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Original article

Substantial anti-gout effect conferred by common and rare dysfunctional variants of *URAT1/SLC22A12*

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Abstract

Objectives. Gout, caused by chronic elevation of serum uric acid levels, is the commonest form of inflammatory arthritis. The causative effect of common and rare variants of *ATP-binding cassette transporter G2* (*ABCG2/BCRP*) on gout risk has been studied, but little attention has been paid to the effect of common (rs121907892, p.W258X) and rare variants of *urate transporter 1* (*URAT1/SLC22A12*) on gout, despite dysfunctional variants of *URAT1* having been identified as pathophysiological causes of renal hypouricaemia.

Methods. To address this important but overlooked issue, we investigated the effects of these *URAT1* variants on gout susceptibility, using targeted exon sequencing on 480 clinically defined gout cases and 480 controls of Japanese males in combination with a series of functional analyses of newly identified *URAT1* variants.

Results. Our results show that both common and rare dysfunctional variants of *URAT1* markedly decrease the risk of gout (OR 0.0338, reciprocal OR 29.6, $P = 7.66 \times 10^{-8}$). Interestingly, we also found that the *URAT1*-related protective effect on gout eclipsed the *ABCG2*-related causative effect (OR 2.30–3.32). Our findings reveal only one dysfunctional variant of *URAT1* to have a substantial anti-gout effect, even in the presence of causative variants of *ABCG2*, a 'gout gene'.

Conclusion. Our findings provide a better understanding of gout/hyperuricaemia and its aetiology that is highly relevant to personalized health care. The substantial anti-gout effect of common and rare variants of *URAT1* identified in the present study support the genetic concept of a 'Common Disease, Multiple Common and Rare Variant' model.

Key words: urate reabsorption transporter, gout susceptibility, genetic risk factor, Common Disease Common Variant hypothesis, Common Disease Multiple Rare Variant hypothesis

CLINICAL SCIENCE

Rheumatology key messages

- Common and rare dysfunctional variants of *URAT1/SLC22A12* have substantial anti-gout effects.
- The anti-gout effect of *URAT1* outweighs the gout-promotive effect of *ABCG2*, a gout gene.
- We show a new 'Common Disease, Multiple Common and Rare Variant' model by two genes.

Introduction

Gout is a form of inflammatory arthritis caused by hyperuricaemia. Several genome-wide association studies have been conducted to identify the genetic loci that influence serum uric acid (SUA) levels [1, 2] and/or gout risk [3–7]. These revealed the pathophysiological importance of urate transporters on urate-related diseases [8]. Recent studies have chiefly focussed on variations that

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increase genetic susceptibility: for example, regarding *ATP-binding cassette transporter G2* (*ABCG2*), a urate exporter excreting urate from the body whose dysfunction is well characterized as a genetic risk factor for hyperuricaemia and gout [9–12], not only common but also rare variants of this 'gout gene' have been examined [9, 13, 14]. Compared with such cases, there is little information about the relationship between the expected gout-protective effect and the genetic variations in urate handling processes contributing to the decrease in SUA levels.

In humans, in addition to endogenous production of uric acid, renal urate re-absorption makes a significant contribution to the regulation of SUA levels. The latter process in urate handling is mediated by a renal urate re-absorber encoded by *urate transporter 1* (*URAT1*, also known as *SLC22A12*) gene, which is a molecular target of urate-lowering drugs [15]. Its dysfunctional variants are also known as pathophysiological causes of renal hypouricaemia (RHUC) [15–17]. However, despite the high prevalence of gout [8], little attention has been paid to the effects of genetic variations of *URAT1* on the risk of gout. We herein address this issue using a clinical genetics approach to gout in combination with functional analyses of the urate transporter URAT1. The findings of this study shed light on the genetic significance of common and rare variants of *URAT1* as anti-gout factors.

