Author's response to reviews

Title: Glutathione S-Transferase P1 (GSTP1) Gene Polymorphism Increases Age-Related Susceptibility of Hepatocellular Carcinoma

Authors:

Yao-Li Chen (ylchen@cch.org.tw)
Hsin-Shun Tseng (91694@cch.org.tw)
Wu-Hsien Kuo (s010002@csmu.edu.tw)
Shun-Fa Yang (ysf@csmu.edu.tw)
Dar-Ren Chen (darren_chen@cch.org.tw)
Hsiu-Ting Tsai (tsaihsiuting@yahoo.com.tw)

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Author's response to reviews: see over
Dear Dr. Scott Edmunds:

First of all, thank you for the interest in our manuscript. We have revised the manuscript with careful consideration of the reviews. The revised parts have been underlined in the manuscript. Below are our responses to the reviewers' comments:

Reviewer #1:

Question 1: Glutathione S-Transferase P1 (GSTP1) Gene Polymorphism Increases Age-Related Susceptibility of Hepatocellular Carcinoma. Yao-Li Chen et al. This study analyzes the effect of two common SNPs in GSTP1 and GSTA1 Genes with regard to hepatocellular carcinoma (HCC) risk in 102 patients and 386 control subjects. Although the study has some merit, a deep review is required before the manuscript can be accepted.

Answer 1: Thank you.

General comments:

Question 2: In the first place, it should be stated clearly what this study adds to the present knowledge. Authors mention that only two previous studies addressed this problem, although actually at least another one did it (Pharmacogenomics 2007 8(8), 895-899), but they argue that this study is important because “no study has been investigated the association between these two gene polymorphisms and hepatocellular carcinoma in Taiwanese”. Authors should explain why results obtained in Taiwanese subjects might be different of those obtained in other oriental populations, and specifically whether a different genetic background, diet or environmental factors or other risk factors for HCC are present in Taiwan but not in other regions.

Answer 2: Thank you. It was suggested that inactivated or down-regulated GSTP1 and GSTA1 genes could increase genomic damage when individuals were exposed to
carcinogens, and the genetic polymorphisms of these two genes are reported to decrease enzyme activity. We therefore hypothesized that the phase II glutathione S-transferase (GST) genes, GSTP1 and GSTA1, were sensitive marker enzymes for preneoplastic and neoplastic liver cells, and that genetic polymorphisms of these two enzymes could facilitate the susceptibility to and clinicopathological development of hepatocellular carcinoma among Taiwanese because they are highly exposed to environmental carcinogens, as well as hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, and habitual alcohol and tobacco consumption, and genetic polymorphism of GSTP1 and GSTA1 could decrease the function of detoxification when individuals are exposed to those risk factors.

We have explained why the results obtained among Taiwanese subjects are important in the background and discussion section of this revised manuscript. Also, the article in “Pharmacogenomics 2007 8(8), 895-899” is included as a relevant reference in this revised manuscript.

Question 3: The manuscript is too long and it has too many references. In addition, some sentences and arguments are repeated in the introduction and in the discussion section.

Answer 3: Thank you, we have reorganized the manuscript.

Question 4: Writing is careless. Some sentences make no sense (for instance in page 5 “Since liver is the major organ of metabolizing endogenous and exogenous toxicant [32, 33].”) and many words are misspelled (for instance in page 5, polymorphisms). Please revise the text carefully and seek the advice of a native English speaker.

Answer 4: Thank you, we have reorganized this revised manuscript carefully and sought the advice of a native English speaker.

Specific comments for compulsory revisions:

Abstract:

Question 5: Abstract: In the sentence “However, in the younger group, age ≤ 56 years old, the individuals with GG alleles of GSTP1 had 11.68 fold risk (95% CI: 1.4-94.9) of inducing hepatocellular carcinoma…”, the adjusted P value should be included here and in the results section. If, as expected, the P value is not significant after multiple comparison analysis, the last sentence in the abstract (and throughout the text) should be modified.
Answer 5: Thank you. We have added case samples to 177 HCC patients and 386 healthy controls in this revised manuscript, and found that in the younger group, individuals with AG or GG alleles of GSTP1 had a 2.18-fold (95%CI=1.09-4.36; p=0.02) and 5.64-fold risk (95%CI=1.02-31.18; p=0.04), respectively, of developing hepatocellular carcinoma, compared to individuals with AA alleles after adjustment for other confounders.

Question 6: The conclusion cannot be that “GG alleles of GSTP1 gene polymorphism is considered as a factor…” if the P value is not statistically significant.

Answer 6: Thank you, the p value is statistically significant in this revised manuscript.

Introduction:

Question 7: Introduction: The sentence “To our knowledge, only two papers and one papers have been investigated the roles of GSTP1 [34, 35] and GSTA1 [34] gene polymorphisms, respectively, on HCC susceptibility” is wrong. Please include and comment all relevant literature on the topic.

Answer 7: We have reorganized the background (introduction) section of this revised manuscript and included and commented on all relevant literature in the discussion section.

