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ORIGINAL ARTICLE

Association of an intronic SNP of *SLC2A9* gene with serum uric acid levels in the Chinese male Han population by high-resolution melting method

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Abstract SLC2A9 is a novel identified urate transporter influencing uric acid metabolism. It has been suggested that the single-nucleotide polymorphisms in *SLC2A9* may affect the serum UA levels. The present study was designed to investigate rs6855911 polymorphism in intron 7 of *SLC2A9* in a total of 372 Chinese male subjects. We examined 166 gout patients, as well as 206 healthy male volunteers in this study. DNA was purified from peripheral blood, and the rs6855911 polymorphism was evaluated using high-resolution melting (HRM) analysis and direct sequencing. Demographic and clinical data obtained from the patients and controls among the genotype groups were analyzed. A/A and A/G genotypes were unambiguously distinguished with HRM technology. The occurrence of the homozygous type (G/G) was completely absent among the study population. The prevalence of the A/A and A/G genotype was 96.0% and 4.0%, respectively. Genotyping based on HRM was fully concordant with sequencing. The G allele frequency was significantly higher in the low-uric-acid group than in the high-uric-acid group. The genotype distribution and allele frequencies were not significantly different between gout and control subjects ($p=0.04$). However, serum uric acid levels in the A/G genotype subjects were significantly lower than those with the A/A genotypes ($p<0.01$). Rapid and accurate genotyping analysis of *SLC2A9* can be done with HRM. The polymorphism rs6855911 in *SLC2A9* may be a genetic marker to assess risk of hyperuricemia among Chinese male Han population.

Keywords Gout · Hyperuricemia · Single-nucleotide polymorphism · *SLC2A9* · Uric acid

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Introduction

The incidence and prevalence of gout or hyperuricemia are rising worldwide [1, 2]. Although higher serum urate levels have been suggested to be beneficial [3], some epidemiological studies also showed an association between high uric acid (UA) levels and cardiovascular disease, hypertension, renal disease, and metabolic syndrome [4, 5]. It was well recognized that genetic factors have an important effect on the incidence of gout. Among family members of patients with gout, the prevalence of asymptomatic hyperuricemia ranges from 25% to 70% [6–8].

Recently, several separate genome-wide association scans have identified common genetic variants of *SLC2A9* to be associated with increased serum urate level and gout [9–11]. SLC2A9 (also known as GLUT9) is a known fructose transporter. Members of this gene family play a

significant role in maintaining glucose homeostasis. However, new findings have unearthed a novel role for SLC2A9 as a modulator of uric acid levels. *SLC2A9* located on chromosome 4 are the most extensively replicated genetic loci associated with serum uric acid levels. The gene effects were larger in women than in men. *SLC2A9* is a causative gene for renal hypouricemia and plays a key role in urate reabsorption on renal proximal tubular cells. The systemic and liver-specific *SLC2A9*-knockout mice develop similar hyperuricemia and hyperuricosuria [12]. Functional impairment of this protein is associated with idiopathic renal hypouricemia. Some loss-of-function mutations of the *SLC2A9* gene are associated with renal hypouricemia by their decreased urate reabsorption on both sides of the renal proximal tubules [13, 14].

The presence of SLC2A9 variants and its disease association has been reported in different populations including Germans [15], Japanese [14], Italians [9], and American Amish people [16]. Two recent studies [9, 11] showed that single-nucleotide polymorphism (SNP) rs6855911 was highly significantly associated with serum uric acid concentrations. Individual homozygous for the rare allele of rs6855911 (minor allele frequency=0.26) had 0.6 mg/dL less uric acid than those homozygous for the common allele.

However, no studies have been done on the presence of the rs6855911 among Chinese Han population. Moreover, after the discovery of the gene, the number of patients with mutations in the *SLC2A9* gene has increased, and, as a result, a rapid method for detecting those mutations would be highly desirable.

