Nov 14, 2012

Dear Editor,

We thank the editors and reviewers of the *World Journal of Gastroenterology* for taking their time to review my article. We have made some corrections and clarifications in the manuscript after going over the reviewers’ comments. The changes are summarized below:

Please find enclosed the edited manuscript in Word format (file name: 762-review.doc).

**Title:** Evaluation of the relationship between dietary factors, *CagA-*positive *Helicobacter pylori* infection, and *RUNX3* promoter hypermethylation in gastric cancer tissue

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**Name of Journal:** *World Journal of Gastroenterology*

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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 A

(1) As serious problem, although most of *H. pylori* in Korea are well known to have *CagA* (more than 90%), prevalence of this study was around 30%. Therefore, detection system of this study may have problems. Authors should re-checked *CagA* status using other primer pairs.

- As the reviewer pointed out, the *CagA* seropositivity rate among Korean gastric cancer patient was reported as 97% (Gwack et al., 2006). The method they used to detect *CagA* status in that study was an immunoblot test, which could detect seral IgG antibodies to *CagA* antigen. Patient who have had the infection in the past, but no current infection, were classified as *CagA* positive by this immunoblot test. The limitation of this immunoblot test is that sensitivity and specificity of the test depend on how high the cut-off value is set; the lower the cut-off value, the higher the positive rate.

To identify *CagA* status of infected *H. pylori*, we used a nested PCR method which amplified highly conserved region of the *CagA* gene of *H. pylori* (ATCC 26695). Patients who were currently infected by a *CagA* positive strain were classified as *CagA* positive irrespective of experiences of past infections. Patients who had the infection in the past, but no current infection, would not be classified as *CagA* positive by this nested PCR method. The difference between the *CagA* positive rates of this and the previous study could be explained by the difference of the detection method.

This study was, as far as we knew, the first one in which current *CagA* positive *H. pylori* infection and *RUNX3* promoter methylation were identified by PCR methods with DNA extracted from Korean gastric cancer tissue. As we used a nested PCR method which amplified highly conserved region of the *CagA* gene of *H. pylori* (ATCC 26695), almost all the *CagA* positive *H. pylori* might have been detected. A Japanese human study in which PCR methods were used to detect *H. pylori* infection and *CagA* status reported a 31.6% *CagA* positive rate in 57 Japanese gastric cancer tissues (Kitajima et al., 2007), which is very close to our data. Therefore, we thought that *CagA* detection system of this study would be acceptable. We have modified discussion on that subjects as follows. .

“We used nested PCR methods with DNA from gastric cancer tissues to detect *H. pylori* infection and *CagA* status. Nested PCR has the advantages of high sensitivity and specificity, quick results, and the ability to type bacteria without the requirement for special transport conditions. In our study, 89% of the gastric cancer patients were found to be positive for *H. pylori* DNA, and 32% were *CagA* positive. A Japanese study in which PCR methods were used to detect *H. pylori* infection and *CagA* status reported a 31.6% *CagA* positive rate in 57 Japanese gastric cancer tissues[27], which is very close to our result. Other Korean studies which document the prevalence of *CagA*-positive *H. pylori* infection generally used an immunoblot method or included smaller sample sizes[46]. Because of the potential of false-positive immunoblot test results, and the fact that individuals who had the infection in the past, irrespective of current infection, were classified as *CagA* positive by this test, *CagA* prevalence is often reported to be higher than it actually is. The *CagA* seropositivity rate among Korean gastric cancer patient was reported as 97%[47]. The difference between the *CagA* positive rates of this present study and the previous one could be explained by the difference in the detection method.”

(2) By reanalysis according to above recommendation, conclusion of this study may change.

- As we did not perform reanalysis, no change in conclusion has been made.

(3) How about endoscopic findings in patients without H pylori infection? If patients had gastric mucosal atrophy, detection system of H. pylori infection may have trouble.

- We could find no difference between endoscopic findings of patients with and those without H. pylori infection. Since we used a PCR method to detect current H. pylori infection, patients who had past, not current, H. pylori infection would be classified as H. pylori negative cases and they could have gastric mucosal atrophy induced by the past infection.

(4) Introduction: In general, vacA is associated with vacuolating toxin, not CagA.

- We have made a correction as the reviewer indicated.