Question 8: The introduction is too long. It can be reduced to one and a half page without loss of relevant information.

Answer 8: Thank you, we have reorganized the background (introduction) section to one and a half page.

Methods:

Question 9: The statistical power of the study, calculated on the basis of the number of participants, allele frequencies and an expected OR of at least 2.0 (alpha 0.05) should be included.

Answer 9: Thank you, we have added “Power and sample size calculations” in the Methods section of this revised manuscript.
Question 10: In the statistical analysis it should be specified how the results were adjusted for multiple comparison testing.

Answer 10: Thank you, we have stated this in the section on statistical analysis.

Results:

Question 11: Authors should explain how the differences in age between patients and controls may bias the findings.

Answer 11: Thank you, we have explained it in the discussion section, because aging is an important risk factor for hepatocellular carcinoma development; however, we controlled this confounding by multiple logistic regression.

Question 12: Page 10, Authors should explain how they calculated the adjusted Odds Ratio (AOR).

Answer 12: Thank you, we have explained in the section on statistical analysis that the odds ratios (ORs) with their 95% confidence intervals (CIs) of the association between genotype frequencies and hepatocellular carcinoma were estimated by multiple logistic regression models, after controlling for other covariates, including age, gender and genotypes for each estimated variable.

Discussion:

Question 13: The sentence “However, GG allele polymorphism of GSTP1 was significantly associated with age-related susceptibility of hepatocellular carcinoma. In the younger group, age ≤ 56 years old, individuals with GG alleles of GSTP1 had 11.68 fold risk (95% CI: 1.4-94.9) of inducing hepatocellular carcinoma” should be modified.

Answer 13: We have modified this sentence.

Question 14: The P values and the statistical power for the comparison should be added here or under Results.

Answer 14: We have added P values in the results and the statistical power for the comparison in the discussion section, respectively.

Question 15: Page 17: Conclusion. Unless a statistically significant P value with a statistical power over 80% is obtained for this comparison, the sentence should be
reformulated.

**Answer 15:** Thank you, we have added P values in the results and the statistical power for the comparison in the discussion section, and based on a 95% confidence interval, a p value of 0.05, and a ratio of cases to healthy controls of 1:2, our sample size has at least 90% power to detect a 2.0-fold risk of gene polymorphisms of GSTP1 and GSTA1 in this revised manuscript.

**Question 16:** The discussion section should be reduced to about one half of its size. Authors should comment their findings, put them into context with other studies and obtain overall conclusions and clinical implications.

**Answer 16:** We have reorganized this revised manuscript.

**Question 17:** Table 1: P values should be included.

**Answer 17:** Thank you, the p values are included in Table 1.

**Question 18:** Table 2: It would be better to view these findings in a graph, including error bars and P values.

**Answer 18:** Thanks, p values are included in all tables, but it's difficult to represent these findings in graphs with error bars because genetic frequencies are categorical variables. However, Table 2 (the sub-group analysis) has been replaced by a brief mention of the results found for the younger group in this revised manuscript.

**Question 19:** Table 3, 4, 5, and 6. Since no statistically significant associations were observed, these tables should be removed and overall findings briefly commented in the text.

**Answer 19:** Thank you, we have removed Tables 3-6 and present the relevant information in a short comment in the text, indicating "data not shown".

**Question 20:** Figure 1: It does not add to the manuscript. It should be removed.

**Answer 20:** We have removed Figure 1.
Reviewer #2:
This is an interesting and quite original report on the possible influence of GSTP1 and GSTA1 polymorphisms on the risk of developing hepatocellular carcinoma. However, some important questions arise after reading the article:

Question 1: Results are negative. The finding of an excess of GSTP1 mutated genotypes among "younger" patients is a post-hoc result and it is not adequately supported: there are very few cases.

Answer 1: Thank you. We have added case samples to 177 HCC patients and 386 healthy controls in this revised manuscript. Based on a 95% confidence interval, a p value of 0.05, and a ratio of cases to healthy controls of 1:2, our sample size has at least 90% power to detect a 2.0-fold risk in gene polymorphisms of GSTP1 and GSTA1 in this revised manuscript. Please see the second paragraph of the discussion section.

Question 2: The proportion of mutated genotypes is greater among "older" than among "younger" controls.

Answer 2: Thank you. The proportion of mutated genotypes was greater among "older" than among "younger" controls, however, the trend of mutated genotypic frequency is consistency in both groups, moreover, the proportion of mutated genotypes was also greater in the case group than in the control group in the younger group. We consider that the significantly different genotypic proportion of wild and mutated genotypes between HCC cases and healthy controls among younger group could provide important information for the mechanism of HCC in different age groups, although it needs further investigation. Please see the second paragraph of the discussion section.

Question 3: The limit of 56 years is arbitrary. The main conclusion of this article is, at least, poorly substantiated.

