Author's response to reviews

Title: Radioactive 125I seeds inhibit cell growth and epithelial-mesenchymal transition in human glioblastoma multiforme via a ROS-mediated signaling pathway

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Author's response to reviews:

Cover Letters

Dear editors and reviewers,

Attached please find the revised version of our manuscript entitled “Radioactive 125I seeds inhibit cell growth and epithelial-mesenchymal transition in human glioblastoma multiforme via a ROS-mediated signaling pathway” (Submission ID: 9693448071054081). We have revised the manuscript according to the reviewers’ comments and our responses are provided following.

Thank you for your kindly consideration on our paper. Please contact us if any further information about this paper is needed.

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Comments and Responses
Responses to Editor
Thank you for editor’s attention. Author ‘He Jie’ has done lots of job in our revised manuscript. Thus, all authors hope you will agree that she was added in our revised manuscript.

Responses to Dr Shen’s Comments

Major Compulsory Revisions

This manuscript seems to be lack of novelty in terms of project design. I noticed that the authors had published a very similar paper in PLoS one (HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed/24040157"PLoS One. 2013 Sep 10;8(9):e74038), where 125I seeds was applied to the treatment of nasopharyngeal carcinoma cells. In this paper, cell growth and EMT pathway was evaluated as well. Meanwhile, the previous study has not been cited in this manuscript. The authors should talk more about what is new of this study by comparing with the previous studies.

Our response:

We have revised the manuscript according to the reviewer’s kindly suggestion. Our previous studies have confirmed that radioactive 125I seed could inhibit the cell growth, migration, and invasion of nasopharyngeal carcinoma (NPC) by triggering DNA damage and inactivating vascular endothelial growth factor-A (VEGF-A) / extracellular-signal-regulated kinase (ERK) signaling. These encouraging results compel us to embark on testing larger numbers of cancer with this treatment modality [1]. Moreover, previous clinical trials have confirmed that 125I seeds treatment is an adjuvant therapy to be effective in GBM, especially recurrent GBM [2, 3]. Thus, GBM cells were also investigated in our previous paper. Results indicated that both NPC cells and GBM cells were more sensitive to 125I seed irradiation than to X-ray irradiation. However, VEGF-A signaling pathway in GBM cells was not affected by 125I seeds (Figure 1). Therefore, other signaling pathways were investigated. Interestingly, ROS pathway was involved with irradiation of 125I seeds on GBM cells.

The previous study has been cited in our revised manuscript.

Comparing with the previous studies, though GBM cell growth and invasion could be inhibited by radioactive 125I seeds in this study, however the signaling pathway via which radioactive 125I seeds inhibit GBM cell growth and EMT in human GBM was different. Moreover, for the lack of E-cadherin expression in GBM cells suggesting a non-classic EMT, only very few recent reports described an EMT phenomenon in GBMs and its association with the poor prognostic mesenchymal subgroup of GBMs [4]. Our study indicated that EMT of GBM cells inhibited by 125I seeds. These findings have clinical implications for the treatment of GBM patients with 125I seeds. These content were added into the revised version of our manuscript.

Figure 1 VEGF-A was measured by western blotting after irradiation with 125I seeds.

2. As a novel approach, 125I seed implantation showed potential success in the study of GBM, many researchers have attempted to assess the efficacy of 125I
seed implants in GBM, which, however, are not fully discussed in this manuscript (For example: http://www.ncbi.nlm.nih.gov/pubmed/?term=J+Neurosurg.+2008+Feb%3B108%282%29%3A236-Neurosurg. 2008 Feb;108(2):236-42).

