Author's response to reviews

Title: Neural protein gamma-synuclein interacting with androgen receptor promotes human prostate cancer progression

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Author's response to reviews: see over
BMC cancer office

Dear editor:

Thank you very much for your letter of May 5, 2012, concerning our manuscript entitled “Neural protein gamma-synuclein interacting with androgen receptor promotes human prostate cancer progression” (Manuscript number: 1116962922695619).

We appreciate the reviewers’ comment that our manuscript is interesting. Based on the constructive comments of the editor and reviewers, we have revised our manuscript and edited it carefully again. For point-by-point responses to the reviewers’ comments, please see the letter to the reviewers on the subsequent pages. We believe that the revised manuscript now is ready for your further consideration for publication in BMC cancer. Please don't hesitate to contact us if you have any problems.

Sincerely yours,

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Comments from Prof Glynn:

The authors present an interesting study on the role of SNCG in prostate cancer progression. They present evidence for the potential role of SNCG in prostate cancer metastasis.

Major Points

1. In the introduction the authors mention that there are three types of SNCG proteins. After this point, there is no mention in the manuscript which SNCG protein they are investigating. The authors need to clarify this in the manuscript, and if they are only investigating one of the proteins, describe why they have focused on this particular one. 

Looking at the primers it suggests that they are researching the gamma version, please clarify.

We mention the synuclein family consists of α, β and γ-synucleins instead of γ-synucleins (synuclein game, SNCG) having three types. The α- and β-synuclein proteins participate in the etiology and pathogenesis of neurodegenerative disorders. Recently, elevated levels of SNCG proteins have been detected in various types of cancer, especially in advanced stages of the disease. The role of SNCG in prostate cancer is not yet clear. We have clarified further.
2. *One major worry is the sole focus of the in vitro work on the LnCap cell line as the only AR dependent cell line, and the focus on only one siRNA (166). It would be preferable to see an additional AR dependent cell line e.g. CWR22 or use of the other siRNAs to see a dose response effect of SNCG inhibition.*

We are very grateful for your critical tips, it is a good idea to see a dose response effect of SNCG inhibition with different siRNAs or other cell line to further verify our result. Now we detected the inhibition efficiency of oligo-166 target to SNCG in CWR22 cells, as expected, we had observed the inhibition effect of SNCG with dose dependent of siSNCG-166 in CWR22 cells. (Supple Figure 1.)

3. *The invasion/migration assays demonstrate a modest inhibition of invasion and migration, considering the level of SNCG knockdown, what is the mechanism for this inhibition? It is unlikely to be proliferation inhibition as migrating/invading cells are usually not proliferating at the same time. This is very interesting but requires further investigation. Is there an inhibition of MMP expression or Rho-GTPases for example?*

Figure 1 suggested that inhibition SNCG in LNCaP cell be associated with the cell-cycle arrest at the G1 phase, which may influence cell
proliferation. Figure 2 indicated that knockdown of SNCG in LNCaP cells inhibit cellular migration and invasion in vitro. Here, we did not further explore the mechanism, some studies in other cancers proved SNCG is implicated in regulation of key steps of cellular invasion and metastasis, which may activate through several cellular mechanisms including reducing BubR1 protein levels [11,26,27], increasing ER-α transcription [28,29], activating RHO GTPase [30], MAPK and ElK1 [31], inducing MMP expression [32], and constitutively activating of ERK1/2 [19].

Given the existence of cell-specific regulation, now we supplement the experiment of the relation between inhibition SNCG and MMP using wild type LNCaP cells and stable cell line of RFP-SNCG-LNCaP and siSNCG-166-LNCaP (Supple Figure 2.). From the result we confirmed the expression of SNCG is involving in the MMP members' regulation.

4. Regarding Table 1 clinical information. The authors were only able to look at 5 cases of androgen independent disease versus 122 of androgen dependent disease. 5 is too low a number to have sufficient power to draw any conclusions of statistical significance. Again this is the same for comparison in normal tissue or prostatitis. These results are indeed intriguing, but it needs to be clarified in the manuscript that solid conclusions can not be made at this time. Instead a larger cohort will need to be examined in the future.
As we all know, it is difficult to collect the human tissue samples of androgen-independent prostate cancer. We hope to increase the amount of the tissue specimen in the future. Thank for your reminding us of modifying “it need a large number specimen to be further explored……”.