(5) Discussion: Authors suggested that H. pylori infection, irrespective of CagA status, does not induce RUNX3 methylation. However, previous studies reported significant associations RUNX3 expression/methylation and H. pylori CagA status in vitro study. Please explain more and discuss comparisons with this study and previous studies adding references.

- As the reviewer wrote, there were some previous studies which reported significant associations between RUNX3 expression/methylation and H. pylori CagA status in vitro study. Tsang et al. (2010) reported that H. pylori infection induces the ubiquitination and degradation of RUNX3, and suppressed RUNX3 expression in cultured gastric epithelial cells as well as mouse gastric epithelial cells infected with H. pylori. Wild type H. pylori strain can down-regulate the cellular level of RUNX3. These results seemed to be different from ours that there was no significant association between H.pylori infection and RUNX3 promoter hypermethylation. It has been reported that there was a marginal significance in the association between H.pylori infection and RUNX3 promoter hypermethylation, however no significance was found between CagA positive H.pylori infection and RUNX3 promoter hypermethylation. (Kitajima et al., 2007) Our results are also supported by a German study that the level of RUNX3 mRNA expression in gastric epithelium was not influenced by H. pylori infection (Fredrich et al., 2006) This discordance can be explained by the facts that, while the studies with the significant association were in vitro ones, our study was a human study, and we tested association of H. pylori infection with RUNX3 promoter hypermethylation, not with RUNX3 expression. There could be a possibility that H. pylori infection suppress the expression of RUNX3 in gastric epithelial cell via pathway other than promoter hypermethylation. Liu et al(2012) reported that CagA can inhibit the expression of RUNX3 via Src/MEK/ERK and p38 MAPK pathway. Considering all these results, more studies are needed on the association between H.pylori infection, CagA status and RUNX3 expression/hypermethylation.

We have added discussion on that subject as follows.

*“H. pylori* infection and *CagA* status did not reveal any significant association with *RUNX3* promoter hypermethylation nor with *RUNX3* expression levels (Table 3). This finding suggests that *H. pylori* infection, irrespective of *CagA* status, does not induce *RUNX3* methylation, and that the ability of *RUNX3* to function as a tumor suppressor is not specific to *H. pylori*-related gastric cancer. A Japanese study reported that there was a marginal significance in the association between H.pylori infection and *RUNX3* promoter hypermethylation, however no significance was found between *CagA* positive *H. pylori* infection and *RUNX3* promoter hypermethylation[27]. Our results are also supported by a German study that the level of *RUNX3* mRNA expression in gastric epithelium was not influenced by H. pylori infection[32]. On the contrary, there was a study that *H. pylori* infection induced the ubiquitination and degradation of *RUNX3*, and suppressed *RUNX3* expression in cultured gastric epithelial cells as well as mouse gastric epithelial cells infected with *H. pylori*. This discordance can be explained by the facts that, while the studies with the significant association were *in vitro* ones, our study was a human study, and we tested association of *H. pylori* infection with *RUNX3* promoter hypermethylation, not with *RUNX3* expression. There could be a possibility that *H. pylori* infection suppress the expression of *RUNX3* in gastric epithelial cell via pathway other than promoter hypermethylation. Liu *et al*[33] reported that *CagA* can inhibit the expression of *RUNX3* via Src/MEK/ERK and p38 MAPK pathway.”

(6) Results section should be shortened in 70%.

- We downsized the results as the reviewer indicated.

Reviewer B

(1) The number of CG is low (184 cases) to draw conclusions about the diet. Furthermore there was not a control group.

- We agree with the reviewer’s opinion that the sample size is not enough to draw conclusions about the causal relationship between dietary factors and RUNX3 methylation/expression. What we intended to make were not conclusions but suggestions. However, insofar as we know a Korean gastric cancer study in which 184 or more gastric cancer tissues were examined for H. pylori infection, CagA status, RUNX3 methylation and expression, and dietary factors has not been presented before. We thought that the results which derived from those data were not meaningless.

This study is not a case-control study; therefore healthy controls were not included in this study. This is one of limitation of this study. Instead, comparisons made in this study were between H. pylori infection positive and negative gastric cancer patients, and between RUNX3 methylation/expression positive and negative gastric cancer patients.

We have added discussion as follows.