Methods

Patients and genetic analyses

This study was approved by the institutional Ethical Committees (National Defence Medical College and Nagoya University, Japan), and written consent was obtained from all of its participants. All the procedures were performed in accordance with the Declaration of Helsinki. Our cases were 480 Japanese males with gout, recruited from the Ryougoku East Gate Clinic (Tokyo, Japan). All had been clinically diagnosed with primary gout according to the criteria established by the American College of Rheumatology [18]. As controls, 480 Japanese males without hyperuricaemia (SUA levels $>$7.0 mg/dl) or history of gout were recruited from participants in the Shizuoka area of the Japan Multi-Institutional Collaborative Cohort Study (J-MICC Study) [19, 20]. The clinical characteristics are shown in Table 1. Additionally, the control group [mean (S.D.) age, 53.0 (7.9) years old] was more elderly compared with the case group [46.2 (9.8) years old]. As aging is one of the important factors to increase SUA and/or gout risk, to avoid over-adjustment bias, we did not conduct age standardization in this study.

To explore non-synonymous variants of *URAT1*, we performed targeted exon sequencing of *URAT1*. The methods of target sequencing as well as variant calling and annotation were conducted as described previously [13]. To confirm one common (W258X) and 10 rare non-synonymous variants, direct sequencing using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was performed according to our previous study [21].

Table 1 Characteristics of the participants

	Case	Control
Number	480	480
Age (years)	46.2 (9.8)	53.0 (7.9)
Body-mass index (kg/m^2)	25.3 (3.7)	23.2 (2.6)

480 gout cases and 480 normouricaemic controls of Japanese males were analysed. Data are expressed as means (S.D.).

Fractional excretion of uric acid (FE_{UA}, calculated as urate clearance/creatinine clearance ratio) and SUA were measured for 480 Japanese male controls. The effects of dysfunctional *URAT1* variants on FE_{UA} and SUA were also investigated.

Materials

Critical materials and resources used in this study are summarized in Supplementary Table S1, available at *Rheumatology* online. All the other chemicals used were commercially available and of analytical grade.

Plasmid construction

The full-length human URAT1 wild-type (WT) (NCBI accession; NM_144585.3) open reading frame (ORF) in the pcDNA3.1(+) plasmid was amplified with the *Xho* I site and the *Xba* I site attached at the 5′- and 3′-end, respectively, by PCR, and then inserted into the pEGFP-C1 vector plasmid (Clontech Laboratories, Palo Alto, CA, USA) for CMV-driven expression of enhanced green fluorescent protein (EGFP)-URAT1 in mammalian cells as in our previous study [22]. Using a site-directed mutagenesis technique, eight URAT1 mutants (P79L, R90H, Y180X, A227T, W258X, Q297X, F379L and Q533K) were constructed with a pEGFP-C1 vector in the same way as in our previous study [13].

Introduction of all mutations was confirmed by full sequencing using BigDye® Terminator v3.1 (Applied Biosystems) with Applied Biosystems® 3130 Genetic Analyzer (Applied Biosystems) following the manufacturer's protocol. Prior to the further experiments described below, all the plasmids were obtained from the same lot.

Cell culture

Human embryonic kidney 293 (HEK293)-derived 293A cells were maintained in Dulbecco's Modified Eagle's Medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% foetal bovine serum (Biowest, Nuaillé, France), and 1% penicillin-streptomycin (Nacalai Tesque), 2 mM L-glutamine (Nacalai Tesque), and 1 × Non-Essential

Amino Acid (Life Technologies, Tokyo, Japan) at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air. Twenty-four hours after the cells had been seeded (0.92×10^5 cells/cm^2), each vector plasmid was transfected into 293A cells using polyethyleneimine 'MAX' (PEI-MAX) (Polysciences, Warrington, PA, USA) as described previously [23].

Preparation of protein lysates and immunoblotting

After preparation of whole-cell lysates, to examine the *N*-linked glycosylation status of each URAT1 protein, the whole-cell lysate samples were treated with Peptide *N*-glycosidase F (PNGase F) (New England Biolabs, Ipswich, MA, USA) as described previously [23]. The protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Kanagawa, Japan) with BSA as a standard according to the manufacturer's protocol. The samples were then separated by SDS–PAGE and transferred to an Immobilon-P polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA) by electroblotting at 15 V for 60 min. Using appropriate antibodies (Supplementary Table S1, available at *Rheumatology* online), immunoblot detection was performed as described in our previous study [23].

