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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 ($p < 5.0 \times 10^{-8}$): urate transporter genes (*SLC22A12* and *SLC17A1*) and *HIST1H2BF-HIST1H4E* for all gout cases, and *NIPAL1* and *FAM35A* for the renal underexcretion gout 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 Caucasian and Polynesian ancestries were also genotyped, and *FAM35A* was associated with gout in all cases. A meta-analysis of the three populations revealed *FAM35A* to be associated with gout at a genome-wide level of significance ($p_{meta} = 3.58 \times 10^{-8}$).

Conclusions Our findings including novel gout risk loci provide further understanding of the molecular pathogenesis of gout and lead to a novel concept for the therapeutic target of gout/hyperuricaemia.

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 causes^{2,3} 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 ROL 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 (GWASs) 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 groups^{5–9} 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 *CNIH-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.¹⁰ 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 (SUA) level ≤ 7.0 mg/dL and without gout history were recruited from BioBank Japan^{11 12} and genotyped with the Illumina HumanOmniExpress-12 v1.0 (Illumina) platform. Finally, 1961 SNPs with 1396 gout cases and 1268 controls were successfully genotyped (see online supplementary table S1). A genome-wide significance threshold was set to be $\alpha=5.0 \times 10^{-8}$ 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 10} 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 m² (600 mg/day/1.73 m²) and patients' urate clearance (urate clearance/creatinine clearance ratio, FE_{UA}) 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 Zealand¹³ and from Europe by the Eurogout Consortium¹⁴ 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.¹⁵ 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 V.3.1.1 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^{16 17} 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).^{18 19} 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.

