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Title：KatA and AhpC *Helicobacter pylori* antibodies as novel biomarkers for gastric cancer

Dear Editor-in-chief and reviewers:

We sincerely thank your encouragement and comments on our study and we highly appreciate the critical review and valuable suggestions on this manuscript. We had revised the manuscript according to the comments and highlighted it with red color. Our response to each comment and the modifications made in the text are summarized as below:

Reviewer 00502831： The authors investigated catalase (KatA), alkyl hydroperoxidase reductase (AhpC) antibodies of Helicobacter pylori (*H. pylori*) as biomarkers for gastric cancer (GC). They concluded that serum KatA and AhpC antibodies are associated with GC risk and KatA may serve as a biomarker for GC. KatA/FlaA combined analysis improved screening accuracy. This study was well designed and considered to contribute to early detection of GC. I have some comments as bellow. #1. The authors should add the results of KatA/AhpC combined analysis to Table1. #2. KatA and AhpC were antibodies of *H. Pylori*. But why there were 21 KatA positive cases and 43 AhpC positive cases even in the control of *H. pylori* negative cases? #3. There is no description of stage of GC in this article. So the effectiveness of KatA and AhpC for early detection of GC is not able to evaluate. #4. The authors should describe about positivity of KatA and AhpC on cardia GC. #5. If possible, the authors had better to investigate the expression of KatA and AhpC on the GC tissues immunohistochemically.

1. The authors should add the results of KatA/AhpC combined analysis to

Table1.

Response: Thank you very much for your kind suggestion. As you suggested, the combined results for KatA and AhpC was calculated and analyzed. An evident association between GC risk and serum positivity of combination of KatA and AhpC was also present with OR = 11.64 (95% CI: 7.12–19.01), 13.39 (6.29–28.53) and 13.91 (6.74–28.74) in all, *H. pylori*-positive and *H. pylori*-negative subjects, respectively ($P < 0.001$). We have complemented these data to Table1 and described this information in the RESULTS section (See lines 9-13 on page 11).

Meanwhile, we plotted the ROC for KatA/AhpC combined analysis (Figure 1). AUC for the combination of KatA and AhpC was 0.806 (95% CI: 0.768–0.848), the optimal cutoff value was 0.4077 with a sensitivity of 71.00% and a specificity of 84.25%. Compared to KatA alone, AUC for combination of KatA and AhpC was not elevated, sensitivity was only increased by 4.19% but specificity was decreased by 2.11% in all subjects. Considering KatA/AhpC combined analysis did not improve screening value compared to KatA alone, the combined results of ROC for KatA/AhpC was not added to our manuscript.

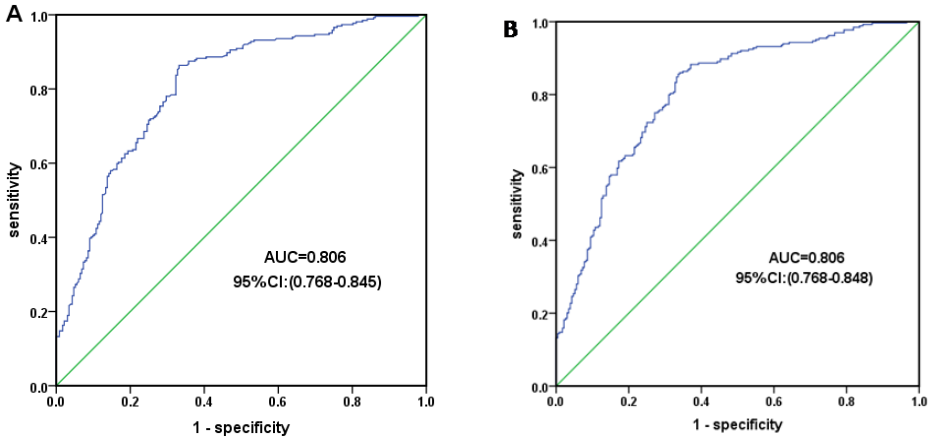

Figure 1 Receiver operating characteristic curve (ROC) for serum antibody in all subjects. A: KatA; B: KatA+AhpC.

2. KatA and AhpC were antibodies of *H. Pylori*. But why there were 21 KatA positive cases and 43 AhpC positive cases even in the control of *H. pylori* negative cases?

Response： Thank you very much for your comments and I am sorry for making this confuse. There are two main reasons for this result. First, commercial ELISA serology may fail to detect previous *H. pylori* infection in GC patients [1]. For example, CagA antibodies may be positive in patients who have a negative *H. pylori* serologic test because CagA antibodies can remain positive for a longer period of time than the anti-*H. pylori* antibody [2-3]. Therefore, a negative *H. pylori* serologic test does not rule out the possibility of a previous exposure to infection. We have made a statement in the DISCUSSION section (See line 21-26 on page 12).

Second, Indirect ELISA method was adopted to detect serum KatA and AhpC antibodies in this study, which might be accompanied by the non-specific signal caused by cross-reactivity. This means that KatA and AhpC will not only react with the corresponding specific antibody but also react with the non-specific antibodies, which eventually led to some *H. pylori*-negative subjects being classified KatA or AhpC as positive. We have also clarified this point in DISCUSSION section (See the last paragraph on page 13).

3. There is no description of stage of GC in this article. So the effectiveness of KatA and AhpC for early detection of GC is not able to evaluate.

Response： Thank you for your kind comment. Since our research group shared the same batch of samples for a couple of years, the stages of GC have been described in our previously published study [4]. In order to avoid repeated report, stage of GC was not described again in this article. We have illustrated this point in the RESULTS section (See the first part of RESULTS on page 10).

