Author's response to reviews

Title: Validation of podocalyxin-like protein as a biomarker of poor prognosis in colorectal cancer

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Version: 2 Date: 8 May 2012

Author's response to reviews: see over
Dear Dr Chap,

Thank you for inviting us to resubmit our paper “Validation of podocalyxin-like protein as a biomarker of poor prognosis in colorectal cancer”. We would also like to thank the reviewers for their helpful and constructive comments, and we have now prepared a revised manuscript taking them into account, with all changes highlighted in yellow. Please see our point-by-point response below.

With these improvements I hope you will find our manuscript acceptable for publication in *BMC Cancer*, and I am looking forward to hearing from you again.

Yours sincerely,

Anna Larsson
Reviewer's report

Title: Validation of podocalyxin-like protein as a biomarker of poor prognosis in colorectal cancer

Version: 1 Date: 29 March 2012

Reviewer: Sanjun Cai

Reviewer's report:

1. Very similar paper has been published in British Journal of Cancer (Br J Cancer. 2011 Aug 23;105(5):666-72). In that paper, they used tissue microarray in 526 colorectal cancer patients. In this paper, the authors (same first and corresponding author) used the same TMA in another group of patients. I don't think it provided any additional information for this biomarker.

Response: Validatory biomarker studies are very important, see also comment from Reviewer 3, if they are ever going to be implemented in prospective trials and, eventually, clinical protocols. Notably, in this study, we had also investigated the effect of PODXL expression on recurrence free and disease-free survival of colorectal cancer patients.

Changes made to the manuscript: A sentence has been added to the Discussion section, page 12: "Moreover, while the results from this study further confirm the association of high PODXL expression with a reduced overall survival, its impact as a biomarker of reduced time to recurrence and disease free survival in curatively treated patients is also demonstrated."

2. I think the author should also clarify if the patients in current study were also included in the paper in BJC.

Response: We feel that it is evident from the manuscript, that this is a validatory study in two additional, independent patient cohorts.

Changes made to the manuscript: To make this even clearer we have reformulated "two independent patient cohorts" in the Abstract (Background and Conclusion) to read "two additional independent patient cohorts"

3. However, the author also provided some other information about the mRNA expression level of PODXL and its relationship to outcomes and clinicopathological characteristics. I think it is useful and interesting to study further in this area. I suggest the author to do more study about the relationship between mRNA of PODXL and protein expression or outcomes. And if possible, further study about the PODXL in colorectal cancer cell lines will be supported.

Response: A valid point as regards further functional studies, which will definitely be of interest. In the clinical setting, though, immunohistochemical assessment of PODXL is and will remain the method of choice, since it is comparatively cheap, can be used even in small pathology laboratories with limited resources, and easy to incorporate into routine diagnostic protocols. To do further functional studies as well as studies related to the correlation of PODXL mRNA and
Changes made to the manuscript: See also response to Reviewer, 2 p 5 and Reviewer 4, p 4.

Level of interest: An article of limited interest

Quality of written English: Acceptable

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:
I declare that I have no competing interests

Reviewer's report

Title: Validation of podocalyxin-like protein as a biomarker of poor prognosis in colorectal cancer

Version: 1 Date: 1 May 2012

Reviewer: ANDREAS LAZARIS

Reviewer's report:

1. The staining of PODXL could be evaluated by using an image analysis programme

Response: This is a valid point and we are indeed planning for sub-studies comparing manual and automated analysis of PODXL within forthcoming prospective studies. The main purpose of such studies will however mainly be to validate the use of automated image analysis approaches, not the clinical utility of PODXL in treatment stratification of CRC patients, since manual assessment of its membranous staining is rather straightforward.

No changes have been made to the manuscript

2. It would be essential to elaborate a little more the PODXL molecule properties

Response: A valid point. This has now been done, not least in light of a recently published study on ovarian cancer related to the prognostic value of PODXL expression on the cell surface (Cipollone et al., 2012).