Confocal microscopic observation

Confocal laser scanning microscopic observation was conducted as previously described [23]. In brief, 48 h after transfection, 293A cells were fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were then treated with a fluorescent wheat germ agglutinin conjugate (WGA, Alexa Fluor® 594 conjugate; Thermo Fisher Scientific). After 0.02% (w/v) Triton-X100 treatment, the nuclei of the cells were visualized with TO-PRO-3 Iodide (Molecular Probes, Eugene, OR, USA). Stained cells were mounted with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA), after which fluorescence was detected using an FV10i Confocal Laser Scanning Microscope (Olympus, Tokyo, Japan).

Urate uptake assay using URAT1-expressing 293 A cells

The urate uptake assay using URAT1-expressing 293A cells was conducted in the same way as in our previous study [23]. In brief, 48 h after plasmid transfection, the cells were washed twice with Cl^--free transport buffer (Buffer T2: 125 mM Na-gluconate, 4.8 mM K-gluconate, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM Ca-gluconate, 25 mM HEPES, 5.6 mM D-glucose and pH 7.4) and pre-incubated in Buffer T2 for 15 min at 37°C. The cells were then further incubated for 20 s in pre-warmed fresh Buffer T2 containing 5 μM [8-^{14}C]-urate. Subsequently, the cells were twice washed with ice-cold Buffer T2 and then lysed with 500 μL of 0.2 M NaOH, then neutralized with 100 μL of 1 M HCl. We measured the radioactivity in the lysate using a liquid scintillator (Tri-Carb 3110TR; PerkinElmer, Waltham, MA, USA). The protein concentrations were determined using the Pierce™ BCA Protein Assay Kit as described above.

The urate-transporting activity was calculated as the incorporated clearance (μL/mg protein/min): [incorporated level of urate (DPM/mg protein/min)/urate level in the incubation mixture (DPM/μL)]. URAT1-dependent urate-transporting activity was calculated by subtracting the urate-transporting activity of mock cells from that of the URAT1-expressing cells.

Statistical analysis

Different statistical tests were used for different experiments as described in the Figure legends, which include the numbers of biological replicates (*n*). All statistical analyses were performed using Excel 2019 (Microsoft, Redmond, WA, USA) in functional analyses. The software R (version 4.0.1) (http://www.r-project.org/) was used for the calculation of Fisher's exact test. Other regression analyses were performed with SPSS v.22.0J (IBM Japan, Tokyo, Japan). Logistic regression analysis with forward selection (likelihood ratio) was conducted to investigate gout susceptibility associated with dysfunctional variants of *URAT1* and *ABCG2*. Linear regression analyses were performed to evaluate the effect of dysfunctional *URAT1* variants on FE_{UA} and SUA. Statistical significance is defined in terms of *P*-values of below 0.05 or 0.01.

Results

Common and rare variants of *URAT1* detected by targeted exon sequencing

To explore *URAT1* variants associated with gout, targeted exon sequencing of *URAT1* was performed with 480 clinically defined gout cases and 480 normouricaemic controls of Japanese males. We identified one common (W258X) and 10 rare non-synonymous variants in *URAT1* (Table 2). Since three variants (Q382L, L418R and V547fsL) are reportedly nonfunctional [16, 24], we conducted a series of expression, localization, and functional analyses of six uncharacterized rare variants (P79L, Y180X, A227T, Q297X, F379L and Q533K) in addition to the W258X (common) and R90H (rare) variants that are frequently observed in Japanese RHUC patients, as described below.

