Author's response to reviews

Title: A synergistic interaction between transcription factors nuclear factor-kappaB and signal transducers and activators of transcription 3 promotes gastric cancer cell migration and invasion

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

We thank the reviewers for their careful evaluation of our manuscript (MS: 6357136518172383) entitled as “A synergistic interaction between transcription factors nuclear factor-κB and signal transducers and activators of transcription 3 promotes gastric cancer cell migration and invasion”.

We agree with the reviewers and tried our best to revise our manuscript in order to address all the comments in the revised manuscript. However, we regret that some of the concerns could not be answered in the revised manuscript because of the lack of time (reviewer 1, comment #9) or the technical problems in our laboratory (reviewer 2, comment #3) at the present time. Major changes are manifested in blue letters. The point-by-point responses to the reviewers’ concerns are described below.

In addition, we listed figure titles after references in the revised manuscript, and removed number of figures in the figure file. We also tried to crop the figures as much as possible.

Again, we highly appreciate the reviewers’ constructive comments. We feel that our manuscript has been greatly improved and hope that it is now acceptable for the publication in the BMC Gastroenterology. We are looking forward to hearing a good news from you.

Thank you.

Yours sincerely,

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Responses to the reviewers’ comments

Reviewer 1

1. Background, page 4. When literature about NF-kB activation in gastric cancer is mentioned, it is important to say whether it happens in cancer cells, infiltrating cells or probably both. Also 1 sentence before, not only cytokines etc can activate the pathway, but also TLR signaling and many other pathways of microbial recognition, relevant for gastrointestinal tumorigenesis.

- As the reviewer recommended, we added the following sentences to the background section (page 4, paragraph 2, line 6-10):

  With respect to gastric cancer, NF-κB is one of the most well-studied transcription factors, and is known to be activated by various factors, including cytokines [6-8], growth factors [9], Toll-like receptor signaling [10] and many other pathways of microbial recognition [11,12]. NF-κB activation has been frequently observed in both gastric cancer cells (20-83%) [13-16] and tumor-infiltrating lymphocytes [17].

2. Page 5. NF-kB deletion is not often used, it is likely IKKβ deletion which results in defective NF-kB activation.

- We agree with the reviewer that our description was not correct. We replaced NF-κB deletion with IKKβ deletion in the revised manuscript (page 5, paragraph 2, line 10).

3. Methods, page 6. Slides after deparaffinization are likely to be rehydrated, not dehydrated.

- As the reviewer pointed out, we changed the word dehydrated to rehydrated (page 6, paragraph 2, line 13).

4. When authors discuss simultaneous activation of NF-kB and STAT3 (Fig1 and elsewhere) it would be nice to see double color (IF) co-staining for both in the same cell/nucleus.

- As the reviewer recommended, we performed double immunofluorescence staining of
pRelA and STAT3 (page 9, paragraph 3) and found that pRelA and STAT3 were colocalized in the nucleus of same gastric cancer cells, which was reduced in IκBαM-overexpressing cells.

- We added these results to Figure 2D and explained the details in the Results section (page 12, paragraph 1, line 15-17) and Figure legends (page 27, paragraph 2, line 12-14).

5. Table 1 data presentation looks a little bit not clear to me. How the data is organized. Table should be explained better. For example, how to find/deduce from the table that NF-KB is activated in 16% of tumors?

- As the reviewer recommended, we showed the percentage of the positive and negative cases of NF-κB activation (manifested by nuclear RelA expression) and STAT3 activation (manifested by nuclear pSTAT3 expression) in Table 1 for the better understanding. In addition, we explained our data more clearly in the Results section (page 11, paragraph 2, line 8-11).

6. Page 11. More references about EMT should be provided with 1-2 specific to the cases of gastric cancer, because not in all types of cancer the existence and importance of EMT has yet been demonstrated.

