High-resolution melting analysis for the rapid detection of an intronic single nucleotide polymorphism in SLC22A12 in male patients with primary gout in China

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Objectives: The human urate transporter 1 (URAT1, encoded by SLC22A12) was recently identified as the major absorptive urate transporter protein in the kidney responsible for regulating blood urate levels. The present study was designed to investigate the rs893006 polymorphism (GG, GT, and TT) in SLC22A12 in a total of 292 Chinese male subjects. Differences of clinical characteristics among the genotype groups were analysed.

Methods: A total of 124 consecutive patients with diagnosis of primary gout and 168 healthy male volunteers were enrolled in this study. Demographic and clinical data were obtained from the patients and controls. DNA was purified from peripheral blood and the rs893006 polymorphism was determined with sequencing analysis. In addition, DNA samples were detected by high-resolution melting (HRM) analysis. Melting curves were analysed as fluorescence difference plots. The shift and curve shapes of melting profiles were used to distinguish the different genotypes.

Results: GG, GT, and TT genotypes were unambiguously distinguished with HRM technology. Genotyping based on HRM analysis was fully concordant with the sequencing. Serum uric acid levels in the TT genotype subjects were significantly lower than those in the GG and GT genotypes. However, no differences among the groups were found in body mass index (BMI), blood pressure, creatinine, total cholesterol, and triglycerides. The TT genotype was observed more frequently among the low uric acid group than the high uric acid group.

Conclusions: HRM analysis is a simple, rapid and accurate one-tube assay for genotyping the SLC22A12 gene. The rs893006 polymorphism in SLC22A12 was confirmed to be a genetic risk for hyperuricaemia among the Chinese male population.

Hyperuricaemia and gout have shown an increase worldwide. One recent study showed that the age-standardized prevalence was 25.3% for hyperuricaemia and 0.36% for gout in adults aged 20–74 years in China (1). Hyperuricaemia is associated with insulin resistance (2). For some high-risk patients hyperuricaemia is likely to be an independent cardiovascular risk factor rather than just an association (3, 4).

It is 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 hyperuricaemia ranges from 25% to 27% (5). The serum level of uric acid in humans is mainly regulated by urate reabsorption in the proximal tubule. Urate transporter 1 (URAT1) is thought to be the major protein responsible for regulating blood urate levels (6, 7). URAT1 is encoded by the SLC22A12 gene (11q13), which is made up of 2642 base pairs in 10 exons. URAT1 is a membrane transporter responsible for the reabsorption of urate in the apical membrane of the renal proximal tubules (7). Mutations in the URAT1 gene, causing functional impairment, are associated with idiopathic renal hypouricaemia (8, 9). The important role of URAT1 in renal urate reabsorption was confirmed by finding polymorphisms of the SLC22A12 gene associated with reduced uric acid excretion and hyperuricaemia (10).

After the discovery of the gene, the detection of mutations in the URAT1 gene in patients increased and therefore a rapid method for detecting such mutations is highly desirable.

High-resolution melting (HRM) analysis was recently introduced as a simple technology for genotyping. An important advantage of HRM...
analysis over many other methods is that it is combined with rapid-cycle polymerase chain reaction (PCR), and is thus particularly suitable for high-throughput DNA diagnostics (11, 12).

In this study, we set out to examine the URAT1 gene for the rs893006 polymorphism in a clinically well-defined Chinese male population of patients with gout and unaffected controls with sequencing analysis. We also developed a sensitive, clinically useful HRM assay to investigate whether the technology has the potential to reduce the sequencing burden and whether it would be suitable for the genotyping of this gene. An association study between genotypes and parameters of urate homeostasis was conducted.

