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## Common genetic variants of the human *UMOD* gene are functional on transcription and predict plasma uric acid in two distinct populations

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### Abstract

Uromodulin (*UMOD*) genetic variants cause familial juvenile hyperuricemic nephropathy, characterized by hyperuricemia, decreased renal excretion of *UMOD* and uric acid; such findings suggest a role for *UMOD* in the regulation of plasma uric acid. We screened common variants across the *UMOD* locus in two populations, one from a community-based Chinese population, the other from California twins and siblings. Transcriptional activity of promoter variants was estimated in luciferase reporter plasmids transfected into HEK293 cells and mIMCD3 cells. By variance components in twin pairs, uric acid concentration and excretion were heritable traits. In the primary population from Beijing, we identified that carriers of haplotype GCC displayed higher plasma uric acid, and 3 *UMOD* promoter variants associated with plasma uric acid. *UMOD* promoter variants displayed reciprocal effects on urine uric acid excretion and plasma uric acid concentration, suggesting a primary effect on renal tubular handling of urate. These *UMOD* genetic marker-on-trait associations for uric acid were replicated in an independent American population sample. Site-directed mutagenesis at trait-associated *UMOD* promoter variants altered promoter activity in transfected luciferase reporter plasmids. These results suggest that *UMOD* promoter variants seem to initiate a cascade of transcriptional and biochemical changes influencing *UMOD* secretion, eventuating in elevation of plasma uric acid.

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### Disclosure

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## Keywords

Uromodulin; uric acid; Tamm-Horsfall protein; UMOD

## INTRODUCTION

Circulating uric acid concentration constitutes a risk factor for both renal and cardiovascular disease<sup>(1)</sup> and unusual mutations at the uromodulin (*UMOD*) locus exhibit disturbances in uric acid metabolism. UMOD is an ~80–90 kDa glycoprotein, consisting of 640 amino acids including 48 cysteine residues, exclusively synthesized by the thick ascending limb (TAL) and early distal convoluted tubule in the kidney<sup>(2)</sup>. UMOD is initially trafficked into the endoplasmic reticulum, then shuttled to the apical membrane of cells as a GPI-linked molecule, and finally released into the urine by proteolytic cleavage<sup>(3–5)</sup>. Healthy individuals typically excrete as much as 20–70 mg of UMOD per day, rendering it the most abundant protein in human urine<sup>(6)</sup>.

*UMOD* mutations have been identified in patients with familial juvenile hyperuricemia nephropathy (OMIM 162000), medullary cystic kidney disease 2 (OMIM 603860) and glomerulocystic kidney disease (OMIM 609886)<sup>(7–9)</sup>, which are characterized by hyperuricemia and progressive kidney disease. Thus UMOD is a candidate regulator of renal uric acid excretion. In a recent genome-wide association study, common genetic variant rs4293393 in the *UMOD* gene promoter was associated with serum uric acid<sup>(10)</sup>.

UMOD may contribute to regulation of sodium reabsorption through the TAL<sup>(11)</sup>; decreased UMOD secretion might lead to a reduction of sodium reabsorption by the TAL<sup>(12)</sup>, perhaps compensated through increased reabsorption of sodium by the proximal tubule. Since sodium reabsorption is coupled to increased urate reabsorption in the proximal tubule<sup>(13)</sup>, plausible mechanisms linking UMOD to serum uric acid are apparent.

In this study, we typed common genetic variants spanning the *UMOD* locus, to establish associations between the *UMOD* gene and plasma uric acid in different populations, and also explored whether the effect of *UMOD* on plasma uric acid was mediated by regulation of renal uric acid or sodium excretion.

## RESULTS

### Primary studies in a community-based cohort of Beijing

#### Association of UMOD haplotypes and common variants with plasma uric acid

—We genotyped 7 common variants (supplementary table 1 and 2) across the *UMOD* locus. We also used 7 common variants to construct haplotype blocks in Haploview<sup>(14)</sup> for all subjects in the Chinese population (Table 1). The analysis revealed 3 blocks across the *UMOD* genes, in which SNPs in *UMOD* promoter region, intron and 3' downstream were all highly correlated within each block (Supplementary table 3). Common promoter haplotypes displayed associations with plasma uric acid levels: carriers of promoter haplotype GCC showed higher plasma uric acid, while carriers of promoter haplotype ATT presented lower plasma uric acid levels (Table 2).

We then tested the association of each of the 7 individual SNPs with plasma uric acid in the cohort. 3 promoter SNPs, rs4293393 C/T, rs6497476 C/T, and rs13333226 G/A, each associated with plasma uric acid. rs13333226 G allele carriers (n=135) have higher plasma uric acid than A/A homozygotes (n=791, 279.2±7.0 vs. 263.4±2.9 μmol/L, p=0.009); rs6497476 C allele carriers (n=114) have higher plasma uric acid than T/T homozygotes

( $n=781$ ,  $281.2\pm 7.7$  vs.  $263.3\pm 2.9$   $\mu\text{mol/L}$ ,  $p=0.005$ ); and finally rs4293393 C allele carriers ( $n=137$ ) have higher plasma uric acid compared with T/T homozygotes ( $n=792$ ) ( $279.3\pm 7.1$  vs.  $264.3\pm 2.9$   $\mu\text{mol/L}$ ,  $p=0.032$ ) (Table 2).

The effects of age, gender, eGFR, BMI, height, weight, waist, SBP and DBP and *UMOD* gene (SNP) on plasma uric acid were tested by linear regression: a model combining age, gender, waist, eGFR, DBP and gene (SNP) predicted plasma uric acid ( $p<0.001$ ); rs13333226 itself accounted for  $\sim 0.4\%$  of plasma uric acid variance ( $p=0.015$ ; table 3), while rs6497476 itself accounted for  $\sim 0.5\%$  of serum uric acid variance ( $p=0.013$ ; table 3), and rs4293393 itself accounted for  $\sim 0.3\%$  of serum uric acid variance ( $p=0.037$ ; table 3).

We employed estimation of the FDR (False Discovery Rate), in order to minimize false negative results while maximizing true positive results, using the Excel calculator of FDRs from p-values, at <http://www.rowett.ac.uk/~gwh/fdr.html>. All the FDR p values for SNPs and haplotypes are listed in Table 2. 3 promoter SNP effects were also confirmed by permutation (non-parametric) tests.

**Association of *UMOD* variant with urinary uric acid excretion**—We also investigated the association of *UMOD* SNPs with urinary uric acid excretion. rs6497476 C allele carriers ( $n=101$ ) have lower urinary uric acid to creatinine ratio than T/T homozygotes ( $n=712$ ,  $282.7\pm 13.5$  vs.  $313.3\pm 6.0$   $\mu\text{mol}/\text{mmol}$ ,  $p=0.023$ ) (Figure 1), consistent with previous results<sup>(15)</sup> that the C allele predicted higher plasma uric acid.

