.2. Restriction enzyme analysis

Sequence variant g.27073insC was analyzed in our control cohort of 300 alleles (75 males, 75 females, randomly selected among patients of General University Hospital in Prague) by PCR RFLP. Twenty-five nanograms of genomic DNA was amplified in 25 µl containing 1.25 U Taq-Purple DNA polymerase, 100 µM of dNTPs and 0.075 µM of primers (the same as for PCR of exon 3). Ten microliters of the PCR reaction mixture was digested by 10 U of BseLI (Fermentas Life Sciences). Twenty microliters of the digestion reaction mixture was analyzed using 2% agarose gel with 1xTAE buffer. Wild type (497 bp) was not digested, p.I118HfsX27 genotype was digested in two segments (220 and 277 bp) (Marker GeneRulerTM 50 bp DNA Ladder (Fermentas Life Sciences)).

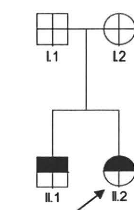
3. Results

3.1. Clinical and biochemical investigations

Detailed purine metabolic investigations were performed and we found the FE-UA of the patient to be consistently elevated (100–380%, normal values: 7.3 ± 1.3%), resulting in low serum uric acid levels (0.08–0.17 mg/dl, reference range: females, 2–5.7 mg/dl; males, 2–7 mg/dl). Urine tests and urinary sediment were within normal limits. No proteinuria or hematuria was found. Renal ultrasonography showed normal morphology of the kidneys, ureters and bladder. Other purine metabolic disorders associated with hypouricemia were excluded. No other secondary causes of hyperuricosuric hypouricemia were found during clinical and laboratory examinations. The brother of the proband (21 years old) had similar findings of hypouricemia and high FE-UA (0.17 mg/dl; 220%), respectively. We also measured cystatin C as an indicator of glomerular filtration rate, the level was below the reference range (0.55–1.15 mg/L), 0.47 in the proband and 0.52 in her brother. Serum uric acid and FE-UA were within normal limits (two measurements) in her parents. Pedigree and data of family are shown in Fig. 1.

3.2. Uric acid transporter genes analysis

Very low blood concentrations of uric acid together with markedly elevated FE-UA caused us to suspect renal hypouricemia and therefore



biochemical investigations, hypouricemia, increased fractional excretion of UA.

Family members	S-UA mg/dl	FE-UA %	S-creatinine µmol/L	S-cystatin C mg/L	U-UA mmol/L	U-Hypoxanthine mmol/molCr	U-Xanthine mmol/molCr	U-Uracil mmol/molCr
I.1*	5.48, 5.63	7, 6	92, 84	N.A.	1.9, 2.8	5.0, 4.1	3.8, 3.6	4.8, 1.5
I.2*	2.46, 3.29	12, 8	78, 65	N.A.	4.0, 1.8	3.8, 3.9	3.5, 4.4	2.8, 8.5
II.1°	0.17	220	80	0.52	2.2	5.5	6.9	3.1
II.2[a]	0.17 ± 0.05	194 ± 99	76 ± 4	0.47°	2.2 ± 0.6	4.0 ± 1.4	5.8 ± 2.4	3.2 ± 1.1

*two measurements, °one measurements, [a]five measurements for S-UA, FE-UA, S-creatinine, U-UA and four measurements for U-Hypoxanthine, U-Xanthine and U-Uracil, means ± S.D.

Fig. 1. Pedigree and data of a family exhibiting clinical features compatible with renal hypouricemia.

mutational analysis of the *SLC22A12* gene was performed for confirmation of the diagnosis. Since no sequence variants were found, we performed a mutational analysis of the other UA transporters *SLC2A9*, *SLC17A3*, as well as *ABCC4* and *ABCG2*. The sequence variations found in the exon regions of the uric acid transporters are shown in Table 1, reference sequence : *SLC2A9* NC_000011, region: 64114688..64126396, *SLC22A12* NC_000004, region 9436946..9650970, *ABCC4* NC_000013.9, region 94470084..94751688, *SLC17A3* NC_000006.10, region 25982450..25953307.

No sequence variants in *ABCC4* and *ABCG2* were found. Though two heterozygous sequence variants, p.A100T and p.G279R were identified in *SLC17A3* of her brother; however, we judged them to be not disease causing, sense no matching mutations was found in the proband. An unpublished one nucleotide insertion was found in *SLC2A9* in exon 3, g.27073insC, heterozygous in parents and homozygous in proband and brother (Fig. 2). The first affected amino acid is isoleucine-118; it is replaced by a histidine and the new reading frame ends in a 'stop' at position 27, p.Ile118HisfsX27. This

Table 1
Sequence variations of coding regions in candidate genes in a family with renal hypouricemia. *Sequencing data available only for proband.

Gene	Region	SNP	Mutation	Haplotype	Family members	Position mRNA	Position aa	refSNP
SLC2A9	exon 3	T>C	synonymous	TC	I.2	383	L108L	ss44549642
				CC	I.1, II.1, II.2			
	exon 3	insC	frame shift	wt/insC	I.1, I.2	415_416	p.I118HfsX27	-
				insC/insC	II.1, II.2			
	exon 3	G>A	synonymous	GA	I.2	436	T125T	ss19582347
				AA	I.1, II.1, II.2			
	exon 5*	T>C	synonymous	CC	II.2	628	L189L	ss44580429
SLC22A12	promoter	A>T	-	AT	I.1, II.2	-	-	-
				TT	I.2, II.1			
	promoter	C>T	-	CT	I.1, II.2	-	-	-
	exon1/5′-UTR	C>T	-	CT	I.1, II.2	30	-	ss39899375
				TT	I.2, II.1			
	exon 1	C>T	synonymous	TT	I.1, I.2, II.1, II.2	1005	H86H	rs3825016
	exon 2	T>C	synonymous	TC	I.1, II.2	1173	H142H	rs11231825
				CC	I.2, II.1			
	exon 7	A>G	synonymous	GG	I.1, I.2, II.1, II.2	1995	R416R	rs1630320
ABCC4	exon 8	A>G	synonymous	GA	I.2, II.1	1070	R317R	rs2274406
				GG	I.1, II.2			
	exon 8	A>G	synonymous	GA	I.2, II.1	1088	S323S	rs2274405
				GG	I.1, II.2			
SLC17A3	exon 3	G>A	missense	GG	I.1, II.1	408	A100T	rs1165165
				GA	I.2, II.2			
	exon 8	G>A	missense	GG	I.1, II.1	945	G279R	rs56027330
				GA	I.2, II.2			
	exon 11	C>T	synonymous	CT	I.1, I.2, II.1, II.2	1454	S448S	rs942379

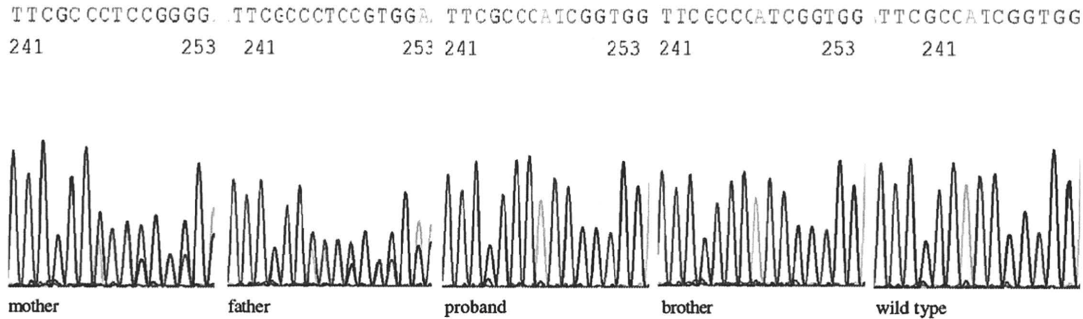


Fig. 2. Electropherograms of partial sequences of exon 3 of *SLC2A9* showing a heterozygous (position 247 in mother, 246 in father) and homozygous one nucleotide insertion g.43412_43413insC (position 247 in proband and brother) in a family with two siblings exhibiting clinical features compatible with idiopathic renal hypouricemia. Ref. sequence: SLC2A9 NC_ 000011, region: 64114688..64126396.

altered protein has only 144 amino acids and only the first extracellular domain, from 12 transmembrane segments, remains. This sequence variant was not detected in a control cohort of 150 randomly chosen individuals (75 males, 75 females; restriction assay with BseLI (Fermentas Life Sciences)), Fig. 3.

