Inhibition of NR2B Phosphorylation Reduces Proton Therapy-Induced Cytotoxicity and Bystander Killing Effect

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Introduction
Proton therapy is known to converge the dose of radiation beams on targets while sparing surrounding normal tissues through Bragg peak optimization. Nevertheless, proton therapy on CNS tumors still leads to patient cognitive changes. To understand the possible mechanism underlying proton therapy-induced neurotoxicity and potential mitigators that reduce the induced neurotoxicity, we designed a coillumination system as the platform to study bystander effects induced and modulated by proton beams and memantine, respectively. NMDA receptor subunits 2B (NR2B) was hypothesized to be associated with proton beam-induced cell lethality and bystander killing effect.

Materials and Methods
Cell line and reagents: Human glioblastoma U87 cells were cultured in Minimum Essential Medium at 37 °C in a humidified atmosphere containing 5% CO₂ in the air. The cells were administrated with 25 μM memantine for two hours prior to irradiation or protein analyses. For immuno-labeling and biotining, antibodies against NR1, NR2A, and NR2B and γH2AX were applied along with secondary antibodies. Cytospin centrifuge, cells were attached, fixed and permeabilized on the slides. The treatment and/or 2-hour post-irradiation were acquired using a 20x objective lens and formulated as below.

CCK8 assay for memantine treatment: Plates to study the bystander effects. The gafchromic EBT3 film was used to validate the dose uniformity for the collimation setup (Fig. 1D). 4 Gy dose was delivered to the eighth day post-irradiation.

Proton (full-field) Collimation (collimated)
Control Memantine + proton (full-field)
Memantine + proton (collimated)
Proton (collimated)
Proton (full-field)

Fig. 2. (A) The effect of two-hour memantine treatment on U87 cell viability and proliferation assessed by the CCK-8 assay. (B) Relative phosphorylation levels of NMDA receptor subunits on the cell surface were evaluated with phos-tag gel electrophoresis. Black and blue arrows indicate the phosphorylated and non-phosphorylated protein bands, respectively. (C) Memantine treatment reduced the relative phosphorylation level of surface NR2B. (D) The number of NR2A puncta exceeded that of NR2B puncta under the same normal condition.

Discussion and Conclusions
Compared with the expression levels of NMDA receptor subunits on normal brain tissues (data not shown), the number of NR2A puncta is significantly greater than that of NR2B puncta on U87 cells. This indicates that proton therapy-mediated excitatory toxicity on normal brain tissues may be effectively abrogated by memantine pretreatment (unshown results from clonogenic and trypan blue exclusion assays revealed that memantine up to 25 μM did not induce significant cytotoxicity). Notedly, memantine is the major content of the FDA-approved drug, Namenda, for treating Alzheimer’s disease.

Today, we confirm that proton therapy can mediate bystander killing effect through the release of potential ROS (reactive oxygen species) and proinflammatory cytokines (passive diffusion, data not shown) as well as the transmission of potential pro-apoptotic cations and factors (active intercellular communication via neurotransmitter activation). We observed that significant increase of DNA damage in the group that received a high dose of proton irradiation evoked cytotoxicity and bystander killing effect. This study provide an insight into minimizing proton therapy-induced neurotoxicity via inactivation of NR2B.

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References