Author's response to reviews

Title: RKIP phosphorylation and STAT3 activation is inhibited by Oxaliplatin and Camptothecin and are associated with poor prognosis in Stage II colon cancer patients

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Version: 2 Date: 31 July 2013

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The Biomed Central Editorial Team

Object: MS: 2041428456984549 - RKIP phosphorylation and STAT3 activation is inhibited by Oxaliplatin and Camptothecin and are associated with poor prognosis in Stage II colon cancer patients. Mr. Sam Cross-Knorr et al.

Thank you for consideration of our manuscript for publication in your journal. We have reviewed and revised the above manuscript according to your reviewer’s comments. We have modified/changed every figure and table in the paper and highlighted changes to text in yellow. We hope that are changes will satisfy the Reviewers and our manuscript will now be accepted for publication in BMC Cancer.

Reviewer's report
Title: RKIP phosphorylation and STAT3 activation is inhibited by Oxaliplatin and Camptothecin and are associated with poor prognosis in Stage II colon cancer patients
Version: 1 Date: 19 May 2013
Reviewer: Viktor H Koelzer
Reviewer's report:

The manuscript by Cross-Knorr and colleagues provides interesting information on the impact of Oxaliplatin and Camptothecin on phosphorylation of RKIP and activation of STAT-3 signaling in vitro. In a first step, evidence of a IL-6 mediated activation of STAT-3 signaling leading to phosphorylation and inactivation of RKIP is provided using a set of well executed experiments including western blotting, apoptosis assays and luciferase reporter assays. Second, treatment with Oxaliplatin and Camptothecin is shown to reduce IL-6 mediated STAT3 activation and RKIP phosphorylation through negative regulation of the gp130/ STAT3 signaling complex. Third, using a set of 140 stage II colorectal cancer patients, the authors demonstrate an increased frequency of lymphovascular invasion and higher tumor grade in patients with increased expression of STAT-3 and nuclear pRKIP by immunohistochemistry. The paper further extends knowledge on the regulation of RKIP phosphorylation,
in particular in the setting of chemotherapeutic treatment and merits publication as an article of importance in its field. However, this reviewer sees room for improvement, in particular in the translational application of pRKIP and STAT3 as biomarkers in respect to adherence to the REMARK guidelines, including the clinicopathological characterization of the patient cohort under study and study design. Further, the manuscript requires substantial proofreading.

- Major Compulsory Revisions
  • The authors should comment on the adherence of the analysis of STAT3 and pRKIP as biomarkers in stage II patients to the REporting recommendations for tumour MARKer prognostic studies (REMARK), British Journal of Cancer (2005) 93, 387–391.

  *Response:* The guidelines in the “REMARK” paper mainly apply to clinical prognostic studies. Our paper summarizes an in vitro investigation of RKIP and STAT3 with an early study of their prognostic values. The REMARK paper noted “Not all of the elements apply to studies conducted in earlier phases of marker development”. However, our paper did address the vast majority of the recommendations in Table 1 of that paper as stated in the Introduction, Materials and Methods, Results, and Discussion.

  • Clinicopathological information including all essential prognostic factors as specified by the UICC TNM-staging system is recommended to be provided in the clinicopathological characteristics. The paragraph describing the patient characteristics of the cohort under study (Results, §7) should be included in the materials and methods section.

  *Response:* Table 1 has been modified for clarity. The cancers are all TNM stage II. They are all N0 M0. They were diagnosed before 2006. K-ras, b-RAF, MSI and budding statuses were not part of the routine workup of colon cancer prior to 2006.

  • The study focuses on the regulation of RKIP by chemotherapeutic agents. As specified by the authors, adjuvant chemotherapeutic treatment is standard of care for CRC patients with stage III disease. Even though recent studies indicate a benefit of adjuvant chemotherapy for high risk stage II patients, chemotherapeutic treatment is not yet standard of care for patients of this stage (American Society of Clinical Oncology Recommendations on Adjuvant Chemotherapy for Stage II Colon Cancer, JCO, (2004) 22: 3408-3419). Further information should be provided, why the authors choose to analyze RKIP and STAT-3 as possible predictive markers in a collective of stage II CRC patients. Alternatively, inclusion of patients with stage III and IV disease with subgroup analysis regarding the prognostic and predictive value of STAT3 and pRKIP is recommended.

  *Response:* We have investigated the role of RKIP/STAT3 in GI malignancies (Clin. Cancer Res., 14:2994-3001, 2008. PMID:18483365; J. of Immunopathological Diseases and Therap., 2, 35-46, 2011) for the past few years. In addition, there have been other
reports on the role of RKIP in metastatic colon cancer (Am J Clin Pathol 2007; 127:820-827; J. Clin. Oncol. 2006; 24: 5672-5679). Therefore, we wanted to determine the association between RKIP expression with clinical outcome in TMA. One of the prerequisites for inclusion of tumors into this stage II colon cancer microarray is the fact that the patients were not treated by adjuvant chemotherapy. Although RKIP function can be altered by chemotherapeutic agents, its prognostic role in stage II colon cancer has not been investigated.

It is correct that our study would be enhanced by the inclusion of stage III and IV disease subgroups. However, we do not have a TMA with such a cohort and constructing one would take considerable time, effort and resources that are currently unavailable.

- Minor Essential Revisions

Methods:
• The authors indicate that three to six cores of tumor were arrayed per colorectal cancer specimen. In light of recent publications highlighting the importance of the geographic expression pattern of RKIP in CRC (BrJC, 4/2013), information on the geographic regions where the samples have been taken should be provided (e.g. Center, Front), and the results should be discussed in context of this literature. The reference provided for TMA construction (Resnick et al, Hum Pathol 2005, 36(8):886-892) describes sampling only of the invasive cancer front (in gastric cancer).

Response: This TMA was used in a previous study (Modern Pathology 2005; 18; 511–518) which stated “Areas of interest which represented the invasive front of the tumor, rich in non-necrotic tumoral glands, were identified and marked on the source block.” Changes have been made to the Materials and Methods to reflect this.

• The authors indicate that the vast majority of the cases have a complete set of staining data and clinicopathologic information upon which statistical analysis was performed. A clear indication of exclusion criteria and numbers of cases evaluated for each marker is recommended for clarification of the number of cases under study.

Response: There are no special exclusion criteria. A small number of cores were missing after sectioning and therefore immunohistochemical staining data could not be obtained. Follow-up data was received from our Tumor Registry and as one would expect certain patients were lost to follow-up. The exact numbers of cases evaluated for each marker is stated in the Table 2. More annotations have been added in Table 2 to minimize the confusion.

Discussion:
§ 6 This paragraph is a repeat from the introduction.
• Please review.

Response: We have removed this paragraph. Sorry for the redundancy.
Conclusion:
§1 In summary, this study examines for the first time, the expression profile of RKIP, phospho-RKIP and STAT3 in Stage II colon cancer.
• Analysis of the expression profile of RKIP in stage II CRC is not performed by the current study. Please explain.

Response: The expression profile of RKIP was performed and no statistically significant correlation was found with any of the clinicopathological features or patient follow-up. This is the reason the study focuses on phosphorylated RKIP.

The results support the role of pRKIP and STAT3 in the provision of clinically prognostic and therapeutic information. …determination of pRKIP and STAT3 levels via IHC may allow for assessment of the risk for recurrence of colon cancer.
• These conclusions cannot be conclusively made based on the current study. pRKIP and STAT3 were examined in a small collective of CRC patients that was limited to stage II disease. Treatment information of the cohort was not provided. It is to be expected that only few patients within the given cohort would be candidates for chemotherapeutic treatment according to the ASCO Recommendations on Adjuvant Chemotherapy for Stage II Colon Cancer. Consequently, the information provided by pRKIP and STAT3 in the cohort under study for therapeutic decision making cannot be conclusively estimated.

Response: We agree. One of the prerequisites for inclusion of tumors into this stage II colon cancer microarray is the fact that the patients were not treated by adjuvant chemotherapy. Nonetheless, we have tempered our conclusion and it now reads:
In summary, this study examines for the first time, the expression profile of RKIP, pRKIP and STAT3 in Stage II colon cancer. Our results strongly suggest the role of pRKIP and STAT3 in the provision of clinically prognostic and therapeutic information. Our data indicate that the current treatment for colon cancer, FOLFOX and FOLFIRI, are both effective in reducing pRKIP levels in vitro. Therefore, examining a larger cohort of patients, in the future, will provide additional data for the assessment of pRKIP and STAT3 for the risk for recurrence of colon cancer. We hope that this is acceptable.

Figure 5 B, C, D:
• Please expand the description of Figures 5 A,B,C,D in the results section.
• The conclusion that nuclear STAT3 expression is associated with worse prognosis is only indirectly supported by more frequent LVI and higher grade. To support this claim, survival data for STAT-3 should be shown in analogy to pRKIP.

Response: Changes have been made to the Results section.
- Discretionary Revisions
• General remarks: Please use consistent labeling (both pRKIP and P-RKIP are currently used) throughout the figures, tables and paper. Please use consistent
labeling for p-values (both p= and P= are currently used).

Response: We have made the labeling consistent.

As mentioned elsewhere in this response, grade II colon cancer has a fairly good long term outcome. We have been following up the patients for up to 250 months (>20 years). Because many patients passed away of diseases other than colon cancer, the number of patients who directly died of colon cancer is few. This limits the power of survival analysis. We are happy that we found pRKIP has a significant impact on overall patient survival. We did not find any significant impact of nSTAT3 in our survival analysis. We do find nSTAT3 is related to high grade and LVI, two indirect indicators of worse overall survival. Our data indicates that a study with a larger case number will very likely show a significant impact of nSTAT3 on patient survival.

Abstract, § 4
• The conclusions in the abstract should be clarified further in context of the aim of the study. In particular, STAT3 and RKIP were already identified as study targets, and were not newly identified in stage II CRC and CRC cell lines as is suggested in the conclusion. A comment on the application of STAT3 and pRKIP as prognostic biomarkers is missing.

Response: We have taken the suggestion and included the suggested statement.

Discussion
• § 1 The authors comment on the work by Huerta-Yepez et al, demonstrating that low levels of pRKIP are related to poor outcome in lung cancer. The current paper provides evidence, that low levels of pRKIP are actually beneficial to CRC patients. A further comment on possible reasons for this discrepancy should be provided.

Response: We have addressed this now in the discussion.

- Quality of written English
• The manuscript requires proofreading before it is ready for publication. Generally, usage of correct greek symbols (e.g. µm to µm) is recommended where appropriate. Further, consistent use of abbreviations and labels is recommended. Please see suggestions in “minor issues”.

Response: We agree and have made the changes

- Minor issues not for publication
- Abstract:
- § 1 The Aim of this study was to evaluate the regulation of RKIP and STAT3 after treatment with clinically relevant chemotherapeutic agents (camptothecin (CPT)
and oxaliplatin (OXP)) and the cytokine interleukin-6 (IL-6) in HCT116 colon cancer cells as well as evaluate the association between RKIP and STAT3 with clinical outcome of Stage II colon cancer patients. 

**Suggestion:**
- The aim of this study was to evaluate the regulation of RKIP and STAT3 after treatment with clinically relevant chemotherapeutic agents (camptothecin (CPT) and oxaliplatin (OXP)) and the cytokine interleukin-6 (IL-6) in HCT116 colon cancer cells as well as to investigate the association between RKIP and STAT3 with clinical outcome of stage II colon cancer patients.

**Response: Corrected**

- § 3 We extended these observations and determined that that STAT3 and nuclear pRKIP are significantly associated with poor patient prognosis in stage II colon cancer patients.

**Suggestion:**
- We extended these observations and determined that STAT3 and nuclear pRKIP are significantly associated with poor patient prognosis in stage II colon cancer patients.