High-resolution melting (HRM) was recently introduced as a simple and reliable technology for genotyping [17]. HRM can precisely monitor the change in fluorescence caused by the release of an intercalating DNA dye from a DNA duplex as it is denatured by increasing temperature [18]. In the present study, we developed an HRM assay to detect an *SLC2A9* polymorphism rs6855911 and conducted an association study between genotypes and parameters of urate homeostasis in Chinese male Han population.

Materials and methods

Patients

One hundred sixty-six non-related, consecutive male outpatients with untreated primary gout according to the American College of Rheumatology diagnostic criteria and 206 age-matched healthy male volunteers without personal or familial history of hyperuricemia or gout were recruited into this study. Any subject with any secondary causes of gout, e.g., any malignancy, and any subject receiving medications which may have potentially affected determination of serum UA levels were excluded. Patients with liver diseases, diabetes mellitus, kidney diseases, and other metabolic disease were excluded to eliminate the influence on uric acid levels. In our study, two patients had to be excluded due to diabetes mellitus and two because of liver diseases. These patients are not contained in our final patient group of 166 individuals. Information about the medical history, condition, and family history of the subjects was obtained from a medical interview of each subject at the time of enrollment. Hyperuricemia was defined as serum uric acid >420 μmol/L for men according to guidelines [19]. All study subjects gave their written consent to participate in the study. The study protocol was approved by the Ethics Committee of the Huashan hospital.

Clinical laboratory parameters

Blood samples were obtained in the morning after an overnight fast. Serum uric acid, blood urea nitrogen (BUN), creatinine, total cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured using a Clinical Analyzer 7600 (Hitachi High-Technologies, Tokyo, Japan).

High-resolution melting analysis

Genomic DNA was isolated from peripheral blood lymphocytes. DNA was extracted with a QIAamp DNA Blood Kit (Qiagen, Valencia, CA, USA). Primer sequences were designed to amplify a small fragment surrounding the polymorphism and avoid the presence of other sequence variations. Primers for the 101-bp amplicon which spanned rs6855911 polymorphism were 5′-tgtctgcccactgtaccact-3′ and reverse primers 5′-gggaagcatctgctccagt-3′. The reaction mixture was made up using HotStarTaq DNA Polymerase (Qiagen) and consisted of 20 ng of genomic DNA, 1× polymerase chain reaction (PCR) buffer, 2.5 mM $MgCl_2$ total, 200 nM of each primer, 200 μM of dNTPs, 5 μM of SYTO 9, 0.5 U of HotStarTaq polymerase, and PCR-grade water in a volume of 20 μL. PCR cycling and HRM were performed on Rotor-Gene 6000™ (Corbett Research, Mortlake, New South Wales, Australia). All the analyses were run according to the following conditions: one cycle of 95°C for 15 min; 40 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 20 s; and a melt from 75°C to 95°C at intervals (ramps) of 0.1°C/s. HRM curve analysis was performed using Rotor-Gene 6000 1.7 software.

Sequencing

Twenty randomly selected samples including 10 A/A genotype samples and 10 A/G genotype samples identified

with HRM were subject to direct sequencing. Amplicons were gel-purified using the QIAquick gel purification kit (Qiagen). DNA sequencing analysis was performed in ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Statistical analyses

Descriptive statistics are given as mean values±standard deviation. Comparison of means was performed by the unpaired Student's *t* test. Genotype and allele frequency distributions were compared using Chi-squared test. *P* values of less than 0.05 were considered statistically significant.

Results

Clinical and laboratory variables

The clinical features of individuals enrolled in the study are summarized in Table 1. Serum concentrations of BUN, total cholesterol, LDL-C, and blood pressure were not significantly different between the gout patients and control subjects. However, the serum concentrations of creatinine, triglyceride, HDL-C, uric acid, and BMI were significantly higher in the gout patients (Table 1) ($p<0.05$).

