April, 18, 2022

Jin-Lei Wang,
Company Editor-in-Chief
World Journal of Clinical Cases

Dear Editor,

We wish to re-submit the manuscript titled "Expression of HNF4α, WNT5a and β-catenin in clinical gastric cancer." The manuscript ID is 76672.

We thank you and the reviewers for your thoughtful suggestions and insights. The manuscript has benefited from these insightful suggestions. I look forward to working with you and the reviewers to move this manuscript closer to publication in *World Journal of Clinical Cases*.

The manuscript has been rechecked and the necessary changes made in accordance with the reviewers' suggestions. The re-submitted manuscript is a revised version. The writing structure has been organized and the writing language has been polished by the professional English language editing company. The pathological pictures in the manuscript have been marked with a scale bar. The revised contents are marked in blue in the revised manuscript. We also responded to each of the reviewers' comments, point by point, along with a clear indication of the location of the revision in the manuscript.

We would like to express our heartfelt gratitude to you for the constructive comments which not only helped us improve the quality and presentation of the paper, but also provided important reference for our future research. The responses to all comments have been prepared and attached herewith/given below.

Thank you for your consideration. I look forward to hearing from you.

Sincerely,

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Reviewer #1: The manuscript shows differential expression of different markers (HTNF4α, WNT5a, etc…) in cancerous lesions vs non-cancerous lesions in patients with gastric cancer. This is potentially interesting, but the manuscript shows several pitfalls and overstatements. In addition, the manuscript needs rewriting and there are some English usage errors.

TITLE (Clinical correlation between HNF4α, WNT5a and β-catenin in gastric cancer) does not match the results shown in the manuscript. True, the expression of the markers analyzed differ depending on the origin of the tissue studied (cancerous vs non-cancerous) or the type of the gastric tumor (tubular, signet ring, mucinous) but it has nothing to do with the clinical evolution of patients, and I think this is major concern with the manuscript.

Response: Thanks for your constructive suggestion. Indeed, we only analyzed the expression of HNF4α, WNT5a, and β-catenin in clinical gastric cancer tissues and non-gastric cancer tissues, and the relationship between these three indicators and clinical types of gastric cancer. After careful consideration, the title of this manuscript is changed to "Expression of Hepatocyte Nuclear Factor 4 alpha, Wingless-Related Integration Site, and β-Catenin in Clinical Gastric Cancer".

The INTRODUCTION is too long and can be shortened. For instance, the sentence "The earliest understanding of HNF4α is that HNF4α mutation is related to several forms of maturity-onset diabetes of the young [8, 9]." can be left out with no detrimental effect on the content of the manuscript. The paragraph "Increasing evidence has shown that HTNF4α….progression of gastric cancer are still unclear" needs to be shortened, if not deleted altogether.

Response: Thanks for your advice. We have deleted this sentence "The earliest understanding of HNF4α is that HNF4α mutation is related to several forms of maturity-onset diabetes of the young" in the INTRODUCTION section. And we retained the paragraph "Increasing evidence has shown that HTNF4α….progression of gastric cancer are still unclear" and simplified it. Because this paragraph explains

why the expressions of HNF4α, WNT5A, and β-catenin will be investigated in clinical gastric cancer tissues. In the existing literature and our previous experiments, HNF4α can regulate the WNT/β-catenin signaling pathway which are involved in the occurrence and development of gastric cancer in preclinical models, and we further explored the expressions of these three molecules in clinical gastric cancer tissues to confirm that HNF4α/WNT5A/β-catenin signaling pathway is related to the clinical gastric cancer tissues.

MATERIALS AND METHODS

1.- There is a discrepancy in the number of paired tissue sections "...158 gastric tumors and 164 matched para-tumor tissue sections…". How can it be paired with different numbers? It is later mentioned that "..6 slices were not included due to quality problems…". The number should match both groups.

Response: Thanks for your comment. I'm sorry for our wrong statement. At the beginning of the experiment, 164 cases of gastric tumoral wax blocks and matched adjacent para-cancerous tissues were selected. However, 6 cases of gastric tumoral tissues were excluded because of their small size and low quality. Therefore, 158 cases of gastric tumoral tissues and 164 cases of para-cancerous tissues were included in this experiment for further study. This sentence has been revised in the new manuscript.

