Author's response to reviews

Title: Decreased expression of RNA-binding motif protein 3 correlates with tumour progression and poor prognosis in urothelial bladder cancer

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

Thank you for your positive response and invitation to resubmit our paper “Decreased expression of the RNA-binding motif 3 protein correlates with tumour progression and poor prognosis in urothelial bladder cancer”. We are grateful for helpful and constructive comments and suggestions from the Reviewers and the Editor. A further revised manuscript has been prepared taking them into account, as outlined in the point-by-point response below, and highlighted in yellow in the revised manuscript. We have also taken the opportunity to check the manuscript for typos and inconsistencies. With these improvements of the manuscript we hope you will find our paper suitable for publication in BMC Urology.

Yours sincerely,

Karolina Boman
EDITORIAL REQUEST:

1. Please state what RBM3 is abbreviated for. Please include the full name of RBM3 in title of manuscript

Response: The title has now been changed to include the full name of RBM3. We have also added a section of Abbreviations to the manuscript.

Reviewer's report

Title: Decreased expression of RBM3 correlates with tumour progression and poor prognosis in urothelial bladder cancer

Version: 1

Date: 19 February 2013

Reviewer: David DeGraff

Minor essential revisions:

1. Representative staining from each tumor stage should be shown.

Response: With due respect, we are not really sure what is meant here, as different modalities of RBM3 staining was observed in all tumour stages (although the proportion of RBM3 negative tumours was significantly higher in more advanced stages and vice versa). Therefore, in addition to the sample images shown in
Figure 1, and the staining distribution according to Stage visualized in Table 2, we do not think that the relationship of RBM3 staining with tumour stage needs additional clarification and no changes have been made to the manuscript.

2. Why was the combined nuclear score used, as opposed to binary positive/negative? What is the rationale for this approach?

Response: Combined scores are frequently used in biomarker studies, as they may provide more accurate information on degree of linear relationship with other factors. In the case of RBM3 expression in urothelial bladder cancer, the group of tumours denoted as having "intermediate" expression also had an "intermediate" prognosis compared to categories with negative and strong expression, although the most evident binary prognostic cutoff was seen for negative vs positive expression.

Changes made to the manuscript:

1. An Additional File 1 has now been added, wherein the associations of nuclear fraction and intensities with disease-specific and overall survival, respectively, are displayed. These KM curves further support the use of a binary "negative vs positive" variable in the Cox proportional hazards analyses.

2. The sentence in the Results section, pp7-8 has been modified from "Analysis of survival according to the fraction and intensity of RBM3 expression, respectively, yielded a similar impact on survival (data not shown)" to "Separate analysis of categories of nuclear fraction and staining intensity yielded similar results for DSS and 5-year OS (Additional File 1)".

3. The authors state that there was no obvious heterogeneity between duplicate cores for RBM3 staining. What was the % heterogeneity observed?

Response: The fraction of positively staining cells was estimated across both sampled cores, and the dominating staining intensity taken into account. Therefore the % of heterogeneity was not denoted. Heterogeneity regarding the nuclear staining fraction between duplicate cores was however only observed in cases with intermediate RBM3 expression and never exceeding 1 category. (Hence, use of "best score" would have yielded similar results.)

Changes made to the manuscript:

1. The following sentence has been added to the M&M section, page 5: "The fraction of positively staining cells was estimated across both sampled cores, and
the dominating staining intensity denoted.”

2. The following sentence has been added to the Results section, page 7: "Some heterogeneity regarding the nuclear staining fraction between duplicate cores was only observed in cases with intermediate RBM3 expression and never exceeding 1 category.”

4. On the bottom of page 8, the authors state “There was a borderline significant association between RBM3 expression and CSS in both univariable analysis....” What is CSS? Cancer specific survival? This is the first time this is used, and is confusing. Did the authors mean DSS?

Response: Thank you, it should indeed be "PFS", and this has now been corrected (now top of p 9). We also corrected another "CSS" that should be "DSS" in the M&M section/Statistics, p 5.

5. In the discussion, the authors state that they examined a larger “prospective cohort.” This is a retrospective analysis of archival tissue.

Response: A valid point, it is a retrospective analysis of samples from a prospective cohort. This has now been corrected (Discussion, p 10, 2nd paragraph) as follows: “In this comparatively large study of retrospectively collected tumours from a prospective cohort of patients with urothelial bladder cancer…”

Major essential revisions:

1. Although this should be less of an issue in pTa disease, examination of different histological subtypes (pure squamous, adeno, small cell, micropapillary, etc) would be informative. Perhaps the strong association between RMB3 loss and advanced tumor stage is because advanced tumors exhibit anywhere from 20-40% mixed squamous differentiation?

Response: This cohort consists of uroepithelial/transitional cell carcinoma, not adenocarcinoma or squamous cell carcinoma. Whether presence of a micropapillary carcinoma component had been denoted in the original pathology report is unknown, as is the extent of squamous differentiation. No such components were however observed in the TMA cores.

Changes made to the manuscript:

The following sentence has been added to the Discussion, page 12: "While the findings in the present study are based on analyses of transitional cell carcinoma,
it would also be of interest to examine RBM3 expression and its possible prognostic implications in other histological subtypes of bladder cancer, e.g. squamous cell carcinoma or adenocarcinoma.

2. Examination of lymph nodes dissected from patients undergoing cystectomy for RMB3 expression would be informative (preferably from matched patient samples used in the TMA; ~20 or so randomly selected.), especially since there may be an association with invasion.

