Author’s response to reviews

Title: The Critical Role of ERK in Death Resistance and Invasiveness of Hypoxia-selected Glioblastoma Cells

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Author's response to reviews: see over
Melissa Norton, MD
Editor-in-Chief
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Dear Dr. Melissa Norton

We are pleased to submit the revised manuscript entitled "The Critical Role of ERK in Death Resistance and Invasiveness of Hypoxia-selected Glioblastoma Cells (MS 7151191092121596)" by Jee-Youn Kim, Yong-Jun Kim, Sun Lee, and Jae-Hoon Park for consideration for publication in BMC Cancer.

In this revised version of manuscript, we have addressed all of concerns.

Neither this submitted paper nor any similar paper has been or will be submitted to or published in any other journal while under consideration by BMC Cancer.

We thank you in advance for your consideration of this manuscript.

With best regards

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Responses to Reviewer

Reviewer 1
1. A previous study selected death resistant cells by repeated episodes of hypoxia and also reported the importance of Bcl-2 protein regulation (Dong and Wang J Biol Chem 2004). The current study should carefully discuss the similarities or consistency between these two studies and also point out the new findings of the current study.

We are thankful to the reviewer for the comments regarding our paper. In previous study by Dong and Wang (J Biol Chem 2004, 279:9215), the authors selected hypoxia-resistant cells by repeated episodes of hypoxia and found that selected cells were cross-resistant to apoptotic stimuli such as staurosporin, azide, and cisplatin. In addition, they showed that death-resistance against different types of insults occurred at the mitochondrial level by up-regulation of Bcl-X\textsubscript{L}, which preserves mitochondrial intergrity by interaction with pro-death protein Bax. Consistent with previous study by Dong and Wang, we detected the increase of Bcl-X\textsubscript{L} in hypoxia-selected cells. In this study, however, we focused on the key signaling molecules responsible for the more invasive phenotypes as well as death resistance. These points were discussed in main text of the manuscript (Page 21, Line 2-10).

2. It would be interesting to determine if the selected T98G cells have undergone epithelial-mesenchymal transition, although Brooks et al. (Am J Physiol 2008) suggested that in their selected cells EMT did not occur. This is important consider the current results of high invasive potential of selected cells.

We totally agree to reviewer’s comment. Epithelial-mesenchymal transition (EMT) plays a critical role for tumor cells to leave an organized epithelial layer and gain the property of invasiveness. Such changes usually initiate cancer metastasis (Kang Y et. Al., Cell, 2004, 118:277). The molecular hallmarks for EMT are downregulation of epithelial cell adherence molecules, such as E-cadherin, a-cathnin, and r-catenin, and up-regulation of molecules of mesenchymal components, including vimentin, fibronectin, and smooth muscle actin (sm-actin) (Yang J, et. Al., Cell, 2004, 117:927).

To gain the informations on EMT, we explored the changes in expression level of E-cadherin, a-catenin, and fibronectin, and sm-actin. However, we could not find the significant alterations in expressions of these molecules between T98G and HRT98G cells. It may suggest that our results on high invasive potential of the selected T98G cells were not caused by EMT. These data was
not described in main text because we could not find the positive results on EMT.

3. Fig 2 should provide representative immunoblots for the analysis of the various proteins.

We regret that the representative immunoblots were not shown in Fig. 2. In this revised manuscript, representative immunoblot pictures were presented in right panels of Fig 2A, B, and C.

4. It is important to examine if ERK inhibition affects Bcl-2 and Bcl-XL expression in the selected cells, therefore sensitise the cells to death.

We thank for the critical comments on the relation between ERK inhibition and Bcl-2 or Bcl-XL expression and agree that this point is very important.

Previous report (Pardo OE, et. al., J Biol Chem, 2002, 277:12040) showed that the activation of ERK pathway rescued small-cell lung carcinoma cells from apoptosis by up-regulation of Bcl-2 and Bcl-XL. To clarify this point in hypoxia-selected cells, we examined the effects of ERK inhibition by U0126 and PD98059 on the expression of Bcl-2 and Bcl-XL. As shown in Fig. 4, ERK inhibition did not affect the expression level of Bcl-2 and Bcl-XL. This suggests that up-regulation of Bcl-2 and Bcl-XL expression by repeated hypoxia did not result from ERK activation. This result was described in main text (Page 15 Line 11 – Page 16 Line 7) and presented in Fig 4.
Reviewer 2.

Major comments

1. Western blots showing Bcl-2, Bcl-XL, DIVA and Mcl-1 protein levels should be presented.

We regret that the representative immunoblots were not shown in Fig. 2. In this revised manuscript, representative immunoblot pictures were presented in right panels of Fig. 2A, B, and C.

2. The effect of Bcl-2 and Bcl-XL expression following inhibition of ERK activation should be done in the T98G cells (both sensitive and resistant cell lines).

As stated above (comment number 4 of reviewer 1), we totally agree that this point is very important. To clarify this point, we examined the effects of ERK inhibition by U0126 or PD98059 on the expression of Bcl-2 and Bcl-XL. As shown in Fig. 4, ERK inhibition did not affect the expression level of Bcl-2 and Bcl-XL. This suggests that up-regulation of Bcl-2 and Bcl-XL expression by repeated hypoxia did not result from ERK activation. This result was described in main text (Page 15 Line 11 – Page 16 Line 7) and presented in Fig 4.