Changes made to the manuscript:

1. The following sentences have been added to the Background, page 5: ”A recent study demonstrated that forced PODXL expression in ovarian cancer cells decreased their adhesivity by altering β1-integrin levels, and that PODXL expression on the cell surface was associated with poor prognosis in high grade serous carcinomas.”
2. The first sentence in the next paragraph, Background page 5 has been modified as follows: “In a previous study, we have demonstrated that membranous expression of PODXL is associated with unfavourable clinicopathological characteristics and independently predicts a poor prognosis in CRC…”

3. In the Discussion section, pp 12-13, the following section has been added: “These observations are also well in line with the study by Cipollone et al, where PODXL expression on the cell surface but not in the cytoplasm was significantly associated with a shorter disease-free survival in patients with high grade serous ovarian carcinoma. In that study, it was also demonstrated that forced expression of PODXL in serous ovarian carcinoma-derived OVCAR-3 cells resulted in localization of PODXL to the cell surface, decreased cell adhesion to mesothelial monolayers and diminished levels of β1-integrin, leading the authors to conclude that PODXL may facilitate transperitoneal metastasis of high grade serous carcinoma. In light of the significant association of high PODXL expression and increased T-stage, in particular stage T4 tumours, observed in our previous study, and in Cohort II in this study, it would be of interest to perform further studies to investigate whether PODXL may have a role in the initiation of serosal invasion also in CRC.”

3. PODXL is a transmembrane protein, why did you declare that it shows a cytoplasmic expression?

Response: Several other studies have demonstrated cytoplasmic expression of PODXL in various cancer forms. However, our study, the study by Cipollone et al. on ovarian cancer, support that its location on the cell surface/membranous expression indeed seems to have most important prognostic implications.

Changes made to the manuscript: See response to p 2 above.

4. It would be appropriate to make a comment on the role of PODXL on pathogenesis of other diseases such as glomerulopathies(similar anti-adhesive properties, "Immunohistochemical evaluation of podocalyxin expression in glomerulopathies associated with nephrotic syndrome" E. Kavoura et al, Hum Pathol. 2011 Feb;42(2):227-35. )

Response: A valid point.

Changes made to the manuscript: This reference, and the following sentence, has now been added to the Introduction, page 4, last paragraph: Loss of PODXL expression has been observed in glomerulopathies primarily associated with the nephrotic syndrome (ref Kavoura)

5. It would be essential to explain your results ie why increased PODXL expression is correlated to worse prognosis of the tumor. We suggest to make a comment on the anti-adhesive role of PODXL

Response: Point taken, please see response to p 2 and 3 above.

Level of interest: An article of importance in its field

Quality of written English: Acceptable
**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

This review was done with Dr. Evangelia Kavoura. 'I declare that I have no competing interests'

**Reviewer's report**

**Title:** Validation of podocalyxin-like protein as a biomarker of poor prognosis in colorectal cancer

**Version:** 1  **Date:** 5 April 2012

**Reviewer:** Caj Haglund

**Reviewer's report:**

This is a very well performed study on an interesting new prognostic marker. The authors have published their preliminary results previously and now they validate the results in two separate validations cohorts. Validation of preliminary findings is extremely important, but is very seldom done. Validation by other groups is still needed before podocalyxin can be recommended for routine use.

The methodology of the paper represents high level immunohistochemistry. Of course IHC has its weakness regarding reliability of scoring. Statistics is appropriate.

This paper is well worth to be published.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

'I declare that I have no competing interests'
Reviewer's report

Title: Validation of podocalyxin-like protein as a biomarker of poor prognosis in colorectal cancer

Version: 1 Date: 11 April 2012

Reviewer: Dermot Leahy

Reviewer's report:

In this paper the authors confirm their previously published finding that overexpression of PODXL is associated with poor prognosis in colorectal cancer. While this validation in two large independent cohorts is useful, the potentially novel aspect of the paper lies in their examination of the correlation between PODXL mRNA and protein levels in a subset of tumours.

In general the paper is well written but there are some points that need to be clarified before I could recommend acceptance.

Major Compulsory Revisions

1. The immunohistochemical staining was dichotomised as low and high staining. Low staining included complete absence of staining and cytoplasmic staining while high staining is membranous. How many cases showed complete lack of staining? Was there any significance in “absence of staining” versus “any positivity in cytoplasm or membrane”.

Response: A valid point. The proportion of tumours lacking PODXL expression was similar in Cohort I in this study and our previous study (Larsson et al., Br J Ca 2011), and the proportion was slightly higher in Cohort II in this study. The prognostic significance did not differ between categories 0, 1 and 2, in neither cohort.