Modification (Sample collection and characterization section):

Human GC tissues were obtained from patients who presented between 2016 and 2018 at Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China). According to the WHO histological classifications, GC comprises signet ring cell carcinoma, and tubular, as well as mucinous adenocarcinoma. We initially selected wax blocks of paraffin-embedded GC and matched para-cancerous tissue blocks (n = 164 each) to reduce the influence of pathological WHO classification of GC on the expression of HNF4α, WNT5a, and β-catenin in GC tissues. However, six small gastric tumor tissues of insufficient quality were excluded. Therefore, we analyzed 158 GC (59 signet-ring cell carcinoma,

and 35 tubular, and 64 mucinous adenocarcinomas) and 164 para-cancerous tissues. The gastric cancer tissues comprised 97, 46, and 15 samples of intestinal, diffuse mixed types according to the Lauren classification.

2.- When explaining the statistical tests run, only the Chi squared is mentioned, but in the supplementary tables, regression analysis and Pearson correlation analysis are cited.

Response: Thanks for your comment. We have revised this paragraph and added Regression analysis and Pearson correlation analysis in this part.

Modification (Statistical analyses section):

Relationships between double-positive HNF4α and WNT5a expression and types of gastric tumor tissues were assessed using regression analysis. Correlations between HNF4α and WNT5a expression at the RNA level in GC tissues found in the TCGA database were analyzed using Pearson correlation coefficients.

3.- The terms used all over the text to identify the origin of the tumoral vs non-tumoral blocks (gastric cancer and para cancerous, respectively) is confusing. Patients suffer from gastric cancer and I thus dare suggest the term “tumoral tissue” or “tumoral sections” for the samples with cancerous lesions, and “peritumoral” or “para-cancerous” for the paired samples with no cancerous lesions from a given patient.

Response: Thanks for your suggestion. In order to express gastric cancer tissues more clearly, we have changed gastric cancer into “gastric tumoral tissues” or “gastric tumor tissues”, including some words in subheadings. See the revised manuscript for details.

4.- Percentages are expressed using two decimal places (91,72%), and one decimal place (91,7%) would be sufficient.

Response: Thank you for your suggestion. All percentages in this manuscript have been changed to keep one decimal point.

5.- Some P values given in the text do not match those given in tables. For instance, in the text it is stated “In Table 3, HNF4α was strongly positive expressed in tubular and mucinous adenocarcinoma but was relatively weak in signet ring cell carcinoma ($P < 0.001$).” But in the footnote to table 3 the P value shown is “***P<0.000.” Same in table 4.

Response: Thank you for pointing out. After checking, we found that the expression of P value in the tables was wrong. In the new tables 4 and 5 "*** $P < 0.000$." has been changed to "*** $P < 0.001$."

6.- In table 4, heading should read “....according to Lauren classification”.

Response: Thanks for your suggestion. The heading of tables 4 and 5 have been corrected. For example, “Table 4. Correlation of HNF4α and WNT5a expression with types of gastric carcinomas according to WHO classification.” and “Table 5. Correlation of HNF4α and WNT5a expression with types of gastric carcinomas according to Lauren classification.”

7.- When commenting on the results of table 5, it is said “Using the X2 test of paired comparison analysis, we found that there was a positive correlation between HNF4α and WNT5a expression in GC ($X2 = 1.5$, $P > 0.05$) (Table 5)”. This P value discards any correlation.

Response: Thanks for pointing out. Indeed, we checked the original data and statistical analysis results ($X^2 = 1.5$, $P > 0.05$) again. The results showed that there was no correlation between the expression of HNF4α and WNT5a in these included gastric cancer tissue sections. This important conclusion has been corrected in this paper.

8.- Results given in tables 7 and 9 are scarcely significant and relevant.

Response: Thanks for your comment. We want to explore whether the expression of HNF4α, WNT5a, β-catenin in gastric cancer is related to sex, age, tumor

differentiation, invasion depth, distinct metastasis and TNM stage. These results indicated that the expression of HNF4α, WNT5a, and β-catenin was not related to the demographic characteristics of patients and the differentiation, metastasis and staging of gastric cancer, except HNF4α, which was the highest expression in modem differentiated gastric tumoral tissues. The effects of these factors on the expression of HNF4α, WNT5a, and β-catenin in gastric cancer tissues were excluded. As most of these results were negative, we decided to place this part of the table in the **supplementary tables**.

9.- The first table should be one describing the characteristics of the population studied.

Response: Thanks for your suggestion. We have adjusted all tables in the results section, and the first table is to describe the characteristics of the people studied. See the **Results-Tables** section for details.

