Author's response to reviews

Title: Expression of endosialin is associated with the progression and with the more aggressive biological characters of rectal cancers with a clinical trial of preoperative radiotherapy

Authors:

Zhi-Yong Zhang (zhiyongzhang1@hotmail.com)
Hong Zhang (hong.zhang@his.se)
Gunnar Adell (gunnar.adell@karolinska.se)
Xiao-Feng Sun (xiao-feng.sun@liu.se)

Version: 2 Date: 14 March 2010

Author's response to reviews: see over
March 14, 2010

Sabina Alam, Senior Scientific Editor
BMC Cancer
Editorial Office

Ms No.: 2042449448319381
Title: Expression of endosialin is associated with the progression and with the more aggressive biological characters of rectal cancers with a clinical trial of preoperative radiotherapy

Dear Editor,

Thank you for your email, along with reviewers’ comments. We have carefully revised the manuscript according to your suggestions and the reviewer’ comments, and resubmit the revised manuscript (the changes highlighted). The detail responds to the comments are following:

Editorial requests:

“Ethics - Experimental research that is reported in the manuscript must have been performed with the approval of an appropriate ethics committee. Research carried out on humans must be in compliance with the Helsinki Declaration (http://www.wma.net/e/policy/b3.htm), and any experimental research on animals must follow internationally recognized guidelines. A statement to this effect must appear in the Methods section of the manuscript, including the name of the body which gave approval, with a reference number where appropriate.”

Response: The study was approved by the ethical committee at the Faculty of Health Sciences, Universities of Linköping and Uppsala, Sweden. We have added the corresponding statement in the Method section on the revised manuscript (page 6, paragraph 1).

Reviewer: Hellmut Augustin

“The authors describe an interesting observation of Endosialin expression in colorectal tumors. Endosialin expression by epithelial tumors is in fact novel and therefore requires rigorous confirmation. In this regard, the manuscript clearly lacks necessary controls to emphatically validate the claims. Importantly, performance of Endosialin expression in the absence of co-staining for carcinoma cells, endothelial cells, pericytes and fibroblasts is not appropriate. Given the extend of the authors’ claims, it is necessary that the authors perform the relevant co-stainings.”

Response: Please see response below.

Major compulsory revisions

“Figure 1 is not at all mentioned in the Results section. The validity of the antibody has been previously demonstrated. The Western blot therefore appears to be irrelevant in this study.”

Response: Taking the reviewer’s suggestion, the Figure 1 has been omitted in this revised manuscript. Correspondently previous Figure 2 becomes Figure 1, and Figure 3 to Figure 2, along with the corresponding changes in the Legends (the page 24) and the text (pages 7, 9-10).

“The detection of ES expression in normal mucosa and in rectal carcinoma which
is the prominent finding in this study as described in the figure (2A-D) is rather incomplete and inconclusive.

A major concern is the finding that Endosialin is expressed by tumor cells, which is not reassuring. The performance of just one staining is not sufficient to draw such a conclusion. Importantly, the co-staining of Endosialin along with relevant tumor cell markers, endothelial markers and stromal markers is required.

For the whole of Figure 2, it is important to stain for the expression of fibroblasts and pericytes. For the fibroblast staining, at least one of the common markers such as vimentin, desmin should be used. In the case of pericytes, NG2 co-staining should be performed. Since many of the above markers are not exclusive for pericytes and fibroblasts and are often expressed interchangeably, it is necessary to co-stain with e.g. endothelial markers. Similarly, in the case of Figure 2B and 2C, it is important to co-stain for the expression of endothelial markers such as CD31 or CD34.

Finally, the authors show a diffuse Endosialin staining pattern in tumors in Figure 2D. Therefore, it is absolutely required that the authors perform co-stainings with a relevant tumor marker.”