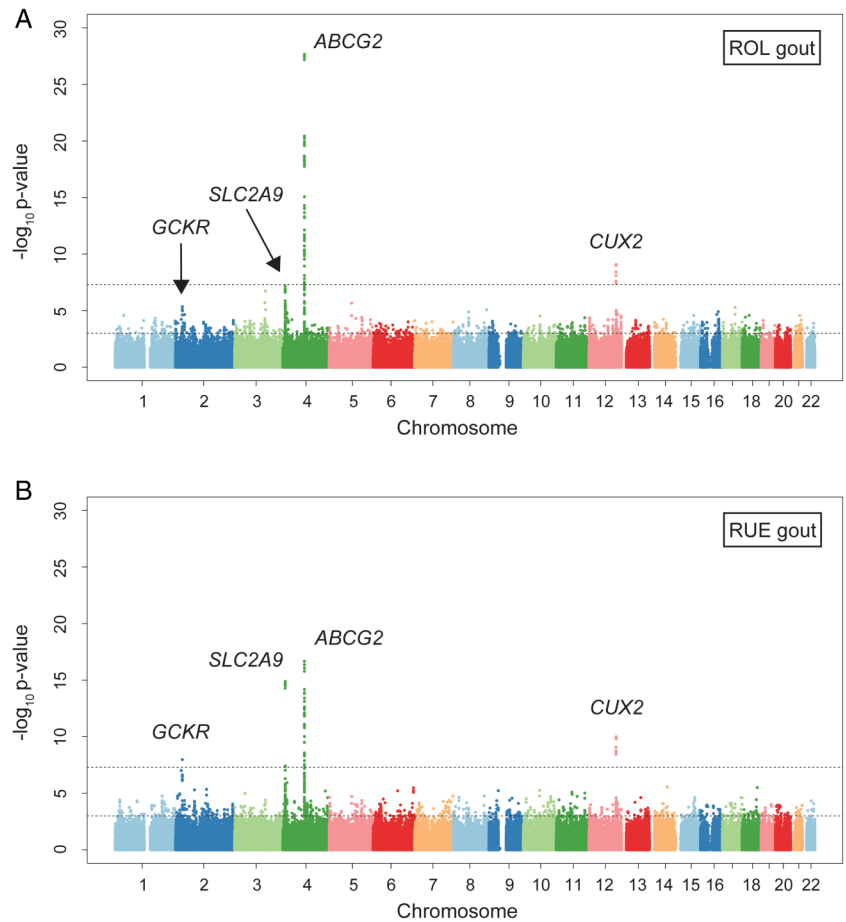
RESULTS

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 males¹⁰ (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* ($p_{\text{meta}}=8.66 \times 10^{-35}$; OR=1.89), rs1014290 of *SLC2A9* ($p_{\text{meta}}=6.50 \times 10^{-26}$; OR=1.57), rs4766566 of *CUX2* ($p_{\text{meta}}=4.03 \times 10^{-20}$; OR=1.51), rs2285340 of *SLC22A12* ($p_{\text{meta}}=4.61 \times 10^{-11}$; OR=1.40), rs1260326 of *GCKR* ($p_{\text{meta}}=7.19 \times 10^{-11}$; OR=1.31), rs1165176 of *SLC17A1* ($p_{\text{meta}}=1.47 \times 10^{-9}$; OR=1.42), rs11758351 of *HIST1H2BF-HIST1H4E* ($p_{\text{meta}}=1.63 \times 10^{-8}$; OR=1.40) and rs4073582 of *CNIH-2* ($p_{\text{meta}}=3.56 \times 10^{-8}$; 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*²⁰ 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* ($p_{\text{meta}}=2.74 \times 10^{-8}$; OR=1.32), which is located downstream of *HIST1H2BF-HIST1H4E* by 4.7 Mb, and is reported to be associated with mitochondrial respiration.²¹ 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 ($p=0.11$).

Figure 1 Manhattan plots of GWASs of subtypes of gout. Manhattan plots of GWASs of (A) ROL gout subtype and (B) RUE gout subtype. X-axis shows chromosomal positions. Y-axis shows $-\log_{10}$ p values. The upper and lower dotted lines indicate the genome-wide significance threshold ($p=5.0\times 10^{-8}$) and the cut-off level for selecting single nucleotide polymorphisms for replication study ($p=0.001$), respectively. GWAS, genome-wide association study; ROL, renal overload; RUE, renal underexcretion.



