Author's response to reviews

Title: Impact of PINCH expression on survival in colorectal cancer patients

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Version: 3 Date: 7 January 2011

Author's response to reviews: see over
Dear Editor,

Thank you for considering our manuscript “Impact of PINCH expression on survival in colorectal cancer patients with or without adjuvant chemotherapy” for further revision, and your valuable comments and suggestions. We have addressed these comments and revised the manuscript, and the detailed responses to the comments of the reviewers are as follows:

**Editorial comments**

“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/en/30publications/10policies/b3/index.html](http://www.wma.net/en/30publications/10policies/b3/index.html)), 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.

Consent - Informed consent must be documented. Manuscripts may be rejected if the editorial office considers that the research has not been carried out within an ethical framework, e.g. if the severity of the experimental procedure is not justified by the value of the knowledge gained.”

**Response:** The study was approved by the local Human Research Ethics committee and performed in accordance with the Helsinki Declaration. Informed consent was obtained from the participants. A statement to this has been added to the methods section under the heading “patients”, page 5, paragraph 1, line 15. The decision for the ethical application is followed as an attachment to this cover letter.

**Reviewer 1**

**Major Compulsory Revisions:**

1. “Is it known by which factors the PINCH expression itself is influenced?”

**Response:** To our knowledge, the factors that influence PINCH expression in cancer are not known. There are a few studies on factors influencing PINCH expression in neurons, however this might not be applicable in the context of our study. What might be of interest is the role...
of PINCH in epithelial-to-mesenchymal transition (EMT) in renal tubuli. TGF-β1 is a main inducer of EMT in several biological systems, including renal tubuli. Further, TGF-β1 induces the expression of PINCH mRNA through Smad signaling. The role of TGF-β1 is well documented in colorectal cancer, so possibly TGF-β1 could be involved in the regulation of PINCH expression also in this context. Further, PINCH is stabilized by its interaction with ILK. Therefore, the abundance of PINCH protein is largely dependent on the levels of ILK.

A section concerning this has been added to the discussion, page 12, paragraph 1, lines 5-11.

2. “In table 4 there are 101 patients with rectal cancer of whom only one patient was treated with radiotherapy. A rather low rate since there are about 100 patients presenting with a stage III disease. Where the others ineligible for adjuvant treatment?”

**Response:** We have asked oncologists and statisticians to re-check the status of the patient treatments. For most of the patients, especially for those from earlier years, we do not have detailed data of the adjuvant radiotherapy.

3. “In the discussion chapter you give some interesting suggestions why a strong PINCH staining could be associated with worse prognosis. Did you also look for a co-expression of the other factors like Nck-2, ILK, MMPs, Fibronectin or VEGF in your tumour samples?”

**Response:** We did not look for co-expression of these factors, although it would certainly be of interest. Because of limited amounts of the patient material, we could not do an additional co-expression experiment for this study. However, at our laboratory, another study (unpublished data) on rectal cancer patients (different material from the present study) investigating angiogenesis and lymphangiogenesis showed PINCH to be related to blood- and lymph vessel density, implicating PINCH as a regulator of angiogenesis. This has been added to the discussion, page 13, paragraph 1, lines 11-13.

**Minor Essential Revisions:**

1. “Please cite the software used for statistical analysis in your materials and methods section."

**Response:** A statement of the software used has been added to the section of the “statistical analysis”, page 7, line 1.

2. “In table 2 you present the data of 146 patients, but in the text, e.g. on page 5 it says 149 samples of adjacent normal mucosa are available. Also in table 2 under differentiation you grouped into well + moderate + poor vs mucinous. In the text on page 5 the groups are good and moderate vs. poor, mucinous and signet-ring cell. Similarly please review the patients numbers in table 4. In the text a number of 251 is given but in the table a maximum of 241 is reached."

**Response:**
Response: Regarding the 146 and 149 patients, since Table 2 shows a multivariate analysis, which is required to be done on the same patients for each factor (i.e., PINCH=146, sex=146... stage=146), we had to omit three cases from this multivariate analysis, as these three cases were graded as stage C or D.

Regarding the groups of the differentiation, the text on page 5 now states “Tumour differentiation was graded as good, moderate, poor, or mucinous (including signet-ring cell carcinomas)”. For all analyses we grouped the cases into good + moderate Vs poor + mucinous, i.e., better Vs worse differentiation, but not for Table 2. In Table 2, if we use the same groups (better Vs worse differentiation), the p value for PINCH would be 0.05. If the editor and the reviewer think that it is more important to keep the same classification of the differentiation, we could make a corresponding change on the Table 2.

Concerning the discrepancy of the number of the primary tumours (251 in the text and in figure 2, but 241 in table 4), not all of the tumours had an assessable tumour margin (Table 4 concerns the staining at the invasive margin).

3. “Page 14, second section: “...29 patients received adjuvant chemotherapy.” Please check the patient numbers since in table 1 only 27 patients had adjuvant chemotherapy.”

Response: In this revised version, 29 patients have been changed to 27 on page 14 as well as the material and methods section, page 5. In table 1, there are 29 patients in total; among them 27 patients with chemotherapy, one patient with radiotherapy and one patient with unknown adjuvant treatment.

4. “Was there also a significant difference in PINCH expression between the distant normal mucosa and the primary tumour or the metastasis?”

Response: There was no significant difference between distant normal mucosa and primary tumour (p=0.16 as stated in the text). Distant normal mucosa did not have a statistical difference from the metastasis either, p=0.074.

Discretionary Revisions:

1. “Please consider in table 4 to write “inflammatory infiltration” as a heading instead of “infiltration” alone.”

Response: We have changed the heading in table 4 according to your suggestion
2. Since there was no significant relationship of PINCH expression with the use of adjuvant chemotherapy in the multivariate analysis you could consider changing your manuscript title into “Impact of PINCH expression on survival in colorectal cancer patients”.

**Response:** We have changed the title according to your suggestion.

**Reviewer 2**

1. Discussion limited mostly to research question but did not appreciate the fact that survival depends on much more than histological grading. I suggest that author mentions also other prognostic and predictive factors and/or markers presently being used or being tested so present research can be put into perspective.

**Response:** Although there are certainly factors other than histological grading influencing survival, the determination of prognosis in colorectal cancer patients still predominantly relies on the histopathological examination. Approaches are being made to improve prognostic methods, such as analyzing additional histopathological factors and molecular markers. Although these markers are promising, they are not routinely used, and the use of molecular markers cannot yet be recommended. Potential molecular markers include allelic imbalances, chromosomal instability, expression of oncogenes, loss of tumour suppressor genes, markers of proliferation, angiogenesis, inflammation, cell adhesion etc. A section concerning this has been added to the discussion, page 10, section 1, lines 1-9.

2. In the regard, limitation of research has not been discussed nor stated adequately.

**Response:** Limitations of the study include the low number of patients receiving adjuvant chemotherapy. Further, the treatment was not randomized. We have now clarified this issue in the discussion, page 15, paragraph 2, lines 7-10.

With warm regards

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