**Minor points**

4. The authors looked at the effects of SNCG inhibition and increased expression in LnCap cells. Inhibition of SNCG resulted in decreased proliferation and accumulation in the G1 phase. Vice versa increased SNCG caused increased proliferation, however the authors do not present corresponding information on Cell cycle progression. Did they not collect this information?

The SNCG plasmid construct was found later than the effective siRNA in our experiment process. Some overexpression experiments were done later, but we haven't repeat for many times. Although these experiments are constructive and positive (Supple Figure 3.), here we primarily show the role of inhibition of SNCG in LNCaP cells. In this way, it avoids the seeming confusion of the entire article structure.

5. In the results section under the heading “SNCG protein interacts with AR inhuman prostate cells”, the authors describe how they investigated SNCG mRNA expression in LnCAP and LnCAP-AI cells.
This section however doesn’t contain any experimental work on the LnCAP-AI cells, which are in the following section. Please delete LnCAP-AI cells and leave until the following section for greater clarity. Thank for your carefulness, it is our mistakes. “LNCaP-AI” has been deleted from the sentence.

6. How do the AR levels compare between LnCAPs and LnCAPs-AI? Can the authors show this?

As to the answer of this question which is also raised by the Pro. Labrie, we have supplemented the relevant data in the “cell lines” of “materials and methods”. Previously, we established an LNCaP-AI subline from androgen-dependent LNCaP cells, which was characterized by the ability of stable growth in an androgen deprivation condition. AR protein in LNCaP-AI is higher than LNCaP (Supple Figure 4.). There was no noticeable difference in proliferation of LNCaP-AI cells cultured in containing androgen or deprived androgen culture medium. However, LNCaP-AI cells showed stronger proliferation ability than LNCaP cells in androgen deprivation culture medium from day 4 after inoculation. PSA secretion was stimulated with increasing concentration of DHT in both LNCaP and LNCaP-AI cells, but the PSA secretion was much higher for LNCaP cells than for LNCaP-AI cells. (Supple Figure 5.)


**Comments from Prof Labrie**

This is an interesting paper in which the authors used complementary approaches (overexpression versus siRNA, LNCaP vs LNCaP-AI cells) to examine the role of SNCG in androgen-dependent proliferation of prostate cancer cells…….

**Major Compulsory Revisions**

1 – *The majority of the experiments rely on SNCG overexpression or knock-down but several of these experiments lack an important control: the authors should provide data showing SNCG protein levels for these experiments. The data should allow the reader to compare SNCG levels in control and over/underexpressing cells. This concerns the following figures: 1C-D, 2A-B, 3C-F and 5A-F.*

Transient transfection was used for cytological experiments. Before the cytological experiment (Fiugre1-3), we repeatedly proved a stable inhibition efficiency of SNCG by oligo-166 (in fact, more than 3 times). In Figure 1B, we show one of these experiments with protein expression. So, we did not show the protein expression in the every following experiment. The stably transfected cells screened by puromycin was used for animal experiments. We will supplement the fluorescence image of
the cells and the protein level of the stable cell line of SNCG (Supple Figure 6.). We are grateful thanks for your critical advice, and we will perform our experiments and prepare our data more carefully in the future study.

2 – In relation to the previous comment, the text of the paper does not always explicitly state if the experiments were performed using stably or transiently transfected cells. Please make this clear.

Transient transfection was used for cytological experiments and the stably transfected cells for animal experiments. The explanation has been added into the “materials and methods”.

3 – The authors used a lentiviral vector to generate stable cell lines. They should specify if the experiments were performed using a pool of stably transfected cells or individual clones.

They were performed using a pool of stably transfected cells.

4 – The authors performed several experiments in LNCaP-AI cells. They should state how these cells compare to LNCaP cells in terms of AR levels, response to androgens, proliferation, etc.

Please refer to the above answer of the sixth question for Pro. Glynn.
5 – The AR-SNCG coimmunoprecipitation experiments were performed using LNCaP cells that stably overexpress SNCG. However, SNCG seems to be quite abundant in untransfected LNCaP cells. The authors should state if they attempted to co-immunoprecipitate AR and SNCG in untransfected LNCaP cells. If so, did they observe an interaction between the two proteins?

