Author's response to reviews

Title: The role of the REG4 gene and its encoding product in ovarian epithelial carcinoma

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Author's response to reviews: see over
Reviewer: Stefan Enroth

Reviewer's report: Chen and colleagues presents a well-written manuscript addressing the role of the REG-4 gene expression and protein abundance in ovarian cancer. The authors study this in a series of cell lines representing different stages of the disease and in tumor material from patients. Higher expression and protein abundance levels are found in some tumors and cell lines compared to normal tissue and higher expression is also found to be associated with poorer survival rate in patient material. The claims made are justified by the data presented.

Major Compulsory Revisions

1. Line 269-271 & Table 2: This is the first mentioning of how many samples that were collected from the patients and the grouping of them based on tumor subtype. Table 2 lists some of the properties of the samples, but the baseline characteristics are missing. How many of the samples were fresh-frozen and how many where fixed in paraffin? Where there any difference in proportion of the tumor types in either of these categories? Where there any differences in age distribution of the samples in the different tumor categories? Why was age = 56 chosen as a divider in Table 2? What does the P-value in the last column in Table 2 denote? Which test was used? How was the classification into the differentiation classes done?

Answer: 123 samples were fresh-frozen, which were used for RT-PCR, while 337 samples fixed in paraffin, which were used for IHC. 56 years was the median age of the samples, as so, it was chosen as a divider in Table 2. P-value in the last column in Table 2 represent for Mucinous adenocarcinoma compared with other pathological subtypes, we had revised it. T-test was used for the calculation. The classification into the differentiation classes of all the samples (HE staining) were read by physiologists, according to the tumor cell differentiation grades.

Minor Essential Revisions

2. Line 114-115: The cell lines where kept in 3 different media with the two cell line with the lowest expression (Figure1A: ES-2 and SKOV3) where kept in the same media (McCoy) different from all others. The media used all have slightly different composition, for example in glucose concentration. Could the different media have an effect on the expression patterns seen?

Answer: Thank you so much for your professional question. The different media may have slightly influence to the REG4 expression in the ovarian cancer cell lines, and we had once tried to use the same medium for all cell lines, however, we found some cell lines can not keep normal growth rate, so we finally decide to use relative medium according to ATCC. In our previous in gastric carcinoma, we found that REG4 expression was low expressed in gastric carcinoma cell lines MKN28 and KATO-III, but they were all maintained in RPMI 1640, while MKN45 had significantly high REG4 expression but also maintained in RPMI 1640, so we suggest different medium may influence, but cannot be easily eliminated.

3. Line 166: Is there anything known about the degradation of the proteins analyzed here over time? The samples collected spans 8 years and this could be an influencing factor. This could be checked by stratifying on tumor type and then performing e.g. regression analysis on the abundance levels with time (e.g. in years) since sample collection date.

Answer: Samples derived from the patients were frozen immediately in -80°C until protein extraction. We used hierarchical test on the abundance levels with time according to your suggestion, and we found that there were no significant difference between different years. Thank you so much for your professional question.

4. Line 168: Serum levels of REG-4 have been showed to be dependent on the subject’s age in
control samples (PMID: 22866070). Since you have a big age-span in your samples, you should check (same procedure as above with sample date) if there is an influence on your abundance levels stemming from age alone.

**Answer:** Spearman’s rank correlation test showed significant correlation between REG4 expression in normal ovary tissues and ages ($r=0.436$, $p=0.026$, Figure 6A), and between ovarian carcinomas and ages ($r=0.144$, $p=0.027$, Figure 6B), when combined together, REG4 expression was also correlated with ages($r=0.0161$, $p=0.09$, Figure 6C). 0 stand for negative REG4 protein expression, 1 stand for +, 2 stand for ++, while 3 stand for +++.

**Figure 6**

5. Line 238: The description of the multivariate analysis is scarce. How many covariates were included? If more than one, the p-value cut-off should be adjusted for multiple hypothesis testing.

**Answer:** Thank you so much for your professional question. Cox’s proportional hazard model contains multivariate factors such as Age, Pathological classification, FIGO staging, Differentiation and Ki-67 expression, the p-value cut-off was used for multiple hypothesis testing just as you told us.

6. Line 263: Please refrain from using statements like “there was no correlation” without providing justification. E.g. calculate $R^2$ for the datasets using the proper method. Note for instance that the Pearson’s statistics is sensitive to outliers and not always appropriate.

