Author's response to reviews

Title: Polymorphisms in GCKR, SLC17A1 and SLC22A12 were associated with phenotype gout in Han Chinese males: a case-control study

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Author's response to reviews: see over
Dear Mr Ervin Cenzon,

Thank you very much for your attention and advice. We also are grateful for the referees’ constructive suggestions on our paper (MS: 1754072544157059). The comments raised are essential to promote the quality of this manuscript and we have learned a lot from them. Thank you very much!

We have addressed the comments according to reviewers and the amendments are highlighted in red in the revised manuscript. The point-by-point response are listed as below. We hope that the revision is acceptable, and would like to re-submit it for your consideration. If there are any concerns regarding our manuscript, please don't hesitate to contact us. I look forward to hearing from you soon.

With best wishes,

Chang-Gui Li (corresponding author; E-mail: licgqd@163.com)
We would like to express our sincere thanks to the reviewers for the constructive and positive comments.

Response to reviewer 1 (Tony Merriman)
Major compulsory revisions:
1. The interpretation (abstract) that there are genetic differences between populations was based on an apparent opposite direction of association at GCKR between Chinese and the Phipps-Green et al study. In fact the direction of association is consistent. In the Zhou et al study the G allele is protective, and the A allele therefore risk. This is the same as Phipps-Green et al (in both Polynesian and European). [Note that 'effect' allele is the one that the OR reported correlates with]

Re: Thanks for your suggestion and instruction. I made a mistake in the meaning of the ‘effect allele’ and thus the effect direction. I have revised the statement in page 13 in the revision.

2. Some of these authors have previously published on GCKR in Chinese (Wang et al. Hum Genet 2011). Why was this study not cited? Was there overlap in data sets?

Re: Thanks for your reminding. “SNP rs780094 in the GCKR gene was found to have a strong association with gout in our samples, of which both p value and effect direction were consistent with our previous study by Wang J et al [23]. The data sets of the two studies were not overlapped, demonstrating its true effect on gout risk in Chinese Han males”. I have added this information in page 13 in the revision.

3. The SNPs selected were based on those reported by the Kolz et al 2009 study. In the field this is not a recent study, and has been superceded by Kottgen et al published 2-years in Nature Genetics. This study should be cited, and the authors should explain why they did not study all 28 variants reported by Kottgen et al. The authors should also cite and compare their results to Urano et al (J Rheumatol 2013). Furthermore, why was ABCG2 not analysed?

Re: Thanks for your remindicings. “Another large-scale GWAS from >140,000 individuals of European ancestry for SUA level also replicated most of these nine significant loci and additional 18 new loci [18].” I have cited this literature by Kottgen et al in page 5 in the revision.

“Besides, we don’t assess the relationship of the 18 new loci with gout considering that the proportion of variance in SUA concentrations explained by these new loci was as low as 1.8% in total [18].” I have explained this in page 7 in the revision.
“SNP rs2231142 in ABCG2 were excluded from this study since our group and other domestic institutions have clearly clarified consistent association between this site and gout [27-29, 31, 36].” I have explained this in the Background section in page 7 and Methods section in page 9 in the revision.

Besides, I also cite and compare our results to that by Urano et al (it was inserted as the 22th literature) in page 6, 13, 15 and 16 in the revision.

4. Based on a HWE P of 0.008 rs1183201 (SLC17A1) was excluded from analysis (although association testing was still done). The FDR approach should be applied, or the P Bonferroni corrected by the 8 SNPs tested, whereupon it would be non-significant. I think this variant should be included in the analysis. It is certainly incorrect to claim throughout the manuscript that SLC17A1 was not replicated. In fact it was the strongest association.

Re: Thank you very much for your valuable suggestion and instruction. HWE p is 0.064 (>0.05) after FDR and thus rs1183201 should be included in the analysis which is the strongest association in the present study. I have added the analysis for rs1183201 in page 15 and 16 in the revision.

5. Discussion of SLC22A12. Flynn et al (2014) (http://www.ncbi.nlm.nih.gov/pubmed/24360580) should be cited, where association of SLC22A12 with gout in European was reported. Thus the conclusion that rs505802 is unique to Chinese is not correct. Also the authors should reconsider the statement that SLC22A12 is a substantial risk factor for gout/SU.

Re: Thanks for your reminding. “SNP rs3825018 (in complete LD with rs505802) also achieved nominal significance in New Zealand case-control sample sets (p=0.002) [51].” I have cited this literature in page 15 and removed the two inaccurate sentences mentioned above in the revision. Thank you very much!

