EXTENDED REPORT

GWAS of clinically defined gout and subtypes identifies multiple susceptibility loci that include urate transporter genes

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ABSTRACT

Objective A genome-wide association study (GWAS) of gout and its subtypes was performed to identify novel gout loci, including those that are subtype-specific.

Methods Putative causal association signals from a GWAS of 945 clinically defined gout cases and 1213 controls from Japanese males were replicated with 1396 cases and 1268 controls using a custom chip of 1961 single nucleotide polymorphisms (SNPs). We also first conducted GWASs of gout subtypes. Replication with Caucasian and New Zealand Polynesian samples was done to further validate the loci identified in this study.

Results In addition to the five loci we reported previously, further susceptibility loci were identified at a genome-wide significance level (p<5.0×10−8): urate transporter genes (SLC22A12 and SLC17A1) and HIST1H2BF-HIST1H4E for all gout cases, and NIPAL1 and FAM35A for the renal underexcretion subtype. While NIPAL1 encodes a magnesium transporter, functional analysis did not detect urate transport via NIPAL1, suggesting an indirect association with urate handling. Localisation analysis in the human kidney revealed expression of NIPAL1 and FAM35A mainly in the distal tubules, which suggests the involvement of the distal nephron in urate handling in humans. Clinically ascertained male patients with gout and controls of Japanese and/or its subtypes. In addition, for the first time, we performed GWASs of gout subtypes to identify

INTRODUCTION

Gout is a common disease characterised by acute painful arthritis, and its global burden continues to rise with the increasingly ageing population.1 Gout is caused by hyperuricaemia, and can be classified according to patients’ clinical parameters reflecting its causes2,3 as renal overload (ROL) gout and renal underexcretion (RUE) gout. As shown in online supplementary figure S1, patients with gout with increased urinary excretion of urate due to overproduction and/or decreased extra-renal underexcretion of urate are classified as having RUE gout, whereas those with decreased renal excretion of urate are defined as having RUE gout.2 Reflecting their causes, almost all patients with gout are divided into those two subtypes. Although these subtypes are important from both genetic and pathophysiological points of view,2,4 genome-wide association studies (GWAS) of gout subtypes have never been performed, partly due to the difficulty in assembling sufficient gout cases with requisite clinical data, including data from a time-consuming urinary collection examination.

We and other groups5–9 recently reported gout/hyperuricaemia to have relatively strong genetic risk factors. More recently, and for the first time, we performed a GWAS with only clinically defined Japanese male gout cases in which 16 single nucleotide polymorphisms (SNPs) were replicated, and five gout-risk loci were identified including two novel loci (MYL2-CUX2 and CN1H-2).10 In the present study (see online supplementary figure S2), we extended our analysis to identify novel susceptibility loci for gout by replicating approximately 2000 SNPs top-ranked in the GWASs of all gout and/or its subtypes. In addition, for the first time, we performed GWASs of gout subtypes to identify

subtype-specific (cause-specific) risk loci. Furthermore, we conducted a replication study with independent Caucasian and Polynesian populations to validate loci.

METHODS

Subjects and genotyping

Genome-wide genotyping was performed with the Illumina HumanOmniExpress-12 v1.0 (Illumina) platform using 946 clinically defined gout cases and 1213 controls, all Japanese males. Detailed methods of genotyping and quality control are previously described.\(^9^\) Ultimately, 570 442 SNPs passed filters for 945 cases and 1213 controls. At the replication stage, 1246 cases were genotyped with a custom genotype platform using iSelect HD Custom Genotyping BeadChips (Illumina) on 1961 SNPs, as described in online supplementary methods and supplementary figure S3, and 150 gout cases were genotyped with the Illumina HumanOmniExpress-24 v1.0 (Illumina) platform. For controls, 1268 Japanese males with a serum uric acid level ≤ 7.0 mg/dL and without gout history were recruited from BioBank Japan\(^1^0\) and 1268 controls were successfully genotyped (see online supplementary table S1). A genome-wide significance threshold was set to be \(\alpha_\text{FWER}=5.0\times10^{-8}\) to claim evidence of a significant association.