Response: We agree with this reviewer that it will be of interest to compare RBM3 expression in matched primary tumours and lymph node metastases. This should however preferably be done in prospective studies, on full-face sections, taking all metastatic nodes into account for each case in order to check for clonality. While such studies would not give additional information on the prognostic value of RBM3, as demonstrated here in a high-throughput setting with a relatively large cohort of retrospectively collected samples, they would indeed provide further information on the role of RBM3 in tumour progression. Moreover, they would also shed light on the clinical utility of assessment of RBM3 expression in metastatic deposits, e.g. if the primary tumour is not available for analysis after neoadjuvant treatment.

No changes have been made to the manuscript.

3. In addition to the observed correlations between RBM3 expression and clinical outcome, the authors should consider screening commonly used human cell lines for RMB3 expression. This would inform other investigators interested in the biological role of RBM3 in bladder cancer.

Response: RBM3 expression has been screened and demonstrated to be expressed in a multitude of human cell lines in the Human Protein Atlas portal, including RT-4 cells (www.proteinatlas.com). A high and specific RBM3 expression in RT-4 cells has also been demonstrated with different RBM3 antibodies, including the antibody used in the present study, in a validatory study by Hjelm et al. (Proteomics Clinical Applications 2011 (11-12):624-35).

Changes made to the manuscript:

The following sentence has been extended in the M&M section, page 5, 1st paragraph: The specificity of the antibody has been validated previously, also in the human bladder cancer cell line RT-4, in which RBM3 was demonstrated to be highly expressed (ref Hjelm, 2011).
1. The potentially organ-specific influence of RBM3 on cell proliferation and invasion is interesting, because it provides an opportunity for the design of a simple experiment. The authors should identify bladder cancer cell lines to knock down and overexpress RBM3. This would enable them to test how RBM3 expression impacts IC50 in cell lines following cisplatin exposure, the mainstay of chemotherapeutic treatment for advanced bladder cancer.

Response: This is indeed an interesting issue but in our opinion not quite within the scope of the present study, where we have examined the prognostic, not treatment predictive, value of RBM3 expression in urinary bladder cancer. We are however planning a prospective clinical study on the potential treatment predictive effect of RBM3, with particular reference to cisplatin sensitivity, and along this line, we will also examine the effects of RBM3 on treatment response in vitro, continuing along the line of earlier studies on ovarian cancer (see Ehlén et al, Journal of Translational Medicine 2010 and Ehlén et al., Translational Oncology, 2011), that implicate a role for RBM3 in DNA integrity and repair.

Notably, to design in vitro studies that explain the mechanisms underlying the prognostic significance of RBM3 expression undoubtedly requires some thinking out of the box in order to apply an appropriate bedside-to-bench approach. While expression of RBM3 has now been demonstrated to be a biomarker of favourable outcome in several major cancer forms, irrespective of adjuvant therapy, hitherto published in vitro data (including our own, see Ehlén et al., JTM 2010) continue to be rather ”contrasting”, as knockdown of RBM3 leads to reduced cellular proliferation etc. We are indeed pursuing this area ourselves, but anticipate that it will take some time to collect appropriate in vitro data, and therefore prefer to let the present paper retain its pure ”in vivo” character.

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

Quality of written English: Acceptable

☐ Statistical review: Yes, and I have assessed the statistics in my report.

Declaration of competing interests: ☐ I declare that I have no competing interests.
**Reviewer's report**

**Title:** Decreased expression of RBM3 correlates with tumour progression and poor prognosis in urothelial bladder cancer

**Version:** 1

**Date:** 26 February 2013

**Reviewer:** Dermot Leahy

**Reviewer's report:**

This manuscript describes a study of a large cohort of patients with urothelial bladder cancer. The authors found that decreased expression of RBM3 is associated with tumour progression and poor prognosis.

Overall, this paper is well written with objectives, methodology and results clearly described. The authors appear to have considerable expertise in this area, having published previously on the significance of RBM3 in various other cancers.

**Major Compulsory Revisions**

However, there appears to be a major problem with the references which needs to be resolved before the "Discussion" can be properly reviewed.

1. References 14, 15 and 16 are cited in relation to RBM3 in other cancer forms, but these three references deal with VEGF, Bcl-2 and the Wnt pathway in colorectal cancer.

2. Reference 14 is cited as describing the upregulation of RBM3 in neoplastic compared to normal tissue, but this reference deals with VEGF in colorectal cancer.

3. Reference 18 is cited to support the idea that urothelial bladder cancer is a recurrent disease but the reference is actually a Wnt signalling review.

4. References 23–26 are cited in relation to platinum-based chemotherapy of bladder cancer, but in fact all deal with Wnt/beta-catenin in colorectal cancer.

**Response:** We truly apologize for this, these references have been exchanged due to an Endnote bug. This has now been corrected.
Discretionary Revisions

1. The second sentence of “Results” states that no heterogeneity in RBM3 expression was seen between duplicate TMA cores. Were any full face sections stained to further check for heterogeneity? Was heterogeneity of expression seen in any of the tumour types in previous studies?

Response: Full-face sections were not available for cases in the present cohort. As regards heterogeneity, we have made some additional clarifications, please see also response to Reviewer 1, p 3, minor essential revisions.

2. In the “Authors’ Contributions”, KJ is mentioned twice but KB is not mentioned.

Response: Thank you, this has now been corrected as follows: ”KB evaluated the immunohistochemical stainings…”

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