**Association of a *UMOD* common haplotype with urinary *UMOD* excretion**—We then investigated whether the common haplotypes or SNPs associated with urinary *UMOD* excretion. Neither single SNPs nor promoter haplotypes was identified to be associated with *UMOD* excretion. Since a local region of high LD at the *UMOD* locus region was identified in the HapMap CHB (China-Han-Beijing) subjects (Supplementary Figure 1), we also inferred longer 7-SNP haplotypes across the *UMOD* locus. The most common haplotype, ATTGGCA (spanning rs13333226, rs6497476, rs4293393, rs7198000, rs11859916, rs4383153, rs4632135) associated urinary *UMOD*-creatinine ratio (Figure 2). Carriers of ATTGGCA displayed higher urinary *UMOD* excretion ( $p=0.016$ ).

**Correlation between urinary *UMOD*, uric acid and sodium excretions**—Urinary uric acid-to-creatinine ratio, urinary *UMOD*-to-creatinine ratio and urinary sodium-to-creatinine ratio were measured in spot urine in 898 cases. We found that plasma uric acid negatively correlated with urinary uric acid-to-creatinine ratio, as well as urinary *UMOD*-to-creatinine ratio. And we also found that the 3 biochemical traits, urinary uric acid-to-creatinine ratio, urinary *UMOD*-to-creatinine ratio and urinary sodium-to-creatinine ratio, positively correlated with each other (Table 4).

### Extension/replication studies in California twins and siblings

Of the 7 SNPs typed at/near *UMOD* in the Beijing cohort, 5 were either genotyped or imputed in the San Diego subjects; each of these 5 variants was significantly ( $p<0.05$ ) associated with plasma uric acid: rs13333226 ( $p=0.0261$ ), rs4293393 ( $p=0.0261$ ), rs7198000 ( $p=0.0492$ ), rs11859916 ( $p=0.0492$ ), and rs4632135 ( $p=0.027$ ). Of note, rs4293393 was previously associated with serum uric acid<sup>(10)</sup>.

Heritability of plasma uric acid and uric acid excretion was also estimated in the twin cohort.  $H^2$  was substantial for both plasma uric acid (at  $64.7\pm 5.5\%$ ,  $p=1.28*10^{-14}$ ) and urinary uric acid excretion (uric acid to creatinine ratio, at  $44.1\pm 6.6\%$ ,  $p=1.33*10^{-8}$ ), suggesting that genetic factors play an important role in regulation of uric acid concentration, metabolism, and excretion (Figure 3).

## Function of *UMOD* promoter variants (common haplotypes and single SNPs)

We constructed luciferase reporter plasmids for the 2 common naturally occurring *UMOD* haplotypes (HAP A-T-T and HAP G-C-C), and then created 3 mutant haplotypes by site-directed mutagenesis at promoter positions rs13333226, rs6497476, rs4293393, from A→G or T→C, forming 3 pairs of haplotypes in which differences only occur at one position: rs13333226, rs6497476, or rs4293393.

First we tested promoter strength by transfection of the 2 natural haplotypes into HEK-293 human kidney cells. Natural promoter haplotype A-T-T showed lower transcription of the reporter gene than haplotype G-C-C (Figure 4A). We then took advantage of mutant haplotypes to evaluate the transcriptional function of individual variants in transfected kidney cells. Higher gene transcription resulted from the G allele at rs13333226 and C allele at rs6497476 (Figure 4A), when tested upon balanced/matched haplotype backgrounds.

We repeated the same procedure in mlMCD3 cells, and obtained similar results which showed that 3-SNP promoter haplotype G-C-C displayed increased transcription compared to haplotype A-T-T (Figure 4B). Individually, the rs13333226 G allele and rs6497476 C allele each elevated reporter gene activity. However the rs4293393 C vs T difference was only identified in mlMCD3 (Figure 4B).

## DISCUSSION

### Overview: *UMOD* and uric acid

Aberrant renal handling of uric acid excretion is a major contributor to serum uric acid elevation in adults with hyperuricemia<sup>(16)</sup>. *UMOD* may be candidate protein to regulate uric acid, based on studies of medullary cystic kidney disease 2 (MCKD2)<sup>(17)</sup> and general cohorts<sup>(18)</sup>. Our study indicated haplotypes across the whole gene associated with plasma uric acid as well as urinary *UMOD* excretion, while only variants in promoter region had effects on plasma uric acid in a community-based Asian population. Carriers of the rs13333226 G allele, rs6497476 C allele or rs4293393 C allele have higher plasma uric acid compared to rs13333226 A/A, rs6497476 T/T or rs4293393 T/T homozygotes, respectively. In an extension/replication study of European-ancestry individuals in San Diego, the same genetic variants across the *UMOD* locus also predicted plasma uric acid. In our study, the correlation between plasma uric acid and *UMOD* excretion, as well as urinary *UMOD* correlation with urinary uric acid/sodium excretion suggested that *UMOD* may play a role in renal tubular handling of uric acid. Studies with the isolated/transfected *UMOD* promoter showed that G allele at rs13333226 and C allele at rs6497476 changed the reporter gene activity compared with A alleles at rs13333226 and T allele at rs6497476. These results suggest a mechanistic chain of events whereby common *UMOD* promoter variants play a role in the determination of human plasma uric acid.

Plasma uric acid concentration is a complex biochemical trait with multiple contributory factors derived from both genes<sup>(19)</sup> and environment<sup>(20)</sup>. In our replication study, high heritability (~64.7%) of plasma uric acid was identified in twins with Caucasian ancestry, while similarly high heritability (~70.5%) in Chinese twins was reported previously<sup>(21)</sup>

The setting of late penetrance of the ultimate disease trait (such as hyperuricemia), as well as likely genetic heterogeneity, the “intermediate phenotype”<sup>(22)</sup> strategy may be a useful approach in the search for disease predisposition loci. In accordance with this pathway concept, we pursued intermediate traits in this study. Excretion of *UMOD*, estimated by the urinary *UMOD*/creatinine ratio, was not only correlated with plasma uric acid but also influenced by *UMOD* haplotype ATTGGCA (based on 7 genotyped *UMOD* SNPs: rs13333226, rs6497476, rs4293393, rs7198000, rs11859916, rs4383153, rs4632135).

Haplotype ATTGGCA predicted higher UMOD excretion (Figure 5), which suggested that ATTGGCA carriers might have more uric acid excretion and lower plasma uric acid. Although we did not identify such correlations, in another haplotype, GCCGGTA as compared with ATTGGCA, which allelic changes in the promoter region (G-C-C versus A-T-T), predicted higher plasma uric acid (Figures 2A/2B).

### UMOD in the kidney

UMOD is exclusively synthesized by the tubular cells of the TAL and early distal convoluted tubule in the kidney<sup>(23)</sup>. It has been speculated that UMOD contributes to colloid osmotic pressure and retardation of sodium/potassium passage through the TAL, thus enhancing active transport in this segment<sup>(23)</sup>. Recently, UMOD has proved to play a role in regulation of the renal outer medullary potassium channel, thus increasing salt re-absorption along the TAL<sup>(24)</sup>. In THP<sup>-/-</sup> mice, urinary sodium/creatinine ratio and FENA/FEUA decreased substantially, without a significant difference in either FENA or FEUA, comparing knockout to wild-type<sup>(25)</sup>. Based on these findings, it appeared that a decrease of UMOD excretion may result in diminished reabsorption of sodium in the TAL, thus triggering an increase of sodium reabsorption in the proximal tubule, with an accompanying increase of uric acid reabsorption. In this study we also found positive correlations among UMOD, uric acid and sodium excretions, indicating functional links among renal excretions of UMOD, uric acid and sodium in the population. Furthermore the correlations between plasma and urinary uric acid, as well as with urinary UMOD, support an influence of UMOD upon plasma uric acid.