- Although the existence and importance of EMT has not been shown in all types of cancers, previous studies have demonstrated that EMT plays a key role in the malignant progression of gastric cancer by using gastric cancer cell lines, orthotopic xenograft tumors and surgical gastric cancer specimens [refs. 41-43 in the revised manuscript].
- We added this discussion in the text (page 16, paragraph 2, line 2-5) along with three references (refs. 41-43), which demonstrated the importance of EMT in the malignant phenotype of gastric cancer.

7. Fig5A would look better and more convincing if fractionated nuclear extracts would be blotted.

- As the reviewer recommended, we obtained nuclear extracts (NE) and performed immunoblotting for pRelA and pSTAT3 (page 8, paragraph 1).
- We added the results to Figure 5A (4th, 5th and 6th rows) and explained in the Figure legends (page 28, paragraph 4 line 4; page 29, paragraph 1, line 1-2).
- We described details of our data in the Results section as follows (page 14, paragraph 3):

To examine the effects of co-transfection of IκBαM and STAT3 siRNA on expressions of pRelA and pSTAT3, we obtained whole cell lysates and nuclear extracts and performed immunoblotting. We found that double knock-down of RelA and STAT3 induced marked down-regulation of pSTAT3 expression in both the whole cell lysates (1st-3rd rows in Figure 5A) and nuclear extracts (4th-6th rows in Figure 5A).

8. Page 12. These data suggest that STAT3 –induced........... I would suggest to correct wording here: Stat3 in this system is induced not only through NF-kB, but also through something else. It is known that Stat3 pathway can be induced by many NF-kB independent pathways including some cytokines and Tyr-kinases.

- As the reviewer pointed out, we corrected wording in the revised manuscript as follows (page 14, paragraph 4, line 6-9):

These data suggest that STAT3 in this system is induced not only through NF-kB, but also through something else. It is known that STAT3 pathway can be induced by many NF-kB independent pathways including some cytokines [38] and tyrosine kinases [39].

9. This study would benefit from at least the most simplistic in vivo metastatic/dissemination assay of injecting Stat3 silenced, NF-kB inactivated cells into mouse and scoring metastasis to prove the role in vivo

- We agree with the reviewer that in vivo metastatic assay would further increase the reliability of our data. Since it has been shown that subcutaneous xenograft tumors derived from gastric cancer cells do not metastasize (Furukawa et al., 1993), orthotopic implantation is required for further analysis as recommended by the reviewer. However, we regret that orthotopic implantation technique is not available in our laboratory at the present time and it will take a year or longer to establish the orthotopic implantation technique.

10. It seems to be important to mention a paper from Hua Yu group Cancer Cell. 2009 Apr 7;15(4):283-9 about the ability of activated Stat3 to maintain NF-kB activation and retention in the nucleus.

- As recommended by the reviewer, we mentioned a paper by Hua Yu group (ref. 27 in the text) in the Background section as follows (page 5, paragraph 2, line 4-6):
STAT3 also maintains NF-kB activation and retention in the nucleus in melanoma cells and prostate cancer cells [27].

11. It is not clear whether indeed MMP9 expression really correlates with NF-kB and Stat3 activation in a biological sense. Table 1 data suggest that there are way more MMP9+ cells than cells with an activated NF-kB and Stat3, therefore MMP9 only sometimes correlates with NF-kB or Stat3, in many other cases it does not look dependent on NF-kB and Stat3, is it correct?

- We agree with the reviewer and added the following comment to the Discussion section (page 17, paragraph 1, line 5-8):

  However, Table 1 showed that there are much more MMP9-positive cells (18%) than cells with activations of both NF-kB and STAT3 (6%). Therefore, we speculate that MMP9 can be induced by many other pathways independent on NF-kB/STAT3 signaling pathway in gastric cancer [47,48].

Reference
Reviewer 2

Minor essential revisions

1. On page 10, the title of the first paragraph needs to be more informative to the reader.

   - As the reviewer recommended, the title of the first paragraph on page 10 was changed to “NF-κB, pSTAT3 and MMP9 are positively correlated with each other in clinical gastric cancer specimens” (page 11, paragraph 2, lines 1-2).