Changes made to the manuscript:

1. The numbers of tumours with negative, low- intermediate and high staining in both cohorts have now been specified in the Results section, page 9 as follows: "In cohort 1, 137 (52.7%) tumours were negative for PODXL (score 0), 98 (37.7%) tumours displayed weak-moderate staining (score 1-2) and 25 (9.6%) tumours displayed high PODXL expression (score 3-4). In cohort 2, PODXL expression was denoted as negative (score 0) in 198 (62.7%) tumours, weak-moderate (score 1-2) in 93 (29.5%) tumours and strong (score 3-4) in 25 (7.9%) tumours."

2. The following sentence has been added to the Results section, page 10: "There was no significant difference in outcome, neither for TTR, DFS or OS, between patients with tumours denoted as having negative, weak or moderate staining; i.e. all categories lacking membranous staining had a similar prognosis (data not shown)."

2. Membranous staining was seen mainly at the invasive tumour front. In the construction of the TMA, was at least one core taken from the invasive front? Is it possible that some positive cases were missed due to this localisation of staining? Were some full face sections analysed to control for this potential problem with the TMA method?
Response: A valid point. In constructions of TMAs we always attempt to take cores from the tumour front and centre, as long as necrotic areas can be avoided. The presence of PODXL high tumour cells, often in conjunction with “tumour budding” is however not necessarily observed in the invasive tumour front, but also within the tumour. Moreover, a comparison of tma-cores vs full-face sections had been performed in our previous study (see also Larsson i al Br J Ca 2011) with an excellent concordance.

Changes made to the manuscript:

1. The following sentence has been added to the Methods section/TMA construction, page 7: “Non-necrotic tumour areas were avoided and, when possible, one core was taken from the centre and periphery of the tumour, respectively.”

2. The Discussion section has been extended on p 14 as follows: “Some limitations related to the TMA-technique must be considered, not least its ability to accurately reflect the expression of heterogenously expressed markers. One way to compensate for this is to, whenever possible, ensure that tumour cores are sampled from different tumour areas, i.e. the invasive front and centre, respectively. While this had been done for the majority of the here analyzed tumours, it should be pointed out that tumour areas denoted as having distinct membranous PODXL expression could not only be found at the invasive front, but also in scattered areas within the tumour. However, we have previously compared results from paired TMA-cores with full-face sections with excellent concordance (ref Larsson BJC 2012). Moreover, assessment of full-face sections from prospectively collected clinical samples have reveled a similar proportion of CRC cases with high PODXL expression as reported here and in our previous study (unpublished observations).”

3. The authors report that no significant correlation was found between mRNA levels and protein expression of PODXL. Similar to No.1, were a group with complete lack of expression compared to a group with any degree of expression.

Response: Yes, and there was no association.

Changes made to the manuscript: The following sentence has been added to Results, page 11: ”mRNA levels did not differ significantly between tumours lacking PODXL expression compared with categories of any degree of expression (data not shown). ”

4. Did the authors consider microdissection of a strongly staining area from a frozen section to provide a definitive answer to the question of correlation with mRNA.

Response: A valid point, but the main purpose of this paper was to further validate the potential clinical utility of PODXL expression in CRC, and, evidently, immunohistochemical analysis is the method of choice. This is an important finding, since even if we had been able to demonstrate a similar, or stronger, prognostic value for PODXL mRNA levels, gene expression analyses are more costly and would still not be an option for all laboratories. Moreover, although microdissection of tumours might well give more accurate information on the association of PODXL protein and mRNA levels, this approach would be far too time- and resource consuming to ever have a place in the clinical setting, and still would not add value to IHC. Please see also response to Reviewer 2, p 5, regarding novel findings of the relevance of membranous PODXL
expression in ovarian cancer.

Nevertheless, in an experimental setting, microdissection and further functional studies, might indeed be of relevance.

Changes made to the manuscript: The following text has been added to the Discussion section, page 14: “A more comprehensive analysis of the correlation between PODXL mRNA and protein expression might be provided by performing microdissection of strongly staining areas from frozen tumour sections. In the clinical setting, however, it is evident that immunohistochemical assessment of PODXL is the method of choice, whereby recognition of its location on the cellular surface should be quite straightforward.”

Minor issues not for publication

In "Evaluation of PODXL staining" the symbol for "less than or equal" should be corrected.

In fourth paragraph of Discussion "molocular" should be "molecular".

Response: Thank you. These typos have now been corrected.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

☐Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests