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ALPK1 genetic regulation and risk in relation to gout

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Background The present study investigated whether single nucleotide polymorphisms (SNPs) in the alpha-protein kinase 1 (*ALPK1*) gene are associated with gout in aboriginal and Han Chinese Taiwanese.

Methods A total of 1351 aborigines from the community (511 cases and 840 controls) and 511 Han people from hospital (104 cases and 407 controls) were recruited. SNPs in potentially functional regions of the 38 genes within 4q25 were identified and genotypes determined by direct sequencing. Quantitation of blood *ALPK1* mRNA expression levels and luciferase assay of gout-associated rs231253 pGL3-SNP constructs cotransfected with *hsa-miR-519e* were examined.

Results We found that *ALPK1* gene was the most determinant of gout. Three SNPs of rs11726117 M861T [C], rs231247 [G] and rs231253 [G] were most associated with gout risk [odd ratios (OR) $\geqslant$1.44, $P \leqslant 3.78 \times 10^{-6}$) in aborigines. A replication set using Han people had risk at rs11726117 and rs231247 (OR $\geqslant$1.72, $P \leqslant 4.08 \times 10^{-3}$). From pooled analysis (Breslow-Day test, $P > 0.33$) assuming an additive model, each increasing copy of the risk allele of rs11726117 [C], rs231247 [G] and rs231253 [G] showed significantly elevated OR for gout $\geqslant$1.42 ($P \geqslant 1.53 \times 10^{-6}$). Consistently, the composite homozygous of linked 3 SNPs (versus wild-type, OR = 1.83, $P = 8.21 \times 10^{-4}$) had strong associations with *ALPK1* mRNA expression. Luciferase showed reduced hybridization between *hsa-miR-519e* and construct carrying gout-associated rs231253 [G] than the wild-type [C] ($P = 6.19 \times 10^{-4}$).

Conclusions Our study found that a newly identified *ALPK1* gene can effectively interfere with microRNA target recognition and modulates the mRNA expression; and the varying distribution of the implicated SNPs among cases and controls in the two studied populations suggests a significant role in gout susceptibility.

Keywords Gout, *ALPK1* gene, SNP, microRNA, mRNA

Introduction

Gout is characterized by monosodium urate (MSU) deposits in the soft tissues of hyperuricaemic patients whose symptoms include recurrent flares of acute gout arthritis, tophaceous deposits of MSU crystals in joints and other tissues, chronic arthropathy, uric acid urolithiasis and renal impairment.[1,2] Gout patients experience very painful attacks due to the precipitation of MSU crystals in joints, along the Achilles tendon and the first metatarsophalangeal joint, as well as the triggering of inflammatory cytokines, chemokines, proteases and oxidants that lead to damage such as chronic synovitis, cartilage loss and bone erosion.[1–3] Hyperuricaemia is present in almost all gout patients, however only a minority (20%) of hyperuricaemic individuals experience gout symptoms.[4] Tophus is a severe form of crystal arthropathy that can occur anywhere in the body. Without treatment, tophi occur in 30% of patients within 5 years of onset of gout,[3] whereas others may continue to have flares of acute arthritis without the tophus occurrence. Therefore, studying a possible role for these familial hyperuricaemia- and gout-causing genes in common gout is justified.[5]

For the past two decades, public health researches have identified the Taiwanese aboriginals as having a high gout prevalence of 12%[6,7] especially male aboriginals, with as high as 64%[8] of gout cases exhibiting familial clustering of chronic tophaceous gout. Linguistic and archaeological evidence suggests that the aboriginals were originally one group of expanding Neolithic *Setaria* farmers who first settled in Taiwan as early as 5500 YBP from the southern coast of China.[9] Their prevalence markedly differs from Taiwanese Han (who immigrated in 300 YBP in large numbers to Taiwan from Guangzhou and Fujian provinces) at 3%[6] and tophi rates that have declined from 34%[10] to 9%[11] of cases during 1948–1984 to 1992–1999.

Following the genome-wide linkage of aboriginal gout multiplex at marker D4S2623 (at 114cM) on chr4q25 (logarithm of odds [LOD] = 4.29), the *Gout Susceptibility1* (*GOUT1, ID %138900*) was a region hypothesized to influence Taiwanese aboriginal gout.[12] The aborigines are likely to have been isolated in small groups for thousands of years, subjected to higher genetic drift and increased homogeneity. Today, the indigenous population is a minority comprising <3% of entire population of Taiwan. This is an opportunity to study gout-related gene variant differences among ethnicities with special demographic histories, adding to the existing suite of major gout genes discovered.