Our response:

Thanks for the reviewer’s carefulness. Effective treatment options are limited for patients with GBM, especially for recurrent GBM, thus, novel treatment approaches are needed. 125I seeds treatment is an adjuvant therapy that has been shown to be effective in recurrent GBM. Chan et al. indicated that GliaSite radiotherapy, in which low-dose-rate radiation was delivered with an aqueous solution of organically bound 125I, is safe and generally well tolerated for recurrent GBM [5]. Wernicke et al. have confirmed that treatment with GliaSite radiotherapy is feasible and safe, while rendering acceptable local control and minimal acute and long-term toxicities for newly diagnosed and recurrent GBM [6]. Moreover, Patel et al. have suggested that permanent placement of 125I seeds for recurrent GBM may prolong survival in patients with recurrent GBM [2]. Similarly, Darakchiev et al. have indicated that the use of adjuvant therapy combining carmustine wafers and permanent 125I seeds was a good treatment option for patients with recurrent GBM who have undergone previous surgery and radiation therapy [3]. In a word, previous clinical trials have confirmed that 125I was an effective treatment for patients with GBM. However, our study was performed to investigate the biological effects of 125I seeds on GBM cells. Results indicated that 125I seed irradiation was more effective than X-ray irradiation in inhibiting GBM cells via the ROS pathway. Obviously, our data are in line with the majority of published clinical trials studies. These results reported here also confirm and extend previous findings. These content were added into the revised version of our manuscript.

- Minor Essential Revisions
1. In the 1st sentence of third paragraph of Materials and methods section, “as according to” should be “according to”.
Our response:
Thanks for the reviewer’s carefulness. “As according to” were revised as “according to”.

2. The last sentence of first paragraph of Results section should be revised.
Our response:

Thanks for the reviewer’s carefulness. The last sentence were revised “Taken together, these assays indicate that GBM cells are more sensitive to 125I seed irradiation than to X-ray irradiation.”

3. The grammar and spelling in this paper should be improved.
Our response:

Thanks for the reviewer’s carefulness. Most of the grammar and spelling in this paper were revised. For example, “Transwell and Boyden chamber assay” in page 11 was revised as “Transwell and Boyden chamber assays”;


“Detection of oxidative stress in intracellular ROS” in page 12 was revised as “Detection of ROS in intracellular”; “We further investigate whether the observed irradiation-induced apoptosis was related to caspase-3” in the 3rd sentence of page 16 was revised as “We further investigated whether the observed irradiation-induced apoptosis was related to caspase-3”.

References:


Responses to Dr William’s Comments

1. It has been previously demonstrate that Stereotactic Brachytherapy (SBT) with I125 represents a safe, minimally invasive, and highly effective local treatment option for pediatric patients with inoperable LGG WHO grades I and II as well as metastatic and meningiomas (Ostertag 1989; Voges J, et al. 1990). It has been use few times in GBM because it’s diffuse and not clean borders characteristics, which doesn’t allow a conformational radiotherapy doses, compromising other structures in vicinity and the difficulty to cover all tumor area besides the risk of complications such as radionecrosis. It has been demonstrate that SBT with
I-125 is a minimally invasive, safe, and effective local treatment when applied as monotherapy or in combination with microneurosurgery for circumscribed cerebral LGGs smaller than 40 to 50 mm in diameter located in functional and anatomically complex brain territories such as midline, brain stem, and/or eloquent cortical areas (Ruge ML 2011). At present it is suggest the management of patients with brain stem glioma by stereotactic biopsy and implantation of I125 seeds for interstitial radiosurgery in a single step procedure as an alternative to external radiation therapy (Lopez WO 2013).

Our response:

We have revised the manuscript according to the reviewer’s kindly suggestion. Low-grade (WHO grades I and II) gliomas (LGGs) are the most common solid tumors in childhood. Because of localization in deep-seated or highly eloquent brain areas, a curable complete tumor resection can be achieved only in a subset of patients. Moreover, either chemotherapy external beam radiation therapy causes considerable toxicity. Thus, it is important to explore effective new modalities for GBM patients to improve the current therapeutic regimens. Ruge et al. have demonstrated that stereotactic brachytherapy (SBT) was an effective and safe treatment for patients with small (< 4 to 5 cm) unresectable or incompletely resected LGGs, localized in deep and/or highly eloquent brain areas, such as midline, brain stem, and/or eloquent cortical areas [1]. Recently, Lopez et al. have indicated that 125I interstitial radiosurgery based on MRI is a safe and effective method to diagnose and treat LGGs of the brain stem in adults. The main advantages of interstitial radiosurgery are that diagnosis and treatment can be performed in one procedure. Moreover, in most of the patients additional therapeutic measures, especially external beam radiation are not influenced by local irradiation [2].