Response: Regarding the comments of co-staining in fibroblasts, endothelia cells and pericytes, we have further discussed with our co-worker, Professor Clare Isacke (Inst. of Cancer Research, Sutton, UK) who provided the endosialin antibody to us for performing the present study, and her group has done several studies on co-staining of endosialin with CD31, CD34 or SMA etc in different types of human tumours and cell lines as well as animal model. Here I have cited the statements of Professor Isacke in an email to me regarding the reviewer’s comments “In tumours we originally looked in breast and colon cancer samples (MacFadyen et al. FEBS Letters 2005;597, 2569-75) where we stained frozen sections. We then followed this up as we developed a method for confocal staining of formalin-fixed paraffin-embedded samples (Robertson et al. BMC Cell Biology 2008;9:13). For that we looked at glioblastomas (Simonavicius et al. Modern Pathology 2008;00,1-8). The reason for choosing glioblastomas is that they have a very well defined vasculature and relatively few tumour fibroblasts. Tumour fibroblasts also express endosialin. Finally we also did the same thing in mouse embryos and the developing mouse retina (MacFadyen et al. Gene Expression Patterns 2007;7:363-9). The bottom line is that in the vasculature we only see expression on the pericytes, not on the endothelial cells.” Based on the studies by Professor Isacke’s group and other related studies, it is clear that the endosialin is expressed in fibroblast and pericytes, but not in endothelial cells. We did not do co-staining but we have done the staining on fibroblast by using the antibody against PINCH (particularly interesting new cysteine-histidine rich protein), (Gao et al., Neoplasia 2004;6:796-801), on pericytes by SMA (unpublished data), CD31 (Gao et al., Dig Liver Dis 2009; 41:116-22) and CD34 (Holmqvist et al., Ann Oncol, 2009, in press) separately, which help us to further confirm the morphology of fibroblast, pericytes and endothelial cells. It indirectly showed that the endosialin expression in the fibroblast and pericytes. In addition, the main aim of the present study was to see if the endosialin was related to the response of radiotherapy, and clinicopathological/biological variables in rectal cancers rather than to further identify the localization of the endosialin expression.

Regarding the comment of the staining in tumour cell, we have described the status of the expression tumour cells in more details in Figure 1, together with the legends (page 24). In Figure 1, we could compare the Figure 1B versus 1D, tumour cells are negative in the former but positive in the latter. We also could compare tumour cells in the same picture of the 1D, the most of tumour cells are positive (black arrow) but some are negative (red arrow). We even could compare the tumour cells in the same gland of the tumour in the Figure 1C, the most of tumour cells are negative (orange arrow) but a few of tumour cells are positive (red
arrow). These results support the evidence that tumour cell staining is specific. In addition, we have added more discussion of the reason for the different results of previous studies regarding tumour cell staining (pages 14-15).

Based on our experiences (we have being worked with IHC for more than 30 years both for clinical diagnosis and research aspect), IHC is a simple, practical, and stable technique although it is, to some certain extent, subjective. In order to limit subjective issue/technique error, in the present study, we did the following things.

- In order to diminish any possible differences between each run of IHC, the same researcher (Dr. Zhi-Yong Zhang) performed the IHC by using an exactly same protocol of IHC at the same laboratory.
- All the runs of IHC were continuously done within three weeks.
- We used both positive and negative controls for each run of IHC, all positive controls are positive, and negative controls are negative. The staining intensity of the positive controls was very similar from one run to another.
- Our procedure for reading IHC slides is as follow:
  1. Two pathologists (Zhi-Yong Zhang and Hong Zhang) read through all slides together with a dual-headed microscopy in order to set the criteria for scoring slides, for example, how to select areas and magnification, how to decide the staining intensity (strong, moderate or weak) and percentage etc.
  2. The two researchers read the slides independently at least twice.
  3. The results are compared, about 2/3 of the slides have the same scores for intensity or the same range for the percentage.
  4. For those with discrepant slides which the two researchers read again independently, and then compare their results. Thus about half numbers of the cases (which were discrepant after step 3) have the same scores.
  5. Repeat step 4.
  6. To the end, there are a few cases which have different scores, the two researchers read them together under a dual-headed microscopy to figure out why they do not have the same scores, and finally meet an agreement.