Methods

Study participants

This study recruited from a community-based Taiwanese aboriginal health cohort study and selected 1351 subjects from 2235 aboriginal highlanders residing in the north central mountains and examined at Township Health Stations since 1994, whom we closely followed up on 511 cases (38.6% had tophi) with chronic gout consistently observed for mean duration of 10 years.[12,13] The general physicians based at health stations and visiting rheumatology medical specialists carefully diagnosed and followed gout in each aborigine using clinical history, physical exam and blood tests performed 2–4 times annually. Because of limited resources of clinical treatment in the area, only a small proportion (<5%) of aboriginal gout patients were treated with uric acid-lowering agents. Additionally, a replication set, 511 hospital-based Taiwanese Han (104 gout and 407 controls), of which cases have chronic gout, were consistently observed for mean duration of 8 years.[14] All gout cases were strictly diagnosed by American College of Rheumatology criteria.[15] Among cases, a high proportion had tophaceous gout, 39% aboriginals and 49% Han; these patients must have demonstrated two or more tophi, clearly visible and palpable from arms, legs, ears, or articular cartilage from other sites, and hyperuricaemia (>8 mg/dl) when their blood was collected. The controls were 840 healthy aboriginals from the same communities as the aboriginal cases, and 407 gout-free Han from the same hospitals as the Han cases, all ascertained to be unrelated, with no history of gout and not taking hypouricaemic agents for other medical conditions. Trained nurses recorded demographic data by standardized questionnaires. Blood specimens were analyzed using standardized hospital protocols. Hyperuricaemia was defined as uric acid exceeding 7.0 mg/dl in males and 6.0 mg/dl in females. Institutional review boards and ethics committees from Kaohsiung Medical University and National Health Research Institutes approved our study design. All participants gave their written informed consent.

DNA resequencing and SNP genotyping

Genomic DNA was extracted from peripheral whole blood by wizard genomic DNA purification kit (QIAGEN-Gentra Puregene Blood Kit) followed standard laboratory protocols. DNA resequencing used Dye Terminator kit (Applied Biosystems) and ABI 3730

DNA sequencers. *ALPK1* gene SNPs were genotyped by TaqMan SNP allelic discrimination assay (Applied Biosystems). TaqMan reactions were based on manufacturer's protocol and samples ran on the ABI7900HT Real-Time polymerase chain reaction (PCR) platform (Applied Biosystems). Allelic discrimination was performed, and analysed by SDS software (v. 2.3) (Applied Biosystems).

mRNA

RNA analysis of 62 aborigines (23 gout and 39 controls) was performed using real-time PCR as described previously.[16] Total RNA from human peripheral blood leukocytes was isolated by PAXgene Blood RNA Kit and generated cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). The expression experiment of *ALPK1* mRNA was performed using pre-designed gene-specific TaqMan probes and primer pairs (Assays-ID: Hs00228473_m1), in triplicate per sample, and a control without template included in each plate. Reference housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified in same plate and equivalent to all samples.

Luciferase assay

The rs231253 allelic differences in promoter activity in the human HEK293 line were confirmed with use of the pGL3-basic vector. Cells were transfected with these reporter constructs and with pRLTK *renilla* (Promega) luciferase vector as a normalization control. We constructed HEK293 cells (2×10^4 cells per 96-well) were cotransfected with 0.1µg of pGL3-SNP (firefly luciferase), 0.02µg of pGL4.73 (*renilla* luciferase) (Promega) and 0.1µg of the *hsa-miR-519e* (Applied Biosystems) plasmid. Cells were lysed 72 h after transfection, in 50 µl of lysis buffer according to the Dual-Glo Luciferase Assay System protocol (Promega) and luciferase activity measured with TopCount NXT luminometer (Packard). Relative luciferase activity of *ALPK1* reporter constructs was calculated as the ratio of firefly luciferase activity to *renilla* luciferase. Reporter assays shown were the average of four independent experiments in triplicates and were analysed by repeated measures analysis.

Statistical analysis

Multipoint analysis was performed using conditional-logistic model implemented in the S.A.G.E. *v*6.0.1 program for linkage fine-map. We used a one-parameter model and default value that constrained relative risks, $\lambda_2 = 3.634\lambda_1 – 2.364$, assuming the expression fix for mode of inheritance was a value approximately halfway between a dominant and a recessive model, corresponding to the Whittemore and Tu minmax model mode of inheritance parameter.[17] The likelihood-ratio statistic was computed by multiplying the LOD (logarithm of odds) score by 4.6. Linkage disequilibrium (LD) coefficients (D′ and r^2) implemented by Haploview *v4.2*[18] and *PLINK v1.07*.[19] Genotype frequencies of the controls of ethnic groups regarding investigated SNPs conformed to Hardy-Weinberg equilibrium and were assessed by χ^2 goodness-of-fit test. The means or proportions for baseline gout risk factors were calculated for cases and controls. Significance of associated risk factors was tested with the χ^2 statistic for categorical variables and the generalized linear regression models for continuous variables. We analysed the association between SNPs and risk of gout using logistic regression analysis. Additive genetic effects were modelled by defining continuous variables with levels 0, 1 and 2 corresponding to genotypes (i.e. rs11726117 was coded as 0 for TT, 1 for CT and 2 for CC). The Breslow-Day test examined the odds ratio (OR) homogeneity between family-based substructures and population-based aboriginal gout subjects (Breslow-Day test, $P = 0.22$) were obtained. OR with 95% confidence intervals (CI) was adjusted for age, gender, body mass index, hypertension, alcohol use, hyperuricaemia, total cholesterol, log (triglycerides) and creatinine using a logistic regression model. Pooled analysis was performed by Cochran-Mantel-Haenszel test method using the *PLINK v1.07*. Case/control traits can be analysed for haplotype associations using *PLINK v1.07*. We categorized composite genotypes into three categories: risk-homozygous (rs11726117 [CC] + rs231247 [GG] + rs231253 [GG]), reference-homozygous ([TT] + [AA] + [CC]) and other-type. *ALPK1* mRNA expression by composite genotypes was calculated.