6. The FDR should be applied to the Table S2 analysis. Reported associations would not be significant. Please also show P values, especially for the serum urate analysis. Association with age does not need be be done.

Re: Thanks for your suggestion and instruction. “We checked all participants’ questionnaires and confirmed that all cases were first diagnosed with gout on our gout clinic without receiving any relevant medication. Therefore, we decided to conduct this genotype–phenotype analysis in all participants to assess the association of these SNPs with collected clinical characteristics. We found significant association of uric acid concentrations with three SNPs (rs780094 in GCKR, corrected p=3.94E−5; rs1183201 in SLC17A1, corrected p=0.005; rs505802 in SLC22A12, corrected p=0.003) and of triglycerides with rs780094 (located in GCKR, corrected p=2.96E−4).” I have added this information in page 12 and a
Table 3-C in page 27 in the revision.

Minor essential revisions:
1. Please put page numbers in.

Re: Thanks for your reminding. I have inserted the page numbers in the revision.

2. Background, para 2. Does the 40% heritability refer to hyperuricaemia or gout/ Sulem et al did not calculate any heritability estimates. Please refer to original study.


3. Background, para 2. Using refs 8-12 it is claimed that the variants are consistently replicated across ethnic groups. However the studies cited are (nearly universally) European.

Re: Thanks for your reminding. I have added the studies “including Europeans, Chinese, Japanese, Koreans and Mexican Americans [8-16].” in page 5 in the revision.

4. Can the authors outline the parameters they used for the power calculations and also reference the software used.

Re: Thanks for your suggestion. “Power was calculated using obtained unadjusted OR and probability of exposure in controls with preestablished α error probability (α=0.05 for a two sided test) using Power and Sample Size Calculation Software [39].” I have added this information in page 10 in the revision.

5. SLC2A9 discussion. The low prevalence of rs734553 minor allele in Chinese most likely relates to a population history that differs from Europeans. The variant is old (it is present in multiple populations). The authors should probably delete this extremely speculative discussion.

Re: Thanks for your suggestion. We recalculate the distributions of allele frequency between case and control groups and find that rs734553 doesn’t reach the significance level after multiple testing correction. Therefore, we delete the positive analysis for rs734553, remove this speculative discussion and explain the potential reason for this negative result in page 16 in the revision.
6. That functional studies weren't done here is not a limitation.

Re: Thanks for your suggestion. I have deleted this statement in the revision.
Response to reviewer 2 (Donia Macartney-Coxson):
Major concerns:
Abstract:
1. Background (3rd sentence) “…… there is no previous association study of these SNPs in a Han Chinese population”. This sentence is misleading. The 9 SNPs (from Koltz et al 2009) to which this refers are located in 9 different genes; at least some of these 9 genes have previously been associated with gout in Han Chinese. For instance, the current study targets 8/9 of these SNPs but does not explain exclusion of 1/9 (rs2231142 in ABCG2) for which an association with gout in Han Chinese has previously been reported (Wang et al 2010). In addition, the authors cite a previous study of their own (Wang et al 2012, Human Genetics) which reported an association between gout in male Han Chinese and SNPs in GCKR (including rs780094 reported in the current manuscript).
Other studies which report associations between variants in the 9 loci (genes) reported by Koltz et al and gout include:
Zhou et al 2014. Functional polymorphisms of the ABCG2 gene are associated with gout disease in the Chinese Han male population.
Li et al 2014. The hURAT1 rs559946 polymorphism and the incidence of gout in Han Chinese men. (URAT1 is also known as SLC22A12).
Li et al 2012. Polymorphisms in the presumptive promoter region of the SLC2A9 gene are associated with gout in a Chinese male population.
Tu et al 2010. The SLC22A12 gene is associated with gout in Han Chinese and Solomon Islanders.
Tu et al 2010. Associations of a non-synonymous variant in SLCA9 with gouty arthritis and uric acid levels in Han Chinese subjects and Solomon Islanders.
While the exact variants investigated in the current manuscript may not always have been analysed in the studies mentioned above, reference to this work should be made, and the linkage disequilibrium between previously reported variants and those examined in this study reported, and discussed.

Re: Thanks for your suggestions!
I revise the original statement into “most of these SNPs have not been studied in a Han Chinese population.” in page 3 in the revision.

“SNP rs2231142 in ABCG2 were excluded from this study since our group and other domestic institutions have clearly clarified consistent association between this site and gout [27-29, 31, 36].” I have explained this in the Background section in page 7 and Methods section in page 9 in the revision.