10.- In several parts of the RESULTS section the notion that HNF4α/WNT5a are biomarkers of cancer with diagnostic capability is stressed ("HNF4α/WNT5a axis can be a biomarker of gastric cancer" or "WNT5a has a high diagnostic accuracy rate of 85.71%"). This is an overstatement. Diagnosis can be readily achieved based on well standardized anatomopathological criteria.

Response: Thanks for your comment, and I'm sorry for our exaggerated statement. We have rewritten the full-text concluding statements, especially the results and conclusions, and tried our best to avoid exaggerated expressions. See the revised manuscript for details.

In **CONCLUSIONS**, it is stated that "...the HNF4α/WNT5a axis could be used as a potential diagnostic tool for gastric cancer." This, in my opinion, is an overstatement. The diagnosis is made upon anatomopathological findings. In this aspect, the results herein shown do not add to already existing diagnostic criteria. English usage needs to be revised. I will show but a few samples

1.- “ Recent studies have showed….” correct to “Recent studies have shown…”

Response: Thanks for your advice. We have revised **Abstract** in new manuscript.

Modification (Abstract-BACKGROUND):

Gastric cancer (GC) is the second most common cause of cancer-related deaths worldwide. Hepatocyte nuclear factor 4 alpha (HNF4α) that belongs to the nuclear hormone receptor superfamily, is overexpressed in GC tissues, and might be involved in the development of gastric cancer by regulating its downstream Wingless-related integration site (WNT)/β-catenin signaling.

2.- “These pathological blocks of gastric cancer patients were selected from Tongji Hospital, and immunohistochemical staining was carried out on 158 gastric tumors and 164 matched para-tumor tissue sections from Tongji Hospital” change to “Pathological blocks of gastric cancer patients were selected from Tongji Hospital, and immunohistochemical staining was carried out on 158 gastric tumors and 164 matched para-tumor tissue sections”

Response: Thanks for your advice. We have revised **Abstract-METHODS** in new manuscript.

Modification (Abstract-METHODS):

We immunohistochemically stained pathological blocks of GC and matched para-cancerous tissues. The intensity of HNF4α, WNT5a and β-catenin staining in the tumor cells was determined according to cell rates and staining intensity. The correlations between gastric cancer and HNF4α, WNT5a, and β-catenin expression using chi-square and paired chi-square tests. Relationships between double-positive HNF4α and WNT5a expression and types of gastric tumor tissues were assessed using regression analysis. Correlations between HNF4α and WNT5a expression at the RNA level in GC tissues found in the TCGA database were analyzed using Pearson correlation coefficients.

3.- “The expression of HNF4α and WNT5a might be used as early prediction biomarkers for prediction in the early stage of GC” correct to “The expression of

HNF4α and WNT5a might be used as early prediction biomarkers in GC"

Response: Thanks for your advice. We have revised **Abstract** again, and the sentence " The expression of HNF4α and WNT5a might be used as early prediction biomarkers for prediction in the early stage of GC " in the **CONCLUSION** part has been corrected to "The expressions of HNF4α and WNT5a could serve as early diagnostic biomarkers for gastric cancer".

4.- "Archival human gastric cancer tissues…" correct to "Archived human gastric cancer tissues…"

Response: Thanks for your advice. We have polished the "**Sample collection and characterization"** section again.

Modification (Sample collection and characterization):

Human GC tissues were obtained from patients who presented between 2016 and 2018 at Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China). According to the WHO histological classifications, GC comprises signet ring cell carcinoma, and tubular, as well as mucinous adenocarcinoma. We initially selected wax blocks of paraffin-embedded GC and matched para-cancerous tissue blocks (n = 164 each) to reduce the influence of pathological WHO classification of GC on the expression of HNF4α, WNT5a, and β-catenin in GC tissues. However, six small gastric tumor tissues of insufficient quality were excluded. Therefore, we analyzed 158 GC (59 signet-ring cell carcinoma, and 35 tubular, and 64 mucinous adenocarcinomas) and 164 para-cancerous tissues. The gastric cancer tissues comprised 97, 46, and 15 samples of intestinal, diffuse mixed types according to the Lauren classification.

5.- "The co-expression of HNF4α and WNT5a in gastric cancer and the correlations with the clinicopathological characteristics in GC" correct to "Co-expression of HNF4α and WNT5a in gastric cancer and correlation with the clinicopathological characteristics". Similar changes in other headings.