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). The meta-analysis of a GWAS of the ROL gout subtype and a replication study revealed significant SNPs in the following four loci: rs2728104 of *ABCG2* ($p_{meta}=5.08\times 10^{-33}$; OR=1.84), rs4766566 of *CUX2* ($p_{meta}=8.14\times 10^{-17}$; OR=1.59), rs3733589 of *SLC2A9* ($p_{meta}=2.25\times 10^{-13}$; OR=1.47) and rs1260326 of *GCKR* ($p_{meta}=5.39\times 10^{-9}$; OR=1.35).

Another subtype analysis, that is, the meta-analysis of a GWAS of RUE gout and a replication study (table 1) demonstrated significant SNPs in the following seven loci: rs1014290 of *SLC2A9* ($p_{meta}=8.71\times 10^{-25}$; OR=1.69), rs1871744 of *ABCG2* ($p_{meta}=2.49\times 10^{-22}$; OR=1.81), rs4766566 of *CUX2* ($p_{meta}=2.17\times 10^{-18}$; OR=1.60), rs2285340 of *SLC22A12* ($p_{meta}=8.79\times 10^{-10}$; OR=1.44), rs780094 of *GCKR* ($p_{meta}=1.62\times 10^{-9}$; OR=1.35), rs11733284 of *NIPAL1* ($p_{meta}=1.13\times 10^{-8}$; OR=1.34) and rs7903456 of *FAM35A* ($p_{meta}=4.29\times 10^{-8}$; OR=1.34). The latter two loci, *NIPAL1* and *FAM35A*, were novel risk loci by the GWAS of the RUE gout subtype (figure 2D, E). In total, 10 loci were identified from the present GWAS of gout and its subtypes (table 1 and see online supplementary table S4).

