Author's response to reviews

**Title:** Knockdown of anterior gradient 2 expression extenuates tumor-associated phenotypes of SNU-478 ampulla of Vater cancer cells

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**Version:** 5  
**Date:** 4 October 2014

**Author's response to reviews:** see over
Dear Dr. Kominsky

RE: Revision of MS #1652078512133134, BMC Cancer

I would like to begin by thanking you for providing us the opportunity to revise our manuscript entitled ‘Knockdown of anterior gradient 2 expression extenuates tumor-associated phenotypes of SNU-478 ampulla of Vater cancer cells’. We greatly appreciate the comments of the reviewers, which have helped us improve the quality of our manuscript significantly.

Major changes in the revised manuscript are as follows:

1. Main text including references.
   The ‘Abstract’ was revised to include new results of BrdU incorporation assay.
   The ‘Background’ was revised to introduce study results of Lepreux et al. (2011) suggested by reviewer #1.
   The ‘Methods’ was revised to include new experimental procedure in the section ‘Cell viability and BrdU incorporation assays’. The section ‘Establishment of stable transfectants’ was also revised to clarify cell clones used in the experiment.
   The ‘Results’ was revised to include new results of the BrdU incorporation assay and western blotting to accommodate recommendations of editor and reviewer #1.
   SNU-869:VEC is now referred as SNU-869:AGR2-N.
   The ‘Discussion’ was modified to comment on the new results and to comply with critiques of reviewer #2.
   Two references (Verma et al., 2012; Hong et al., 2013) were newly added according to the modified text.
   The main text and figure legends were also modified in wording upon suggestions of peers to improve readability.

2. Figures and figure legends
   Figure 1B was modified to include new western blot result.
   Legends of individual bars were inserted inside the Figure 2F as suggested by reviewer #1.
   Figure 4D for BrdU incorporation assay was newly added to accommodate recommendation of reviewer #1.
   Figure 4D in the original manuscript is referred as Figure 4E.
   Figure legends were revised so that they could correspond to the modified figures.
According to your directions and review’s recommendations, we have revised the manuscript extensively. On the attached pages you will find detailed responses to the reviewers’ comments.

We have endeavored to address the reviewers’ concerns in our revised manuscript. Considering the novelty and potential impact of the current report, we believe that this article is suited well for publication in ‘BMC Cancer’. Moreover, with the revisions suggested by the reviewers’ we believe our paper is significantly improved. We trust that you and the reviewer’s will consider our modified paper worthy of publication in ‘BMC Cancer’.

Once again, we appreciate your generosity to allow us to revise this manuscript. We also thank you in advance for handling our paper.

Sincerely yours,

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Responses to Editor and Reviewer Comments:

1. **Reviewer #1 comment 1-minor**: The authors claim in the introduction (lines 118-121) that there have not been any studies regarding AGR2 expression in biliary tract cancers, but Lepreux et al. (Liver Int 2011, 31:322-328) describe AGR2 expression during morphogenesis and carcinogenesis of the biliary tree with human tissue specimens. The results of this study should be included in the introductory material. This is important because without the knowledge that AGR2 is indeed expressed in human biliary tree cancer these in vitro studies have limited relevance.

   Thank you for this suggestion. The paragraphs have been modified as follows in order to address this concern:

   “Despite that AGR2 is implicated in tumorigenesis and tumor progression of various cancers, AGR2 expression and its tumor-promoting role in biliary tract cancers have not yet been studied in detail. AGR2 is reported to be expressed in normal tissues of the biliary tract and the expression pattern is conserved in biliary tract cancer [23]. However, the expression and tumor-promoting function of AGR2 in biliary tract cancer cells have not been investigated to date. Thus, this study aimed to analyze the expression and functional role of AGR2 in development and maintenance of tumor phenotypes of biliary tract cancer cells. To this end, we determined AGR2 expression in six biliary tract cancer cell lines. In addition, tumor-promoting activity of AGR2 was examined by knockdown of AGR2 expression with shRNA and its overexpression in AGR2-positive SNU-478 and AGR2-negative SNU-869 ampulla of Vater cancer cell lines, respectively.”

2. **Reviewer #1 comment 2-discretionary**: In Figure 1, do the authors know how the levels of AGR2 compare to those found in well described AGR2-expressing cells such as MCF-7 after E2 treatment? Without any kind of reference it is difficult to know whether these levels are very low or reasonably robust.

   Thank you for pointing this out. In response to this comment, we have compared AGR2 protein level in the six biliary tract cancer cells and MCF-7 breast cancer cell. Fig. 1B has been modified to include the new western blot result. We found that AGR2 protein level in SNU-245 was comparable to that in MCF-7 cells. We have modified ‘Methods’ and ‘Results’ to accommodate the result.

3. **Reviewer #1 comment 3-major**: The authors describe an effect of AGR2 on cell number in both MTT and colony forming assays. This could be due to an effect on cell proliferation or cell death. The authors should determine whether AGR2 controls cell proliferation in these cells. In the mammary gland and in breast cancer cells, AGR2 controls cell proliferation as shown by Verma et al. (Dev Biol. 2012, 369:249-260).