Response: Thanks for your advice. All subheadings of the results section have been

corrected.

Modification (Results-subheadings):

1. Demographic characteristics of patients and pathological characteristics of gastric cancer sections
2. Expression of HNF4α in gastric tumoral tissues and para-cancerous tissues
3. Expression of WNT5a in gastric tumoral tissues and para-cancerous tissues
4. Relationships between HNF4α, WNT5a expression and different types of gastric cancer
5. Co-expression of HNF4α and WNT5a in gastric tumoral tissues and correlation with the clinicopathological characteristics
6. Expression of β-catenin in gastric tumoral tissues and para-cancerous tissues

6.-" And it still suggests that the expression of the two factors in gastric cancer is positively correlated both at RNA and protein level[17, 29, 32]" Unclear sentence.

Response: Thanks for pointing out. This sentence "And it still suggests that the expression of the two factors in gastric cancer is positively correlated both at RNA and protein level" has been corrected to " whereas others have found that HNF4α and WNT5a expression in GC positively correlates at the RNA and protein levels", in the new manuscript.

7. Legend to figure 1 "Figure 1. The expression of HNF4α in gastric cancer (GC) and para-cancerous tissue (PC). A、B: The representative figures of HNF4α expressed at different grades of pathological scores in GC (A) and PC (B), which is nuclear staining. C: The numbers of samples expressing HNF4α in GC and PC groups. Specimens were examined under a light microscope (200×)." correct to "Figure 1. Expression of HNF4α in gastric cancer (GC) and para-cancerous tissue (PC). Representative figures of HNF4α expression (nuclear staining) at different grades of pathological scores in GC (A) and PC (B). C: Numbers of samples expressing HNF4α in GC and PC groups. Specimens were examined under a light microscope (200×)" Similar changes are required in other figure legends.

Response: Thanks very much for your useful advice. We have corrected all figure legends as you suggested.

Modification (Figure legends):

Figure 1. Expression of HNF4α in gastric cancer tissues and para-cancerous tissues. Representative figures of HNF4α expression (nuclear staining) at different grades of pathological scores in gastric cancer tissues (GC) (A) and para-cancerous tissues (PC) (B). C: Numbers of samples expressing HNF4α in GC and PC groups. Specimens were examined under a light microscope (200× magnification). The same as Figure 2 and 3.

Reviewer #2: Comments to Authors: Authors explored the relationship between HNF4α/WNT/β-catenin signal and the development of gastric cancer in clinical patient specimens. Generally, it is an interesting study, however there are some comments and questions the authors should address all were detailed below:

Major corrections:

1. The introduction is too long and needs to be summarized.

Response: Thanks for your advice. We have rewritten the introduction and simplified it. See the revised manuscript for details.

2. Provide the refence for the used scoring system for interpretation of immunohistochemistry.

Response: Thanks for your useful advice. Our immunohistochemical scoring system was established according to the reference "HER2 in Gastric Cancer: a Digital Image Analysis in Pre-neoplastic, primary and metastatic lefties. Mod Pathol. 2013jun; 26(6):816-24". And this reference has been cited in the text at corresponding locations.

3. What was the criteria for selecting specimens from patients? Which type of gastric cancers was the aim of the study? What was the size of explored specimens?

Response: Thank you for your question. According to WHO classification criteria,

gastric cancer can be divided into tubular adenocarcinoma, signet-ring cell carcinoma and mucinous adenocarcinoma. In order to reduce the impact of gastric cancer classification (WHO classification) on the expression of HNF4α, WNT5A, and β-catenin in gastric cancer tissues, we tried to select relatively more and equivalent amounts of wax blocks of various types of gastric cancer tissues. However, since some gastric cancer tissues are too small to be selected for the next experiment, finally, 35 tubular adenocarcinomas, 59 signet-ring cell carcinomas and 64 mucinous adenocarcinomas were included in the experiment, and this study did not focus on which type of gastric cancer was involved. It is worth mentioning that when making pathological sections, we choose the largest cross-section of the wax block of gastric cancer tissue to ensure a large enough area.