In our study, we systemically genotyped several common variants across the *UMOD* gene, and thereby identified additional SNPs potentially contributing to control of plasma uric acid in two populations of different ancestries. In our Asian population, promoter SNPs appeared to play especially important roles, including rs4293393, which was reported previously related to serum uric acid and reduced risk of kidney stone formation<sup>(10)</sup>; additional trait-associated *UMOD* promoter SNPs upstream of rs4293393 were also identified. Although these additional variants were not previously reported to be associated with uric acid in earlier studies, they have been associated with sodium excretion<sup>(15)</sup>, hypertension<sup>(15)</sup> or chronic kidney disease progression<sup>(10, 26,27)</sup>. Such associations might begin to explain why serum uric acid, hypertension and chronic kidney disease are tightly linked together in many cohort studies<sup>(28)</sup>. In the California subjects, SNPs across the *UMOD* locus (including the promoter) predicted uric acid. In the Third National Health and Nutrition Examination Survey (NHANES III), interactions between race (white, black, Hispanic and others) and abnormal levels of uric acid were observed<sup>(29)</sup>. Previous GWAS studies found that *UMOD*-region tagging-SNP rs12917707 associated with CKD and eGFR<sup>(27)</sup>, however the position of rs12917707 is far 5' of *UMOD*, indeed >5 kbp upstream of the *UMOD* 5'-UTR. Thus we did not include tagging-SNP rs12917707 in our functional studies.

### Functional genetic variation in the *UMOD* promoter

To investigate whether the promoter SNPs associated with plasma uric acid are functional, we constructed *UMOD* promoter/reporter plasmids, focusing on 3 common promoter variants: rs13333226 → rs6497476 → rs4293393. We tested luciferase reporter activity in two cell lines: human embryonic kidney cell HEK-293 and mouse inner medullary collecting duct cell mIMCD-3. Since Hepatocyte Nuclear Factor 1b (HNF1b) is a transcription factor expressed in tubular epithelial cells as a regulator of *UMOD* transcription<sup>(30,31)</sup>, we verified expression of *UMOD* and HNF1b mRNAs in renal/kidney cell lines at NCBI-GEO <<http://www.ncbi.nlm.nih.gov/geo/>> transcriptome database. Both *UMOD* and HNF1b were shown to be expressed in both human HEK-293 and mouse mIMCD3 cells.

In vivo chromatin immunoprecipitation experiments demonstrated that HNF1b binds to two sites close to the *UMOD* gene (at  $-1.1$  and  $-0.58$  kb with respect to the transcriptional start/cap site) in the mouse<sup>(32)</sup>. Although SNP rs4293393 (from this study) is located at around  $-0.55$  kb in the human *UMOD* promoter, it is unclear whether HNF1b binds to a similar site in the human *UMOD* promoter region.

In both cell lines we obtained similar results wherein 3-SNP promoter haplotype G-C-C displayed increased transcription compared to haplotype A-T-T. Individually, the rs13333226 G allele and rs6497476 C allele each elevated reporter gene activity, suggesting functional transcriptional roles for these variants in both cell lines. The difference between rs4293393 C vs T alleles was only identified in mlMCD3 cells.

We were initially surprised to note that *UMOD* promoter haplotypes and individual variants had opposite effects on transcription in cella (reporter gene activity in transfected cells) and urinary uromodulin excretion in vivo. Thus, there might be several explanations for this paradox.

First, although we constructed luciferase reporter plasmids by inserting a 1,794 bp promoter fragment of *UMOD*, this length might still not be sufficient to reflect the promoter regulation in vivo. However the luciferase activity difference between genotypes at same background indicated that all 3 SNPs were functional in the regulation of *UMOD* transcription. *UMOD* is a GPI-anchored cell membrane protein, whose biosynthesis and trafficking involve a complex secretory pathway: *UMOD* is cleaved, glycosylated and glypiated in the ER, and then N-glycan moieties are further trimmed in the Golgi apparatus, after which the mature moieties and GPI modification act as sorting signals, routing uromodulin to the apical membrane, from which it is released into the urine. Urinary uromodulin may thus be influenced by several steps: synthesis, glycosylation, trafficking and cleavage. Alternatively, negative feedback mechanisms can be proposed to explain the contrasting results in vivo and in cella: for example, after synthesis, accumulation of *UMOD* in the ER might delay glycosylation and thus *UMOD* expression on the apical cell membrane, or cleavage of the *UMOD* anchor for release into the urine.

## Conclusions and perspectives

In summary, our data suggest that common genetic variation in the promoter region of *UMOD* confers a series of changes upon gene transcription and secretion of *UMOD*, renal tubular sodium/uric acid reabsorption, and finally serum uric acid concentration. Our observations are consistent with the “intermediate phenotype”<sup>(33)</sup> framework for complex traits as well as with the “common disease/common allele” hypothesis for frequent traits in the population<sup>(34)</sup>, and suggest new molecular strategies for probing the pathophysiology, risk, and rational treatment of hyperuricemia (Figure 5).

## METHODS

### Subjects

#### Primary studies in a community-based cohort in an urban area of Beijing—

Individuals were from a community-based cohort in an urban area of Beijing. Briefly, individuals aged  $\geq 40$  years were evaluated for chronic kidney disease (CKD) and associated factors in 2004<sup>(35)</sup>. As a consequence of urban renewal in Beijing, part of the participants moved out of the area. Survival status and indicators of kidney damage were re-evaluated in 67.7% ( $n = 1563$ ) of them in 2008<sup>(36)</sup>. We were able to ascertain, consent, and prepare genomic DNA from blood leukocytes obtained from 1000 of the original 1563 participants of 2008 in this study.

We were able to ascertain, consent, and prepare genomic DNA from 1000 of the original participants of 2008 in this study. The subset reported here (n=1000) did not differ from the complete cohort in age (p=0.603), gender (p=0.718), blood pressure (SBP p=0.35, DBP p=0.207), eGFR (p=0.308), or plasma uric acid (p=0.622). Characteristics of the cohort are listed in Table 1. eGFR was estimated with the equation developed from data based on Chinese patients with CKD<sup>(37)</sup> and the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation<sup>(38)</sup>. Since eGFR calculated with these two equations were highly correlated, we used the CKD-EPI equation to generate eGFR for both populations (Table 1).