4. Discussion

Even with much research into renal uric acid transport, a complete understanding for the regulation of renal uric acid handling has not yet been fully elucidated. It has been established that URAT1 is mainly involved in UA reabsorption and sequence variants in the *SLC22A12* gene cause renal hypouricemia. More than a hundred cases with a loss-of-function mutation in the *SLC22A12* gene have been found in Japan [18,19]. However, there have been three reports suggesting defects in other undiscovered UA transporters in Japanese patients [18,23] and one Israeli-Arab patient with renal hypouricemia; these patients were observed to have no mutation in the *SLC22A12* gene [24]. Recently results revealed a significant correlation between genetic variants in another causative gene of renal hypouricemia,

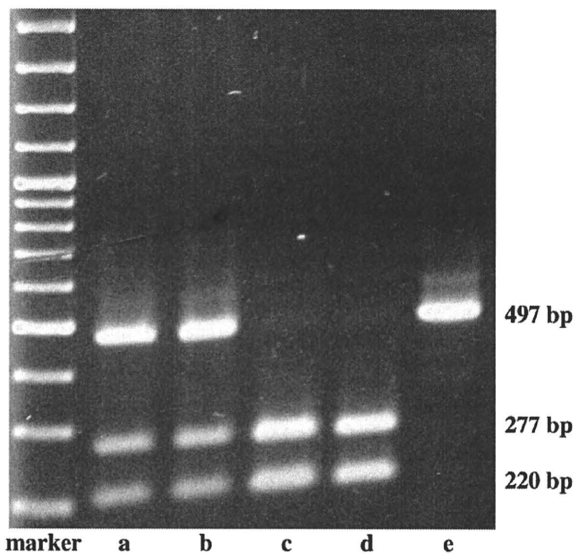


Fig. 3. The p.Ile118HisfsX27 mutation results in the gain of a BseLI restriction site. Wild type (497 bp) was not digested, allela with p.Ile118HisfsX27 was digested in two segments (220 and 277 bp); a) mother, b) father, c) proband, d) brother, e) control (normouricemic subject from cohort of 150 randomly chosen individuals).

SLC2A9, and serum UA level, FE-UA, gout [4,5] and body mass index [8]. Two new loci were recently identified, *ABCG2* and *SLC17A3*, which show an association with UA concentrations [25] even though there have been no reports of hypouricemia patients with defects in these genes. These data suggest a new concept of renal UA transport – multimolecular complex "transportsome" that probably allows cooperation between multiple transporters [17].

Currently, 23 mutations in the *SLC22A12* coding region (15 for renal hyperuricemia phenotype and five for primary gout) and seven mutations in *SLC2A9* (five for renal hypouricemia phenotype, two associated with primary gout) have been described worldwide (https://portal.biobase-international.com/hgmd/pro). Characterization of the above-mentioned allelic variants have only been partially performed [20,21,25] and their frequency and influence on levels of serum uric acid have not been studied in a sufficiently sized population cohort. Additionally, interference associated with URAT1, GLUT9a and GLUT9b has not yet been studied. Missense heterozygous mutations in *SLC2A9* were described in four patients with renal hypouricemia. Mutations in p.R198C have been described in a 32-year-old female (plasma UA level 2.1 mg/dl) and mutations in p. R380W have been described in a 70-year-old female (plasma UA level 1.5 mg/dl, FE-UA 15.7%) and her 43-year-old son (plasma UA level 2.7 mg/dl, FE-UA 14.6%) [20]. A p.P412R mutation was found in a 36-year-old female with plasma UA levels of 2.4 mg/dl [21]. These phenotypes are similar to the values found in renal hypouricemia patients with a URAT1 mutation p.W258X, which is common in Japanese renal hypouricemia [18,21].

Recently homozygous loss-of-function mutations causing severe hypouricemia, complicated by nephrolithiasis and AKI, in two families, were described [22]. Mutation p.L75R, in the homozygous state, was found in six patients from one family, with plasma UA levels ranging from 0.01 to 0.67 mg/dl and FE-UA>150%. Heterozygous relatives had plasma UA levels of 2.0–4.5 mg/dl and FE-UA between 5.4 and 21.7%. From six patients, two presented with nephrolithiasis and three presented with AKI. Only one patient, a 10-year-old female, was without clinical history. A homozygous 36-kb deletion was found in another patient with nephrolithiasis, with a plasma UA level of 0.10 mg/dl and fractional excretion of uric acid>150%.

Serum creatinine concentration was measured as a marker for glomerular filtration rate; however, production depends on several factors such as gender, age and muscle mass. Of eight patients with homozygous mutations and extremely low serum UA (0.01–0.67 mg/dl) only two patients had elevated serum creatinine levels (178 and 282 mg/dl). Clinical data from patients with *SLC2A9* mutations did not find a relationship between serum UA and creatinine levels and renal failure. Cystatin C is an alternative marker for glomerular filtration rate that provides increased sensitively. During early renal dysfunction, when glomerular filtration rate is still

near normal, Cystatin C is more accurate than serum creatinine [26]. However, the level of serum cystatin C was below the reference range (0.55–1.15 mg/L) in the proband (0.47 mg/L) and in her brother (0.52 mg/L). This result shows that glomerular filtration rate was not impaired in our patient.

As mentioned above, URAT1 and GLUT9 are expressed in proximal tubule cells. Staining of polarized MDCK cells, stably expressing GLUT9, showed a difference in localization. Whereas GLUT9 was found exclusively in the basolateral membrane, GLUT9b was found in the apical membrane [6]. Malfunction of both GLUT9 isoforms results in a pathological condition. In RHUC1, the loss of URAT1 function produces a partial uric acid absorption defect. In RHUC2 there are two clinical distinctions with important differences: values found in renal hypouricemia patients are similar to those with URAT1 mutations; serum uric acid levels are much lower (near 0) and renal excretion is markedly higher (>100%). Heterozygous GLUT9 mutations cause hypouricemia haplo-insufficiency rather than dominant-negative effects [20]. Homozygous GLUT9 mutations cause severe hypouricemia through loss-of-function and preclude uric acid absorption by all apical transporters (including URAT1) by completely blocking uric acid efflux [22].

The frequency of AKI in RHUC1 was about 10% in a cohort of over a hundred cases [18,19]. AKI was reported in three probands (homozygous loss-of-function mutations) from a sample of 10 patients with RHUC2. These investigations suggested that the frequency of AKI might be different between RHUC1 and heterozygous and homozygous RHUC2. Two mechanisms have been proposed to explain how renal hypouricemia causes AKI: i) UA nephropathy results from an increase in UA production during exercise [27], ii) renal reperfusion injury due to vasoconstriction results from an exercise-induced increase in oxygen free radicals potentiated by a lack of UA [28]. However, the two patients, described here, with homozygous mutations in GLUT9, never had AKI in their medical history and their life styles were full of athletic activity.

Extremely low hypouricemia is also a characteristic of xanthine dehydrogenase (XDH, E.C. 1.1.1.204) deficiency (OMIM #278300, OMIM 603592). Xanthinuria is a rare autosomal recessive disorder of purine catabolism. The affected individuals are characterized by strongly diminished production of serum uric acid and high urinary excretion of hypoxanthine and xanthine. Patients may develop calculi in the urinary tract, AKI and myopathy due to deposits of xanthine, some patients are clinically asymptomatic. We diagnosed four non-consangiune probands with significantly low concentrations of UA in the serum (0.16 ± 0.28 mg/dl). Neither of the probands with xanthinuria had AKI in their medical history. On the other hand, AKI was reported in patients with defects in URAT1 and GLUT9 with levels of serum uric acid similar to patients with xanthinuria. It suggests, that hypouricemia alone, probably, could not contribute to renal failure in patients with primary renal hypouricemia and the most likely cause of AKI was UA overproduction.

