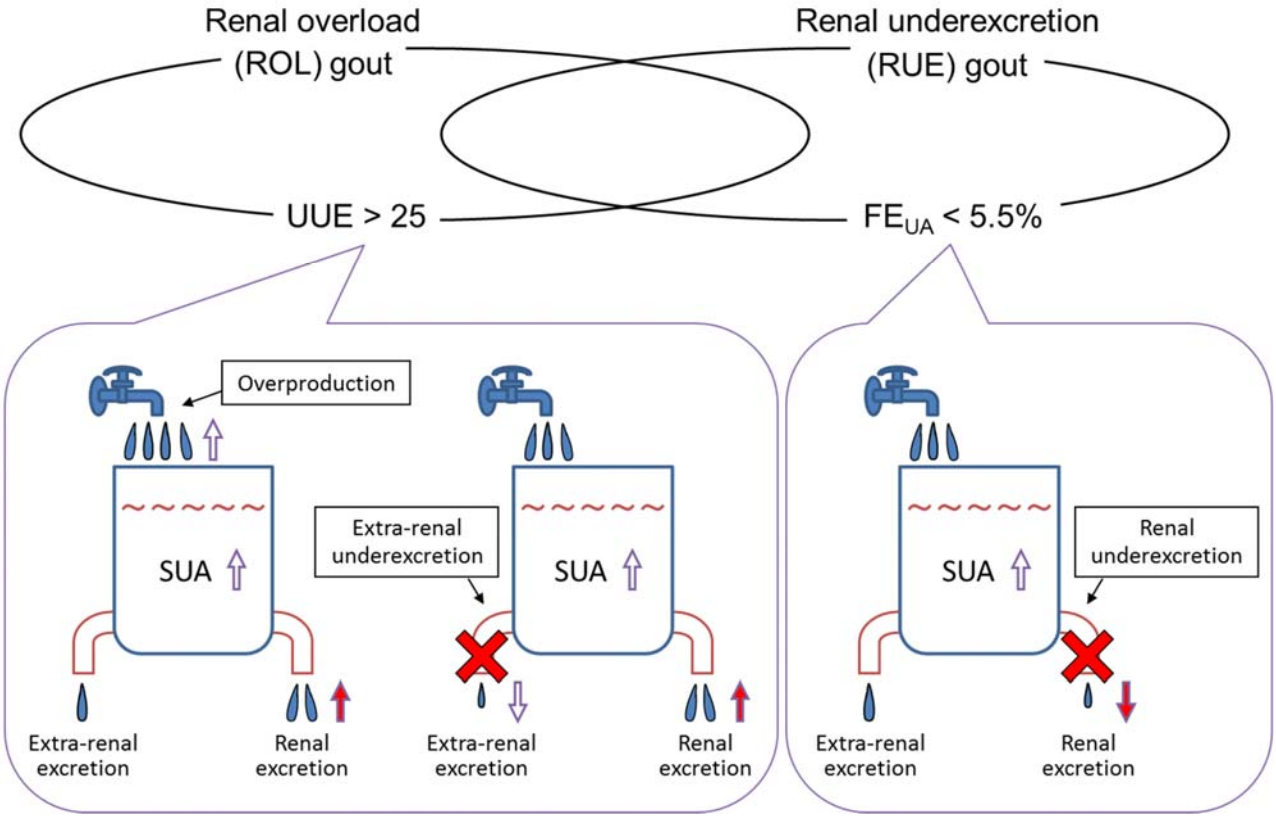
Supplementary Materials

GWAS of clinically-defined gout and subtypes identifies multiple susceptibility loci including urate transporter genes.

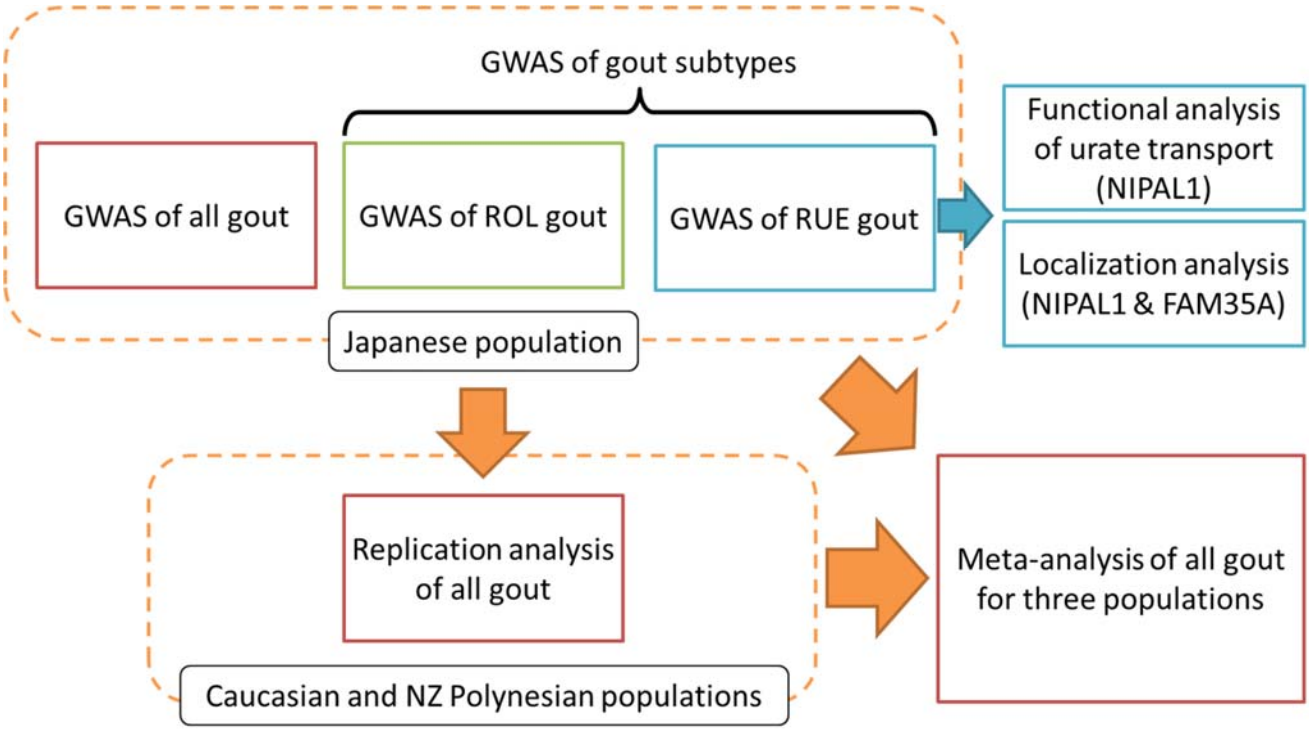
A. Nakayama, H. Nakaoka, K. Yamamoto, M. Sakiyama, A. Shaukat, Y. Toyoda, *et al.*
Correspondence to H. Matsuo. (email: hmatsuo@ndmc.ac.jp).



Supplementary Figure S1　Classification of gout.

Gout is a sequela to hyperuricemia which is characterized by an elevated serum uric acid level. In human, urate is produced in organs such as liver, and mostly excreted from the kidney and intestine.

According to its causes, gout can be classified into renal overload (ROL) gout and renal underexcretion (RUE) gout. ROL gout (or formerly-named “urate overproduction gout”) is caused by genuine urate overproduction and/or by extra-renal underexcretion, both of which are characterized by increased urinary urate excretion [UUE; over 25.0 mg/hr/1.73m^2 (600 mg/day/1.73m^2)]. On the other hand, RUE gout is caused by renal urate underexcretion, which features decreased urate clearance [urate clearance/creatinine clearance ratio, FE_{UA}; under 5.5%]. SUA, serum uric acid levels.

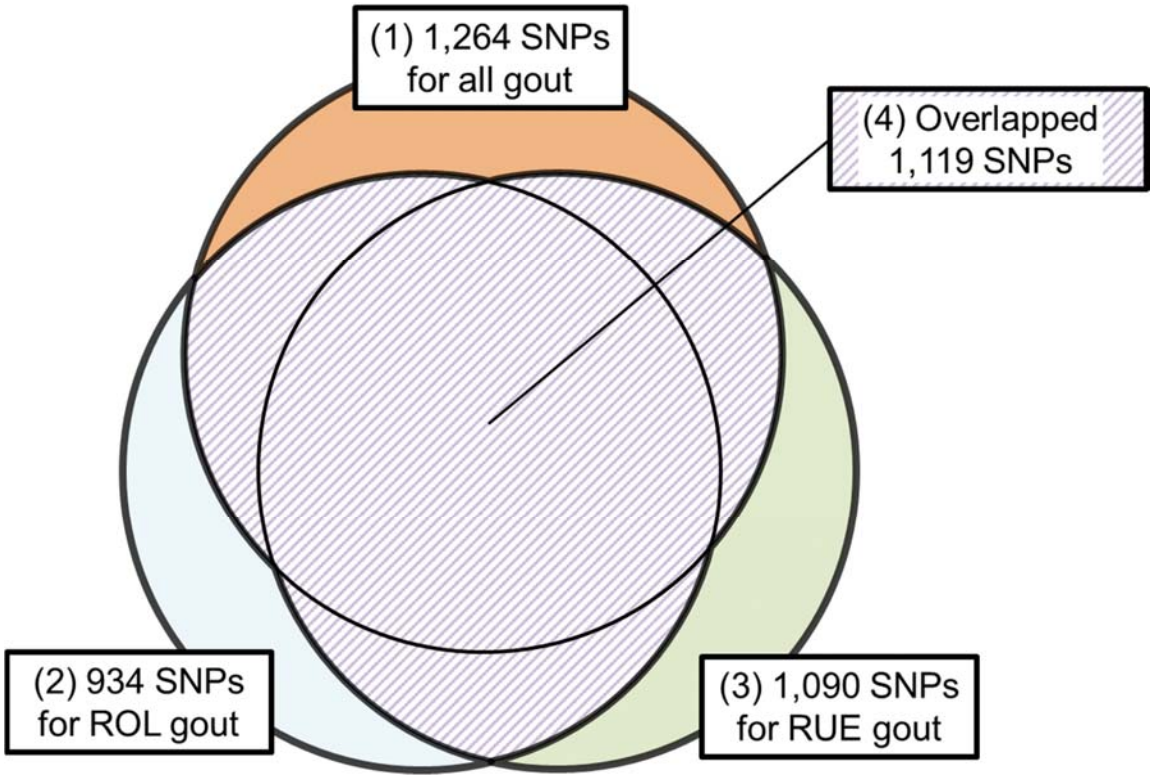


Supplementary Figure S2　Flow chart of the present study.

