Author's response to reviews

Title: Self-renewal and chemotherapy resistance of p75NTR positive cells in esophageal squamous cell carcinomas

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Author's response to reviews: see over
April 16, 2008

Dear Dr. Michael B. Kastan,

Please consider this revised submission of our manuscript # (4783751217747018), entitled “Self-renewal and chemotherapy resistance of p75NTR positive cells in esophageal squamous cell carcinomas”, for publication in the Journal of BMC cancer. We greatly appreciate the thoughtful reviews received from you and from the reviewers. In response, we have prepared and included (below) a detailed description of all changes made to the manuscript, referenced to each of the reviewers’ comments. With reference to the manuscript file, we submit a revised, marked version of the paper with additions indicated in blue bold text and deletions using strikethrough, and a revised, unmarked version with all changes incorporated.

Again we would like to thank the reviewers for their constructive and professional comments, which help us to improve our manuscript. Your re-consideration of this revised manuscript is much appreciated.

Yours sincerely,
Zhi-Yun XU, MD, PhD
(corresponding authors)
Reviewer: Maria Grazia Daidone

Reviewer's report:

This paper is focused on a very popular and up-to-date topic, that is the possibility to isolate putative cancer stem cells from human tumors.

The Authors applied different approaches to investigate the relationship between p75 expression and stemness in esophageal squamous cell carcinomas, and to characterize p75-positive cells compared to the other cells subpopulations, unsorted or p75-negative.

We thank the reviewer for their positive comments.

Question 1: However, as a gold standard for the identification of putative stem cells, the Authors did not provide any information about the in vivo growth advantage of cells expressing p75 compared to those unsorted and/or not expressing the putative stem cell marker.

Answer: We injected $10^2$ to $10^8$ p75\textsuperscript{NTR+} and p75\textsuperscript{NTR-} cells into NOD/SCID mice to determine whether p75\textsuperscript{NTR} status could distinguish between tumorigenic and nontumorigenic cells. When $>1 \times 10^3$ p75\textsuperscript{NTR+} cells or p75\textsuperscript{NTR-} cells were injected, tumors formed within 10–16 weeks. This result was in contrast to the generality of the cancer stem cell hypothesis. Several mouse tumor models also challenge the generality of the cancer stem cell hypothesis (Li et al, Mol Cancer Ther. 2008;7(3):721-9. Inoue et al, Cancer Immunol Immunother. 2008), and more compelling tests with human tumors presumably will require transfer into mice installed with all the requisite human support cells and support factors. Recently, Kennedy et al (Kelly et al, Science. 2007;317(5836):337.) reported that current xenotransplantation systems seriously underestimate the frequency of cells that can maintain the growth of human tumors. Adams et al (Adams et al, Science.2007;318(5857):1722.) further showed that much of the excitement about the cancer stem cell hypothesis arises from the possibility that the putative stem cell population will prove to be uniquely responsible for the relapses that so frequently follow conventional therapy.

Therefore, we regarded that p75\textsuperscript{NTR+} cells displayed cancer stem cells properties of self-renewal and chemotherapy resistance and we have addressed this issue in the Discussion sector of this revision.

Question 2: In addition, in consideration of the large number of patients with esophageal squamous cell carcinomas in which p75 expression has been evaluated, some information on the prognostic role of this putative stemness marker could have been provided.
Answer: We thank the reviewer for the constructive suggestions, and we have replenished the revised manuscript with clinical data analysis and the results obtained have been added to the Results sector in the revised manuscript. However, we do not have sufficient survival and therapy data to be included in this paper.
Reviewer: Gabriela Dontu

Reviewer's report:
The study has a scientifically sound experimental design and a logical, clear presentation of the data. With several exceptions, discussed below, the results are clear-cut and support the author’s conclusions. Overall the work is interesting and relevant for esophageal cancer research, although not a particularly novel contribution compared with previous publications by Okumura et al. Chemoresistance of p75NTR positive cells is probably the most significant result of this work which was not previously reported.

We thank the reviewer for their positive comments.

Question 1: The study would be considerably more convincing if in vivo functional data would be added, in addition to the in vitro results, to show difference in tumorigenicity between the p75NTR positive and negative cell populations. This would also represent an important contribution and a step forward compared to previous findings by Okumura et al.

