ANSWERING REVIEWERS

May 12, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 17424-revised.doc).

Title: β-elemene enhances radiosensitivity of gastric cancer cells through inhibition of Pak1 activation

Author: Jun-Song Liu, Xiang-Ming Che, Shuai Chang, Guang-Lin Qiu, Shi-Cai He, Lin Fan, Wei Zhao, Zheng-Liang Zhang, Shu-Feng Wang

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We appreciate the reviewers' comments and suggestions. All of the revisions we make to the revised manuscript are highlighted in the updated vision. The responses to reviewers' comments are as below.

The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated.

Response: Format has been updated according to "Format for basic study" and the editor's advice.

2 Revision has been made according to the suggestions of the reviewer

(1) Reviewer 1: # 03270395

Comments: This manuscript is interesting and valuable to explore the potential of β-elemene as a radiosensitizer for gastric cancer cells. However, I have a question about your experiment. MTT assay was adopted to determine that which gastric cancer cell line was not sensitive to the IR, and at the same time Clonogenic survival assay was adopted to observe whether β-elemene could promote the cell killing effect of IR on the cancer lines. My question is that why don't you use the same kind of method, such as MTT assay, to study the two experiments, which would make the results more convincing.

Response: Thanks for your review and suggestions. We agree with your view of using the same kind of method to study radiosensitivity of gastric cancer cells in the two experiments. And as a matter of fact, we did it as such. We used a same method, clonogenic survival assay, to screen for relatively radioresistant gastric cancer cell lines and to evaluate radiosensitization effects of drugs. The details were presented in the results (**Page 10, line 3-4 and line 9-10; Page 12, line 15-17**), corresponding figures and figure legends (**Page 20, Figure 1; Page 20, Figure 2; Page 22, Figure 7**).

(2) Reviewer 2: # 03004155

The authors present data suggesting that β-elemene enhances the radiosensitivity of gastric cancer cells via up-regulation of PAK1IP1 (p21-activated protein kinase-interacting protein 1) and down-regulation of phospho-Pak1 (T423) and phospho-ERK1/2. The therapeutic potential of β-elemene as a radiosensitizer for gastric cancer may be worth pursuing. This is an interesting work.

Comments: 1) However, one main issue should be addressed before resubmission: The manuscript is devoid of direct evidences indicating that β-elemene enhances the radiosensitivity of gastric cancer cells via up-regulation of PAK1IP1. It is recommended to detect the effects of alteration of PAK1IP1 expression (overexpression and knockdown) on the β-elemene-induced radiosensitivity of gastric cancer cells.

Response: Thanks for your valuable suggestion. Indeed, PAK1IP1 was a molecule directly screened out by the proteomic assay when we searched for molecules underlying the mechanism of radiosensitization effects of β-elemene in gastric cancer cells. And we have validated that β-elemene up-regulates PAK1IP1 in gastric cancer cells (**Figure 5A**). It seems rational to alter the expression of PAK1IP1 in the latter part of the study. However, two aspects of evidence led us to think that targeting Pak1 was probably more valuable to the present study after we made a cautious evaluation.

Firstly, Pak1 (p21-activated protein kinase 1) is a more pivotal molecule in tumorigenesis and cancer progression, and regulates diverse cellular biological behaviors including cytoskeletal dynamics, survival, proliferation and transcription in cancer **[reference 16 and 17 in the manuscript: [16] Manser E, Leung T, Salihuddin H, Zhao ZS, Lim L. A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature 1994; 367(6458): 40-46 [PMID: 8107774 DOI: 10.1038/367040a0]; [17] Kumar R, Gururaj AE, Barnes CJ. p21-activated kinases in cancer. Nature reviews Cancer 2006; 6(6): 459-471 [PMID: 16723992 DOI: 10.1038/nrc1892]]**. Targeting Pak1 is an important and promising method in cancer therapy. Actually, PAK1IP1 was initially found in the research for Pak1 inhibitors **[reference 28: Xia C, Ma W, Stafford LJ, Marcus S, Xiong W-C, Liu M. Regulation of the p21-activated kinase (PAK) by a human Gβ-like WD-repeat protein, hPIP1. P Natl Acad Sci USA 2001; 98(11): 6174-6179 [PMID: 11371639 DOI: 10.1073/pnas.101137298]]**. Through immunoprecipitation assay and western blot analysis, Xia and his colleagues have demonstrated that PAK1IP1 interacts with Pak1 *in vitro* and *in vivo*. PAK1IP1 specifically binds to the N-terminal regulatory domain of Pak1 and transfection of PAK1IP1 in mammalian cells inhibits the activation of Pak-mediated signaling pathways. The results acquired broad agreement in other researchers' reviews **[reference 19 and 29: [19] Dummler B, Ohshiro K, Kumar R, Field J. Pak protein kinases and their role in cancer. *Cancer Metast Rev* 2009;**

