Author's response to reviews

Title: Therapeutic effects of STAT3-decoy oligodeoxynucleotide on human lung cancer in xenograft

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Author's response to reviews:

Dear Editor,

Submitted are the revised manuscript and Figures, entitled "Therapeutic effects of STAT3-decoy oligodeoxynucleotide on human lung cancer in xenograft mice", by Xulong Zhang and colleagues for your re-consideration for publication in BMC Cancer.

The revised manuscript displays the correction in the guidance of editor's and reviewers' comments. The changes in the article are described point by point as followed:

To #1 Reviewer Brent Cochran's Comments:

1. Grammatically the paper needs significant improvement throughout.

Reply: The manuscript has been critically reviewed (including language-editing) by Dr. Bin Gao, NIAAA, NIH, who, as correspondent author, published more than 70 articles in peer-reviewed journals.

2. In general, the results section and figure legends need to be expanded as they do not adequately describe the experiments performed. The background section needs to be expanded. A more detailed explanation of the previously identified role of STAT3 in lung cancer should be provided. The statement in the introduction about "in vivo evidences." is misleading. The Grandis lab has previously demonstrated that STAT3 ODNs do work in vivo. There is no reference for the STAT3 ODN sequence used given in the methods. Is this the same sequence the Grandis lab uses or a different one? Does it compete for binding to other STATs? No data is even presented or referenced that STAT3 is even activated in the A549 cells though others have shown this. The authors also do not give the concentration of primary antibody used the Western blot section.

Reply: We have expanded some description of results and figure legends which labeled with red color. The STAT3 ODN sequence is the same sequence as the Grandis lab uses (reference27, 29). As shown in the new Figure 1, STAT3 is constitutively activated both in A549 and PG cells. The concentration of primary antibody used in the Western blot section was presented in MM.

3. In Figure legend 2a, the authors state that they are performing assays to measure cell proliferation. However, they may be seeing largely effects of increased apoptosis or a balance between apoptosis and inhibition of proliferation. This should be stated or measured in some way. In Figure 4, the western blot of bcl-xl is not convincing. In Figure 6, the name of the protein examined should be put directly in the figure in both panels. It is not at all clear how the quantitation was obtained in panel B.

Reply: We added a new data on cell proliferation with [3H]-thymidine up-take method (new Figure 3B), which demonstrated that STAT3-decoy, at least partly, inhibited the proliferation of the treated cells.
Meanwhile, as indicated in Figure 4-7 (previous Fig 3-6), STAT3-decoy also strongly induced the apoptosis of this cancer cells. In the new Figure 5B (previous Figure 4B) with new western blot data, the expression levels of bcl-xl in different groups were significantly different. The expression levels of bcl-xl in different groups were calculated by the densitometry using AlphaEaseFC software (Version 4.0.0, Alpha Innotech Corporation) with normalization of each band to their corresponding loading control, which have been added to MM. The names of the proteins examined in Figure 7 (previous Figure 6) have been directly labeled.

4. In any case, a western blot and RT-PCR of the entire tumor sample would provide a more accurate indication of the overall effect of the ODNs in vivo. How long after ODN treatment were these experiments performed? Data on the percentage of cells that take up the STAT3 decoy ODN in vivo should also be presented.

Reply: The whole cell populations after transfection or the entire tumor sample were used in westerns and RT-PCR assays. ODN treatment protocol was described in MM. Since the STAT3 decoy ODN for in vivo administration was not labeled with fluorescent, we did not examine the STAT3 decoy-ODN up-take percentage.

To #2 Reviewer Bart JL Eggen's Comments:

1. On page 12, the authors claim that they demonstrated in this study that STAT3 was constitutively activated in several human lung cancer cell lines (data not shown). This is not shown by the authors, they show that introduction of a STAT3-decoy oligodeoxynucleotide resulted in reduced proliferation, induction of apoptosis, reduced tumor growth and reduced STAT3 target gene expression. The evidence provided that inhibition of STAT3 activity is responsible for the effects observed is circumstantial. It would make the manuscript stronger if the authors show that in these cells STAT3 indeed is constitutively phosphorylated on Tyr705 and possibly also on Ser727. An alternative would be to transfecct these cells with a STAT3-responsive reporter construct in combination with increasing amounts of the STAT3 decoy, to show progressive loss of STAT3-reporter activation.

