Author's response to reviews

Title: High expression level and nuclear localization of Sam68 are associated with progression and poor prognosis in colorectal cancer

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Author's response to reviews: see over
Dear editors:

Thank you for your decision letter dated Dec 10, 2012, regarding our manuscript (MS: 2642303377933807), entitled “High expression level and nuclear localization of Sam68 are associated with progression and poor prognosis in colorectal cancer” authored by Wen-Ting Liao et al. We have taken all the points raised by the reviewers and added necessary data as the reviewers suggested. We are now re-submitting a revised manuscript with point-to-point responses to the critiques (please find it below). I hope that you will find that the current version of our manuscript is suitable for publication in *BMC Gastroenterology*. If you have any question regarding the re-submitted manuscript, please do not hesitate to contact me.

Sincerely yours,

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Response to reviewers’ comments and suggestions

Reviewer #1
Reviewer: Per Pfeiffer

Reviewer's report:

1. It is impressing that the authors have evaluated cell lines and FFPE tissue at the RNA and protein level. They have compared normal tissue and tumor tissue. However, the study is not acceptable for publication at least not in the present form for several reasons.

   What was the primary aim of the study?
   Why did the authors include stage 1 to 4? The patients did not receive pre-op therapy but the authors do not report on post-op therapy.

   Response: We appreciate the reviewer’s points and take them into good consideration.

   First, the biosynthesis and metabolism of RNA have been proved to play important roles in regulating gene expression. RNA expression profiling is differentially between cancer and normal cells, suggesting the possibility that aberrant regulation of RNA metabolism might be associated with the pathogenesis of cancer. Sam68 is an RNA-binding protein that regulates RNA processing. Functional researches have revealed the oncogenic property of Sam68. However, the deregulation of Sam68 in human cancer tissues has only been found in limited cancer types, including prostate cancer, breast cancer, renal cell carcinoma and cervical cancer. Thus, the primary aim of the study was to explore whether deregulation of Sam68 is a prevalent event in human cancer. Here, we investigate the deregulation of Sam68 in human colorectal cancer.

   Second, staging of colorectal cancer refers to how far a cancer has spread on a scale from 0 to 4, with 0 meaning a cancer that has not begun to invade the colon wall and 4 describing cancer that has spread beyond the original site to other parts of the body (Reference 1). Tumors are staged or graded for severity, according to evidence
of invasion into the intestinal wall, or evidence of spread. There is a close correlation between advancing stage and cancer mortality. Tumor size does not appear to be important in terms of outcome. Our study revealed a significant relationship of Sam68 expression in patients categorized according to Dukes stage, strongly suggesting that Sam68 can be used as a marker to identify subsets of CRC cancer patients with more aggressive disease. In addition, these results indicate that Sam68 might play an important role in the progression and invasion of CRC.


Third, the prognostic value of Sam68 expression was evaluated in patient with adjuvant chemotherapy. We analyzed the recurrence-free survival of patients who underwent adjuvant chemotherapy. Interestingly, we found that patients with weak Sam68 expression had a much higher risk of recurrence than patients with high Sam68 expression (Figure 5B; \( P = 0.004 \), log-rank test). These results were added into the revised manuscript.

2. Page 3 “However, lymph node metastases and/or liver metastases are present in approximately 20% of CRC patients at diagnosis”

Not correct

Response:

The sentence “However, lymph node metastases and/or liver metastases are present in approximately 20% of CRC patients at diagnosis” has been revised and replaced by “about 15%-25% of patients have liver metastases at the time of initial diagnosis, and an additional 20% of patients will develop liver metastases during the course of their disease.” (Reference 1)

3. Page 7
How was 231 pts changed to 224. How was patients selected?
“Prior patient consent and approval were obtained from the Institutional Research Ethics Committee”
Did the patients give written informed consent in 2000-2003 at the time of resection?
Patients who died of cancer (or other causes) were classified as dead. ????

Response:
1) We are sorry for the mistake. Actually, there were 231 samples which were involved in our initial experiment. However, 7 cases of tissues sections (6 cases were male, 1 case was female) were damaged and detached from the slides during the process of immunohistochemistry staining. Therefore, a total of 224 cases of patient samples were involved in statistical analyses. We have corrected “231” to “224” in the revised manuscript.
2) The patients were randomly selected.
3) For the use of clinical materials for research purposes, prior patients’ consents and approval were obtained from the Sun Yat-sen University and Cancer Center Institutional Board. All samples were collected and analyzed with prior written informed consents from the patients. We have added this content in Patients and tissue specimens section in the revised manuscript.
4) In this cohort of 224 patients, 80 cases (35.7%) were died due to CRC cancer, and 8 cases (3.6%) were died of uncertain cause. These Patients (both died of cancer and other causes) were all classified as dead.