Base on these reports and the reviewer’s kindly suggestion, we can conclude that 125I seed treatment may be more effective for LGGs, though reports about interstitial radiosurgery for LGGs of the brainstem are rare. This may be due to that the long potential doubling time of slow-growing tumor cells inside LGGs requires elongating the tumor killing over several weeks or months. 125I seed irradiation can achieve a cumulative reference dose of more than 60 Gy on the surface of the tumor for approximately 9 months until the decay of activity. Furthermore, because of the inverse square law of radiation, using 125I seeds that emit low-energy radiation results in a steep falloff of the dose of highly conformal irradiation toward the periphery within a millimeter range, also considerably reducing the radiation burden on surrounding (functionally relevant) tissue, thus allowing an almost strictly local treatment regimen and greater doses delivered. In the future studies, we will focus on LGGs. Some of these contents were added into the revised version of our manuscript.

2. The paper wrote by Yunhong Tian et al. states that radioactive 125I seeds are more effective than X-ray irradiation in inhibiting GBM cell growth. Moreover, EMT was effectively inhibited by 125I seed irradiation. Such concept may bring additional information of the feature of GBM which currently is resistance to treatment; however the experiments realized to bring such conclusion seem to be insufficient. According to the methods what is the primary target of the 125I seed
irradiation? They show that apoptosis is more induced, cell survival is less but don’t show any profound underlying mechanism (i.e. DNA repair enzyme activation), that would be very interesting.

Our response:

We have revised the manuscript according to the reviewer’s kindly suggestion. GBM is the most common and most aggressive type of primary brain tumour. Current treatment consists of tumour resection (where possible), followed by ionising radiotherapy combined with concomitant and adjuvant temozolomide (TMZ) chemotherapy. The TMZ is a methylating agent that creates lesions in DNA, the most cytotoxic of which is O6-methylguanine [3].

O6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein, which removes O6-methylguanine adducts from damaged DNA. This reaction is irreversible and once bound to the alkyl group MGMT is ubiquitinated and destroyed by the proteasome [4]. Studies have confirmed MGMT promoter methylation is an independent favorable prognostic factor in patients with GBM. Previous work has shown that patients with tumors displaying MGMT promoter hypermethylation or low expression of MGMT protein [i.e., MGMT(−)] are more likely to benefit from TMZ treatment, compared with patients with tumors displaying unmethylated MGMT, or high MGMT expression [5, 6]. Therefore, we evaluated the effect of radioactive 125I seeds on MGMT. The results showed that 125I treatment significantly decreased MGMT protein in U251 cells (Figure 1A), whereas U87 showed no detectable MGMT gene expression (Figure 1B). Consistently, previous studies have confirmed that U251 cell lines expressed the MGMT gene transcripts, whereas U87 showed no detectable MGMT gene expression [7]. Moreover, results indicated that these decreased MGMT in U251 were dependent on the irradiation doses (Figure 1C). Our results also indicated that MGMT in GBM cells decreased by 125I seeds may not be associated with ROS, for it was not recovered by GSH (Figure 1D). However, previous study have confirmed that MGMT as a key determinant of resistance toward alkylating chemotherapy can be transcriptionally activated by X-ray [8]. Considering the observed decrease of MGMT levels under irradiation, 125I seeds may enhance TMZ effect, by decreasing MGMT expression. Nevertheless, MGMT alone can’t be an indispensable factor for 125I seed irradiation because MGMT-negative U87 cells also become more sensitive to 125I seeds than to X-ray. These data suggest that there may be additional mechanisms involved in the 125I seeds - provoked MGMT and sensitivity to irradiation in GBM. Some of these contents were added into the revised version of our manuscript.