**Response: Corrected**

**Results**

- §1 Treatment with IL-6 causes phosphorylated RKIP levels to increase IL-6 has been shown to lead to STAT3 activation in colon cancer [26, 27]. HCT116 cells were treated for 1, 3 and 6 hrs with 40ng/ml IL-6. As expected we observed an increase in pY705STAT3 but were surprised to also note an increase in pRKIP (Figure 1A). HCT116 cells were treated with IL-6 and examined for STAT3 and RKIP phosphorylation. To our knowledge this is the first report to show cytokine-mediated phosphorylation of RKIP.

**Suggestion:**
- Treatment with IL-6 causes phosphorylated RKIP levels to increase IL-6 has been shown to lead to STAT3 activation in colon cancer [26, 27]. HCT116 cells were treated for 1, 3 and 6 hrs with 40ng/ml IL-6 and examined for STAT3 and RKIP phosphorylation. As expected, we observed an increase in pY705STAT3 but were surprised to also note an increase in pRKIP (Figure 1A). To our knowledge this is the first report to show cytokine-mediated phosphorylation of RKIP.

**Response: Corrected.**

- Results, §2 Oxaliplatin inhibits IL-6 signaling Previous studies have shown that treating colorectal cancer CT26 cells with 300 uM OXP for 24 hours leads to about 50% of the cells showing signs of apoptosis [49]. In our experiment treatment with OXP induced approximately 32% of the cells to undergo apoptosis which was lowered to 19% after cotreatment with IL-6 (Figure 1B)…
Western blot analysis showed that co-treatment of HCT116 cells with IL-6 and 300 uM OXP for 18 hours inhibited the increase in pY705 STAT3 and pRKIP caused by IL-6 (Figure 1C). OXP induced apoptosis was confirmed with Western blot analysis by measuring PARP (Poly-ADP-ribose polymerase) cleavage and DNA damage by H2AX (Histone 2AX) phosphorylation [10, 50, 51] (Figure 1C).

Results, §3 CPT (ST2461) reduces IL-6 induced RKIP phosphorylation and STAT3 Transcription … (p<0.0002) in STAT3 transcription when cells were treated with IL-6 and CPT treatment (Figure 2D).

Suggestion:

Results, §4 STAT3 overexpression increases pRKIP IL-6 treatment enhances STAT3 phosphorylation, transcription and pRKIP (Figures 1,2).

Results, § 7 Clinicopathologic features of cancer patients luciferase reporter assay luciferase reporter assay

Suggestion:

Results, § 8 Expression of phosphorylated RKIP in colon cancer and its prognostic value

Suggestion:
than the population with LVI (Figure 4C)

Suggestion:
• ...The percentage of patients with low levels of pRKIP and no LVI was much
greater than the population with LVI (Figure 4C)

Response: This has been changed.

... Twenty six percentage (26%) cytoplasmic pRKIP-low (< 3+) tumors are high
grade comparing with 11% cytoplasmic pRKIP-high (3+) tumors being high
grade...
Suggestion:
• Twenty six percent (26%) cytoplasmic pRKIP-low (< 3+) tumors are high grade
compared with 11% cytoplasmic pRKIP-high (3+) tumors being high grade

Response: This has been changed.

... Thus, low expression of cytoplasmic pRKIP is associated with higher grade
tumor...
Suggestion:
• Thus, low expression of cytoplasmic pRKIP is associated with higher tumor
grade...

Response: This has been changed.

Discussion:
§ 3 Previous studies show that protein kinase C (PKC) is responsible for the
direct phosphorylation of RKIP...
Suggestion:
• Previous studies show that protein kinase C (PKC) is responsible for the direct
phosphorylation of RKIP...

Response: This has been changed.

Figures:
• Including the percentages of each gate within Figure 1 C may make the data
easier to interpret for the reader.

Figure 1C:
• Axis labels are missing. Including the percentages of each gate within the
Figure may make the data easier to interpret for the reader.

Response: Changes have been made to Figures 1 and 2.

Figure 4A:
• Please include scale bars. It is recommended to use images with the same
magnification for better comparison.
- Figure 4B:
  - Please use a correct axis label for the Kaplan-Meier survival analysis
  - (“surviving” is unclear). Please indicate which survival curve corresponds to low
  - and high levels of pRKIP in the figure.

