PEER REVIEW HISTORY

BMJ Open publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form (see an example) and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below. Some articles will have been accepted based in part or entirely on reviews undertaken for other BMJ Group journals. These will be reproduced where possible.

ARTICLE DETAILS

TITLE (PROVISIONAL)	Genetic polymorphism of NFKB1 and NFKBIA genes and liver cancer risk: a nested case-control study in Shanghai, China
AUTHORS	Xiang, Yong-bing; Gao, Jing; Xu, Hong-Li; Gao, Shan; Zhang, Wei; Tan, Yu-Ting; Rothman, Nat; Purdue, Mark; Gao, Yu-Tang; Zheng, Wei; Shu, Xiao-Ou

VERSION 1 - REVIEW

REVIEWER	Lingeng Lu Yale University School of Public Health, USA Research Scientist
REVIEW RETURNED	10-Dec-2013

GENERAL COMMENTS	With a minor revision, the manuscript can be accepted for publication. In this study, Gao and colleagues investigated the associations of inflammation-related genes NFKB1 and NFKBIA variants and liver cancer risk in Shanghai, China using a nested case-control study design. The eligible participants of two well-established cohort studies, the Shanghai Women's Healthy study enrolled during the period of 1996 to 2000, and the Shanghai Men's Health study enrolled during the period of 2002-2006, were included in this study. They selected 8 tSNPs of NFKB1 and NFKBIA using Hapmap, and performed Taqman® SNP assays on these subjects. Unconditional logistic regression models were used in the data analyses. They found 2 variants (one 5'-near region, and another intronic) in NFKB1 increased the risk of liver cancer in a dominant model, and the haplotypes of 2 intronic SNPs in NFKB1 were associated with liver cancer risk in men but not in women. None of tSNPs in NFKBIA was found in association with liver cancer risk. The results are quite interesting and were well presented. However, the quality of the manuscript would have been substantially improved if the issues were addressed as follows: 1. Which kind of Taqman SNP assays did the authors use in the detection of the tSNPs, pre-designed or custom-designed? If the authors used custom-designed assays, please provide the sequences of primers and probes. If they ordered the probes and primers for some tSNPs based on the literature, please cite the references. 2. The finding in this study on the SNP rs28362491 was opposite to the results of the previous reports on Chinese population. It would be expected to explain why this finding differs from these two studies. Also on page 13 lines 30-32, the authors referred 'Cao and his colleagues….'. It should be 'He and colleagues….' or maybe reword as 'A study reported.....'. Otherwise, it will be confusing compared to the reference 29.

	3. Two tSNPs in NFKB1 are associated with increased risk of liver cancer in a dominant model. However, only the heterozygote but not the homozygous mutant was associated with liver cancer risk. The discussion would be expected. 4. One of the significant tSNPs is an intronic variant, and the haplotype of two intronic tSNPs is significant too. A short paragraph or several sentences should be seen in the discussion on what potential mechanisms of these intronic SNPs are underlying the associations. Based on the two recently published literature (Lu et al., Carcinogenesis; 2013;34:2024-30; PMID: 23677070; Lu et al., Carcinogenesis. 2012;33:2119-25; PMID: 22822098), intronic SNPs can affect either DNA or RNA secondary structure, thereby are functional. In addition, the authors could check how the intronic tSNPs examined in this study affect the theoretical RNA secondary structure. Also for the 5'-near SNP, one recently published reference (Heinz et al. Nature, 2013;503:487-92, PMID: 24121437), SNPs in 5'-near region may locate in enhancers, thereby affecting gene function. 5. page 3 lines 34-38, please clarify the sentence. It is confusing. The same problem on page 13, lines 19-21.

REVIEWER	Chao-Wen Cheng Taipei Medical University, Taiwan
REVIEW RETURNED	20-Dec-2013