**Answer:** Thank you so much and we had revised it into “$R^2= 0.022$, $P = 0.278$” using Spearman’s rank correlation test according to your suggestion.

7. Line 271: What does “expression was statistically higher” refer to? Please provide the proper statistics for this statement.

**Answer:** Sorry, and we had revised it into “expression was significantly higher”.

8. Line 276: Please refrain from stating that there are correlations without providing the statistics on it (including p-values).

**Answer:** We had revised it into “REG4 expression was significantly higher in well- and moderately differentiation than poor differentiation ($P = 0.009$), and in age $\geq 56$ years than $< 56$ years ($P = 0.03$), but no significantly differences in FIGO staging or expression of Ki-67, a proliferative marker ($P > 0.05$)”, thank you so much.

9. Line 280: Which criteria were used for stratifying the REG-4 expression?

**Answer:** Age, Pathological classification, FIGO staging, Differentiation, Ki-67 expression were used for stratifying the REG-4 expression.

10. Line 291-292. These two sentences don’t make any sense. Please rephrase.

**Answer:** We had deleted it according to your suggestion.

11. Line 479: The legend specifies “positive correlation”, although the ordering of the bars in the figure (from “Well” to “Poorly”) suggests a negative correlation in the sense that poor is low and good is high. Please also provide statistics and measures on the correlation.
Answer: Sorry for our written mistake, we had revised it into REG4 mRNA expression is seen to be higher expressed in well- and moderately differentiation than poor differentiation. Mann-Whitney U test was used to differentiate the means between the different groups.

Answer: We had revised the sentence according to your suggestion.

13. Figures: It’s hard to read the labels inside the panels. The small text in Figure 2A is impossible to make out. Please use larger fonts when the text is essential and remove otherwise.
Answer: Thank you so much for your reminding us that, we had revised the figure according to your suggestion.

14. Throughout: Please write out the actual p-values instead of P < 0.05. Since many tests are being made it is of interest to the reader to know if the statistics are clear or borderline.
Answer: We had written out the actual p-values according to your suggestion.

Discretionary Revisions

15. Line 33-34: last part of the sentence (starting with “subjected to phenotypes’ measurement”) doesn’t make sense to me and should be rewritten.
Answer: We had revised it into “subjected to cell phenotypes’ measurement”, which includes cell cycle, cell apoptosis, cell invasion and metastasis, and so on.

16. Line 78: REG-4 have also been shown to be up-regulated in HepG2 after 1h stimulation of TGF-B (PMID: 24771338).
Answer: We had referenced this article and revised our manuscript, thank you so much.

17. Line 81: There is a “:” right after “[8]”, typo?
Answer: Sorry for our mistake, and we had revised it into “.”, thank you so much.

18. Line 206: Consecutive. Where these samples collected using consecutive sampling? None was excluded?
Answer: Thank you for your reminding us our mistake, we had deleted “Consecutive”.

19. Line 245: Space missing in “ 50nM/ml(Figure 1)”
Answer: We had revised it according to your suggestion.

20. Line 246: Verb missing. Perhaps “SKOV3 cells where chosen to be transfected”?
Answer: Thank you for your reminding us that and we had revised it into “SKOV3 cells where chosen to be transfected”.

21. Line 465-466: There seem to have been a change in font size?
Answer: We had revised it into “Times New Roman” according to your suggestion.

Reviewer: Rebecca T Marquez

Major Compulsory Revisions:

1. Your study suggests significant differences in histotype associated with Reg4 expression. According to your cell line data, ES-2 cells (clear cell carcinomas) do not have upregulation of REG4. Can you further detail the “miscellaneous subtypes” you examined for Table 2. Are any of these clear cell? Because Huang et al, has already shown that mucinous tumors have an increase in REG4. This paper would be strengthened by showing a novel finding where clear cell cancers have low REG4 and correlate this will survival rates comparing clear cell and mucinous cancers.
Answer: Thank you so much for your professional question, among the 10 clear cell cancers in the miscellaneous subtypes, only one of them had survival result, so it’s mysterious does it correlate with survival rates, more samples were needed to make a conclusion.
2. This study would be strengthened by measuring REG4 in metastatic lesions in order to confirm the author’s conclusion that REG4 expression correlates with poor outcome. This is especially important since there are not differences in REG4 expression between stage I and stage IV cancers and is less expressed in tumors versus benign.

**Answer:** We measured REG4 expression in metastatic lesions and relative primary cancer lesions, and we found that there was no significant difference of REG4 expression between primary cancer and relative metastatic lesions. P=0.076, n=34 (Table 1). We had revised the manuscript and results part according to your suggestion.