Uric acid is powerful scavenger of peroxyl radicals, hydroxyl radicals and singlet oxygen in human biological fluids [29,30]. UA accounts for up to 60% of plasma antioxidative capacity and presumably protects not only erythrocytes, but also DNA-contained in long-lived T and B lymphocytes and macrophages. UA may also protect other tissues since they are, presumably, in equilibrium with blood UA [29]. UA has been hypothesized to protect against oxidative stress, a prominent contributor to dopaminergic neuron degeneration in Parkinson's disease. Studies have evaluated a potential association between serum UA and the risk of developing Parkinson's disease, finding a lower risk among the individuals with higher levels of serum UA [31,32]. A case-control study (1052 Parkinson's disease patients and 6634 controls) and population-based cohort study (11,258 gout patients, 56,199 controls) showed protective effects of gout on the risk of developing Parkinson's disease [33,34].

Observation of neurological symptoms in severe hypouricemia patients might provide a new information about a relation between UA and neurologic diseases [29].

Our finding of a defect in the *SLC2A9* gene, in two siblings, shows the following: a) it provides further evidence that *SLC2A9* is a causative gene in renal hypouricemia RHUC2 with clinical distinctions; b) it supports the prediction that normal function of both URAT1 and GLUT9 are essential for normal UA reabsorption in the renal proximal tubule. In contrast to previously reported hypouricemic heterozygous patients with RHUC2 [20,21], we did not find even slight hypouricemia and found no decrease in the FE-UA of the heterozygous parents of the reported siblings.

In conclusion, we have found a Czech family with renal hypouricemia caused by a homozygous insertion in the *SLC2A9* gene with no sequence variants in *SLC22A12*, *SLC17A3*, *ABCC4* or *ABCG2*. Homozygous loss-of-function mutations in *SLC2A9* cause massive renal hypouricemia *via* total loss of uric acid absorption, however, they do not necessarily lead to nephrolithiasis and AKI. Detailed studies concerning uric acid transporters *SLC2A9* and *SLC22A12* and their interactions could clarify the pathogenesis of acute kidney injury in renal hypouricemia; the relationship between URAT1 and GLUT9 (including the interaction between isoforms), hypouricemia, hyperuricemia, and gout and improvements in early and effective diagnosis of hypo/hyperuricemia.

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RESEARCH ARTICLE

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Serum uric acid distribution according to *SLC22A12* W258X genotype in a cross-sectional study of a general Japanese population

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Abstract

Background: Although *SLC22A12 258X* allele was found among those with hypouricemia, it was unknown that serum uric acid distribution among those with *SLC22A12 258X* allele. This study examined serum uric acid (SUA) distribution according to *SLC22A12* W258X genotype in a general Japanese population.

Methods: Subjects were 5,023 health checkup examinees (3,413 males and 1,610 females) aged 35 to 69 years with creatinine < 2.0 mg/dL, who were participants of a cohort study belonging to the Japan Multi-Institutional Collaborative Cohort Study (J-MICC Study). *SLC22A12* W258X was genotyped with a polymerase chain reaction with confronting two-pair primers.

Results: The genotype frequency was 4,793 for *WW*, 225 for *WX*, and 5 for *XX*, which was in Hardy-Weinberg equilibrium ($p = 0.164$) with *X* allele 0.023 (95% confidence interval [0.021-0.027]). Mean (range) SUA was 6.2 (2.1-11.4) mg/dL for *WW*, 3.9 (0.8-7.8) mg/dL for *WX*, and 0.8 (0.7-0.9) mg/dL for *XX* among males, and 4.5 (1.9-8.9) mg/dL, 3.3 (2.0-6.5) mg/dL, and 0.60 (0.5-0.7) mg/dL among females, respectively. Six individuals with SUA less than 1.0 mg/dL included two males with *XX* genotype, one male with *WX* genotype, and three females with *XX* genotype. Subjects with *WX* genotype were 14 (77.8%) of 18 males with a SUA of 1.0-2.9 mg/dL, and 28 (34.6%) of 81 females with the same range of SUA. The corresponding values were 131 (25.1%) of 522 males and 37 (3.5%) of 1,073 females for SUA 3.0-4.9 mg/dL, and 8 (0.4%) of 2,069 males and 5 (1.1%) of 429 females for SUA 5.0-6.9 mg/dL. The *X* allele effect for SUA less than 3 mg/dL was significantly ($p < 0.001$) higher in males (OR = 102.5, [33.9-309.8]) than in females (OR = 25.6 [14.4-45.3]).

Conclusions: Although *SLC22A12* W258X was a determining genetic factor on SUA, SUA of those with *WX* genotype distributed widely from 0.8 mg/dL to 7.8 mg/dL. It indicated that other genetic traits and/or lifestyle affected SUA of those with *WX* genotype, as well as those with *WW* genotype.

Background

It is well known that the mean serum uric acid (SUA) is lower in females than in males. In addition, it is fully documented that age, menopause, food consumption, alcohol intake, obesity, a sedentary lifestyle, dyslipidemia, insulin resistance, blood pressure, renal function, and drug use for hypertension were associated with SUA levels [1-6]. Meanwhile, recent studies have elucidated that genotypes are also influential factors of SUA.

SUA is reabsorbed in renal tubules through uric acid transporter 1 (URAT1) encoded by *SLC22A12* in chromosome 11q13 [7,8]. *SLC22A12* was documented to have functional polymorphisms, among which W258X was found among those with renal hypouricemia [9-11]. However, the distribution of SUA among those with *258X* allele was not reported in a general population. This study aimed to examine SUA distribution according to *SLC22A12* W258X genotye, elucidating the overall effect of *SLC22A12* W258X on SUA among Japanese.

Methods

This study was approved by the Ethics Committee of Nagoya University School of Medicine (approval

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number 288). Subjects who gave written informed consent to participate in the study were enrolled.

Subjects and data collection

Subjects were derived from 5,040 examinees aged 35-69 years who visited a health checkup center in Hamamatsu, Japan in 2006-2007. They were enrolled as participants of a cohort study belonging to the Japan Multi-Institutional Collaborative Cohort Study (J-MICC Study) [12,13]. As of October, 2010, one participant was found to be ineligible in terms of age (34 years old at enrollment), and 11 participants withdrew from the study. Blood sample was not available for one participant, and genotyping was not successful for another. Three participants with creatinine of 2.0 mg/dL or over were excluded from the analysis, leaving 5,023 subjects for the analysis.

Health checkup data including blood tests were used for this study. Peripheral blood was drawn in the morning from those fasting overnight. Biochemical analysis of the sampled sera was performed using an auto-analyzer in the health checkup center.

Genotyping

DNA was extracted from buffy coat conserved at -80°C using a BioRobot® M48 (QIAGEN Group, Tokyo). *SLC22A12* W258X polymorphism was genotyped by a polymerase chain reaction with confronting two-pair primers (PCR-CTPP) [14]. Each 25 μl reaction tube contained 30-80 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 10x PCR buffer including 15 mM $MgCl_2$. The PCR-CTPP was conducted with initial denaturation at 95°C for 10 minutes, 35 cycles of denaturation at 95°C for 1 minute, annealing at 62°C for 1 minute, and extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The primers were F1: 5'- TCC ATG CAG GCT CCA GG -3', R1: 5'- ACC ACC AGC TGC AGC AGT GTT -3', F2: 5'- TAC GGT GTG CGG GAC TGG -3', and R2: 5'- GGC AGG ATC TCC TCT GAG G -3'. The amplified DNA fragments were 117-base pairs (bp) for the *W* allele (*G* allele), 176-bp for the *X* allele (*A* allele), and 255-bp for a common band, as demonstrated in Figure 1.

