**Author’s response to reviews**

**Title:** Estrogen receptor (ER) signaling regulates the expression of the breast tumor kinase (BRK) in breast cancer cells

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**Version:** 2 **Date:** 21 Sep 2018

**Author’s response to reviews:**

September 20, 2018

Dr. Catherine Rice

Editorial board

BMC Cancer

RE: Resubmission BCAN-D-18-01530R1
Dear Dr. Rice,

Thank you for providing reviewers comments for our manuscript (BCAN-D-18-01530R1) titled: “Estrogen receptor (ER) signaling regulates the expression of the breast tumor kinase (BRK) in breast cancer cells”, on September 04, 2014. You gave us until October 04, 2018 to submit a revised version of the manuscript.

We are pleased to inform you that we have revised the current manuscript and have addressed and/or justified all 15 comments issued by the reviewers. Please find our response after every comment made by the reviewers. We hope that our accepted manuscript is now suitable for publication in BMC Cancer.

Sincerely,

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Response to Reviewer’s Comments

We would like to thank the reviewers for their helpful comments and suggestions to improve the manuscript. To address the reviewer’s concerns, we have revised the manuscript, and we believe this revision has improved the manuscript.

Reviewer #1 (Guy Leclercq, PhD)

Overall, objective outlined in the abstract of the manuscript are satisfied, taken into account that most of the reported data refer to ERa as specified in this abstract. Even if ERb transfections within ER-negative cells may enhance BRK level (Fig. 5 D), one may consider that most observations reported in the manuscript relate to ERa or eventually to an ERa/ERb ratio favorable to ERa, ERb presenting an antagonistic activity against the ERa action. The very significant BRK expression in BRCA vs normal mammary tissue supports this primacy of ERa (Suppl. Table 1; normal tissue express mainly ERb rather than ERa). Hence, ERa rather than ER frequency written in the manuscript would better fit.

On the other hand, studies conducted on breast cancer cell lines clearly show that BRK expression relates to the level of ERa subjected to activation. Tamoxifen known to provoke accumulation of ERa in an inactive form fails to enhance BRK expression. Hence, presence of ERa is required but not sufficient for such an enhancement. Note in this context that the very significant correlation between ERa and BRK in breast cancer cell lines (p11; line 1/2) would most probably not similarly hold if the analysis had been restricted to ERa-positive cells. Some ERa-positive cells display indeed relatively low amounts of BRK. (Fig 5 B vs C). Reason for such a property would probably be related to a basal growth condition which may limit BRK expression. Test with E2 stimulated cells would logically validate this view (as well as other ERa activators).

In this context of ERa activation, one may wonder upon a possible contribution of BRK in a rapid phosphorylation of the membrane bound form of ERa required for activations of signal transduction pathways as well as subsequent ERE-dependent and independent transcriptions (see p14). Even if BRK-shRNA fail to affect ERa levels, would such receptors still able to generate such responses. Assessment of this question seems quite easy. It would not necessary be included within this study but proposed for further investigations.

Other findings of the study were not addressed here: they are sufficiently commented and do not suffer of criticisms.

In brief, this study is original, well conducted and interesting.
1. ERα rather than ER frequently wrote in the manuscript would better fit.

Response to the reviewer’s comment: We agree with reviewer’s comment and ER is replaced with ERα when referring to ERα specifically in the lines 80 and 341 of example. The change was not made when referring ER-positive breast cancer subtype since this is the standard way of referring to the subtype.

2. In this context of ERα activation, one may wonder upon a possible contribution of BRK in rapid phosphorylation of the membrane-bound form of ERα required for activations of signal transduction pathways as well as subsequent ERE-dependent and independent transcriptions (see p14). Even if BRK- shRNA fail to affect ERα levels, would such receptors still able to generates such responses. Assessment of this question seems quite easy. It wound not necessary be included within this study but proposed for further investigations.

Response to the reviewer’s comment: We have added the following in the Discussion section as suggested: “Unliganded ERα can be phosphorylated by membrane receptor tyrosine kinases including the epidermal growth factor receptor (EGFR), HER2, and IGF1-R or non-receptor kinases such as Src, leading to dimerization and the activation of transcription {Viedma-Rodriguez, 2014 #9674}. A possible contribution of BRK in the rapid phosphorylation of the membrane-bound form of ERα is currently under investigation.”

3. Complementary remark: Text p9/fig 3, bottom of the page should be simplified. It looks to be an incorrectly modified form of a previous manuscript (Fig 3 A/B; Fig 4 not existing)

Response to the reviewer’s comment: We have corrected the figure numbering and modified text and figure numbering for clarifications. Pages 9 and 10 lines 210-218.

Reviewer #2

The overall objective of the manuscript is made clear right from the beginning which is great. Background section is drafted very well.
In the Methods section, I suggest that the authors provide a listing of all cell lines, as opposed to mentioning 'all breast cancer cell lines…'. The section should also include culture conditions etc.