Genotyping with HRM and sequencing

Amplicon melting analyses in the presence of the saturating HRM dye were used to detect SNP using the Roter-Gene 6000 System. HRM analysis allows clear discrimination between the homozygous (A/A) and heterozygous (A/G) genomic DNA samples based on differences in melting curve shapes (Fig. 1a), which is consistent with the lower thermal stability of A/G base pairs compared with AA base pairs. Heterozygotes were readily distinguished by their characteristic biphasic melting patterns (Fig. 1b). However, in some cases, A/A genotype may produce a curve that was similar in shape to the G/G type. To avoid mis-clarification of genotyping between A/A and G/G, exogenous DNA sample with AA genotype confirmed by sequencing was added (in a 1:1 ratio) to every homozygous samples. If unknown samples are A/A type, their melting curves do not change after the addition of exogenous A/A DNA template. If the unknown samples are G/G type, heteroduplexes are produced, and samples can be correctly identified as homozygous G/G type. We did not identify any G/G genotype in all samples. The genotype analysis by HRM was 100% concordant with the results obtained by sequencing for the all selected samples.

Distribution of *SLC2A9* genotypes

Among the 372 subjects, 357 cases (96.0%) had the A/A genotype, and 15 cases (4.0%) had A/G genotype. The frequency of the A allele was 98.0% and that of the G allele was 2.0%. The occurrence of the homozygous type (G/G) was completely absent among the study population. Genotype distributions for SNP rs6855911 were in Hardy–Weinberg equilibrium in both gout patients and healthy controls. Testing for departure from Hardy–Weinberg equilibrium was performed separately for the gout and control groups and revealed *p* values of 0.81 and 0.75, respectively, as determined by χ^2 statistics. Differences in the distribution of both genotypes ($p=0.71$) and alleles ($p=0.72$) between gout patients and normal controls did not reach statistical significance (Table 2). Furthermore, the presence of rs6855911 genotypes did not change the risk for gout disease (odds ratio (OR)=1.21, 95% confidence interval (CI)= 0.43~3.44, $p=0.72$).

Table 1 Clinical and biochemical profile of gout patients and controls

Index	Gout patents	Controls	*p*
Subjects (%)	166 (44.6)	206 (55.4)	
Age (year)	56.1±16.0	54.6±19.6	NS
BMI (kg/m^2)	27.8±4.1	23.4±2.7	<0.01
Systolic blood pressure (mmHg)	126.1±10.3	124.5±11.6	NS
Diastolic blood pressure (mmHg)	75.9±7.9	74.8±5.6	NS
BUN (mmol/L)	5.9±2.4	5.6±2.1	NS
Creatinine (μmol/L)	104.7±18.7	96.4±14.3	<0.01
Total cholesterol (mmol/L)	5.4±1.2	5.1±0.9	NS
Triglyceride (mmol/L)	2.4±1.4	1.5±0.8	<0.01
HDL-C (mmol/L)	1.4±0.4	1.2±0.5	<0.01
LDL-C (mmol/L)	3.6±1.1	3.4±0.9	0.05
Uric acid (μmol/L)	438.4±133.1	324.3±91.4	<0.01

