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Emodin coupled with high LET neutron beam—a novel approach to treat on glioblastoma

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ABSTRACT

The primary motivation of this investigative study is trying to find an alternative treatment that can be used to slow down or treat glioblastoma due to the witnessed toxic side effects of the current drugs coupled with limited effectiveness in overall treatment. Consequently, a Chinese plant extract emodin proves to play a critical role in this investigative study since results from the Western blot and the other accompanying assays for anti-cancer effects indicate that it cannot work a lot to suppress cell migration and possible invasion, but rather emodin can be combined with radiation to give desired outcomes. Our result shows that the kind of radiation which acts well with emodin is neutron radiation rather than gamma radiation. Emodin significantly enhanced the radiosensitivity of LN18 and LN428 cells to γ-rays through MTT assay and cell counting. Accordingly, exposure to neutron radiation in the presence of emodin induced apoptotic cell death and autophagic cell death to a significantly higher extent, and suppressed cell migration and invasiveness more robustly. These effects are presumably due to the ability of emodin to amplify the effective dose from neutron radiation more efficiently. Thus, the study below is one such trial towards new interventional discovery and development in relation to glioblastoma treatment.

Keywords: emodin; neutron beam; glioblastoma; apoptosis; enhancement ratio

INTRODUCTION

Gliomas are the most common malignant brain tumors with a high mortality rate [1]. Chemotherapy plays a more important role in the treatment and prevention of gliomas as gliomas usually locate at many important brain function areas, which makes the surgical resection very difficult [2]. However, most of the current anti-glioma drugs such as temozolomide (TMZ), carmustine, lomustine, and procarbazine are DNA cytotoxic alkylating agents with limited efficacy and serious toxicity and side-effects [3]. Thus, the discovery and development of new anti-glioma drugs with unique mechanism of action is the current top priority.

Emodin, a common traditional Chinese medicine, is an anthraquinone derivative present in the roots or rhizomes of rhubarb. Emodin has various medical applications, including anti-cancer, anti-oxidation, anti-inflammation or allergy, anti-diabetes, and anti-viral or bacterial activities [4–6]. Many types of biologically active compounds that are used widely for cancer treatment, such as doxorubicin and paclitaxel, are derived from nature. Similarly, recent studies have shown that emodin also has anti-cancer effects in different types of cancers, including leukemia, lung cancer, colon cancer, gallbladder cancer, pancreatic cancer, breast cancer and HCC [7, 8]. Interestingly, several recent studies have shown that emodin could synergistically improve the anti-cancer efficacy of conventional chemotherapeutic drugs, such as gemcitabine, paclitaxel, cisplatin, and etoposide, in pancreatic cancer, malignant melanoma and HER-2/neu-overexpressing lung cancer [9–11]. Further, this agent has the ability to inhibit metastasis and increase the efficacy of other anti-cancer agents with minimal toxicity to normal cells [12–19]. Nevertheless,

the ability of emodin to sensitize cells to the anti-cancer efficacy of molecular targeted cancer therapies, such as radiation, especially high Linear energy transfer (LET), has not been investigated in glioblastoma multiforme (GBM).

Thus, we have investigated whether emodin exerted beneficial effects to improve the anti-cancer efficacy of neutron beam therapy among high LET radiations in GBM treatment.

MATERIALS AND METHODS

Antibodies and chemicals

Anti-cleaved PARP1(#5625), anti-caspase 3 (#9668) and anti-cleaved caspase 3 (#9664) antibodies were purchased from Cell Signaling Technology (Danver, MA, USA). Anti-β-actin (sc-47 778) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Emodin was purchased from Sigma, dissolved in DMSO to make a 10 mmol/l stock solution and stored at 4°C.

Cell culture

Human glioma LN18 cancer cells were obtained from the Korean Cell Line Bank (Seoul, South Korea) and were cultured in Dulbecco's minimal essential medium (DMEM; GIBCO, Gaithersburg, MD, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS; GIBCO), 0.1 mM non-essential amino acids, glutamine, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid (HEPES), and antibiotics at 37°C in a 5% CO_2-humidified incubator. Human glioma LN428 cells were obtained from the KCLB and were grown in RPMI 1640 medium supplemented with 10% FBS, glutamine, HEPES, and antibiotics at 37°C in a 5% CO_2 humidified incubator.