Bioinformatics analysis

Binding site of 3′ untranslated region (UTR) polymorphism to miRNA was indicated by MicroCosm Targets version 5 at EMBL-EBI. RNAhybrid[20] calculated miRNA::SNP interaction induced allele-induced minimum free energy (MFE) changes. Signal peaks of NF-κB (p65) within 100-10 kb upstream of the transcription initiation site of *ALPK1* was shown to be present in GM12878 (lymphoblastoid) cell-line of ENCODE Yale TFBS by ChIP-seq Yale/UC-Davis/Harvard (release 3; May 2010). MOTIF Search explored all putative transcription binding sites in the *ALPK1* promoter region.

Results

Clinical characteristics of study participants are presented in Table 1. Gout cases had a mean onset age of 40.8 years for aborigines and 45.2 years for Han. Cases showed high urate levels ($\geqslant$8.9 mg/dl), $\geqslant$85.5% hyperuricaemia and $\geqslant$38.5% tophaceous gout, and higher mean total cholesterol, triglycerides and creatinine levels ($P < 0.01$). Gout cases also had significantly increased alcohol intake ($P < 0.001$). Aboriginal controls showed borderline-high uric acid ($\geqslant$7 mg/dl) and triglycerides (199 mg/dl), and 31%

Table 1 Characteristics of the study participants

Characteristic	Taiwanese aborigines		P	Taiwanese Han		P
	Cases	Controls		Cases	Controls	
Number	511	840		104	407	
Age (SD), years	51.1 (14.4)	53.5 (16.5)	0.0065	52.8 (13.7)	55.2 (14.5)	0.1308
Men, *n* (%)	392 (76.7)	419 (49.9)	<0.0001	104 (100.0)	405 (99.5)	0.4738
Age of onset (SD), years	40.8 (15.0)			45.2 (12.3)		
Duration of gout (SD), years	9.9 (7.9)			8.2 (6.3)		
Tophi, *n* (%)	197 (38.6)			51 (49.0)		
Systolic pressure (SD), mmHg	139.1 (21.7)	131.5 (20.7)	<0.0001	136.0 (18.0)	131.7 (19.4)	0.0535
Diastolic pressure (SD), mmHg	88.9 (14.1)	83.3 (12.5)	<0.0001	85.6 (13.3)	83.4 (11.7)	0.1344
Body mass index (SD), kg/m^2	26.4 (4.2)	26.5 (4.1)	0.9322	26.0 (4.0)	24.6 (3.4)	0.0002
Hypertension, *n* (%)	225 (44.0)	262 (31.2)	<0.0001	32 (30.8)	71 (17.4)	0.0025
Type 2 diabetes mellitus, *n* (%)	41 (8.0)	62 (7.4)	0.6661	2 (1.9)	13 (3.2)	0.4931
Alcohol use, *n* (%)	393 (76.9)	446 (53.1)	<0.0001	35 (33.7)	98 (24.1)	0.0470
Total cholesterol (SD), mg/dl	186.9 (48.4)	183.8 (46.3)	0.2381	210.7 (48.1)	190.9 (38.1)	<0.0001
Triglycerides (SD), mg/dl	268.5 (275.4)	192.2 (245.3)	<0.0001	225.3 (121.4)	144.2 (136.8)	<0.0001
Log(triglycerides) (SD), mg/dl	5.3 (0.7)	4.9 (0.7)	<0.0001	5.3 (0.5)	4.7 (0.6)	<0.0001
Creatinine (SD), mg/dl	1.2 (0.6)	1.0 (0.2)	<0.0001	1.4 (0.4)	1.2 (0.2)	<0.0001
Uric acid (SD), mg/dl	9.3 (2.4)	7.0 (2.0)	<0.0001	8.9 (1.8)	6.1 (1.3)	<0.0001
Hyperurcaemia, *n* (%)	437 (85.5)	490 (58.3)	<0.0001	91 (87.5)	99 (24.3)	<0.0001

SD, standard deviation. *P*-values from generalized linear regression models for continuous variables and from chi-square tests for categorical variables.

were hypertensive. Type 2 diabetes, often comorbid with gout in Caucasians, was insignificant in the aborigines.