In the Japanese analysis, all 945 gout cases and 1,213 controls were included in the GWAS stage, and 1,961 cases and 1,268 controls were included in the replication stage (shown as "GWAS of all gout"). For "GWAS of ROL gout", 560 and 618 ROL gout cases were analyzed in the GWAS stage and replication stage, respectively. For "GWAS of RUE gout", 619 and 696 RUE gout cases were studied for the GWAS stage and replication stage, respectively (see also Supplementary Table S1 and S2). Because *NIPAL1* and *FAM35A* were found as novel risk loci from "GWAS of RUE gout", additional analyses were performed for these two loci.

Sample sets of Caucasian (1,319 cases and 514 controls; see Supplementary Table S1) and NZ Polynesian (971 cases and 565 controls) were also genotyped as a replication analysis, and meta-analysis was performed along with the Japanese sample sets.

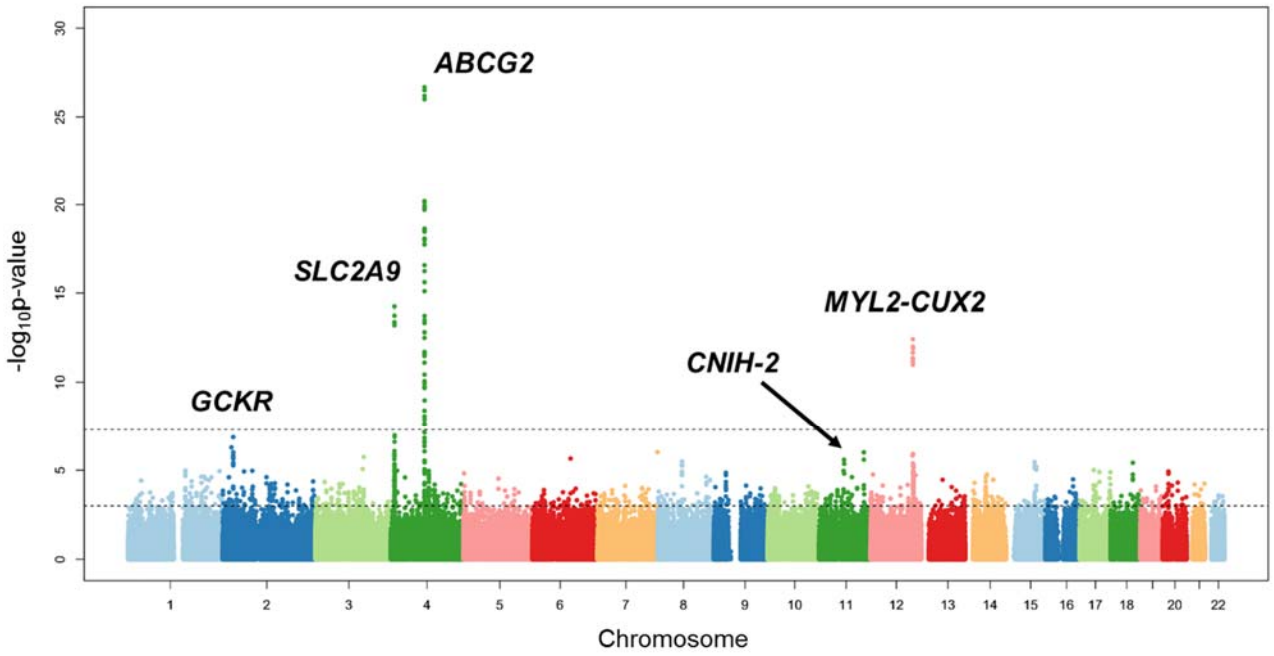
ROL, renal overload; RUE, renal underexcretion; NZ Polynesian, New Zealand Polynesian.



Supplementary Figure S3 Selected 1,961 SNPs for the replication stage by a custom chip.

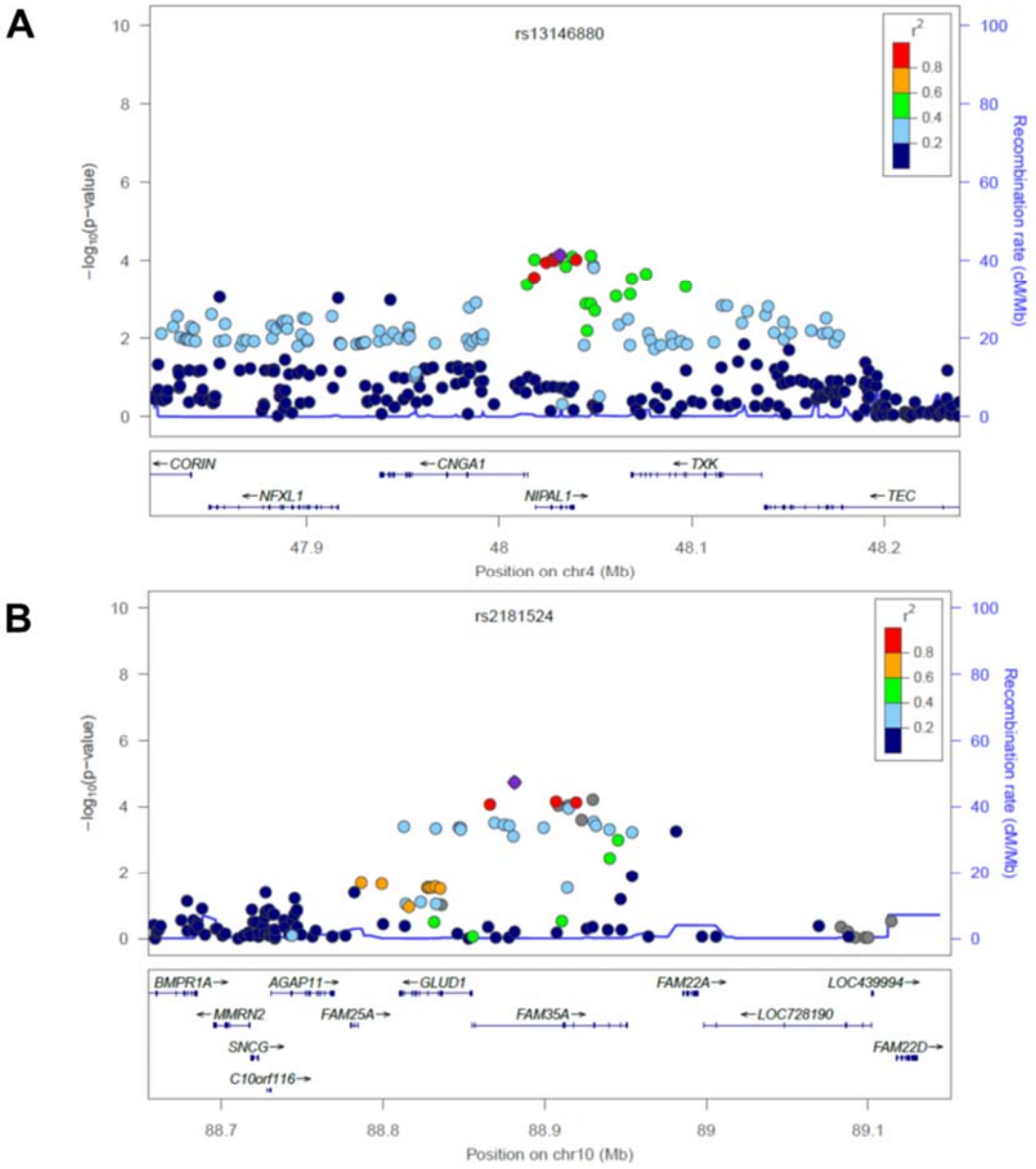
1,961 SNPs were selected for the custom chip using the following criteria: (1) 1,264 SNPs were included based on an association ($p < 0.001$ with Fisher's exact test) in the GWAS stage with all gout cases and controls. (2) 934 SNPs were selected as having an association ($p < 0.001$ with Fisher's exact test) in the GWAS stage with ROL gout cases and controls. (3) 1,090 SNPs were extracted with an association ($p < 0.001$ with Fisher's exact test) in the GWAS stage with RUE gout cases and controls. (4) After the overlapping 1,119 SNPs and undesignable 208 SNPs were eliminated, 1,961 SNPs were finally included.

SNP, single nucleotide polymorphism; ROL, renal overload; RUE, renal underexcretion.



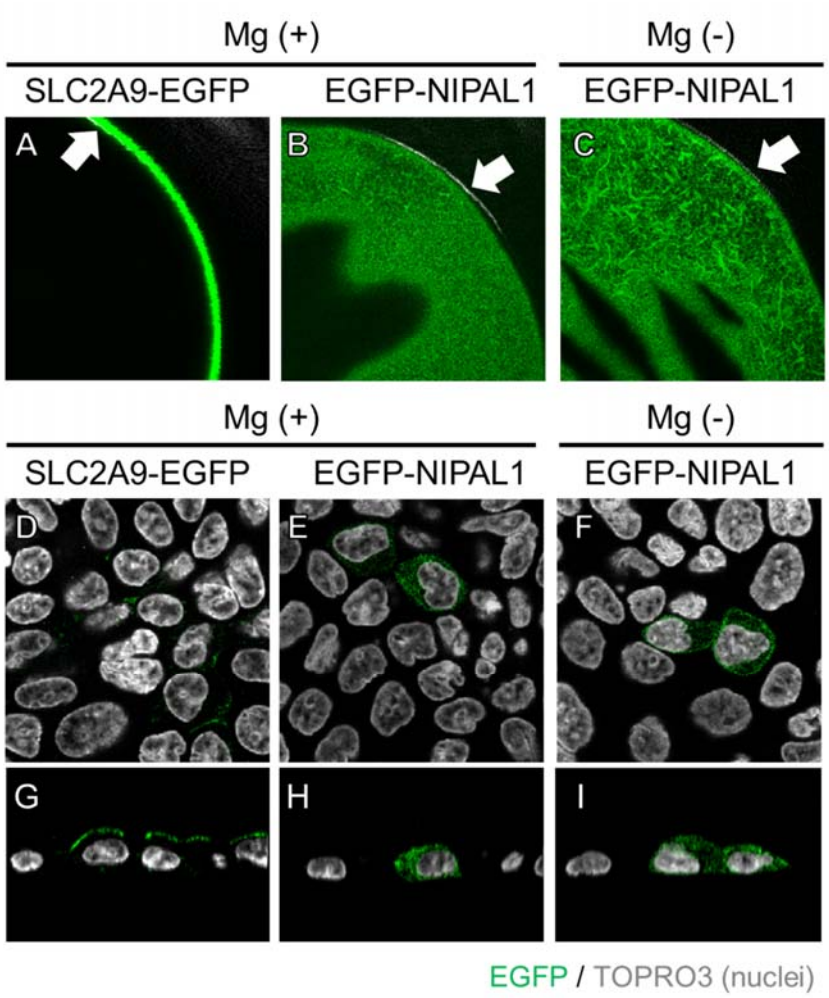
Supplementary Figure S4　Manhattan plot of GWAS with all gout cases.

