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Professor. Andrzej S Tarnawski

President and Editors-in-Chief “*World Journal of Gastroenterology*”

Title: Anoctamin 5 regulates the cell cycle and affects prognosis in gastric cancer

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Dear Prof. Andrzej S Tarnawski,

Thank you very much for your review of our manuscript listed above. Thank you for giving us an opportunity to submit a revised version. I hope that we have answered the reviewer comments adequately. I attach below the responses to reviewers' comments in a point-to-point manner.

We hope that our revised manuscript is suitable for publication in *World Journal of Gastroenterology*. I look forward to hearing from you soon.

Best Regards,

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Comments from Reviewer #1 (5177597):

In this study, the author performed a comprehensive discovery of Anoctamin 5 (ANO5) in

the regulation of tumor progression and the clinicopathological significance of its expression in gastric cancer. However, there are several major issues to be addressed.

1. In the selection of gastric cancer cell lines, the author selected five cell lines, but only two of them were used in the study (part of the study was only applied to one cell line), the author did not show or discuss the experimental results of other cell lines in the discussion.

Response:

We do appreciate the Reviewer's important points. To identify cell lines with high ANO5 expression, we first examined ANO5 expression in five human GC cell lines, MKN7, MKN45, MKN74, HGC27, and NUGC4, using RT-qPCR and western blotting. Among the five cell lines, NUGC4 and MKN45 cells with high ANO5 expression were selected for knock down study, MKN7 cells with ANO5 low expression were selected for overexpression study. However, the other two cell lines were not studied, so we should mention as a limitation.

According to the comment, we added the following sentences to the Discussion section (page 23):

“Secondary, in the selection of gastric cancer cell lines, we selected five cell lines, but only three of the them were used in the study.” (Discussion section, page 23)

2. The description of the experimental results on “ANO5 regulates proliferation and cell cycle in GC cells” and “ANO5 inhibits apoptosis in GC cells” seems repetitive and could be shortened without affecting content.

Response:

We do appreciate the Reviewer's important points. In accordance with the suggestion, we revised the heading of the Results section (page 14), as follows.

“*ANO5 regulates cell growth and survival in GC cells*” (Results section, page 14)

3. As for the “Effects of low-chloride conditions”, the author's experimental results can only prove the relevance between ANO5 and chloride, but can not demonstrate causality.

Response:

We do appreciate the Reviewer's important points. Our previous study identified that the culture in the Cl⁻-replaced medium (replacement of Cl⁻ by NO₃⁻) decreased the intracellular chloride concentration ([Cl⁻]_i) and inhibited cell growth in GC cells [28]. In the present study, the same experimental procedure was used as in the previous study, which may indicate a causal relationship between the expression of ANO5 and intracellular chloride levels.

According to the comment, we revised the sentences to the Results section (page 18), as follows.

“We previously reported that the culture in the Cl⁻-replaced medium (replacement of Cl⁻ by NO₃⁻) decreased the intracellular chloride concentration ([Cl⁻]_i) and inhibited cell growth in GC cells. [28]” (Results section, page 18)

4. FIG. 6a shows “Non-cancerous gastric epithelia were immunohistochemically stained using an anti-ANO5 antibody”, while FIG. 6b shows “Primary human GC samples were immunohistochemically stained using an anti-ANO5 antibody”. However, about the immunohistochemical results, FIG. 6A is deeper than FIG. 6b, which can not prove that ANO5 was predominant in the cell membranes and cytoplasm of GC tissue.

Response:

We do appreciate the Reviewer's important points. We sincerely apologize for the misleading wording. Immunohistochemical staining detected ANO5 expression in both non-cancerous stomach (Fig. 6A) and cancerous epithelium (Fig. 6B). In the present study, the expression of ANO5 in the plasma membrane and cytoplasm was assessed and scored in GC tissues. In addition, we also investigated the expression of ANO5 mRNA in normal gastric and GC tissue, and found that ANO5 expression was significantly increased in GC tissue compared to normal gastric tissue (Supplementary Fig. 1).

According to the comment, we revised the following sentences to the Results section (page 19):

“ANO5 was expressed in the cell membranes and cytoplasm of GC tissue.” (Results section, page 19)

5. The criteria for staining intensity score for the expression of ANO5 is not specified the specific location of gastric cancer tissue.

Response:

Thank you very much for your important comment. It would be appreciated if you could confirm the following in the manuscript (Materials and methods, page 11).

“ANO5 expression levels in immunohistochemically stained samples were semi-quantitatively graded based on the staining intensity and proportion of cytoplasm in stained cancer cells.” (Materials and methods, page 11).

‘Semi-quantitatively graded based’ means that both the staining intensity and the proportion of cytoplasm in stained cancer cells are taken into account. Furthermore, the staining intensity score for the expression of ANO5 was assessed the proportion of stained tumor cells as a percentage of the stained area in the whole cancer, rather than specific location of gastric cancer tissue.