GWASs of the two subtypes of gout, ROL gout and RUE gout (see online supplementary figure S1), were also performed, followed by replication studies with a custom SNP chip (see online supplementary figure S3) and a subsequent meta-analysis. As described previously,\(^2\)\(^1^0\) and shown in online supplementary figure S1 and supplementary methods, ROL gout and RUE gout are defined when patients’ urinary urate excretion is over 25.0 mg/hour/1.73 m\(^2\) (600 mg/day/1.73 m\(^2\) ) and patients’ urate clearance (urate clearance/creatinine clearance ratio, FEUA) is under 5.5%, respectively. For GWASs of gout subtypes, 1178 cases were classified as ROL gout (560 cases at GWAS stage and 618 cases at replication stage) and 1315 cases as RUE gout (619 cases at GWAS stage and 696 cases at replication stage), respectively (see online supplementary table S2).

A replication study with independent Caucasian and New Zealand (NZ) Polynesian sample sets was also performed to validate the genetic risk loci identified in the present study. This replication was done in a data set recruited from New Zealand\(^1^3\) and from Europe by the Eurogout Consortium comprising 1319 male cases and 514 male controls of European ancestry and 971 male cases and 565 male controls of NZ Polynesian ancestry. SNPs were genotyped by an allelic discrimination assay (TaqMan) with a LightCycler 480 Real-Time PCR (RT-PCR) System (Roche Applied Science, Indianapolis, Indiana, USA). Detailed information of clinical characteristics and genetic analysis is shown in online supplementary methods and tables S1–S3.

Statistical analyses

The inverse-variance fixed-effects model was used for meta-analysis. In the meta-analysis with Japanese, Caucasian and NZ Polynesian populations or in the presence of heterogeneity (\(P_{\text{het}} < 0.05\) or \(I^2 > 50\%\)), we implemented the DerSimonian and Laird random-effects model for meta-analysis.\(^1^5\) For the replication analysis with Caucasian and NZ Polynesian sample sets, ORs were adjusted by age and ancestral group. All the meta-analyses were performed using the R V3.1.1 and 3.2.2 (R Development Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing, 2006) with meta package. All calculations of linkage disequilibrium (LD, measured in \(r^2\)) were conducted using the Japanese population. The detailed information for statistical analyses is described in online supplementary methods.

Functional and localisation analyses

Urate transport analysis of NIPAL1 was performed with an oocyte expression system\(^1^6\)\(^1^7\) with high potassium (HK) buffer or HK buffer without magnesium. For immunohistochemical analysis, the human kidney sections (3 μm) incubated with anti-human NIPAL1 antibody (1:50) (LS-C164878; LifeSpan BioSciences, Washington, USA) or with anti-human FAM35A antibody (1:75) (HPA036582; Sigma-Aldrich, Missouri, USA) were used, and then visualised with diaminobenzidine (0.8 mM).\(^1^8\)\(^1^9\) Intracellular localisation of NIPAL1 was also studied in *Xenopus* oocytes and Madin-Darby canine kidney II (MDCKII) cells. Detailed information for the functional and localisation analyses is described in online supplementary methods.

RESULTS

GWAS of all gout and its subtypes

In addition to the GWAS stage previously performed with 945 patients with clinically defined gout and 1213 controls, all Japanese males\(^1^0\) (see online supplementary figure S4), the replication stage for all cases of gout was carried out by genotyping 1961 SNPs (see online supplementary figure S3 and supplementary note) in a further 1396 male patients and 1268 male controls, and a meta-analysis then conducted (see online supplementary figure S2). Furthermore, GWASs of two subtypes of gout, ROL gout (figure 1A) and RUE gout (figure 1B), were also performed in the present study, followed by replication studies with a custom SNP chip and a subsequent meta-analysis.