**Replication/extension studies: American twins and siblings**—642 American individuals of white (European or Hispanic) ancestry, from 242 nuclear families ascertained at UCSD were subjected to genome-wide genotyping with the Illumina-610-Quad array, encompassing ~590K SNP genotypes, after which an additional ~2 million Hap-Map-2 SNPs were imputed with MACH v. 1.0.16 (<http://www.sph.umich.edu/csg/abecasis/MaCH/>). This population included 24% Males, with age 40.6+/-0.8 years, SBP 123.0+/-0.6mmHg, DBP 75.6+/-0.4mmHg, plasma uric acid 414.3+/-0.1umol/L and eGFR 97.8+/-1.0 ml/min/1.73m<sup>2</sup>. SNP-on-phenotype effects with tested in MERLIN v1.1.2 (<http://www.sph.umich.edu/csg/abecasis/merlin/>) in order to explicitly account for family structure. For the plasma uric acid trait, the Q-Q plot of -log<sub>10</sub> expected (theoretical uniform distribution) versus observed significance (p-values) across the genome did not show deviation across the range from -log<sub>10</sub> = 0 to 5.5. Heritability (h<sup>2</sup>, or the fraction of trait variance accounted for by genetic variance) of uric acid traits was estimated by variance components in twin pairs, using SOLAR, available at <<http://txbiomed.org/departments/genetics/genetics-detail?r=37>>.

## Genomics

Genomic DNA was isolated from whole blood using a modified salt extraction technique<sup>(39)</sup>. Single nucleotide polymorphisms were obtained from public SNP databases (<http://www.ncbi.nlm.nih.gov/SNP>). Genomic DNA was typed using a matrix assisted laser desorption ionization time-of-flight mass spectrometry system<sup>(40)</sup>, or by Taqman assay on an ABI PRISM Sequence Detection System 7500. Reproducibility of genotyping was verified with 50 blinded replicate samples. We selected 7 UMOD SNPs according to previously published research and the dbSNP database in NCBI. SNPs were selected for minor allele freq (>=5%), and to span functional domains in the gene, as well as LD blocks according to pertinent HapMap CHB results. SNPs evaluated in this study were in Hardy Weinberg equilibrium (p>0.05). Genotyping failing rates were less than 10%. In order to prove that the missing data were randomly distributed, we compared clinical features between subgroups (whole population group, successfully genotyped group and genotype failed group) for each SNP and no differences were identified. Allele distribution of these SNPs were listed in supplementary table 1.

## UMOD measurement

Measurement of UMOD in urine was achieved by ELISA according to the Lau protocol<sup>[37]</sup>. 96-well microtiter plates were coated with 100 µl of 0.1mol/ml polyclonal mouse anti-human THP antibody (Biomedical Technologies Inc BT-590) overnight at 4°C. Plates were washed and treated with 200 µl blocking buffer (3% BSA in PBS) for 1 hour at room temperature. Urine samples and standards (Biomedical Technologies) were diluted in TEA buffer and added to wells in duplicate. After two hours incubation at 37°C, wells were washed and anti-UMOD antibody was added. After another one-hour incubation at 37°C, wells were again washed and goat anti-rabbit IgG horseradish peroxidase (CEDARLANE CL1032AP) was added. After 0.5 hour incubation at 37°C, wells are washed and color was

developed by the addition of TMB substrate solution and incubation at room temperature for 15 minutes. The reaction is stopped by adding 2N H<sub>2</sub>SO<sub>4</sub>, followed by reading immediately at OD<sub>490</sub> and OD<sub>570</sub>. Urinary UMOD concentration was determined by interpolation on the standard curve.

### Plasmid construction for luciferase reported assays

Human *UMOD* promoter/reporter plasmids were constructed as described<sup>(41)</sup>. We isolated genomic DNA from human blood, from which we excised 1,794 bp of *UMOD* promoter region by PCR, containing common polymorphic sites for insertion into the upstream region polylinker of the firefly luciferase reporter plasmid pGL3-Basic (Promega). Synthetic replacements were made by site-directed mutagenesis (QuikChange; Stratagene) to produce the common haplotypes showed in Table 2. All inserts were sequence-verified before use.

HEK-293 (human embryonic kidney) cells and mlMCD3 (mouse inner medullary collecting duct) cells were both used to test transcriptional activity of transfected *UMOD* promoter/luciferase reporter plasmids. Cells were transfected (at ~50–60% confluence) with 1 µg of pGL3-Basic haplotype/firefly luciferase reporter plasmid and 10 ng of Renilla luciferase plasmid pRL-TK (Promega) as an internal control per well, by the liposome method (Superfect; Qiagen). The firefly and Renilla luciferase activities in the cell lysates were measured 48 hours after transfection, and results were expressed as the ratio of firefly/Renilla luciferase activities (“Stop & Glo®”; Promega). Each experiment was repeated a minimum of three times.

### Statistical analyses

Typing 7 common variants (also 3 promoter common variants) across the *UMOD* locus allowed the inference of haplotypes from diploid genotypes in PLINK (version 3.0) <<http://pngu.mgh.harvard.edu/purcell/plink/>><sup>(42)</sup>. Haplotype distribution was listed in supplementary table 3.

Statistical Package for the Social Sciences (SPSS v13.0; Chicago, IL) was used routinely. Baseline characteristics were reported as mean ±SEM or median (inter-quartile range [IQR]) for continuous variables. One-way ANOVA (using Bonferroni comparison tests) was performed in SPSS to test *in vitro* reporter activity assays. Genotypes and haplotypes were grouped by the number of copies of the particular allele or haplotype (0, 1, 2 copies). Mode of inheritance emerged from visual inspection of the marker-on-trait plots–scatterplots or bar-graphs -- to determine whether two of the three diploid genotypes (Major/Major, Major/Minor, or Minor/Minor) displayed the same trait clustering. In this study, we thus pooled the minor allele homozygotes with heterozygotes, in a minor-allele-dominant model for single SNPs or Haplotypes.

Univariate ANOVA analyses and multiple linear regression in SPSS were computed to evaluate the effects of genotype or haplotype on uric acid. Age, gender, eGFR, BMI, height, weight, waist, SBP or DBP were added as covariates in univariate ANOVA analysis, and as independent variables in multiple linear regression.

In consideration of multiple testing of the effects, we employed estimation of the FDR (False Discovery Rate), in order to minimize false negative results while maximizing true positive results, using the Excel calculator of FDRs from p-values, at <<http://www.rowett.ac.uk/~gwh/fdr.html>>.

Permutation tests was performed with “Resampling Stats Excel add-in version 3.2” (<http://www.resample.com/content/software/excel/index.shtml>) to confirm genotype effect on plasma uric acid.



## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

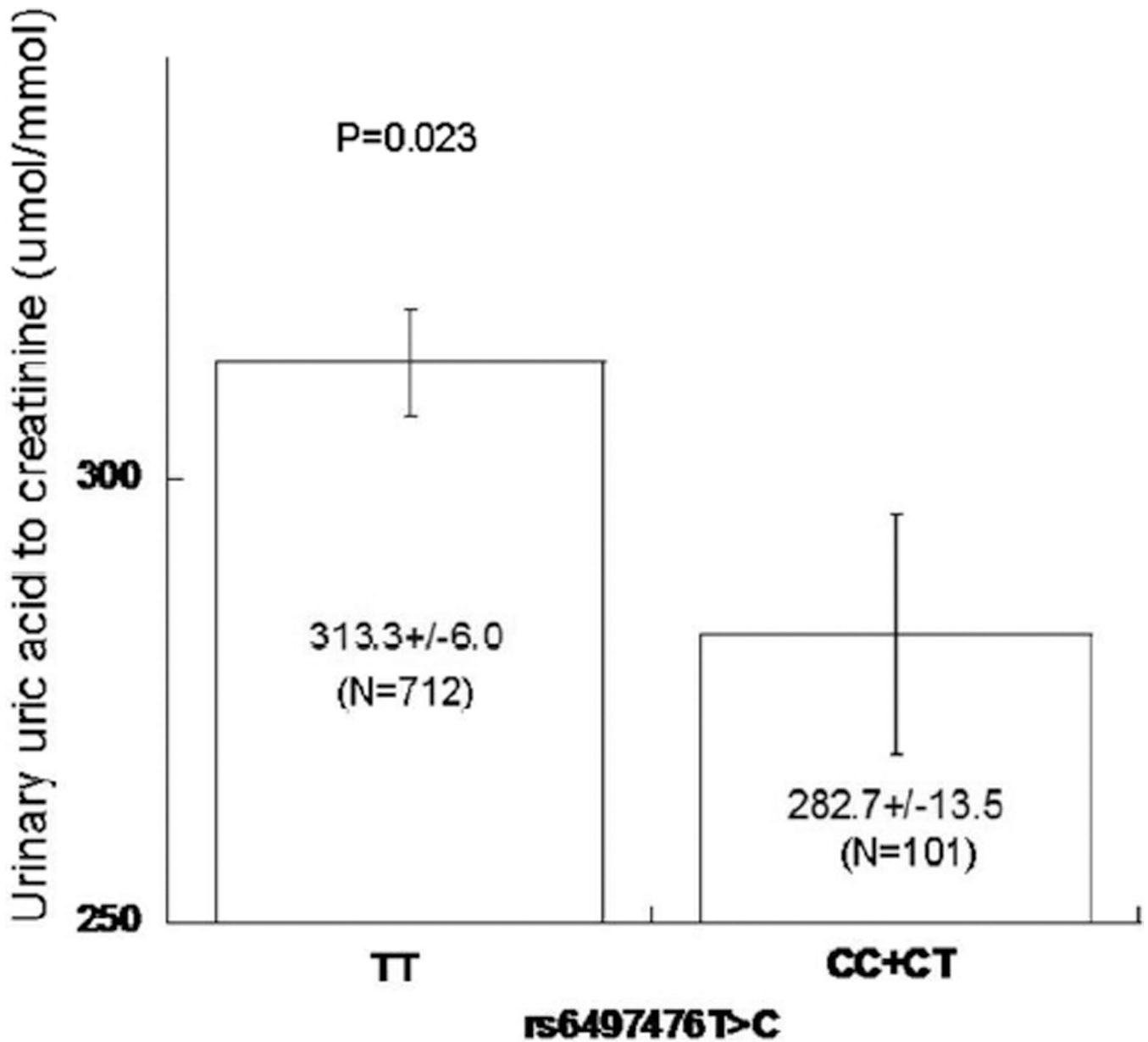
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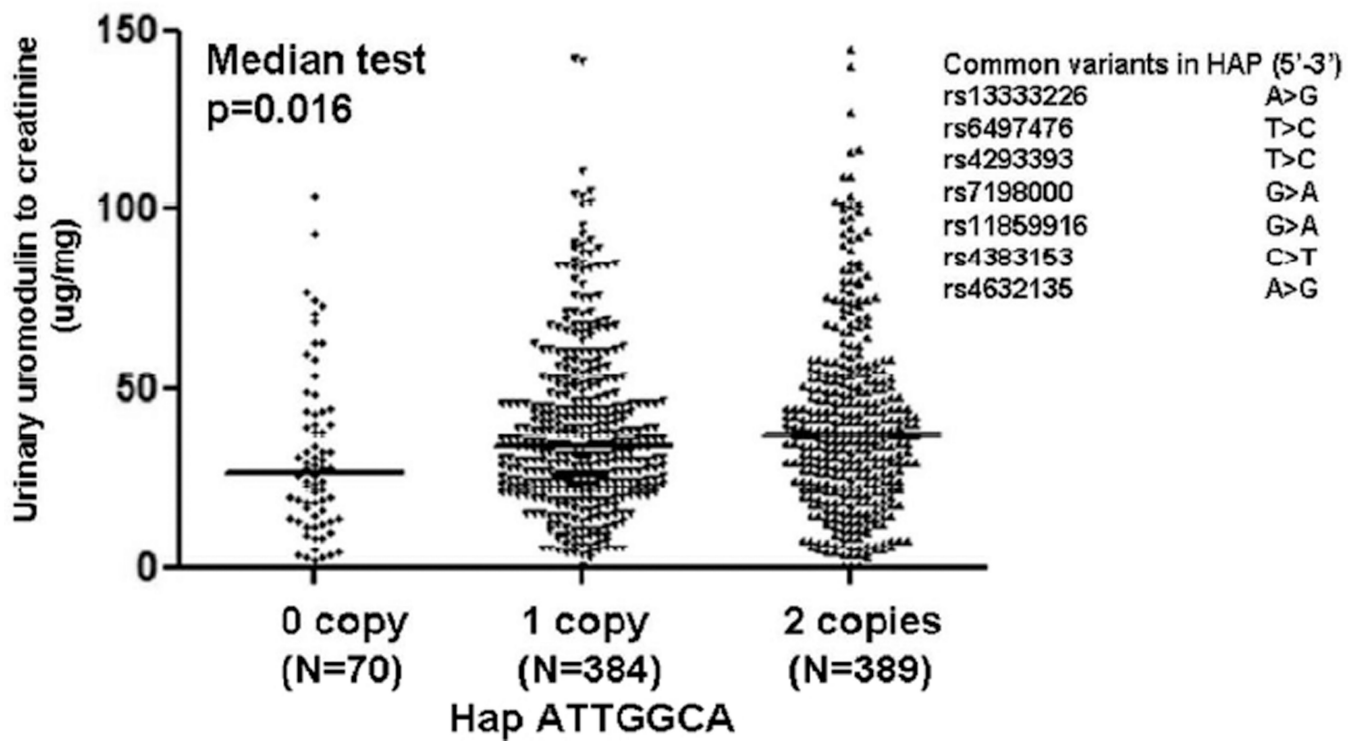
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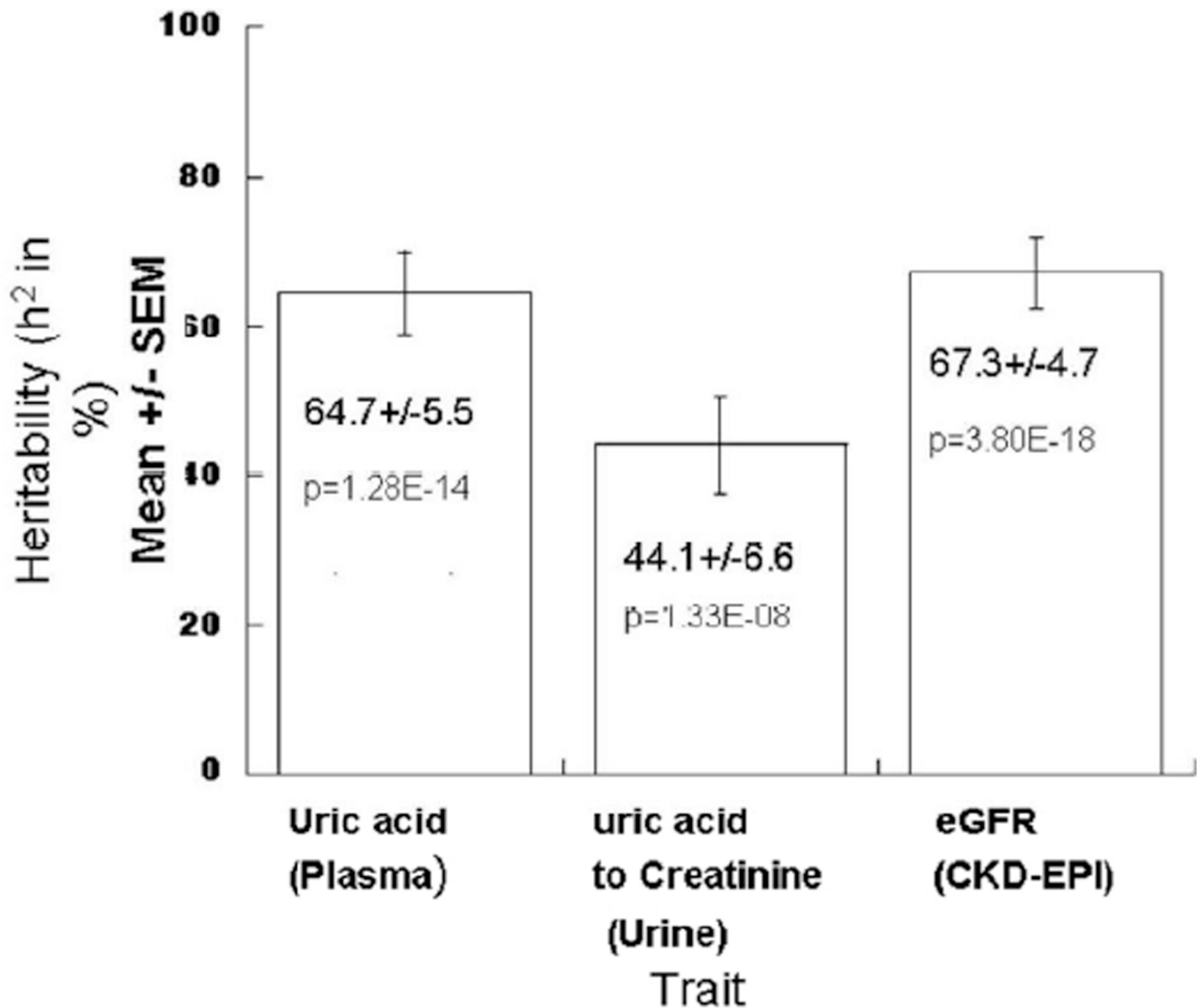
**Figure 1. *UMOD* promoter common genetic variation: Effect on urine uric acid excretion in a community based cohort of the Chinese population**