GENERAL COMMENTS	The statistics of haplotypes analysis in the data are not described fully. With significance or not? Gao and colleagues effects of NFKB1 and NFKBIA gene polymorphisms on genetic susceptibility to liver cancer in a nested case-control study. They found rs28362491 ins/del or del/del and rs230496 AG and GG genotypes were associated with higher risk of liver cancer. In addition, no association was observed between NFKBIA variants and risk of live cancer. Main points 1. There are only five authors listed in the contributors section? Please specifically describe other five authors' contributions. 2. In the current study, the liver cancer cases were defined according to ICD-9, codes of 155.0 (primary malignant neoplasms), 155.1(malignant neoplasms of the intrahepatic bile ducts), or 155.2 (unspecified malignant neoplasms of the liver). In this definition, liver cancer cases with different cell origins were all included; however, the biologic and pathologic characteristics and the clinical course are largely different. For example, in compare with the worldwide incidence, Shanghai had higher incidence of cholangiocarcinoma (7.55/100,000) (Transl Gastrointest Cancer 2012;1:21-32), intrahepatic cholangiocarcinoma will take part of the current cases. Do NFKB1 and NFKBIA gene polymorphisms present on genetic susceptibility in these cancer types? 3. As author mentioned, there are many limitations of the current study include relatively small sample size, unmeasured HBV infection, HCV infection and aflatoxin exposure. However, without detail discussion in the genetic susceptibility and the pathogenesis, the findings are also be limited. 4. Does any statistical significance present in the haplotypes analysis? 5. In addition, this study focused on only two genes involved in

	canonical pathway of NF-κB. The authors did not describe in detail why they only selected these two genes? 6. What are the evidences to support that the presents of HCV infection and aflatoxin exposure are very low in “this” study? Minor suggestions 1. There are about 10 typos in the whole text, for example, page 2, line 41: “No assiciation was observed…”; page 14, line 21: ” … with colonrectal cancer” and etc. 2. Is this sentence correct? In page 8, line 33: “Carriers of rs230525 AG or GG genotypes had about 30% percent increased risk of liver cancer, but the risk was not insignificant.”

VERSION 1 – AUTHOR RESPONSE

In this study, Gao and colleagues investigated the associations of inflammation-related genes NFKB1 and NFKBIA variants and liver cancer risk in Shanghai, China using a nested case-control study design. The eligible participants of two well-established cohort studies, the Shanghai Women’s Healthy study enrolled during the period of 1996 to 2000, and the Shanghai Men’s Health study enrolled during the period of 2002-2006, were included in this study. They selected 8 tSNPs of NFKB1 and NFKBIA using Hapmap, and performed Taqman® SNP assays on these subjects. Unconditional logistic regression models were used in the data analyses. They found 2 variants (one 5’-near region, and another intronic) in NFKB1 increased the risk of liver cancer in a dominant model, and the haplotypes of 2 intronic SNPs in NFKB1 were associated with liver cancer risk in men but not in women. None of tSNPs in NFKBIA was found in association with liver cancer risk. The results are quite interesting and were well presented. However, the quality of the manuscript would have been substantially improved if the issues were addressed as follows:

1. Which kind of Taqman SNP assays did the authors use in the detection of the tSNPs, pre-designed or custom-designed? If the authors used custom-designed assays, please provide the sequences of primers and probes. If they ordered the probes and primers for some tSNPs based on the literature, please cite the references.

Answer: Thanks for the comments.
All of the probes and primers in this study were ordered from Applied Biosystems by life technology, Foster City, CA, USA. Among the 8 SNPs, rs28362491 was custom-designed and other 7 SNPs were pre-designed. The sequences of custom-designed were listed in the following table.
Assay name Forward Primer Sequence Reverse Primer Sequence Reporter 1 Sequence Reporter 2 Sequence
rs28362491 GCCTCCGTGCTGCCT AGGGAAGCCCCCAGGAA TTCCCCGACCATTGG CCGACCATTGATTGG
For pre-designed assays, they didn’t provide sequences of primer and probes. We also contacted the technical supporter of ABI and they said they had never offered this kind of service. So we did not provide the sequences of primers and probes in the section of material and methods.

2. The finding in this study on the SNP rs28362491 was opposite to the results of the previous reports on Chinese population. It would be expected to explain why this finding differs from these two studies. Also on page 13 lines 30-32, the authors referred ‘Cao and his colleagues….’. It should be ‘He and colleagues….’ or maybe reword as 'A study reported.....'. Otherwise, it will be confusing compared to the reference 29.

Answer: Thanks for the question and correction.
Actually, we have discussed this issue in the manuscript, which could be found in the third paragraph

of the discussion section.
As Cao was the corresponding author of the cited reference, so we used his name in the previous manuscript. And we have modified this sentence to “He and his colleagues……” as your suggestion. Thank you.

3. Two tSNPs in NFKB1 are associated with increased risk of liver cancer in a dominant model. However, only the heterozygote but not the homozygous mutant was associated with liver cancer risk. The discussion would be expected.