Comments from Reviewer #2 (06152876):

This study explored the role of ANO5 in GC progression by conducting retrospective clinical study, in vitro functional assays as well as a brief mechanism investigation. IHC staining indicated the prognostic role of ANO5 in GC patient while functional assays indicated its oncogenic role in GC progression. Further microarray analysis suggested that ANO5 regulated the cell cycle progression by mediating the expression of cyclin-associated genes. Personally I find this manuscript both interesting and informative. However, several points have to be addressed before acceptance.

1. Regarding functional assays, there are a few concerns:

1). For the knockdown-based functional assay, it is more common to use two pairs of siRNA instead of one;

Response:

We do appreciate the Reviewer’s important points. In accordance with the suggestion, we performed knockdown-based functional assay using another siRNA (HSS137120).

We added new Supplementary Figure 3. We described the following sentences to the Materials and methods section (page8). Further, we added the sentences to the Results section (page 15), as follows.

“ANO5 siRNA (Stealth RNAi siRNA; HSS137119, Stealth RNAi siRNA; HSS137120) and control siRNA (Stealth RNAiTM siRNA Negative Control) were obtained from

Invitrogen.” (Materials and methods section, page 8)

“Another ANO5 siRNA (HSS137120) was used to assess the impact on cell growth and survival, with similar results to HSS137119 (Supplementary Figure 3).” (Results section, page 15)

2). To evaluate cell proliferation, I’m wondering if you could introduce CCK-8 to evaluate cell proliferation in the long run?

Response:

We do appreciate the Reviewer’s important points. In accordance with the suggestion, we additionally performed proliferation assay using Cell Counting Kit-8.

We revised new figure 2A. We described the following sentences to the Materials and methods section (page 8). Further, we added the sentences to the Results section (page 14), as follows.

“The cell proliferation activity was measured using the water-soluble tetrazolium salts-8 assay with Cell Count Reagent SF (Nacalai Tesque). NUGC4, MKN45, and MKN7 cells were seeded at density of 1.0×10^4 , 1.0×10^4 , 1.5×10^4 cells/well, respectively, on 24-well plates and incubated at 37°C in a 5% CO₂ incubator. siRNA was transfected 24 h after seeding. Cell proliferation was evaluated every 24 h by measuring the absorbance at 450 nm using Thermo Scientific Multiskan FC (Thermo Fisher Scientific).” (Materials and methods section, page 8)

“The results of the cell proliferation assay showed that the relative absorbance of GC cells with the control siRNA (NUGC4 and MKN45) was significantly lower than that of GC cells with ANO5 siRNA (HSS137119) (NUGC4 and MKN45) (Figure 2A, right panel).” (Results section, page 14)

3) ANO5 is dramatically down-regulated in MKN7. Have you ever considered performing overexpression-based functional assays?

Response:

We do appreciate the Reviewer’s important points. According to the comment, we additionally performed overexpression-based functional assay. We added new Supplementary Figure 2. We described the following heading and sentences to the Materials and methods section (page 8) and Results section (page 14, 16).

“Overexpression study

Control-HaloTag[®] plasmid (Promega, G6591) and ANO5-HaloTag[®] plasmid (pFN21AE5809) were transfected using P3000TM (Invitrogen) and lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. After passaging cells, ANO5-expressing cells were used for cell proliferation assay.” (Materials and methods section, page 8)

“We also conducted an overexpression study in MKN7 cell. ANO5 plasmid increased ANO5 mRNA levels (Supplementary Figure 2A, left panel).” (Results section, page 14)

“Whereas, ANO5 plasmid increased the relative absorbance of MKN7 cell (Supplementary Figure 2B).” (Results section, page 14)

“Further, ANO5 plasmid decreased CDKN1A/p21 mRNA levels (Supplementary Figure 2A, right panel).” (Results section, page 16)

2. Have you ever checked the online database (GEO, TCGA and etc.) or used tools (such as GEPIA2) to validate the significance of ANO5 in GC? If ANO5 is shown to be oncogenic in GC, these findings help consolidate the observations reported in the present study.

Response:

Thank you very much for your important comment. In the present study, we used the Kaplan Meier plotter as a tool to assess the correlation between gene expression and survival. The sources for this database include GEO, EGA, and TCGA. It would be appreciated if you could confirm the Supplementary materials (Supplementary Figure 7).

3. As both MKN45 and NUGC4 cell lines were used in the functional assays. I'm wondering why you didn't include MKN45 into the microarray analysis. As we could see in the mechanism studies, down-regulation of ANO5 significantly reduced not only phosphorylation level of Rb but also the expression level of normal Rb in MKN45 cell line, which was different from NUGC4 (Figure 4C). This indicates that there might be some inherent difference between these two cell lines when their ANO5 expression is ectopically dysregulated.

Response:

We do appreciate the Reviewer's important points. In addition to NUGC4 cells, the gene expression profiles of MKN45 cells following ANO5 silencing were examined using a microarray analysis.

We added new Supplementary Figure 4. We described the following sentences to the

Materials and methods section (page 9). Further, we added the sentences to the Results section (page 17), as follows.