Irradiation

Cells were plated in 60-mm dishes and incubated at 37°C under humidified conditions and 5% CO_2 to 70–80% confluence. Cells were irradiated with a ^{137}Cs γ-ray source (Atomic Energy of Canada, Ltd, Ontario, Canada) at a dose rate of 3.81 Gy/min. Fast neutrons (9.8 MeV, 30–40 keV/μm) were produced by the bombardment of beryllium by proton 9 Be (p, n)10B, as a nuclear reaction in the cyclotron (MC-50; Scanditronix, Uppsala, Sweden). The distance from the source to the surface (source to surface distance [SSD]) was 150 cm, the size of the irradiation field was 26 × 26 cm^2, the current was 20 μA, and the thickness was 2 cm. The direction of the beam was vertical, and the cell dishes were irradiated with media without surrounding substances. For measuring the absorbed dose and dose distribution of fast neutron beams or γ-rays, we used the dosimetry method of paired ionization chambers. Dosimetry data was measured before *in vitro* study and the neutron dose was calculated using RBE, 2.2, which has been used for neutron therapy in our institute and represents an equivalent cell-killing efficacy to γ-rays, as determined by clonogenic assay.

Colony-forming assay

Cells (500–1000) were seeded into 6-well plates in triplicate and maintained at the indicated doses of emodin before IR. After 14–20 days, colonies were fixed with 100% Methanol and stained with 0.4% crystal violet (Sigma, St Louis, MO, USA).

Cell death detection assay

The treatment, harvesting, and staining of cells were done with a cellular death detection reagent in line with the manufacturer's instruction. Multiskan EX (Thermo Fisher Scientific, Germany) was used to calibrate cell mortality at 450 nm.

ROS assay

Cells were grown and collected at the periods specified by the producer, and ROS was quantified at 450 nm through a Multiskan EX (Thermo Fisher Scientific, Germany). The fluorescent ROS indicator C2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; 5 M; Molecular Probes) was used to observe the ROS. FACS was used to identify fluorescence linked with the cells by engaging a FACSort™ flow cytometer and CellQuest™ software (BD Biosciences).

Cell viability assay

Cells were seeded at a density of 5000 cells/well in a 96-well plate and incubated for 24 h, in accordance with the indicated experimental conditions. To quantify cell viability, an equal volume of culture medium containing EZ-Cytox reagent (EZ3000, Daeillab Service, Chungcheongbuk-do, Republic of Korea) was added to the cells, and the mixture was incubated for 4 h. Cell viability was determined by measuring the absorbance at 450 nm using a Multiskan EX (Thermo Fisher Scientific; Waltham, MA, US).

Autophagy Assay

Cells were treated, harvested, stained with Cyto-ID® Green detection reagent (Cyto-ID® Autophagy Detection Kit 2.0, Enzo Life Science, Farmingdale, NY, US) and Hoechst 33342 in accordance with the manufacturer's protocols, and observed under a confocal laser scanning microscope (LSM 880).

Flow cytometry

Cells were cultured and treated with radiation or emodin. They were harvested at the indicated times, stained with propidium iodide (1 μg/mL, Sigma) according to the manufacturer's protocol, and then analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). A minimum of 10 000 cells was counted for each sample, and data analysis was performed with the use of CellQuest software (BD Biosciences).

Western blot analysis

Total proteins from cells were extracted in RIPA buffer (50 mM Tris-Cl, pH 7.4; 1% NP-40; 150 mM NaCl, and 1 mM EDTA) supplemented with protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM Na3VO4) and quantified using the Bradford method. Protein samples (30 μg) were separated by SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking non-specific antibody binding sites, the membrane was incubated overnight at 4°C with primary antibodies. After incubation with peroxidase-conjugated secondary antibodies at 37°C for 1 h, the protein bands were visualized using enhanced chemiluminescence reagent (GE Healthcare Biosciences, Pittsburgh, PA, USA) and detected using the Amersham Imager 680 (GE Healthcare Biosciences).

