Author’s response to reviews

Title: Eukaryotic Elongation Factor-2 Kinase Expression is an Independent Prognostic Factor in Colorectal Cancer

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Author’s response to reviews:

Jean-Philippe Brosseau, PhD
Handling Editor
BMC Cancer

9th May 2019

Dear Dr Brosseau,

RE: Your submission to BMC Cancer - BCAN-D-19-00327R1

Thank you for your interest and giving us the opportunity to revise our manuscript. Below is an itemized summary of the reviewers’ comments, followed by our responses and additions to the
manuscript. Changes to the manuscript in response to comments are highlighted in yellow in the text of our revised paper to facilitate your review.

We believe that your comments and those from the reviewers have improved our manuscript. We sincerely hope that you feel our manuscript worthy of publishing. All authors have seen and approved the final manuscript submitted.

Yours Sincerely,

Christopher H.K. Cheng and William K.K. Wu, on behalf of all co-authors

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Point-by-point response

Reviewer 1

Zhang Xiaoli (Reviewer 1): In this paper, the authors used 20 patients to investigate the EEF2K gene expression at mRNA and protein level comparing the tumor to adjacent normal. After they found there was decreased expression of EEF2K in tumor, they used another cohort of 151 patients to test their hypothesis that reduced EEF2K associates with worse survival. The authors also used the TCGA CRC data to support their finding that reduced EEFK2 expression is associated with worse survival. However there are some major issues the authors didn't cover:

Was the expression of EEF2K correlated with patients' age, gender, especially patient clinical characteristics such as tumor stage, node status and metastasis status in the cohort of 151 patients? Without answering this, it is not appropriate to conclude that EEF2K is an independent prognostic factor.

Response: Patients’ clinical characteristics including age, gender, tumor grade and TNM staging were included in multivariate Cox regression analysis when assessing the prognostic potential of EEF2K expression (Table 3). Their potential confounding effects were already controlled in this analysis. To further support this, correlation analysis between EEF2K expression and patients’ clinical characteristics (including age, gender, tumor location, tumor grade, TNM staging and MSI status) was also performed (Page 14, line 246-248 and Table 2). No significant correlation was identified.

On page 13 line 231-234 the sentence is confusing "that patients in the low expression group had worse overall survival in obvious than those in the high expression group in patients with Stage IV". Did the authors mean that the tested patients were all at stage IV, or patients with high expression were at stage IV? Please clarify.

Response: We have now clarified that the clinical relevance with survival outcome of CRC patients was only noted in patients with stage IV CRC in TCGA dataset. It is aware that the
methodologies in assessing EEF2K expression level, and also the ethnicity of patients and underlying genetic background were different and may account for the discrepancy (Pages 16-17, line 296-302).

The authors used the TCGA data to validate their finding, however the result is not convincing. What is the rational for only using patients with high and low expression? A survival analysis using all the data should be done instead of only using the patients with the highest and lowest expression or with a specific stage. Correlation of EEF2K with patient tumor stage should be tested to indicate whether the low expression group was patients with high tumor stage. In addition, a comparison between tumor and normal is needed to confirm the author's point that EEF2K was decreased in CRC.

Response: Our dataset exhibits normal distribution. Cases centered at the middle will mask the underlying difference between high and low EEF2K expression. In Additional File 2, we assessed the significance level using different cut off values to group the patients (from highest 50% vs lowest 50% down to highest 10% vs lowest 10%). The p values progressively decreased with more extreme comparison which support our findings that low expression of EEF2K correlate with worse clinical outcomes (p value increased at 10% cut-offs due to limited sample size). Therefore, we compared high expression vs low expression.

Our results could only be validated in patients with stage IV CRC but not stage I-III. However, the methods used to assess the EEF2K expression level are different, in which in our cohort we assess protein expression by IHC whereas in TCGA cohort it’s mRNA expression by RNAseq. Also, the patients of the two cohorts experience genetic difference (mainly Caucasian in TCGA vs Asian in our cohort). These may account for the differences.

Response: The association of EEF2K expression with TNM staging was studied (Figure 5). EEF2K expression was not correlated with advanced TNM staging (Page 15, line 274-278). Also, we compared the EEF2K expression level between tumor and normal tissues. EEF2K expression was downregulated in tumor tissues in both unpaired and paired comparisons (Page 15, line 272-274 and Figure 4).

Reviewer 2

Veronique Giroux (Reviewer 2): BCAN-D-19-00327R1

Eukaryotic Elongation Factor-2 Kinase expression is an independent prognostic factor in colorectal cancer

The authors and other have previously shown that EEF2K could act as a tumor suppressor in colorectal cancer using cell lines and in vivo models. Herein, they have performed some expression experiments and clinical data analysis to suggest that EEF2K expression could be
used as a prognostic factor. They have used several approaches that all lead to the same conclusions. Overall, it is a well-conducted study that suggests a new prognostic tool for colorectal cancer. However, further in-depth analysis would strengthen the manuscript.