Functional characterization of URAT1 variants

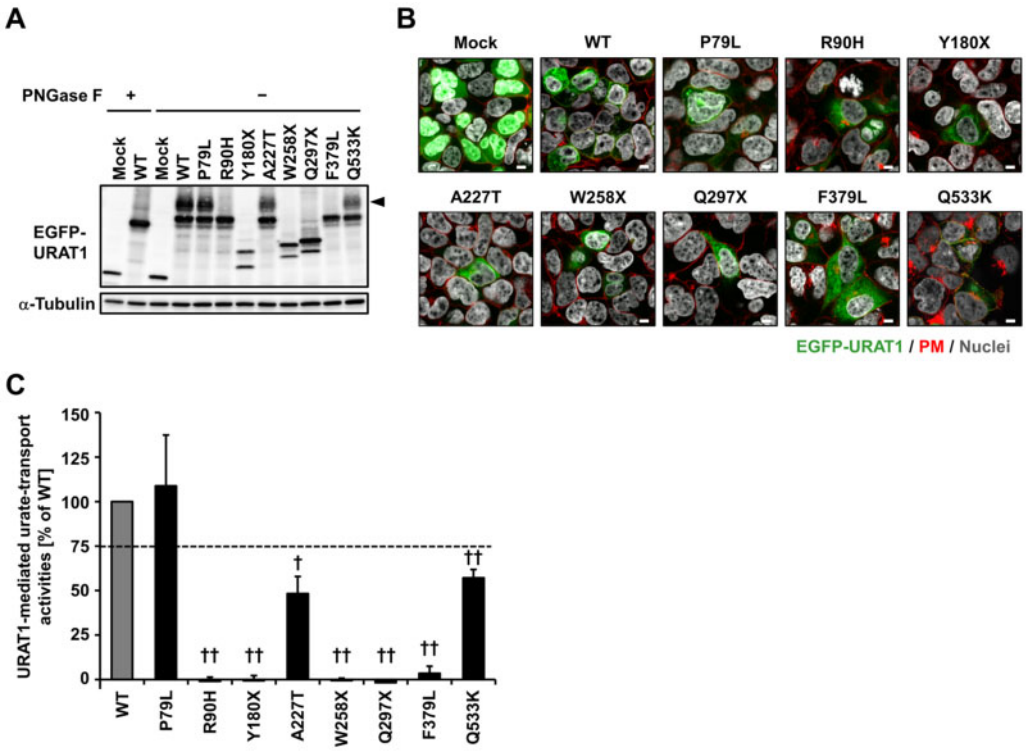
To examine the effect of the eight variants found in our genetic analysis on the URAT1 function as a urate transporter, we expressed each URAT1 variant tagged with EGFP in 293A cells, a human renal cell line. First, to confirm the protein expression, we performed immunoblot analysis of whole-cell lysate samples using the anti-EGFP antibody (Fig. 1A). As expected, URAT1 WT was matured as a glycoprotein evidenced by the presence of PNGase F-sensitive immunoreactive bands at high molecular weight ranges. Our results revealed only three

Table 2 All common and rare non-synonymous variants of *URAT1* identified in the present study and their urate transport function

Type of variant	rs number	Position[a]	DNA sequence change[b]	AA change	Case[c] (*n*)	MAF in case (%)	Control[c] (*n*)	MAF in control (%)	Transport function (%)	MAF in Japanese[d] (%)
Common variant	rs121907892	64361219	G774A	W258X	0	0	16	1.77	[b]0	2.28
Rare variant	rs757926758	64359264	C236T	P79L	1	0.104	0	0	108.8	NA
	rs121907896	64359297	G269A	R90H	0	0	2	0.208	0[f]	0.339
	rs1286450383	64360910	C540A	Y180X	0	0	2	0.208	0[f]	NA
	rs201136391	64361124	G679A	A227T	0	0	2	0.208	48.3	0.248
	NA	64366046	C889T	Q297X	0	0	1	0.104	0[f]	0.0558
	NA	64367214	C1137G	F379L	0	0	1	0.104	3.5	NA
	rs765990518	64367222	A1145T	Q382L	0	0	2	0.208	3.8[g]	NA
	rs121907895	64367330	T1253G	L418R	0	0	1	0.104	1.9[g]	0.167
	NA	64368409	C1597A	Q533K	1	0.104	0	0	57.2	0.0418
	NA	64368997	1639_1643del	V547fsL	0	0	1	0.104	5.7[g]	NA
Total of rare variant carriers[e]					2		12			
Total number of participants					480		480			