Transwell chamber assay

The invasive ability of GBM cells was measured using Transwell chambers. Briefly, the cells were seeded onto the membrane of the upper chamber at a concentration of 4×10^5 cells/ml in 150 μl serum-free medium and were either left untreated or treated with radiation for 24 h. The medium in the lower chamber contained 10% (v/v) FBS as a source of chemoattractants. For the invasion assay, cells that passed through the Matrigel®-coated membrane (coating time, 30 min at 37°C) were stained with Cell Stain Solution containing Crystal violet (MilliporeSigma) for 30 min, and for the invasion assay, cells that passed through the gelatin-coated membrane were stained and examined after 24-h incubation. The wells were evaluated under a light microscope (Olympus CK40; Olympus Corporation).

Statistical analysis

Statistical significance was determined using Student's t-test. Differences were considered significant if the *P* value was less than 0.05 or 0.001. ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

RESULTS

Effect of emodin on GBM cell proliferation

Initially, MTT analysis was employed to determine the ideal emodin concentration level. The highest efficient level of cytotoxicity detected in multiple LN18, and LN428 cells were 20 μM and about 30 μM, respectively, which is comparable to 20% inhibitory concentration after 48 hours of treatment (Fig. 1A). Subsequently, a cell viability assessment was conducted on LN18 and LN428 cells to determine the emodin-induced cytotoxicity stimulating radiation. Cell sustainability of two GBM cancer cell lines was significantly reduced in neutron beams and cells treated with emodin (Fig. 1B). Furthermore, colonies generated by emodin-treated 3D cultures in association with γ-ray were bigger in comparison to the ones created by these 3D cultures paired with neutron beams (Fig. 1C), thus demonstrating the equal size of colonies developed by 3D cultures treated with γ-ray as well as those treated with neurons.

Emodin boosts neutron-Induced Apoptosis

To ascertain the occurrence of cellular apoptosis caused due to emodin and radiation, annexin V and PI staining were used to detect early apoptosis. In the context of GBM cell lines, the ratio of early apoptotic cells increased significantly after 48 hours of being subjected to emodin and radiation (Fig. 2A). The comparable findings of flow cytometry revealed the higher sensitivity of emodin and neutron irradiation blended together in comparison to the mix of emodin and γ-ray irradiation (Fig. 2A). Neutron irradiation and emodin induced the largest proportion of apoptotic cells. The cell death assay assisted the assessment of early apoptosis for determining the induction of apoptosis in GBM cells treated with emodin and radiation. Seventy-two hours of emodin treatment caused a significant rise in the number of GBM's apoptotic cells (Fig. 2B). Subsequently, flow cytometry was conducted to investigate the impact of emodin on cell cycle progression solely or in conjunction with radiation (Fig. 2C). Sub-G1 cells, signifying apoptotic cells, were raised marginally by emodin though significantly by the combination irradiation therapy of emodin and neutron beam in comparison to γ-ray irradiation. Furthermore, in line with this finding, the apoptosis rate was raised after the employment of emodin and neutron beam therapy, as assessed by the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) test (Fig. 2D).

Emodin combined neutron treatment augments the apoptosis of Glioblastoma via cleaved caspase 3 and enhanced ROS generation

The existence of emodin causes a dramatic hike in the number of apoptotic cells post-treating the two GBM cell lines with radiations (Fig. 3A). When compared to radiation treatment alone, the combined therapy promoted PARP1 fragmentation and caspase-3 expression, corroborating the relevance of emodin in promoting apoptosis. Furthermore, as compared to radiation, the advantages of combined emodin with neutrons were far more apparent. The next step was to explore if the enhanced cytotoxicity caused by the emodin and neutron beam therapy was driven by higher caspase activation, which caused a higher number of apoptotic cell deaths. Precisely, the combination of neutron beam therapy and emodin leads to a higher rate of caspase-3 activation in comparison to the control group (Fig. 3B). Furthermore, both emodin and neutron beam therapy enhanced the creation of ROS in GBM cancer cells (see Fig. 3C), showing that ROS generating emodin and neutron beam therapy boost the signaling of intracellular caspase thus causing apoptosis.