Of the seven loci newly identified by GWAS of the RUE gout subtype, only *NIPAL1* and *FAM35A* had not been implicated previously in the GWASs of SUA levels or gout. Analysis with data from previously reported GWAS²² of SUA in Caucasians revealed the association with *NIPAL1* and *FAM35A* loci (see online supplementary figure S5).

Urate transport analysis of *NIPAL1* transporter

NIPAL1 and *FAM35A* 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 *FAM35A* 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 *FAM35A*

By immunohistochemical analysis, *NIPAL1* and *FAM35A* 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 *FAM35A*) 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 ($r^2=0.99$), we performed the following analyses using rs1165196, assuming that the causal SNP in this locus was rs1165196 of *SLC17A1*. Among these five loci, the

Table 1 Single nucleotide polymorphisms (SNPs) associated with gout and its subtypes at a genome-wide level of significance in the Japanese population

Gout types	SNP*	Chr. (bp)†	Position	GWAS‡				Replication study**				Meta-analysis††					
				Frequency of A1		Frequency of A1		Frequency of A1		Frequency of A1		Heterogeneity		Heterogeneity			
				A1/A2§	Cases	Controls	OR (95% CI)	p Value	Cases	Controls	OR (95% CI)	p Value	OR (95% CI)	p Value	Cochran's Q	I ² (%)	
All gout	rs1260326	2	27730940	GCKR	T/C	0.616	0.535	1.39 (1.23 to 1.57)	1.34×10 ⁻⁷	0.611	0.557	1.25 (1.12 to 1.39)	6.10×10 ⁻⁵	1.31 (1.21 to 1.42)	7.19×10 ⁻¹¹	0.20	38.2
	rs1014290	4	10001861	SLC2A9	T/C	0.678	0.564	1.63 (1.44 to 1.85)	1.75×10 ⁻¹⁴	0.673	0.576	1.51 (1.35 to 1.69)	2.97×10 ⁻¹³	1.57 (1.44 to 1.70)	6.50×10 ⁻²⁶	0.39	0.0
	rs3114020	4	89083666	ABCG2	C/T	0.842	0.724	2.03 (1.75 to 2.37)	1.17×10 ⁻²⁰	0.844	0.752	1.78 (1.55 to 2.04)	7.74×10 ⁻¹⁷	1.89 (1.71 to 2.09)	8.66×10 ⁻³⁵	0.20	38.9
	rs1165176	6	25830298	SLC17A1	G/A	0.874	0.834	1.38 (1.16 to 1.64)	2.89×10 ⁻⁴	0.872	0.824	1.46 (1.25 to 1.69)	1.08×10 ⁻⁶	1.42 (1.27 to 1.59)	1.47×10 ⁻⁹	0.63	0.0
	rs11758351	6	26203910	HIST1H2BF-HIST1H4E	G/T	0.158	0.121	1.37 (1.15 to 1.63)	4.22×10 ⁻⁴	0.158	0.116	1.43 (1.22 to 1.67)	1.01×10 ⁻⁵	1.40 (1.25 to 1.57)	1.63×10 ⁻⁸	0.72	0.0
	rs2285340	11	64435906	SLC22A12	A/G	0.228	0.174	1.40 (1.21 to 1.63)	1.09×10 ⁻⁵	0.227	0.174	1.40 (1.22 to 1.61)	9.96×10 ⁻⁷	1.40 (1.27 to 1.55)	4.61×10 ⁻¹¹	1.00	0.0