Our study scheme is summarized in Figure 1. A pilot study involved gene-centrically resequenced 666 polymerase-chain-reaction (PCR) amplicons including 38 gene exons, and flanking intron sequences of genes between D4S1647 and D4S2937 region in *GOUT1* were typed and 404 SNPs exposed, using 23 unrelated male aboriginal gout case-control pairs to produce the association SNPs (Supplementary Table 1, available as Supplementary data at *IJE* online). Although, as for *ALPK1*, only one SNP is reported as significant ($P = 0.041$), we cannot rule out the possibility that *ALPK1* variants contribute to the gout risk. Increased cohort size had narrowed these SNPs to those belonging to four genes (*SCYE1*, *DKK2*, *FLJ39370*, *ALPK1*; Supplementary Table 2, available as Supplementary data at *IJE* online). Separately, a confirmatory study by adding five dense microsatellite markers into the linkage peak at 114cM ($P = 4.40 \times 10^{-6}$, LOD = 4.29) moved the maximal signal to a new peak at 117cM ($P = 5.00 \times 10^{-7}$, LOD = 5.17; Supplementary Table 3 available as Supplementary data at *IJE* online). After re-examining many of previous genes that had been associated with smaller cohort sizes (e.g. *INTS12* and *FLJ39370*), only *ALPK1* SNPs remained consistently associated in all cohorts and at ~113.3 Mb were closest to revised signal at ~114 Mb on the physical map. The candidates that were not significant in the final cohort or closest to revised signal, such as intronic SNPs of *INTS12* and *FLJ39370*, were discontinued.

Thus, *ALPK1* was selected as a Taiwanese aboriginal gout susceptibility gene and a linkage disequilibrium plot (44 SNPs) for 445 aborigines was constructed (Supplementary Figure 1 available as Supplementary data at *IJE* online). Furthermore, nine SNPs [located one in intron 7-8; four nonsynonymous in exon 11; two synonymous in exon 13 and 14; and two in 3'untranslated region (3'UTR)] were found to be significant with gout (Table 2) and in strong linkage disequilibrium (LD: D' $\geqslant$ 0.85 and $r^2 \geqslant 0.75$ in controls). Three loci particularly, a nonsynonymous rs11726117 M861T [C], a synonymous rs231247 [G] and 3'UTR rs231253 [G] showed most significance with gout ($P = 3.78 \times 10^{-6}$, 2.00×10^{-6}, 3.48×10^{-6}, respectively) with odds ratios for gout after adjustment for hyperuricaemia and other covariates being 1.45 (95% CI = 1.21–1.73), 1.48 (95% CI = 1.24–1.77) and 1.43 (95% CI = 1.20–1.72), respectively. Independently using these three SNPs, we replicated a similar risk in Taiwanese Han (adjusted OR = 2.41 in rs11726117, adjusted OR = 2.15 in rs231247 and adjusted OR = 1.36 in rs231253). The haplotype-block showed high gout risk in two studied ethnicities