Emodin increases neutron-stimulated autophagic cell death

For additional research on the anti-tumor properties of emodin and radiation, we evaluated various cellular reactions linked with cell death after neutron or emodin therapy; we specifically looked at the impact on autophagy since it is promoted both by neutron as well as emodin. The enhanced buildup of an autophagy biomarker, namely Cyto-ID Green, was detected surrounding the emodin and neutron beam treated groups, as portrayed in (Fig. 4A).

Impact of combined emodin with neutron beam on GBM cell migration and invasiveness

With the aid of transwell chamber experiments and wound-healing tests, we then explored the influence of neutron beam and emodin on the invasive and migratory properties of GBM cancer cells. The findings demonstrated considerable prevention of cell invasion and migration in the case of emodin and neutron therapy in comparison to the sole treatment or emodin treatment along with γ-rays (Fig. 5A–B). The findings of the wound healing experiment revealed that neutron coupled emodin therapy hindered cell migration on the LN428 cells (Fig. 5C). These findings imply that combining emodin with a neutron beam inhibits GBM cell migration and invasion.

DISCUSSION

When cancer drugs are adopted for use, their efficacy in stopping malignancy is usually tested over time to determine the extent to which they can be used. However, most cancers defy the drug effect, and therefore, they progress regardless. Consequently, gliomas that affect the brain are no exception in this, as they are equally fast-spreading in the brain cells of patients. As early indicated, these types of brain tumors occur in