Data are expressed as mean±SD

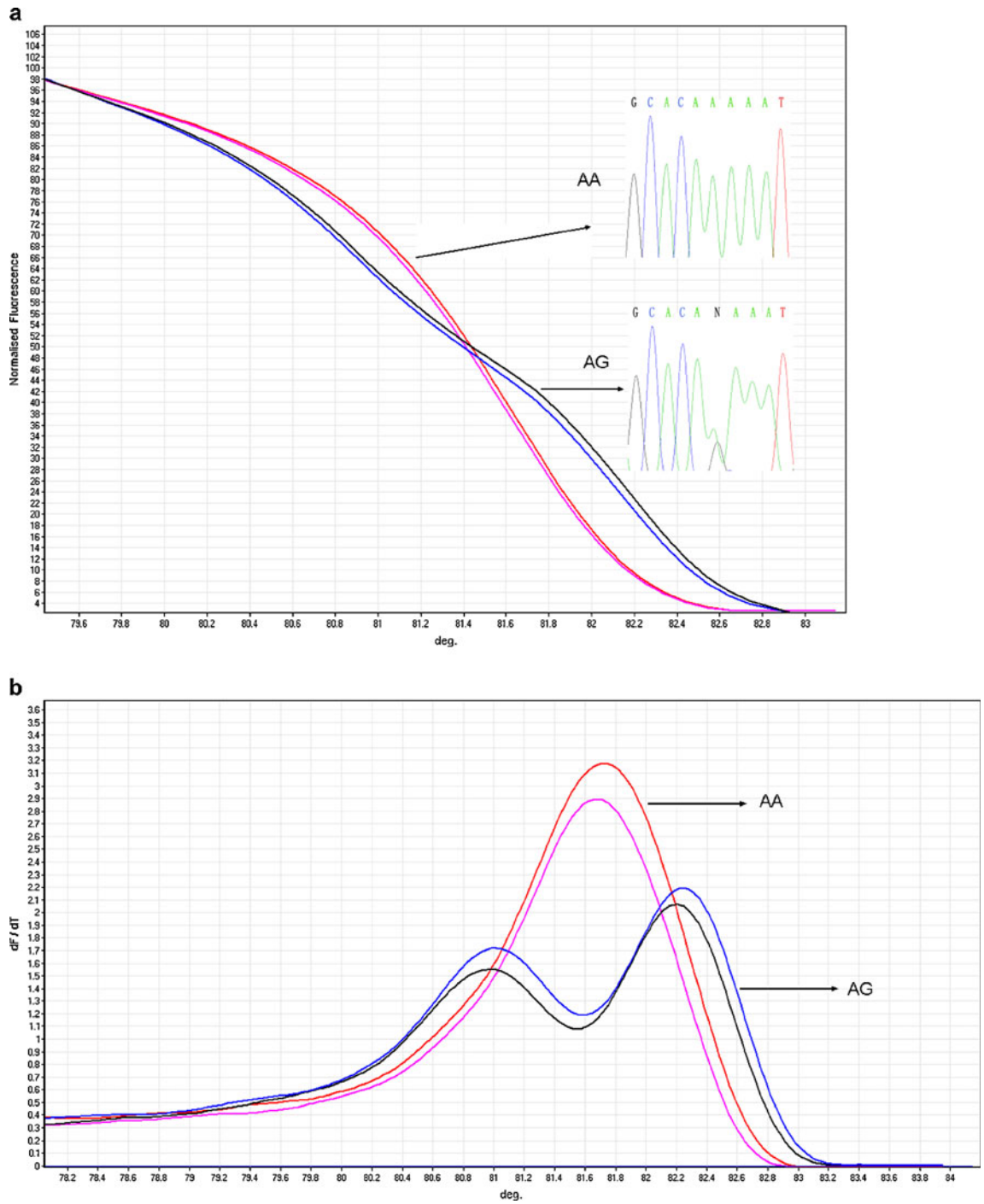

Fig. 1 Discrimination of rs6855911 SNP genotypes (A/A and A/G) using SYTO 9 intercalation dye. Amplification and HRM analysis were done using a Rotor-Gene 6000 instrument, and genotypes were automatically assigned by the Rotor-Gene software. **a** Two samples with homozygous type (A/A) (*red* and *pink*) and two samples (*blue* and *black*) with heterozygous type (A/G) are shown on a standard normalized melt plot. **b** Dissociation curve analysis of A/A and A/G type

SLC2A9 genotypes and uric acid levels

The individuals were divided into two groups according to their uric acid levels: a normal-uric-acid group (≤420 μmol/L) and a high-uric-acid group (>420 μmol/L). The genotype frequencies differed significantly between these two groups (Table 2) (p=0.04). The G allele frequency was also higher in the normal-uric-acid group than in the high-uric-acid group (p=0.04). Results from the odds ratio analysis have shown that the risk of hyperuricemia was 4.1 times greater in male with AA genotype compared with individuals with A/G genotype (OR=4.1, 95% CI=0.92~18.31).