	rs4073582	11	66050712	CNIH-2	C/T	0.950	0.915	1.78 (1.39 to 2.29)	4.32×10 ⁻⁶	0.943	0.920	1.44 (1.16 to 1.79)	8.47×10 ⁻⁴	1.58 (1.34 to 1.86)	3.56×10 ⁻⁸	0.21	36.1
	rs4766566	12	111706877	CUX2	T/C	0.735	0.633	1.60 (1.41 to 1.83)	1.22×10 ⁻¹²	0.741	0.665	1.44 (1.28 to 1.62)	2.07×10 ⁻⁹	1.51 (1.38 to 1.65)	4.03×10 ⁻²⁰	0.22	33.8
ROL gout	rs1260326	2	27730940	GCKR	T/C	0.611	0.535	1.36 (1.18 to 1.58)	2.43×10 ⁻⁵	0.626	0.557	1.33 (1.16 to 1.53)	6.12×10 ⁻⁵	1.35 (1.22 to 1.49)	5.39×10 ⁻⁹	0.81	0.0
	rs3733589	4	9987324	SLC2A9	G/A	0.662	0.570	1.48 (1.28 to 1.71)	2.00×10 ⁻⁷	0.668	0.580	1.46 (1.26 to 1.68)	2.05×10 ⁻⁷	1.47 (1.32 to 1.63)	2.25×10 ⁻¹³	0.88	0.0
	rs278104	4	88973006	ABCG2	C/T	0.505	0.346	1.93 (1.67 to 2.23)	3.28×10 ⁻¹⁹	0.496	0.359	1.75 (1.53 to 2.01)	1.56×10 ⁻¹⁵	1.84 (1.66 to 2.03)	5.08×10 ⁻³³	0.35	0.0
	rs4766566	12	111706877	CUX2	T/C	0.737	0.633	1.62 (1.39 to 1.90)	8.42×10 ⁻¹⁰	0.757	0.665	1.57 (1.34 to 1.83)	7.55×10 ⁻⁹	1.59 (1.43 to 1.78)	8.14×10 ⁻¹⁷	0.76	0.0
RUE gout	rs780094	2	27741237	GCKR	T/C	0.633	0.543	1.45 (1.26 to 1.67)	2.43×10 ⁻⁷	0.615	0.559	1.26 (1.10 to 1.44)	6.47×10 ⁻⁴	1.35 (1.22 to 1.48)	1.62×10 ⁻⁹	0.16	48.8
	rs1014290	4	10001861	SLC2A9	T/C	0.699	0.564	1.80 (1.55 to 2.08)	1.58×10 ⁻¹⁵	0.685	0.576	1.60 (1.39 to 1.84)	1.72×10 ⁻¹¹	1.69 (1.53 to 1.87)	8.71×10 ⁻²⁵	0.26	21.8
	rs11733284	4	48028097	NIPAL1	A/G	0.346	0.281	1.35 (1.17 to 1.57)	6.48×10 ⁻⁵	0.342	0.280	1.34 (1.16 to 1.54)	6.36×10 ⁻⁵	1.34 (1.21 to 1.49)	1.13×10 ⁻⁸	0.91	0.0
	rs1871744	4	89039629	ABCG2	T/C	0.834	0.723	1.93 (1.62 to 2.29)	3.85×10 ⁻¹⁴	0.824	0.733	1.71 (1.45 to 2.01)	7.04×10 ⁻¹¹	1.81 (1.60 to 2.04)	2.49×10 ⁻²²	0.33	0.0
	rs7903456	10	88919319	FAM35A	A/G	0.303	0.248	1.32 (1.13 to 1.53)	4.32×10 ⁻⁴	0.296	0.235	1.37 (1.18 to 1.59)	3.09×10 ⁻⁵	1.34 (1.21 to 1.49)	4.29×10 ⁻⁸	0.72	0.0
	rs2285340	11	64435906	SLC22A12	A/G	0.236	0.174	1.47 (1.25 to 1.74)	8.04×10 ⁻⁶	0.228	0.174	1.41 (1.20 to 1.66)	4.04×10 ⁻⁵	1.44 (1.28 to 1.62)	8.79×10 ⁻¹⁰	0.72	0.0
	rs4766566	12	111706877	CUX2	T/C	0.738	0.633	1.63 (1.40 to 1.89)	1.58×10 ⁻¹⁰	0.759	0.665	1.58 (1.36 to 1.83)	9.51×10 ⁻¹⁰	1.60 (1.44 to 1.78)	2.17×10 ⁻¹⁸	0.78	0.0

*dbSNP is 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.