“Despite a correlation with markers, which positively correlate with poor prognosis, the authors state that the Endosialin expression does not correlate with long term survival of patients in either of the studied group. This is rather surprising and should be part of the Discussion. Additionally, this conclusion should likely be incorporated into the title of the article.”

Response: Taking the comments, we have added more discussion (page 13-14), and changed the title (on cover page). One of the reasons for not seeing the endosialin in relation to survival may partly depend on the limited numbers of the patients. Another possible reason may be that the endosialin plays a survival role in certain groups of the patients where the endosialin was related to the clinicopathological variables such as TNM, Cox-2, p73 and PRL. We did find the endosialin presented different pictures in such subgroups but it is hard to draw a conclusion due to limited numbers. Here I would like to present the survival curves as an attached file (PDF), as you could see the curve separated better in the subgroups defined by RT, TNM stage and p73, compared to the whole group.

“In Table 3 and Table 4, the number of patients used by the authors is not clear. For example, the authors use in the non radiotherapy group a cohort of either 43 or 46 patients. In the radiotherapy, it fluctuates from 40 to 35. The derivation of percentages for variables needs to be clarified.”
Response: In Table 3, we used McNemar’s test the percentage for 4 numbers (24, 24, 3 and 22) of the non-radiotherapy cases are 100, namely, 33%+33%+4% +30%=100%, we have added “total (%)” in the revised Table 3. We have added more columns in the Table 4, which makes clearer to readers regarding the percentages.

The different numbers of the cases are dependent on the material available for each study. As we have mentioned in the material section “The data for expression of Cox-2 [16], p73 [17], and phosphates of regenerating liver (PRL) [18] were taken from the previous studies carried out at our laboratory,” for each study we used paraffin-embedded sections provided by Dept. of Pathology for immunohistochemistry, the staff at the Dept. of Pathology made the sections according to how much tissue (tumour) left on the blocks. We just took the cases and sections available for each study, therefore the number of the cases differed from one study to another.

Minor Essential Revisions

“It is recommended that the authors rearrange sentences in some paragraphs for better flow and readability. In general, the authors should describe each of the group (RT and the non RT group) separately and completely prior to describing the other group. For example on page 3, the fourth sentence can be placed at the end of the paragraph.”

Response: We have rearranged the manuscript to describe non-RT first followed by RT group including text, tables and figures, while in the abstract since non-RT is less interesting, we lifted RT first, the sentences (pages 2-3) have been rearranged as the review’s suggestion.

“The Figures 2 A-D have different magnifications and the magnification needs to be shown. Additionally, the stage of tumor needs to be mentioned for the pictures whenever relevant. It is important to mention if the pictures in Figure 2 were obtained from the same patient or if those are representative images from either group.”

Response: The Figure 1 (previous Figure 2) was taken from the representative images from either group, stage II or III (they are not from stage I and IV, but we could not tell if which is II or III). The magnifications have been added (page 24).

“A legend (footnote) for the tables should be include”.

Response: It has been done on the Tables 1-4.

Discretionary Revisions

“In table 3, the authors have used Dukes’ stage for determining the status of the tumor. It is advisable that the authors use the latest AJCC/TNM system.”

Response: We have gotten the TNM data from Depts. of Pathology and Surgery, and done a new statistical analysis in this revised manuscript (Tables 1-2, pages 7, 9, 12 and 14).

“For fibroblasts, it would be advisable to co-stain for the expression of alpha SMA which would reflect the myofibroblast population.”
Response: Please see the response above regarding this comment.

Additionally, the written style could be improved. The article contains a number of sentences which are either not clearly written or are in complete or incoherent. Quality of written English: Needs some language corrections before being published”

Response: We have re-written the manuscript, and the English has been carefully revised in the revised manuscript.

Reviewer: Carolyn Staton

“The authors present an interesting study on the effects of radiotherapy on the expression of endosialin in both the normal mucosa and in rectal tumours. This is a follow up study from others previously published by this group with the same cohort of patients and data presented are correlated with those from previous studies. In general the paper is very well written and presents some interesting new findings.”