This figure is modified from our previous GWAS[1] of clinically-defined gout in which five loci were demonstrated as gout risk loci at a genome-wide significance level. The upper and lower dotted lines indicate the genome-wide significance threshold ($p = 5.0\times10^{-8}$) and the cut-off level for selecting single nucleotide polymorphisms for the replication study ($p = 0.001$), respectively.



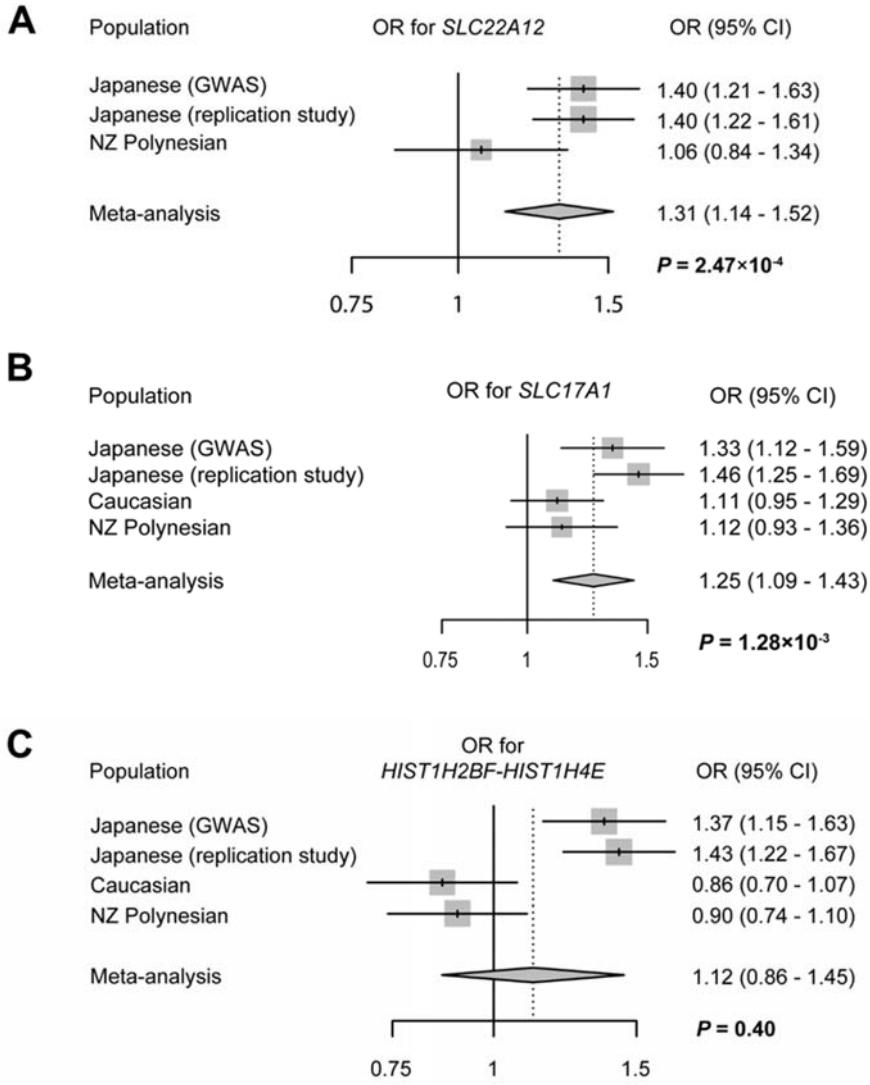
Supplementary Figure S5 Regional association plots of *NIPAL1* and *FAM35A* loci in the Köttgen *et al.* GWAS of serum uric acid levels.

Of the loci newly identified by GWAS of the RUE gout subtype, only *NIPAL1* and *FAM35A* had never before been associated with serum uric acid (SUA) levels or gout. From a previously-performed GWAS of SUA in Caucasian[2] (available from http://metabolomics.helmholtz-muenchen.de/gugc/), possible association of (**A**) *NIPAL1* and (**B**) *FAM35A* with SUA was evaluated and this revealed evidence for the association of each locus with SUA in Caucasian [SNP with the lowest p value is depicted as a purple diamond; rs13146880 of *NIPAL1*, β=0.022 mg/dl (relevant to A allele), $p=7.41\times10^{-5}$; rs2181524 of *FAM35A*, β=0.027 mg/dl (relevant to T allele), $p=1.85\times10^{-5}$].



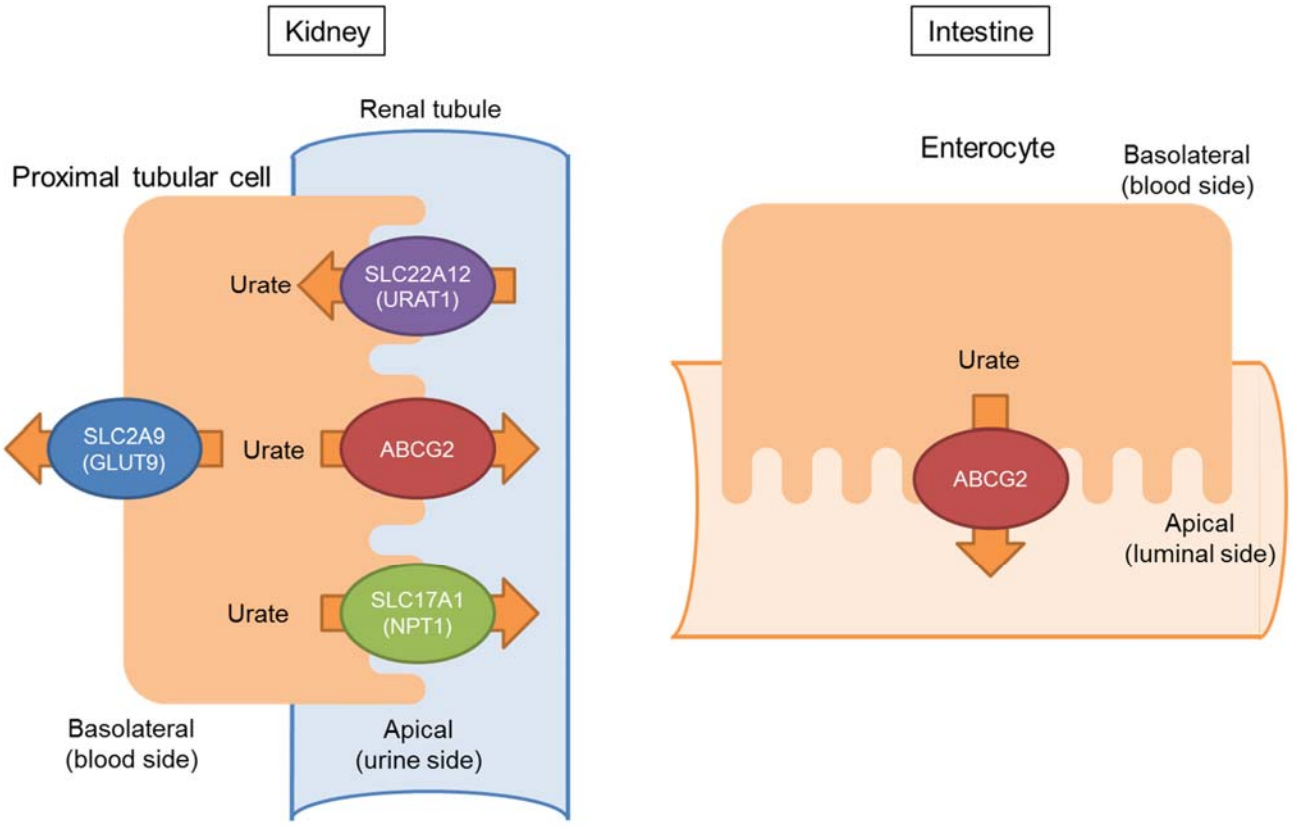
Supplementary Figure S6 Intracellular localization of NIPAL1.

(**A**-**C**) *Xenopus* oocytes expressing SLC2A9-EGFP or EGFP-NIPAL1 were cultured in ND-96 buffer, and imaged by confocal microscopy 48 hours after cRNA injection. For the Mg^{2+}-free condition, incubation buffer was replaced by ND-96 buffer without Mg^{2+} 24 hours after the cRNA injection. SLC2A9-EGFP was localized to the plasma membrane of the oocyte. EGFP-NIPAL1 exhibited cytosolic localization in the oocyte. Arrows indicate the oocyte contour in bright field. Thus, we succeeded in the localization analysis of transporters (SLC2A9 and NIPAL1) using live oocytes without fixation. (**D**-**I**) Polarized MDCKII cells were transiently transfected with SLC2A9-EGFP or EGFP-NIPAL1, and imaged by confocal microscopy 90 hours after the transfection. Twelve hours prior to the observation, culture medium was replaced by HBSS with or without Mg^{2+}. SLC2A9-EGFP was localized on the apical membrane of the MDCKII cells. EGFP-NIPAL1 exhibited the intracellular localization in the MDCKII cells. Nuclei were stained with TO-PRO-3 iodide (gray). The upper panels (**D**-**F**) are *en face* images focused at the middle of the cells, respectively. The bottom panels (**G**-**I**) show the Z-sectioning images.



Supplementary Figure S7 Forest plots of *SLC22A12*, *SLC17A1* and *HIST1H2BF-HIST1H4E* for all gout in all three populations.

