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)
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.

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.

- 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).

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.

Discussion:

§ 6 This paragraph is a repeat from the introduction.

• Please review.

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.

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.

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.

- 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).

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.

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.

- Quality of written English
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.

§ 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.

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

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.

Results, §2 Oxaliplatin inhibits IL-6 signaling Previous studies have shown that treating colorectal cancer CT26 cells with 300 μM 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)...

• This data seems to refer to Figure 1C/c vs. 1C/e not to Figure 1B. Please clarify.

Western blot analysis showed that co-treatment of HCT116 cells with IL-6 and 300 μM 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).

• This data seems to refer to Figure 1B, not to Figure 1C. Please clarify.

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:
• … (p<0.0002) in STAT3 transcription when cells were treated with IL-6 and CPT (Figure 2D).

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

Suggestion:
• 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:
• § 7 Clinicopathologic features of cancer patients

…correlate our cell based studies with the colon cancer patient clinical outcome we examined TMA of 140 patients.

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• …correlate our cell based studies with the colon cancer patient clinical outcome we examined a TMA of 140 patients.

Results, §8 Expression of phosphorylated RKIP in colon cancer and its prognostic value
... The percent of patients with low levels of RKIP and no LVI was much greater 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)

... 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...

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

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

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

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Figures:

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

Figure 2C:
• Axis labels are missing. Including the percentages of each gate within the Figure may make the data easier to interpret for the reader.

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.

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.

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.

Level of interest: An article of importance in its field

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