Urine uric acid excretion was measured in spot (untimed) urine samples from year 2008. Promoter rs6497476 results indicated that C allele carriers had lower urinary uric acid-to-creatinine ratios than T/T homozygotes.



**Figure 2. *UMOD* haplotype ATTGGCA: Effect on urinary *UMOD* excretion in a community-based cohort of Chinese population**

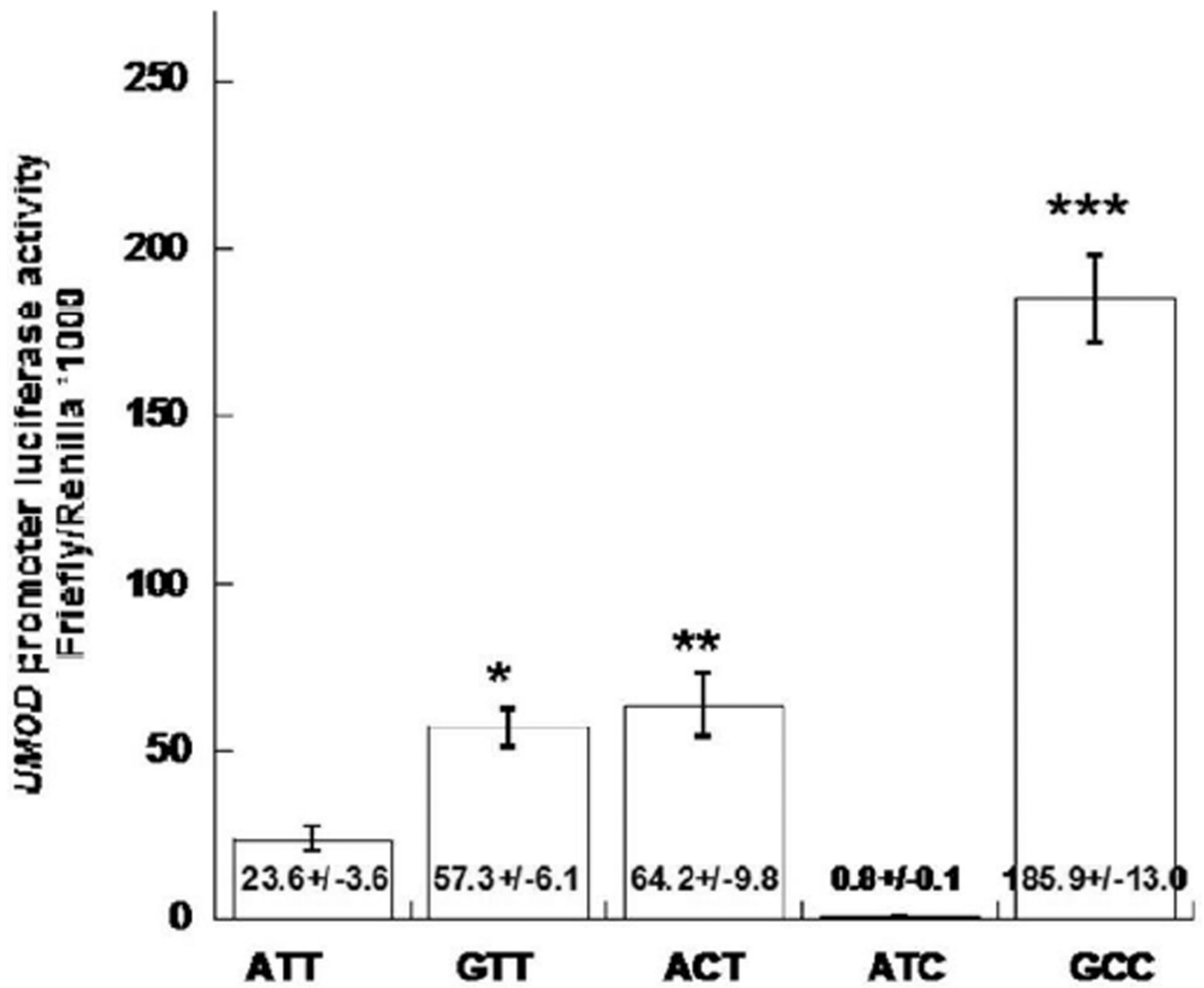
Haplotype homozygosities were confirmed by PLINK [URL: <http://pngu.mgh.harvard.edu/purcell/plink/>]<sup>[14]</sup>. Urinary *UMOD*-to-creatinine ratio was presented as  $\mu\text{g}/\text{mg}$ . The most common haplotype ATTGGCA associated urinary *UMOD*-to-creatinine ratio.