Meta-analysis of both the GWAS and the replication study for all gout cases (table 1) identified eight loci which showed evidence for associations at the genome-wide significance level: rs3114020 of ABCG2 (\(P_{\text{meta}}=8.66\times10^{-15}\); OR = 1.89), rs1014290 of SLC2A9 (\(P_{\text{meta}}=6.50\times10^{-26}\); OR = 1.57), rs4766566 of CUX2 (\(P_{\text{meta}}=4.03\times10^{-20}\); OR = 1.51), rs2283540 of SLC22A12 (\(P_{\text{meta}}=4.61\times10^{-11}\); OR = 1.40), rs1260326 of GCKR (\(P_{\text{meta}}=7.19\times10^{-11}\); OR = 1.31), rs1156176 of SLC17A1 (\(P_{\text{meta}}=1.47\times10^{-9}\); OR = 1.42), rs11758351 of HIST1H2BF-HIST1H4E (\(P_{\text{meta}}=1.63\times10^{-3}\); OR = 1.40) and rs4073582 of CNHI-2 (\(P_{\text{meta}}=3.56\times10^{-8}\); OR = 1.58). Among these eight loci, SLC22A12, SLC17A1 and HIST1H2BF-HIST1H4E (figure 2A–C) were first identified as gout-risk loci by the GWAS approach at the genome-wide significance level. SLC17A1 was identified here by the GWAS approach for the first time, while Hollis-Moffatt et al.\(^2^0\) reported that rs1183201, another SNP of SLC17A1, is strongly associated with gout in Caucasians and NZ Polynesian sample sets by the candidate gene approach. While rs11758351 of HIST1H2BF-HIST1H4E is located 374 kb downstream from rs1165176 of SLC17A1, they are not in LD with each other (\(r^2=0.03\)), demonstrating them to be independent susceptibility loci for gout. There was also a significant signal from rs2532941 of VARS2 (\(P_{\text{meta}}=2.74\times10^{-12}\); OR = 1.32), which is located downstream of HIST1H2BF-HIST1H4E for 4.7 Mb, and is reported to be associated with mitochondrial respiration.\(^2^1\) Since rs2532941 of VARS2 showed mild LD with rs11758351 of HIST1H2BF-HIST1H4E (\(r^2=0.37\)), its significance did not remain for the GWAS stage samples after adjustment with rs11758351 of HIST1H2BF-HIST1H4E (\(P=0.08\)) or with both rs1165176 of SLC17A1 and rs11758351 (\(P=0.11\)).
For GWASs of gout subtypes, 1178 cases were classified as ROL gout (560 cases at GWAS stage and 618 cases at replication stage) and 1315 cases as RUE gout (619 cases at GWAS stage and 696 cases at replication stage), respectively. GWAS, genome-wide association study; ROL, renal overload; RUE, renal underexcretion.

Urate transport analysis of NIPAL1 transporter
NIPAL1 and FAM3SA were revealed to be associated with RUE gout in the present study. NIPAL1 has been reported to be a magnesium transporter,23 which has nine transmembrane domains (figure 3A), whereas FAM3SA is predicted to be a soluble protein. In this context, we hypothesised that NIPAL1 could be involved in the regulation of urate handling as a renal urate efflux transporter. However, our functional analysis using Xenopus oocytes did not show urate transport via NIPAL1, regardless of the presence of magnesium (figure 3B).

Localisation analysis of NIPAL1 and FAM3SA
By immunohistochemical analysis, NIPAL1 and FAM3SA showed cytosolic expression in the renal distal tubules of human kidney (figure 4A, B). Both proteins were also weakly detected in the cytoplasm of collecting ducts. NIPAL1-expressing Xenopus oocytes and MDCKII cells also showed intracellular localisation of NIPAL1 (see online supplementary figure S6).