Figure 1 MGMT were measured by measured by western blotting. U251 (A) and U87 (B) were measured. (C) Down-regulated MGMT in dose dependent manner. (D) MGMT without significant changes after pretreatment of U251 cells with GSH.

3. The authors speculate “EMT is modulated” testing this with altered motile behavior of the cells. They used e-Cadherin and Vimentin as markers, however all GBM express Vimentin and it is well know that E-Cadherin is only rarely
expressed in glial tumors and in fact there are reports even saying it is a negative prognostic marker in GBMs (Lewis Tuffin et al., PlosONE 2011). It is also known that the E-to N-Cadherin switch is not a hallmark event in EMT-like process in GBMs (Kahlert et al., Cancer Letters, 2013). It worth to mention that U251 and U87 do not express e-Cadherin in Gliomas. I would suggest testing ZEB1 and SNAI1 as core factors of EMT to test if there is any regulation in mesenchymal transformation after radiation. If the authors talk about EMT in GBMs they miss to cite the pioneer paper of EMT in gliomas from Kahlert et. al (Cancer Letters 2012).

Our response:

We have revised the manuscript according to the reviewer’s kindly suggestion. Despite the discoveries made in tumors of other tissue origins, until now the assumption whether aggressive mesenchymal GBMs always underwent epithelial-to-mesenchymal conversion had not been widely asserted [9]. An important feature of EMT is that the expression of the epithelial Ecadherin is reduced in exchange for increased expression of mesenchymal cadherins such as N-cadherin [10]. However, unlike in epithelial cells, #catenin was expressed in all lines and most lines expressed N-cadherin. Only the one cell line, derived from a recurrent anaplastic astrocytoma, was found to express E-cadherin [11]. Above all, the lack of E-cadherin expression in GBM cells suggests that a classic EMT is not involved in GBMs progression.

To date, many transcription factors have been said to drive EMT, including members of the Snail and basic Helix Loop Helix (bHLH) families, and zinc finger and homeodomain (ZEB) factors [12]. Snail, ZEB, and KLF8 (Kruppel-like factor 8) can bind to E-cadherin promoter and repress its transcription, whereas factors such as Twist, transcription factor 4 (TCF4), homeobox protein SIX1 and fork-head box protein C2 (FOXC2) repress E-cadherin indirectly [13]. Although many transcription factors can trigger it, the full molecular reprogramming occurring during an EMT is mainly orchestrated by three major groups of transcription factors: the ZEB, Twist and Snail [14]. Moreover, in GBM cell lines, the capabilities of motility and invasion have been ascribed to Snail, Twist, and ZEB1 expression [15]. Thus, our studies investigated the levels of ZEB1, Snail and Twist. Our results indicated that the levels of ZEB1 were involved in EMT inhibited by 125I seeds, with no significant changes in Snail and Twist in U251 (Figure 2A) and U87 (Figure 2B). Furthermore, we measured ZEB1 expression after irradiation by immunofluorescent assay. As expected, ZEB1 protein levels in U251 cell nuclear decreased significantly 24 hours after 125I seed irradiation (Figure 2C). As shown in Figure 2D, 125I seeds inhibited ZEB1 in a dose-dependent manner, indicating that seed irradiation activates ZEB1-mediated EMT, whereas ZEB1 was up-regulated by X-ray irradiation. Consistently, previous studies have confirmed that ZEB1 bound to E-CADHERIN promoter and suppresses its expression eventually leading to loss of cell - cell contact and therefore promotes increased motility in GBMs [16, 17]. These content were added into the revised version of our manuscript.

Figure 2 125I seed irradiation inhibits the EMT in GBM cells via ZEB1. (A) Western blotting analysis of the expression levels of ZEB1, Twist and Snail in
U251 (A) and U87 (B) cells exposed to 125I seeds. (C) Suppression of ZEB1 expression by 125I seed irradiation as measured by immunofluorescent assay. (D) Western blotting analysis of the expression levels of ZEB1 in U251 cells treated with 125I seeds (upper panel) and X-ray irradiation (lower panel).