†SNP positions are based on NCBI human genome reference sequence build 37.4.

‡Five discovered loci are shown in bold.

§A1 is risk-associated allele and A2 is non-risk-associated allele.

¶945 cases for all gout, 560 cases for ROL gout, 619 cases for RUE gout with 1213 controls from Japanese male population.

**1396 cases for all gout, 618 cases for ROL gout, 696 cases for RUE gout with 1268 controls from Japanese male population.

††Meta-analysis of GWAS and replication samples.

Chr., chromosome; GWAS, genome-wide association study; ROL, renal overload; RUE, renal underexcretion; SNP, single nucleotide polymorphism.

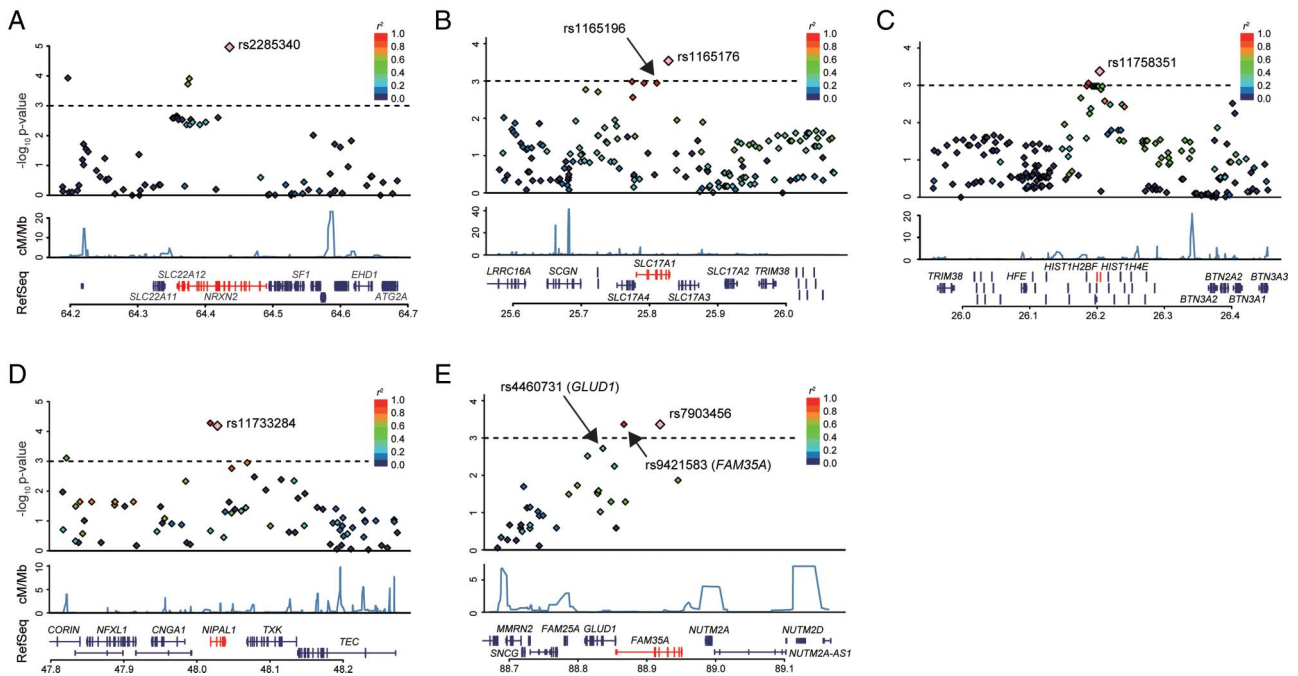
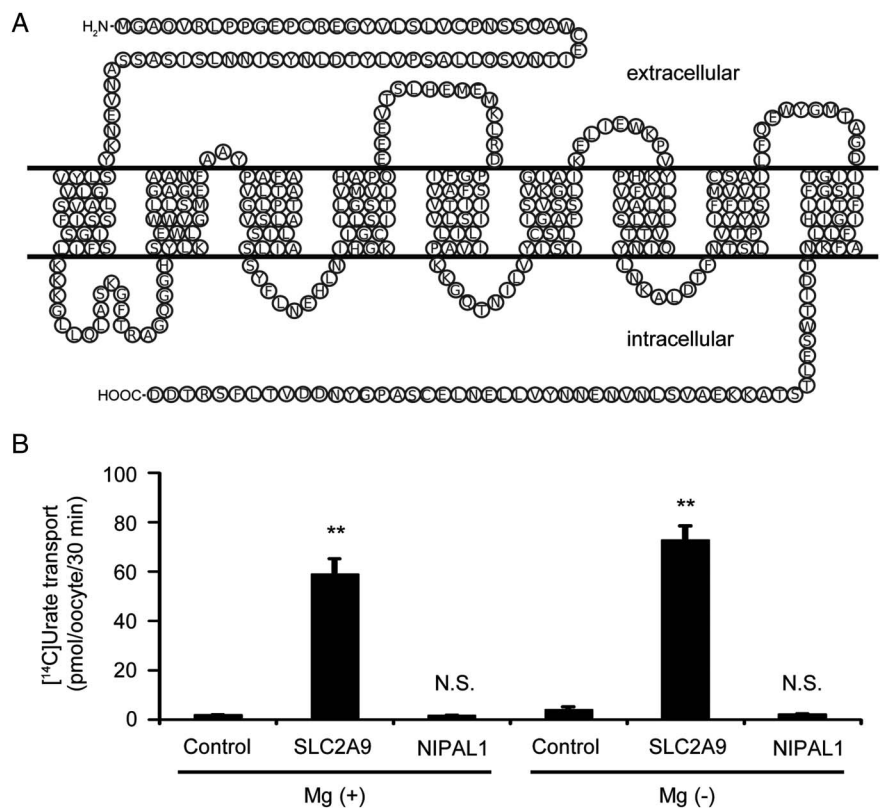