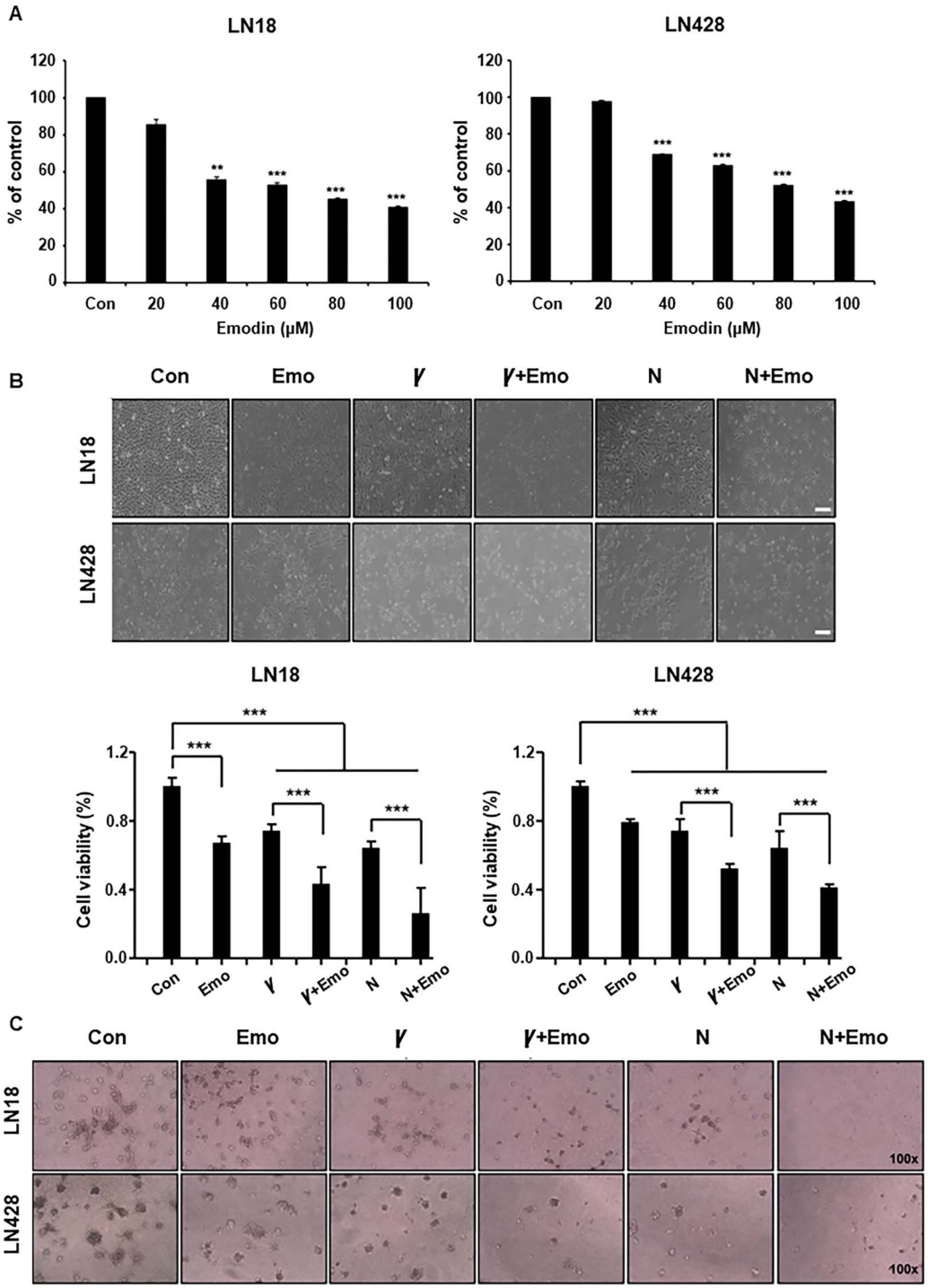

Fig. 1. The radiosensitizing effects of emodin combined radiation on GBM cells. (A) Emodin inhibited GBM cell viability in a dose-dependent manner. Cell viability was evaluated by MTT assay for LN18 and LN428 cells treated with the indicated doses of emodin; $^{**}P < 0.01$, $^{***}P < 0.001$. (B) MTT assays of LN18 and LN428 cells treated with 25 μM emodin and irradiated with γ-rays (6 Gy) and neutrons (6 GyE). Values are the means ± SD from three experiments; $^{*}P < 0.05$, $^{***}P < 0.001$. (C) 3D spheroid growth assay of LN18 and LN428 cells treated with emodin and radiation (6 Gy) for four days.