Answer: Thanks for the comments.
We have noticed this issue when we did the analysis and this might resulted from the relatively small sample size of the study, especially in the homozygous group. Compared to the heterozygote group, the frequency of corresponding homozygous group was much lower which might therefore reduce the statistical power. In the original manuscript, we have discussed this limitation and the potential influence to our study in page 14 lines 52~57.

4. One of the significant tSNPs is an intronic variant, and the haplotype of two intronic tSNPs is significant too. A short paragraph or several sentences should be seen in the discussion on what potential mechanisms of these intronic SNPs are underlying the associations. Based on the two recently published literature (Lu et al., Carcinogenesis; 2013;34:2024-30; PMID: 23677070; Lu et al., Carcinogenesis. 2012;33:2119-25; PMID: 22822098), intronic SNPs can affect either DNA or RNA secondary structure, thereby are functional. In addition, the authors could check how the intronic tSNPs examined in this study affect the theoretical RNA secondary structure. Also for the 5’-near SNP, one recently published reference (Heinz et al. Nature, 2013;503:487-92, PMID: 24121437), SNPs in 5’-near region may locate in enhancers, thereby affecting gene function.

Answer: Thank you so much for the helpful comments.
According to your suggestions, we have added several sentences in the discussion section to address this issue, which could be found in the third paragraph of discussion section. Please find from the sentence “In addition …” to the end of this paragraph.

5. page 3 lines 34-38, please clarify the sentence. It is confusing. The same problem on page 13, lines 19-21.

Answer: Thank you so much for the corrections.
For page 3 lines 34-38: We have modified the sentence to “NF-κB, a collection of dimeric transcription factors, was originally identified as a nuclear factor bound to the enhancer of the immunoglobulin κ-light chain gene specific to B cells and presents in all cell types.”
For page 13, lines 19-21: We have modified the sentence to “This gene encodes for two proteins-p105 and p50. p105 is a none-DNA binding protein and is activated to p50, a DNA binding protein by proteasome-mediated degradation.”

Authors’ Responses to Reviewers’ Comments of Chao-Wen Cheng

Gao and colleagues effects of NFKB1 and NFKBIA gene polymorphisms on genetic susceptibility to liver cancer in a nested case-control study. They found rs28362491 ins/del or del/del and rs230496 AG and GG genotypes were associated with higher risk of liver cancer. In addition, no association was observed between NFKBIA variants and risk of live cancer.

The statistics of haplotypes analysis in the data are not described fully. With significance or not?

Answer: Thanks for the question.
For NFKB1 haplotypes, rs230525-rs230530 GA or AA haplotypes were significantly associated with liver cancer in men. For NFKBIA, none of the haplotypes were associated with liver cancer.

Main points
1. There are only five authors listed in the contributors section? Please specifically describe other five authors' contributions.

Answer: Thanks for the question.
In acknowledgement, please see sentence of "All authors critically reviewed and approval manuscript." which means all authors reviewed the paper and contributed their comments or suggestions, including other authors you mentioned.

2. In the current study, the liver cancer cases were defined according to ICD-9, codes of 155.0 (primary malignant neoplasms), 155.1(malignant neoplasms of the intrahepatic bile ducts), or 155.2 (unspecified malignant neoplasms of the liver). In this definition, liver cancer cases with different cell origins were all included; however, the biologic and pathologic characteristics and the clinical course are largely different. For example, in compare with the worldwide incidence, Shanghai had higher incidence of cholangiocarcinoma (7.55/100,000) (Transl Gastrointest Cancer 2012;1:21-32), intrahepatic cholangiocarcinoma will take part of the current cases. Do NFKB1 and NFKBIA gene polymorphisms present on genetic susceptibility in these cancer types?

Answer: Thank you so much for the comments.
In genetic studies and other small epidemiological studies, ideally, we should confine our cases to certain pathological types because cancers originated from different cells might have different characteristics and causes. But this is very difficult for the large-scale, population-based study.
In our study population, 61.3% of the cases were primary malignant neoplasms, 8.7% were malignant neoplasms of the intrahepatic bile ducts and 30.0% were unspecified malignant neoplasms of the liver. And unfortunately, the proportion of liver cancer cases with pathological diagnosis was below 20% in the studied population. So we could not analyze the data in subgroups.
Furthermore, in China (mainland), the proportion of pathological diagnosis is much lower which you can see in some publications of liver cancer incidence from China cancer registration data. Our study is a population-based cohort study, not a specific genetic research, so we did not focus on the specific cell type of liver cancer. If so, the sample size will be so small. If possible, we may consider it in future after long time follow-up of our cohorts.