[a]SNP positions are based on the GRCh37 assembly. [b]Nucleotide numbering is based on the DNA reference sequence from GenBank (accession code NM_144585). [c]Summary count of homozygous and heterozygous carrier participants. Except for one W258X homozygous participant, only heterozygous or wild-type carriers were observed. There were no individuals with more than one rare variant. [d]MAF in Japanese refers to the Human Genetic Variation Database (ver. 2.30) [33]. [e]Count of rare variant carriers of *URAT1*. Only P79L was excluded from dysfunctional variants in the subsequent analyses (see Table 3). [f]Values were calculated as under 0. [g]Values are from Wakida *et al.* [16]. AA: amino acid; OR: odds ratio; MAF: minor allele frequency; NA: not assigned.

FIG. 1 Functional characterization of common and rare variants of *URAT1* identified in this study



(A) Immunoblot detection. Arrowhead, matured URAT1 as a glycoprotein; α-Tubulin, loading control. Three variants characterized by an acquired stop codon (Y180X, W258X and Q297X) resulted in the production of truncated proteins; two variants (R90H and F379L) were not matured; wild-type (WT) and three variants (P79L, A227T and Q533K) were expressed as matured forms. **(B)** Confocal microscopy. Nuclei were stained with TO-PRO-3 iodide (grey); plasma membrane (PM) was labelled with Alexa Fluor® 594-conjugated WGA (red). Bars, 5 μm. The five variants (R90H, Y180X, W258X, Q297X and F379L) were only barely localized on the PM. **(C)** Functional assay. Incorporation of radiolabeled urate into the cells expressing each URAT1 variant was measured and URAT1-dependent urate transport activities were calculated. Data are shown as % of WT and expressed as means (S.D.), $n = 3$. ††, $P < 0.01$; †, $P < 0.05$ *vs* WT control (one sample *t*-test).

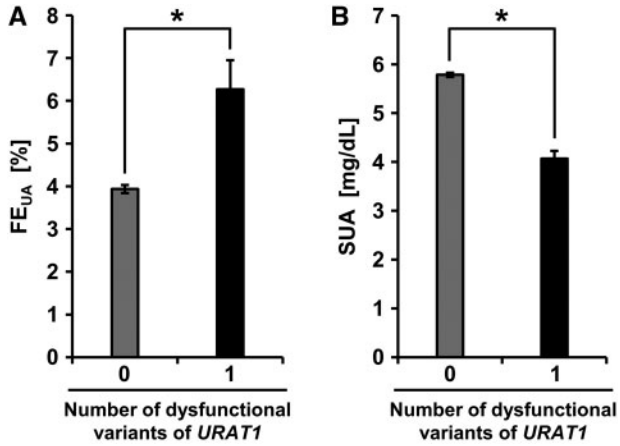
(P79L, A227T and Q533K) variants to be expressed as a matured glycoprotein in addition to WT protein, while the others (R90H, Y180X, W258X, Q297X and F379L) showed little in the way of signals corresponding to the matured form. Next, with confocal laser microscopy, we examined the intracellular localization of each URAT1 variant in 293A cells (Fig. 1B). The five variants (R90H, Y180X, W258X, Q297X and F379L), which were not maturated as a glycoprotein, hardly localized on the plasma membrane, contrary to the WT.

Last, we investigated the urate-transporting activity of each URAT1 variant in the cell-based uptake assay (Fig. 1C). As expected, the five immature URAT1 variants (R90H, Y180X, W258X, Q297X and F379L) were functionally null as a urate transporter. The urate transport activities of A227T and Q533K were approximately half that of the URAT1 WT. Conversely, the P79L mutation hardly affected URAT1 function; we therefore excluded this variant from subsequent association analyses.

