ANSWERING REVIEWERS

October 27, 2014

Dear Editor,

Title: Combined detection of plasma GATA5 and SFRP2 methylation is a valid noninvasive biomarker for colorectal cancer and adenomas

Author: Xie Zhang, Yu-Fei Song, Hong-Na Lu, Dan-Ping Wang, Xue-Song Zhang, Shi-Liang Huang, Bei-Lei Sun, Zhi-Gang Huang

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 13962

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 reviewers.

Part A (reviewer 02908153)

(1)The details of the TNM stage should be mentioned in the materials and methods section because the reader will not be able to interpret the data.

Response: Tumor stage was determined according to the tumor node metastasis (TNM) criteria of the Union for International Cancer Control/American Joint Committee on Cancer, 2010. We have added a reference in the materials and methods section of manuscript: [20] Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manualand the future of TNM. Ann Surg Oncol; 2010,7: 1471-4. [PMID: 20180029 DIO:10.1245/s10434-010-0985-4]

(2)How did you combined ROC analyses using the two genes, and please show us the predicted values of logistic regression equation, and provide AUC.

Response: In this study, We used a methylation-specific PCR (MSP) technique to detect the methylated DNA at CPG islands in the plasma of clinical samples. MSP is a kind of qualitative method, rather than quantitative method(such as Q-MSP). Methylation judgment standard: A gene was deemed methylated if

unmethylated in plasma samples of healthy controls but methylated in plasma samples of CRC and adenomas patients, or methylated in plasma of CRC and adenomas patients was more than methylated in normal controls. If neither, it was unmethylated. SO, there is no exact values, only the number of methylation or unmethyaltion samples, we can't provide the values of logistic regression equation, and AUC.

(3) In Figure 2, (A), (B), and (C) may refer to GATA5, SFRP2, and ITGA4.

Response: It is really ture. we have made correction in the manuscript. The statements of "Representative MSP results of GATA5, SFRP2, and ITGA4 aberrant methylation in colorectal cancer(A), adenomas(B), and control patients(C). " were corrected as "Representative MSP results of GATA5 (A), SFRP2 (B), and ITGA4 (C) aberrant methylation in colorectal cancer, adenomas, and control patients. "

Part B (reviewer 02906811)

(1)Abstract: I think "prognosis" should be removed from the conclusion based on the results of this study.

Response: we hade removed "prognosis" from the conclusion.

(2)Methods: A: How did the authors estimate the sample size? B: The methylation status of GATA5, SFRP2 and ITGA4 genes should be detected both in solid tissue from CRC, adenomas and normal colorectal tissue and their corresponding plasma, especially in the early stage of research on this issue.

Response: A: The sample size was evaluated by the power of statistical test. We are planning a study with 57 CRC patients and 47 control patients. If the true odds ratio for disease in exposed subjects relative to unexposed subjects is **, we will be able to reject the null hypothesis that this odds ratio equals 1 with probability (power) **(listed in the following table). The Type I error probability associated with this test of this null hypothesis is 0.05. We will use a Fisher's exact test to evaluate this null hypothesis.

plasma	Odds ratio	power
Colorectal cancer		
GATA5 methylation	5.89	0.98
SFRP2 methylation	3.12	0.75
ITGA4 methylation	2.46	0.51

B: It is a good suggestion. Since many previous studies have proved that GATA5, SFRP2, and ITGA4 were higher methylated in CRC tissues than para-carcinoma tissue, we just did a small number of tissues samples in the preliminary study. In our preliminary study, we had detected these three genes in a small number of tissues to verify they were indeed highly methylated(the data was not showed in paper), then we detected these three genens in blood. The objective of this article was to investigate non-invasive biomakers for CRC and adenomas screening. Therefore, we took much more time in blood study, rather than tissues. Our preliminary experimental design was really not rigorous. In future, We will follow the reviewer's suggestion, supplied these part content in the experiments to make it more meaning information.

(3)Results：A: The title "Plasma GATA5 methylation as a coordinate marker for CRC and adenomas with SFRP2 methylation" can not reflect the content of this paragraph. B: What is "OR analysis"? C: The results of sensitivity and specificity for GATA5, SFRP2 and ITGA4 methylation and their combination testing should be shown in a table. D: With which method did the authors calculate the sensitivity and specificity? E: How did the authors determine the cutoff values ?

Response: A: Considering the reviewer's suggestion, we have revised the statements of "Plasma GATA5 methylation as a coordinate marker for CRC and adenomas with SFRP2 methylation" as follows: "Effect of combining measurement of plasma GATA5, SFRP2, and ITGA4 methylation for CRC and adenomas determination. " in the manuscript.

B: OR, abbreviation of odds ratios, which is the analysis index of the relation between disease and the degree of exposure factors. OR=ad/bc (The below table can be understood as follows).

	Methylation	Unmethylation
CRC/adenomas	a	b
Control	c	d

C: we have shown the results of sensitivity and specificity for GATA5, SFRP2 and ITGA4 methylation and their combination testing in table III in the manuscript.

D: The χ^2 test or Fisher's exact test was uesd to calculate the sensitivity and specificity.

E: In this study, We used a methylation-specific PCR (MSP) technique to detect the methylated DNA at CPG islands in the plasma of clinical samples. MSP is a kind of qualitative method, rather than quantitative method(Q-MSP). Methylation judgment standard: A gene was deemed methylated if unmethylated in plasma samples of healthy controls but methylated in plasma samples of CRC and adenomas patients, or methylated in plasma of CRC and adenomas patients was more than methylated in normal controls. If neither, it was unmethylated.

