Author’s response to reviews

Title: Association of NTCP polymorphisms with clinical outcome of hepatitis B infection in Thai Individuals

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Author’s response to reviews:

Dear the editors

We appreciate the opportunity to submit a revision of our manuscript entitled “Association of NTCP polymorphisms with clinical outcome of hepatitis B infection in Thai Individuals” (MGTC-D-18-00503) for publication in BMC Medical Genetics. It is gratifying that the reviewers consider our study interesting and contains information of potential importance.

Please find the revised manuscript with changes highlighted in red color and a point-by point response to the reviewers’ comments. Please also see the response to the editor’s comments.

This manuscript is not currently under consideration elsewhere, and all authors have approved the submission of this manuscript for publication in BMC Medical Genetics.

Thank you for considering this revised manuscript. We look forward to your kind reply.

Best regards,

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Point-by-Point Response to the Editor and Reviewers’ Comments

Editor Comments:

Besides the points raised by reviewers, these issues need to be addressed:

1. Clarify if there were any cirrhosis patients (how diagnose made?) among CHB; if so and numbers permitting, sub-group analyses with/without cirrhosis vs. HCC should be performed, and discussed.

Ans. In the non-HCC group, there were 61 patients diagnosed with cirrhosis based on histologic examinations and/or imaging studies (see page 4, first paragraph of Methods, and page 6, second paragraph of Results). Supplement Table1 shows allele and genotype frequencies the studied SNPs between HCC vs. non-cirrhosis and HCC vs. cirrhosis. The additional data have also been discussed (see page 11, second paragraph of Discussion).

2. Table 2 listed results for Spontaneous Clearance vs. Healthy Controls. No justification was provided for this comparison, nor interpretation of its significant results. Clearance and CHB have similar NTCP frequency distribution. Provide an appropriate explanation or consider removing.

Ans. We appreciate the comment. The data between Spontaneous Clearance vs. Healthy Controls showed significant results because the frequencies of GA, GA+AA genotypes and A allele (rs2296651) decreased significantly in the clearance group compared with healthy controls. Although the Spontaneous Clearance and CHB groups had similar NTCP frequency distribution, they both differed significantly compared with healthy controls. These data suggest that the SNP is associated with increased resistance to HBV infection probably by reduced viral entry. However, the SNP might not play a role in host immune clearance against HBV after established an infection (see page 9, first paragraph of Discussion). Together, we consider not removing any data in Table 2.

Reviewer #1

Comments to Author

This interesting article conducted by research group of Pisit Tangkijvanich et al. examines the potential role of NTCP polymorphisms (rs2296651 and rs4646287) in nature course HBV infection as well as the progression of liver disease in a large cohort of HBV patients (n=816) and healthy controls (n=205). The authors found that rs2296651 polymorphism was associated with a decreased risk of susceptibility to HBV infection and the development of HCC. The article is easy to read and represent results. However, I do have some concerns as below.
Major points:

1. The authors need to clarify the definition of spontaneous HBV clearance and to distinguish between this term and occult HBV infection. As the authors stated that HBV DNA (table 1) was not performed in spontaneous HBV clearance subjects, it is hard to distinguish between two categories.

Ans. Spontaneous HBV clearance, an event in the natural history of HBV infection, is defined by the loss of serum HBsAg with or without the appearance of anti-HBs in infected individuals who do not receive any antiviral treatment. (see page 5, first paragraph of Methods). As the review’s comment, it is sometimes difficult to differentiate from those who achieve HBsAg loss but detectable HBV DNA in the serum or in the liver (so-called occult HBV infection). We agree that this is a limitation of our study and it has been mentioned in Discussion (see page 13, second paragraph).

2. Did CHB patients receive antiviral therapy? Several factors that contribute to the development of HCC in chronic hepatitis patients. Was HDV infection checked? Because HDV infection is an important factor in this context

Ans. The minority of non-HCC patients (8.2%) received antiviral therapy at the enrollment. Thus, this factor might not influence the natural history and progression of disease in our cohort. We did not examine the serological testing for HDV because this type of viral hepatitis is very rare in Thailand. However, we have mentioned this limitation in Discussion (see page 13, second paragraph).

3. How many CHB cases exhibit liver cirrhosis (LC)? It is interesting to see the role of NTCP SNPs in development of advanced liver disease by examining the distribution of the genotype/allele frequencies between subgroups of chronic hepatitis B patients including CHB, LC and HCC patients.

Ans. Among CHB patients without HCC, 61 patients were diagnosed with cirrhosis. Supplement Table1 shows allele and genotype frequencies the studied SNPs between HCC vs. non-cirrhosis and HCC vs. cirrhosis. The additional data have also been discussed (see page 11, second paragraph of Discussion).