Table 2 Genotype and allele distribution of the rs6855911 in patients with gout and hyperuricemia

Group	Genotypes frequencies (%)		*p*	Allele frequencies (%)		*p*
	A/A	A/G		A	G	
Gout	160 (96.4)	6 (3.6)	0.71	326 (98.2)	6 (1.8)	0.72
Normal	197 (95.6)	9 (4.4)		403 (97.8)	9 (2.2)	
Hyperuricemia (level>420μol/L)	140 (98.6)	2 (1.4)	0.04	282 (99.3)	2 (0.7)	0.04
Normal uric acid (level≤420 μmol/L)	217 (94.3)	13 (5.7)		447 (97.2)	13 (2.8)	

Association between genotypes and biochemical indexes

The distribution of physical and biochemical variables, in subjects classified by rs6855911 genotype, was shown in Table 3. The unpaired *t* test showed that the serum uric acid levels differed significantly between the two genotypes ($p<0.01$). A/G group (284.5 mmol/L) had a significantly lower mean uric acid level than AA group (380.7 mmol/L). Other urate homeostasis parameters were not affected by the rs685591 genotype.

Discussion

There is overwhelming evidence that genetic factors influence the regulation of blood uric acid levels. It is therefore of importance to identify with certainty those variants which are associated with increased UA levels.

In 2002, SLC22A12 has been identified as a urate anion exchanger in the human kidney [20]. Inactivating mutations in *SLC22A12* have been shown to cause renal idiopathic hypouricemia [21, 22]. Previously, we examined the *SLC22A12* gene for the rs893006 SNP in a Chinese population. We found that the presence of T/T allele was associated with lower uric acid levels, which resulted in significantly different serum uric acid levels among the three genotypes in male subjects [23]. However, the fact that patients with renal hypouricemia had no mutation in *SLC22A12* [21, 24] implies the existence of another important urate transporter in the human kidney.

Recent genome-wide association studies have revealed that the most significant SNPs associated with urate concentrations map within *SLC2A9*. In the present study, we identified the rs6855911 SNP of *SLC2A9* in a Chinese cohort of unrelated patients with primary gout and unaffected controls. The A and G allele frequencies were found to be 98% and 2%, respectively among Chinese male population. There were no individuals homozygous for the G/G genotype in the study population. No statistically significant difference of genotype distribution existed between gout patients and normal individuals ($p=0.71$). Our results are inconsistent with the findings of Stark et al. [15], who found that the polymorphism had highly significant association with gout. The discordant results may reflect, at least partly, the widely different genotype distributions among different populations. According to the records in dbSNP (NCBI), the frequencies of the G allele are 30.8% in Caucasian subjects and 45.0% in sub-Saharan Africans. Therefore, the G allele frequency in our cohort was one of the lowest reported in the literature (2%).

Table 3 Clinical data for subjects grouped by rs6855911 SNP genotypes

Indexes	rs685591 genotypes		*p*
	A/A	A/G	
Subjects (%)	357 (96.0)	15 (4.0)	
Age (year)	55.3±13.5	53.5±14.4	0.61
BMI (kg/m^2)	25.4±5.7	24.5±4.0	0.54
Systolic blood pressure (mmHg)	125.4±13.8	120.8±14.6	0.21
Diastolic blood pressure (mmHg)	74.8±8.9	73.2±7.8	0.50
BUN (mmol/L)	5.7±2.4	5.6±3.1	0.81
Creatinine (μmol/L)	100.3±21.3	94.5±19.6	0.30
Total cholesterol (mmol/L)	4.8±2.0	4.6±1.8	0.66
Triglyceride (mmol/L)	1.9±0.9	1.9±1.1	0.86
HDL-C (mmol/L)	1.3±0.5	1.3±0.3	0.82
LDL-C (mmol/L)	3.5±1.0	3.2±1.2	0.31
Uric acid (μmol/L)	380.7±130.8	284.5±97.6	<0.01