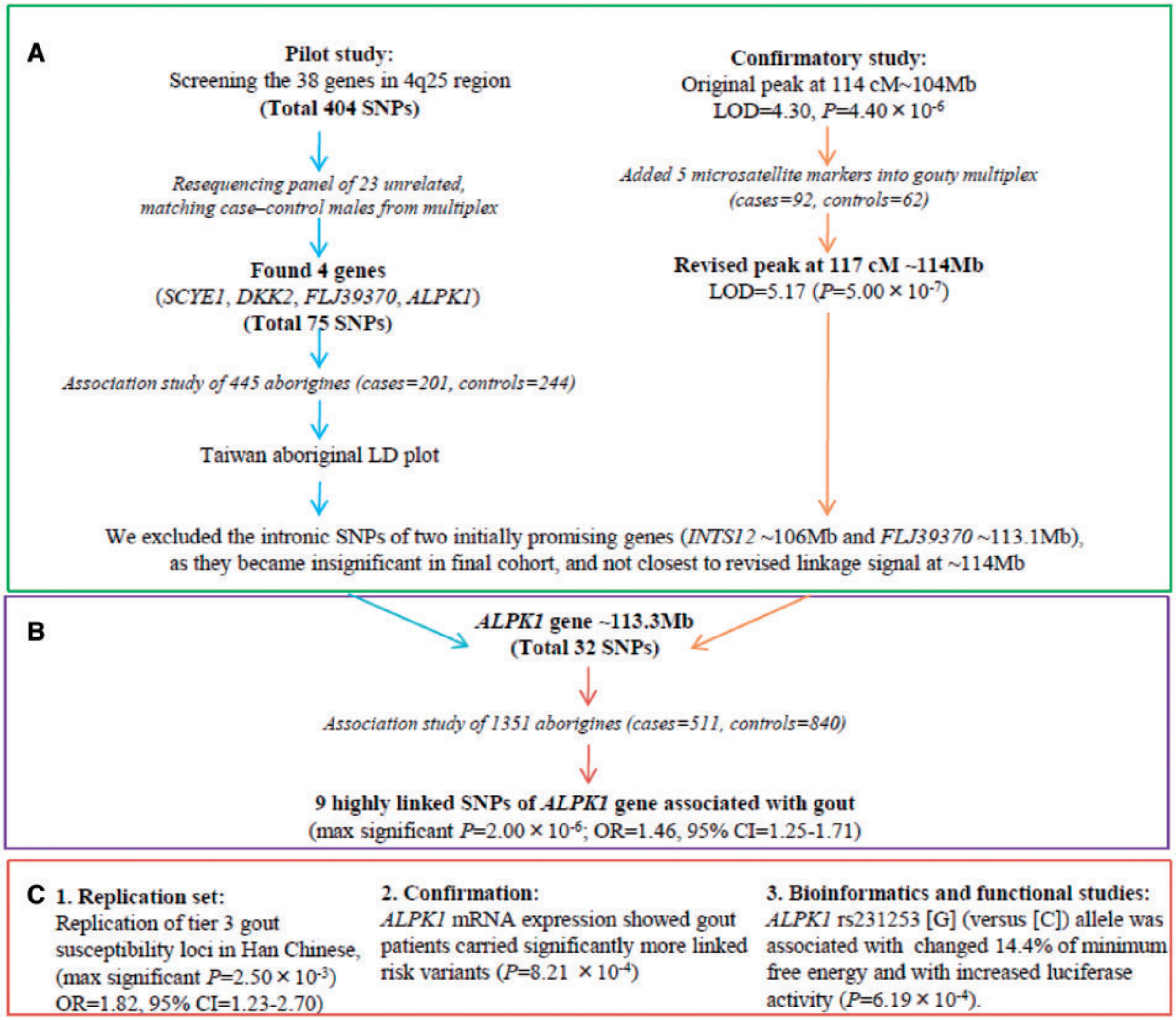

Figure 1 Region-wide association fine-map of chr4q25 to identify *ALPK1*. (**A**) We commenced with a pilot study that gene-centrically resequenced 38 genes (total 404 SNPs) in the 4q25 region using 23 unrelated male aboriginal gout case-control pairs that produced the association SNPs; a confirmatory study was made by adding five dense microsatellite markers into linkage peak at 114cM, then maximal signal migrated to a new peak at 117cM ($P = 5.00 \times 10^{-7}$, LOD = 5.17); raising the cohort size had narrowed down SNPs further to those within four genes (*SCYE1*, *DKK2*, *FLJ39370* and *ALPK1*). (**B**) Using the final 1351 cohort, which consisted 511 gout cases and 840 controls, we isolated nine SNPs which were significantly associated with gout. (**C**) We replicated independently similar risk in the Han Chinese people. Interestingly, the linked variant at 3′UTR is predicted to be a binding site polymorphism of *hsa-miR-519e*, suggesting loss of gene regulation among carriers of the affected, a result consistent with luciferase activity *in vitro* and *ALPK1* mRNA expression from blood samples findings

(Supplementary Table 4 available as Supplementary data at *IJE* online).

For aborigines, those aforementioned three most important SNPs were within 10.2 k of each other (113571846-113582071 base pair), having an $r^2 > 0.74$. Model comparison test was significant for at-risk haplotype [CGG] versus reference haplotype [TAC] (OR = 1.43, 95% CI = 1.22–1.67) and the likelihood ratio test ($\chi^2 = 20.5$, degree of freedom [df] = 1, $P = 5.86 \times 10^{-6}$). For Han Chinese people, of those three SNPs generated, four were common haplotypes (rs11726117/rs231247, $r^2 = 0.88$; rs11726117 or rs231247/rs231253, $r^2 > 0.41$). Model comparison test was significant for at-risk haplotype [CGG] versus reference haplotype [TAC] (OR = 2.05, 95% CI = 1.29–3.26) and the likelihood ratio test ($\chi^2 = 28.9$, df = 3, $P = 2.34 \times 10^{-6}$). SNPs of rs11726117, rs231247 and rs231253 showed homogeneity in associating with gout (Ethnic Breslow-Day test, $P > 0.33$). Therefore, pooled analysis of the three SNPs by Cochran-Mantel-Haenszel assuming an additive model was used to test for associations (Table 2). When individuals were combined into a pooled analysis, each increasing copy of the [C] allele at rs11726117, [G]