Answer: We injected \(10^2\) to \(10^8\) \(p75^{NTR+}\) and \(p75^{NTR-}\) cells into NOD/SCID mice to determine whether \(p75^{NTR}\) status could distinguish between tumorigenic and nontumorigenic cells. When \(>1 \times 10^3\) \(p75^{NTR+}\) cells or \(p75^{NTR-}\) cells were injected, tumors formed within 10–16 weeks. This result was in contrast to the generality of the cancer stem cell hypothesis.

Several mouse tumor models also challenge the generality of the cancer stem cell hypothesis (Li et al, Mol Cancer Ther. 2008;7(3):721-9. Inoue et al, Cancer Immunol Immunother. 2008), and more compelling tests with human tumors presumably will require transfer into mice installed with all the requisite human support cells and support factors. Recently, Kennedy et al (Kelly et al, Science. 2007;317(5836):337,) reported that current xenotransplantation systems seriously underestimate the frequency of cells that can maintain the growth of human tumors. Adams et al (Adams et al, Science.2007;318(5857):1722.) further showed that much of the excitement about the cancer stem cell hypothesis arises from the possibility that the putative stem cell population will prove to be uniquely responsible for the relapses that so frequently follow conventional therapy.

Therefore, we regarded that \(p75^{NTR+}\) cells displayed cancer stem cells properties of self-renewal and chemotherapy resistance and we have addressed this issue in the Discussion sector of this revision.

Question 2: Self-renewal was not demonstrated by the experiments performed. In order to demonstrate self-renewal one should separate and analyze the \(p75NTR\) positive and
negative cell populations in several serial passages. The same analysis shown in Figure 2, with respect to the phenotype of the progeny population should be performed. The same applies to sphere formation. In addition, information about the sphere formation of the p75NTR negative population should be shown.

Answer: We thank the reviewer for the insightful comments. Indeed, we realized the importance to demonstrate self-renewal of p75NTR+/- cells in serial passages; so we performed the experiments, which has been added to the Results sector in the manuscript.

Question 3: Figure 1 B, C, D pictures should be replaced with better quality versions of same sections, larger details should be shown.

Answer: We’d like to thank the reviewers for their constructive suggestions. We have replaced with better quality pictures (Figure. 1 B, C, D) in this revision.

Question 4: Figure 4. It would be useful to label flow charts with marker name (instead of FL1-height)

Answer: We have revised FL1-height as marker name according to the comments of the reviewer in this revision.

Question 5: There are numerous language, grammar, spelling errors. Manuscript should be checked and some sections re-written.

Answer: The manuscript has been reviewed by an English expert.
Reviewer: Steffen Hauptmann

Reviewer's report:
The study submitted for publication contains immunostains (p75, p63, involucrin, MIB) of 60 esophageal squamous cell carcinomas and data of in vitro experiments, leading to the message that p75+ tumor cells have stem cell properties associated with platin resistance.

We thank the reviewer for their professional comments.

Question 1: The first part is purely descriptive and suggest that p75 and p63 are coexpressed. I have a problem with the quality of the immunostains, particularly with the double stainings which do not justify the conclusion mentioned above.

Answer: As requested by the reviewer, we have replaced with better quality pictures (Figure. 1 B, C, D) in this revision.

Question 2: There are neither follow-up data nor any information of the therapy. To see a correlation between the amount of p75+ tumor cells and patients outcome would significantly improve the importance of the study.

Answer: We thank the reviewer for the constructive suggestions, and we have replenished the revised manuscript with clinical data analysis and the results obtained have been added to the Results sector in the revised manuscript. However, we do not have sufficient survival and therapy data to be included in this paper.

Question 3: Regarding the in vitro-part I am doubtful regarding the purity of the sorted cell cultures. The percentage of p75+ cells was mentioned to be 1.6-3.7%, which is extremely low. Therefore, I would be interested to know the percentage of p75+ cells within your sorted cell culture.

Answer: We agree with the reviewer on this point, the percentage of p75NTR+ cells was very low in each tested cell line. At first, we used magnetic cell sorting to isolate p75NTR+ cells, however, the purity of sorted p75NTR+ population was only 50%±. So we used FACS to isolate p75NTR+ cells in further experiments, which raised the purity of sorted p75NTR+ populations to 90%±.