Major compulsory revisions:

“I. There is very clear vascular endosialin staining seen which, from the few photographs presented appears to be stronger in normal mucosa than in the stroma associated with the tumours. It would greatly add to the paper to quantify this vascular staining and relate it to tumour progression and radiotherapy treatment. This could be presented as an additional panel in Figure 3.”

Response: We have done two studies on CD31, CD34, D2-40 in relation to clinical pathological variables including patients survival in the same material and other materials (Gao et al., Dig Liver Dis 2009; 41:116-22; Holmqvist et al., Ann Oncol, 2009, in press). In the present study, our main aim was to see if the endosialin staining, no matter of the localization, had any impact on clinical, pathological and biological issues. Therefore we semi-quantified the endosialin in the stroma including fibroblasts and pericytes etc., and determined its significance of the patients. To take your suggestion, we will do a separate study and see if vascular staining of the endosialin in normal mucosa or in tumour was related to cancer development and response to radiotherapy.

“2. In Table 3 why are the tumours grouped according to Dukes A and then Dukes B, C & D? It would be better to use an alternative statistical method (e.g. Kruskall-Wallis) to see if there is a true relationship between endosialin expression with increasing severity of tumour by assessing each Dukes stage as a separate group.”

Response: As the changes in Table 2 (previous Table 3), we analyzed the endosialin expression with TNM stage I, II, III and IV (Dukes’ stage has been replaced by TNM stage in the revised version) separately. In RT group, there was a trend to be significant (p=0.07). If we compared TNM stage I (45%) with the rest (II+III+IV, 74%), the difference was statistical significant (p=0.03), (page 9).

Regarding Kruskal-Wallis test suggested the reviewer, we have consulted a statistician, professor John Carstensen at Linköping University (Sweden) and he said “The standard chi-square test for
trend is an often used technique which makes use of ordinal nature of a variable (and the usual scoring is 1, 2, 3, etc) (Kirkwood & Sterne, 2003, pp 173-175). The Kruskal-Wallis test (in this case equivalent to the Mann-Whitney U-test/Wilcoxon rank sum test) is another option to capture the ordinal nature of the TNM classification, but the scoring ('mean of ranks') of the TNM categories depends on frequency distribution and will be different between radiotherapy and non-radiotherapy group (due to a somewhat different stage distributions in the two treatment groups).” He even tested Kruskal-Wallis (Mann-W) p = 0.67 for non-RT and p = 0.06 for RT group.

“3. The discussion is largely a re-iteration of the results section and should be much more tightly focused on the relevance of the findings to other factors, the relationships between them etc. It was a little unclear as to the interpretation of your results and this needs to be clarified.”

Response: We have re-written the discussion (pages 13-15).

Minor essential revisions:

“1. When describing the association with Cox-2 etc it was unclear what the values in brackets were (e.g. 44% vs 73%). It would be better to simply put the p value in brackets and refer to the table where this is much clearer.”

Response: we have changed the description on the Result section (pages 9 and 11). We have also added more data of the weak staining on Table 4, which makes clearer for readers.

Discretionary revisions:

“1. It was stated that there were only a few positive tumour cells in entire slides in many cases. If that is the case an additional data set measuring the percentage of tumour cells expressing endosialin within a tumour section may generate more meaningful data and add to that already presented.”

Response: Since only 25 cases in the non-RT group and 21 cases in the RT group had tumour cell positive for the endosialin (if positive cells >5% of tumour cells as positive) it is hard to further subgroup the positive cases by percentage considering statistical analysis.

We would appreciate if this revised manuscript could be reconsidered for publication in the BMC Cancer.

Yours Sincerely,

Xiao-Feng Sun, Prof., MD, PhD
Department of Oncology
Institute of Biomedicine and Surgery
Linköping University
S-581 85 Linköping, Sweden
Tel: +46-13-222066, Fax: +46-13-222846
Email: xiao-feng.sun@liu.se