The GC patients involved in previously studies of our group were

distinguished into four clinical stages according to TNM staging system. Among the 232 cases, 14 (7.22%) were classified as stage Ⅰ, 16 (8.25%) as stage Ⅱ, 143 (73.71%) as stage Ⅲ, and 21 (10.82%) as stage Ⅳ, respectively, data were missing in 38 cases.

4. The authors should describe about positivity of KatA and AhpC on cardia GC.

Response: Thank you for your considerate suggestion. Our study included nine (3.88%) cardia GC cases. The positivity of both KatA and AhpC was 88.89% (8/9) among cardia GC cases. Considering the sample of cardia GC was too small, this positivity of KatA and AhpC can not accurately reflect the positivity of the whole cardia GC population. Also, those 9 cardia GC cases did not affect the overall results and conclusion. Therefore, positivity of KatA and AhpC on cardia GC was not described in our manuscript. We have also discussed it in the DISCUSSION section (See lines 4-5 on page 14).

5. If possible, the authors had better to investigate the expression of KatA and AhpC on the GC tissues immunohistochemically.

Response: Thank you very much for your excellent comments. It is significant to investigate the expression of KatA and AhpC on the GC tissues immunohistochemically, which will strengthen our findings. Unfortunately, we could not complete relevant study at present since GC tissues was deficiency. When the conditions permit we will consider doing immunehistochemistry tests as soon as possible.

Reference:

1 Annibale B, Lahner E, Santucci A, *et al.* CagA and VacA are immunoblot markers of past *Helicobacter pylori* infection in atrophic body gastritis. *Helicobacter* 2007; 12: 23-30.

2 Ekstrom AM, Held M, Hansson LE, *et al*. *Helicobacter pylori* in gastric cancer

established by CagA immunoblot as a marker of past infection. *Gastroenterology* 2001; 121: 784-91.

3 Sorberg M, Engstrand L, Strom M, *et al*. The diagnostic value of enzyme immunoassay and immunoblot in monitoring eradication of *Helicobacter pylori*. *Scand J Infect Dis* 1997; 29: 147-5

4 Tian W, Jia Y, Yuan K, Huang L, Nadolny C, Dong X, Ren X, Liu J. Serum antibody against *Helicobactor pylori* FlaA and risk of gastric cancer. *Helicobactor* 2014; 19: 9-16 [PMID: 24118166 DOI: 10.1111/hel.1209]

Reviewer 00039368： This well done and written hospital-based case-control study considers the investigation of possible use of evaluation of antibodies to catalase (KatA) and alkyl hydroperoxide reductase (AhpC) of *H. pylori* as biomarkers for gastric cancer. The study was performed in 232 gastric cancer patients and 264 controls subjects using indirect ELISA for detection of aforementioned antibodies in sera. The main finding of the study was that KatA and AhpC antibodies are associated with gastric cancer risk and Kat A may serve as a novel biomarker for gastric cancer screening. Introduction gives a sufficient overview of the study background and the authors raised clearly the aim of the study. The Tables and Figure give a sufficient overview about the results. This study make a contribution to studies of better understanding the mechanisms of carcinogenesis and evaluation the possible non-invasive diagnostic markers of gastric cancer patients. However, the following point needs to be considered: 1. It would be useful to compare the presence and the level of KatA and AhpC antibodies also in patients with gastric ulcer and/or with chronic atrophic gastritis cased by *H. pylori*. 2. How the authors do explanation of relatively high frequency of KatA seropositivity (70.79%) in gastric cancer patients in H. pylori negative subjects? It should be discussed a possibility of some cross-reactivity, especially taken into account the very high serum dilution used in ELISA (1:32000).

1. It would be useful to compare the presence and the level of KatA and AhpC antibodies also in patients with gastric ulcer and/or with chronic atrophic gastritis caused by *H. pylori*.

Response： Thank you very much for your excellent comments. To compare the presence and the level of KatA and AhpC antibodies in gastric ulcer and/or with chronic atrophic gastritis patients caused by *H. pylori* is meaningful, which will help us to evaluate the ability of our biomarkers for differential diagnosis of GC. Unfortunately, the serum samples of patients with gastric ulcer and/or with chronic atrophic gastritis are not available at present. We will carry out the relevant exploration in the near future.

2. How the authors do explanation of relatively high frequency of KatA seropositivity (70.79%) in gastric cancer patients in *H. pylori* negative subjects? It should be discussed a possibility of some cross-reactivity, especially taken into account the very high serum dilution used in ELISA (1:32000).

Response： I am truly sorry that I make a mistake about the serum dilution-fold. Serum sample used in ELISA was diluted **3200-fold** instead of 32000-fold in this study. I have made correction in the ELISA part of the MATERIALS AND METHODS section (See line 15 on page 9).

As you suggested, we reconsidered the possibility of cross-reactivity. Indirect ELISA method might be accompanied by the non-specific signal caused by cross-reactivity. This means that KatA and AhpC will not only react with the corresponding specific antibody but also react with the other non-specific antibodies, which may be the reason for the relatively high frequency of KatA seropositivity in gastric cancer patients in *H. pylori* negative subjects. We discussed this point in DISCUSSION section (See the last paragraph on page 13).

I hope we have satisfactorily addressed the reviewers' concerns and the

revised manuscript has met the requirement and expectation of the journal. Thanks for your consideration and we look forward to a favorable decision.

Sincerely yours,

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