Materials and methods

Patients

A total of 124 non-related, consecutive male outpatients with primary gout according to the American College of Rheumatology (ACR) diagnostic criteria and 168 healthy male without a personal or familial history of hyperuricaemia or gout were enrolled in this study. All patients and controls underwent a standardized questionnaire that contained questions on demographic, dietary, drinking, smoking information, personal and family history of diabetes, gout, and hyperuricaemia. The patients with gout did not receive any anti-hyperuricaemia therapy. Blood pressure, body weight, and height were obtained and body mass index (BMI) was calculated. Hyperuricaemia was defined as serum uric acid >420 µmol/L for men according to the epidemiologic study (13). The study was approved by the Ethics Committee of Huashan Hospital.

Clinical laboratory parameters

Blood samples were obtained in the morning after an overnight fast. Serum uric acid, creatinine, total cholesterol, and triglycerides were measured using a Clinical Analyzer 7600 (Hitachi High-Technologies, Tokyo, Japan).

Sequencing

Genomic DNA was isolated from peripheral blood lymphocytes. DNA was extracted with a QIAamp DNA Blood Kit (Qiagen, Valencia, CA, USA). Primers for the 137-bp amplicon that spanned the rs893006 polymorphism were 5'-CTGAGG-ACCCCGAAGGCGTTGTGCAGCC-3' and 5'-ATCAGCCAGACCCAGAATGAGACAGCAT-3'. PCR products were purified with a PCR purification kit (Qiagen II, Qiagen). DNA sequencing analysis was performed in an ABI PRISM 310 genetic analyser (Applied Biosystems, Foster City, CA, USA).

HRM analysis

PCR amplification and HRM analysis were carried out on Rotor-Gene 6000 (Corbett Research, Mortlake, Australia) with the same pair of primers. The intercalating dye used was SYTO 9 (Invitrogen, Carlsbad, CA, USA). The reaction mixture was made up using HotStarTaq (Qiagen) and consisted of 30 ng of genomic DNA, 1 × PCR buffer, 1.5 mM MgCl₂, total, 200 nM of each primer, 200 µM of dNTPs, 1.5 µM of SYTO 9, 0.5 U of HotStarTaq polymerase, and PCR-grade water in a volume of 20 µL. All PCR reactions were performed in duplicate. The 137-bp amplicon was 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 15 s, 72°C for 15 s; one cycle of 95°C for 1 s, 72°C for 90 s, and a melt from 75°C to 95°C rising at 0.2°C/s. Fluorescence difference curves were generated from normalized temperature-shifted data by selecting a control for comparison and subtracting the fluorescence of the control from all other melting curves. The fluorescence difference between all other curves and the comparison curve was then plotted against temperature.

Statistical analyses

The association between demographic and clinical variables and genotypes was calculated by an analysis of variance (ANOVA) and the χ²-test. p-values <0.05 were considered statistically significant.

Results

Distribution of SLC22A12 genotypes

The frequency of the G allele was 0.821 and that of the T allele was 0.279. Among the 292 subjects, 154 (52.7%) had the GG genotype, 113 (38.7%) had the GT genotype, and 25 (8.6%) had the TT genotype. Genotype distributions for the rs893006 single nucleotide polymorphism (SNP) were in Hardy–Weinberg equilibrium in both gout patients and healthy controls. The genotypic and allelic frequencies of the SNP in gout patients and in controls are shown in Table 1. There was no statistically significant difference in genotypic frequencies between patients and controls (p=0.308).

Association between genotypes and biochemical indexes

The distribution of the physical and biochemical variables in the subjects classified by the SLC22A12 genotype is shown in Table 2. An ANOVA showed that the serum uric acid levels differed significantly among the three genotypes (p=0.018). The TT group had a significantly lower mean uric acid level (310 µmol/L) than the GG (422 µmol/L) or the GT
Table 1. Genotype distributions of the rs893006 SNP in gout patients and controls.

<table>
<thead>
<tr>
<th>rs893006 genotype</th>
<th>Cases</th>
<th></th>
<th></th>
<th></th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG (%)</td>
<td>GT (%)</td>
<td>TT (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gout patients</td>
<td>67 (54.0)</td>
<td>50 (40.3)</td>
<td>7 (5.6)</td>
<td>0.308</td>
<td></td>
</tr>
<tr>
<td>Normal controls</td>
<td>87 (51.8)</td>
<td>63 (37.5)</td>
<td>18 (10.7)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Clinical data for subjects grouped by SLC22A12 genotype.

