ANSWERS FOR REVIEWERS

March 25, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 9161-revision.doc).

Title: Lower serum folate is associated with development and invasiveness of gastric cancer

Author: Teng-Yu Lee, En-Pei Chiang, Yin-Ting Shih, Hsien-Yuan Lane, Jaw-Town Lin, Chun-Ying Wu

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 9161 (invited manuscript: ID 00058438)

The manuscript has been improved according to the suggestions of reviewers:
1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

Reviewer (No. 7096):

(1) This is useful research which addresses an important topic. The work uses a NCC design from a cohort that the authors have previously used with success. The study design has important limitations, and the authors address these clearly and fairly. Their conclusions are not overstated. Though not likely available at the time of submission, it might be noted that their results appear to conflict with Xiao Q et al BJ Cancer Jan 2014. The authors may wish to address this manuscript as a minor revision.
Answer: Thanks for your comments. As the interesting issue mentioned by the reviewer, although increasing evidence suggests low folate status is involved in the development of gastric cancer, the results of studies on dietary folate intake and risk of gastric cancer are inconsistent. This phenomenon may be explained by a variety of conditions may affect the processes of folate absorption and metabolism. For examples, complex host-environment interactions, defects in the uptake system, gastrointestinal diseases, gene polymorphism for folate metabolism, or rapid folate consumption. We have addressed this issue in the discussion of this manuscript (page. 15, line 23 to page. 16, line 15), and the content of discussion is enriched by the recent cohort study (Xian Q, et al. Br J Cancer. 2014 Mar 4;110(5):1328-33).

Reviewer (No. 11431):

(1) In the Core Tip, the authors wrote that this is a case-control study, but from the other part of the ms, it seems a nested case-control study. Since the author did not indicate in the Material and Methods when the blood samples were collected in this study, it is not clear whether this is a case-control study or nested case-control study. Please state the experiment design clearly.
Answer: Thanks for your reminding. The controls in this study were not recruited from a subset of the cohort, so we considered that this was a case-control study. We apologized for the confusion in the manuscript, and we have clearly revised the statements of experiment design in the Materials and Methods section (page. 9, line 1-21).

(2) The authors reported that low serum folate level is associated with increased gastric cancer risk. This is consistent with a previous Chinese study, but contrary to a European study. The authors considered that this discrepancy could be resulted from the timing of blood collection. Is there

any other factor such as the different SNP genotype of folate metabolic enzymes between Chinese and European populations may be involved?
Answer: Thanks for your reminding. Other risk factors may be also involved in the difference between Chinese and European populations. For example, in a recent meta-analysis (Int J Cancer. 2012;131(9):2103-16), the gene polymorphism in thymidylate synthase, an important enzyme involved in folate metabolism, was associated with an increased risk of gastroesophageal cancer among Asians, but not among Caucasians. However, the interaction between other risk factor and blood folate in different populations need further evaluation. We have enriched the discussion of this discrepancy according to your suggestion (page. 14, line 21 to page. 15, line 4).

(3) The authors should indicate the parameters which have been adjusted for when estimating the OR in table 3 and 4.
Answer: We have indicated the adjusted parameters that are age and gender for the ORs in the tables as your recommendation (page. 26, 27).

(4) To access the association between serum folate levels and invasiveness of gastric cancer, the authors set a cutoff value (?2.61) to divide the study subjects into two groups. Did they ever compare the mean level of serum folate between the patients with invasive and non-invasive gastric cancer?
Answers: In the patient cohort of gastric cancer of this study, we considered that multivariate logistic regression analysis may be a better way to study the association between serum folate levels and invasive phenotypes, and the cutoff values in various clinicopathological features should be different. Thus, we used ROC analysis to calculate the best cutoff values of serum folate, and the differences in serum folate levels between invasive and non-invasive phenotypes could be well presented.

Reviewer (No. 54951):
(1) Given the number of limitations of the present study, authors should interpret more cautiously their findings. For example, several future studies should confirm the present findings before authors can conclude that serum folate is an important biomarker for gastric cancer.
Answer: Thanks for your comments. Due to limitations in this association study, we have reviewed and revised related statements in the manuscript as your recommendation (page. 15, line 14-20), and softer conclusions were made (page. 18, line 2-6).

(2) Description of methods lacks of important data, including the period of enrolment of both cases and controls, how cases and controls were selected (are those all consecutive patients?), etc.
Answer: Thanks for your reminding. Related statements have been added in the description of Materials and Methods section as your recommendation (page. 9, line 1-21).

(3) Follow-up and survival analysis should be carefully described in the methods section.
Answer: Thanks for your reminding. Related statements have been added in the description of Materials and Methods section as your recommendation (page. 4, line 17 in the Abstract, page. 9, line 17-18 in the Study subjects and page. 11, line 3-5 in the Statistical analysis).