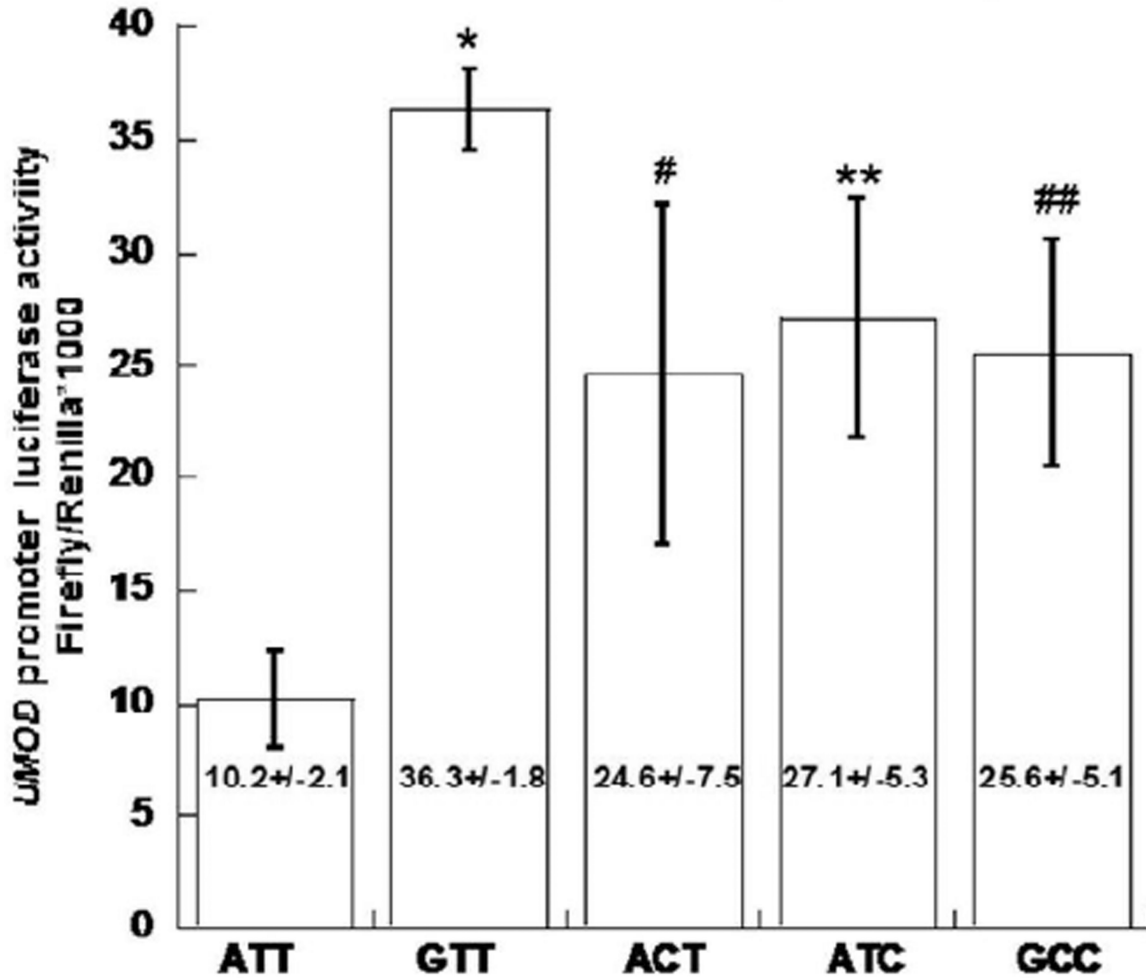
**Figure 3. Uric acid trait heritability in twin pairs**

Heritability ( $h^2$ ) of plasma uric acid and uric acid excretion, as well as eGFR.  $h^2$ , the fraction of trait variance accounted for by genetic variance ( $h^2 = V_G/V_P$ ) was established by study of variance components in California 173 twin pairs (346 individuals). Plasma uric acid is in  $\mu\text{mol/L}$ , while urinary uric acid to creatinine ratio is in  $\mu\text{mol/mmol}$ , and eGFR was calculated by the CKD-EPI algorithm (normalized to  $\text{ml/min}/1.73\text{m}^2$ ).