<table>
<thead>
<tr>
<th></th>
<th>rs893006 genotype</th>
<th></th>
<th></th>
<th></th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>Subjects, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age (years)</td>
<td>54.05</td>
<td>52.58</td>
<td>54.00</td>
<td>0.133</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.18</td>
<td>23.77</td>
<td>24.32</td>
<td>0.561</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure (mg/dL)</td>
<td>124.81</td>
<td>124.39</td>
<td>125.33</td>
<td>0.474</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure (mg/dL)</td>
<td>82.14</td>
<td>81.36</td>
<td>82.51</td>
<td>0.320</td>
<td></td>
</tr>
<tr>
<td>Uric acid (µM)</td>
<td>422</td>
<td>417</td>
<td>310</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>Creatinine (µM)</td>
<td>92.18</td>
<td>93.27</td>
<td>85.73</td>
<td>0.889</td>
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</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>4.76</td>
<td>4.46</td>
<td>4.21</td>
<td>0.215</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.87</td>
<td>1.87</td>
<td>1.36</td>
<td>0.549</td>
<td></td>
</tr>
</tbody>
</table>

SLC22A12 genotypes and uric acid levels

As a decreasing trend in uric acid level was observed as the number of T allele copies increased (shown in Table 2), subjects were divided into two groups based on the uric acid level: a low uric acid group (<420 µmol/L) and a high uric acid group (>420 µmol/L). Among these, 21 gout patients had normal uric acid levels and 11 normal controls had the high uric acid levels. The genotype frequencies differed significantly between these two groups (Table 3) (p=0.017). The T allele frequency was also higher in the low uric acid group than in the high uric acid group, although it did not reach statistical significance (p=0.07).

Genotyping with HRM analysis

HRM analysis revealed differences in the melting curve shape that correlated to the genotype and the presence of SNP heterozygotes. Figure 1A shows the melting curves from homozygous and heterozygous 137-bp products overlaid at high temperature to aid comparison visually. The TT genotype produced a curve that was similar in shape to the GG type but with earlier melting of the amplified product compared to the GG-type samples, which is consistent with the lower thermal stability of the AT base pairs compared with GC base pairs. Heterozygotes were easily distinguished from homozygotes based on the shape of the melting curves. In addition, homozygote discrimination is based on differences in melting temperature (Tm). The Tm difference between the homozygous G and T variants was approximately 0.5°C and proved to be sufficient for discrimination between the GG and the TT type. The amplified fragments from the GG type had a specific peak at a Tm of 87.9°C, whereas the amplified fragments from TT and GT had a specific peak at a Tm of 88.4°C (Figure 1B). All samples were of known genotypes and were grouped correctly by the Rotor-Gene 6000 Software. The difference plots normalized with the TT type shown in Figure 1C clearly separate the homozygous genomic DNA samples (GG and TT) from the heterozygous one (GT) for the HRM assays.

Discussion

Hyperuricaemia is the most important risk factor for gout and, in 90% of patients, is secondary to renal uric acid underexcretion. The human URAT1 has been identified as a highly specific renal urate/anion exchanger and was recently identified as the major absorptive urate transporter protein in kidney responsible for regulating blood urate levels. The important role of URAT1 in renal urate reabsorption was subsequently confirmed by the finding that the mutations in the human URAT1 (hURAT1) gene dramatically increased uric acid excretion and the development of idiopathic renal hypouricaemia (6, 14, 15).
Figure 1. Discrimination of human SLC22A12 rs893006 SNP genotypes (GG, TG, and TT) using the SYTO 9 intercalation dye. Amplification and HRM analysis were conducted using a Rotor-Gene 6000 instrument and genotypes were automatically assigned by the Rotor-Gene software. (A) Homozygous types (GG and TT) and heterozygote samples (GT) are shown on a standard normalized melt plot. (B) Dissociation curve analysis of GG, TT, and GT types. (C) Homozygous types (GG and TT) and heterozygote samples (GT) are shown on a difference plot normalized to TT genotype samples.
The rs893006 genetic polymorphism is located on intron 4 of SLC22A12. This SNP resulting in a G to T transversion is associated with higher urate levels in Japanese subjects (16). There is also substantial ethnicity-related diversity at this genetic polymorphism. In the present study, we examined the URAT1 gene for the rs893006 SNP in a clinically well-defined Chinese population of unrelated patients with primary gout and unaffected controls. An association study between this SNP and parameters of urate homeostasis was performed. We found that the presence of the TT allele was associated with lower uric acid levels, which resulted in significantly different serum uric acid levels among the three genotypes in the male subjects. Female subjects were not included in this study because of the lower incidence of gout in females. Compared with the elevated uric acid group, the prevalence of the TT genotype and the frequency of the T allele were higher in the group with low uric acid levels.

