Author's response to reviews

Title: Genetic variant I148M in PNPLA3 is associated with the ultrasonography-determined severity of steatosis in a Chinese population

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Author's response to reviews: see over
Dear Editor,

I am pleased to submit the revised manuscript, “Genetic variant I148M in PNPLA3 is associated with the ultrasonography-determined severity of steatosis in a Chinese population” to BMC Medical Genetics for review and possible publication.

In the revision we made important changes according to the reviewers’ suggestions, addressed issues brought up by reviewers, clarified unclear parts, and updated with latest progress in the field. All the changes are marked in red ink. Please find enclosed our point-by-point responses to the concerns raised by the reviewers.

We thank you and reviewers’ energy and time on our manuscript, and look forward to your favorable reply.

Sincerely,

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Reviewer 1:
In this paper, Yiling Li and coworkers evaluated the association between the I148M variant of PNPLA3 and the severity of steatosis in 203 Chinese subjects with nonalcoholic fatty liver disease (NAFLD) with ultrasonographic estimation of liver fat content. They confirmed in this population that the frequency of the 148M variant was higher than in 202 matched controls, and found that the frequency of the 148M variant increased with the severity of steatosis, and that there was a significant interaction between PNPLA3 genotype and BMI in determining ALT levels in patients with NAFLD. They conclude that the I148M PNPLA3 variant is associated with the severity of NAFLD in Chinese population. The study, though severely limited by the ultrasonographic evaluation of liver fat content, and by the fact that the association between the PNPLA3 I148M variant and NAFLD risk in China has already been reported by some of the Authors (ref. 16) and other groups (ref. 15), and the association with the severity of steatosis was confirmed in multiple population by a recent meta-analysis (ref. 18), represents a nice confirmation of the association of the 148M with the susceptibility to steatosis in a Chinese population, and adds interesting novel information on the association with severe steatosis and on the interaction of PNPLA3 genotype with body mass.

Major compulsory revisions:
1. The methodology adopted to quantify liver steatosis by ultrasonography, and inter- and intra-operator variability of the determination, should be reported in details, as this is a key outcome the study.

   We now describe in details the criteria used to quantify the liver steatosis by ultrasonography in the Methods section (the first paragraph, highlighted in red). We also point out all the examinations were performed by the same radiologist on the same ultrasound machine.

   One potential limitation is that there was not a second radiologist to validate the results; however, considering the diagnosis was performed by an experienced radiologist strictly following the established protocol, we believe the results are valid.

2. As “severity of NAFLD” usually denotes the histological severity of liver damage including necroinflammation, hepatocellular damage, and fibrosis, the conclusions should be modified to, e.g. “the PNPLA3 I148M variant is associated with US determined severity of steatosis in a Chinese population”.

   We now use “the ultrasonography-determined severity of steatosis” instead of “severity of NAFLD” throughout the paper.

3. As severity of steatosis was the main outcome of the study, it would be important to know whether there was a significant interaction between PNPLA3 and BMI towards the severity of US determined steatosis.

   We tested the interaction between I148M and BMI on the ultrasonography-determined severity of steatosis using an ordered logistic regression model, as now described in the Methods section. However, the result was insignificant (P-value > 0.10), as reported in the Results section.
4. Discussion: most of the patients with NAFLD included in the study had increased liver enzymes and/or moderate/severe steatosis, accepted indications for liver biopsy; therefore it cannot be stated that biopsy could not be performed for ethical reasons.

We admit it is inappropriate to state that biopsy could not be performed because of ethical reasons. This statement is deleted in the manuscript, and we acknowledge that assessment based on ultrasonography instead of liver biopsy is a major limitation of the study.

However, we also discuss that ultrasonography showed an accuracy of 88% in the diagnosis and staging of fatty liver in the literature, and argue that the association between I148M and the ultrasonography-determined severity of steatosis in this study was unlikely false positive due to technology limitations.

Minor discretionary revisions:
1. It is worth noting that the 148M variant was associated with reduced LDL levels in patients with NAFLD. Can the Authors comment on that based on recent findings by other groups (e.g. Kollerits 2009, Palmer 2012).