Response: The changes have been made to Figure 4.

- Figure 5A:
  - Please include scale bars. It is recommended to use images with the same
  - magnification for better comparison. The STAT3-positive case seems to be a
  - rare signet cell subtype of CRC. For better comparison with other studies, use of
  - a common case of intestinal type CRC is recommended.

Response: We have taken the suggestion and used images with the same
magnification and included scale bars.

Tables:
- Table 1:
  - Please correct spelling error in “High gade” to High grade
- Table 2:
  - Please correct Cytoplasmic, Nuclear and high Grade to cytoplasmic, nuclear and
  - high grade in the label.

Response: Changes have been made to Tables 1 and 2.

- Level of interest: An article of importance in its field
- Quality of written

Reviewer's report
Title: RKIP phosphorylation and STAT3 activation is inhibited by Oxaliplatin and
Camptothecin and are associated with poor prognosis in Stage II colon cancer
patients
Version: 1 Date: 29 May 2013
Reviewer: Fahd Al-mulla
Reviewer's report:
The paper by et al., entitled “RKIP phosphorylation and STAT3 activation is
inhibited by Oxaliplatin and Camptothecin and are associated with poor
prognosis in Stage II colon cancer patients” describes an interesting observation
that IL-6 induced STAT3 activation and phosphorylation of RKIP. co-treatment of
HCT116 cells with IL-6 and 300 uM OXP for 18 hours inhibited the increase in
pY705 STAT3 and pRKIP caused by IL-6. Similarly, Camptothecin reduces IL-6
induced RKIP phosphorylation and STAT3 transcription. Moreover, the levels of
pRKIP were reduced after STAT3 overexpression. The authors then studied STAT3 and pRKIP expression in stage II CRC and correlate these with clinicopathological characteristics of the patients. These are interesting data. However, this reviewer sees ample room for improvement. Both RKIP and pRKIP actions may be cell specific. The authors present data based on one single cell line, which really compromised these novel findings. Illustrating the same consequences in another cell line will strengthen their conclusions significantly.

Response: We have now included similar results obtained from another colon cell line (HT29) cells as a supplemental figure. We currently have a paper under revision in Plos One that shows these effects in prostate and breast cancer cells.

As stated, Chemotherapy induced the expression of RKIP. The authors did not comment on why RKIP expression in the Western blots did not increase after OXP or Camptothecin treatment? Moreover, if the RKIP antibody detects total RKIP (RKIP and pRKIP) why it is not elevated in the Western blots?

Response: We have shown the increase of RKIP protein after treatment with the CPT analog, 9NC (J Biol Chem 2004, 279(17):17515-17523). However, in this study we are using a structurally different CPT congener. The induction of RKIP by 9NC occurred by a transcriptional mechanism and this did not occur with the current derivative. This could be due to numerous reasons such as lack of recruitment of transcription factors, alteration in Pol II activity, protein degradation etc. We have studied various DNA damaging compounds in our laboratory and have determined that not all agents induce RKIP, especially taxane derivatives (Molecular Cancer, submitted). This is also apparently the case with OXP.

We have observed the increase in pRKIP without the concomitant increase in RKIP (PLoS One 2012, 7(5):e37819). This was due to an increase of proteosome-mediated RKIP degradation. We suspect that a similar mechanism may occur in this study but did not pursue it because our focus was on pRKIP.

RKIP reduced or loss of expression has been well documented in a variety of cancers especially stage II CRC. The authors provided no data on this but rather focused their findings on p-RKIP, which (overexpression) had been previously shown to be associated with good prognosis not worse. Can the authors provide data on RKIP survival in stage II CRC?