It was our mistake, we did not state clear. LNCaP cells were used in co-immunoprecipitate experiments, for it itself expressed SNCG protein. “stable SNCG-expressed” in the sentence has been deleted.

6 – The figure legends need to be completely rewritten for two reasons. First, they generally do not contain enough pertinent information to enable the reader to understand the figure/experiment. Second, most of the figure legends contain statements that interpret the data. With the exception of general figure titles, these statements should be removed.

We have rewritten the figure legends, hoping it will meet the publication demand.

7 – Experimental details are lacking for several experiments including the transwell chamber assays and the quantitative RT-PCR assays. In the Methods section the authors do not state which antibodies were used to immunoprecipitate AR and SNCG. In reference to the nude
mouse experiments the authors mention “tumour imaging”. What is this exactly? Please ensure Methods are sufficiently detailed.

We have supplemented experiment methods, including quantitative RT-PCR, cell migration assay, cell invasion assay, cell proliferation assays, cell-cycle analysis and dual-luciferase reporter assay. The antibodies of AR and SNCG for immunoprecipitate were the same as Western blot. We have supplemented the explanation in the section of “Co-immunoprecipitation and Western blot analyses”.

8 – In reference to the experiment presented in Fig. 5A-C, the authors state that “a significant delay” in tumour growth was observed in siRNA-producing tumours compared to controls. This is surprising given the overlap in the error bars. Do the authors stand by their interpretation of the data?

Thanks for your careful observations and comments. After 4 weeks, we can obviously distinguish the experiment group from the control group with eyes. Due to some individual mouse was not sensitive, a larger standard deviation was observed. Fortunately, statistics are still meaningful.

9 – I do not fully understand the rationale for evaluating the effect of SNCG overexpression on LNCaP tumour growth in castrated male mice
(Fig. 5D-F). In reference to this experiment the authors state that “there is no significant difference between two groups with different expression levels of SNCG, indicating that SNCG regulates androgen-dependent prostate tumorigenesis.” I am not certain that this experiment was designed to address this question. Please clarify.

Without androgen, the over-expression SNCG of LNCaP cells have no advantage over the LNCaP cell in tumorigenesis. However, there is difference between the LNCaP cell and the low-expression of SNCG LNCaP cell in male mice with androgen. It suggested that SNCG involved in the role of tumorigenesis be associated with androgen.

10 – The authors should define what they consider to be “androgen-independent” tumours (Ref. Table 1 and Fig. 6). Are these relapsing tumours?

Most prostate cancers initially are androgen dependent tumors. They become refractory after one to three years and resume growth despite hormone therapy. At this time, they are called androgen-independent prostate cancer which is a relative advanced stage. We have added the explanation in the text of “Tissue specimens and a prostate tissue microarray (TMA)”.

Minor Essential Revisions
11 – There are a few typographical/spelling errors such as “SCNG”, “Ablate-Shen”. Please revise.

Thanks, we have substituted “SNCG”, “Abate-Shen” for the wrong ones.

12 – If appropriate, please correct the y-axis labels of Figs. 5C and E to read “Mean Tumor Volume (cm3)”. 

Accepted and thanks.

13 – The manuscript is generally well written and while sentence structure could be improved in some areas, the language is generally acceptable.

Thanks for your approval, we have try our best to improve it.

Discretionary Revisions

14 – Did the authors examine the effect of SNCG overexpression on invasion?

We had examined the effect of SNCG inhibition or up-regulation in LNCaP cells. Up-regulation exogenous expression of SNCG in LNCaP cells enhanced the in vitro invasion and migration capacity. Here, we did not show the results, for we mainly focused on the role of inhibition SNCG in this paper.

15 – The Introduction could be shortened by removing the first
paragraph that discusses the management of prostate cancer patients.

We have shortened the first paragraph.

16 – The discussion is lengthy and could probably be shortened to avoid repeating what is already stated in the Results section. On the other hand, the authors should consider discussing the possible mechanism(s) whereby SNCG enhances AR activity.

We discretionarily revised the discussion by deleting some repeated sentences in the results section. In the paragraph 4 of discussion, we demonstrated that AR signaling could be modulated by AR cofactors. SNCG, as the other cofactors, interacted with AR and enhanced the AR transcriptional activity. And the mechanism need further exploration, including how they interacted with each other, what’s downstream factor they promote or suppress, and so on.