   Thank you for this recommendation that helped us clarify the mechanism of increased cell viability in AGR2-overexpressing SNU-869 cells. We have carried out pulsed BrdU incorporation assay as recommended by you and included the result in Fig. 4 (Fig. 4D). We have modified ‘Methods’, ‘Results’, ‘Discussion’ and ‘Figure legends’ according to
the change. We have also added a new reference in ‘References’ that we used as a protocol guide (Verma et al., 2012)

**In ‘Methods’ section:**

“Cell proliferation was analyzed by BrdU incorporation assay as described in Verma et al. [26] with minor modification. SNU-869 stable transfectants were plated at 3 X 10^5 cells in a 10-cm culture plates. The cells were grown for four days and pulsed with 20 μM BrdU for 30 min. Harvested cells by trypsinization were washed with PBS containing 1% FBS twice and fixed in 70% ethanol for one hour at -20°C. After washing with PBS, the fixed cells were permeabilized by incubation in 2 M HCl for one hour at room temperature and then washed with PBS three times. The cells were divided into two aliquots and treated with FITC-α-BrdU (BD Biosciences, San Jose, CA, USA) or FITC-mouse IgG1 κ isotype control (eBioscience, San Diego, CA, USA) in dark for 45 min at room temperature. Then, the cells were washed with PBS twice and resuspended in 500 µL of 1.5 µg/mL 7-AAD in PBS. Fluorescence signals of FITC-α-BrdU and 7-AAD were measured by flow cytometry using a FACSCalibur™ (BD Bioscience), and the data were analyzed with CellQuest Pro software (BD Bioscience).”

**In ‘Results’ section:**

“In order to determine the mechanism of the elevated cell viability in the SNU-869:AGR2 cells, cell proliferation rate was measured by pulsed BrdU incorporation assay. Percentage of BrdU-positive cells was 8.4% higher on average in 7-AAD-positive SNU-869:AGR2 cells compared to 7-AAD-positive SNU-869:AGR2-N (P = 0.053). Moreover, percent BrdU-positive cells normalized against SSC were 8.8% higher on average in SNU-869:AGR2 cells compared to SNU-869:AGR2-N (21 ± 2.4 in AGR2-N vs. 30.0 ± 5.9, 26.9 ± 4.1 and 32.4 ± 6.3 in AGR2-1~3, respectively, P = 0.036). These results suggest that the enhanced proliferation rate could account for the viability increase of the SNU-869:AGR2 cells.”

**In ‘Discussion’ section:**

“AGR2 expression in SNU-869 enhanced cell proliferation rate that should result in the increased cell viability measured by MTT assay as in normal mammary epithelial cells and in various cancer cells [9, 13, 26, 30].”

**In Figure legends:**

**Figure 4. D.** The effect of AGR2 overexpression on cell proliferation. Pulsed BrdU incorporation into the SNU-869 stable transfectants was measured by flow cytometry. Percentage of BrdU-positive cells was shown in mean ± standard deviation of four experiments (ANOVA P = 0.053; post hoc analysis by Bonferroni testing: AGR2-2 vs. AGR2-N, P = 0.027). Results shown are representatives of four experiments.

4. Reviewer #1 comment 4-minor: Figure 2F would be improved with a “legend” inside the figure itself to describe what each bar represents.

Thank you for this suggestion. In response to this suggestion, we have modified the Fig. 2F to locate legends for individual bars inside the figure.
5. Reviewer #1 comment 5-minor: The authors should explain the rationale for only using the SNU-478:KD2 cells for xenografts. Did the authors also inject SNU-478:KD1 and KD3 cells, and if so, what were the results of these xenografts?

Thank you for pointing this out. In fact, we did not include SNU-478:KD1 and KD3 cells in the xenogeneic tumor model because we thought that SNU-478:KD2 could be a representative among the AGR2-silenced SNU-478 clones. We apologize for not being more extensive. Instead, we have attempted to provide a rationale for the experiment in ‘Results’ as follows.

“Finally, we examined the effect of AGR2 expression on in vivo tumor formation by injecting an equal number of SNU-478:VEC and SNU-478:KD2 cells into BALB/c-Sl-cnu/nu immunocompromised mice. SNU-478:KD2 manifested all of the phenotypic characteristics resulted from knockdown of AGR2 expression in SNU-478:KD cells representatively (Fig. 2A).”

6. Reviewer #2, comment 1: The only concern this reviewer has is a minor one. Basically, the last 3 paragraphs of the discussions should be strengthened. As currently written, they do not convey a clear message, and this minor essential revision will be helpful to the readers of the journal and will strengthen the paper.

Thank you for your thoughtful comment that helped us improve the logic of the paragraphs. Based on your comment, we have massively modified the last three paragraphs of ‘Discussion’ as follows.

