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Novel role of reactive oxygen species in regulating proliferation and migration of osteosarcoma by NHE-1 activation

Hua Bai[1,†], Guojing Chen[2,†], Congwen Fang[1,†], Xuekang Yang[3], Sixun Yu[1], Chunxu Hai[1*]

Corresponding author:
Chunxu Hai, Department of Toxicology, Shaanxi Provincial Key Lab of Free Radical Biology and Medicine, the Ministry of Education Key Lab of Hazard Assessment and Control in Special Operational Environment, School of Public Health, Fourth Military Medical University, Xi'an, Shaanxi 710032, China

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Michal Heger
Department of Experimental Surgery, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

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Novel role of reactive oxygen species in regulating proliferation and migration of osteosarcoma by NHE-1 activation
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Dear Dr. Bai,

Two expert reviewers have commented on your paper, their comments can be found below. You will see that they are advising that you considerably revise your manuscript. Reviewer 1 is very hesitant about accepting the manuscript in its current form, so I kindly ask you to be particularly forthcoming to this reviewer's suggestions and implement the requested changes (especially regarding the methodological concerns) to the fullest possible extent. If you are prepared to undertake the work required, I will help you with the language polishing and figures in accordance with my editorial (http://www.jctres.com/en/201501005/).

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you resubmit your work.

Your revision is due by Sep 30, 2015.

To submit a revision, go to http://jctres.edmgr.com/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely,

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

********Reviewer comments********

Reviewer #1: This manuscript reports on the up-regulation of NHE1 expression by low concentrations of ROS and its contribution to the regulation of proliferation and invasion in osteosarcoma.

The topic of the study is of interest and the paper is potentially containing new information. Nevertheless, the manuscript presents significant methodological and textual deficiencies.

Collectively, the experiments are feeble and not completely satisfactory. The western blots are not of publication quality. Authors' claims are based mainly on descriptive studies. The results are not so robust and exhaustive to support the statements and conclusions of the Authors.

Moreover, the bibliography is not completely adequate and updated.

Overall, my opinion is that Authors turn one's attention to many subjects, so that they lack to approach fully each of them.
Should the Authors be able to reduce the number of addressed issues, the link between results and conclusions would be made easier, and very likely the quality and reliability of this study improved.

In addition, the manuscript is in great need of textual editing to improve its English expression and to avoid grammatical errors and misreadings throughout the text that make the meaning of some sentences conflicting and unclear.

Reviewer #2: The authors say (page 13 lines 37-42): "Staining of a representative sample is presented in Figure 1A. MI (n = 9) and MC (n = 14) biopsies had significantly lower expression of NHE1 (P = 0.029 and P = 0.0087, respectively) than NB (n = 5) (Figure 1B)". Is it correct? Please compare to the Fig 1 legend. (B) "The level of NHE1 protein expression was significantly higher in MI and MC tissues compared with NB tissue".

Fig. 1B If the data shown here are based on experiments displayed in Fig. 1A I wonder how β-actin content was tested.

Fig. 1C Do U2OS and HOS cells belong to MI category (while KHOS and 143B to M2)? It should be clarified as MI and MC abbreviations pertained to muscle biopsies.
Fig.2A The abbreviations MI and MC are listed in Materials and methods section (as "Summary of OS patients") and explained in the paragraph 3.1, but what about OS? It is also not shown in Fig. 1. This issue should be clarified.
Fig. 2B The representative graphs are indistinct and therefore not convincing. More detailed description and interpretation is needed.
Part 3.2 OS cells produce more ROS than OB cells that is correlated with increased proliferative activity. If so, an effect of artificially increased ROS (with tBHP) should also be tested on OB cells. OS cells have increased endogenous ROS level within pathophysiological range while tBHP can induce an additional effects related to abnormally high ROS generation. Such approach would allow distinguishing "pure" effect of tBHP on normal cells from that possibly modified by cancerous background of OS cells.
Fig. 3 More details about cell viability assay, please. (cell proliferation? It is not a synonym of viability). How to explain significantly (but slightly) increased viability after 4 and 24 hours and lack of effect or even significant inhibition after 8h of the treatment with tBHP?
Effects of DMTU and cariporide on control OS cells not treated with tBHP should also be tested.
Cell cycle and apoptosis - information about number of independent experiments (independent biological samples) should be given in the figure legend. Cell apoptosis - data from one experiment have been shown. If so, it is definitely not enough.
Page 17/18 Why the authors expected decreasing effect of cariporide on tBHT-derived ROS generation?
Page 18 lane 31 Cariporide inhibits sodium/proton exchanger activity but not affects tBHT-induced ROS generation. It is not obvious why this compound reduces HNE1 expression. The final conclusion presented in this part of the manuscript bases on correlative data. Possible mechanism of such regulation would be desirable.
Fig. 5C Increased wound healing ability and increased proliferation; How did the authors distinguish between them. Laser scanning cytometry would be recommended. How many independent experiments have been done?
Part 3.5 Very preliminary data.
In sum, this manuscript contains interesting data, however in my opinion they need to be seriously improved.