4. Page 7
Please use median

Response: We appreciate the reviewer’s points. Appropriate correction has been made in the revised manuscript.

5. Page 8 Immunohistochemistry was performed as previously described [24, 25].
The degree of Sam68 staining was observed and evaluated as previously
described [21, 23]. Sam68 staining was not described in ref 21 and 23. What is the difference between ref 21 and 24?

Response: We are sorry for the inappropriate citation of these references. We have removed these references from the manuscript, and described the method in detail in the revised manuscript.

6. Page 11

“To further investigate whether upregulation of Sam68 is linked to the clinical progression of CRC, 224 paraffin-embedded, archived primary CRC tissue samples and 43 matched lymph node metastases derived from relapsed CRC patients were examined using immunohistochemistry.” What do the authors mean?

Response: We are sorry for the confused description. Here we mean “we further performed immunohistochemical analysis to determine the expression pattern of Sam68 in 224 paraffin-embedded CRC tissues and 43 lymph node metastatic tissues.” We have improved the description in the revised manuscript.

7. Page 12 and 16

Sma68?

Response: We are sorry for the mistakes in spelling. They have been corrected in the revised manuscript.

8. Page 12

“In univariate Cox regression analysis, age, gender and tumor histology had no prognostic value for overall survival in CRC”

Not surprising that histology is not prognostic because to few pts.

Response: We agree with the reviewer’s point. We have make revision: “In univariate Cox regression analysis, tumor differentiation, Dukes stage, T stage, N stage and distant metastasis were significant prognostic factors in this cohort of CRC patients”.

9. Page 12
Spearman’s correlation analysis indicated that a high Sam68 expression level correlated significantly with poorer survival in CRC.

Do not compare survival with Spearman

**Response:** We thank the reviewer for the comments. We have made correction in the revised manuscript.
Reviewer #2
Reviewer: Claudio Sette
Reviewer's report:

In this work Liao and colleagues investigate the clinical relevance of the expression of Sam68, an RNA binding protein frequently upregulated in human cancers, in colorectal cancer. The authors describe a positive correlation between high levels of Sam68 expression and its nuclear localization with colorectal cancer progression and poor outcome. The work relies on standard techniques, such as Western Blot, RT-PCR and immunohistochemistry and it is not particularly original, as the authors have reported very similar analyses of Sam68 and of its upregulation in renal, breast, cervical and oral tongue carcinomas in 4 previous publications from October 2009 to now. Nevertheless, the large number of patient specimens analysed by immunohistochemistry and the possible prognostic value of the expression and subcellular localization of Sam68 represent solid aspects of the study. The manuscript is not very accurate in the description of what is known about Sam68 and is flawed by an excessive weight given to the work of the authors with respect to that of other scientists in the field. This negligence may negatively affect the information that the general reader receives on the role(s) of this specific protein in human cancer.

Major Points:

1. Introduction: the literature cited in regard to the function of Sam68 is very old and largely incomplete, especially regarding the potential role of this protein in cancer. The authors mainly refer to studies focused on the signal transduction function of Sam68 in the cytoplasm. However, Sam68 is mainly localized in the nucleus of most cancer cells and articles in the last 10 years have clearly demonstrated that its activity in transcriptional (Babic et al., Oncogene 2006; Rajan et al J Pathol 2008) and post-transcriptional (Matter et al., Nature 2002; Paronetto et al., J Cell Biol 2007; Paronetto et al., Cancer Res 2010; Valacca et al., J Cell Biol 2010) regulation of gene expression is relevant to human cancer. Moreover, reference 15 suggested that ablation of Sam68 promoted neoplastic transformation, thus suggesting that Sam68 functions as a tumor suppressor, not a pro-oncogene as stated by the authors in
reference to this work. Since this manuscript is mainly descriptive, the description of the literature should be accurate and complete.

Page 6: The first evidence that Sam68 was upregulated in human cancers was shown in prostate carcinomas (Busà et al., Oncogene 2007; Rajan et al., J Pathol 2008). These articles preceded the many articles published by the authors on the upregulation of Sam68 in several other human cancers and should be correctly mentioned in the text when upregulation of Sam68 in human cancers is first described;

**Response:** We appreciate the reviewer’s points and take them into good consideration. We have rewritten the introduction section in the revised manuscript according to the reviewer’s suggestion.