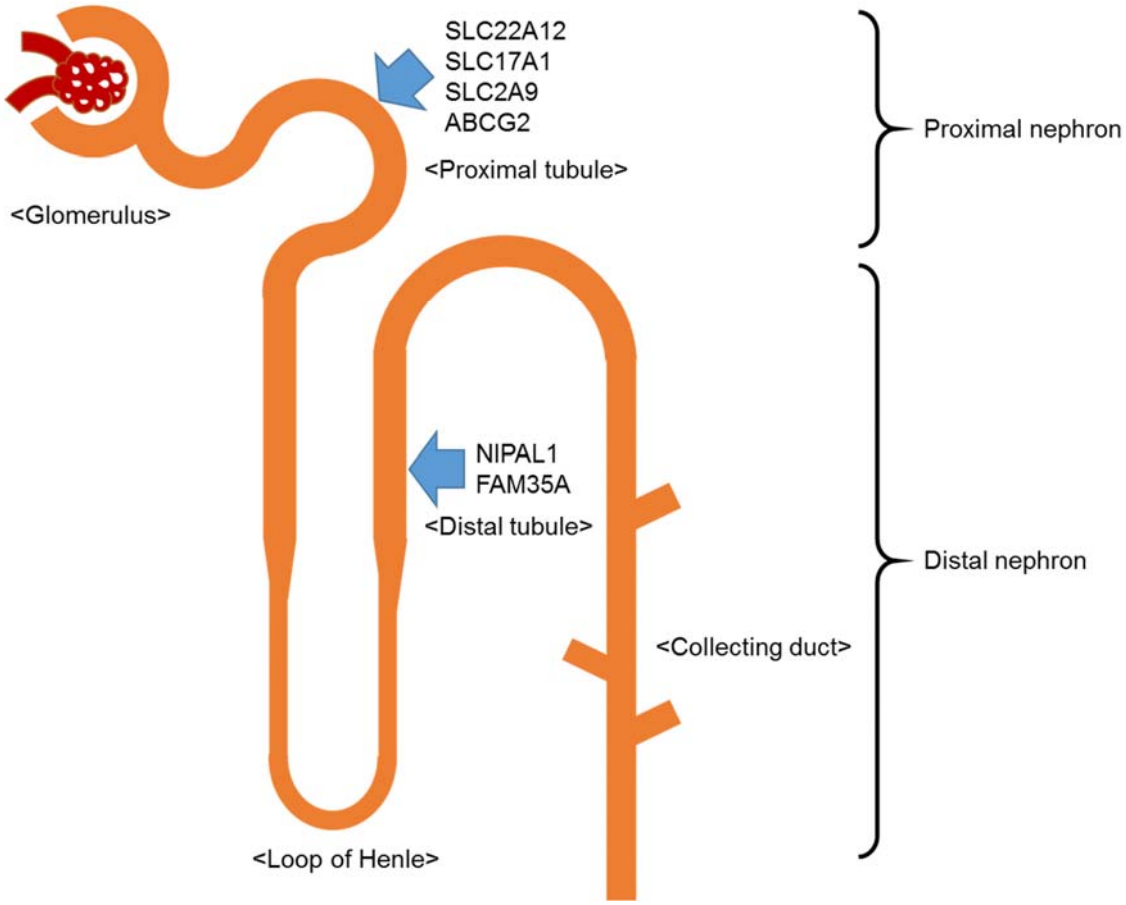
(**A**) rs2285340 of *SLC22A12*, (**B**) rs1165196 of *SLC17A1*, and (**C**) rs11758351 of *HIST1H2BF-HIST1H4E*. rs2285340 of *SLC22A12* is monomorphic in Caucasians. rs11758351 of *HIST1H2BF-HIST1H4E* did not show significant association with gout (p_{meta} = 0.40; OR = 1.12), because the direction of effect for rs11758351 of Caucasian and NZ Polynesian was inverted (OR<1) from that of Japanese. Although rs2285340 of *SLC22A12* and rs1165196 of *SLC17A1* did not reach a genome-wide significance level, they displayed a significant association with all gout (p_{meta} = 2.47×10^{-4}; OR = 1.31; and p_{meta} = 1.28×10^{-3}; OR = 1.25, respectively). NZ Polynesian, New Zealand Polynesian; OR, odds ratio; CI, confidence interval.



Supplementary Figure S8　Urate transporters in human kidney and intestine.

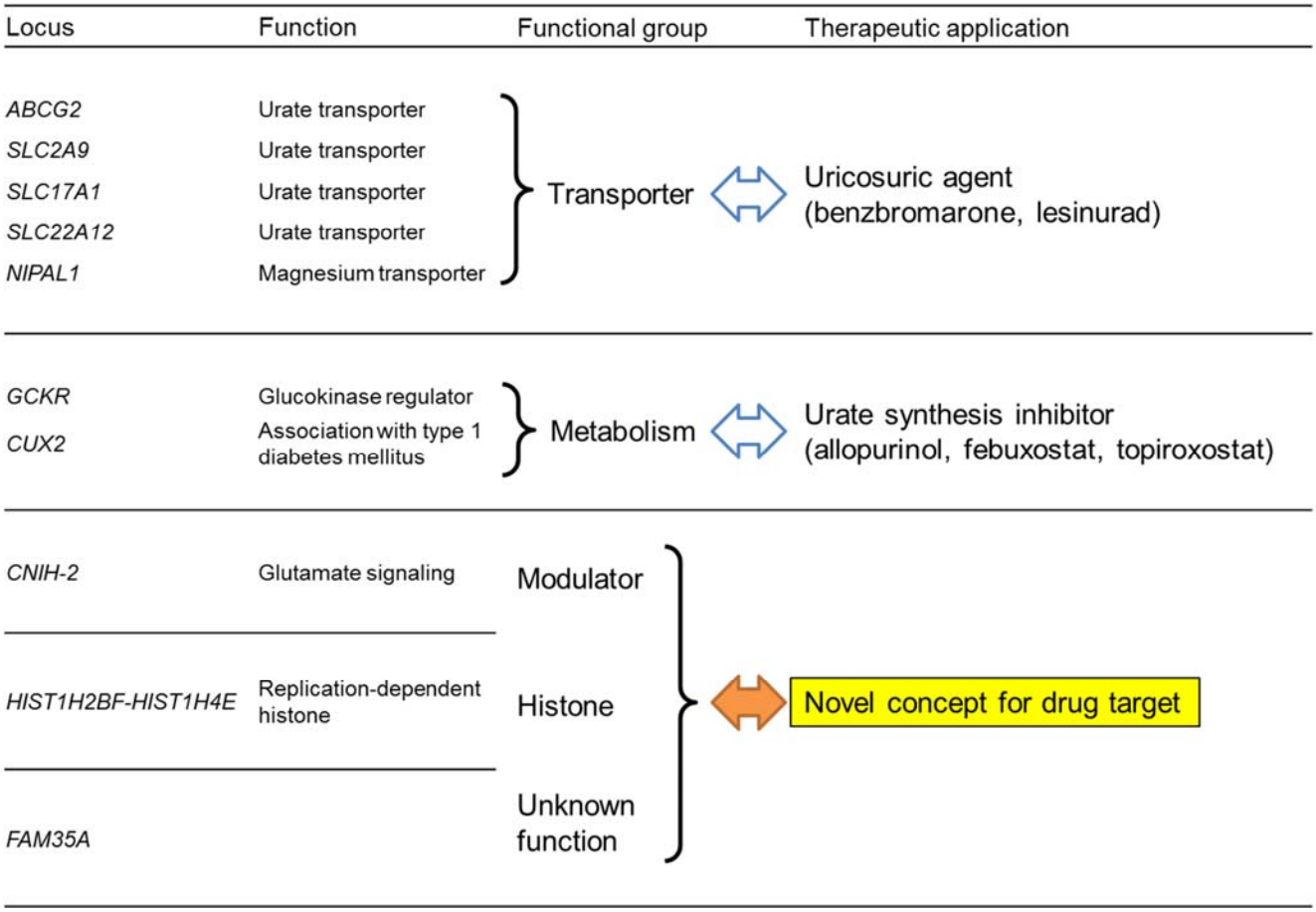
The urate transporters shown in this figure have a significant association with gout in the present GWAS.

In human, serum uric acid levels are mainly regulated by reabsorption of urate in kidney, and excretion in the kidney and intestine. Urate transporter is one of the key molecules for this urate handling. In proximal tubular cells of the kidney, SLC22A12[3] and SLC2A9[4,5] reabsorb urate from primary urine to blood, and ABCG2[6] and SLC17A1[7] excrete urate from blood to the urine (renal excretion). ABCG2[8] is also expressed in the intestine and excretes urate into the intestinal lumen (gut excretion, or extra-renal excretion). Given that four of ten significant gout risk loci encode urate transporters, urate transporters should indeed have an important role in the progression of gout.



Supplementary Figure S9 Renal expression of identified genes in proximal and distal nephron.

It is well-known that urate handling takes place in proximal tubules of the human kidney. As shown in online supplementary figure S8, four urate transporters, SLC22A12[3], SLC2A9[4,5], SLC17A1[7] and ABCG2[9], are expressed in proximal tubules of the human kidney. In the present study, *NIPAL1* (a magnesium transporter gene) and *FAM35A* are revealed to be associated with gout and to be expressed in distal tubules of the human kidney. Immunoreactivities of NIPAL1 and FAM35A are also weakly detected in the collecting ducts, where the short isoform of SLC2A9 (SLC2A9S, also known as GLUT9S[5]) is reportedly expressed[10]. These findings suggest the involvement of the distal nephron in gout progression including urate handling dysfunction.



Supplementary Figure S10 Ten loci identified in the present gout GWAS.

Supplementary Table S1 Clinical characteristics of all cases and controls

	Japanese (GWAS stage)		Japanese (replication stage)		Caucasian		NZ Polynesian	
	Case	Control	Case	Control	Case*	Control	Case*	Control
Number	945	1,213	1,396	1,268	1319	514	971	565
Age (year)	46.6 ± 11.3	65.8 ± 8.4	44.9 ± 11.5	48.2 ± 17.6	62.2 ± 12.7	53.6 ± 18.0	52.4 ± 12.8	40.9 ± 14.8
Body-mass index (kg/m^2)	25.1 ± 3.6	21.8 ± 3.3	24.9 ± 3.4	23.2 ± 3.5	30.3 ± 6.7	27.5 ± 4.7	35.8 ± 7.8	32.5 ± 6.6
Serum uric acid (mg/dl)	8.46 ± 1.19	5.46 ± 1.05	8.56 ± 1.26	5.59 ± 1.00	6.64 ± 1.87	6.42 ± 1.62	7.28 ± 1.87	6.88 ± 1.34

Plus-minus values are means ± SD.

*Serum uric acid data for these populations include those under urate lowering therapy.

GWAS, genome-wide association study; NZ Polynesian, New Zealand Polynesian.