Authors' rebuttal:

Dear Editor,
We would like to thank you for giving us a chance to resubmit the paper, and also thank the reviewers for their constructive comments and suggestions on our manuscript entitled "A novel role of reactive oxygen species in regulation of osetosacroma cell proliferation and migration via NHE-1 activation." We have studied the reviewers' comments carefully and have made the necessary corrections, which we hope meet your standards. The revised text is marked in red.
Sincerely yours,
Chunxu Hai, Ph.D. Professor

The following is a point-by-point response to the reviewers' comments.

Reviewer# 1
Comments to the Author
This manuscript reports on the up-regulation of NHE1 expression by low concentrations of ROS and its contribution to the regulation of proliferation and invasion in osteosarcoma.
The topic of the study is of interest and the paper is potentially containing new information. Nevertheless, the manuscript presents significant methodological and textual deficiencies. Collectively, the experiments are feeble and not completely satisfactory. The western blots are not of publication quality. Authors' claims are based mainly on descriptive studies. The results are not so robust and exhaustive to support the statements and conclusions of the Authors.
Answer: Another representative graph of ERK, p-ERK, Ki67, and laminB are presented in Fig. 4D to address this issue. As to the remark about the descriptive nature of our work, we somewhat share that view but like to reiterate that this study aimed to unveil the essential basics in OS biology, as this study was the first one to address this topic. The reviewer should know that no precedent on this topic exists in the context of OS. Accordingly, we studied the most logical, rudimentary hypotheses in a translational setting. Our interventions with DMTU and cariporide are, in our opinion, adequate to provide first-hand compelling evidence for the role of ROS and NHE1 in OS biology, further backed up by the in vivo validation study. Moreover, our approach is exactly in line with the journal's philosophy as addressed in the editor-in-chief's editorial [Heger, J Clin Transl res 2015(1): 1-5], where it is stated that "JCTR considers the mechanistic underpinning of a medical intervention, especially a novel one, to be of inferior value compared to the provision of solid empirical evidence demonstrating that the intervention is effective in vivo." We believe we have provided that to a certain extent.

Moreover, the bibliography is not completely adequate and updated.
Answer: The references have been updated or added as requested.

Overall, my opinion is that Authors turn one's attention to many subjects, so that they lack to approach fully each of them. Should the Authors be able to reduce the number of addressed issues, the link between results and conclusions would be made easier, and very likely the quality and reliability of this study improved.
Answer: We thank the reviewer for this valuable comment. We concur and have revised the "Introduction" and "Discussion" parts. The graphical abstract has also been modified. We tried to focus on the role of ROS and NHE1 this time as a starting point for further studies.

In addition, the manuscript is in great need of textual editing to improve its English expression and to avoid grammatical errors and misreadings throughout the text that make the meaning of some sentences conflicting and unclear.
Answer: The manuscript has been considerably revised and the language has been polished. We have received much help from JCTR's editorial board in achieving the format of the resubmitted version.