Data are expressed as mean±SD

Since rs6855911 was reported to be strongly associated with serum uric acid levels [9, 11], to determine whether the SNP might also affect the circulating uric acid levels in Chinese male Han population, we measured serum uric acid concentrations as well as other biomedical parameters in 372 subjects of the population studied. Homozygous carriers of the A allele has higher serum uric acid levels than the carriers of the A/G genotypes (A/A 380.7 μmol/L; A/G 284.5 μmol/L). Compared with the elevated uric acid group, the frequency of the G allele was higher in the group with low uric acid levels. In agreement with previous studies [9, 15], our results also provide evidence that there is a significant negative effect of *SLC2A9* polymorphism rs6855911 on serum uric acid levels, resulting from a protective effect of the minor alleles. Female subjects were not included in this study due to the lower incidence of gout in female. Therefore, this SNP marker could be helpful for stratification of risk for hyperuricaemia in the Chinese male population.

Recently, it was proven that SLC2A9 functions as a urate reabsorption transporter on human renal proximal tubular cells and plays a role in the direct regulation of serum urate levels [14]. Other genetic variants within the *SLC2A9* gene have been identified to be associated with susceptibility to hyperuricemia and gout. McArdle et al. [16] revealed a nonsynonymous coding SNP (rs16890979) within *SLC2A9* that was correlated with serum urate levels in a cohort of Amish population and also significantly associated with gout in the Framingham Heart Study. SNP rs3733591 variant within *SLC2A9* gene from two geographically diverse populations (Taiwan and Solomon Islands) served as an important genetic checkpoint for tophaceous gout and increased uric acid levels [25]. Another study by Stark and colleagues showed that four SNPs within or near the *SLC2A9* demonstrated highly significant association with 665 gout patients and 665 healthy controls in Germany [15]. These studies suggest that genetic polymorphisms in *SLC2A9* may be implicated in the development of gout, although further studies are needed to understand how the identified SNPs affect urate handling by *SLC2A9*.

This study focused on using HRM analysis of relatively small amplicons for rs6855911 detection and identification without the use of unlabeled probes. HRM analysis was 100% accurate in distinguishing heterozygous type from homozygous type samples. Compared with TaqMan-based SNP genotyping and pyrosequencing, the HRM approach is much more cost-effective. Therefore, HRM may become the method of choice for rs6855911 genotyping, as it is a novel, accurate, and simple technique, and no processing or separations are required.

However, not all homozygotes can be distinguished by curve shape or Tm when the base pair is inverted or neutral (A:T to T:A or G:C to C:G). In such a case, a known homozygote can be mixed into each unknown homozygote, and the mixture is melted again for complete genotyping.

To rule out the possibility that homozygous A/A type and homozygous G/G could not be exactly discriminated, each homozygous sample was spiked with the DNA sample of A/A type as internal standard [26, 27], no extra peak appeared in the dissociation curve, indicating that the sample was identical to the A/A type.

It might be considered a limitation of our study that we did not know how the intronic SNP affects UA levels. This might be partly explained by the presence of strong linkage disequilibrium with a nonsynonymous SNP in exon 8 causing a Val253Ile amino acid change [9]. Further analyses are necessary to identify the causal variants in the region.

In conclusion, the findings of this study revealed that the G/G genotype of rs6855911 SNP was completely absent among male Chinese Han population. Our results showed for the first time that rs6855911 SNP was associated with uric acid level, which might be a genetic marker to assess risk of hyperuricemia in male Chinese Han population. However, larger sample sizes are required to corroborate this finding. The HRM we established herein may become a useful technology in providing this SNP screening information.

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Disclosures None

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