Supplementary Table S2 Clinical parameters of Japanese gout cases

		Number	SUA (mg/dl)	FE_{UA} (%)	UUE (mg/hr/1.73m^2)
GWAS stage	All gout	945	8.46 ± 1.19	4.93 ± 1.51	28.5 ± 8.7
	ROL gout	560	8.52 ± 1.20	5.39 ± 1.53	33.1 ± 7.4
	RUE gout	619	8.63 ± 1.20	4.16 ± 0.80	26.4 ± 7.7
Replication stage	All gout	1,396	8.56 ± 1.26	4.84 ± 1.62	29.2 ± 9.9
	ROL gout	618	8.63 ± 1.27	5.25 ± 1.69	33.9 ± 8.8
	RUE gout	696	8.62 ± 1.24	4.14 ± 0.80	27.0 ± 8.4

Plus-minus values are means ± SD.

SUA, serum uric acid; FE_{UA}, fractional excretion of urate clearance; UUE, urinary urate excretion; GWAS, genome-wide association study; ROL, renal overload; RUE, renal underexcretion.

Supplementary Table S3 Concomitant diseases of gout cases

Number (%)	Hypertension	Dyslipidemia	Diabetes mellitus	Ischemic heart disease	Stroke	Renal impairment*
Japanese - Total	621 (26.5%)	111 (4.7%)	87 (3.7%)	54 (2.3%)	23 (1.0%)	20 (0.85%)
- GWAS stage	249 (26.3%)	42 (4.4%)	37 (3.9%)	19 (2.0%)	9 (0.95%)	11 (1.2%)
- Replication stage	372 (26.6%)	69 (4.9%)	50 (3.6%)	35 (2.5%)	14 (1.0%)	9 (0.64%)
Caucasian	696 (58.2%)	529 (49.3%)	178 (16.0%)	275 (35.9%)	62 (7.6%)	197 (14.0%)
NZ Polynesian	512 (54.9%)	437 (49.3%)	222 (23.9%)	269 (28.6%)	28 (4.2%)	159 (11.0%)

*Renal impairment is defined with high serum creatinine (≥1.5 mg/dl).

NZ Polynesian, New Zealand Polynesian.

Supplementary Table S4 Association of ten SNPs with gout and its subtypes

Gout types	SNP*	Chr.	Position (bp)†	Gene‡	A1/A2§	GWAS‖ Frequency of A1 Cases	GWAS‖ Frequency of A1 Controls	GWAS‖ OR (95% CI)	GWAS‖ p Value	Replication study¶ Frequency of A1 Cases	Replication study¶ Frequency of A1 Controls	Replication study¶ OR (95% CI)	Replication study¶ p Value	Meta-analysis# OR (95% CI)	Meta-analysis# p Value	Heterogeneity Cochran's Q	Heterogeneity I^2 (%)
All gout	rs1260326	2	27730940	*GCKR*	T/C	0.616	0.535	1.39 (1.23-1.57)	1.34×10^{-7}	0.611	0.557	1.25 (1.12-1.39)	6.10×10^{-5}	1.31 (1.21-1.42)	7.19×10^{-11}	0.20	38.2
	rs1014290	4	10001861	*SLC2A9*	T/C	0.678	0.564	1.63 (1.44-1.85)	1.75×10^{-14}	0.673	0.576	1.51 (1.35-1.69)	2.97×10^{-13}	1.57 (1.44-1.70)	6.50×10^{-26}	0.39	0.0
	rs11733284	4	48028097	***NIPAL1***	A/G	0.330	0.281	1.26 (1.10-1.43)	6.55×10^{-4}	0.324	0.280	1.24 (1.10-1.39)	4.29×10^{-4}	1.24 (1.14-1.36)	9.05×10^{-7}	0.85	0.0
	rs3114020	4	89083666	*ABCG2*	C/T	0.842	0.724	2.03 (1.75-2.37)	1.17×10^{-20}	0.844	0.752	1.78 (1.55-2.04)	7.74×10^{-17}	1.89 (1.71-2.09)	8.66×10^{-35}	0.20	38.9
	rs1165176	6	25830298	***SLC17A1***	G/A	0.874	0.834	1.38 (1.16-1.64)	2.89×10^{-4}	0.872	0.824	1.46 (1.25-1.69)	1.08×10^{-6}	1.42 (1.27-1.59)	1.47×10^{-9}	0.63	0.0
	rs11758351	6	26203910	***HIST1H2BF-HIST1H4E***	G/T	0.158	0.121	1.37 (1.15-1.63)	4.22×10^{-4}	0.158	0.116	1.43 (1.22-1.67)	1.01×10^{-5}	1.40 (1.25-1.57)	1.63×10^{-8}	0.72	0.0
	rs7903456	10	88919319	***FAM35A***	A/G	0.286	0.248	1.21 (1.06-1.39)	5.38×10^{-3}	0.285	0.235	1.30 (1.15-1.47)	3.22×10^{-5}	1.26 (1.15-1.38)	6.45×10^{-7}	0.47	0.0
	rs2285340	11	64435906	***SLC22A12***	A/G	0.228	0.174	1.40 (1.21-1.63)	1.09×10^{-5}	0.227	0.174	1.40 (1.22-1.61)	9.96×10^{-7}	1.40 (1.27-1.55)	4.61×10^{-11}	1.00	0.0
	rs4073582	11	66050712	*CNIH-2*	C/T	0.950	0.915	1.78 (1.39-2.29)	4.32×10^{-6}	0.943	0.920	1.44 (1.16-1.79)	8.47×10^{-4}	1.58 (1.34-1.86)	3.56×10^{-8}	0.21	36.1
	rs4766566	12	111706877	*CUX2*	T/C	0.735	0.633	1.60 (1.41-1.83)	1.22×10^{-12}	0.741	0.665	1.44 (1.28-1.62)	2.07×10^{-9}	1.51 (1.38-1.65)	4.03×10^{-20}	0.22	33.8
ROL gout	rs1260326	2	27730940	*GCKR*	T/C	0.611	0.535	1.36 (1.18-1.58)	2.43×10^{-5}	0.626	0.557	1.33 (1.16-1.53)	6.12×10^{-5}	1.35 (1.22-1.49)	5.39×10^{-9}	0.81	0.0
	rs1014290	4	10001861	*SLC2A9*	T/C	0.658	0.564	1.49 (1.28-1.72)	1.17×10^{-7}	0.663	0.576	1.44 (1.25-1.66)	3.82×10^{-7}	1.46 (1.32-1.62)	2.63×10^{-13}	0.78	0.0
	rs11733284	4	48028097	***NIPAL1***	A/G	0.320	0.281	1.20 (1.03-1.40)	1.94×10^{-2}	0.337	0.280	1.30 (1.13-1.51)	4.21×10^{-4}	1.25 (1.13-1.39)	2.72×10^{-5}	0.45	0.0
	rs3114020	4	89083666	*ABCG2*	C/T	0.863	0.724	2.39 (1.98-2.90)	6.14×10^{-21}	0.853	0.752	1.91 (1.59-2.29)	5.79×10^{-13}	2.13 (1.70-2.66)	3.08×10^{-11}	0.09	65.0
	rs1165176	6	25830298	***SLC17A1***	G/A	0.875	0.834	1.40 (1.14-1.72)	1.33×10^{-3}	0.855	0.824	1.26 (1.04-1.52)	1.73×10^{-2}	1.32 (1.15-1.52)	8.83×10^{-5}	0.47	0.0
	rs11758351	6	26203910	***HIST1H2BF-HIST1H4E***	G/T	0.156	0.121	1.35 (1.10-1.65)	4.64×10^{-3}	0.139	0.116	1.23 (1.00-1.50)	5.13×10^{-2}	1.28 (1.11-1.48)	5.79×10^{-4}	0.52	0.0
	rs7903456	10	88919319	***FAM35A***	A/G	0.276	0.248	1.16 (0.99-1.36)	7.55×10^{-2}	0.282	0.235	1.28 (1.09-1.49)	2.03×10^{-3}	1.22 (1.09-1.36)	4.80×10^{-4}	0.39	0.0
	rs2285340	11	64435906	***SLC22A12***	A/G	0.220	0.174	1.34 (1.13-1.60)	1.21×10^{-3}	0.221	0.174	1.35 (1.14-1.60)	5.48×10^{-4}	1.35 (1.19-1.52)	1.65×10^{-6}	0.97	0.0
	rs4073582	11	66050712	*CNIH-2*	C/T	0.948	0.915	1.71 (1.27-2.31)	3.39×10^{-4}	0.938	0.920	1.30 (0.99-1.71)	6.33×10^{-2}	1.48 (1.21-1.81)	1.35×10^{-4}	0.19	42.7
	rs4766566	12	111706877	*CUX2*	T/C	0.737	0.633	1.62 (1.39-1.90)	8.42×10^{-10}	0.757	0.665	1.57 (1.34-1.83)	7.55×10^{-9}	1.59 (1.43-1.78)	8.14×10^{-17}	0.76	0.0
RUE gout	rs1260326	2	27730940	*GCKR*	T/C	0.634	0.535	1.50 (1.31-1.73)	1.06×10^{-8}	0.617	0.557	1.28 (1.12-1.46)	3.00×10^{-4}	1.39 (1.18-1.62)	5.00×10^{-5}	0.10	62.3
	rs1014290	4	10001861	*SLC2A9*	T/C	0.699	0.564	1.80 (1.55-2.08)	1.58×10^{-15}	0.685	0.576	1.60 (1.39-1.84)	1.72×10^{-11}	1.69 (1.53-1.87)	8.71×10^{-25}	0.26	21.8
	rs11733284	4	48028097	***NIPAL1***	A/G	0.346	0.281	1.35 (1.17-1.57)	6.48×10^{-5}	0.342	0.280	1.34 (1.16-1.54)	6.36×10^{-5}	1.34 (1.21-1.49)	1.13×10^{-8}	0.91	0.0
	rs3114020	4	89083666	*ABCG2*	C/T	0.837	0.724	1.95 (1.64-2.32)	1.31×10^{-14}	0.832	0.752	1.63 (1.38-1.92)	6.09×10^{-9}	1.78 (1.49-2.12)	2.33×10^{-10}	0.14	53.7

rs1165176	6	25830298	***SLC17A1***	G/A	0.875	0.834	1.40 (1.15-1.71) 9.25×10^{-4}	0.858	0.824	1.29 (1.08-1.55) 5.20×10^{-3}	1.34 (1.17-1.53) 1.87×10^{-5}	0.57	0.0
rs11758351	6	26203910	***HIST1H2BF-HIST1H4E***	G/T	0.169	0.121	1.47 (1.22-1.79) 8.88×10^{-5}	0.150	0.116	1.34 (1.11-1.62) 2.76×10^{-3}	1.41 (1.23-1.61) 8.74×10^{-7}	0.49	0.0
rs7903456	10	88919319	***FAM35A***	A/G	0.303	0.248	1.32 (1.13-1.53) 4.32×10^{-4}	0.296	0.235	1.37 (1.18-1.59) 3.09×10^{-5}	1.34 (1.21-1.49) 4.29×10^{-8}	0.72	0.0
rs2285340	11	64435906	***SLC22A12***	A/G	0.236	0.174	1.47 (1.25-1.74) 8.04×10^{-6}	0.228	0.174	1.41 (1.20-1.66) 4.04×10^{-5}	1.44 (1.28-1.62) 8.79×10^{-10}	0.72	0.0
rs4073582	11	66050712	*CNIH-2*	C/T	0.946	0.915	1.63 (1.22-2.16) 6.80×10^{-4}	0.936	0.920	1.27 (0.98-1.64) 7.47×10^{-2}	1.42 (1.18-1.72) 2.88×10^{-4}	0.20	38.7
rs4766566	12	111706877	*CUX2*	T/C	0.738	0.633	1.63 (1.40-1.89) 1.58×10^{-10}	0.759	0.665	1.58 (1.36-1.83) 9.51×10^{-10}	1.60 (1.44-1.78) 2.17×10^{-18}	0.78	0.0