“The sample size is another limitation. It is not enough to draw firm conclusions, especially on the effects of dietary factors on RUNX3 promoter methylation.”

(2) Define if a prospective or retrospective. If is retrospective, the conclusions on the diet should be eliminated.

- This study is a retrospective one. As the review pointed out, if dietary intake pattern after cancer diagnosis were included in the analyses, we should not draw any conclusions from those data. As dietary data of this study were collected about patients’ food intake patterns during the 12 months preceding the diagnosis of gastric cancer, these were expected to reflect dietary patterns prior to the cancer development. Therefore we could make some suggestions according to the analysis results of the dietary data.

We have added discussion as follows.

“Secondly, being as this study is retrospective, we could not confirm the temporal sequence of H. pylori infection and RUNX3 promoter methylation.”

(3) In materials and methods must define Who does the questionnaire on diet? How is it done? What is considered high or low consumption of a food? Types of alcoholic beverages? Does the questionnaire was validated? For example in study EPIC and gastric cancer, The usual diet over the previous 12 months was at EPIC study recruitment Measured With The use of country-specifi c Questionnaires validated (20, 23).

- More concrete descriptions were made on the questionnaire survey as follows.

“Patient demographics, as well as other known and potential risk factors for gastric cancer were collected by direct interviews. Trained interviewers interviewed subjects using a structured questionnaire within one month after the diagnosis of gastric cancer or benign diseases or at the time of the hospital visit for control subjects undergoing the routine medical examination. Dietary data were collected using a semiquantitative food frequency table previously evaluated for validity and reliability[18]. The average frequencies of intake and portion sizes of 89 common food items were documented. These items were classified into 21 food groups having similar ingredients: cereals, potato; nuts; noodles; breads and cakes; vegetables; mushrooms; fruits; red meats; egg; fish and shellfish; stews; chicken; kimchi; soybean foods; soybean pastes; milk and dairy products (butter, cheeses, and margarine); jams, honey, sweets and chocolates; coffee and tea; seaweeds; and alliums. Each food item was divided into high and low groups according to the median value of its distribution.

The amount of calories, nutrients, vitamins, and minerals consumed for each food item was estimated by multiplying the intake amount of the food item and its nutrient value. The total intake of calories, nutrients, vitamins, and minerals was calculated for each subject by summing the respective calories, nutrients, vitamins, and minerals for each food item[19]. The intake amounts of these factors were adjusted for caloric intake using the method of Willett *et al*[20].”

(4) Is unacceptable smoker defined as smoking 200 cigarettes in lifetime . You must correct

- As the reviewer A recommended to shorten the Results section in 70%, we have deleted all the description about smoking.

(5) In results define how many patients were CAG - HP - CAG - HP, CAG HP - and HP CAG?

- We have added table 2 for those data in the results.

(6) Analyze the results of methylation by staging (TNM), survival (at 1, 3 and 5 years) and gastric cancer (intestinal and diffuse).

- We sincerely appreciate the reviewer’s suggestions for future studies. We would like to present follow-up results of these study subjects in near future.

(7) Discussion. You should review articles related to diet-Medline Pubmed gastric cancer and EPIC group. See references below

- We have added following sentences in the discussion.

“The European Prospective Investigation into Cancer and Nutrituion (EPIC-EURGAST) has reported similar results that fresh fruit and citrus fruit consumption may protect against diffuse and cardia gastric cancer, respectively[45], but a consistent result is yet to be achieved”

(8) Add the limitations of the study in the discussion. There are many.

- We have added the limitations of the study in the discussion as follows.

“This study has some limitations. Since corresponding non-cancerous mucosa from gastric cancer patients or normal mucosal tissues from healthy controls were not included in this study, we could not check RUNX3 hypermethylation and RUNX3 expression level in those tissues. Secondly, being as this study is retrospective, we could not confirm the temporal sequence of H. pylori infection and RUNX3 promoter methylation. The sample size is another limitation. It is not enough to draw firm conclusions, especially on the effects of dietary factors on RUNX3 promoter methylation.”

(9) Introduction. Remove surface, "H. pylori is associated with chronic gastritis and . "

- Corrections have been made as the reviewer’s recommendation.

(10) Table 4 is repeated alliums

- Corrections have been made as the reviewer’s recommendation.

3 References and typesetting were corrected

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

Sincerely yours,



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