Lowering effect of dysfunctional *URAT1* variants on SUA levels

We next further investigated the pathophysiological impact of identified variants of *URAT1* with a focus on renal urate handling in humans (Fig. 2). In our cohort, 15 heterozygous carriers and one homozygous carrier of the common dysfunctional variant W258X were found in 480 controls (3.33%); no carriers were found in 480 gout cases. Of the nine dysfunctional variants (R90H, Y180X, A227T, Q297X, F379L, Q382L, L418R, Q533K and V547fsL), 12 control subjects were heterozygous carriers for either of the rare variants (2.50%, 12 heterozygotes among 480 controls). Only one gout patient was a Q533K heterozygous carrier (0.208% of 480 gout cases). Carriers with these dysfunctional variants had significantly higher FE_{UA} (Fig. 2A) and lower SUA levels (Fig. 2B) than those of controls without these variants ($P = 6.14 \times 10^{-8}$ and 2.98×10^{-20}, respectively). Our results suggest that these common and rare heterozygous dysfunctional variants in *URAT1*

FIG. 2 Physiological impact of common and rare variants of *URAT1* identified in this study



The dysfunctional variants of *URAT1* significantly increased FE_{UA} **(A)** and decreased SUA **(B)** among 480 Japanese male controls. *$P < 0.001$. Bars show means (S.E.).

drastically reduce the net amount of re-absorbed urate from urine into blood, resulting in a marked decrease in SUA level that would provide a substantial gout-protective effect.

Anti-gout effect conferred by common and rare variants of *URAT1*

Finally, we addressed the effect of the *URAT1* variants on gout risk. Stratified association analyses (Table 3) demonstrate the nine rare dysfunctional variants to significantly decrease gout risk (OR 0.0788, reciprocal OR 12.7, $P = 1.47 \times 10^{-3}$). All the common and rare dysfunctional variants of *URAT1* drastically decreased the risk of gout (OR 0.0338, reciprocal OR 29.6, $P = 7.66 \times 10^{-8}$). Furthermore, to gain a quantitative insight into the anti-gout effect conferred by the dysfunctional variants of *URAT1* against the gout-promotive effect caused by those of *ABCG2* (a gout gene), we performed multivariate logistic regression analysis of gout with the dysfunctional variants of *URAT1* and *ABCG2*. The results revealed the dysfunctional variants of *URAT1* to have a substantial protective and beneficial effect (OR 0.0339, reciprocal OR 29.5) that outweighed the gout-promotive effect of *ABCG2* (OR 2.30–3.32) (Table 4). These findings indicate that common and rare variants of *URAT1* have a substantial anti-gout effect against the common disease of gout.

Discussion

In the present study, using a Japanese cohort of 480 patients with clinically defined gout and the same number of controls without hyperuricaemia or gout history, we explored the exonic non-synonymous variants of *URAT1* (Table 2). A series of functional assays, together with the previous studies, revealed that all variants, except for the P79L variant found in this study, are functionally null or reduced (Fig. 1); such variants of *URAT1* have an anti-gout effect that is significant enough to cancel out the gout risk conferred by dysfunctional variants of *ABCG2* (Table 4). Additionally, since we addressed the Japanese population, we successfully identified several novel and pathophysiologically important rare variants of *URAT1* that were not found in European and African American populations in a previous study [25]. A recent study addressing a cohort of gout/hyperuricaemia in the Czech Republic also did not find such *URAT1* variants [26], which supports the importance of conducting genetic studies in various ethnic populations. To the best of our knowledge, this is the first study to make a quantitative comparison of genetic effects of common and rare variants between *URAT1* and *ABCG2* in combination with functional validation.

