ANSWERING REVIEWERS

January 14, 2014

Dear Prof. Lian-Sheng Ma,
President and Editor-in-Chief *"World Journal of Gastroenterology"*

Please find enclosed the edited manuscript in Word format (file name: ESPS Manuscript No. 6811 WJG.doc).

Title: Roles of the $Na^+/K^+/2Cl^-$ cotransporter NKCC1 in cell cycle progression in human esophageal squamous cell carcinoma

Author: Atsushi Shiozaki, Yoshito Nako, Daisuke Ichikawa, Hirotaka Konishi, Shuhei Komatsu, Takeshi Kubota, Hitoshi Fujiwara, Kazuma Okamoto, Mitsuo Kishimoto, Yoshinori Marunaka, Eigo Otsuji

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 6811

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
Comments from Reviewer 1:
1) The results demonstrated that no correlation was found between NKCC1 expression and Ki-67 labeling index in IHC of ESCC, however, the NKCC1 really plays a role in cell proliferation as shown in vitro experiment, so this is why? Which need to give a reasonable explanation in discussion section.
Response:
According to the reviewer's comment, we revised the Discussion part (page 15-16), as follows. Further, we added the new reference, No.30.
"Our results demonstrate that no correlation was found between NKCC1 expression and the Ki-67 labeling index in immunohistochemical studies of ESCC expression. Ki-67 is commonly used to assess cell proliferation, and this factor reacts with a nuclear antigen present throughout the cell cycle (late G_1, S, G_2, and M phase) of proliferating cells but is absent from quiescent (G_0) cells [30]. In the present study, we found that NKCC1 plays an important role in the G_2/M phase of the cell cycle. The different rates of progression through each phase of the cell cycle may explain why no correlation was found between NKCC1 and Ki-67 expression, although further studies will be needed with a larger sample size to confirm these observations." Discussion part (page 15-16)

2) The manuscript just demonstrated the down-regulation of NKCC1 in KYSE170 cells, if the work of NKCC1 up-regulation in other low expression cells such as TE2 and TE5 had been done, and the results of which indicated the upregulation of NKCC1 could promote the cell proliferation, the work might be more perfect. Of course, this is just a suggestion for further research. For this paper, the work is enough to prove the relationship between NKCC1 and cell cycle progression.

Response:
Thank you for the precious comments and suggestions. We also realize the importance of the work of NKCC1 up-regulation, and try to perform further research in future.

Comments from Reviewer 2:
1) Recommend subgroup analysis for survival. Since pathologic stage has very large impact in survival, the no difference in 5 year survival may be due to difference in the two groups with different percentage of stage I patients. Analysis of just stage I patients may provide insight into the clinical relevance of this finding.
Response:
According to the reviewer's comment, we performed subgroup analysis of pStage I patients. We revised the Result part (page 11), as follows.
"Subgroup analysis of pStage I patients showed that the 5-year survival rate of the high grade group (86.5 %) tended to be lower than that of the low grade group (100.0 %), although no significant difference was observed (p=0.403, the log-rank test)." Result part (page 11)

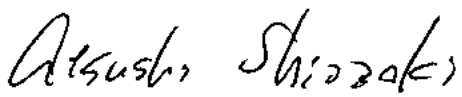
2) Recommend adding information about the impact of NKCC blocker on proliferation of other cell lines. Is this effect just on the KYSE170 cells or is it also seen in other five cell lines.
Response:
We examined ESCC cell lines with NKCC1 expression, TE9, TE13 and KYSE70 to determine the inhibitory effect of the NKCC blocker, furosemide on the proliferation (Supplementary Fig. 2). We revised the Result part (page 12), as follows. Further, we added new Supplementary Fig. 2 and Supplementary Figure legend.
"Similar trends were found in several cell lines, including TE9, TE13 and KYSE 70, which expressed NKCC1 (Supplementary Fig. 2)." the Result part (page 12)

3) Recommend determining the RT-PCR results of six genes for other five cell lines. Overall, the manuscript can be improved if the authors show that this is a generalized phenomenon with all ESCC rather then just KYSE170. If it is just shown with KYSE170, please limit the conclusion to just this cell line. Can not make a generalized statement that NKCC1 played a role in proliferation of ESCC cells if it only applies to one cell line.
Response:
We examined ESCC cell lines with NKCC1 expression, TE9, TE13 and KYSE70 to determine the expression change of six selected genes (MAD2L1, DTL, BLM, CDC20, BRCA1, and E2F5) by the knocking-down of NKCC1 (Supplementary Fig. 4). We revised the Result part (page 13), as follows. Further, we added new Supplementary Fig. 4 and Supplementary Figure legend.
"Similar trends were found in several cell lines, including TE9, TE13 and KYSE 70 which expressed NKCC1 (Supplementary Fig. 4)." the Result part (page 13)

3 References and typesetting were corrected

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

Sincerely yours,



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