Modification (Sample collection and characterization section):

Human GC tissues were obtained from patients who presented between 2016 and 2018 at Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China). According to the WHO histological classifications, GC comprises signet ring cell carcinoma, and tubular, as well as mucinous adenocarcinoma. We initially selected wax blocks of paraffin-embedded GC and matched para-cancerous tissue blocks (n = 164 each) to reduce the influence of pathological WHO classification of GC on the expression of HNF4α, WNT5a, and β-catenin in GC tissues. However, six small gastric tumor tissues of insufficient quality were excluded. Therefore, we analyzed 158 GC (59 signet-ring cell carcinoma, and 35 tubular, and 64 mucinous adenocarcinomas) and 164 para-cancerous tissues. The gastric cancer tissues comprised 97, 46, and 15 samples of intestinal, diffuse mixed types according to the Lauren classification. Pathologists from the Department of Pathology at Tongji Hospital determined the histological classification. The study was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Approval no: TJ-IRB20190501).

4. In results section: authors said" Gastric cancers are divided into tubular adenocarcinoma, signet ring cell carcinoma and mucinous adenocarcinoma

according to the WHO histological classification. In Table 3, HNF4α was strongly positive expressed in tubular and mucinous adenocarcinoma but was relatively weak in signet ring cell carcinoma" the total number of specimens presenting each type of these cancers wasn't mentioned in except in table 3??

Response: Thank you for pointing out our error. We have already mentioned the number of tissue sections for each type of gastric cancer in the **Sample collection and characterization section** in the newly revised manuscript. See the Methods-Sample Collection and characterization section in the revised manuscript for details.

5. Discussion is too long and also needs to be summarized

Response: Thanks for your advice. We have rewritten the discussion and simplified it. See the revised manuscript for details.

Reviewer #3: Scale bar for the figure picture may be added in Figures.

Response: Thank you for your suggestion. We have added the scale in all Figures. See the revised figure for more details.

Reviewer #4: This is an interesting and well-designed manuscript. However, this reviewer has some minor concerns,

1. There are some grammars throughout the text.

Response: Thank you for your comment. We have polished the manuscript again and corrected the grammatical errors, for example, the "prediction biomarker" was changed to "predictive biomarker", the "accurate targeted therapy" was changed to "accurately targeted therapy", and so on. See revised manuscript for more details.

2. Please provide all product codes in order to facilitate the reproducibility of these results, by other researchers.

Response: Thank you for your useful advice. Because this experiment mainly focuses

on immunohistochemistry of tissue sections, we have added all the detailed information of products used in the **MATERIALS AND METHODS** section.

Modification (**MATERIALS AND METHODS**--Immunohistochemistry staining section):

The paraffin-embedded tissues were cut into 4-μm-thick sections and mounted on triplicate APES-coated slides (**AR0001; Boster Biological Technology Co., Ltd., Wuhan, China**). The tissues were deparaffinized in xylene (**Hubei Jing-Hengye Technology Co., Ltd., Wuhan, China**) and rehydrated in graded ethanol (**National Medicine Group Chemical Reagent Co., Ltd.**). Endogenous peroxidase activity was quenched with a 3% hydrogen peroxide in methanol (**National Medicine Group Chemical Reagent Co., Ltd.**) at room temperature for 30 min, then the tissues were rinsed in phosphate-buffered saline (PBS) at pH 6.0 after antigen retrieval in 10 mmol/L citrate buffer (pH 6.0) at 94℃ for 8 and 10 min, the slides were immediately cooled for 20 min at room temperature. Nonspecific antigen binding sites were blocked by incubation with wash buffer containing 10% normal goat serum (**YJ0130; Shanghai Yanjin Biology Science and Technology Co., Ltd., Shanghai, China**) at 37℃ for 30 min. The sections were then incubated overnight at 4℃ with primary antibodies against HNF4α diluted 1:200 (**#3113; Cell Signaling Technologies, Danvers, MA, USA**), WNT5a diluted 1:80 (**abs113167; Abgent, San Diego, CA, USA**), and β-catenin diluted 1:100 (**#8480; Cell Signaling Technologies)**. Positive tissues were stained brown with the peroxidase substrate 3-3'-diaminobenzidine tetrahydrochloride (DAB) (**Hubei Biossci Biological Co., Ltd., Wuhan, China**) and all tissues were lightly counterstained with hematoxylin. Specimens were examined under a light microscope (200×magnification).

3. This reviewer missed a more detailed explanation about the significance in the context of the pathophysiology of gastric cancer of the results obtained.

Response: Thank you for your constructive advice. We have filled in the discussion section about the mechanism by which HNF4α and WNT5a affect the occurrence of gastric cancer and their importance. See the blue part of the DISCUSSION section for

details.