We cite other studies which reported associations of variants in the 9 loci (genes)
with gout mentioned above and also report the linkage disequilibrium between them in the Background section in page 6 and 7 in the revision. “Other replication studies from Han Chinese origin also reported additional associations for variants in these loci with gout disease, especially for SLC2A9 [25-27], ABCG2 [28-31] and SLC22A12 [32-34]. LD structure between these variants may differ among different ethnics. Both rs16890979 and rs6855911 were in strong LD with rs734553 in HapMap-CEU (r2=0.957 and 1.0, respectively) and were confirmed to be associated with gout in Europeans [8, 20]. In Chinese population, rs6855911 (in strong LD with rs734553 in HapMap-CHB, r2=1.0) was not identified in gout-control groups but was replicated in high-uric-acid and normal-uric-acid groups [26] and rs16890979 was in much lower LD with rs734553 from HapMap-CHB (r2=0.494). GCKR rs780093 (in strong LD with rs780094 in HapMap-CEU and HapMap-CHB, r2=1.0) was nominally associated with gout in Europeans and Chinese [12, 23]. SLC17A1 rs1165196 (in strong LD with rs1183201 from HapMap-CEU, r2=0.889) was associated with SUA level in whites [8, 12]. However, both rs1165196 and rs1183201 (in strong LD with r2=0.904 in HapMap-CHB) showed no significant difference between the case-control groups [35].”

Introduction
2. This is a study of Han Chinese and the SNPs selected for analysis were based on previously reported associations with serum uric acid. More mention should be made of previous related studies involving Han Chinese.

Notably, a recent GWAS of serum uric acid in 3473 Chinese with validation analyses of 10 SNPs in a further 8830 Chinese individuals should be referred to in more detail. (Yang et al 2014, reference 11).

Other Han Chinese studies include:
Guan et al 2011. Association of an intronic SNP of SLCA29 gene with serum uric acid levels in the Chinese male Han population by high-resolution melting method.
Li et al 2010. Multiple single nucleotide polymorphisms in the human urate transporter (hURAT1) gene are associated with hyperuricaemia in Han Chinese. It would also seem appropriate to mention GWAS related to serum uric acid in other Asian populations (Okada et al 2012 and Kamatani et al 2010).

Re: Thanks for your suggestions!
“Noticeably, a recent GWAS in Chinese population identified two previously reported SUA loci of SLC2A9 (rs11722228) and ABCG2 (rs2231142, rs4148152 and rs3114018, rs4148155), but failed to replicate the remaining loci which were associated with SUA level in Europeans [11].” We cite this in page 6 in the revision.

The studies by Guan et al and Li et al are also cited in the revision as the 26th and 32th reference.
“Besides, another GWAS and meta-analysis both for SUA level in Japanese population also corroborated the three well-known loci of SLC2A9 (rs11722228 and rs3775948), ABCG2 (rs4148155 and rs2725220) and SLC22A12 (rs506338 and rs504915) [13, 14].” We mention these GWAS related to serum uric acid in Japanese (Okada et al 2012 and Kamatani et al 2010) in page 7 in the revision.

Material and Methods
Statistical Analyses
3. The authors state that Hardy-Weinberg equilibrium analyses was evaluated on the control group. This should have been performed in both cases and controls.

Re: Thanks for your suggestion! We recalculate the HWE in both case and control cohorts. The data is shown in Table 3-A in page 25 in the revision.

Results
SNP associations with gout risk
4. A clear explanation of why only 8/9 loci reported by Koltz et al were selected for analyses is required.

Re: Thanks for your suggestions!
“SNP rs2231142 in ABCG2 were excluded from this study since our group and other domestic institutions have clearly clarified consistent association between this site and gout [27-29, 31, 36].” I have explained this in the Background section in page 7 and Methods section in page 9 in the revision.

5. In the section on phenotype details and Table 1 the authors clearly present data showing that the cases were significantly different from controls for a number of phenotypes including gout (i.e. age, BMI, diastolic blood pressure, blood glucose, triglycerides, creatinine and cholesterol). Table 3 presents the association data with p values adjusted for these covariates. As the study is designed as a case control analysis of gout, association analyses for this trait alone should be performed and presented, and then sequential analyses which consider each of the potential confounding co-variates. Evidence needs to be provided which indicates that gout is (or isn’t) the major contributor to the associations. The authors have attempted to address this by performing an analysis of the clinical data with respect to genotype in the control individuals, arguing that medical treatment of the cases is a confounder. However, only a small number of significant associations were found suggesting that they may have over fitted the model for data presented in Table 3 which is adjusted for the multiple co-variates. Further clarification and detail is required.