Table 2 Result of the gout-associated *ALPK1* SNPs

		Cases				Controls							
SNP	R/NR	11	12	22	1	11	12	22	1	HWE *P*	*P* (trend)	OR (95% CI)	Adjusted OR (95% CI)[a]
Taiwan aborigines													
rs9994944	G/A	229	221	61	0.66	324	392	124	0.62	0.76	1.87×10^{-2}	1.21 (1.03–1.43)	1.26(1.05–1.52)
rs2074388 G565D	A/G	180	243	88	0.59	214	414	212	0.50	0.68	9.03×10^{-6}	1.43 (1.22–1.67)	1.41(1.18–1.69)
rs13148353 H642R	G/A	191	240	80	0.61	244	404	192	0.53	0.32	1.04×10^{-5}	1.36 (1.17–1.59)	1.39(1.16–1.66)
rs2074379 M732I	A/G	184	246	81	0.60	237	405	198	0.52	0.33	1.03×10^{-4}	1.36 (1.17–1.59)	1.37(1.14–1.63)
rs11726117 M861T	C/T	198	237	76	0.62	244	396	200	0.53	0.11	3.78×10^{-6}	1.44 (1.23–1.69)	1.45(1.21–1.73)
rs231247 R1084R	G/A	195	233	83	0.61	225	414	201	0.51	0.70	2.00×10^{-6}	1.46 (1.25–1.71)	1.48(1.24–1.77)
rs55840220 T1145T	A/G	23	131	357	0.17	21	125	694	0.10	<0.01	1.89×10^{-7}	1.76 (1.42–2.18)	1.71 (1.33–2.18)
rs231253, 3′ UTR	G/C	190	239	82	0.61	223	416	201	0.51	0.80	3.48×10^{-6}	1.45 (1.24–1.70)	1.43(1.20–1.71)
rs960583, 3′ UTR	A/G	25	159	327	0.20	19	224	597	0.16	0.71	1.37×10^{-3}	1.39 (1.13–1.69)	1.33(1.06–1.66)
Taiwanese Han													
rs11726117 M861T	C/T	70	31	3	0.82	209	168	30	0.72	0.64	2.50×10^{-3}	1.82 (1.23–2.70)	2.41 (1.41–4.12)
rs231247 R1084R	G/A	70	28	6	0.81	204	167	36	0.71	0.83	4.08×10^{-3}	1.72 (1.18–2.50)	2.15 (1.31–3.50)
rs231253, 3′ UTR	G/C	63	35	6	0.77	215	164	28	0.73	0.66	1.92×10^{-1}	1.27 (0.89–1.83)	1.36 (0.84–2.20)
Pooled analysis[b]													
rs11726117 M861T	C/T	268	268	79	0.65	453	564	230	0.59		2.89×10^{-8}	1.51 (1.31–1.75)	1.53 (1.30–1.81)
rs231247 R1084R	G/A	265	261	89	0.64	429	581	237	0.58		2.05×10^{-8}	1.52 (1.31–1.75)	1.55 (1.31–1.83)
rs231253, 3′ UTR	G/C	253	274	88	0.63	438	580	229	0.58		1.53×10^{-6}	1.42 (1.23–1.65)	1.42 (1.21–1.68)

R, risk allele; NR, non-risk allele; 11 indicates at-risk homozygote, 12 indicates heterozygote; 22 indicates wild-type homozygote; HWE, Hardy-Weinberg equilibrium; 3′ UTR, three prime untranslated region; OR, odd ratios; CI, confidence interval.

ORs and *P*-values were for each case-control study separately under an additive model of inheritance.

[a]OR with 95% CI in parentheses was adjusted for age, gender, body mass index, hypertension, alcohol use, total cholesterol, log (triglycerides), creatinine and hyperuricaemia by a logistic regression model.

[b]Pooled analysis was performed by the Cochran-Mantel-Haenszel test method using the *PLINK v1.07*. Three SNPs revealed homogeneity in the association with gout (Breslow-Day test, $P > 0.33$). OR with 95% CI in parentheses was adjusted for ethnicity, hyperuricaemia and other covariates using a logistic regression model.

allele at rs231247 and [G] allele at rs231253 conferred a significantly elevated OR for gout of 1.51 ($P = 2.89 \times 10^{-8}$), 1.52 ($P = 2.05 \times 10^{-8}$) and 1.43 ($P = 1.53 \times 10^{-6}$) after controlling for ethnicity only. The above results were similar even after controlling for ethnicity and hyperuricaemia and other covariates concerning the risk estimate for rs11726117 [C] (OR = 1.53, 95% CI = 1.30–1.81), rs231247 [G] (OR = 1.55, 95% CI = 1.31–1.83) and rs231253 (OR = 1.42, 95% CI = 1.21–1.68). From a 2-*df* model, the results were consistent with an underlying additive model. For instance, compared with reference allele homozygotes, the OR for heterozygotes in the full multivariable model was 1.68 (95% CI = 1.19–2.36) for rs11726117, and the risk allele homozygotes had the highest risk (OR = 2.43, 95% CI = 1.70–3.47), again remaining consistent with an additive genetic model.

To quantitate the expression differences in genotype carriers of these three SNPs, a real-time PCR of *ALPK1* mRNA was performed on peripheral blood leukocytes of 62 aborigines (Figure 2, and Supplementary Figure 2 available as supplementary data at *IJE* online). The rs11726117 [CC] and [CT] genotypes when compared with AA genotype were found to be 1.22 to 1.49 significantly higher (*P-trend* = 0.0007). Composite genotype analyses of the rs11726117 [CC], rs231247 [GG] and rs231253 [GG] showed association with gout. *ALPK1* transcribed significantly higher in gout cases, specifically the homozygous of linked three SNPs [CC+GG+GG] versus wild-type [TT+AA+CC] (OR = 1.83, 95% CI = 1.24–2.68; $P = 8.21 \times 10^{-4}$) with *post hoc* compared Dunnett's test. At-risk homozygous [CC+GG+GG] was also significantly more highly expressed in cases than its composite genotype in controls (OR = 1.40, 95% CI = 1.01–1.93, $P = 4.15 \times 10^{-2}$).