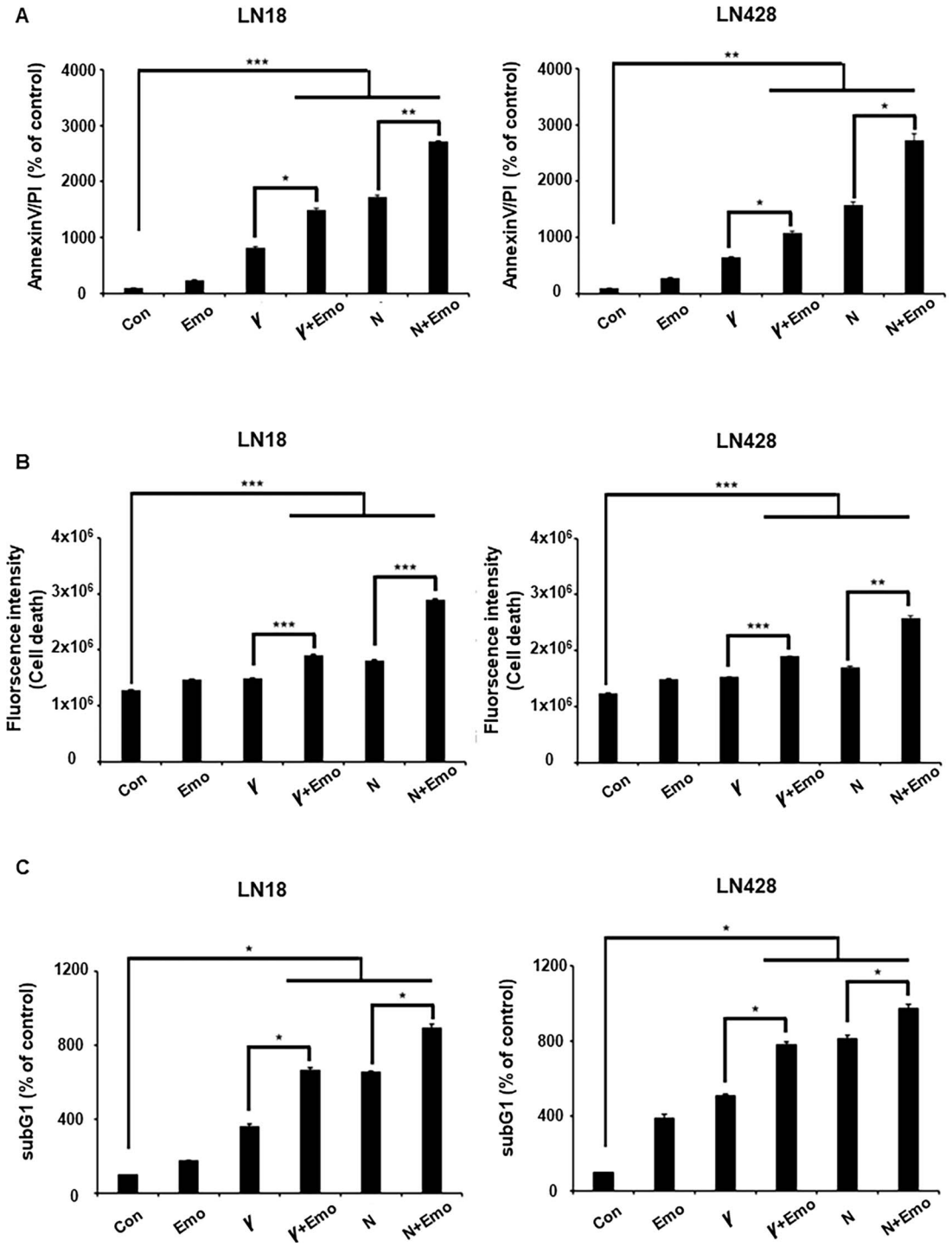

Fig. 2. Effects of emodin and radiation on apoptosis in GBM cells. (A) LN18 and LN428 cells were exposed to emodin (25 μM) and/or γ-ray (6 Gy) or neutron (6 GyE) radiation for 48 h for Annexin V staining. Values represent means of three experiments $\pm$ SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (B) Analysis of cell death in two GBM cell lines 72 h after treatment with emodin using a cell death detection kit; $^{**}P < 0.01$, $^{***}P < 0.001$. (C) LN18 and LN428 cell was treated with γ-ray (6 Gy) or neutron (6 GyE) and/or emodin for 24 h. Cell cycle distribution (subG1) was analyzed quantitatively by flow cytometry; $^{*}P < 0.05$. (D) TUNEL staining of LN18 and LN428 cells with and without neutron beam (6 GyE) or with and without emodin treatment. Values represent means of three experiments $\pm$ SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

