Author's response to reviews

Title: Diet Folate, DNA Methylation and Genetic Polymorphisms of MTHFR C677T in Association with the Prognosis of Esophageal Squamous Cell Carcinoma

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Author's response to reviews: see over
Dear editors and reviewers,

Thanks a lot for the comments on our manuscript entitled with “Diet Folate, DNA Methylation and Genetic Polymorphisms of MTHFR C677T in Association with the Prognosis of Esophageal Squamous Cell Carcinoma”. We carefully read and revised it again. Here are the responses.

**Reviewer: Karen Curtin**

Minor essential revisions:

1. Experiments section of Methods and throughout, human gene names should appear in italicized font; also include the dbSNP reference SNP id number (rs#) for the C677T polymorphism.

   Answer: In the revised version, we added the reference number (rs1801133) for the SNP of MTHFR C677T. The font of gene names has been expressed in italic throughout the manuscript.

2. In the Statistical Analysis paragraph of the Methods, the authors state they examined differences between subjects in folate intake categories and survival using Kaplan-Meier curves and Log-rank test. Were the K-M curves/survival times significantly different in Figure 1 between high, moderate, and low intake based on the log-rank test? Please state in the results. It appears survival may be significantly higher for those with high intake compared to moderate and low, but that moderate did not differ significantly from low; the authors might consider comparing high intake to moderate and low intake combined.

   Answer: Figure 1 illustrates the KM survival curves for patients in the prognosis of esophageal squamous cell carcinoma. According to the reviewer’s suggestion, in the revised version we graphed the curves by comparing high intake of folate to moderate and low intake combined. Furthermore, in the part of the results, we stated the P value based on the Log-rank test.

3. In the Methods and/or Results, the authors need to describe in more detail how aberrant DNA methylation was determined.

   Answer: In the revised version we briefly described how the aberrant DNA methylation was determined.

   “The CpG island methylation at the promoter region of P16, MGMT and hMLH1 was
determined by methylation specific PCR (MSP) after sodium bisulfite modification of DNA. In brief, genomic DNA was incubated with NaOH at 37°C for 10 minutes and then treated with freshly prepared hydroquinone and NaHSO₃. Samples were incubated under mineral oil at 50°C for 16 hours. Modified DNA was purified and eluted with 50ul preheated TE solution. Modification was completed by the treatment of 5.5ul 3M NaOH (final concentration 0.3 M) for 15~20 minutes at room temperature. DNA was precipitated by ethanol and resolved in TE. The CpG island methylation at the promoter region of P16, MGMT and hMLH1 genes was assessed by MSP using methylation-specific primer and relevant annealing temperature. A methylation positive DNA control sample was made in vitro by SssI methylase (New England Biolabs). PCR products were loaded onto 3% gels, stained with ethidium bromide, and directly visualized under UV illumination.”

4. Discussion section: Is it possible that those patients in the highest category of intake also exhibited other healthy behaviors (no tobacco use, no or low alcohol); do the aHRs in Table 4 differ substantively if the hazard ratios are also adjusted for alcohol or tobacco use?
Answer: In this study, no significant association was found between smoking or drinking and the prognosis of ESCC after esophagectomy (as showed in Table 1). The aHRs weren’t different substantively if the hazard ratios were also adjusted for alcohol or tobacco use, thus we didn’t regard them as the confounders in the COX regression model.

5. The authors should tone down their assertion, which the data from a small study provide suggestive evidence of but do not clearly support, that “This finding indicates that the role of genetic variants in the carcinogenesis depends on the dose of folate intake” to more clearly state that ESCC prognosis may be influenced by folate intake in relation to MTHFR C677T genotype.
Answer: We revised the statement as the reviewer suggested.
“This finding indicates that ESCC prognosis may be influenced by folate intake in relation to \textit{MTHFR C677T} genotypes”.

6. Cut-points and units (e.g. micrograms) for tertile of folate intake should be
explicitly stated in the Results or alternately a footnote in Table 4. Also, please state explicitly if there is a government-mandated folate fortification of processed foods similar to the United States.

**Answer:** In Table 4, we added a footnote to state cut-points and units for tertile of folate intake. “The cut point of folate intake is tertile (µg/d). Low: <30.3; moderate: 30.3-95.4; high:≥95.5”.

No programs of government-mandated folate fortification of processed foods have been performed in the study site.

Discretionary revisions:
1. In the Experiments section of the Methods, please describe the methylation at the promoter regions in more detail, e.g. CpG island methylation.
   **Answer:** We corrected it in the revised version.

2. In the Statistical Analysis section, the authors define time of survival as time between diagnosis and death; was this death from all causes, or primary cause of death esophageal cancer? Please explicitly state and which and if all cause mortality, indicate if any patients died of a primary cause other than cancer.
   **Answer:** We explicitly stated it in the revised version.

   “The primary death of esophageal cancer was defined as the failure event and the time of survival was the time between diagnosis and death. The cause of death was defined by specialists based on the clinical documents and reports by patient’s family members. If the patient died of other causes rather than esophageal cancer, he/she was censored at the date of death.”

3. Results section, paragraph 2, rather than describe single or unmarried patients as “sole patients,” it might be clearer to describe them as “Those patients divorced or living alone.”.
   **Answer:** We corrected it in the revised version.