We now devote one paragraph in the Discussion section to discuss the association between I148M and lipoprotein metabolism. In particular, we tested association between I148M and LDL levels adjusting for ALT levels, as did in Kollerits et al. (2009), and found the signal became more significant, suggesting this association was not simply a consequence of liver function impairment. We also made a hypothesis that I148M interacts with BMI / body fat distribution on triglyceride levels given that there was no association between I148M and triglycerides either in normal populations (Romeo et al. 2008) or in our NAFLD cases (P–value > 0.10), but there were reports on their association in obese populations (Kollerits et al. 2009; Palmer 2012).
Reviewer 2

Major compulsory revisions:

1. A major concern is that the authors considered disease severity in NAFLD as the degree of steatosis by ultrasonography. This is a major mistake—disease severity in NAFLD only can be assessed by liver biopsy. The “severity of NAFLD” is not studied in this work. At best, grade of steatosis, but even this is not appropriately measured because it is a variable hardly quantified by ultrasound, which is very subjective. In fact, the PNPLA3 variant and disease severity was associated with disease severity (simple steatosis vs NASH) in biopsy-proven patients for the first time in 2009 (cite 24) soon after the first report done by Romeo et al in their GWAS (cite 10).

   We agree that it is inappropriate to take the degree of steatosis by ultrasonography as the severity of NAFLD. We thank the reviewer for pointing out this incorrect use of terms. In the revision, we use “the ultrasonography-determined severity of steatosis” instead of “severity of NAFLD” throughout the paper.

2. The genotyping method should be better described and Hardy-Weinberg equilibrium data provided. In the statistical section, some paragraphs required clarification, in particular, how adjusted p values are calculated. The major outcome is the figure showing that the effect of the gene variant seems to be dependent on BMI, but number of patient should be clearly indicated in each genotype group for every BMI percentile. Owing to the small number of patients enrolled, a false positive is likely; therefore an a priori power estimation should be done.

   The detailed genotyping method is now described in the Methods section.
   The distribution of each genotype in cases and controls (i.e., counts in each category) is now summarized in the first row of Table 2. The method to test HWE—Pearson’s chi-squared goodness-of-fit test—is described in the Methods section, and the result is presented in the Results section. In particular, in controls it was in HWE (P-value=0.59), which guaranteed the genotyping quality, but in cases it was out of HWE (P-value=0.02), which indicated its association with the disease status (Wittke-Thompson et al. 2005).

   We adjusted multiple testing by Bonferroni correction, as we now indicate in the Methods, Results, and Tables sections.
   We now indicate the number of patients in the figure under each bar that represents a genotype group in a quarter of cases stratified by BMI.

   We performed power analysis calculating both power to detect the interaction at the level of 0.05 given data and sample sizes required to detect the interaction with a power of 0.8 at the level of 0.05. In particular, for a continuous trait, we used the method by Luan et al. (2001); for a case-control design we used the method by Lubin and colleagues (1990 & 1999); and for a case-only design we used the method by VanderWeele (2011).

3. In the discussion, again, authors used “severity of the NAFLD” and affirmed wrongly that its association with the variant is inconsistent in the literature. The effect of I148M PNPLA3 variant is one of the strongest and most replicated ever reported for a common variant in any complex disease.
In the revision, we use “the ultrasonography-determined severity of steatosis” instead of “severity of NAFLD” throughout the paper, delete incorrect statements, and rephrase relevant parts emphasizing what we studied was the ultrasonography-determined severity of steatosis.

Minor essential revisions:
1. In the background section some phrases are confusing, i.e. “multiple genes have been associated with NAFLD”. This reviewer assumes that authors referred to GWAS because the list of citations is very limited and there is a number of candidate genes studies also performed. Please add additional evidence or rephrase.

We thank the reviewer for pointing out our neglect of candidate gene studies in the Introduction. Due to existence of many candidate gene studies in the literature, we point audience to a recent review paper by Hernaez (2012). The sentence is now rephrased as “Multiple genes have been reported to be associated with NAFLD by candidate gene studies (for a review see [10]), and, more recently, by genome-wide association studies [11-13]”.