Figure 2 Regional association plots of five discovered loci. Three loci were revealed to exceed the genome-wide significance level from the meta-analysis with all gout cases, and two loci with renal underexcretion (RUE) gout cases. The highest association signal in each panel is located on (A) *SLC22A12*, (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 \pm 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.).



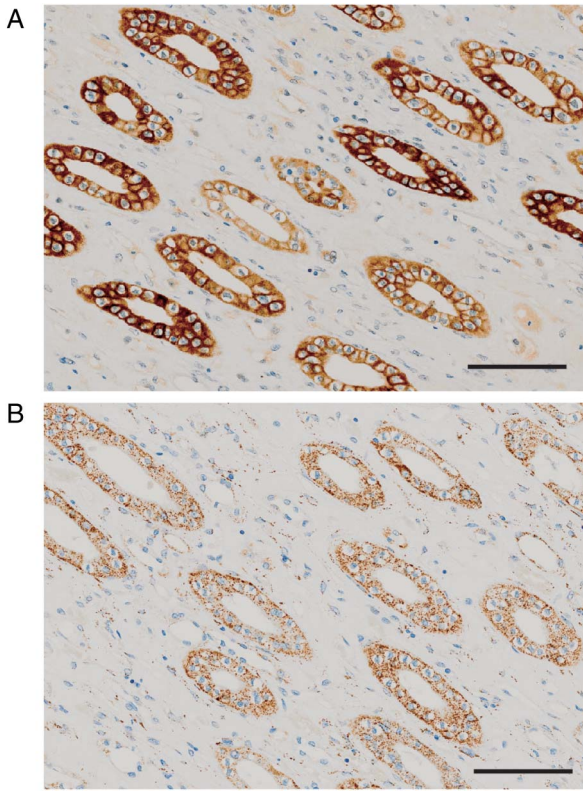


Figure 4 Localisation analysis of NIPAL1 and FAM35A in the human kidney. Cytosolic expression was detected strongly in distal tubules and weakly in collecting ducts in human kidney for (A) NIPAL1 protein and (B) FAM35A protein. Bar=100 µm.

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 paper²⁰ 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,¹⁰ 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 FE_{UA} 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.

Table 2 Replication study of all gout for five discovered loci in Caucasian and NZ Polynesian sample sets

SNP*	Chr.	position (bp)†	Gene	Caucasians‡				NZ Polynesians§				Meta-analysis**				
				Frequency of A1		p Value	OR (95% CI)	Frequency of A1		p Value	OR (95% CI)	p Value	OR (95% CI)	Heterogeneity		
				Cases	Controls			Cases	Controls					Cochran's Q	I ² (%)	
rs11733284	4	48028097	<i>NIPAL1</i>	A/G	0.362	0.356	1.01 (0.86 to 1.18)	0.896	0.251	0.270	0.92 (0.77 to 1.10)	0.355	0.97 (0.86 to 1.09)	0.603	0.43	0.0
rs1165196	6	25813150	<i>SLC17A1</i>	T/C	0.614	0.583	1.11 (0.95 to 1.30)	0.271	0.731	0.711	1.12 (0.93 to 1.35)	0.266	1.11 (0.98 to 1.25)	0.119	0.88	0.0
rs11758351	6	26203910	<i>HIST1H2BF-HIST1H4E</i>	G/T	0.141	0.158	0.86 (0.70 to 1.07)	0.173	0.192	0.199	0.90 (0.74 to 1.10)	0.334	0.88 (0.77 to 1.02)	0.0941	0.77	0.0
rs7903456	10	88919319	<i>FAM35A</i>	A/G	0.737	0.699	1.18 (1.00 to 1.40)	0.0462	0.351	0.333	1.16 (0.98 to 1.38)	0.0997	1.17 (1.04 to 1.32)	9.72×10 ⁻³	0.85	0.0
rs2285340††	11	64435906	<i>SLC22A12</i>	A/G	-	-	-	-	0.158	0.143	1.06 (0.84 to 1.35)	0.634	-	-	-	-

* dbSNP rs number.
 †SNP positions are based on NCBI human genome reference sequence build 37.4.
 ‡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.