It was found that a microRNA, *hsa-miR-519e* (chr19q13.42), shared binding site complementarily with 3′UTR rs231253, and that gout-associated risk [G]-allele creates a slight kink in the mRNA structure with respect to [C]-allele thus becoming less negative in free energy state, denoting a less stable hybridization [MFE change: −25.4 kcal/mol to −22.2 kcal/mol, (Supplementary Figure 3 available as supplementary data at *IJE* online). There was a 2-fold increase in luciferase activity of rs231253 on the construct carrying [G] allele following an overexpression of the interacting *hsa-miR-519e* ($P = 6.19 \times 10^{-4}$; Figure 3].

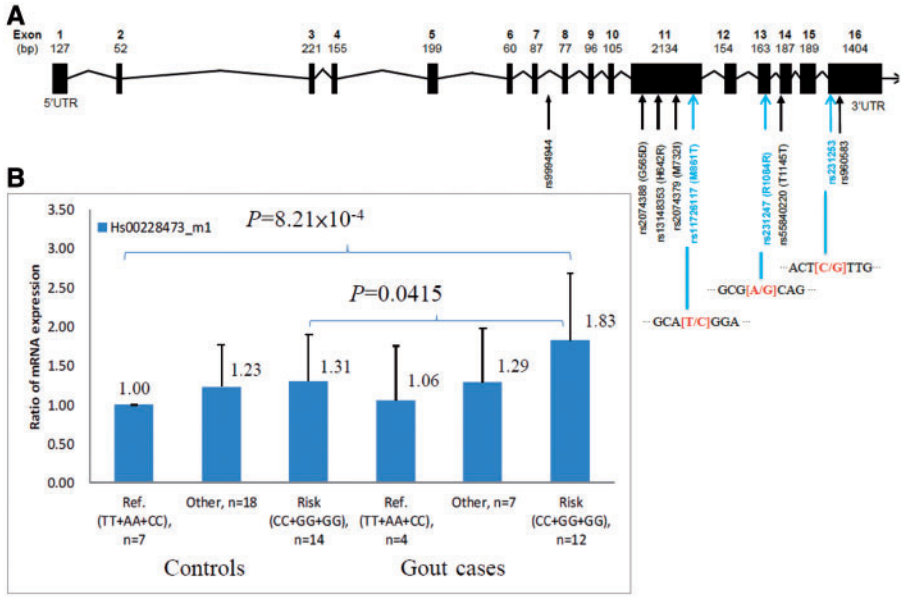

Figure 2 Genomic structure of *ALPK1* with nine linked loci and mRNA expression in gout aboriginal cohort (**A**) Exons are indicated by black boxes. Most significant three loci are indicated by blue text and alleles in red text. SNP rs9994944 is located in intron 7-8, followed by four nonsynonymous variants in exon 11, two synonymous variants in exon 13 and exon 14 located within alpha-kinase domain, and rs231253 and rs960583 located in three prime untranslated region. SNP rs11726117 [C], rs231247 [G] and rs231253 [G] have most significant *P* values from association study. Therefore, three SNPs were entered into real-time PCR validation. (**B**) Real-time PCR result of *ALPK1* mRNA (Hs00228473_m1) on peripheral blood leukocytes of 62 gout aborigines demonstrated elevated *ALPK1* mRNA (at-risk [CC+GG+GG] in cases compared with wild-types [TT+AA+CC] in controls) with *post hoc* compared Dunnett's test (OR = 1.83, 95% CI = 1.24–2.68; $P = 8.21 \times 10^{-4}$). Error bar indicates 95% confidence interval; PCR, polymerase chain reaction

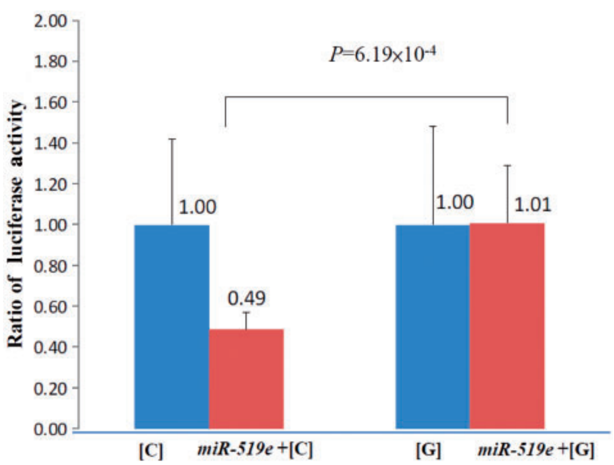

Figure 3 Higher expression from miRNA hybridized with gout-associated *ALPK1* rs231253 [G] allele. Approximately 2-fold differential luciferase activity ($P = 6.19 \times 10^{-4}$) between pGL3-SNP constructs of gout-associated rs231253 [G] allele and wild-type [C] allele cotransfected with *hsa-miR-519e* that shared complementary binding. Values represent mean ± SD of four independent experiments performed in triplicate. SD, standard deviation