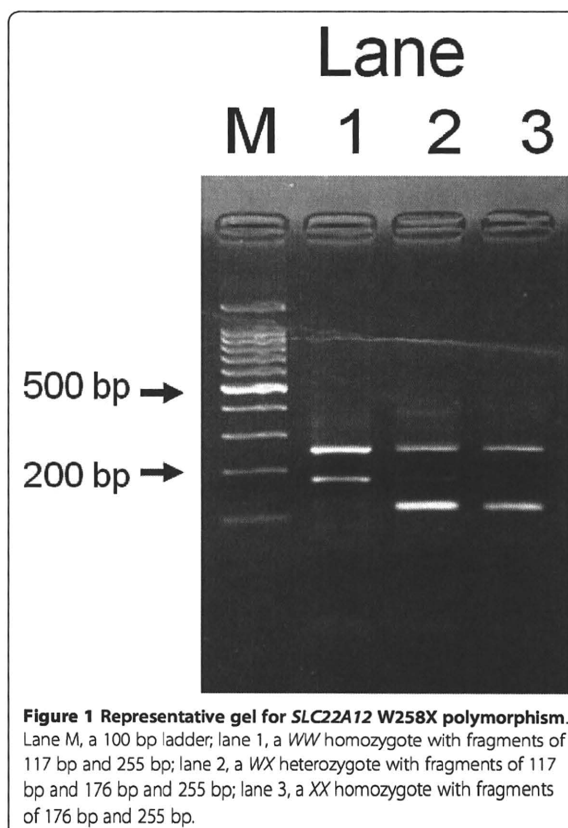

Figure 1 Representative gel for *SLC22A12* W258X polymorphism. Lane M, a 100 bp ladder; lane 1, a *WW* homozygote with fragments of 117 bp and 255 bp; lane 2, a *WX* heterozygote with fragments of 117 bp and 176 bp and 255 bp; lane 3, a *XX* homozygote with fragments of 176 bp and 255 bp.

Statistical analysis

Body mass index (BMI) was calculated by weight (kg)/squared height (m^2). Hardy-Weinberg equilibrium was examined with a chi-square test. Binomial distribution was used to estimate 95% confidence interval (CI) of proportions. Means among the three genotype groups were tested with analysis of variance (ANOVA). Adjusted odds ratio (OR) and 95% CI were estimated using an unconditional logistic model. All statistical analyses were performed using STATA software version 11 (STATA, College Station, TX).

Results

Table 1 shows the characteristics of the 5,023 subjects (3,413 males and 1,610 females). Those with a SUA less than 3.0 mg/dL were 0.6% in males and 5.2% in females, while those with a SUA of 7.0 mg/dL or over were 23.5% in males and 1.5% in females. The genotype frequency was 4,793 for *WW*, 225 for *WX*, and 5 for *XX*, which was in Hardy-Weinberg equilibrium (p = 0.164); the *X* allele frequency was 0.023 (95% CI, 0.021-0.027).

The means of age, BMI, blood pressure, and blood tests according to the genotype and sex are listed in Table 2. The null hypothesis that the means were equal among the three genotypes was rejected with one-way ANOVA for creatinine and glucose in males, and for age and gamma-glutamyltransferase (GGT) in females. When the *WX* and *XX* were combined, the difference in the mean between the combined and the *WW* became nonsignificant for GGT in females, but still significant for creatinine and glucose in males and age in females.

Mean SUA was 6.21 mg/dL for *WW*, 3.95 mg/dL for *WX*, and 0.80 mg/dL for *XX* among males, and 4.50 mg/dL,

Table 1 Characteristics of subjects according to sex

Characteristics	Males		Females	
	N	(%)	N	(%)
Total	3,413	(100)	1,610	(100)
Age (years)				
35-39	265	(7.8)	192	(11.9)
40-49	983	(28.8)	495	(30.7)
50-59	1,366	(40.0)	630	(39.1)
60-69	799	(23.4)	293	(18.2)
SUA (mg/dL)				
-3.0	21	(0.6)	84	(5.2)
3.0-4.9	522	(15.3)	1,073	(66.6)
5.0-6.9	2,069	(60.6)	429	(26.6)
7.0-	801	(23.5)	24	(1.5)
BMI (kg/m^2)*				
-18.4	90	(2.6)	160	(9.9)
18.5-24.9	2,423	(71.0)	1,195	(74.2)
25.0-	899	(26.3)	255	(15.8)
Creatinine (mg/dL)				
0.0-0.4	0	(0.0)	33	(2.1)
0.5-0.9	2,710	(79.4)	1,574	(97.8)
1.0-1.4	701	(20.5)	3	(0.2)
1.5-1.9	2	(0.1)	0	(0.0)
SLC22A12 W258X				
WW	3,256	(95.4)	1,537	(95.5)
WX	155	(4.5)	70	(4.3)
XX	2	(0.1)	3	(0.2)

SUA, serum uric acid; BMI, body mass index.
*BMI was not available for one male.

3.31 mg/dL, and 0.60 mg/dL among females, respectively. The difference in mean SUA between *WX* and *WW* genotypes was significantly (p = 8E-12) higher in males (2.26 mg/dL) than in females (1.19 mg/dL). The difference among three genotypes was highly significant both in males (p < 1E-40) and females (p = 1E-33). Table 3 demonstates the SUA distribution in percentage according to the genotype. All five individuals with *XX* genotype had a SUA of less than 1.0 mg/dL; the range was 0.7 mg/dL to 0.9 mg/dL in males and 0.5 mg/dL to 0.7 mg/dL in females. The SUA of those with *WX* genotype varied from 0.8 mg/dL to 7.8 mg/dL in males and from 2.0 mg/dL to 6.5 mg/dL in females, while the corresponding values for those with *WW* genotype were 2.1 mg/dL to 11.4 mg/dL in males and 1.9 mg/dL to 8.9 mg/dL in females. Subjects with *WX* genotype were 14 (77.8%) of 18 males with SUA 1.0-2.9 mg/dL, and 28 (34.6%) of 81 females with the same range of SUA. The corresponding values were 131 (25.1%) of 522 males and 37 (3.5%) of 1,073 females for SUA 3.0-4.9 mg/dL, and 8 (0.4%) of 2,069 males and 5 (1.1%) of 429 females for SUA 5.0-6.9 mg/dL, as depicted in Figure 2.

The age-adjusted OR (95% CI) of SUA < 3 mg/dL for the *X* allele was significantly (p < 0.001) higher in males (OR = 102.5, 95% CI, 33.9-309.8) than in females (OR = 25.6, 95% CI, 14.4-45.3). The corresponding ORs were reduced for SUA < 4 mg/dL, as shown in Table 4. Among those with BMI < 25 kg/m^2, the age-adjusted OR (95% CI) was 116.3 (32.5-416.8) in 2,514 males and 23.3 (12.7-42.9) in 1,355 females, while they were 78.6 (8.4-731.5) in 899 males with BMI > 25 kg/m^2 and 56.2 (9.9-320.4) in 255 females with BMI > 25 kg/m^2. When these ORs were adjusted for creatinine and glucose in males and for GGT in females, no substantial differences were observed; for example, the OR of SUA < 3 mg/dL for *X* allele was 89.8 instead of 102.5 in males and 30.7 instead of 25.6 in females.

Discussion

This study demonstrated the SUA distribution according to *SLC22A12* W258X genotype in a general Japanese

Table 2 Characteristics of subjects according to *SLC22A12* W258X genotype

Characteristics	Males				Females			
	WW n = 3,256	*WX* n = 155	*XX* n = 2	P	*WW* n = 1,537	*WX* n = 70	*XX* n = 3	p
Age (years)	50.6	52.0	52.5	0.166	49.1	51.4	58.3	0.017
Body mass index (kg/m^2)	23.5	23.7	20.9	0.240	22.0	21.9	19.7	0.407
Systolic blood pressure (mmHg)	121.0	120.5	105.0	0.319	114.4	115.1	119.3	0.811
Diastolic blood pressure (mmHg)	76.3	76.0	66.0	0.350	69.9	70.4	66.7	0.798
Total cholesterol (mg/dL)	202.3	202.7	195.5	0.944	207.2	208.9	197.3	0.793
HDL cholesterol (mg/dL)	57.7	56.0	63.5	0.338	71.1	69.7	88.3	0.159
Triglyceride (mg/dL)	124.7	129.0	66.0	0.460	85.1	85.2	73.3	0.895
AST (U/dL)	22.4	22.4	18.0	0.890	19.5	20.5	27.0	0.071
ALT (U/L)	25.5	26.6	18.5	0.732	17.1	19.5	24.7	0.066
GGT (U/L)	47.0	45.9	24.5	0.817	23.2	25.0	56.3	0.023
Creatinine (mg/dL)	0.86	0.82	0.90	0.001*	0.61	0.62	0.63	0.750
Blood urea nitrogen (mg/dL)	14.3	14.5	16.0	0.567	13.1	14.0	15.0	0.057
Blood glucose (mg/dL)	101.9	107.0	90.5	0.003*	93.7	95.2	89.7	0.434

HDL, high-density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase.
p-value was calculated from one-way analysis of variance among *WW*, *WX*, and *XX* genotypes.