Recently, Shima et al (16) investigated the effects of the rs893006 polymorphism of the hURAT1 gene on serum uric acid levels in a Japanese population. Our results are consistent with their studies, which show that the TT group has the lowest uric acid level, indicating that this SNP could be a genetic risk factor for hyperuricaemia in Chinese patients as well. The genotype frequencies, however, were different between the two populations. The prevalence of the CC, CT, and TT polymorphisms in the Japanese male subjects was 70.0, 26.7, and 3.3%, compared with 52.7, 38.7, and 8.6% in the Chinese cohort, respectively. In addition, our study failed to reveal any difference in uric acid levels between the GG and GT subjects. This discrepancy may indicate their high specificity for the Japanese population.

Other SNPs in URAT1 have been implicated in an increase in uric acid in a German population (17). A significant association with reduced fractional excretion of uric acid (FEUA) was shown for the −788T>A (promoter), C258T (exon 1), and C426T (exon 2) polymorphisms. The analysis of association of the URAT1 C426T polymorphism with urate homeostasis showed that serum uric acid concentration was significantly lower in individuals with the CC genotype in comparison with individuals with the CT genotype and the TT genotype. These studies suggest that genetic polymorphisms in SLC22A12 may be implicated in the development of gout, although further studies are needed.

Patients who have homozygous loss-of-function mutations in SLC22A12 do not respond to pyrazinamide and benzbromarone (14), suggesting that the effect of these drugs targeting URAT1 is sequence dependent in vivo. Although genetic factors affecting urate metabolism are mostly unclear, mutations or SNPs in this protein could potentially be involved and may prove interesting targets for future gout therapy agent development.

HRM analysis was recently introduced as a new technique to genotype SNPs within small amplicons (18–20), which was enabled by novel saturation dyes and high-resolution instruments. Difference plots are the best way to compare melting profiles visually. 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 cases, a known homozygote can be mixed into each unknown homozygote and the mixture is melted again for complete genotyping. In this study we chose a sample from the TT genotype samples as the normalization sample for the construction of the difference plots to standardize the interpretation. The presence of the TT genotype produced difference plots that were readily discriminated from the GG and GT genotype samples. The successful use of HRM to detect SNP or mutations has been reported for several other genes, including FGFR3 (21), apolipoprotein B-100 (22), and TP53 (23). In the current study the HRM results showed 100% concordant with the results obtained by sequencing, so we suggest that HRM may become the method of choice for rs893006 genotyping, as no processing or separations are required and the cost is minimal.

In conclusion, our results show for the first time an association between the TT allele in the rs893006 SNP and uric acid levels in a Chinese male population, which might be involved in the pathogenesis of primary hyperuricaemia and gout. The role of this SNP in URAT1 activity needs to be further studied. The HRM analysis that we used here may become a useful technology for providing this SNP screening information.

Acknowledgements
This work was supported in part by the Natural Science Foundation of China (No. 30872590). We thank Xinju Zhang, Xiaoye Gu, and Weizhe Ma (Central Laboratory of Huashan Hospital) for providing technical support.

References
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