4. Were HCC patients in this study cohort performed histological modality (biopsy) to confirm the final diagnosis? The authors need to characterize the clinical features of patients with HCC such as HCC staging (BCLC), treatment etc…

Ans. According to current international guideline (reference#14), the diagnosis of HCC is based on tumor characteristics obtained from dynamic CT or MRI studies. In general, biopsy is rarely required for the diagnosis because seeding of tumor in the needle tract can occur in 1-3%. As the
reviewer’s suggestion, we have added patients’ characteristics regarding tumor staging and treatment modalities (see page 6, second paragraph of Results).

5. The author stated that "Survival analysis was determined by using the Kaplan-Meier method and compared with the log-rank test." But in this manuscript, I could not see the corresponding results generated from this kind of analysis please correct.

Ans. We have provided the results of survival analysis in supplement Figure 1.

6. This study tried to evaluate the association between NTCP polymorphism and progression of HBV infection, some concern arise, especially regarding subgroup analysis and multiple tests. Adjusted p values for multiple comparisons should be considered to be calculated and interpreted.

Ans. We appreciate the comment. Adjusted P values for multiple comparisons have been applied in Table 2, 3 and Supplement Table 1 as suggested. Overall, the results were comparable with previous data.

7. In this study, the authors found that in HCC patients (but not in non-HCC patient), the rs2296651 GG (or CC) genotype had significantly increased rate of HBV replication indicated by HBeAg positivity and HBV DNA levels in HCC patients compared to non-GG genotypes. The authors should discuss this point in the discussion section. Did authors analyze the correlation between rs2296651 genotypes with any other liver function parameters (ALT, AST, bilirubin, Albumin, AFP)? Among 305 HCC cases, how many antiviral treatment naive cases were included in this study?

Ans. We have already discussed the reason why HCC patients with non-GG genotypes have decreased HBeAg positivity and viral load. (page 12, third paragraph and page 13 first paragraph of Discussion). In addition, we have analyzed the correlation between rs2296651 genotypes and clinical parameters (Supplement Table 2). In this cohort, 217 (71.1%) patients with HCC received oral antiviral treatment at the enrollment.

Minor points:

In introduction section: line 53, 54. Please clarify in details which mutations mentioned?

Title of figure 1 should be changed

Ans. We have revised the MS in Introduction and Title of Figure 1 as the reviewer’s suggestion.
Reviewer #2:

Comments to Author

In the present study, Chuaypen N. et al., study the association between two polymorphisms (SNP) in the sodium taurocholate co-transporting polypeptide (NTCP) gene with the risk of infection by the hepatitis B virus (HBV) and with the clinical outcome of infection, in a population of Thai individuals. To this end, the authors genotyped these two SNPs in a sample of 1021 individuals; 205 healthy controls; 206 subjects with spontaneous HBV clearance; and in 610 patients with chronic HBV infection (CHB), 305 of them with hepatocellular carcinoma (HCC) and 305 without HCC. The controls are age and gender matched.

The authors describe a lower frequency of both the A allele and the GA + AA genotypes of the SNP rs2296651 in the CHB patients, there being no differences between the CHB and HBV clearance groups. Within the group of CHB patients, those with HCC also showed lower allelic and genotypic frequencies than patients without HCC. The CHB patients with GA + AA genotype showed lower frequencies of positivity for HBeAg and the HCC patients of the same genotype also showed lower levels of HBV DNA.

The study demonstrates an association previously described in other populations, mainly Asian. In this sense, it is not novel. However, given the high frequency of the A allele in the Thai population and the endemic character of HBV infection, the present study is relevant. However, the paper presents some issues that should be explained as they reduce the interest in this work.

Specific comments

Authors showed that PNPLA3 and TM6SF2 polymorphisms were independently associated with NBNC-HCC but not viral related-HCC in Thai populations. However numbers of samples were small and it was well known PNPLA3 and TM6SF2 associated with fatty liver disease. Therefore, they must show how these SNPs were clinical usefulness and need to analyze in detail.

Major points:

1. Much of the possible readers of the journal BMC Medical Genetics, are not experts in the subject of this work so some data should be explained conveniently. For example, it should be explained why there is a percentage of CHB patients who are seronegative for HBeAg, if the positivity for this antigen has been used to define CHB patients (page 5, lane 39). In the same line, the HBV clearance group was defined as negativity for HBeAg (and positivity for HBV core and anti-HBs). However, Table 1 indicates that there is no HBeAg data for this group of individuals. Please correct this.

Ans. We appreciate the comments. HBsAg positivity represents HBV infection, while another viral protein, HBeAg, serves as a surrogate marker of viral replication. For example, HBsAg
positivity represents ongoing HBV infection; regardless of viral load (HBV DNA) in the serum. HBeAg positive usually represents high serum HBV DNA levels. HBV clearance is defined as negativity for HBsAg. We confirm that the results in Table 1 are correct.