Table 3 Serum uric acid (SUA) distribution (%) according to *SLC22A12* W258X among Japanese health checkup examinees

Genotype	N	SUA (mg/dL)											
		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-	Mean	S.D.
Males													
WW	3,256	0.0	0.0	0.1	1.6	10.4	31.4	31.9	17.4	6.1	1.0	6.21	1,12
WX	155	0.6	0.0	9.0	40.0	44.5	3.2	1.9	0.6	0.0	0.0	3.95	0.83
XX	2	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.80	0.14
Total	3,413	0.1	0.0	0.5	3.3	12.0	30.1	30.5	16.7	5.9	0.9	6.10	1.21
Females													
WW	1,537	0.0	0.1	3.4	24.9	42.5	22.8	4.7	1.2	0.3	0.0	4.50	0.94
WX	70	0.0	0.0	40.0	38.6	14.3	5.6	1.4	0.0	0.0	0.0	3.31	0.89
XX	3	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.60	0.10
Total	1,610	0.2	0.1	5.0	25.5	41.2	22.0	4.6	1.2	0.3	0.0	4.44	0.98

*95% CI: 95% confidence interval for SUA mean.

population. The SUA of all five subjects with *XX* genotype was less than 1 mg/dL, while SUA of those with *WX* genotype distributed widely; from 0.8 mg/dL to 7.8 mg/dL in males and from 2.0 mg/dL to 6.5 mg/dL in females. The difference in the mean SUA between *WX* and *WW* genotypes was 2.26 mg/dL in males and 1.19 mg/dL in females, indicating that the reduction of the mean SUA due to possessing *X* allele was significantly larger in males than in females. Since the distribution among the males with *WX* genotype was closer

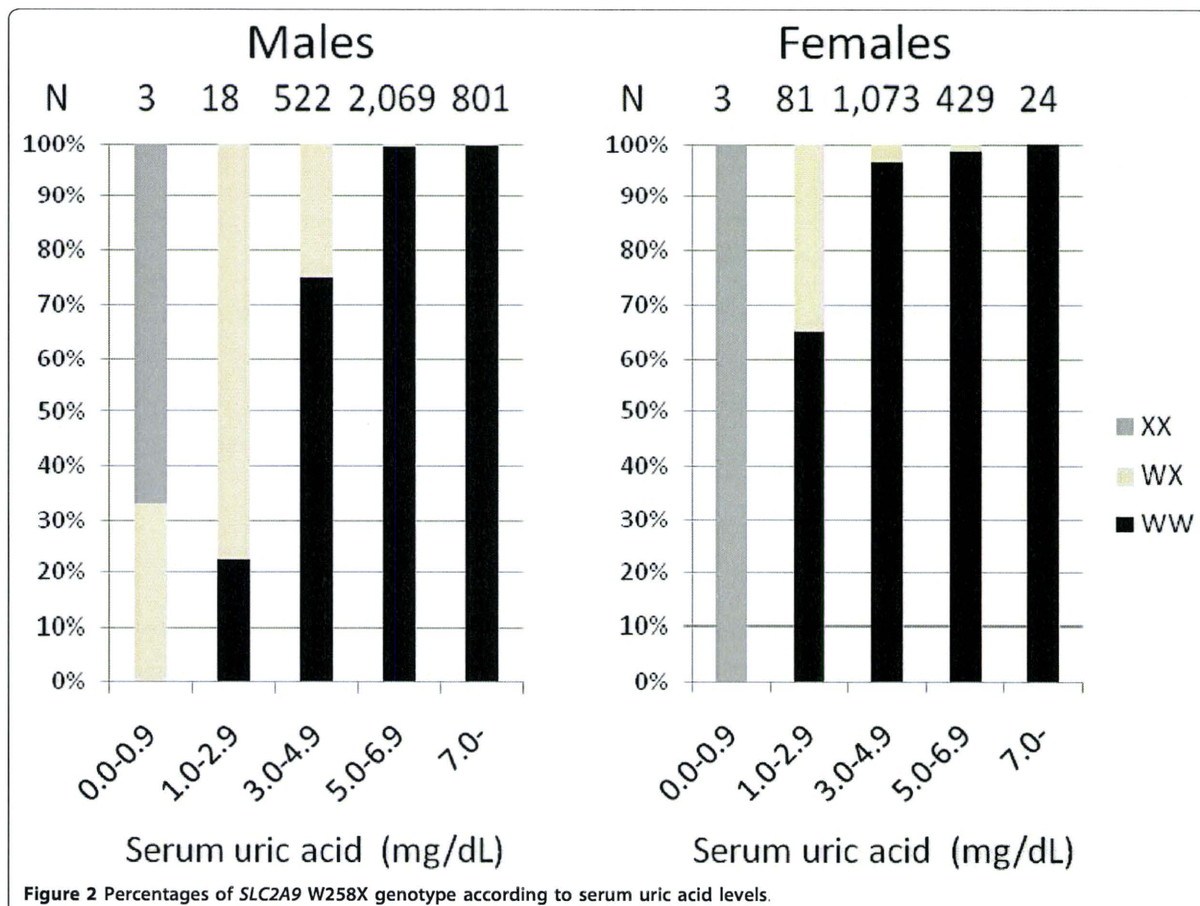

Figure 2 Percentages of *SLC2A9* W258X genotype according to serum uric acid levels.

Table 4 Odds ratio (OR) and 95% confidence interval (95% CI) of low serum uric acid (SUA) for *SLC22A12* W258X among Japanese health checkup examinees

	SUA < 3 mg/dL				SUA < 4 mg/dL			
Genotype	**< 3 mg/dL**	**3 mg/dL ≤**	**OR**	**95% CI**	**< 4 mg/dL**	**4 mg/dL ≤**	**OR**	**95% CI**
Males								
WW	4	3,252	1	Reference	55	3,201	1	Reference
WX or *XX*	17	140	102.5	33.9-309.8	79	78	58.2	38.6-87.9
Total	21	3,392			157	3,279		
Females								
WW	53	1,484	1	Reference	436	1,101	1	Reference
WX or *XX*	31	42	25.6	14.4-45.3	58	15	11.9	6.6-21.5
Total	84	1,526			494	1,116		
Both sexes								
Males with *WW*	4	3,252	1	Reference	55	3,201	1	Reference
Males with *WX* or *XX*	17	140	106.5	35.3-321.6	79	78	63.7	42.0-96.5
Females with *WW*	53	1,484	27.2	9.8-75.4	436	1,101	22.4	16.8-29.9
Females with *WX* or *XX*	31	42	672.6	225.4-2006.8	58	15	246.3	130.9-363.7
Interaction*			0.23	0.07-0.80			0.17	0.08-0.35
Total	105	4,918			628	4,395		

*Interaction between females and *X* allele for low SUA.

to that among the females with *WW* genotype (Table 3), the effect of *X* allele on SUA was similar to the effect of sex difference. BMI did not significantly modify the effect of the *X* allele on SUA both for males and females. These findings were actually new for the associations between *SLC22A12* W258X and SUA.

Among Japanese, the frequency of *258X* allele was 0.024 among 1,875 participants of a cohort study [11], 0.023 among 980 controls in a case-control study [15], and 0.025 among 5,165 participants from another cohort study [16], which were quite similar to the estimate in the present study (0.023). Among Koreans, *258X* allele was found in 3 of 5 hypouricemia patients [17], and was 1.1% in a general population [18]. Since this allele has not been reported among other ethnic groups to date, the origin was thought to be in East Asia [19].