'Stable cell preparation and Transfection' should ideally be 'Transfection and Stable cell line preparation' because transfection precedes selection for stably transfected cells. This subsection should also briefly describe how the stable cells were selected. I understand that reference to earlier publication(s) is provided but same basic details should be provided right here.

The authors mention that plasmids were gift from Dr. Michael Mancini. I suggest that the Institution details of Dr. Michael Mancini should be provided.

Are there any specific identifiers for the 24 tumor samples the data for which was downloaded from TCGA?

The very first results are assessment of BRK levels in 24 patients (TCGA). The authors look at samples that represent different human cancers. Are the observations statistically reliable, given that there are just a few samples representing individual cancer types? Also, since the samples represent different cancers, in this reviewer's opinion it is not appropriate to describe results in the way they have been presented. For example, instead of mentioning that 15 of the 24 cancer had higher BRK, they should mention which cancers had high levels, which cancers had moderate levels and which ones had lower levels, compared to normal. Irrespective of outcome, this is not a very convincing piece of data, given the sample size.

In continuation of my above comment, it is possible that the authors have not presented their case very well. Instead of 24 patients, what they actually meant was perhaps 24 different human cancers. If that is the case, the results are good! But the authors need to make it explicitly clear that they are talking about analyses of 24 different cancers and not 24 patients!! Please note that the description in Methods definitely seems to suggest that authors are talking about 24 samples (patients). They need to re-phrase sentences to make this very clear.

When checking for the correlation of BRK with breast cancer subtypes, the authors seem to convey a message that BRK levels were higher in ER+ breast cancers, compared to HER2+ or TNBCs. In this reviewer's opinion, the comparison should be made with normal controls as the primary analyses. The comparison between subtypes is also informative but the diagnostic value can only be assessed by comparing levels with normal tissues.

In the Results section 'BRK protein expression correlates with tumor progression' the authors state in the very first sentence that their intent was to corroborate BRK at protein levels so as confirm mRNA results. This is not correct. The intent of the results presented in the section was actually to correlate BRK levels with tumor metastasis and progression (as rightly mentioned in section title). I would suggest a careful editing of all the text to minimize such errors and improve presentation.
The heading 'BRK protein expression is generally higher in ER-positive breast carcinomas compared to other subtypes' does not entirely reflect the contents. The real goal of the analyses in this section is to establish that metastatic samples from ER+ breast cancers have higher BRK protein levels than metastatic samples representing other breast cancer subtypes.

The data from cell lines is too dramatic and seems to suggest that ER+ cells have higher levels than all other cells. Is this also the case with human samples? Do all breast cancer patients with ER+ disease have higher BRK levels than HER2+ and TNBCs?

I commend the authors that they have provided a lot of information and data. However, this is not accurately and adequately reflected in the Abstract. I recommend that the authors re-write the Abstract to reflect on all the contents and to make it more impressive.

Finally, a tight editing for the manuscript is needed. I have pointed out some concerns above. Additionally, improvements are needed in the language / grammar at many points. A few examples are - Please remove 'any' from this sentence in the Abstract - 'Herein, we investigated the correlation of BRK with any breast cancer subtypes…' and the sentence 'As shown in Figure 1B, the log2 fold change of the BRK mRNA in different subtypes of breast cancers' is not grammatically correct.

1. In the Methods section, I suggest that the authors provide a listing of all cell lines, as opposed to mentioning 'all breast cancer cell lines…'. The section should also include culture conditions etc.

Response to the reviewer’s comment: All the breast cancer cell lines were listed in the manuscript. Please see page number 4 and lines 85-90.

2. 'Stable cell preparation and Transfection' should ideally be 'Transfection and Stable cell line preparation' because transfection precedes selection for stably transfected cells. This subsection should also briefly describe how the stable cells were selected. I understand that reference to earlier publication(s) is provided but same basic details should be provided right here.

Response to the reviewer’s comment: The title has been changed, and a brief description of the procedure has been added to the manuscript. Please visit pages 6 and 7 and lines 127-146.

3. The authors mention that plasmids were gift from Dr. Michael Mancini. I suggest that the Institution details of Dr. Michael Mancini should be provided.

Response to the reviewer’s comment: The detail affiliation of Dr. Michael Mancini has been provided. Please visit pages 6 and line 128.
4. Are there any specific identifiers for the 24 tumor samples the data for which was downloaded from TCGA?

Response to the reviewer’s comment: It is actually 24 tumor types and not 24 tumor samples. Thus each tumor type has hundreds of samples.

5. The very first results are assessment of BRK levels in 24 patients (TCGA). The authors look at samples that represent different human cancers. Are the observations statistically reliable, given that there are just a few samples representing individual cancer types?