(4) How is that h-pylori infection, the most important risk factor for gastric cancer, is not associated to the risk in this case-control study? This should be further discussed.
Answer: Thanks for your reminding. We have revised and enriched the discussion regarding H. pylori infection in the manuscript (page.16, line 16 to page. 17, line 2) as follows: The association between H. pylori infection and folate depletion has been evaluated in previous studies. In a recent meta-analysis (Helicobacter 2012, 17:1-15.), no significant association between H. pylori infection and folate levels was observed, and cure of H. pylori infection had no effect on serum

folate levels. In this case-control study, the impact of H. pylori infection on serum folate levels was also not significant. However, the patient proportions of H. pylori infection were not different between the gastric cancer group and the matched control group, and the prevalence of H. pylori infection in the gastric cancer group may be underestimated due to H. pylori eradication therapy and lower sensitivity of H. pylori test in severe atrophic gastritis (Helicobacter. 2009;14: 512-9). Further study may help to clarity the effect of H. pylori infection on folate levels among gastric cancer patients.

(5) Given that serum folate is not a recognized biomarker for gastric cancer, it is a nonsense to evaluate the best cut-off to identify gastric cancer cases. I strongly suggest authors to avoid the use of ROC curves. Authors can show the association between serum folate and gastric cancer risk using the median value (or tertiles) of serum folate computed among controls or among the overall population of cases and controls.

Answer: We have avoided the use of ROC curves to show the association between serum folate and gastric cancer according to your suggestion. Using the median value of serum folate computed among the overall population of cases and control as the cutoff value, the associations between serum folate and gastric cancer in all cases and different age and gender subgroups were analyzed by multivariate logistic regression analysis. Low serum folate was significantly associated with gastric cancer risk in the whole population (OR 19.77) and all strata (age < 60 years [OR 17.39], age ≥ 60 years [OR 21.67], males [OR 17.95], and females [OR 20.95]; all P < 0.001). The changes have been revised throughout the manuscript including the Abstract (page. 4, line 10-13), Materials and Methods section (page. 10, line 17-22), Result section (page. 11, line 19-22)) and Table 2 (page. 26).

Reviewer (No. 33061):

(1) This case-control study (or better nested case-control study) can only suggest an association between low levels of folate and presence of gastric cancer, but it cannot demonstrate a cause/effect relationship. In fact, it could be possible that the low folate status could be secondary to gastric cancer-related malnutrition, instead of being the cause.

Answer: As your comments, a causal relationship between folate depletion and gastric cancer evolution could not be determined in this clinical study, and one possibility was that low folate status could be secondary to gastric cancer-related malnutrition. However, in our preliminary data in animal model (unpublished data), folate depletion is related to gastric cancer metastasis. Further studies to confirm the causal relationship will be crucial. We have addressed this study limitation in the discussion section (page. 15, line 9-10; page. 17, line 5-7; page. 17, line 5-6).

(2) Significant and important information are missing in the description of study population, as BMI that could give an idea on the nutritional study of cases as well as of controls.

Answer: Thanks for your comments. Some important information could not be fully obtained in this retrospective study; for example, body mass index (BMI). Even though the mean BMI values of study subjects with available data were not significantly different between the gastric cancer group and the control group, we did not present the findings because BMI data for some subjects were missing. A prospective study could provide a more complete description of study population. We have addressed this limitation in the discussion (page. 17, line 11-16).

(3) H. Pylori status was not analyzed as a possible risk factor.

Answer: We have revised and enriched the discussion regarding H. pylori infection in the manuscript (page.16, line 16 to page. 17, line 2) as follows: The association between H. pylori infection and folate depletion has been evaluated in previous studies. In a recent meta-analysis (Helicobacter 2012, 17:1-15.), no significant association between H. pylori infection and folate levels was observed, and cure of H. pylori infection had no effect on serum folate levels. In this case-control study, the impact of H. pylori infection on serum folate levels was also not

significant. However, the patient proportions of H. pylori infection were not different between the gastric cancer group and the matched control group, and the prevalence of H. pylori infection in the gastric cancer group may be underestimated due to H. pylori eradication therapy and lower sensitivity of H. pylori test in severe atrophic gastritis (Helicobacter. 2009;14: 512-9). Further study may help to clarity the effect of H. pylori infection on folate levels among gastric cancer patients.

(4) ROC curves in this context (and obtained from a case-control study) seems inappropriate.
Answers: We have avoided the use of ROC curves to show the association between serum folate and gastric cancer according to your suggestion. Using the median value of serum folate computed among the overall population of cases and control as the cutoff value, the associations between serum folate and gastric cancer in all cases and different age and gender subgroups were analyzed by multivariate logistic regression analysis. Low serum folate was significantly associated with gastric cancer risk in the whole population (OR 19.77) and all strata (age < 60 years [OR 17.39], age ≥ 60 years [OR 21.67], males [OR 17.95], and females [OR 20.95]; all P < 0.001). The changes have been revised throughout the manuscript including the Abstract (page. 4, line 10-13), Materials and Methods section (page. 10, line 17-22), Result section (page. 11, line 19-22)) and Table 2 (page. 26).

(5) In this study, a significant recall bias is present.
Answer: In retrospective studies, recall bias could be one of the most important limitations. In this study, we analyzed objective factors such as age, gender and lab findings, and we have avoided recall bias as possible as we could.

(6) The conclusions are inappropriate, in particular the conclusion regarding the association between serum folate and patients survival: the folate levels were already of borderline significance at the univariate analysis, but this lost any significance in multivariate analysis: the conclusion in this regard should be that it is not associated with survival.
Answer: Thanks for your recommendations. Due to limitations in this association study, we have reviewed and revised related statements regarding the association between serum folate and patient survival to avoid overstatements (page. 15, line 14-20; page. 18, line 2-6).

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

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