Major comments:

1. Since colorectal cancer exists in several different types (sporadic vs hereditary; adenocarcinoma vs serrated vs …), more information regarding the tissues used for the expression experiments would provide insightful data. Also, does EEF2K expression correlate with any subtypes of colorectal cancer?

Response: It is a pity that the clinical information of patients in the expression experiments is limited. We only have the information regarding patients’ age, gender, tumor location, tumor grade, T stage and N stage. We grouped patients based on expression patterns of EEF2K between paired tissues (Upregulation vs Downregulation) and performed correlation analysis with these variables. No significant association was found (Page 13, line 232-234 and Table 1).

The same approach was applied in our TMA cohort. We performed correlation analysis on different variables (age, gender, tumor location, tumor grade, TNM stage, MSI status) but no significant correlation was found (Page 14, line 246-248 and Table 2).

Regarding TCGA cohort, we obtained EEF2K expression levels from RNA sequencing data and performed correlation analysis by comparing means of EEF2K expression with patients’ information on age, gender, tumor location, CRC subtypes (sporadic vs familial) and TNM staging, and similarly, we did not find any significant correlation (Page 15, line 274-278 and Figure 5).

For correlation analysis with CRC subtypes (adenocarcinoma and serrated CRC), we do not have related information in all of the assessible datasets. Yet, it is noted that serrated CRC predominantly occurs in the right colon whereas adenocarcinoma is usually in the left colon. No significant correlation between EEF2K expression with tumor location could, to some extent, suggested that EEF2K expression is not associated with either CRC subtypes.

2. Figure 1C: Is there anything common between the 5 samples that did not show a decrease in EEF2K expression? Subtypes, mutation, localization, differentiation status, treatment regiment, etc.

Response: It is a pity that the clinical information of patients in the expression experiments is limited. We only have the information regarding patients’ age, gender, tumor location, tumor grade, T stage and N stage. We grouped patients based on expression patterns of EEF2K
between paired tissues (Upregulation vs Downregulation) and performed correlation analysis with these variables. No significant association was found (Page 13, line 232-234 and Table 1).

3. Due to the published role of EEF2K in cell proliferation of APC-deficient enterocytes, is there any correlation between EEF2K expression and APC mutation status in their samples as well as TCGA data? Any correlation with other common mutations (TP53, KRAS, SMAD4, MSI vs MSS)

Response: In our TMA cohort, EEF2K expression was not correlated with MSI status (Table 2).

In TCGA cohort, we compared means of EEF2K expression with mutational status on TP53, KRAS, APC, BRAF and MSI status (Page 15, line 274-278, Figure 5 and Additional File 1). No significant association was identified, indicating that EEF2K expression was not associated with any of these mutations.

Please note that KRAS and BRAF mutation and MSI status were examined based on tests adopted in clinical settings so that sample sizes were relatively larger, while mutational status on TP53 and APC were obtained from whole-genome sequencing data, in which only 7 cases could be matched with EEF2K expression from RNA sequencing data for analysis. Meanwhile, no SMAD4 mutation was found in those 7 cases.

4. Due to the relation between EEF2K and chemotherapy response, is there any correlation between EEF2K expression and previous cancer treatments in the patients analyzed (naïve vs chemo-treated) in their own cohort as well as TCGA data?

Response: In TCGA cohort, there is only one patient receiving previous treatment and the rest are all treatment naïve. Therefore, the sample size is not enough for statistical analysis. In our TMA cohort, it is a pity that we lack the information on the treatment history of patients prior to operation and thus no analysis can be done to address this question.

Minor comments:

5. Page 4, line 75: should read as "up to 50% patients whom underwent"

Response: Amended accordingly (Page 6, line 99)

6. Page 5, top paragraph: some references should be included to support that the clinical value system currently in used could be improved to better pinpoint the best treatment for the different patients.

Response: References were added accordingly (Pages 6-7, line 108-115 and References 3 and 4)
7. Page 9, line 169: What was the cut-off value (intensity and/or percentage of stained cells) used to delineate positive vs negative expression?

Response: It has now been elaborated in METHODS (Page 11, line 202-205)

8. Page 11, line 198: should read as "subjected"

Response: Amended accordingly (Page 13, line 239)

9. Page 13, top paragraph, last sentence: Sentence should be revised to make it clearer.

Response: Revised accordingly. (Page 16 top paragraph)