*dbSNP rs number. The associations of ten SNPs (eight significant SNPs of all gout and two novel SNPs of *NIPAL1* and *FAM35A* on RUE gout in Table 1) are shown in order to compare the effects of ten SNPs among all gout, ROL gout and RUE gout.

†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, and 619 cases for RUE gout with 1,213 controls from Japanese male population.

¶1,396 cases for all gout, 618 cases for ROL gout, and 696 cases for RUE gout with 1,268 controls from Japanese male population.

#Meta-analysis of GWAS and replication samples.

Chr., chromosome; GWAS, genome-wide association study; OR, odds ratio; CI, confidence interval; ROL, renal overload; RUE, renal underexcretion.

Supplementary Table S5 Replication analysis of all gout for *CNIH-2* and *CUX2* loci in Caucasian and NZ Polynesian populations

					Caucasian				NZ Polynesian				Meta-analysis[#]			
		Position			Frequency of A1		OR (95% CI)	p Value	Frequency of A1		OR (95% CI)	p Value	OR (95% CI)	p Value	Heterogeneity	
SNP[*]	Chr.	(bp)[†]	Gene	A1/A2[§]	Case	Control			Case	Control					Cochran's Q	I^2 (%)
rs4073582	11	66050712	*CNIH-2*	C/T	0.648	0.631	1.05 (0.89-1.23)	0.545	0.902	0.897	0.89 (0.67-1.17)	0.424	1.00 (0.87-1.15)	0.915	0.32	0.0
rs4766566	12	111706877	*CUX2*	T/C	0.240	0.220	1.11 (0.93-1.34)	0.235	0.617	0.564	1.22 (1.04-1.44)	0.0133	1.17 (1.04-1.32)	8.30×10^{-3}	0.44	0.0

*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.

#Meta-analysis of Caucasian and NZ Polynesian samples.

NZ Polynesian, New Zealand Polynesian; Chr., chromosome; OR, odds ratio; CI, confidence interval.

Supplementary Table S6 Replication study for five discovered loci with gout cases and normouricemic controls of Caucasian and NZ Polynesian populations

					Caucasian[§]				NZ Polynesian[‖]				Meta-analysis[¶]			
					Frequency of A1		OR (95% CI)	p Value	Frequency of A1		OR (95% CI)	p Value	OR (95% CI)	p Value	Heterogeneity	
SNP[*]	Chr.	Position (bp)[†]	Gene	A1/A2[‡]	Case	Control			Case	Control					Cochran's Q	I^2 (%)
rs11733284	4	48028097	*NIPAL1*	A/G	0.360	0.348	1.06 (0.85-1.33)	0.555	0.246	0.264	0.92 (0.74-1.16)	0.522	0.99 (0.85-1.16)	0.978	0.38	0.0
rs1165196	6	25813150	*SLC17A1*	T/C	0.613	0.583	1.14 (0.91-1.43)	0.256	0.722	0.745	0.99 (0.77-1.27)	0.949	1.08 (0.86-1.27)	0.426	0.43	0.0
rs11758351	6	26203910	*HIST1H2BF-HIST1H4E*	G/T	0.146	0.160	0.85 (0.64-1.14)	0.271	0.193	0.219	0.80 (0.63-1.02)	0.0771	0.82 (0.68-0.99)	0.0389	0.76	0.0
rs7903456	10	88919319	*FAM35A*	A/G	0.738	0.696	1.20 (0.95-1.52)	0.105	0.351	0.284	1.39 (1.10-1.74)	4.71×10^{-3}	1.30 (1.10-1.51)	1.56×10^{-3}	0.40	0.0
rs2285340[#]	11	64435906	*SLC22A12*	A/G	-	-	-	-	0.315	0.117	1.34 (0.99-1.85)	0.0582	-	-	-	-

*dbSNP rs number.

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

‡A1 is the effect allele.

§1,319 cases for all gout and 272 normouricemic controls (SUA ≤ 7.0 mg/dl) from Caucasian male population.

||971 cases for all gout and 281 normouricemic controls (SUA ≤ 7.0 mg/dl) from NZ Polynesian male population.

¶Meta-analysis of Caucasian and NZ Polynesian samples.

#rs2285340 is monomorphic in Caucasians.

NZ Polynesian, New Zealand Polynesian; Chr., chromosome; OR, odds ratio; CI, confidence interval.

Supplementary Table S7 Association analysis between five SNPs and clinical parameters (SUA and FE_{UA}) in gout cases

				SUA				FE_{UA}			
				95% CI				95% CI			
Population*	**SNP†**	**Gene**	**A1/A2§**	**Coef.**	**LL**	**UL**	**p Value‖**	**Coef.**	**LL**	**UL**	**p Value‖**
Japanese	rs11733284	*NIPAL1*	A/G	0.068	-0.014	0.149	0.103	-0.120	-0.225	-0.016	**0.0238**
	rs1165196	*SLC17A1*	T/C	-0.018	-0.135	0.099	0.766	0.063	-0.086	0.213	0.405
	rs11758351	*HIST1H2BF-HIST1H4E*	G/T	0.047	-0.061	0.155	0.389	-0.044	-0.183	0.094	0.529
	rs7903456	*FAM35A*	A/G	0.122	0.033	0.212	**7.56×10^{-3}**	-0.194	-0.309	-0.080	**9.22×10^{-4}**
	rs2285340	*SLC22A12*	A/G	0.159	0.065	0.253	**9.07×10^{-4}**	-0.121	-0.242	-0.001	**0.0484**
Caucasian	rs11733284	*NIPAL1*	A/G	0.114	-0.309	0.538	0.594	-0.046	-1.302	1.210	0.941
	rs1165196	*SLC17A1*	T/C	0.0477	-0.323	0.419	0.799	0.829	-0.114	1.774	0.0837
	rs11758351	*HIST1H2BF-HIST1H4E*	G/T	0.487	-0.068	0.043	0.0846	-0.719	-2.017	0.578	0.269
	rs7903456	*FAM35A*	A/G	0.444	-0.0404	0.928	0.0720	-0.914	-2.198	0.369	0.158
	rs2285340	*SLC22A12*	A/G	-	-	-	-	-	-	-	-
NZ Polynesian	rs11733284	*NIPAL1*	A/G	-0.145	-0.529	0.237	0.452	0.034	-0.997	1.066	0.946
	rs1165196	*SLC17A1*	T/C	0.184	-0.174	0.543	0.311	0.854	0.064	1.644	**0.0346**
	rs11758351	*HIST1H2BF-HIST1H4E*	G/T	-3.65×10^{-3}	-0.418	0.411	0.986	-0.101	-1.104	0.901	0.839
	rs7903456	*FAM35A*	A/G	0.028	-0.346	0.402	0.883	0.062	-0.876	1.002	0.893
	rs2285340	*SLC22A12*	A/G	0.354	-0.149	0.858	0.166	-0.516	-1.822	0.790	0.431

*For Japanese gout cases, 1,910 cases and 1.904 cases were examined for SUA and FE_{UA}, respectively. For Caucasian gout cases, 158 cases and 77 cases were examined for SUA and FE_{UA}, respectively. For NZ Polynesian gout cases, 250 cases and 124 cases were examined for SUA and FE_{UA}, respectively. Data were obtained from those not taking urate lowering therapy nor diuretics.

†dbSNP rs number.

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

‖p Values <0.05 are shown in bold.

SUA, serum uric acid (unit: mg/dl); FE_{UA}, fractional excretion of urate clearance (unit: %); Coef., regression coefficient; CI, confidence interval; LL, lower limit; UL, upper limit. We performed multivariate linear regression analyses with BMI and alcohol intake. For NZ Polynesian, adjustment was also done by the number of self-reported Polynesian grandparents.

Supplementary Note

SLC22A12/URAT1

SLC22A12 (figure 2A, table 1), also known as *URAT1* (urate transporter 1), encodes a well-known urate transporter which reabsorbs urate at the apical side of the renal proximal tubule[3] (online supplementary figure S8). Dysfunctional variants of *SLC22A12* are known to be the cause of renal hypouricemia type 1 (RHUC1),[3,11] which shows the physiological role of SLC22A12 in regulation of SUA in humans. Its association with SUA has also been reported in previous GWASs of SUA.[2,12-14] Therefore, it is reasonable that SNPs around this locus would display a significant association with gout, or a sequela of hyperuricemia.

SLC17A1/NPT1

SLC17A1 (also known as *NPT1* [sodium-dependent phosphate cotransporter type 1]) was also first identified by GWAS approach to have a genome-wide significant association with gout in the present study. It is not surprising that *SLC17A1* is associated with gout, because the association with this locus and SUA has been reported in several studies since Dehghan *et al.*[15] first reported the association of the *SLC17A1-A3-A4* locus by the GWAS approach. By the candidate gene approach, Hollis-Moffatt *et al.*[16] reported that rs1183201, a SNP of *SLC17A1*, is strongly associated with gout in Caucasian and NZ Polynesian sample sets. Iharada *et al.*[17] showed that SLC17A1 transports urate and that rs11165196, another SNP of *SLC17A1*, exhibited 32% change in urate transport by the proteoliposome system. Moreover, SLC17A1 is expressed on the apical membrane of proximal tubules in human kidney[7] (online supplementary figure S8), and its missense variant (rs1165196; p.Ile269Thr) decreased gout risk because this variant was found to be a gain-of-function polymorphism which increased urate transport function in our previous report by an oocyte expression system.[7] Because rs1165196 is in strong LD with rs1165176 (r^2=0.99) (figure 2B, table 1; the most significant SNP in this locus in the present study), this gain-of-function variant (rs1165196)[7] could be the causal variant at this locus. Further replication analyses were required to investigate the significance of *SLC17A1* locus on gout in other populations.