4. Did they see WNT- pathway modulation? In fact it is strange that after conventional radiation EMT is accumulated (as tested by Vimentin expression) and after 125I seed irradiation it goes down. This is intriguing would that seed radiation more effectively kills the EMT-like GBM cells. Those cells are thought to be very resistant to conventional radiation (Mahabir et al., Neuro-Oncology 2014) and the author’s seed-technology would be from highest clinical interest. The paper should focus on that and proof with further adequate experiments.

Our response:

Thanks for the reviewer's carefulness. Cell migration and invasion are fundamental components for tumor cell metastasis. EMT seems to be crucial for tumor progression and metastasis, as well as for resistance against chemotherapy or radiation and thereby for tumor relapse after treatmen [9]. Previous studies have reported that a sublethal dose of X-ray irradiation promoted glioma cell migration and invasion in the border area of postoperative radiotherapy [18]. It has also been shown that sublethal irradiation promotes migration and invasion of cells through the TGF-β [19], and vascular endothelial growth factor (VEGF) in GBM [20]. Moreover, Mahabir et al. have clarified that irradiation induced mesenchymal phenotypes in GBM and that long-term elevation of Snail may contribute to transition to a mesenchymal phenotype of MG possibly via ERK activation, together with TGF-β-dependent signaling pathway [15]. In contrast, other studies reported that both proton and carbon ion irradiation significantly decreased cell migration and invasion [21, 22]. Our previous study have confirmed that 125I seed irradiation inhibited CNE2 cell line migration and invasion by inactivating vascular endothelial growth factor-A (VEGF-A) / extracellular-signal-regulated kinase (ERK) signaling [23]. Similarly, Kahlert et al. have confirmed that in a subgroup of GBMs the activity of the canonical WNT/β-catenin signaling pathway is directly associated with increased motility of the tumor cells [16].

The WNT signal transduction cascade controls myriad biological phenomena throughout development and adult life of all animals. Currently, three different pathways are believed to be activated upon Wnt receptor activation: the canonical Wnt/β-catenin cascade, the noncanonical planar cell polarity (PCP) pathway, and the Wnt/Ca2+ pathway. Of these three, the canonical pathway is best understood [24]. Previous studies have shown that the canonical Wnt/β-catenin pathway is crucial for the development and progression of breast cancer. High levels of Wnt receptor and co-receptor expression, as well as aberrant activation of β-catenin, have been detected in breast cancer tissues. Downregulation of the Wnt/β-catenin pathway can inhibit the epithelial-mesenchymal transition (EMT) and reduce spontaneous invasion of breast cancer cells [24]. Moreover, canonical WNT/β-catenin pathway is a critical regulator of GBM invasion [16].
Vascular endothelial growth factor A (VEGF-A) is an important VEGF family member that is essential for cell proliferation and migration [25-28]. Overexpression of VEGF-A can augment cell proliferation and migration through extracellular-signal-related kinase signaling. VEGF-A overexpression is associated with poor prognosis in cancer patients [29-31]. A previous report described a post-radiation increase in VEGF-A enhanced glioma cell motility in vitro [32]. Previous study have demonstrated that radioactive 125I seeds nasopharyngeal carcinoma (NPC) cell migration was effectively inhibited by 125I seed irradiation through inactivation of VEGF-A/ERK signaling. Thus, the role of canonical WNT/#-catenin network and VEGF-A/ERK in an in vitro model of GBM invasion inhibited by 125I seeds was measured. However, WNT signaling decreased was not observed in GBM cells treated with 125I seeds, without different #-catenin and Axin expression (Figure 3A). Moreover, irradiation did not increase the expression and phosphorylation of VEGF-A (Figure 3B). Therefore, 125I seed irradiation might regulate ZEB1 through other signaling pathway at the transcriptional level. Thus, further study is necessary to address this issue in the future.

Figure 3 Western blotting analysis of the expression levels of #-catenin, Axin expression (A) and VEGF-A expression (B) in U251 cells treated with 125I seeds