28(1-2): 51-63 [PMID: 19165420 DOI: 10.1007/s10555-008-9168-1] [29] Bokoch GM. Biology of the p21-activated kinases. Annual review of biochemistry 2003; 72: 743-781 [PMID: 12676796 DOI: 10.1146/annurev.biochem.72.121801.161742]]. **Secondly**, recent studies indicate that Pak1 closely relates to radiation response in cancer cells. Pak1 was found to be activated by ionizing radiation (IR) and participated in DNA damage repair after IR **[reference 20: Li DQ, Nair SS, Ohshiro K, Kumar A, Nair VS, Pakala SB, Reddy SD, Gajula RP, Eswaran J, Aravind L, Kumar R. MORC2 signaling integrates phosphorylation-dependent, ATPase-coupled chromatin remodeling during the DNA damage response. Cell Rep 2012; 2(6): 1657-1669 [PMID: 23260667 DOI: 10.1016/j.celrep.2012.11.018]]**. More recently, Mona Motwani reported that the genes regulated by Pak1 in response to IR were mainly involved in DNA damage responsive events, such as cell cycle arrest and apoptosis **[reference 21: Motwani M, Li DQ, Horvath A, Kumar R. Identification of novel gene targets and functions of p21-activated kinase 1 during DNA damage by gene expression profiling. *PloS one* 2013; 8(8): e66585 [PMID: 23950862 DOI: 10.1371/journal.pone.0066585]]**. These preliminary results indicate that Pak1 signaling are potential targets for enhancing tumor radiosensitivity. Therefore, Pak1 is probably a core molecule that works in radiation response and is the molecular more worthy of further research.

Based on the evidence above, we hypothesized that inhibition of Pak1 underlies radiosensitization effect of β-elemene in gastric cancer cells, and chose to directly target Pak1, rather than alter PAK1IP1 expression in the latter part of the present study. As a result, our data showed that selective inhibition of Pak1 signaling using small molecule inhibitor IPA-3, enhanced radiosensitivity of gastric cancer cells, indeed, increasing radiation-induced cell death and decreasing clonogenic survival. So, our results proved to support our initial hypothesis.

Comments: 2) Additionally, some grammar errors were occurred in the manuscript. For example, In Page 2 Line 9 "β-elemene pretreatment with decreased clonogenic survival following ionizing radiation (IR) and increased radiation-induced cell death in MKN45 and SGC7901 gastric cancer cell lines." The word "with" in this sentence should be deleted. It is strongly recommended to double check the whole manuscript before resubmission.

Response: Thanks for your careful review. We have corrected the grammar errors in the manuscript. Before resubmission, we have carefully and repeatedly checked the whole manuscript to make sure there are no grammar errors any more.

(3) Reviewer 3: # 03270499

This is an interesting work. However, some issues should be addressed before resubmission:

Comments: 1) From Figure1, the clonogenic survival of SGC7901, MKN28, and N87 are quite similar, would you please show all data of three cell lines which would make the results more convincing.

Response: Thanks for the precious suggestion. We have shown the data of this experiment as suggested **(Page 20, Table 1)** and described the results in corresponding part of the manuscript **(Page 10, line 6).**

Comments: 2) Figure 5, β-elemene increased the expression of Pak1-interacting protein 1 (PAK1IP1) in gastric cancer cells. Since PAK1IP1 was demonstrated to be a negative regulatory molecule of Pak1, here is the question, how about the expression level of PAK1IP1 after ionizing radiation treatment? Would you please show PAK1IP1 expression level in figure 5B and 5C?

Response: Thanks for the suggestion. We have shown PAK1IP1 expression level in corresponding results and revision has been made according to the suggestions of the reviewer. **(Page 12, line 2-3; Page 25, Figure 5B and 5C**)

Comments: 3) You did clonogenic survival assay and apoptosis detection assay in MKN45 and SGC7901, But there is no western blot result of MKN45.

Response: Indeed. The cytology experiments were to confirm the effect of radiosensitization and western blots were to explain the potential underlying mechanisms. So we performed cytology experiments in two cell lines to make sure the drug worked. While western blots of SGC7901 cell line would be sufficient to explain the phenomenon.

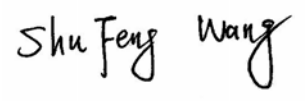
Comments: 4) For Figure 8 and 9, please clarify the cell type for those two experiments.

Response: Actually, the cytology experiments showed that the SER (sensitization enhancement ratio) was 1.53 for SGC7901 cell line and 1.26 for MKN45 cell line, respectively **[Page 10, line 25-28 (line 2-6 from backwards); Figure 2]**. It means the effeciency of radiosensitization of β-elemene in SGC77901 cell line was better than that in MKN45 cell line. Similar effects were seen in targeting Pak1 using IPA-3 **(Figure 7)**. Consequently, we chose SGC7901 cell line in subsequent study to investigate the expression of potential proteins underlying radiosensitization.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,



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