(4)Figure 2: There are several mistakes and the quality of the images should be improved.

Response: we have made correction in the manuscript. The statements of "Representative MSP results of

GATA5, SFRP2, and ITGA4 aberrant methylation in colorectal cancer(A), adenomas(B), and control patients(C). " were corrected as "Representative MSP results of GATA5 (A), SFRP2 (B), and ITGA4 (C) aberrant methylation in colorectal cancer, adenomas, and control patients. "

Part c (reviewer 02451547)

(1)In this paper, the mean age of the patients in the CRC, adenoma, and control groups was 56.64 ± 8.27, 57.00 ± 11.27, and 61.40 ± 12.41 years, respectively. Why the authors selected 60 years old as cut-off point when stratified by age.

Response: Becaues 60 is the median of all subjects' years old, We selected 60 years old as cut-off point.

(2)GATA5 methylation in CRC tissues and in normal colon tissue samples from controls has been explored in other ethnic population. Why the authors did not detect it in CRC tissues when they detected it in plasma. If the data were obtained, the paper might provide more meaning information. The authors could compare the sensitivity and specificity between in plasma and in tissues and compare them with previous studies.

Response: It is a good suggestion. Since many previous studies have proved that GATA5, SFRP2, and ITGA4 were higher methylated in CRC tissues than para-carcinoma tissue, we just did a small number of tissues samples in the preliminary study. In our preliminary study, we had detected these three genes in a small number of tissues to verify they were indeed highly methylated(the data was not showed in paper), then we detected these three genens in blood. The objective of this article was to investigate non-invasive biomakers for CRC and adenomas screening. Therefore, we took much more time in blood study, rather than tissues. Our preliminary experimental design was really not rigorous. In future, We will follow the reviewer's suggestion, supplied these part content in the experiments to make it more meaning information.

(3)In this paper, the authors declared that most of these studies only analyzed a small number of plasma samples. Do the authors think the sample size is enough large to detect the difference among different groups, would you please give the power of statistical test.

Response: As reviewer's requirement, we have give the power of statistical test in the follow table:

plasma	power	plasma/serum	Other Reference power
Colorectal cancer			
GATA5 methylation	0.98	SEPT9 methyaltion[17]	1.00
SFRP2 methylation	0.75	SFRP2 methylation[29]	0.99
ITGA4 methylation	0.51	*SMAD4* methylation[34]	0.65
		FHIT methylation[34]	0.71

DAPK1 methylation[34]	0.65
APC methylation[34]	0.75
E-cad methylation[34]	0.75

[17] Warren JD, Xiong W, Bunker AM, Vaughn CP, Furtado LV, Roberts WL, Fang JC, Samowitz WS, Heichman KA. Septin 9 methylated DNA is a sensitive and specific blood test for colorectal cancer. BMC Med 2011; 9:133 [PMID: 22168215 DOI: 10.1186/1741-7015-9-133]

[29] Tang D, Liu J, Wang DR, Yu HF, Li YK, Zhang JQ. Diagnostic and prognostic value of the methylation status of secreted frizzled-related protein 2 in colorectal cancer. Clin Invest Med 2011; 34: E88-95 [PMID: 21463549]

[34] **Pack SC**, Kim HR, Lim SW, et al. Usefulness of plasma epigenetic changes of five major genes involved in the pathogenesis of colorectal cancer. Int J Colorectal Dis 2013; **28**:137-47 [PMID: 22990173 DIO: 10.1007/s00384-012-1566-8]

According to the table above, the power of ITGA4 methylation is the lowest. We are very sorry for our negligence to estimate the sample size before. In our paper, the statement of "Furthermore, most of these studies only analyzed a small number of plasma samples." have been removed. Because of it is not rigorous.

(4) In this paper, the authors found the methylation frequency of GATA5 gene was significantly higher in CRC plasma samples and in adenomas than in normal plasma samples and GATA5 methylation in the plasma significantly correlated with larger tumor size (p = 0.019), differentiation status (p = 0.038), TNM stage (p = 0.008), and lymph node metastasis (p = 0.008), but there was no statistical difference in the incidence of GATA5 gene in the plasma of CRC and adenoma patients. Would you please give the reasonable guess and possible clinical meanings.

Response: Firstly, the methylation of GATA5 might be occurred in the whole process of colorectal carcinogenesis, and might be involved in the very early of CRC, even in precursor adenomas. Secondly, MSP method is a kind of qualitative method, rather than quantitative method(such as Q-MSP). Methylation judgment standard: A gene was deemed methylated if unmethylated in plasma samples of healthy controls but methylated in plasma samples of CRC and adenomas patients, or methylated in plasma of CRC and adenomas patients was more than methylated in normal controls. If neither, it was unmethylated. SO, there was no exact values, only the number of methylation or unmethyaltion. The percent of GATA5 methylation in CRC (61.40%) was larger than the percent of GATA5 methylation in adenomas (43.33%), but it did not reach statistical significance (P=0.10). It might be our sample size was not big enough, or existed statistical deviation, could not reach statistical significance. In the future, we will using Q-MSP combined pyrosequencing method and expand the sample size to improve sensibility and specificity of detection methylation of genes in CRC. I have added the above content in the dicussion.

3 References and typesetting were corrected

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

Sincerely yours,

Xie zhang

Peter Laszlo LAKATOS, MD, PhD

Dept. of Gastroenterology

Ningbo Medical Treatment Center Li Huili Hospital

Zhejiang, China

TEL: +86-18857401592

E-mail: rennie22@12