Several genotypes affecting SUA have been reported to date. *ABCG2* in chromosome 4q22 coding ATP-binding cassette subfamily G member 2 has functional polymorphisms, Q126X (rs72552713) and Q141K (rs2231142), with a minor allele frequency of 0.018 and 0.281 in Japanese, respectively [20]. Although the genotypes with reduced function increase the risk of hyperuricemia [20-22], *126X* was rare and *141K* was less influential on SUA. *SLC2A9* in chromosome 4p16-p15.3 coding glucose transporter 9 (GLUT9) was reported to have mutations (R380W and R198C in Japanese [23], L75R in an Israeli-Arab family, and exon 7 deletion in Ashkenazi-Jewish [24]) causing hypouricemia. Their allele frequency was very rare. Common polymorphisms including *MTHFR* C677T have been reported to have an association with SUA [25,26], but the impact was limited in comparison with the above genotypes. Accordingly, *SLC22A12 258X* seemed to be one of the important genetic traits influencing SUA among Japanese.

The present study discovered that the *X* allele had a significantly larger impact in males than in females. The OR of possessing the *X* allele was larger in males than in females; 102.5 vs 25.6 for SUA < 3 mg/dL and 58.2 vs 11.9 for SUA < 4 mg/dL. The differences in mean SUA between males and females were 0.20 mg/dL among those with *XX* genotype, 0.63 mg/dL among those with *WX* genotype, and 1.71 mg/dL among those with *WW* genotype. There was no biological explanation for these phenomena.

In the present study, since the subjects with *XX* genotype were few, the distriubtion of those with *XX* genotype might not reflect the distribution of the population with *XX* genotype. Another limitation was that the medication influencing SUA was not taken into account for the genotype frequency according to the SUA level. Since the medication was common for hyperuricemia, but not for hypouricemia, the effect due to the medication might be limited for low SUA.

Conclusions

In conclusion, this study demonstrated the SUA distribution according to *SLC22A12* W258X genotype in a large study. The effect of *X* allele was larger in males than in females. Since the *X* allele was influential and relatively common among Japanese, the information on the genotype would be useful for the interpretation of individual SUA. Since SUA distributes widely among Japanese with *WX*

genotype, further studies are warranted to elucidate the determinants of the SUA distribution among those with *WX* genotype, as well as among those with *WW* genotype.

List of abbreviations
BMI: body mass index; bp: base pairs; CI: confidence interval; GLUT9: glucose transporter 9; OR: odds ratio; PCR-CTPP: polymerase chain reaction with confronting two-pair primers; SUA: serum uric acid; URAT1: uric acid transporter 1.

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Authors' contributions
NH conceived of the study, participated in the design and coordination, and drafted the manuscript. MN and KW participated in the design and coordination, edited the data, and drafted the manuscript. RO contributed the genotyping, establishing PCR primers and PCR conditions for *SLC22A12* W258X. AH and YA participated in the coordination, organizing the informed consent process and data/sample collection. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Study Profile

Profile of Participants and Genotype Distributions of 108 Polymorphisms in a Cross-Sectional Study of Associations of Genotypes With Lifestyle and Clinical Factors: A Project in the Japan Multi-Institutional Collaborative Cohort (J-MICC) Study

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ABSTRACT

Background: Most diseases are thought to arise from interactions between environmental factors and the host genotype. To detect gene–environment interactions in the development of lifestyle-related diseases, and especially cancer, the Japan Multi-institutional Collaborative Cohort (J-MICC) Study was launched in 2005.
Methods: We initiated a cross-sectional study to examine associations of genotypes with lifestyle and clinical factors, as assessed by questionnaires and medical examinations. The 4519 subjects were selected from among

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participants in the J-MICC Study in 10 areas throughout Japan. In total, 108 polymorphisms were chosen and genotyped using the Invader assay.

Results: The study group comprised 2124 men and 2395 women with a mean age of 55.8 ± 8.9 years (range, 35–69 years) at baseline. Among the 108 polymorphisms examined, 4 were not polymorphic in our study population. Among the remaining 104 polymorphisms, most variations were common (minor allele frequency ≥0.05 for 96 polymorphisms). The allele frequencies in this population were comparable with those in the HapMap-JPT data set for 45 Japanese from Tokyo. Only 5 of 88 polymorphisms showed allele-frequency differences greater than 0.1. Of the 108 polymorphisms, 32 showed a highly significant difference in minor allele frequency among the study areas ($P < 0.001$).

Conclusions: This comprehensive data collection on lifestyle and clinical factors will be useful for elucidating gene–environment interactions. In addition, it is likely to be an informative reference tool, as free access to genotype data for a large Japanese population is not readily available.

Key words: allele frequency; cross-sectional studies; gene–environment interactions; Japan Multi-institutional Collaborative Cohort Study; polymorphism

INTRODUCTION

Although the etiology of many diseases is not completely understood, most are likely to be caused by interactions between hazardous environmental factors and the host genome. Recent advances in genotyping techniques have allowed many epidemiologic studies to investigate gene–environment interactions in chronic diseases.[1–4] Cohort and case–control studies focusing on such interactions are ongoing worldwide, and these investigations use DNA from established and new cohorts.[5–8] Understanding gene–environment interactions requires long-term cohort studies to clarify the temporality of associations and to avoid information and selection biases that are inevitable in cross-sectional and case–control studies.[9] For most multifactorial diseases, such cohort studies must be conducted on a large scale to ensure significant results.

The Japan Multi-institutional Collaborative Cohort (J-MICC) Study is a new cohort study that was launched in 2005 to examine gene–environment interactions in lifestyle-related diseases, especially cancers. It is supported by a research grant for Scientific Research on Special Priority Areas of Cancer from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT).[10,11] The J-MICC Study group is composed of 10 cohorts surveyed by 10 independent research teams.[12,13]

Although the long-term aim of this study is to elucidate gene–environment interactions in the whole cohort, some of its research objectives will be achieved by cross-sectional studies. In 2009, we started a cross-sectional study to examine correlations between lifestyle and medical factors, as assessed using questionnaires and medical examinations, and the distribution of possible related genotypes. Here we describe the recruitment and profile of the participants, including their genotype analysis, and selected demographic, lifestyle, and medical characteristics.

METHODS

Study participants, data collection, and blood sampling

The participants in this study completed questionnaires on lifestyle factors and diseases and donated blood samples at the time of the baseline survey for the J-MICC Study. The details of the J-MICC Study have been described elsewhere.[10] The participants were enrolled in 9 study areas throughout Japan between 2005 and 2008, and in 1 area in 2004, under the supervision of an associate member of the J-MICC Study. The study participants were enrolled from the community by mailing invitation letters or distributing leaflets (3 areas), or by recruiting patients at their first visit to a cancer hospital (1 area) or at health checkups (6 areas). The response rates for the baseline survey were 7.0%, 36.5%, 25.9%, 58.4%, 60.1%, 37.6%, 14.0%, 24.0%, 19.7%, and 65.5% for the Chiba, Shizuoka, Okazaki, Aichi Cancer Center, Takashima, Kyoto, Tokushima, Fukuoka, Saga, and Amami areas, respectively. For cases in which the baseline survey is still ongoing in a cohort, the latest response rate (as of 30 September 2010 or later) was used. Anthropometry, blood pressure, and blood chemistry data obtained from health checkups were available in 8 of the study areas. The subjects for the cross-sectional study comprised 500 to 600 participants enrolled consecutively in each area of the J-MICC Study, except in 2 areas, where fewer participants had been recruited. The recruitment period for the present study, however, was arbitrarily defined by the researchers in each area after the enrollment.

Of the 5108 men and women initially selected, we excluded participants for whom we did not have sufficient DNA ($n = 442$), appropriate informed consent ($n = 8$), questionnaire data ($n = 9$), or local government registration of residence in the study area ($n = 7$), as well as anyone who had declined follow up ($n = 2$) or withdrew from the study ($n = 1$), and the 120 participants who were younger than 35 years or older than 69 years. Thus, our final study group comprised 4519

participants aged 35 to 69 years.