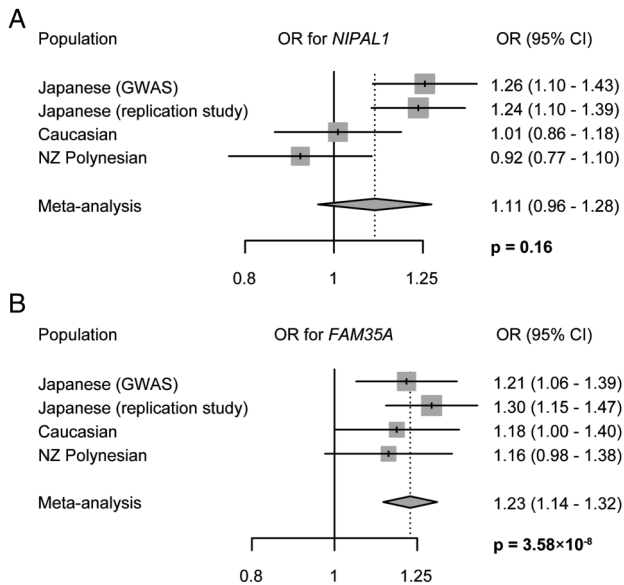


Figure 5 Forest plots for all gout among Japanese, Caucasian and New Zealand (NZ) Polynesian populations. Although rs11733284 of *NIPAL1* (A) did not show significant association with all gout, rs7903456 of *FAM35A* (B) revealed an association with all gout at a genome-wide significance level ($p_{meta}=3.58 \times 10^{-8}$; 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. rs11758351 of *HIST1H2BF-HIST1H4E* did not show a significant association with gout ($p_{meta}=0.40$; OR=1.12). rs2285340 of *SLC22A12* and rs1165196 of *SLC17A1* did not reach a genome-wide level of significance ($p_{meta}=2.47 \times 10^{-4}$; OR=1.31; and $p_{meta}=1.28 \times 10^{-3}$; OR=1.25, respectively) partly due to statistical fluctuation in relatively small sample sets, although the effects were consistently in the same direction.

DISCUSSION

With clinically defined gout cases, we previously performed a GWAS¹⁰ and revealed that *ABCG2*, *SLC2A9*, *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*²⁴ with clinically ascertained gout cases revealed three novel loci (*BCAS3*, *RFX3* and *KCNQ1*) in Han Chinese. In the present study, we performed a gout follow-up study focused on loci not reaching the genome-wide level of significance in the previous GWAS,¹⁰ genotyping 1961 SNPs in an additional 1396 cases and 1268 controls. We revealed a total of eight loci to be associated with all gout cases in Japanese males (table 1). Among them, three loci (*SLC22A12*, *SLC17A1* and *HIST1H2BF-HIST1H4E*) were first identified as gout risk loci at a genome-wide significance level by the present GWAS approach.

Both *SLC22A12* and *SLC17A1* encode urate transporters at the apical side of the renal proximal tubule^{16 25} (see online supplementary figure S8) and are reportedly associated with SUA level in humans by previous GWASs of SUA.^{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).

The *HIST1H2BF* and *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*, *SLC2A9*, *ABCG2* and *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, *SLC22A12*, *NIPAL1* and *FAM35A*, were identified to be associated at a genome-wide significance level. As described above, it is compatible for *SLC22A12* to be associated with RUE gout, because *SLC22A12* (like *SLC2A9*) encodes a renal urate reabsorption transporter.^{25 28}

Of note, *NIPAL1* and *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 *NIPAL1* and *FAM35A*. Furthermore, to our knowledge, there is no study reporting an association between any diseases and *NIPAL1* or *FAM35A*.

NIPAL1, also known as *NIPA3*, is reportedly expressed on the membrane of some organs including kidney, and to be a magnesium transporter,²³ as another magnesium transporter *NIPA2*.²³ Because *NIPAL1* was associated with RUE gout (ie, gout with renal urate underexcretion), we hypothesised that *NIPAL1* is a urate transporter in the human kidney. However, our functional study did not show urate transport via *NIPAL1*, regardless of the presence of magnesium (figure 3B). Moreover, localisation to the membrane was not detected for *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 *NIPAL1*-expressing oocytes showing intracellular localisation of *NIPAL1* protein (see online supplementary figure S6). These findings suggest that *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,²⁹ serum magnesium level³⁰ and magnesium excretion.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 *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.

FAM35A is ubiquitously expressed in organs including the kidney, and our immunohistochemical analysis of human kidney also revealed cytosolic immunoreactivity of the *FAM35A* protein mainly in the distal tubules (figure 4B). Our findings from *FAM35A* and *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 *FAM35A* is totally unknown. Although further studies are necessary to confirm this, it is possible that genes near *FAM35A* including *GLUD1* (figure 2E)

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, rs7903456 of *FAM35A* was replicated with a significant association with gout (table 2). *CUX2*, 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 5B), 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/hyperuricaemia. 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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Competing interests TT, KIchida, NS and HM have a patent pending based on the work reported in this paper.

Patient consent Obtained.

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