## A. In Human embryonic kidney HEK-293



## B. In Mouse inner medullary collecting duct mlMCD-3

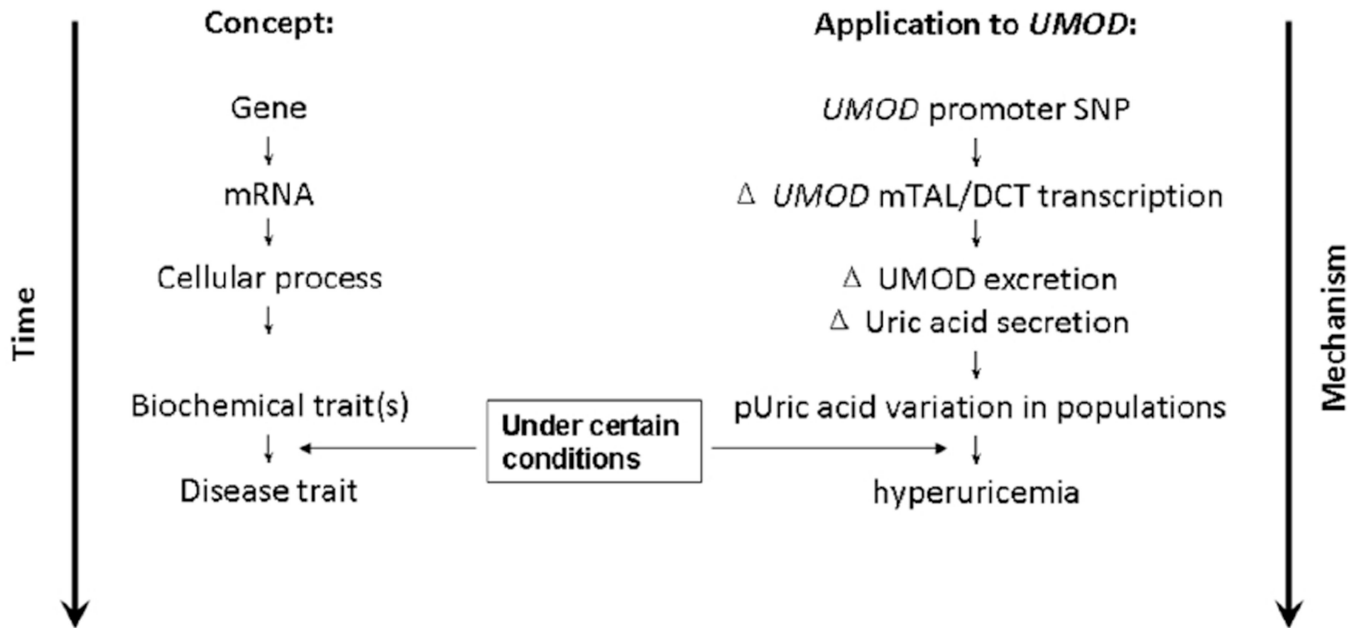


**Figure 4. *UMOD* promoter haplotype/luciferase reporter functional studies**

Transfection results for human *UMOD* promoter haplotype/luciferase reporters are shown in human embryonic kidney (HEK-293) cells as well as in mouse inner medullary collecting duct (mlMCD-3). Results are expressed as the ratio of firefly/Renilla luciferase activity. As a negative control, the ratio of firefly/Renilla luciferase activities for the promoter-less luciferase reporter vector (empty vector pGL3-Basic) co-transfected with transfection efficiency control plasmid Renilla plasmid (pTK-RL) was  $0.002 \pm 0.001$  for HEK-293 cells and  $8.44 \times 10^{-5} \pm 1.84 \times 10^{-6}$  for mlMCD-3. The absolute firefly luminescence for *UMOD* haplotype ATT was  $22826 \pm 1739$  RLU/sec in HEK293 cells;  $235 \pm 28$  RLU/sec in mlMCD-3. 50  $\mu$ l from each 500  $\mu$ l cell lysate were used for luciferase assay. Each experiment was performed in triplicate, and such experiments were repeated at least three times. Statistical significance refers to the haplotype effects. Results are compared by ANOVA followed by post-hoc LSD.



**Uromodulin (*UMOD*, Tamm-Horsfall protein):  
Working hypothesis for transcriptional and pathophysiological  
consequences of common genetic variants in the promoter**



**Figure 5.** Uromodulin (*UMOD*, Tamm-Horsfall protein): Working hypothesis for transcriptional and pathophysiological consequences of common genetic variants in the promoter.

**Table 1**

Characteristics of the Chinese community-based cohort in this study.

<b>Variables</b>	<b>Participants (n=1000)</b>
Age (years)	63.7±0.3
Male (%)	48.5%
BMI (kg/m <sup>2</sup> )	25.3±0.1
SBP (mmHg)	137.8±0.6
DBP (mmHg)	81.0±0.3
Plasma uric acid (μmol/L)	265.7±2.6
Plasma creatinine (μmol/L)	138.33±0.6
eGFR (mL/min/1.73 m <sup>2</sup> )	70.8±0.5
Cholesterol (mmol/L)	5.3 (4.7–6.0)
Triglycerides (mmol/L)	1.3 (1.0–1.8)
Urinary ACR (median [IQR])	2.6 (1.3–4.9)
Hypertension(%)	46.3
Anti-hypertension medication(%)	81.6

Values shown are mean ± SEM, or median (inter-quartile range [IQR]). Samples shown here were collected in 2008. eGFRs presented in this table were estimated from plasma creatinine using the equation developed from data on the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI)<sup>[34]</sup>. ACR: albumin to creatinine ratio in urine.

UMOD promoter common haplotypes and SNPs: Effects on plasma uric acid in the Chinese population (by univariate analysis of variance).

**Table 2**

HAP/ SNP	Copy (N)/ Allele (N)	pUA (umol/L) (mean+/-SEM)	model	gene	P value for			FDR p	
					Age	Gender	Waist		
				significant covariate			BMI		
GCC	0 (812)	263.9+/-2.8	<0.0001	0.017	0.006	<0.0001	<0.0001	0.013	0.023
	1+2 (114)	279.4+/-7.6							
ATT	0+1 (138)	278.7+/-6.8	<0.0001	0.021	0.006	<0.0001	<0.0001	0.012	0.021
	2 (788)	263.8+/-2.9							
rs1333226									
rs 6497476	AA (791)	263.4+/-2.9	<0.0001	0.009	0.020	<0.0001	<0.0001	>0.05	0.018
	GA+AA(135)	279.2+/-7.0							
R <sup>s</sup> 4293393	TT (781)	263.3+/-2.9	<0.0001	0.005	0.027	<0.0001	<0.0001	>0.05	0.020
	CC+CT (114)	281.2+/-7.7							
TT (792)	264.3+/-2.9		<0.0001	0.032	0.042	<0.0001	<0.001	>0.05	0.026
	CC+CT (137)	279.3+/-7.1							

HAP: haplotype. pUA: plasma uric acid. The haplotypes located 5' near gene region of UMOD, consisted of rs1333226 (A>G) → rs6497476 (T>C) → rs4293393 (T>C). Univariate analysis of variance was performed to test the association of haplotypes with plasma uric acid level. Age, gender, eGFR, BMI, height, weight, waist, SBP and DBP were selected as covariates. Covariates with significant p values (p<0.05) are listed in the Table.

**Table 3**

Regression: Fractional effects of UMOD common variants on plasma uric acid in the Chinese cohort.

<b>MLR model</b>			
<b>Predictors: SNP (one SNP at a time), Gender GFR DBP, waist</b>			
<b>Dependent variables: Plasma uric acid level</b>			
RefSNP	F	P	Adjusted R <sup>2</sup>
rs13333226	86.005	3.72* [0–74]	0.316
rs6497476	83.789	4.27* [0–72]	0.317
rs4293393	86.183	3.34* [0–74]	0.318
Model component (with all independent variables) p values			
RefSNP	SNP	R <sup>2</sup> for SNP	Gender GFR DBP Waist
rs13333226	0.015	0.4%	<0.001 <0.001 0.027 <0.001
rs6497476	0.013	0.5%	<0.001 <0.001 0.036 <0.001
rs4293393	0.037	0.3%	<0.001 <0.001 0.04 <0.001

MLR: Multiple linear regression, by the stepwise regression method. Plasma uric acid was the dependent variable. Single SNP, age, gender, eGFR, BMI, height, weight, waist, SBP and DBP were selected as independent variables. A model combining UMOD SNP genotype, gender, DBP and waist predicted plasma uric acid level.

**Table 4**

UMOD correlations (non-parametric, Spearman rank) with sodium and uric acid traits.

	<b>uNa/Cr</b>	<b>uUA/Cr</b>	<b>uUMOD/Cr</b>	<b>pUA</b>
<b>uNa/Cr (μmol/mmol)</b>				
Correlation coefficient	1.0	0.645	0.239	-0.161
Sig. (2-tailed)		5.24*10 <sup>-106</sup>	6.56*10 <sup>-13</sup>	1.53*10 <sup>-6</sup>
<b>uUA/Cr (μmol/mmol)</b>				
Correlation coefficient		1.0	0.348	-0.187
Sig. (2-tailed)			1.66*10 <sup>-26</sup>	2.11*10 <sup>-8</sup>
<b>uUMOD/Cr (μg/mg)</b>				
Correlation coefficient			1.0	-0.253
Sig. (2-tailed)				3.72*10 <sup>-14</sup>
<b>pUA (μmol/L)</b>				
Correlation coefficient				1.0
Sig. (2-tailed)				