Reply: The expression and activation of STAT3 of a human pulmonary giant cell carcinoma cell line (PG) were added as indicated in the new Figure 1, together with those of a human non-small-cell-lung cancer line (A549). As indicated in the new Figure 1, in both A549 and PG cells STAT3 indeed is constitutively phosphorylated on Tyr705 and Ser727. Since time limitation, we are sorry that we did not successfully establish the STAT3-responsive reporter construct, and could not supply the alternative explanation.

2. The western blot presented in Figure 4, panel B does not support the conclusion that the decoy results in a downregulation of Bcl-xl expression. There is no difference in Bcl-xl protein levels between the decoy oligo and scrambled oligo treated cells. Are the westerns and RT-PCR assays shown in Fig 4 performed on transfected cells purified by flow cytometry or on whole cell populations? This is unclear from the manuscript and this information should be included. If the latter is the case, how high was the transfection efficiency (%) and did that potneiutally lead to an underestimation of the decoy effect.

Reply: In the new Figure 5B (previous Figure 4B) with new western blot data, the expression levels of bcl-xl in different groups were calculated by the densitometry using AlphaEaseFC software (Version 4.0.0, Alpha Innotech Corporation) with normalization of each band to their corresponding loading control. The results showed that the expression of bcl-xl was reduced by STAT3 decoy ODN. The whole cell populations after transfection were used in westerns and RT-PCR assays and were not purified by flow cytometry, because the ODN in the assay were not labeled with FITC, and the transfection efficiency was very high. In the previously assay as shown in the new Figure 2A (previous Figure 1A), we detected the transfection efficiency was 93%, and the MFI mean was 53.36 at 25nM. We think it did not lead to much underestimation of the decoy effect, because the transfection efficiency was as high as 93%.

3. The STAT3 targets c-myc and Survivin are not downregulated by treatment with the STAT3 decoy. The authors should discuss this.

Reply: Regarding regulation of the c-myc and surviving by STAT3-decoy, we added a paragraph into
Discussion as "Inhibition of STAT3 signaling resulted decreased survivin and c-myc expression in many tumor cell lines. However, in A549 cells, the transcription levels of surviving and c-myc were not down-regulated following STAT3 decoy ODN treatment. One of the reasons was that the expressions of c-myc and survivin were also controlled by other transcription factors such as STAT5 and STAT1, which may compensate it when the STAT3 was blocked [31,32]. These results suggested that STAT3 inhibition induces a selective down-regulation of surviving and c-myc in A549 cells".

To #3 Reviewer Salih Sanlioglu's Comments:

1. Authors stated on Discussion page (line five from the top) that "the present study demonstrates that STAT3 constitutively activated in several human lung cancer cell lines (data not shown)." This data has to be shown in the manuscript to make generalizations about lung cancer cells.

Reply: The expression and activation of STAT3 of a human pulmonary giant cell carcinoma cell line (PG) were added as indicated in the new Figure 1, together with those of a human non-small-cell-lung cancer line (A549).

2- Only a single cell line (A549) was tested in the manuscript. In order to rule out the cell line effect, the efficacy of STAT3 decoy ODN application has to be shown at least in another non small cell lung cancer cell line with constitutive STAT3 DNA binding activity. Here, an in vitro assay would be sufficient so there is no need to repeat in vivo assays.

Reply: Unfortunately, we didn't have second non-small-cell-lung cancer other than A549 cells in our storage, and could not use this cell line within the suggested interval by editor although we tried our best to introduce this cell line from other lab.

3- Despite the fact that experiments are well designed, the manuscript was written very poorly since there is a major concern with the language. So the authors are strongly advised to get help in writing the manuscript. The ideas have to be presented in a more concise and clear way.

Reply: The manuscript has been critically reviewed (including language-editing) by Dr. Bin Gao, NIAAA, NIH, who, as correspondent author, published more than 70 articles in peer-reviewed journals..

In conclusion, all comments of the reviewers and editors are considered in the new revision, and the revised manuscript adheres to the Instructions to Authors (length, layout and format).

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Sincerely,

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