Replication study of all gout cases with Caucasian and Polynesian populations
A replication study for the discovered loci (SLC22A12, SLC17A1, HIST1H2BF-HIST1H4E, NIPAL1 and FAM3SA) was performed for all gout cases with males drawn from Caucasian (1319 cases and 514 controls) and NZ Polynesian populations (971 cases and 565 controls). Because a gain-of-function SNP of SLC17A1, rs1165196 (Ile269Thr),16 was in strong LD with rs1165176 (r2=0.99), we performed the following analyses using rs1165196, assuming that the causal SNP in this locus was rs1165196 of SLC17A1. Among these five loci, the
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*dbSNP rs number. SNPs having associations for all gout, ROL gout and RUE gout at the lowest p value in each locus by meta-analysis are shown in this table.
SNPs positions are based on NCBI human genome reference sequence build 37.4.
\(d\)Five discovered loci are shown in bold.
\(\dagger\)rs1 is risk-associated allele and \(\dagger\) is non-risk-associated allele.
\(\ddagger\)G45 cases for all gout, 560 cases for ROL gout, 619 cases for RUE gout with 1213 controls from Japanese male population.
\(\dagger\)1396 cases for all gout, 618 cases for ROL gout, 696 cases for RUE gout with 1268 controls from Japanese male population.
\(\dagger\)Meta-analysis of GWAS and replication samples.
\(\dagger\)Chr., chromosome; GWAS, genome-wide association study; ROL, renal overload; RUE, renal underexcretion; SNP, single nucleotide polymorphism.
Figure 2 Regional association plots of five discovered loci. Three loci were revealed to exceed the genome-wide significance level from the meta-analysis with all gout cases, and two loci with renal underexcretion (RUE) gout cases. The highest association signal in each panel is located on (A) SLC22A12, (B) SLC17A1 and (C) HIST1H2BF-HIST1H4E for all gout cases, and (D) NIPAL1 and (E) FAM35A for RUE gout cases. The region within 250 kb from the single nucleotide polymorphism (SNP) indicating the lowest p value is shown. (Top panel) Plots of $-\log_{10} p$ values for the test of SNP association with gout in the genome-wide association study stage. The SNP showing the lowest p value in the meta-analysis is depicted as a pink diamond. Other SNPs are colour-coded according to the extent of linkage disequilibrium (measured in $r^2$) with the SNP showing the lowest p value. (Middle panel) Recombination rates (centimorgans per Mb) estimated from HapMap Phase II data are plotted. (Bottom panel) RefSeq genes. Genomic coordinates are based on NCBI human genome reference sequence build 37.

Figure 3 Functional analysis of NIPAL1 transporter. (A) The topological model of the NIPAL1 transporter. NIPAL1 is predicted to have nine transmembrane regions. The amino acid sequences of NIPAL1 were obtained from GenBank (accession code NM_207330). (B) Urate transport analysis of NIPAL1. SLC2A9 (also known as GLUT9) is a renal urate transporter and is used for a positive control for the urate transport analysis. In contrast to SLC2A9, urate transport via NIPAL1 was not detected, regardless of the presence of magnesium. Data are expressed as mean±SEM (n=8). Statistical analyses for significant differences were performed according to Student’s t-test. (**p<0.01; N.S., not significantly different as compared with control.).
meta-analysis of those populations for all gout revealed a significant association with rs7903456 of FAM35A ($p_{meta} = 9.72 \times 10^{-3}$; OR = 1.17) (table 2). Although SLC17A1 did not show significance ($p_{meta} = 0.119$) in the present study of those populations (table 2), a previous paper revealed a significant association of SLC17A1 with gout in Caucasian and NZ Polynesian sample sets, indicating the necessity of further replication studies to investigate the ancestral differences in the significance of other genetic loci including SLC17A1. Genotyping the CUX2 and CNIH-2 loci, which were identified in both our present and previous GWASs of Japanese, was also performed, and the CUX2 locus was replicated successfully for the first time in other populations (see online supplementary table S5). The results of further association analyses and expression quantitative trait locus (eQTL) analysis are shown in online supplementary note and tables S6 and S7. Significant effects on FEUA were detected in NIPAL1, FAM35A and SLC22A12 loci in the Japanese population, and were also observed at SLC17A1 in NZ Polynesian population.

A further meta-analysis of all gout cases with Japanese, Caucasian and NZ Polynesian populations was performed for NIPAL1 and FAM35A, which were at a genome-wide significance level in the Japanese population only for the RUE gout subtype, and not for all gout cases. rs11733284 of NIPAL1 was not associated with all gout ($p_{meta} = 0.16$; OR = 1.11), suggesting the presence of ancestral differences in genetic effects of this locus, or a subtype-specific effect. On the other hand, rs7903456 of FAM35A showed an association with all gout at a genome-wide level of significance ($p_{meta} = 3.58 \times 10^{-8}$; OR = 1.23) (figure 5), indicating that rs7903456 is a susceptibility locus for all gout as well as the RUE gout subtype.

![Figure 4](image_url)

**Figure 4** Localisation analysis of NIPAL1 and FAM35A in the human kidney. Cytosolic expression was detected strongly in distal tubules and weakly in collecting ducts in human kidney for (A) NIPAL1 protein and (B) FAM35A protein. Bar=100 μm.