2. In the present study, 2 SNPs and several comparisons between groups are analyzed, so a test for multiple comparisons should be considered. The p-values described are sufficiently low so that the results are substantially affected by the application of this test. On the other hand, the manuscript would gain in statistical solidity.

Ans. We appreciate the comments and have re-analyzed the data accordingly by using adjusted P values for multiple comparisons (see Table 2, 3 and Supplement Table 1).

3. The authors state that the whole cohort were not deviated from Hardy-Weinberg Equilibrium (HWE). The authors must provide the p-value for the HWE. The most common causes of HWE departure are errors in the genotyping or association of an allele to a phenotype in a subgroup or in the whole population. In this sense, what was the value of HWE in healthy controls? And in the patients?

Ans. We have provided the P values of both SNPs in this revised MS (see page 7, first paragraph of Results). The P values of rs2296651 and rs4646287 in healthy controls were 0.252 and 0.906, respectively. The corresponding values in patients were 0.770 and 0.411, respectively.

4. The SNP rs2296651 is a polymorphism for which functionality has been studied. The authors should have more emphasis on the description of this functionality in relation to the genetic association detected.

Ans. We have provided more data that emphasize the functional role of this genetic variant in Discussion (see page 12, second paragraph).

5. The authors should discuss why the frequency of allele A for SNP rs2296651 is so high in the Thai population. They should also discuss a possible evolutionary advantage in carriers of the A allele in that geographical area.

Ans. The reason regarding high frequency of A allele in our cohort is not clear. However, the prevalence of HBV infection in Thailand before universal HBV vaccination was extremely high (approximately 8-10%). Thus, it is speculated that such high frequency of the minor allele might display the advantage in conferring resistance to infection in areas with previously high HBV prevalence (see page 10, second paragraph and page 11, first paragraph of Discussion).

6. Although the number of homozygotes for the A allele is very low in both patients and controls to draw conclusions, it is striking that this frequency is higher in CHB patients although the
differences are not significant. That is, there seems to be no protection for this genotype. Have
the authors considered or tested an over-dominant inheritance model? On the other hand, the
formation of homo- and heterodimers for this protein has been described. A possible explanation
for this could be that the polymorphism had a kind of negative dominant effect so that the
heterodimer (G / A) is less functional for the entry of the virus than either of the two homodimers
G / G or A / A.

Ans. We agree with the reviewer that homozygotes for this NTCP variant are not protective to
HBV infection, as already mentioned in other reports. We have analyzed the data and the results
do not support an over-dominant model in our cohort. Whether a negative dominant effect might
play a role in viral entry needs to be further investigated.

7. The authors should discuss the causes of the lack of association of rs4646287 SNP with the
risk of infection, since this association has been described in other populations and rs4646287
SNP seems functional being associated with lower expression of the NTCP gene.

Ans. We have discussed the lack of association of rs4646287 with the risk of infection as
reviewer’s suggestion (see page 9, first paragraph of Discussion).

8 Please correct the SNP of the conclusions. Such SNP has not been analyzed in the present
work!

Ans. We would like to apologize for the mistake. The SNP has already been corrected in
Conclusion.

Minor points:

1. Please avoid the use of non-defined acronyms in the abstract (e.g. HCC), or define it
previously.

Ans. In this revised MS, hepatocellular carcinoma (HCC) has been defined in Abstract.

2. In order to gain clarity, the abstract should contain less numerical information (odds ratio,
95% CI, etc.).

Ans. In this revised MS, odds ratio and 95% CI have been deleted from the abstract.

3. Please indicate if the TaqMan assays were commercial or designed by the authors. In relation
to this, the abstract indicates that the SNPs were analyzed by allelic discrimination. In this case it
would be necessary to indicate the technique used (e.g. "... by using TaqMan probes"). Also,
briefly comment on the genotyping technique since the reference provided is not easily found.
Ans. In this study, we used commercial TaqMan probes as mentioned in a previous report. The abstract and Methods have been modified accordingly (see second paragraph of Abstract and page 5, fifth paragraph of Methods).

4. The aminotransferase levels of Table 1 are not discussed in the text of the manuscript. Please indicate the information provided or delete this data in Table 1.

Ans. In this revised MS, AST and ALT have been deleted from Table 1.

5. The data in Table 1 have been analyzed by means of an ANOVA. However, the use of this test does not appear in the corresponding section on Material and Methods.

Ans. We have added ANOVA test in the Methods.

6. English must be reviewed throughout the manuscript

Ans. Thank you for the recommendation. We have corrected grammar, spelling, and punctuation errors throughout the manuscript.