Reviewer: 2
The authors say (page 13 lines 37-42): "Staining of a representative sample is presented in Figure 1A. MI (n = 9) and MC (n = 14) biopsies had significantly lower expression of NHE1 (P = 0.029

and P = 0.0087, respectively) than NB (n = 5) (Figure 1B)". Is it correct? Please compare to the Fig 1 legend. (B) "The level of NHE1 protein expression was significantly higher in MI and MC tissues compared with NB tissue".
Answer: The corrections have been made in the revised manuscript as “Staining of a representative sample is presented in Figure 1A. MI (n = 9) and MC (n = 14) biopsies had significantly higher expression of NHE1 (P = 0.029 and P = 0.0087, respectively) than NB (n = 5) (Figure 1B).”

Fig. 1B If the data shown here are based on experiments displayed in Fig. 1A I wonder how actin content was tested.
Answer: The corrections have been implemented in the y-axis of Fig. 1B. The part “Immunohistochemistry” has been rewritten in the Materials and methods section.

Fig. 1C Do U2OS and HOS cells belong to MI category (while KHOS and 143B to MC)? It should be clarified as MI and MC abbreviations pertained to muscle biopsies.
Answer: Yes. The sentence “U2OS and HOS cells belong to MI category, while KHOS and 143B cells belong to MC category” has been added in the Results section in the revised version.

Fig.2A The abbreviations MI and MC are listed in Materials and methods section (as "Summary of OS patients") and explained in the paragraph 3.1, but what about OS? It is also not shown in Fig. 1. This issue should be clarified.
Answer: The abbreviations OS has been rewritten as “osteosarcoma” in the revised version.

Fig. 2B The representative graphs are indistinct and therefore not convincing. More detailed description and interpretation is needed.
Answer: Fig. 2B and Fig. 4A have been replaced to make the symbols clear and a more detailed description has been provided.

Part 3.2 OS cells produce more ROS than OB cells that is correlated with increased proliferative activity. If so, an effect of artificially increased ROS (with tBHP) should also be tested on OB cells. OS cells have increased endogenous ROS level within pathophysiological range while tBHP can induce an additional effects related to abnormally high ROS generation. Such approach would allow distinguishing "pure" effect of tBHP on normal cells from that possibly modified by cancerous background of OS cells.
Answer: We thank the reviewer for this valuable comment. Now it is well known that different cellular levels of ROS can induce different cellular effects regardless of whether these cells are tumor cells or normal cells. Low levels of ROS have a physiological role in cell signaling such as proliferation, differentiation, and migration, as addressed and referenced throughout the manuscript. A constitutive increase in the cellular levels of ROS has been associated with the carcinogenic process. Higher levels of ROS can induce cell death. We believe that the biphasic effect of ROS is also applicable to OB cells. However, in non-malignant cells the basal levels of ROS are relatively low, while in tumor cells the basal levels of ROS are relatively high. The higher background of ROS constitutes a possible explanation to the high susceptibility of tumor cells to exogenous ROS. Accordingly, tumor cells may require lower exogenous ROS levels than non-transformed cells to increase their proliferation rate or to induce cell death. In this paper we

focused mainly on OS rather than OB, which is why we did not investigate this further, but your suggestion is indeed a valuable one for future studies.

Fig. 3 More details about cell viability assay, please. (cell proliferation? It is not a synonym of viability).
Answer: Cell viability was detected by WST-1 method. The 3.1 part “cell proliferation assay” has been changed to “cell viability assay” in the “Materials and methods” section.

How to explain significantly (but slightly) increased viability after 4 and 24 hours and lack of effect or even significant inhibition after 8 h of the treatment with tBHP?
Answer: Thank you for pointing this out. We have studied the time-response relationship and dose- response relationship between many kinds of oxidants (ROS or free radicals) and cells in previous studies using MTT or WST-1 assays. A complex relationship between these parameters was found because different cells react variably to different pro-oxidative stimuli. Sometimes unexpected responses are observed and it is hard to explain these objectively. In most cases, these unexpected data have no effect on the objective of our study. We focused on cell proliferation in this study, but combined the viability method used with other methods (e.g., cell cycle analysis) to further corroborate the interrelatedness of findings and underlying hypotheses. Moreover, the reviewer should note that we only performed these experiments to determine the optimal tBHP concentration to conduct the rest of the experiments, focusing on the proliferative signaling aspects rather than the cell death induction aspects of ROS.