We previously reported dysfunctional common and multiple rare variants of *ABCG2* to be major contributors to gout as a common disease, supporting the 'Common Disease, Common Variant (CDCV)' and 'Common Disease, Multiple Rare Variant (CDMRV)' hypothesis. This led us to propose the genetic concept of a 'Common Disease, Multiple Common and Rare Variant (CDMCRV)' model [13]. Conversely, the dysfunctional rare variants of *URAT1* have been reported to be the pathophysiological causes of RHUC, a hereditary disorder [27]. Via the stratified association analyses and the multivariate logistic regression analysis of gout susceptibility with dysfunctional variants of *URAT1* and *ABCG2,* the present study revealed that common and multiple rare dysfunctional variants of *URAT1* significantly decrease gout susceptibility, and that their protective effects independently overwhelm the causative effect of common (Q126X and Q141K) and multiple rare variants of *ABCG2*. In addition to recent studies reporting common protective variants against some common diseases [28, 29], this study is the first to reveal multiple rare protective variants of *URAT1* against the common disease

Table 3 Common and rare dysfunctional variants of *URAT1/SLC22A12* strongly decrease gout susceptibility

Dysfunctional variants of *URAT1*	Case			Control					
	Number	Carrier[a]	Freq[b] (%)	Number	Carrier[a]	Freq[b] (%)	*P*-value	OR (95% CI)	Reciprocal OR (95% CI)
All rare variants	480	1	0.208	464	12	2.59	1.47×10^{-3}	0.0788 (0.00184, 0.536)	12.7 (1.86, 543.4)
All common and rare variants	480	1	0.208	480	28	5.83	7.66×10^{-8}	0.0338 (0.000827, 0.206)	29.6 (4.85, 1209.0)

One common variant W258X and nine rare variants (R90H, Y180X, A227T, Q297X, F379L, Q382L, L418R, Q533K and V547fsL) were identified as dysfunctional variants of *URAT1/SLC22A12* (details are in Table 2). A non-synonymous variant P79L of *URAT1* is excluded here due to its non-altered function. Fisher's exact test was used for the calculation for the *P*-values. [a]The number of carriers with dysfunctional non-synonymous variants in *URAT1*. [b]The percentage of carriers in case or control populations. Freq: frequency, OR: odds ratio.

Table 4 Multivariate logistic regression analysis of gout susceptibility with dysfunctional variants of *URAT1* and *ABCG2*

Variables	β	OR (95% CI)	*P*-value
Dysfunctional variants of *URAT1*	−3.39	0.0339[a] (0.00454, 0.253)	9.68×10^{-4}
Q126X (Common variants of *ABCG2*)	1.20	3.32 (1.97, 5.59)	6.20×10^{-6}
Q141K (Common variants of *ABCG2*)	0.83	2.30 (1.88, 2.82)	1.26×10^{-15}
Rare variants of *ABCG2*	0.90	2.47 (1.29, 4.73)	6.62×10^{-3}

[a]The reciprocal OR (odds ratio) of common and rare dysfunctional variants of *URAT1* is 29.5. β is for per copy of the allele.

of gout. Our findings can also strengthen the CDCV and CDMRV hypothesis as well as the CDMCRV model. Furthermore, while our previous study proposed the CDMCRV model for the association between only *ABCG2* and gout [13], the present study shows that multiple common and rare variants of not only *ABCG2* but also *URAT1* are markedly associated with gout, demonstrating a new CDMCRV model by two genes (*ABCG2* and *URAT1*).

Also, the results of this study will deepen the understanding of genetic variations associated with the risk of RHUC. While the frequency of RHUC is relatively high in East Asia, especially in Japan (~0.3%) [27], this hereditary disorder is also reported in Jewish [30] and European Roma [31] populations. Moreover, RHUC patients have been described in a variety of ethnic groups including Arab Israelis, Iraqi Jews, Caucasians, and European Roma and in geographically non-contiguous countries [32]. In addition to RHUC risk, in this context, our findings will also be meaningful to evaluate gout risk in non-Asian populations.

The limitations of this study include the following. To confirm the CDMCRV model more robustly, the identification of more rare variants of *URAT1* will be needed. The expected protective effects of rare dysfunctional variants of *URAT1* against gout should also be assessed in future studies, which will assist with the development of personalized genome medicines for subjects with *URAT1* rare variants against gout.

In conclusion, common and multiple rare dysfunctional variants of *URAT1* have substantial anti-gout effects. Our findings provide significant insights into genetic factors that protectively and substantially influence the risk of gout.

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Data availability statement

Data are available upon reasonable request.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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