4. Results section, paragraph 2, authors state that median survival was >4.59 years for high folate consumption. Does the > sign indicate the majority these patients were alive at the end of the follow up period, given the maximum follow up was 4.66 years.
   **Answer:** The median survival time was estimated by the STATA software. For
patients with high folate intake, more than half of them were alive till the end of last follow-up and exact MST couldn’t be estimated. Based on the longest survival time of patients in this group, we stated that the median survival time was >4.59 years for high folate consumption.

5. How do the authors see their findings influencing future research, i.e. a randomized clinical trial of folate supplementation in pre/post-esophagectomy patients? It would strengthen the manuscript if the authors more fully address the impact of their findings in terms of future studies, either planned or suggested.

Answer: In the part of Conclusion, we added “A randomized clinical trial of folate supplementation among post-esophagectomy patients is suggested to measure the effect of supplement use of folate.”.

Minor issues not for publication: Authors should correct numerous minor typographical and grammatical errors throughout their manuscript. Some examples: In the Background, paragraph 2 “More and more evidences have indicated..” should read “More and more evidence has indicated..”. Methods paragraph, “All patients were followed by staffs of Yangzhong Cancer Research Institute..” should read “All patients were followed by staff of Yangzhong Cancer..”. Results, paragraph 1, “Among 125 ESCC patients, 5 were loss to follow up..” should read “..lost to follow up..”. Discussion, last paragraph, “This finding indicates that the role genetic variants..” should read “..role of genetic variants..”.

Answer: We corrected these errors in the revised version.

Reviewer: Suminori Kono

Compulsory comments
1. The study subjects were 80% of ESCC patients who underwent the surgery. For what reasons, were 20% of the eligible patients excluded. What were the eligibility criteria? They may be described in reference 13, but should be briefly explained.

Answer: We briefly explained the inclusion and exclusion criteria in the revised version.

“The eligible cases were Han Chinese living in Yangzhong for more than five years, with pathologically confirmed ESCC and informed consent. The recrudesced cancer patients or cases with the secondary ESCC from a primary cancer located elsewhere
were excluded.”

2. It is described in the Discussion (page 11) that dietary intake of folate at the time of 1-2 years before the diagnosis was ascertained. This should be described in the Methods section.

Answer: In the Methods section, we described such information.

How many food items were included in the FFQ? Describe validity and reproducibility of estimated folate intake if such information is available.

Answer: The FFQ we used in the present study was referred to NIH questionnaires and modified according to Chinese food items and cooking habits. It has been applied in several studies conducted by Harvard School (USA) and Nanjing Medical University (China).

The following table is an example of the FFQ we used in this study.

<table>
<thead>
<tr>
<th>Food items</th>
<th>How long do you eat per year (months)</th>
<th>How often (times)</th>
<th>How much do you eat each time (gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>/year /month /week /day Never</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

What is strange, only fruit folate was used in the study (page 11). Such information should have been described in the Methods.

3. If only fruit folate was used, the protective association was probably due to high fruit intake, which may be an indicator of socioeconomic status or better care after the surgery.

Answer: FFQ has been widely used to measure dietary folate and has been proved to be valid and economic in western countries. However, as the diet habits are significantly different between China and western countries, the application of FFQ in Chinese population is facing some difficulties. (1) The recipe in China is very complex compared to that in western countries. So it is difficult to recall and calculate the type and amount of each food item. (2) As we know, the main sources of dietary folate are fruits, vegetables, legumes, cereals, and liver [1]. The folate is susceptible to
heat, pH, and oxidation. Incorrect cooking methods would influence the actual intake of folate. Estimated folate intake from total consumed food using FFQ may not accurately reflect the true intake of total dietary folate [1]. According to the traditional habits in Yangzhong County, people like to fry vegetables under higher temperature or boil it for a long time which will destroy the folate easily. It might not be appropriate to calculate folate intake by summarizing all consumed food. So, in this study, we try to use folate from fresh fruits for the indicator of total dietary folate intake. Some evidences from other studies also support it. For example, Flood [2] reported that there existed a significant correlation between fruit intake and folate intake. With the increase of fruit intake, the folate level also increased. In the present study, we used fruit folate values to estimate the total folate intake. It may not reflect the truth and more studies are needed to prove the association between high dose folate and protective effects of prognosis after esophagectomy.

4. Five patients were lost to follow-up, and they were excluded. They should have been included and treated as censored cases in the analysis.
Answer: Five patients were lost to follow-up since they left hospitals after surgery. They have been treated as censored cases, but gave no contribution to the length of survival time.

5. This reviewer is not well informed of the effect of folate on cancer survival in general. The authors imply that such information is almost null. Please describe briefly results for other sites of cancer, if any.
Answer: We added some information about the effects of folate on other sites of cancer.
“The role of dietary folate in cancers is still controversial and the studies on the relation between folate intake and ESCC in Chinese population were scarce till now. Only several studies reported that dietary folate was negatively associated with the risk of endometrial cancer, breast cancer and rectal cancer. However, few concerned their effects in the prognosis of cancers. High folate intake has found to be significantly related with better survival of patients with advanced gastric cancer who were treated with first-line fluorouracil-based chemotherapy”.

6. The hazard ratio was adjusted for TNM stage (Table 4). What categories of the
stage were used in the adjustment? Was it dichotomous as used in Table 2?
Answer: There was only one patient with M1 stage, thus we didn’t adjust HR for M stage. T and N stage were regarded as dummy variables and used in the adjustment.

7. English can be understood, but is not grammatically correct in many places.
Consult a language expert.
Answer: We asked a language expert to help edit this manuscript.

References