All the participants included in this analysis had provided written informed consent. The ethics committees of Nagoya University School of Medicine (the affiliation of the former principal investigator, Nobuyuki Hamajima) and the other participating institutions approved the protocol for the J-MICC Study.

Genotyping

We chose 107 single nucleotide polymorphisms (SNPs) and 1 insertion/deletion polymorphism for genotyping, based on their potential relevance to the lifestyle and medical factors described in the next section ("Lifestyle and clinical data"). Researchers from all participating cohorts proposed potentially relevant polymorphisms, and those selected for inclusion in the present study were determined through discussion among the members of the J-MICC Study Group.

In all study areas except Fukuoka, buffy coat fractions were prepared from blood samples and stored at −80°C at the central J-MICC Study office. DNA was extracted from all buffy coat fractions using a BioRobot M48 Workstation (Qiagen Group, Tokyo, Japan) at the central study office. For the samples from the Fukuoka area, DNA was extracted locally from samples of whole blood, using an automatic nucleic acid isolation system (NA-3000, Kurabo, Co., Ltd, Osaka, Japan). The buffy coat fractions or DNA samples were anonymized in a linkable manner[14] and then sent to the central office.

The selected polymorphisms were genotyped using the multiplex polymerase chain reaction (PCR)-based Invader assay[15] (Third Wave Technologies, Madison, WI, USA) at the Laboratory for Genotyping Development, Center for Genomic Medicine, RIKEN.

Lifestyle and clinical characteristics

The lifestyle factors considered were smoking and drinking habits, coffee consumption, sleep, and mental stress, while the clinical characteristics were height, weight, blood pressure, blood glucose, glycated hemoglobin (HbA1c), serum triglyceride, total and high-density lipoprotein (HDL) cholesterol, uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (γ-GT), C-reactive protein (CRP), creatinine, and bone mineral density. Ages at menarche and menopause were also ascertained.

We used a standard questionnaire in all study areas except the Fukuoka area, where some questions are slightly different from those of other areas. Furthermore, a validated food-frequency questionnaire was used for the dietary assessment.[16–19] We were unable to directly control the quality of information from health examinations because most data were obtained at routine health checkups offered by other institutions. However, the J-MICC Study Group is now collecting information on participation in the Japan Medical Association's quality control program for clinical laboratories and the instruction manuals used for measurement of blood pressure, height, and weight. For the current report, participants whose blood was drawn less than 3 hours after their last meal were excluded from the analysis of serum lipids and blood glucose.

Statistical analysis

We tabulated selected baseline characteristics by sex and 10-year age group or by sex and study area. In this analysis, body mass index (BMI; kg/m^2) was calculated on the basis of self-reported height and body weight, as independent measurements were not available in some study areas. In the case of educational attainment, participants from the Fukuoka area were excluded from the analysis because the questionnaire used there had not included this item. Participants who consumed alcohol at least once a week were classified as drinkers. To compare characteristics among participating cohorts, we attempted to adjust for age by using the direct method (for proportions) or the general linear model (for means). The variations among study areas, however, were not significantly altered after adjusting for age. Thus, in this report, we present only crude figures by sex and study area. The difference in the minor allele frequency (MAF) among the cohorts was tested by the chi-square test for contingency tables. The MAF of the *ABCC11* Arg180Gly (T/C) polymorphism by study area is not presented here because the inter-area variation in the distribution of this genotype will be reported in a separate article.

Genotypes with distributions that departed from the Hardy–Weinberg equilibrium were assessed using the exact test[20] with the genhwi command of Stata version 8.0 (Stata Corp, College Station, TX, USA). Other statistical analyses were performed using Statistical Analysis System version 9.1 (SAS Institute Inc, Cary, NC, USA).[21] To compare the allele frequencies of genotypes in our study with those in another Japanese population, we used data from HapMap, which is an open access database that includes allele frequencies for 45 Japanese in Tokyo (HapMap-JPT, http://www.ncbi.nlm.nih.gov/snp). Of the 108 polymorphisms of interest, we made comparisons for 88. The 20 polymorphisms excluded from our analysis showed no minor alleles in our study group ($n = 4$), were not represented ($n = 15$), or had invalid data ($n = 1$, 100% heterozygotes) in the HapMap-JPT data set.

RESULTS

Our analysis included 2124 men (47.0%) and 2395 women (53.0%) with a mean age ± standard deviation at baseline of 55.8 ± 8.9 years (range, 35–69 years). There were considerable differences in the age and sex distributions of different study areas (Table 1). In Fukuoka and Saga, the

Table 1. Sex and age distribution of study participants by study area

Study area	Men										Women									
	Age (years)								Total		Age (years)								Total	
	35–39		40–49		50–59		60–69				35–39		40–49		50–59		60–69			
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Chiba	4	2.7	22	14.8	56	37.6	67	45.0	149	100.0	30	8.4	138	38.7	118	33.1	71	19.9	357	100.0
Shizuoka	21	5.0	122	29.3	175	42.1	98	23.6	416	100.0	16	10.1	35	22.0	70	44.0	38	23.9	159	100.0
Okazaki	13	4.8	29	10.6	66	24.2	165	60.4	273	100.0	12	4.7	45	17.6	85	33.3	113	44.3	265	100.0
Aichi Cancer Center	12	4.4	32	11.6	115	41.8	116	42.2	275	100.0	33	10.9	88	29.0	102	33.7	80	26.4	303	100.0
Takashima	7	4.2	18	10.7	45	26.8	98	58.3	168	100.0	27	7.2	59	15.8	102	27.3	186	49.7	374	100.0
Kyoto	37	30.3	31	25.4	48	39.3	6	4.9	122	100.0	9	23.7	19	50.0	9	23.7	1	2.6	38	100.0
Tokushima	8	11.0	21	28.8	24	32.9	20	27.4	73	100.0	1	4.5	9	40.9	10	45.5	2	9.1	22	100.0
Fukuoka	0	0.0	0	0.0	60	31.9	128	68.1	188	100.0	0	0.0	0	0.0	96	37.5	160	62.5	256	100.0
Saga	0	0.0	31	12.7	82	33.5	132	53.9	245	100.0	0	0.0	64	19.3	127	38.4	140	42.3	331	100.0
Amami	1	0.5	53	24.7	82	38.1	79	36.7	215	100.0	1	0.3	77	25.7	135	45.0	87	29.0	300	100.0
Total	103	4.8	359	16.9	753	35.5	909	42.8	2124	100.0	129	5.4	534	22.3	854	35.7	878	36.7	2395	100.0

participants originally enrolled in the J-MICC Study were limited to adults aged 50 years or older and 40 years or older, respectively.

Table 2 summarizes selected demographic, lifestyle, and medical characteristics of the participants by sex and age. Within our sample, 29.1% of men and 7.1% of women were current smokers. More than two thirds (71.4%) of men drank alcoholic beverages at least once a week, as did 27.7% of the women. Table 3 presents data on selected lifestyle and medical variables of the participants by sex and study area. Considerable variations were found among the participating cohorts.

The genotype distributions and allele frequencies of the analyzed genetic polymorphisms are summarized in Table 4. The call rate ranged from 99.40% to 99.98%. Of the 108 polymorphisms, the 4 SNPs for which we found no different alleles were *APOA1* Arg184Pro (G/C), *ESR1* IVS1-351A/G (*Xba* I), *LCAT/SLC12A4* Ser232Thr (T/A), and *SCARB1* Val135Ile (G/A). For the remaining 104 polymorphisms, the MAF varied from 0.016 (*PTGS2(COX2)* C-163G) to 0.492 (*CETP* Ile405Val (A/G)), and most of the variations were common (MAF ≥0.05 for 96 polymorphisms).

The *P* value for departures from the Hardy–Weinberg equilibrium was less than 0.05 for 19 polymorphisms. However, the only genotypes for which the difference between the observed and expected frequencies exceeded 3% were the *CETP* Ile405Val (A/G) heterozygote and the *SLC30A8* Arg325Trp (C/T) heterozygote. As shown in Table 5, some polymorphisms demonstrated a considerable difference in MAF among the participating cohorts; for 32 of the 108 polymorphisms, including *ABCC11* Arg180Gly (T/C), there was a highly significant difference in MAF among study areas ($P < 0.001$).