**Minor Essential Revisions:**

1. Figure 1 is too tight. Especially figure 1d and 1e are overlapping. 1b needs labels on western. Figure 1c needs to have the amount of REG4 protein in legend in increasing order to match graph. page 13 line 245. 50nM/ml needs to be changed to 50 nM. nanomolar concentration is nanomoles/ml. Also occurs in figure legend. Change throughout manuscript.

**Answer:** We had revised the Figure and Manuscript according to your suggestion, thank you so much, we really appreciate your helping us that.

2. Figure legends and methods need to be in more detail. Please include the definition of controls. Please describe how the experiment was carried out in more detail in the results section. i.e. what was compared.

**Answer:** We had revised the Figure legends, results and methods according to your suggestion.

3. Figure 3. What is CTR(SKOV3)? How did you perform your mock treatment? Why is there such a difference in migration between CTR and Mock? please make mock image brighter. This does not appear to be reflected in the migration index. The labels are spelled wrong for migration index graph. I believe it would be better to separate the graphs based on the similar experiment that is being performed. i.e. mock and Reg4 protein should be next to each other since these are what is being compared. Make separate figures or place a space between these two pairs. Discuss in text in more detail.

**Answer:** CTR means normal SKOV3 cells without any treatment, mock cells means cells transfected with pCI-REG4-Mut plasmid. Our data showed that there were no significant difference in migration between CTR and Mock. We had revised the labels into REG, thank you for your reminding. According to your suggestion, we had separated the graphs into Ctr vs. REG4 protein, Mock vs. SKOV3/REG4.

4. It is not necessary to list the “n” for each experiment in the results section or the figure labels. Instead please move this to the figure legend for easy reference.

**Answer:** We had revised it and move this to the figure legend according to your suggestion.

5. Figure 4. please state the statistical tests you performed in the figure legend and what is being compared. Also, please discuss why benign lesions have a higher Reg4 expression than cancer. Are these differences statistically significant? please rewrite the results section describing this figure, for Example, page 14 line 260. “Expression of REG4 mRNA were also higher in mucinous benign tumors and carcinomas compared to serous ones (P < 0.05; Figure 4B). are the mucinous combined benign tumors and cancers? if so, these should be separated. Please clarify.

**Answer:** Thank you so much for your professional question. According to your suggestion, we had revised Figure 4B. Expression of REG4 mRNA was higher in mucinous benign tumors (n=7) compared to serous benign tumors (n=6), besides, REG4 mRNA expression was also higher in mucinous carcinomas (n=7) compared to serous carcinomas (n=52), (P < 0.05; Figure 4B).
However, there was no significant difference between mucinous benign tumors and mucinous carcinomas, and between serous benign tumors and serous carcinomas. We guess the reason why benign lesions have a higher Reg4 expression than cancer is that mucinous contains most of the benign tumors (7/13), while serous contains most of the cancers (52/66), which may lead to the difference between benign and cancer, more mucinous cancer and serous benign tumors were needed in our further study. We had rewritten the results section according to your suggestion.

6. Figure 5. Please include a magnified image in order to identify the difference in cytoplasmic staining and an arrow to point out the differences. Make the scale legend readable and include what the size is in the figure legend.
Answer: We had revised Figure 5 according to your suggestion, we also include the size right in the figure, and which may better benefit the reader understand, thank you so much for your suggestion, which help making our manuscript better and better.

7. Table 1, 2 and 3. Please indicate what the bold text indicates. It appears to indicate statistically significant p-values, however in table 3, the correlation with cumulative survival is 0.084 which is not statistically significant, however, it is bold. Table 1 and 3 need to be detailed by histotype. Table 1 needs to state that it is measuring REG4 protein expression and not gene expression. Also, use same descriptions in table as in the definition of p-values. This is confusing.
Answer: The bold text indicates statistically significant p-values, we had indicated it in the Tables part, thank you so much to your suggestion. We had corrected 0.084 without bold, sorry for our mistakes. We had detailed the histotype in Table 2 and revised the descriptions in order to make them clear, thank you so much for your professional question.

8. Conclusion. Please explain in more detail what “phenotype-related complexes” (page 18 line 348) are. More detail about REG4 mechanism would be helpful. How does it regulate gene expression?
Answer: We had revised it into “Wnt5a, p70s6k, survivin, VEGF and Bax”. The mechanism how REG4 regulated these genes was complex and needs further research in the future.