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* dbSNP rs number.
† SNP positions are based on NCBI human genome reference sequence build 37.
‡ A1 is risk-associated allele, and A2 is non-risk-associated allele.
§ 1319 cases for all gout and 514 controls from Caucasian male population.
¶ 971 cases for all gout and 565 controls from NZ Polynesian male population.
** Meta-analysis of Caucasian and NZ Polynesian samples.
†† rs2285340 is monomorphic in Caucasians.
Chr., chromosome; NZ, New Zealand; SNP, single nucleotide polymorphism.
The \textit{HIST1H2BF} and \textit{HIST1H4E} genes encode histone 1 H2bf and histone 1 H4e, respectively, both of which have a role of binding DNA to form a chromatin structure. Both are replication-dependent histone proteins with expression dependent on cell cycle. Therefore, functional SNPs in this locus might affect the stability of the chromatin structure, varying the cell cycle, cell amount or reaction to inflammation by changing the expression level of histones in the kidney and/or intestine. Since it is also possible that rs11758351 is a surrogate marker near these histone genes, further studies concerning this locus will be necessary.

In this study, we first performed GWASs of gout subtypes, that is, RUE gout and ROL gout (figure 1). From the results of meta-analysis for GWASs of both ROL gout and RUE gout, four shared loci of GCKR, \textit{SLC2A9}, \textit{ABCG2} and \textit{CUX2} were identified at a genome-wide significance level, showing the importance of these loci for the pathogenesis of both gout subtypes. Especially for RUE gout, three more loci, \textit{SLC22A12}, \textit{NIPAL1} and \textit{FAM35A}, were identified to be associated at a genome-wide significance level. As described above, it is compatible for \textit{SLC22A12} to be associated with RUE gout, because \textit{SLC22A12} (like \textit{SLC2A9}) encodes a renal urate reabsorption transporter.\textsuperscript{23} 28

Of note, \textit{NIPAL1} and \textit{FAM35A} were identified as novel loci by performing GWAS of the RUE gout subtype. Associations with gout and SUA have never been previously reported with \textit{NIPAL1} and \textit{FAM35A}. Furthermore, to our knowledge, there is no study reporting an association between any diseases and \textit{NIPAL1} or \textit{FAM35A}.

\textit{NIPAL1}, also known as \textit{NIPAL3}, is reportedly expressed on the membrane of some organs including kidney, and to be a magnesium transporter,\textsuperscript{24} as another magnesium transporter \textit{NIPA2}.\textsuperscript{23} Because \textit{NIPAL1} was associated with RUE gout (ie, gout with renal urate underexcretion), we hypothesised that \textit{NIPAL1} is a urate transporter in the human kidney. However, our functional study did not show urate transport via \textit{NIPAL1}, regardless of the presence of magnesium (figure 3B). Moreover, localisation to the membrane was not detected for \textit{NIPAL1} protein, which was mainly expressed within the distal tubules of human kidney, as revealed by immunohistochemical analysis (figure 4A). A similar result was obtained in confocal microscopic observation, with \textit{NIPAL1}-expressing oocytes showing intracellular localisation of \textit{NIPAL1} protein (see online supplementary figure S6). These findings suggest that \textit{NIPAL1} is not a urate transporter and that it might be involved in the indirect regulation of urate transport kinetics. Nevertheless, recent studies have revealed associations between hyperuricaemia and magnesium intake,\textsuperscript{29} serum magnesium level\textsuperscript{30} and magnesium excretion.\textsuperscript{31} Together with previous reports, our findings support the hypothesis that there could be some relationship between gout and magnesium handling via magnesium transporters including \textit{NIPAL1}, and that the present study could well provide new insights into the genetic background of urate and magnesium handling in patients with gout/hyperuricaemia.

\textit{FAM35A} is ubiquitously expressed in organs including the kidney, and our immunohistochemical analysis of human kidney also revealed cytosolic immunoreactivity of the \textit{FAM35A} protein mainly in the distal tubules (figure 4B). Our findings from \textit{FAM35A} and \textit{NIPAL1} suggest the involvement of the distal nephron in gout progression as well as dysfunction in urate handling in humans (see online supplementary figure S9). To date, the molecular function of \textit{FAM35A} is totally unknown. Although further studies are necessary to confirm this, it is possible that genes near \textit{FAM35A} including \textit{GLUD1} (figure 2E)