“The mechanism underlying the AGR2-associated tumor promotion has been investigated recently. AGR2 silences UV-induced p53 transactivation activity by attenuating p53 phosphorylation in H1299 cells [6]. In addition, overexpression of AGR2 upregulates genes that are involved in cell proliferation, invasion and angiogenesis [9]. Meanwhile, knockdown of AGR2 expression results in downregulation of cyclin D1, survivin, and c-Myc [30]. Moreover, AGR2 expression is suppressed by SMAD4 that is a tumor suppressor of pancreatic ductal adenocarcinoma [31]. In addition, induction of AGR2 expression by tamoxifen through AKT or Src has been implicated in tamoxifen resistance of breast cancer cells [32]. Both AGR2-mediated inhibition of p53 tumor suppressor activity and AGR2-associated expression of genes that regulate cell growth and survival may directly contribute to the tumor-promoting activity of AGR2 through augmented cell proliferation and survival. Meanwhile, increased invasiveness and metastasis associated with AGR2 expression might be the result of regulation of cathepsins B and D expression, enhanced angiogenesis and modulation of extracellular matrix by AGR2 [31, 33, 34]. It is plausible that changes in the AGR2-associated gene expression regulate the tumor phenotypes of the biliary cancer cells including viability, anchorage-independent growth and invasiveness. Therefore, initial clues to determine the mechanism for the AGR2-enhanced tumorigenic potential and drug resistance of the biliary cancer cells can be obtained by detailed investigation in the AGR2-associated alterations in gene...
Although AGR2 expression was found to enhance tumor-associated phenotypes of the biliary tract cancer cells, caution should be taken to correlate these results with tumor progression and prognosis of biliary tract cancer patients directly. An immunohistochemical analysis of AGR2 expression pattern demonstrated that AGR2 expression in the biliary tract is not tumor specific, but is associated with anatomical location and mucin-secreting phenotype [23]. An independent study on AGR2 expression in tissue specimens of biliary tract cancer patients revealed that AGR2 expression was higher in tumors of lower stage and advanced differentiation (manuscript under revision). Such discrepancy between in vitro tumor-promoting role of AGR2 and tumor progression in patients has also been reported in pancreatic cancer studies. Whereas knockdown of AGR2 in pancreatic cancer cells decreases cell proliferation and invasion, and increased drug sensitivity [19], AGR2 expression in tissue samples of pancreatic cancer patients is positively correlated with differentiation status of the cancer [35]. In this case, aberrant AGR2 expression in poorly differentiated cancer is correlated with worse prognosis of the patients, suggesting that tumor promoting role of AGR2 is specific to tumor type and stage [35]. Lack of correlation between in vitro tumor-promoting activity of AGR2 and pathologic findings in biliary tract cancer patients is an issue to be resolved. It is conceivable that uncoupling of AGR2 expression from tumor progression of biliary tract cancers can be ascribed to peculiarity of biliary tract cancers including cancer microenvironment. Since AGR2 manifested tumor promoting activity in ampulla of Vater cancer cells, pathologic relevance of AGR2 expression in carcinogenesis of the ampulla of Vater should also be investigated in detail. An orthotopic biliary tract cancer model in which AGR2 expression can be modulated specifically in the biliary tract might be required to provide more definitive evidences on in vivo tumor-promoting function of AGR2 in biliary tract cancers.

By analyzing AGR2 expression and its tumor-promoting role in vitro and in vivo, we have provided evidences for tumor-promoting activities of AGR2 in ampulla of Vater cancer cells for the first time. AGR2 is thought to promote tumor formation by augmenting cell viability, anchorage-independent growth, and invasive properties of the biliary tract cancer cells. The mechanisms underlying the AGR2-associated tumor promotion in the biliary tract cancer cells and the observed discrepancy in the tumor promoting potential of AGR2 in different cells remain to be elucidated in future studies in which AGR2 knockdown SNU-478 or AGR2 overexpressing SNU-869 could be utilized as a valuable model system.”

7. Editor's comment: We would be happy to consider a revised version of this manuscript following amendment in accordance with the reviewer's suggestions. I would also like to echo one reviewer comment concerning Figure 1, which is to include the expression of AGR2 in a reference sample(s) (eg."normal" biliary cells) for comparison to that in the biliary cancer cell lines.

First of all, thank you for providing us the opportunity to revise our manuscript. We also thank you for this suggestion. It was not successful for us to obtain normal biliary cells. Instead, we decided to use AGR2 protein level in MCF-7 breast cancer cells as a
reference amount. Human AGR2 was identified in MCF-7 cells for the first time by Thompson and Weigel (1998). AGR2 protein level in six biliary cancer cell lines and MCF-7 cells was compared by western blot analysis and Fig 1B in the previous version has been replaced with the new result.

8. Quality of written English-Reviewer #1: The manuscript is generally well written, but would benefit from editing for grammar and spelling (for example, pancreatic is misspelled on line 371).

Thank you for pointing this out. We apologize for this oversight on our part. In order to avoid grammatical error and to improve readability, we have endeavored to obtain advices from two of our peers. We have made corrections based on their comments. We hope that you will find the readability and flow of the revised manuscript to be greatly improved.