HIST1H2BF-HIST1H4E and VARS2

The *HIST1H2BF* and *HIST1H4E* genes encode histone 1 H2bf and histone 1 H4e, respectively, which are replication-dependent histone proteins with expression dependent on cell cycle. rs11758351 of *HIST1H2BF-HIST1H4E* showed an association with all gout at a genome-wide significance level ($p_{meta}=1.63\times10^{-8}$; OR=1.40) (figure 2C, table 1). The detailed discussion is also

described in the Discussion section of the main text.

There was also a significant signal from rs2532941 of *VARS2* ($p_{meta}=2.74\times10^{-8}$; OR=1.32), which is located downstream of *HIST1H2BF-HIST1H4E* by 4.7 Mb. Since rs2532941 of *VARS2* showed mild LD with rs11758351 of *HIST1H2BF-HIST1H4E* ($r^2=0.37$), its significance did not remain for GWAS stage samples after adjustment with rs11758351 of *HIST1H2BF-HIST1H4E* (p=0.08). (Also see the Result section of the main text.)

NIPAL1/NIPA3

NIPAL1 (non-imprinted in Prader-Willi/Angelman syndrome (NIPA)-like domain containing 1) was revealed to be associated with RUE gout, that is, renal underexcretion gout in the present study (figure 2D, table 1). NIPAL1, also known as NIPA3, was reported to be a magnesium transporter,[18] as another magnesium transporter NIPA2.[18] Based on the amino acid sequence of NIPAL1, a total of nine transmembrane helices were predicted by SOSUI algorithm[19] (figure 3A), which is consistent with NIPAL1 as a membrane transporter whereas FAM35A is predicted to be a soluble protein. The amino acid sequences of NIPAL1 shown in figure 3A and FAM35A were obtained from GenBank (accession code NM_207330 and NM_019054, respectively). In the present study based on the findings of GWAS, we hypothesized that NIPAL1 could be involved in the regulation of urate handling as a renal urate efflux transporter. To verify this hypothesis, we evaluated urate transport activities using exogenously NIPAL1-expressing *Xenopus* oocytes in a high potassium buffer. In this buffer condition, urate efflux activity via NIPAL1 transporter could be detected as an influx activity,[7] since plasma membranes are depolarized by high levels of external potassium. As shown in figure 3B, SLC2A9-mediated ^{14}C-urate transport was detected. However, no significant difference was observed in the urate transport between the control and NIPAL1 groups. In addition, the absence of Mg^{2+} in the incubation buffer had no effect on the urate transport activities via NIPAL1, whose function was reportedly modified by the presence of magnesium.[18] These results suggested that NIPAL1 could not excrete urate from cells, at least in this typical experimental condition. These findings also suggest that NIPAL1 is not a urate transporter and might be involved in the indirect regulation of urate transport kinetics through the magnesium handling. In the present study, we succeeded in the localization analysis of transporters (NIPAL1 and SLC2A9) using live oocytes without fixation (online supplementary figure S6).

FAM35A and GLUD1

To date, the molecular function of FAM35A (family with sequence similarity 35, member A) is totally unknown. In the present study, *FAM35A* and *NIPAL1* were revealed to be associated with RUE

gout (figure 2D, E, table 1), showing that these genes would play important roles in renal urate handling. Interestingly, both FAM35A and NIPAL1 proteins were expressed in the distal tubules of the human kidney (figure 4A, B), suggesting the involvement of the distal nephron in the urate handling. Furthermore, rs7903456 of *FAM35A* showed an association with all gout at a genome-wide significance level ($p_{meta}=3.58\times10^{-8}$; OR=1.23) (figure 5) by a meta-analysis among Japanese, Caucasian and NZ Polynesian populations, indicating that rs7903456 is a susceptibility locus for all gout as well as the RUE gout subtype.

Although further studies are necessary to confirm this, it is possible that genes near *FAM35A* have some relationship with gout. For example, *GLUD1* (glutamate dehydrogenase 1) locates near *FAM35A*, and some SNPs of *GLUD1* such as rs4460731 are in mild LD with rs7903456 of *FAM35A* (figure 2E). *GLUD1* encodes glutamate dehydrogenase that regulates amino-acid-induced insulin secretion, and mutations in this gene are known to be a common cause of congenital hyperinsulinism.[20] Because hyperinsulinemia is correlated with SUA and is inversely related with FE_{UA} (fractional excretion of uric acid),[21] SNPs of *GLUD1* could be associated with RUE gout.

Results of the replication stage with 1,961 SNPs

The replication stage by genotyping 1,961 SNPs over 1,396 male patients and 1,268 male controls was carried out. Among them, 135 SNPs showed evidence for associations at the genome-wide significance level ($p_{meta}<5.0\times10^{-8}$) after subsequent meta-analysis for all gout cases, and eight independent SNPs were finally identified as gout loci after their linkage disequilibrium (LD) was examined. For ROL and RUE gout, 77 and 100 SNPs were revealed to have an association, which was finally specified to four and seven independent SNPs, respectively, after LD was examined.

Expression quantitative trait locus (eQTL) analysis

The lead SNP from each of the five loci (rs11733284 of *NIPAL1*, rs1165196 of *SLC17A1*, rs11758351 of *HIST1H2BF-HIST1H4E*, rs7903456 of *FAM35A*, and rs2285340 of *SLC22A12*) was tested for association with gene expression in the Genotype-Tissue Expression (GTEx: http://www.gtexportal.org/) database. Specifically liver, stomach and pancreas were examined, since renal and immune expression data sets were not available in GTEx. Setting a significance level of $p < 1.0\times10^{-4}$, two eQTL were detected. The first was in liver between rs11733284 and *NIPAL1* (Pos 4_48028097_G_A_b37; $\beta=0.59$, $p=4.0\times10^{-10}$) and the second in pancreas, also between rs11733284 and *NIPAL1* (Pos 4_48028097_G_A_b37; $\beta=0.39$, $p=5.4\times10^{-5}$). These data support the hypothesis that rs11733284 is associated with gout through an influence on the expression of *NIPAL1* and that *NIPAL1* is the causal gene at this locus. The “A” allele that increases the risk of gout, associates with increased

NIPAL1 expression. It will be important to test for association of rs11733284 with *NIPAL1* expression in kidney when a suitable eQTL data set is available.

Additional analyses with Caucasian and NZ Polynesian populations

In the present study, the differences between controls as well as between ancestries might affect the result of replication study. That is, the controls of Japanese consisted of those whose SUA are ≤ 7.0 mg/dl, whereas those of Caucasian and NZ Polynesian include hyperuricemic controls in addition to normouricemic controls. Therefore, we also performed the replication study with normouricemic controls only. With gout cases and normouricemic controls of Caucasian and NZ Polynesian populations, *HIST1H2BF-HIST1H4E* as well as *FAM35A* showed a significant association (online supplementary table S6), while the direction of the effect for *HIST1H2BF-HIST1H4E* was inverted (OR<1) from that of Japanese. Since it is possible that the heterogeneity of the results derives from fewer participants, larger-scale replication studies should be performed in the future.

Association analysis between FE_{UA} and five novel loci

We also examined the association between novel loci and clinical parameters (SUA and FE_{UA}) in gout cases of three populations (online supplementary table S7). Data of clinical parameters were obtained from those not taking urate lowering therapy nor diuretics. Among the novel five loci (*SLC22A12*, *HIST1H2BF-HIST1H4E*, *NIPAL1*, *SLC17A1* and *FAM35A*), *SLC17A1* showed significant effects on FE_{UA} in NZ Polynesian gout cases, whereas *NIPAL1*, *FAM35A* and *SLC22A12* revealed significant effects on FE_{UA} in Japanese gout cases. For Japanese gout cases, *FAM35A* and *SLC22A12* also showed significant effects on SUA. Because SLC22A12 is a renal urate transporter in proximal nephron, it is reasonable that a variant of *SLC22A12* significantly decreases FE_{UA} and increases SUA, and FAM35A might be involved in the similar mechanism of SLC22A12, but in distal nephron (online supplementary figure S9). These findings are also consistent with the fact that these loci increase the risk of RUE gout. In supplementary table S4, three loci (*NIPAL1*, *FAM35A* and *SLC22A12*) which have associations with RUE gout at genome-wide significance showed higher odds ratios for RUE gout than those for all gout or ROL gout. These results also support their association with FE_{UA} shown in supplementary table S7.

Supplementary Methods

Subjects

In the present study, we collected only clinically-defined cases of gout according to the criteria established by the American College of Rheumatology[22]. All Japanese patients were assigned from male outpatients at the gout clinics of Midorigaoka Hospital (Osaka, Japan), Kyoto Industrial Health Association (Kyoto, Japan), Ryougoku East Gate Clinic (Tokyo, Japan), or Jikei University Hospital (Tokyo, Japan). A total of 2,342 male patients with gout (946 at GWAS stage and 1,396 at replication stage) were registered as valid case participants. By the quality control described below, 2,341 patients (945 at GWAS stage and 1,396 at replication stage) finally passed filter (online supplementary table S1). For the pathophysiological understanding of urate/uric acid handling, gout is classified into two subtypes[23]: "renal overload (ROL)" gout and "renal underexcretion (RUE)" gout (online supplementary figure S1). ROL (or previously-named "urate overproduction") gout was defined as when urinary urate excretion (UUE) is over 25.0 mg/hr/1.73 m^2 (600 mg/day/1.73 m^2)[23-26], and RUE gout was determined to be when urate clearance (urate clearance/creatinine clearance ratio, FE_{UA}) is under 5.5%[23,26-28]. Clinical parameters including UUE and FE_{UA} before taking medications for hyperuricemia or antidiuretics were used in this study. Among those with gout, 1,178 cases were classified as ROL gout (560 cases at GWAS stage and 618 cases at replication stage) and 1,315 cases as RUE gout (619 cases at GWAS stage and 696 cases at replication stage), respectively (online supplementary table S2). For controls, 2,481 Japanese males with a SUA level $\leq$ 7.0 mg/dl and without gout history (1,213 controls for GWAS stage and 1,268 for replication stage) were obtained from BioBank Japan[13,29] (online supplementary table S1). The clinical characteristics of Japanese participants in this study are shown in online supplementary tables S1 and S2.