The Figure shows a comparison of the allele frequencies in our study population and the HapMap-JPT data set. Among 88 polymorphisms, only 5 (*ABCA1* rs2230808, *COMT* rs4680, *IL-6* rs1800796, *NOS3* rs2070744, and *VDR* rs2228570) showed a difference in allele frequencies of more than 0.1 between the 2 populations.

DISCUSSION

The present report describes the profiles of participants in a cross-sectional study within the J-MICC Study data set and the allele frequencies of 108 polymorphisms, with potential relevance to lifestyle and clinical factors, in their genomes. The allele frequencies for most polymorphisms in our study population were comparable to those in the HapMap-JPT data set.

It has been suggested that polymorphisms for *APOA1* 184Pro (C), *ESR1* IVS1-351G, *LCAT/SLC12A4* 232Thr (A), and *SCARB1* 135Ile (A) do not exist in the Japanese population (http://www.ncbi.nlm.nih.gov/snp and personal communication); however, we included them in the present study to test this notion in a large sample (>4000 people). Our results confirmed that these minor alleles were indeed absent among Japanese.

Of the remaining 104 polymorphisms, 19 showed departures from the Hardy–Weinberg equilibrium with *P* values <0.05. In most cases, however, the absolute differences between the actual and expected frequencies were minimal. Thus, these apparently small *P* values could be accounted for by the large sample size and the multiple tests used in our study, and any errors in genotyping seemed unlikely to have resulted in substantial misclassification.

Although genotype data for only 45 people, at most, are available in the HapMap-JPT data set, the allele frequencies in the HapMap-JPT population and our study population were remarkably similar for most of the polymorphisms examined (Figure). For 45 individuals, the 95% confidence intervals

Table 2. Selected demographic, lifestyle, and medical characteristics of participants by sex and age

	Men					Women				
	Age (years)				Total	Age (years)				Total
	35–39	40–49	50–59	60–69		35–39	40–49	50–59	60–69	
n	103	359	753	909	2124	129	534	854	878	2395
Educational attainment (%)[a]										
Elementary/junior high school	1.0	3.1	10.0	20.9	12.6	2.3	2.1	9.8	31.6	14.7
High school	42.7	37.8	41.9	43.7	41.9	41.9	38.4	50.7	46.4	45.6
Vocational school	14.6	9.5	6.8	3.2	6.3	15.5	13.9	12.7	10.6	12.6
Junior college	3.9	4.8	3.9	2.2	3.4	24.8	26.5	16.1	7.4	16.3
University	33.0	39.5	34.8	27.6	32.7	15.5	17.7	10.2	3.8	10.2
Postgraduate school	4.9	5.0	2.2	1.7	2.6	0.0	1.5	0.5	0.0	0.6
Others	0.0	0.3	0.4	0.7	0.5	0.0	0.0	0.0	0.1	0.0
Current smokers (%)	39.8	32.3	34.8	21.9	29.1	11.6	10.3	7.4	4.1	7.1
Ex-smokers (%)	25.2	35.7	41.8	48.6	42.9	5.4	7.9	4.8	2.8	4.8
Current drinkers (%)[b]	60.2	70.8	75.1	69.9	71.4	32.6	34.6	27.5	22.8	27.7
Exercise ≥1/month (%)	75.7	81.6	79.7	86.8	82.9	62.0	70.4	74.9	82.5	76.0
Body mass index ≥25.0 (%)	37.9	32.2	32.6	24.9	29.5	9.5	16.5	20.0	21.8	19.3
History of disease (%)										
Diabetes	1.9	4.6	8.4	13.9	9.7	0.0	0.6	3.4	7.1	3.8
Hypertension	3.9	8.9	23.2	36.2	25.2	0.0	4.3	17.1	30.5	17.9
Coronary heart disease	0.0	1.1	2.1	7.3	4.0	0.8	0.6	2.8	5.3	3.0
Stroke	0.0	1.1	2.7	3.9	2.8	2.3	0.9	1.6	2.9	2.0
Cancer	6.1	2.0	7.7	12.6	8.6	2.8	5.0	9.3	6.4	6.9
Blood pressure and blood chemistry[c]										
Systolic blood pressure (mmHg)	120.4 ± 14.3	121.1 ± 15.4	129.7 ± 17.9	135.4 ± 19.3	130.1 ± 18.8	109.6 ± 11.4	116.8 ± 18.3	125.0 ± 18.9	133.4 ± 19.0	126.5 ± 19.9
Diastolic blood pressure (mmHg)	73.6 ± 10.3	77.6 ± 11.7	81.9 ± 12.6	82.1 ± 11.1	80.8 ± 11.9	65.4 ± 7.4	72.0 ± 11.9	76.7 ± 11.6	79.0 ± 10.8	76.4 ± 11.7
Total cholesterol (mg/dl)	191.3 ± 28.9	205.3 ± 30.2	206.6 ± 32.5	205.2 ± 33.1	205.2 ± 32.3	185.6 ± 25.2	202.4 ± 31.0	223.3 ± 34.2	223.1 ± 33.5	217.9 ± 34.6
HDL-cholesterol (mg/dl)	56.2 ± 16.0	57.6 ± 15.4	59.1 ± 15.8	59.7 ± 16.2	58.9 ± 15.9	73.1 ± 15.7	68.7 ± 15.3	68.9 ± 15.0	66.2 ± 16.0	68.0 ± 15.5
Triglyceride (mg/dl)	128.6 ± 74.9	146.5 ± 96.5	135.1 ± 93.0	132.0 ± 92.9	135.9 ± 93.0	64.8 ± 28.4	88.0 ± 61.2	104.9 ± 64.2	113.7 ± 69.2	103.4 ± 65.7
Blood glucose (mg/dl)	98.0 ± 27.2	98.3 ± 15.7	103.2 ± 17.6	103.9 ± 23.3	102.1 ± 20.3	86.8 ± 7.1	92.4 ± 19.7	95.9 ± 16.8	97.1 ± 15.8	95.1 ± 16.9
HbA1c (%)	4.96 ± 0.32	5.12 ± 0.48	5.32 ± 0.86	5.30 ± 0.75	5.27 ± 0.74	4.86 ± 0.27	4.96 ± 0.36	5.17 ± 0.64	5.26 ± 0.57	5.17 ± 0.57

Plus-minus values are means ± SDs.
[a]Participants in Fukuoka area were excluded from the analysis because they were not asked about educational attainment in the questionnaire.
[b]Individuals who drank alcoholic beverages ≥1 day/week.
[c]Not available in some study areas, as shown in Table 3.

were 0.047 to 0.181, 0.208 to 0.406, and 0.393 to 0.607 for MAF values of 0.1, 0.3, and 0.5, respectively, based on a binomial distribution.

A major strength of the current study was that it provided a comprehensive collection of data on lifestyle and clinical factors. Because it is not easy to gain access to data on genotype distributions in a large Japanese population, our data might also be useful as a reference tool. However, because the participants in this study were recruited from various sources throughout Japan, associations of genotypes with lifestyle and clinical factors might vary between populations. There might also have been differences between institutions in terms of the measurement methods used in the clinical examinations, because we could not directly control the quality of the health examinations. These differences must be taken into consideration when analyzing and interpreting the data. In addition, some polymorphisms showed a substantial difference in MAF among the participating cohorts (Table 5). Yamaguchi-Kabata et al suggested that individuals from the Ryukyu Islands, including the Amami Islands, had genetic characteristics that differed considerably from those of individuals from the main islands of Japan,[22] which was consistent with our present results. Genetic variations among study areas should be taken into account in the data analysis. Furthermore, the generalizability of the study findings should be considered because the response rates were low in some areas. In most cases, however, the underlying biological mechanisms are unlikely to differ between the respondents and members of the general population. The low response rate might have been due to the recruitment methods (mailing invitation letters or distributing leaflets to the general populations of 3 areas) or the strict procedures used to obtain informed consent.

In conclusion, this comprehensive data collection on lifestyle and clinical factors will be useful in elucidating gene–environment interactions and could provide an informative reference tool, particularly because free access to genotype data for a large Japanese population is not readily