Response to the reviewer’s comment: 24 different types of cancer sample from more than 11,000 patients were used (and not 24 patients sample). We have clarified this in the manuscript. Please see page no 7, 179 and lines 157, 179 respectively. The observations are statistically reliable and indicated in the figure and the figure legends. We noted in Figure 1A legend that “...Data obtained from The Cancer Genome Atlas database, median ± one quartile; *p <0.05; **p <0.01; ***p <0.001; ns=not significant (also see Table 1 for details)...”. In Figure 1B legend we indicated that: “…Statistical significance was calculated against the normal tissue: p-value 8.1 x 10^-45 (ER-positive); p-value 2.3 x 10^-11 (HER2-positive); p-value 0.002 (TNBC). P < 0.005=significant.”

6. Also, since the samples represent different cancers, in this reviewer's opinion it is not appropriate to describe results in the way they have been presented. For example, instead of mentioning that 15 of the 24 cancer had high BRK, they should mention which cancers had high levels, which cancers had moderate levels and which ones had lower levels, compared to normal. Irrespective of outcome, this is not a very convincing piece of data, given the sample size.

Response to the reviewer’s comment: Our intention was to highlight the global expression pattern of BRK across cancer type which has been shown in the box plot. Additionally, a supplementary table was also provided which contained the detailed analysis of the expression pattern of different cancer types. Since we have emphasized breast cancer, a detail description and comparison has been made on it.

7. In continuation of my above comment, it is possible that the authors have not presented their case very well. Instead of 24 patients, what they actually meant was perhaps 24 different human cancers. If that is the case, the results are good! But the authors need to make it
explicitly clear that they are talking about analyses of 24 different cancers and not 24 patients!! Please note that the description in Methods definitely seems to suggest that authors are talking about 24 samples (patients). They need to re-phrase sentences to make this very clear.

Response to the reviewer’s comment: We agree with the reviewer’s comment. A necessary change has been made to remove the confusion. Please see the explanation in point 5.

8. When checking for the correlation of BRK with breast cancer subtypes, the authors seem to convey a message that BRK levels were higher in ER+ breast cancers, compared to HER2+ or TNBCs. In this reviewer's opinion, the comparison should be made with normal controls as the primary analyses. The comparison between subtypes is also informative, but the diagnostic value can only be assessed by comparing levels with normal tissues.

Response to the reviewer’s comment: We agree with the reviewer. We have compared the expression levels of BRK across the subtypes of breast cancer along with normal tissues what reviewer suggested. Please visit pages 10 and 11 and lines 224-240.

9. In the Results section 'BRK protein expression correlates with tumor progression' the authors state in the very first sentence that their intent was to corroborate BRK at protein levels so as confirm mRNA results. This is not correct. The intent of the results presented in the section was actually to correlate BRK levels with tumor metastasis and progression (as rightly mentioned in section title). I would suggest a careful editing of all the text to minimize such errors and improve presentation.

Response to the reviewer’s comment: mRNA expression is not always reflected in the protein level. Since we have observed higher BRK mRNA expression in breast tumor samples, we have examined whether this higher BRK mRNA is reflected in BRK protein level. We have found the elevated expression pattern of BRK mRNA and protein in those tumor tissue samples. Please see pages 9, 10 and line202-222.

10. The heading 'BRK protein expression is generally higher in ER-positive breast carcinomas compared to other subtypes' does not entirely reflect the contents. The real goal of the analyses in this section is to establish that metastatic samples from ER+ breast cancers have higher BRK protein levels than metastatic samples representing other breast cancer subtypes.
The data from cell lines is too dramatic and seems to suggest that ER+ cells have higher levels than all other cells. Is this also the case with human samples? Do all breast cancer patients with ER+ disease have higher BRK levels than HER2+ and TNBCs?

Response to the reviewer’s comment: We have analyzed over a hundred breast tumor samples, and representative IHC images were provided in Fig. 2 and Fig. 3 and observed that ERα+ breast tumor tissues exhibited higher BRK levels than HER2+ and TNBCs breast tumor tissues. Additionally, we have examined 17 breast cancer cell lines and observed a pattern that ERα+ cells showed higher expression of BRK (Fig. 4A). Thus, we could not claim that all breast cancer patients with the ERα+ disease have higher BRK levels than HER2+ and TNBCs.

11. I commend the authors that they have provided a lot of information and data. However, this is not accurately and adequately reflected in the Abstract. I recommend that the authors re-write the Abstract to reflect on all the contents and to make it more impressive.

Response to the reviewer’s comment: We have re-written part of the abstract. Please see line no. 46-50.

12. A tight editing for the manuscript is needed. I have pointed out some concerns above. Additionally, improvements are needed in the language/grammar at many points. A few examples are - Please remove 'any' from this sentence in the Abstract - 'Herein, we investigated the correlation of BRK with any breast cancer subtypes….' and the sentence 'As shown in Figure 1B, the log2 fold change of the BRK mRNA in different subtypes of breast cancers' is not grammatically correct.

Response to the reviewer’s comment: “Any” has been removed from that sentence line 35. Obvious grammatical errors have been corrected including some in lines 193, 237, 251, 285 and 332.