Effects of DMTU and cariporide on control OS cells not treated with tBHP should also be tested.
Answer: We agree. In preliminary experiments we tested the effect of different doses of DMTU and cariporide on cell viability, cell cycle, apoptosis, and NHE1 expression of U2OS. No notable effects were observed at the concentrations employed in this manuscript, which has been added to the manuscript.

Cell cycle and apoptosis - information about number of independent experiments (independent biological samples) should be given in the figure legend. Cell apoptosis - data from one experiment have been shown. If so, it is definitely not enough.
Answer: The figure legends of Fig.4-6 have been revised to address this issue. A column graph is added in Fig. 3C.

Page 17/18 Why the authors expected decreasing effect of cariporide on tBHP-derived ROS generation? Page 18 lane 31 Cariporide inhibits sodium/proton exchanger activity but not affects tBHP-induced ROS generation. It is not obvious why this compound reduces HNE1 expression. The final conclusion presented in this part of the manuscript bases on correlative data. Possible mechanism of such regulation would be desirable.
Answer: Little is known about the interplay between ROS and NHE1. We expected a cariporide-decreasing effect of on tBHP-induced ROS generation because cariporide has been shown to actively suppress cell death caused by oxidative stress. But in this study cariporide did not show any antioxidant activity. Cariporide reduced NHE1 expression because it is a specific and powerful inhibitor of NHE1.
One important action of ROS appears to be the inhibition of protein tyrosine phosphatases (PTPs) through stepwise oxidation of a critical cysteine residue at the catalytic site. PTPs make

up a large family of enzymes involved in many cellular signaling pathways, and a recent study suggests that PTPs also affect the regulation of pH by interacting physically with NHE. These mechanisms may explain some of the regulatory effect of ROS on NHE1 in our study. We acknowledge, however, that aside from the limited mechanistic conjecture here and in the manuscript, we did not perform additional mechanistic experiments for reasons indicated in the response to point 1 of reviewer 1. More focused studies should be performed to further determine the pharmacodynamics of cariporide in the context of OS and NHE1 signaling.

Fig. 5C Increased wound healing ability and increased proliferation; How did the authors distinguish between them. Laser scanning cytometry would be recommended. How many independent experiments have been done?
Answer: We thank the reviewer for this valuable comment. The wound healing assay is simple, inexpensive, and one of the earliest developed methods to study directional cell migration in vitro. The growth of cells is arrested by contact inhibition when this assay begins. In the serum free condition, cells are more likely to move to fill the uncovered areas instead of proliferating. Five independent experiments have been done and a column graph is added in Fig. 5C.

Part 3.5 Very preliminary data.
Answer: We concur and have already carried out more in vivo experiments in mice, which will be published in a separate paper.

In sum, this manuscript contains interesting data, however in my opinion they need to be seriously improved.
Answer: We hope that the improvements were sufficient to solidify the study and paper and to steer more research into this important subject, which lies at the basis of why a considerable fraction of young OS patients dies far too early or loses limbs.

2nd editorial decision:

Date: 2-Dec-2015

Ref.: Ms. No. JCTRes-D-15-00007R1
A novel role of reactive oxygen species in regulation of osetosacroma cell proliferation and migration via NHE-1 activation
Journal of Clinical and Translational Research

Dear Dr. Bai,

Reviewers have submitted their critical appraisal of your paper. The reviewers' comments are appended below. Based on their comments and evaluation by the editorial board, your work was FOUND SUITABLE FOR PUBLICATION AFTER MINOR REVISION.

If you decide to revise the work, please itemize the reviewers' comments and provide a point-by-point response to every comment. An exemplary rebuttal letter can be found on at http://www.jctres.com/en/author-guidelines/ under "Manuscript preparation." Also, please use

the track changes function in the original document so that the reviewers can easily verify your responses.

Your revision is due by Jan 01, 2016.

To submit a revision, go to http://jctres.edmgr.com/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Reviewers' comments:

Dear authors, please address the comments I placed in your resubmission draft. Also, continue with the proofread version of the paper I sent to you separately. Once this has been completed and my comments have been properly addressed, please resubmit the final version, after which your manuscript can be accepted for publication.

There is additional documentation related to this decision letter. To access the file(s), please click the link below. You may also login to the system and click the 'View Attachments' link in the Action column.