3. As author mentioned, there are many limitations of the current study include relatively small sample size, unmeasured HBV infection, HCV infection and aflatoxin exposure. However, without detail discussion in the genetic susceptibility and the pathogenesis, the findings are also be limited.

Answer: Thank you so much for the comments.
We totally agreed with your comment that our findings are limited and we also tried our best to minimize the possible influence of these limitations. For example, we designed a nested case-control study with case to control ratio of 1: 2 to improve the statistical power. Furthermore, we adjusted for the participants' history of hepatitis and liver cirrhosis in the statistical analysis.
Although the underlying mechanisms of the studied SNP and liver cancer susceptibility are still obscure, our findings could still provide some suggestions and additional evidence that genetic variations in NFKB1 gene might play a role in liver cancer susceptibility.

4. Does any statistical significance present in the haplotypes analysis?

Answer: Thanks for the question.

For NFKB1 haplotypes, rs230525-rs230530 GA or AA haplotypes were significantly associated with liver cancer in men. For NFKBIA, none of the haplotypes were associated with liver cancer. We have added further description in the last paragraph of the result section.

5. In addition, this study focused on only two genes involved in canonical pathway of NF-κB. The authors did not describe in detail why they only selected these two genes?

Answer: Thanks for the question.
Please see the following reasons for the selection of these two genes in our study.
The major reason is based on the contributions of the genes involved in NF-κB signaling pathway (which could be found in the 3rd paragraph of introduction section).
Among the seven members in the NF-κB family, the major form of NF-κB is a heterodimer of the p50 and p65/RelA subunits and P50 is encoded by NFKB1 gene. Of the three inhibitors (IκBα、IκBβ、IκBε) of NF-κB, the IkBα protein encoded by NFKBIA is the main regulator of NF-κB activation through conjugation with the NF-kB protein in cytoplasmic sequestration and inhibition of its transcriptional activation.
Then, our liver cancer study was in combination with several parts of the aims except for above SNPs part. So based on our limited funds we got , we could not study all the genes involved in NF-κB signaling pathway which may be considered next.

6. What are the evidences to support that the presents of HCV infection and aflatoxin exposure are very low in “this” study?

Answer: Thanks for the question.
As we known that in China, currently, the role of HCV in the development of liver cancer is relatively minor. We provided a reference [Yuan JM, et al.1995] in the manuscript to support that the present of HCV infection was very low in our study because this study was conducted in urban Shanghai and had the same source population with us. In the cohort study, Dr. Yuan JM and his colleagues tested the anti-HCV in serum and results showed that the only 1 in 76 patients with liver cancer and 1 in 402 control subjects in Shanghai, China, were positive for anti-HCV in prediagnostic serum. In another paper published later with the same population and larger sample size, the positive rate of HCV was 1.3% in liver cancer and 0.2% in control [Yuan JM, et al.2006]. Therefore the role of HCV infection in liver cancer development in this study population was not as that from HBV infection.
Yuan JM, Ross RK, Stanczyk FZ, et al. A cohort study of serum testosterone and hepatocellular carcinoma in Shanghai, China. Int J Cancer 1995; 63: 491-3.
Yuan JM, Gao YT, Ong CN, et al. Prediagnostic level of serum retinol in relation to reduced risk of hepatocellular carcinoma. J Natl Cancer Inst 2006; 98:482-90.

Regarding the aflatoxin exposure, although Yuan’s study found a positive association, we can still infer that the contribution of aflatoxin exposure to liver cancer is limited in this population based on the following evidence. In order to decrease the aflatoxin contamination in food, the Chinese government took several effective measures since 1980s including: improvement of storage methods to protect food from mildew, updating of the extracting process of oil to reduce the aflatoxin level, development of food safety standards to control the food quality and increased publicity of healthy lifestyles [Wang RK, et al.1981, Chen JG, et al. 1995, Ren JS, et al. 2008]. Wang et al. reported the exposure of dietary aflatoxin in urban residents was lower than rural residents and the level in urban residents was much lower than ever [Wang J, et al. 2007, Yeh FS, et al,1986]. Moreover, with tremendous economic progress and increased health knowledge, people paid more attention to the quality and safety of food therefore substantially reduced the intake of aflatoxin.
Wang RK. Progress in liver cancer research in China. Journal of Medical Research 1981; 6:1-6
Chen JG, Zhu YR. Current status and perspectives in liver cancer prevention. Journal of Medical Research 1995, 24:1-5.