For the replication analysis with male Caucasian and NZ Polynesian populations, two case-control sample sets were used, consisting of one of Caucasian ancestry (1,319 cases and 514 controls) and one of NZ Polynesian ancestry (971 cases and 565 controls). The Caucasian cases were recruited from NZ and by the Eurogout Consortium[30] and the Caucasian controls were recruited from NZ. The NZ Polynesian sample set comprised 359 cases and 174 controls of Western Polynesian (WP; Samoa, Tonga, Niue and Tokelau) ancestry, 361 cases and 256 controls of Eastern Polynesian (EP; NZ Māori and Cook Island Māori) ancestry, 28 cases and 36 controls of mixed EP and WP ancestry, and 223 cases and 99 controls of Ngati Porou Hauora Eastern Polynesian (NZ Māori) ancestry. All gout cases recruited had their diagnosis of gout confirmed according to the criteria established by the American College of Rheumatology[22].

Controls self-reported as having no history of gout. The clinical characteristics of participants in the replication study are shown in online supplementary table S1.

Genotyping and quality control

Genome-wide genotyping was performed with Illumina HumanOmniExpress-12 v1.0 (Illumina) with 946 cases and 1,213 controls from among Japanese males. Detailed methods of genotyping and quality control are described as done previously[1]. Finally, 570,442 SNPs passed filters for 945 cases and 1,213 controls.

At the replication stage, 1,246 cases were genotyped with a custom genotype platform using iSelect HD Custom Genotyping BeadChips (Illumina) on 1,961 SNPs, and another 150 gout cases were genotyped with Illumina HumanOmniExpress-24 v1.0 (Illumina). As shown in online supplementary figure S3, 1,961 SNPs were selected using the following criteria: (1) 1,264 SNPs were selected as they showed association ($p < 0.001$ with Fisher's exact test) in GWAS stage with all gout cases and controls. (2) 934 SNPs were selected for their association ($p < 0.001$ with Fisher's exact test) in GWAS stage with ROL gout cases and controls. (3) 1,090 SNPs were selected for their association ($p < 0.001$ with Fisher's exact test) in GWAS stage with RUE gout cases and controls. (4) After overlapped 1,119 SNPs and undesignable 208 SNPs were eliminated, 1,961 SNPs were finally selected. As quality control, the data set was filtered individually on the basis of SNP genotype missing call rates (>1%). In addition, we excluded subjects with low genotype call rates (<98%). Quality controls for 150 gout cases genotyped with Illumina HumanOmniExpress-24 v1.0 (Illumina) were performed as described previously[1]. All controls from BioBank Japan[13,29] had been genotyped with Illumina HumanOmniExpress-12 v1.0 (Illumina) and filtered by the Hardy-Weinberg equilibrium (HWE) ($p < 1.0\times10^{-4}$). Finally, 1,961 SNPs with 1,396 cases and 1,268 controls were successfully replicated and applied to subsequent statistical analysis.

Replication was done in a data set recruited from New Zealand[31] and from Europe by the Eurogout Consortium[30] comprising 1,319 cases and 514 controls of European ancestry and 971 cases and 565 controls of NZ Polynesian ancestry. For the replication analysis with the Caucasian and NZ Polynesian samples, SNPs were genotyped by an allelic discrimination assay (TaqMan) with a LightCycler 480 Real-Time Polymerase Chain Reaction (RT-PCR) System (Roche Applied Science, Indianapolis, IN, USA).

Statistical analyses

The results from GWAS and replication stages for Japanese population were combined by

meta-analysis[32]. The inverse-variance fixed-effects model meta-analysis was used for estimating summary OR. Cochran's Q test[33] and I^2 statistic[34,35] were examined to assess heterogeneity in ORs between GWAS and replication study. If heterogeneity was present by the statistical test ($p_{het} < 0.05$) or measurement ($I^2 > 50\%$), we implemented DerSimonian and Laird random-effects model meta-analysis[36]. For the replication analysis with Caucasian and NZ Polynesian populations, ORs were adjusted by age and ancestral group, and the inverse-variance fixed-effects model meta-analysis was used unless p_{het} was < 0.05 for the meta-analysis with Caucasian and NZ Polynesian populations. For the meta-analysis with Japanese, Caucasian, and NZ Polynesian populations, we implemented only DerSimonian and Laird random-effects model meta-analysis[36]. All the meta-analyses were performed using the R version 3.1.1 and 3.2.2[37] with meta package. Genome-wide significance threshold was set to be $\alpha = 5.0 \times 10^{-8}$ in order to claim evidence of a significant association. All calculations of LD (r^2) were conducted with Japanese population.

Topology model and urate transport analysis of NIPAL1 transporter

The amino acid sequences of NIPAL1 shown in figure 3A and FAM35A were obtained from GenBank (accession code NM_207330 and NM_019054, respectively). Transmembrane helices of these proteins were predicted by SOSUI algorithm[19], whereas FAM35A was predicted to be a soluble protein. The topology model of NIPAL1 was generated using $LAT_EX2\varepsilon$ with a macro package T_EXtopo (v1.5)[38]. For the functional analysis, the cDNA of human NIPAL1 wild-type in pCI-Neo vector was inserted into the pCS2(+) vector between the restriction enzyme sites of *EcoR*I and *Xba*I. In a similar way, EGFP-NIPAL1/pCS2(+) vector and SLC2A9-EGFP/pCS2(+) vector were constructed in order to better visualize each protein. For the SLC2A9 clone, we used SLC2A9 short isoform, also known as SLC2A9S or GLUT9S[5] (accession code NM_001001290). The EGFP/pCS2(+) vector for the control study was kindly provided by Dr. Shinji Kanda (the University of Tokyo, Japan). SLC2A9 is a renal urate transporter[5] and was used as a positive control for this functional and localization analysis. To synthesize cRNA, each cDNA construct was linearized in the downstream region of the SV40 polyA site, and then transcribed with SP6 polymerase in the presence of cap analog using a mMESSAGE mMACHINE SP6 Transcription Kit (Thermo Fisher Scientific, Yokohama, Japan) according to the manufacturer's instructions. The obtained cRNAs were purified using a MEGAclear™ Transcription Clean-Up Kit (Thermo Fisher Scientific), and stored at -80ºC until use.

Oocytes were prepared from *Xenopus laevis* (Kato-S-Science, Chiba, Japan) anesthetized with ethyl 3-aminobenzoate methanesulfonate (1 g/L) (Sigma Aldrich, St. Louis, MO, USA) and

treated with collagenase (1.5 mg/mL in calcium free OR2 buffer: 82 mM NaCl, 2.5 mM KCl, $MgCl_2$ 1.0 mM, HEPES 5.0 mM, and pH 7.6) (Wako Pure Chemical Industries Ltd., Tokyo, Japan) for 2 hours at room temperature, followed by defoliculation in ND-96 buffer (96 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM HEPES, and pH 7.5). Stage V-VI oocytes were injected with 50 ng cRNA in 50 nL of RNA-free water using a microdispenser as described previously[3,39]. The oocytes were incubated at 18ºC for 2-3 days in glass bottles containing fresh ND-96 buffer. The medium was changed per 12 hours. For the Mg^{2+}-free experiment, the buffer was replaced by ND-96 buffer without Mg^{2+} 24 hours after the injection.

Sixty hours after the cRNA injection, urate transport activity in each oocyte was determined. In brief, cRNA-injected oocytes were washed with high potassium (HK) buffer (2.0 mM Na-gluconate, 96 mM K-gluconate, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, and pH 7.5) or HK buffer without Mg^{2+}, and pre-incubated in the HK buffer for 10 min at 18ºC. Subsequently, the buffer was exchanged with a fresh one containing 40 μM [8-^{14}C]urate (American Radiolabeled Chemicals, St. Louis, MO, USA) and 100 μM non-radiolabeled urate, and incubated for 30 min at 18ºC. After the incubation, the oocytes were washed with ice-cold HK buffer with or without Mg^{2+} six times, then dissolved in 200 μL of 62.5 mM NaOH. Radioactivity in the resulting lysate of each oocyte was measured by liquid scintillator[7,39].

Immunohistochemical analysis of human kidney

For microscopic immunohistochemical analysis, three-micrometer paraffin sections of human kidney were processed. The kidney sections were incubated with affinity-purified rabbit anti-human NIPAL1 antibody (1:50) (LS-C164878; LifeSpan BioSciences, WA, USA) or with affinity-purified rabbit anti-human FAM35A antibody (1:75) (HPA036582; Sigma-Aldrich, MO, USA) at 4°C overnight for immunostaining. Thereafter, they were treated with anti-rabbit peroxidase-labeled polymer (EnVision+; Dako, Tokyo, Japan) for 30 minutes. The immunoreactions were detected with diaminobenzidine (0.8 mM)[40,41].

Confocal laser scanning microscopic observation

Madin-Darby canine kidney II (MDCKII) cells were maintained in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France), and 1% penicillin-streptomycin (Nacalai Tesque) at 37ºC in a humidified atmosphere of 5% (v/v) CO_2 in air. Each vector plasmid was transfected into MDCKII cells by using polyethyleneimine MAX (PEI-MAX) (Polysciences Inc., Warrington, PA, USA) as described previously[42]. Subsequently, the cells were seeded onto a collagen-coated glass bottom

dish (cover size 22 × 22 mm and 0.16-0.19 mm thick; Matsunami Glass Inc., Tokyo, Japan), followed by incubation for 84 hours. Then, the culture medium was replaced by Hank's Balanced Salt Solution (HBSS) (Wako Pure Chemical Industries Ltd., Tokyo, Japan) or HBSS Mg^{2+}-free. Twelve hours after the further incubation, the cells were fixed by ice-cold methanol, washed with PBS (-) three times, and then treated with TO-PRO-3 Iodide (Molecular Probes, Eugene, OR, USA) diluted 250-fold in PBS (-) for 10 min at room temperature. After the nuclear DNA staining, the cells were mounted in VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA). To analyze the localization of EGFP-fused NIPAL1 protein, fluorescence was detected using Fluoview FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). For the localization analysis of transporters in oocytes, cRNA-injected oocytes at the indicated time were transferred onto the collagen-coated glass bottom dish containing ND-96 buffer, and then observed by confocal laser scanning microscope. By this method, we investigated the cellular localization transporters (NIPAL1 and SLC2A9) using live oocytes without fixation.

Supplementary References

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