2. Figure 1, scale bars are missing.

   - As the reviewer recommended, we added scale bars to Figure 1.

Major compulsory revisions

3.

(i) While Figure 2 shows that SNU-638 cells over-expressing IκBαM displayed reduced levels of STAT3 protein expression and activation, the mechanism by which this is occurring has not been addressed. For instance, is NF-κB directly regulating the transcription of STAT3 and are there any NF-κB binding sites in the STAT3 promoter? To address this, STAT3 luciferase reporter assays should be conducted.

   - Since we agree with the reviewer’s comment, we performed STAT luciferase reporter assays. Our results were added to Figure 2C and explained in Results section in the revised manuscript as follows (page 12, paragraph 1, line 13-15):
     
     STAT luciferase reporter assay also showed that STAT transcriptional activity was decreased in IκBαM-overexpressing cells (Figure 2C).

(ii) In addition, given that a study has shown that STAT3 and p65 can heterodimerize to transcriptionally regulate NF-κB-dependent genes (Yang et al, Genes and development, 21, 1396-1408), it is therefore important to investigate this by performing co-immunoprecipitation experiments on these cell lines.

   - Although we agree with the reviewer that it is important to investigate the relationship between STAT3 and RelA, we were not able to succeed in co-immunoprecipitation of these two molecules at the present time. Thus, we added the reviewer’s comment to the
Discussion section (page 15, paragraph 3, line 9; page 16, paragraph 1, line 1-2) as follows:

Our observations contrast with a report by Yang et al. (2007), which showed that STAT3 and RelA can heterodimerize to transcriptionally regulate NF-κB-dependent genes [40].

(iii) Furthermore, the 2nd paragraph on page 13, the authors state that their findings are consistent with Wani et al. (2011), however they have not shown that IL-6 is reduced in the SNU-638 cells over-expressing IκBαM, which may account for the reduced STAT3 levels.

- We agree with the reviewer. Thus, we changed the sentences as follows (page 16, paragraph 1, line 2-6):

  Although Wani et al. (2011) reported that NF-κB activation induced STAT3 activation mediated by IL-6 [26], the present study did not show whether IL-6 is reduced in the SNU-638 cells overexpressing IκBαM, which may account for the reduced STAT3 levels. Thus, further investigations are needed to obtain a better understanding of the mechanism involved in NF-κB-induced STAT3 activation.

4. Figure 3B and 4B show that IκBαM and siSTAT3 over-expressing SNU-638 cells are significantly less invasive. Given that their immunoblot assays showed that IκBαM and siSTAT3 reduced pRelA and pSTAT3 by approximately 50%, respectively, the marginal decrease in the percentage of invasion does not look convincing. Therefore, I would recommend showing these experiments in a second gastric epithelial cell line.

- As recommended by the reviewer, we performed additional wound-healing assay and invasion assay using a second gastric cancer cell line, MKN1. Our results were added to Figure 2 (G and H), Figure 3 (D and E) and Figure 4 (D and E).

5. While Figure 3C and 4C shows that EMT markers are altered in IκBαM and siSTAT3 over-expressing SNU-638 cells, this was not looked at in SNU-638 cells that over-express both IκBαM and siSTAT3. Since EMT is linked to cell migration and invasion, and Figure 5B and 5C suggest that a reduction of pRelA and pSTAT3 synergistically contributed to cell migration and invasion, it is important to show that these EMT markers are also altered in SNU-638 cells that over-express both IκBαM and siSTAT3.
- As the reviewer pointed out, we performed immunoblotting for E-cadherin and Snail after co-transfection of IkBaM and STAT3 siRNA into the SNU-638 cells. We found that E-cadherin expression was increased whereas Snail expression was decreased in cells with down-regulation of both NF-kB and STAT3 compared with those with down-regulation of either alone. These data are added to Figure 5D and explained the details in the Results section (page 14, paragraph 4, line 9-10; page 15; paragraph 1; line 1-2) and Figure legends (page 29, paragraph 1, line 6-8) in the revised manuscript.

Thank you very much!