Ren JS, Qiao YL. Recent advance in risk factor and prevention for primary liver cancer. China Cancer 2008; 17: 293-296.
Wang J, Liu XM. Assessment of dietary aflatoxins exposure in Chinese residents. Chin J Food Hygiene 2007; 19:238-40.
Yeh FS, Shen KN. Epidemiology and early diagnosis of primary liver cancer in China. Adv Cancer Re. 1986; 47:297-329.

Although HCV infection and aflatoxin exposure are very important for liver cancer in certain population, they are not the main concerns of this study now, so they were not described in detail in the manuscript. But we agree with you, especially for the contribution of HCV infection on the development of liver cancer, which needs to pay more and more attentions in the future in Shanghai (or China).

Minor suggestions
1. There are about 10 typos in the whole text, for example, page 2, line 41: “No assiciation was observed…”; page 14, line 21: ” … with colonrectal cancer” and etc.

Answer: Thank you so much for the corrections. We are sorry for that and corrected them all.

2. Is this sentence correct? In page 8, line 33: “Carriers of rs230525 AG or GG genotypes had about 30% percent increased risk of liver cancer, but the risk was not insignificant.”

Answer: Thanks for the question. The sentence is correct.

VERSION 2 – REVIEW

REVIEWER	Lingeng Lu Yale University School of Medicine, Yale School of Public Health
REVIEW RETURNED	14-Jan-2014

GENERAL COMMENTS	It is acceptable but need revision (as in the comments to the authors) by the authors before accepted. Thank the authors for their efforts to address the concerns I raised in the manuscript. Most of my concerns have been addressed appropriately. Two questions, I think, need further to take care. 1. In the response to Q1, the authors stated they used the pre-design and custom-design probes and primers for the genotyping. However, this information was not included in the revised manuscript. The authors should include the information of which one(s) is pre-designed, which one is custom-design. For the custom design, probe and primers sequence should be provided in the materials and methods of manuscript. 2. In the response to Q5, page 3 lines 34-38, the rewording is okay although it is not very clear. However, the sentence on page 13 line 19-21 is not okay at all. Still confusing. The authors may have misunderstood references. Actually, NFkB encodes p105, a precursor of p50. p105 is an inactive form, which has to be activated via degradation to active form p50 by proteasome. p50 is a DNA bidnign subunit of the NFkB protein complex. (NFkB only encodes p105, but not two proteins of p105 and p50). Please reword this sentence.

REVIEWER	Chao-Wen Cheng

	Graduate Institute of Clinical Medicine, Taipei Medical University, Taipei, Taiwan
REVIEW RETURNED	26-Jan-2014

- The reviewer completed the checklist but made no further comments.

VERSION 2 – AUTHOR RESPONSE

Authors' Responses to Reviewers' Comments of Lingeng Lu

Thank the authors for their efforts to address the concerns I raised in the manuscript. Most of my concerns have been addressed appropriately. Two questions, I think, need further to take care.

1. In the response to Q1, the authors stated they used the pre-design and custom-design probes and primers for the genotyping. However, this information was not included in the revised manuscript. The authors should include the information of which one(s) is pre-designed, which one is custom-design. For the custom design, probe and primers sequence should be provided in the materials and methods of manuscript.

Answer: Thanks again for the suggestion.
We have added the required information under your suggestion which could be found in the paragraph titled "Genotyping" from "rs28362491 was genotyped……" to "……using pre-designed assays (Applied biosystems)." (see: pages 5 and 6).

2. In the response to Q5, page 3 lines 34-38, the rewording is okay although it is not very clear. However, the sentence on page 13 line 19-21 is not okay at all. Still confusing. The authors may have misunderstood references. Actually, NFkB encodes p105, a precursor of p50. p105 is an inactive form, which has to be activated via degradation to active form p50 by proteasome. p50 is a DNA bidnign subunit of the NFkB protein complex.
(NFkB only encodes p105, but not two proteins of p105 and p50). Please reword this sentence.

Answer: Thanks a lot for the correction.
I am so sorry for the confusing expression of the sentence, and grateful thanks for your detailed explanation, that was exactly what we intended to express. We have modified the sentence to "This gene encodes for p105 which is a none-DNA binding protein. As an inactive precursor, it was activated to p50, a DNA binding protein by proteasome-mediated degradation." (see: page 13)