2. Page 8-9: please specify what antibody (catalogue number) was used for the immunohistochemistry; please, provide also the details of the methods used for the evaluation and classification of Sam68 staining in tissues. The authors refer to two of their articles (ref 21 and 23) that deal with different proteins (Centromere protein H and AEG-1), not with Sam68. Moreover, reference 21 and reference 24 refer to the same article. This Referee does not see any reason to cite these two articles, which refer to different proteins analysed in different types of cancers and should be deleted from the reference list. All is needed here is to specify in detail the methods used to analyze Sam68 expression in colorectal cancer tissues. Again, this manuscript is a descriptive study and the description of the results should be accurate and easy to understand for the reader.

**Response:** We thank the reviewer for the comments. We have removed these inappropriate references from the reference list and provided the intimate methods of Western blot and immunohistochemistry in the revised manuscript. The details of the methods used for the evaluation and classification of Sam68 staining in tissues have also been described in detail.

3. Results: page 12, in line 3 the authors state: “nuclear localization of Sam68 correlated significantly with tumor differentiation (P = 0.002) and advanced Dukes
stage (P= 0.013) and T stage (P = 0.021), but not with N stage or distant metastasis.

whereas in line 12 they state “However, no significant associations were observed between the subcellular localization of Sam68 and any other clinicopathological feature including age, gender or tumor histology.” Do the authors mean with tumor histology only the type of epithelium (i.e. mucinous, columnar etc.) and not the differentiation status of the cells?

Please specify better in the text.

Response: We thank the reviewer for the comments. We have made modifications in demonstrating these observations in the revised manuscript.

4. Results: what is the relevance of the upregulation of Sam68 in CRC cells? Since the authors have tested Sam68 overexpression in several CRC cell line models, they could investigate the molecular contribution of this protein in the process of tumoral transformation, as author have already performed for other cancer types (e.g. breast cancer, renal cancer etc.).

Response: We appreciate the reviewer’s points and take them into good consideration. As suggested by the reviewer, we compared the Sam68 expression levels between seven CRC cell lines and two cases of normal intestine tissues. The results showed higher levels of Sam68 in CRC cell lines than that in normal intestine tissues (new Figure 1).

5. Discussion: the first page repeats the same concepts just examined in the Results section and could be deleted or largely shortened.

Response: We agree with the reviewer’s point and deleted the first paragraph in the discussion, according to the reviewer’s suggestion.

6. Page 15 lines 5-9: Taylor et al., (2004) (ref 5 in the text) demonstrated that up-regulation of Sam68 in fibroblasts, not its down-regulation, caused cell cycle arrest and apoptosis; reference 7 reports the phenotype of Sam68 knockout mice, and does not deal with the role of Sam68 in oncogenesis as stated by the authors; as mentioned
already for the Introduction, the authors refer to the literature incorrectly and inappropriately.

Page 15, lines 13-16: Busà et al 2007 and Rajan et al., 2008 (refs 20 and 26 in the text) did not report a significant correlation with poorer prognosis in prostate carcinomas, as incorrectly stated by the authors;

**Response:** We are sorry for the mistakes. We have re-written the discussion according to the reviewer’s suggestion.

7. Page 16, lines 3-7: the authors state “Interestingly, patients with cytoplasmic Sam68 localization had a better clinical outcome than patients with Sam68 nuclear localization. In agreement with these findings, cytoplasmic localization of Sam68 correlated significantly with known risk factors for progression and poor prognosis in human renal cell carcinoma and breast cancer [17, 19].” How can they state that the two lines of evidence are in agreement? In one case, the present work, cytoplasmic localization is a favourable prognostic value; in the other two studies it was presented as a negative prognostic value. The authors should seriously consider the differences in the two studies and discuss here possible scenarios in which Sam68 promotes oncogenesis more when it is localized in the nucleus (CRC) or in the cytoplasm (breast and renal carcinomas) depending on the cell type or tissue of the lesion. For the same reasons a more detailed description of Sam68 nuclear functions in the Introduction and in the Discussion is necessary. The authors conclude this paragraph stating that “The role of nuclear Sam68 in tumorigenesis is largely unknown…” This is quite a peculiar statement, as the majority of the articles published in high profile journals on Sam68 in the last 10 years have focused on its nuclear functions, such as transcription, alternative splicing and nuclear export of target RNAs. The fact that the authors have not addressed the nuclear function of Sam68 in their previous papers does not mean that nothing is known.

**Response:** We thank the reviewer for the comments. We have re-written the discussion according to the reviewer’s suggestion.
Minor Points:

page 3, third last line, page 11, line 8 and in many other parts of the manuscript:
Sam68 should read Sam68

Response: We are sorry for the mistakes in spelling. We have made corrections in the revised manuscript.