“NUGC4 and MKN45 cells were transfected with control and ANO5 siRNA.” (Materials and methods section, page 9)

“In addition, the gene expression profile of MKN45 cells transfected with ANO5 siRNA were also investigated using a microarray. Gene expression changes in ANO5-depleted NUGC4 and MKN45 cells are shown in Supplementary Figure 4. Among the 21,440 genes, 7,246 genes were upregulated and 6,622 genes were downregulated in both cell lines, for a total of 13,868 genes (64.7%) with identical expression direction in NUGC4 and MKN45 cells. The direction of gene expression changes of gene related to “Cell Cycle: G1/S Checkpoint Regulation” was consistent in both cell lines (Supplementary Table 1). Furthermore, all 40 genes displayed in Table 1 showed the identical expression direction in ANO5-depleted MKN45 cells (Supplementary Table 2). These results support that ANO5 affects the cell cycle by similar mechanisms in both NUGC4 and MKN45 cell lines.” (Results section, page 17)

4. The resolution of figure 4A is too low to read.

Response:

Thank you very much for your important comment. In accordance with the suggestion, we revised the resolution of Figure 4A.

5. Legend of figure 4D, “The down-regulation of ANO5 suppressed the phosphorylation of the JNK protein in NUGC4 and MKN45 cells. “I think it should be “increase”.

Response:

We do appreciate the reviewer’s important points. According to the comment, we revised the legend of figure 4D, as follows.

“The down-regulation of ANO5 increased the phosphorylation of the JNK protein in NUGC4 and MKN45 cells. Mean $\pm$ SEM. n = 3. *p<0.05 (significantly different from control siRNA).” (Legend of figure 4D, page 38)

6. As you have focused on CDK2 and JNK phosphorylation and proposed that ANO5 regulated the cell cycle via the up-regulation of p21 through activating JNK cascade, have you ever considered conducting further validation studies by introducing reverse

approaches?

Response:

We do appreciate the Reviewer's important points. According to the comment, we performed RT-qPCR using NUGC4 and MKN45 cells incubated with JNK inhibitor.

We added new Supplementary Figure 5. We described the following heading and sentences to the Materials and methods section (page 12-13). Further, we added the heading and sentences to the Results section (page 17-18), as follows.

"JNK signaling pathway inhibitor treatment"

To block JNK signaling pathway, the NUGC4 and MKN45 cells were incubated with JNK inhibitor SP600125 (10 μ m, ab120065, Abcam) according to manufacturer's instructions. The cells were divided into 3 groups: control group, ANO5 siRNA group, JNK inhibitor group (ANO5 siRNA + SP600125). The cell proliferation was detected every 24 h after ANO5 silencing." (Materials and methods section, page 12-13)

"ANO5 inhibits the JNK pathway" (Results section, page 17)

"To elucidate the regulatory role of ANO5 on JNK signaling pathway in GC cells, the phosphorylation of JNK protein was examined." (Results section, page17)

"Furthermore, the increase of CDKN1A/p21 mRNA expression induced by ANO5 silencing in NUGC4 and MKN45 cells was suppressed by JNK inhibition (Supplementary Figure 5, lower panel). Whereas, treatment with JNK inhibitors made no difference in ANO5 mRNA expression (Supplementary Figure 5, upper panel)." (Results section, page 17-18)

Comments from Reviewer #3 (05469117):

Thank you for giving me a chance to review this research regarding the role of Anoctamin 5 (ANO5) in the regulation of tumor progression and the clinicopathological significance of its expression in gastric cancer (GC). The results obtained indicate that ANO5 plays a significant role in the cell cycle of GC cells, especially in the progression of G1/S checkpoint, and a high expression level of ANO5 was identified as a poor prognostic factor in GC patients. The present study demonstrated that ANO5 has potential as a poor prognostic biomarker and a novel therapeutic target for GC. This paper is a well-written, My major comments are as follows:

1. On page 10, in the last paragraph "Tumor staging was conducted according to the International Union Against Cancer (UICC)/TNM Classification of Malignant Tumors (8th

edition) [39]. "The reference 39 here is marked incorrectly. It should be 38. Please check the correctness of other references.

Response:

We do appreciate the reviewer's important point. According to the comment, we revised the following sentences to the Materials and methods section (page 11) and Results section (page 17).

"Tumor staging was conducted according to the International Union Against Cancer (UICC)/TNM Classification of Malignant Tumors (8th edition) [38]." Materials and methods section, page 11)

"JNK has been shown to stabilize the p21 protein through phosphorylation [39]." (Results section, page 17)

In addition, we checked the correctness of other references.

2. In Table 3, why is the 5-year overall survival rate the lowest (73.9 %) when the cutoff value is 1.3?

Response:

We do appreciate the reviewer's important point. The histograms of ANO5 IHC score did not show normal distribution and the median score is low (0.9). Therefore, we chose the minimum P-value method to determine the cut-off value. With regard to the method for determining cut-off values, we added a reference reviewing about minimum P-value method [41].