3. RNA interference against ERK1/2 should also be used to confirm chemical inhibitors of MEK results.

We are thankful to the reviewer for the critical comments regarding our paper and agree that knock-down of ERK1/2 by siRNA is more specific than by chemical inhibitors. Thus, we introduced ERK siRNA (siERK) to HRT98G cells and examined the effect on cell death and invasiveness. Consistent with data using chemical inhibitors, siERK significantly increased the cell death rate and decreased invasive potential of HRT98G cells, compared to siControl. This result was described in main text (Page 17 Line 2-3, Page 18 Line 3-4) and presented in Fig. 5 & 6.

3. The amount of RAS activation and the level of reactive oxygen species in the cell lines should be measured.

As reported previously (Haddad JJ, Int Immunopharmacol, 2004, 4:1249), Hypoxia/reoxygenation rapidly induces activation of RAS, which is the first step for the
activation of the MAPK cascades. In addition, ROS is known as a triggering factor for the ERK activation. In this view of point, we agree that determination of the amount of RAS activation and the level of ROS is very important.

As described in text (Page 15 Line 4-9) and depicted in Fig 3A, the Ras/Raf/MEK pathway was significantly activated in HRT98G cells. These results suggest that RAS pathway is a key regulator of ERK activation in HRT98G. Next, we determined the levels of ROS using ROS-sensitive fluorescence dye, DCFH-DA. As shown in Fig. 3B, ROS level was increased in HRT98G compared to T98G. This result was described in main text (Page 15 Line 4-9) and shown in Fig. 3B.

4. Finally, the tumors should be stained for a hypoxia marker (for example Glut-1) to correlated ERK activation to increased hypoxia regions of viable cells in tumors.

We immunostained the tumor tissues with hypoxia markers such as BNip3 and HGTD-P and the results from BNip3 stainings were correlated with those of HGTD-P. We showed data obtained from HGTD-P immunostaining.

Minor comments

Spelling mistakes in manuscript should be corrected.

We apologize that there were spelling errors in the manuscripts. We checked and made corrections in this revised manuscript.
Reviewer 3

Major comment

The authors showed there results using on the cell type namely T98G. Although their results are interesting and also represent an important contribution to the development of cancer treatment approaches, we feel that performing comparative experiments with another glioblastoma cell line that bear other characteristics like GaMG (Akslen LA, Andersen KJ, Bjerkvig R, Anticancer Res. 1988) can give clear conclusive evidence for their finding. If necessary we are ready to provide the research group with that cell line.

We are greatly thankful to the reviewer for the comments and GaMG cell line with different characteristics provided by reviewer. We will perform the comparative experiments with GaMG. Regrettably, however, it takes much time over 6 months for the comparative experiments. So, we cannot meet deadline for revision of manuscript. Please consider this situation.

Minor comment

Within the classification in Table 1 of the manuscript, where a comparison of p-ERK expression in different tumour grade in astrocytic glial tumors was shown, here they should divide analysed samples when plotting it against WHO grade in all four stages of human glioma, since oligodendrome (stage I) has completely different characteristics than stage II and can not be “pooled” together. The same is true for stage three and stage four.

We are thankful to the reviewer for the critical comments regarding our paper. We re-classified the glial tumor tissue according to WHO grade (grade I, II, III, and IV) and showed it in Table 1.
Reviewer 4

1. In figure 1 and all blotting/gel data the authors need to show molecular weight markers and the relative size of the indicated band. In addition in Panel B of Fig 1 the authors need to indicate if this is cleaved PARP or total PARP.

We apologize for missing the molecular weight or band marker. This was corrected in the revised manuscript.

2. The authors need to show the blots for figure 2. It is helpful to summarize the data in a bar graph and these could be left in.

Representative immunoblot images were shown in right panels of Fig 2A, B, and C.

3. In figure 3 the authors need to specify either in the results section or methods what is the IC50 for each of the ERK inhibitors used and then detail in the figure legend the concentration of the inhibitor relative to the IC50 (i.e. 2-fold the IC50). Also in figure 3 is PMA activating PKC and resulting in increased Erk activity?

We are thankful to the reviewer for the critical comments regarding our paper. IC50 for each of the ERK inhibitor was shown Page 16 Line 3-4. In addition, we used PMA to activate PKC and ERK. Previous reports showed that PMA induces PKC activation, which in turn activates ERK (Chow, et. al., Cytometry 2001, 46:72). These cascades were confirmed by western blots (data not shown).

4. The authors need to specific for the invasion assay how many replicas they used for each condition and how many times the experiment was repeated.

We performed four independent invasion assays in triplicates. This was described in methods section (Page 10 Line 2) and legend for figure 6.

5. It looks like the immunohistochemical stain in Fig 5 is nuclear. If this is correct the authors need to shown a serial section for each tumor that is stained with hemotoxin and eocin to help
the reviewer

The immunohistochemical staining for p-ERK and HGTD-P are nuclear and cytoplasmic, respectively.

6. The authors need to define each abbreviation in the manuscript someplace.

We added ‘abbreviation’ for definition of each abbreviation (Page 25, Line 6-12)