Discussion

Region-wide association fine-map of 4q25 showed the *ALPK1* gene to be most associated with gout in Taiwanese aboriginals and Han and closest to newly revised linkage signal at 117cM (LOD = 5.2) in this study. Particularly, three *ALPK1* loci of the nonsynonymous rs11726117 M861T [C], synonymous rs231247 [G] and 3'UTR rs231253 [G] were most associated with gout risk. These could cause alterations to the normal physiological function of the ALPK1 of the alpha-kinase family although the functions of *ALPK1* are less known. For example, the missense rs11726117 M861T is a threonine substitution located forward of the catalytic domain, and potentially a new phosphorylation site for ALPK1 with preferentially phosphorylate threonine residues.[21,22] Synonymous rs231247 (R1084R) codes for an amino acid in the alpha catalytic domain, located adjacent to a conserved invariant glutamine, which structurally maintains the alpha-helix C of subdomain III and near a polar residue that binds H_2O to help orientate the ATP γ-phosphate in the binding groove.[21,23] We identified 3'UTR rs231253 [G] showing association with risk of gout (pooled analysis, OR = 1.42). Although the rs231253 [G] is marginal associated with gout (OR = 1.36) in Han people, we cannot rule out the possibility that rs231253 [G] contributes to the gout risk. One replication study is needed to clarify the genetic effects in the future. With functional testing, we found that the gout cases, carrying risk rs231253 [G], showed

83% increased *ALPK1* mRNA expression ($P = 8.21 \times 10^{-4}$), and *in vitro* experimentation for verification of the rs231253 [G] showed increased expression in the luciferase assay ($P = 6.19 \times 10^{-4}$). With bioinformatic prediction, SNP rs231253 [G] presented a less stable hybridization of *hsa-miR-519e*/target duplex, as suggesting disruption in gene silencing (Supplementary Figure 3, available as supplementary data at *IJE* online).

Uric acid, generated by degradation of purines, is further cleared by degradation to allantoin catalyzed by hepatic uricase, or by excretion largely in the kidneys. Humans, unlike most mammals, lack uricase, and therefore rely on renal excretion as a critical determinant of systemic urate levels.[2] Genome-wide association studies have identified the common polymorphisms in several genes involved in the renal urate transport that are associated with hyperuricaemia and gout, including *SLC2A9*, *SCL22A12*, *SCL22A11*, *SLC17A3*, *ABCG2* and *SLC17A1* genes.[24,25] *ABCG2* rs2231142 Q141K has been suggested as a major causative gene variant for gout.[25] However, renal urate transporter gene variants explain only about 5.3% of the total variation of serum urate concentrations in people of Caucasian ancestry.[24,25] Therefore, many other genes with partial effects and accompanied by high urate levels contribute to formation of MSU crystals and the clinical presentation of acute gout arthritis and chronic tophaceous disease.

We found *in vitro* that the ALPK1 and MSU may synergistically induce ERK1/2 and p38 phosphorylation, then regulate cytokine expressions through the activation of NF-κB pathway.[16] The activated NF-κB complexes are known to translocate into the nucleus and bind to NF-κB DNA binding motifs to trigger transcription of genes that are critical to inflammation, such as cytokines, chemokines and cell adhesion molecules.[1,26] The ENCODE 'Yale TFBS' track has revealed the entire 10 kb of *ALPK1* promoter region to contain several signal peaks of NF-κB transcription factor binding motifs suggested in an overall positive feedback (Supplementary Figure 4, available as supplementary data at *IJE* online).

In conclusion, the ethnic replication of association findings between *ALPK1* loci and gout has led us to consider *ALPK1* as a significant new candidate susceptible gene for gout. Uniquely, a 3'UTR rs231253 variant on *ALPK1* has been found to disrupt miRNA gene regulation and contribute to a higher mRNA expression among the patients affected.

Supplementary Data

Supplementary data are available at *IJE* online.

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Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr Ko had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design and final approval of the version: Ying-Chin Ko; drafting the article: Albert Min-Shan Ko, Hung-Pin Tu; acquisition of samples with information: Hung-Pin Tu, Shang-Lun Chiang, Shun-Jen Chang; performance of experiments: Tze-Tze Liu, Chung-Yee You, Jan-Gowth Chang; analysis and interpretation of the data: Ying-Chin Ko, Albert Min-Shan Ko, Jan-Gowth Chang, Hung-Pin Tu, Shun-Jen Chang, Allen Min-Jen Ko, Chien-Hung Lee, Chi-Pi Lee, Chung-Ming Chang and Shih-Feng Tsai; miRNA bioinformatics: Albert Min-Shan Ko, Yu-Fan Liu.

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Conflict of interest: None declared.

KEY MESSAGES

- *ALPK1* is a gout-associated gene in Taiwan aborigines and with replication in Han Chinese people.
- *ALPK1* mRNA transcription is transcribed more highly among gout cases with variants of this gene. Luciferase activity assay showed *in vitro* that the 3'UTR variant affected the complementary binding motif to a microRNA, leading to this increased expression.
- *ALPK1* recognizes phosphorylation sites related to regulation of cytokine expression, but the molecular mechanism and function need further study.

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