Response: According to the manufacturer, the RKIP antibody does not recognize pRKIP and the pRKIP antibody does not recognize RKIP. We have used a blocking peptide to pRKIP in our previous publication (BMC Cancer 2011, 11:259) and did not detect RKIP. Based on our preliminary data, RKIP did not have significant prognostic value on survival. This is why we focused on phosphorylated RKIP in this paper.
This reviewer is concerned about the term ‘Limited’ used to designate the number of patients used in the survival calculation. What are the numbers? Also, the authors did not attempt a multivariate analysis on the dataset. This is extremely important to exclude other confounding factors associated with poor prognosis in stage II (Like T-Stage, which has not been mentioned, MMR-status etc)

All of the patients had stage II colon cancer. Cancer at this stage in general has good prognosis. Most patients are either still alive or died of other causes and therefore are censored according to statistics. The survival statuses of patients who died of cancer are plotted in the K-M curve. Multivariate analysis will be not useful given the small number of un-censored cases.

It is not clear which p-RKIP antibody was used in the Immunohistochemistry (Although in the method section STAT3 antibody source was stated clearly, the same was not true for pRKIP ) If the same anybody was used for the Western blotting then there is a problem because the sensitivity and the specificity has not been determined for the pRKIP in immunohistochemistry. Santa Cruz pRKIP clone sc-32623 is a polyclonal antibody and in our hands proved problematic on immunohistochemistry. This reviewer suggests that the staining be performed again with monoclonal antibodies for RKIP and pRKIP on the same section (Epitomics).

Response: We have now stated the source in the Methods the sources of all the antibodies. We have used the pRKIP antibody for our previous publication (BMC Cancer 2011, 11:259) and this is the reason it was used for the current manuscript. The IHC was completed 2 years ago. It is a valid point to evaluate another antibody. Unfortunately, we currently do not have the resources to re-analyze the study with another antibody. We will certainly consider it for our future research. Both polyclonal and monoclonal Abs have advantages and disadvantages. For example, the use of polyclonals has the added benefit of recognizing multiple epitopes. This can increase sensitivity of detection - while not necessarily reducing specificity. The polyclonal Abs for both RKIP and pRKIP have been widely used and validated in the literature. Therefore, we feel there is no reason to doubt others results and no reason to assume the Abs are not specific.

This reviewer is also concerned that scoring of the sections appeared different for cytoplasmic and nuclear stains. For example, 0,+1,-2 were grouped together in cytoplasmic and designated low expression, while only 0 scores were designated as low in nuclear scoring. This is not appropriate and should be discouraged. Similarly, what happens when p-RKIP is +3 for both nuclear and cytoplasmic (Figure 4A)? These may be a totally different and important subclass?
Response: Please see the diagram below regarding cytoplasmic pRKIP expression levels:

![Diagram](image.png)

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>0.01563</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>0.10938</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
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</tr>
<tr>
<td>3</td>
<td>66</td>
<td>0.51563</td>
</tr>
<tr>
<td>Total</td>
<td>128</td>
<td>1.00000</td>
</tr>
<tr>
<td>N Missing</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>4 Levels</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Response: These are all excellent points made by the reviewer.

pRKIP was expressed in the cytoplasm of almost all the cancers. +1 and +2 constitute 48% of the cases, and +3 constituted 51%. To simplify the data presentation and to obtain a relative even distribution of expression levels, 0, +1, and +2 were grouped together.

As mentioned above, pRKIP is expressed in the cytoplasm of almost all the cancers at variable levels. The different localization likely indicates a different function. This is one of the points of focus of the paper.

We feel that it is not wrong to use different scales. We do not claim that the scoring means the same thing in each compartment. The scoring is only meant to be relative intensity for each cellular compartment - not absolute. It would be extremely hard, if not impossible, to claim that a score of 3+ in the nucleus (which is much smaller) is the same as a 3+ in the cytoplasm.

Minor changes:
There are numerous grammatical and spelling errors that needs to be corrected
Response: We have corrected the mistakes

The introduction and discussion need to refer to recent papers showing the prognostic value of RKIP and lymphovascular invasion in stage II CRC (Raf kinase inhibitor protein expression combined with peritoneal involvement and lymphovascular invasion predicts prognosis in Dukes' B colorectal cancer patients. Doyle B. et al) and the relationship between RKIP and chemotherapeutic resistance (A new model for raf kinase inhibitory protein induced chemotherapeutic resistance. Al-Mulla F, et al.)

Response: These are now included.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Needs some language corrections before being published
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