**DISCUSSION**

With clinically defined gout cases, we previously performed a GWAS\textsuperscript{10} and revealed that ABCG2, \textit{SLC2A9}, \textit{MYL2-CUX2}, GCKR and CNIH-2 were associated with gout at a genome-wide significance level (see online supplementary figure S4). A more recent GWAS by Li et al\textsuperscript{25} with clinically ascertained gout cases revealed three novel loci (\textit{BCAS3}, \textit{REFX} and \textit{KCQ1}) in Han Chinese. In the present study, we performed a gout follow-up study focused on loci not reaching the genome-wide significance level of \textit{GWAS10} and revealed that \textit{ABCG2}, \textit{CUX2}, \textit{SLC2A9} (table 1). Among them, three loci (\textit{SLC22A12}, \textit{SLC17A1} and \textit{HIST1H2BF-HIST1H4E}) were first identified as gout risk loci at a genome-wide significance level by the present GWAS approach.

Both \textit{SLC22A12} and \textit{SLC17A1} encode urate transporters at the apical side of the renal proximal tubule\textsuperscript{16 23} (see online supplementary figure S8) and are reportedly associated with SUA in humans by previous GWASs of SUA.\textsuperscript{12 22 26 27} Therefore, it is reasonable that SNPs around these loci would display significant associations with gout or sequelae of hyperuricaemia (see also online supplementary note for detail).

**Figure 5** Forest plots for all gout among Japanese, Caucasian and New Zealand (NZ) Polynesian populations. Although rs11733284 of \textit{NIPAL1} (A) did not show significant association with all gout, rs7903456 of \textit{FAM35A} (B) revealed an association with all gout at a genome-wide significance level ($p_{meta}=3.58\times10^{-4}$; OR=1.23). GWAS, genome-wide association study.

**Meta-analysis of all gout for the other three loci (SLC22A12, SLC17A1 and HIST1H2BF-HIST1H4E) was also performed with Japanese, Caucasian and NZ Polynesian populations as shown in online supplementary figure S7.** The histone genes shown in online supplementary figure S9. We revealed a total of 1396 cases and 1268 controls. We revealed a total of 1396 cases and 1268 controls. We revealed a total of 1396 cases and 1268 controls.
have some relationship with gout (see also online supplementary note for details).

In addition to studying the Japanese population, we performed a replication study with male Caucasian and NZ Polynesian sample sets for the five newly discovered loci. Since they were not divided into subtypes, further evaluations by meta-analysis were conducted with all gout groups. While other loci were not replicated, rs7903436 of FAM35A was replicated with a significant association with gout (table 2). CX2, which was reported by both our present and previous gout GWAS in Japanese,10 was also replicated in these sample sets (see online supplementary table S5).

A meta-analysis of all gout with Japanese, Caucasian and NZ Polynesian populations for these five SNPs revealed FAM35A to be associated with all gout at the genome-wide significance level (figure S5B), and that rs2285340 of SLC22A12 and rs1165196 of SLC17A1 showed a significant association but did not reach a genome-wide significance level (see online supplementary figure S7). rs11758351 of HIST1H2BF-HIST1H4E and rs11733284 of NIPAL1 were not associated by this meta-analysis, although these loci showed a genome-wide significant association in the Japanese population. Since this might be due to the differences in LD structure among these populations, a replication analysis with East Asian populations will be necessary for these loci. rs2285340 of SLC22A12 was monomorphic (only G allele) in Caucasians and not associated with NZ Polynesians. Therefore, replication studies of this locus in East Asian populations would also be insightful for future analysis. Although the underlying molecular mechanism of gout by FAM35A is unknown, this locus seems to have a common pathophysiological risk of gout for Japanese, NZ Polynesians and Caucasians.

In summary, we performed GWASs of all gout as well as gout subtypes and identified five loci in addition to the five loci that we reported previously.10 Furthermore, the FAM35A locus showed an association with all gout by meta-analysis among the Japanese, Caucasian and NZ Polynesian sample sets at a genome-wide level of significance. Together with their expression in the renal distal tubules, the identification of NIPAL1 and FAM35A as gout loci suggests the involvement of the distal nephron (see online supplementary figure S9) in the urate handling of the human kidney and in the pathogenesis of gout/hyperuricemia. These findings could well provide a clue leading to a novel concept for the therapeutic target of gout (see online supplementary figure S10).

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