Answer 3: Thank you. Since the mean (±SE) and median age of our 488 recruited subjects was 56.57±0.68 and 56.0 years, respectively, we classified the individuals aged ≤ 56 years as one subgroup and regarded the individuals aged > 56 years as another subgroup. However, in this revised manuscript, the mean (±SE) and median age of our 565 recruited subjects was 57.36±0.62 and 57.0 years, respectively, so we...
classified the individuals ages \( \leq 57 \) years as one subgroup and regarded individuals aged \( > 57 \) years as another subgroup in this revised manuscript. We have added this illustration in the results section of the revised manuscript. Also, we have reformulated the conclusion conservatively.

**Question 4:** The control group is not age-matched. A control group composed of patients with liver disease matched by age, sex, causal agent and type stage of liver disease should be more adequate.

**Answer 4:** Thank you, your suggestion is what we wanted to attain, but we had some limitation in realizing this goal. Originally, we designed the study based on matched ages between the HCC cases and healthy controls. However, this ideal was difficult to realize, so finally, we adjusted this confounder using multiple logistic regression analysis. We have illustrated the limitations in this revised manuscript.

**Question 5:** There is, at least, another report on this subject: Pharmacogenomics. 2007 Aug;8(8):895-9. Glutathione S-transferases pi 1, alpha 1 and M3 genetic polymorphisms and the risk of hepatocellular carcinoma in humans. Ladero JM, Martinez C, Fernandez JM, Martin F, Garcia-Martin E, Ropero P, Villegas A, Diaz-Rubio M, Agundez JA. Results of this study are fully negative and the study group is larger than that included in this report.

**Answer 5:** The article of “Pharmacogenomics 2007 8(8), 895-899 Pharmacogenomics 2007 8(8), 895-899” is included as a relevant reference in this revised manuscript.

**Question 6:** I think that this study merits to be published, but it needs a revision in deep and it must be substantially shortened. Many data on tables are not relevant because are negative. These tables may be substituted by a short comment in the text indicanting "data not shown".

**Answer 6:** Thank you, we have revised the manuscript, removed Tables 3-6, and presented the relevant information in a short comment in the text, indicating "data not shown".

**Reviewer # 3:**
Major Revision:

**Question 1:** Now, for the epidemiological study publication, there is need large sample size (at least 200 cancer patients). Authors control number is ok but there is need to add more cancer patient samples so that concrete result will come in statistical analysis. When it stratified (stage, grade, age strata etc.) in analysis there is less in number (2, to 8 etc) so the statistical power will not come for the concrete results in logistic regression model. Beside that there is other major limitation related to risk factor information because this cancer is affected by genetic and environmental factors (alcohol, smoking etc.).

**Answer 1:** Thank you, your suggestion is what we wanted to attain, but we had some limitations in realizing this goal; we have illustrated the limitations in this revised manuscript. However, we have added case samples to the 177 HCC patients and 386 healthy controls in this revised manuscript. Based on a 95% confidence interval, a p value of 0.05, and a ratio of cases to healthy controls of 1:2, our sample size has at least 90% power to detect a 2.0-fold risk in gene polymorphisms of GSTP1 and GSTA1 in this revised manuscript. Please see the second paragraph of the discussion section. As well, please consider that we have provided a novel finding, that the GSTT1 gene polymorphism is considered to be associated with hepatocellular carcinoma susceptibility in Taiwanese aged ≤ 57 years.

Minor revision:

**Background**

**Question 1:** Background, line 3, phase II glutathione s-transferases should be change in phase II enzymes such as glutathione s-transferases (GSTP1, GSTA1).

**Answer 1:** Thank you, we have changed it.

**Question 2:** In background, method part, PCR-RFLP full form initial capital letter should be change in small letter.

**Answer 2:** Thank you, we have changed it to lower case letters.

**Question 3:** In background, conclusion is not much clear, re-write again.

**Answer 3:** Thank you, we have re-written it.

**Introduction**
Question 4: Line 14, electrophilic should be change in electrophilic metabolites.

Answer 4: Thank you, we have re-organized introduction section.

Question 5: Line 15, you have written about 2 papers but mention only one, WHY? Re-write this sentence again in effective way.

Answer 5: Thank you, we have re-written the introduction and discussion sections in this revised manuscript.

Material and Method

Question 6: Subject’s part, last line, informed written consent should be change in written informed consent.

Answer 6: Thanks, we have changed it.

Discussion

Question 7: Paragraph 1, is not clear according to your hypothesis because you did not cited the articles related to genetic factor. So you should re-write it.

Answer 7: Thanks, we have re-written it.

Question 8: Para 2, Line 11, should be change in this way: these two studies demonstrate non-significant association.

Answer 8: Thanks, we have re-written it.

Question 9: Line 11-14 is not clear. Rewrite in effective ways.

Answer 9: Thank you, we have re-written it.

Question 10: Line 25 is not clear, after the year old put full stop (.) then write next sentence in scientific way. Change whole manuscript in place of no significantly different into non-significant.

Answer 10: Thanks, we have re-written it.