Re: Thanks for your instructions!
As the study is designed as a case control analysis of gout, association analyses for this trait alone is performed and presented in Table 3-A in page 25 in the revision.
We then perform sequential logistic analyses which consider each of the potential confounding co-variates. “Notably, the logistic regression which considered each of the potential confounding covariates at a time did not change the genetic associations substantially except for uric acid concentrations, indicating that gout was the major contributor to the associations independent of such clinical and biochemical characteristics (shown in Table 3-B).” We add this information in page 11 in the revision.

Besides, “We checked all participants’ questionnaires and confirmed that all cases were first diagnosed with gout on our gout clinic without receiving any relevant medication. Therefore, we decided to conduct this genotype–phenotype analysis in all participants to assess the association of these SNPs with collected clinical characteristics. We found significant association of uric acid concentrations with three gout-related SNPs (rs780094 in GCKR, corrected p=3.94E^-5; rs1183201 in SLCL7A1, corrected p=0.005; rs505802 in SLC22AI2, corrected p=0.003) and of triglycerides with rs780094 (located in GCKR, corrected p=2.96E^-4). The remaining SNPs showed no significant associations with all collected clinical data (shown in Table 3-C). The results provide further evidence indicating that gout is the major contributor to the associations.” We add this information in page 12 in the revision.

Discussion
6. The authors state that the observed association of rs780094 in GCKR with gout is consistent with a New Zealand study (but opposite effect allele) but a German one. They then go on to discuss the possibility that the different population backgrounds may provide an explanation. The recent Han Chinese GWAS of serum uric acid (published in BMC Medical Genomics and therefore I assume that the data is publically available) and the Han Chinese HapMap data provide the authors with the possibility of exploring genomic structure and LD across the region and comparing this with Europeans (HapMap) to generate potentially supportive data for their suggestion at least for these two populations (to my knowledge genomic data for NZ Polynesian populations is not available).

Re: Thanks for your suggestions and instructions!
“Besides, we extracted the genotype data across the gene for Han Chinese and Europeans from the HapMap database and explored the difference of genomic structure and LD between Chinese and Europeans using HaploView software (version 4.2). LD plot for the two populations is shown in Fig. 1. SNP rs780093 in the GCKR gene was in very high LD with rs780094 (r^2=1.0) in both Chinese and Europeans. However, the linkage degree around rs780094 in Europeans was obviously higher than that of Chinese. Therefore, the differences of genetic background in different populations indeed exist and more validation tests with larger sample size are needed across various populations.” We add this analysis
Minor essential Revisions

Introduction

On occasion the translation in to English would benefit from editing/refining.

“With the improvement of living conditions, the incidence of hypertension and gout is increasing rapidly……”

Re: Thanks for your suggestion! “There are extensive data which suggests that the incidence and prevalence of hyperuricemia and gout increased markedly over the past decades worldwide [2-5].” We use this sentence in page 4 in the revision.

“Gout has imparted a considerable financial burden resulting from severe pain and complications……”

Re: Thanks for your suggestion! “Gout is a lifelong disease inflicting a considerable burden of illness upon employers in terms of treatment costs as well as other work-related “benefits” [6].” We use this sentence in page 4 in the revision.

Material and Methods

Participants and Phenotypes.

• While the title indicates that the study was of males only, this should be stated within the methods section.

Re: Thanks for your reminding! “Notably, only males were recruited in this study due to less than 5% of our outpatients were female, not sufficient to conduct a statistical analysis.” We state this in the Methods section in page 8 in the revision.

• Plink is a “gold standard” for genetic association studies. While it was used for the SNP: SNP interaction analyses it is unclear why SHEsis was selected for the association analyses. Plink version 19. Is this correct or should it be 1.9?

Re: Thanks for your reminding!

“All these association analyses were performed using the online software SHEsis (http://shesisplus.bio-x.cn/) [38], a powerful and user-friendly software platform for genetic association analysis. SNP-SNP interactions were conducted using a logistic regression analysis with the second SNP as a covariate also by SHEsis (http://shesisplus.bio-x.cn/) [38].” We revise this in page 10 in the revision. SHEsis is powerful and user-friendly, which was published in Cell research. The result is almost consistent with PLINK version 1.9. Considering SHEsis can provide HWE p value, genotype/allele count and FDR value at the same time, we
list all the output of SHEsis in the manuscript.