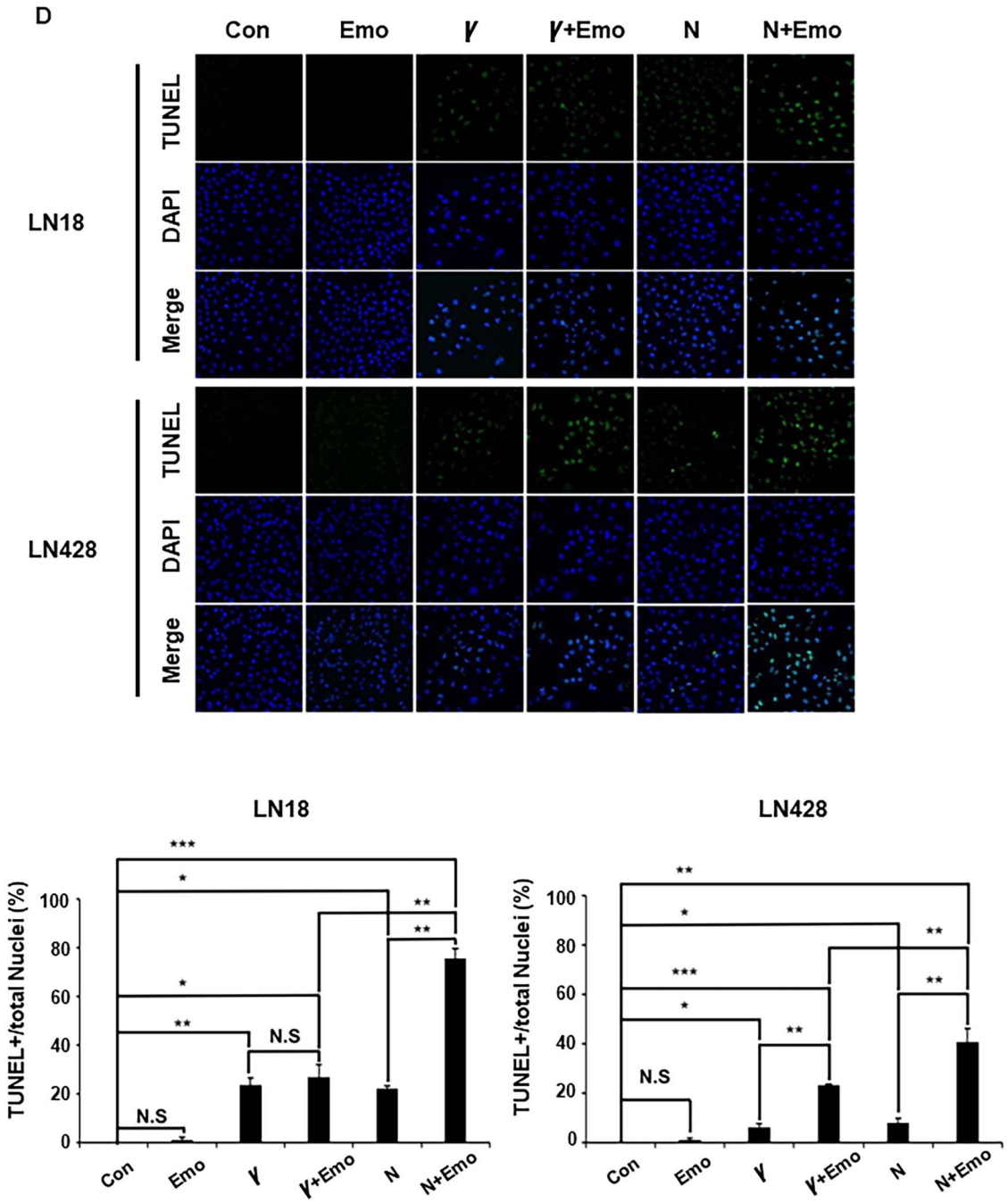

Fig. 2. Continued.

such a manner that it becomes difficult for surgeons to perform surgical operations on the remote locations of the brain where they tend to start and later spread to more delicate areas that carry out complex and vital functions. Since most cancers are treated either by chemotherapy, drug administration, and removal of the tumors, gliomas have been handled in a similar manner in the past with little or no success at all. This makes the search for unique drugs that can help in reducing the spread and cure of the cancer, like the study featured above revolving around Emodin, urgent and highly fundamental. As indicated, above the most important discovery from the investigation is that combining emodin with radiation restrained the migration and invasion of the cells.

Emodin has been reported to effectively reduce the growth of ovarian cancer cells by reducing the speed of growth and destroying malignant cells by targeting ILK [20]. Emodin has also been reported to inhibit migration and invasion through the CXCR4 modulation process in an orthotopic model of human hepatocellular carcinoma

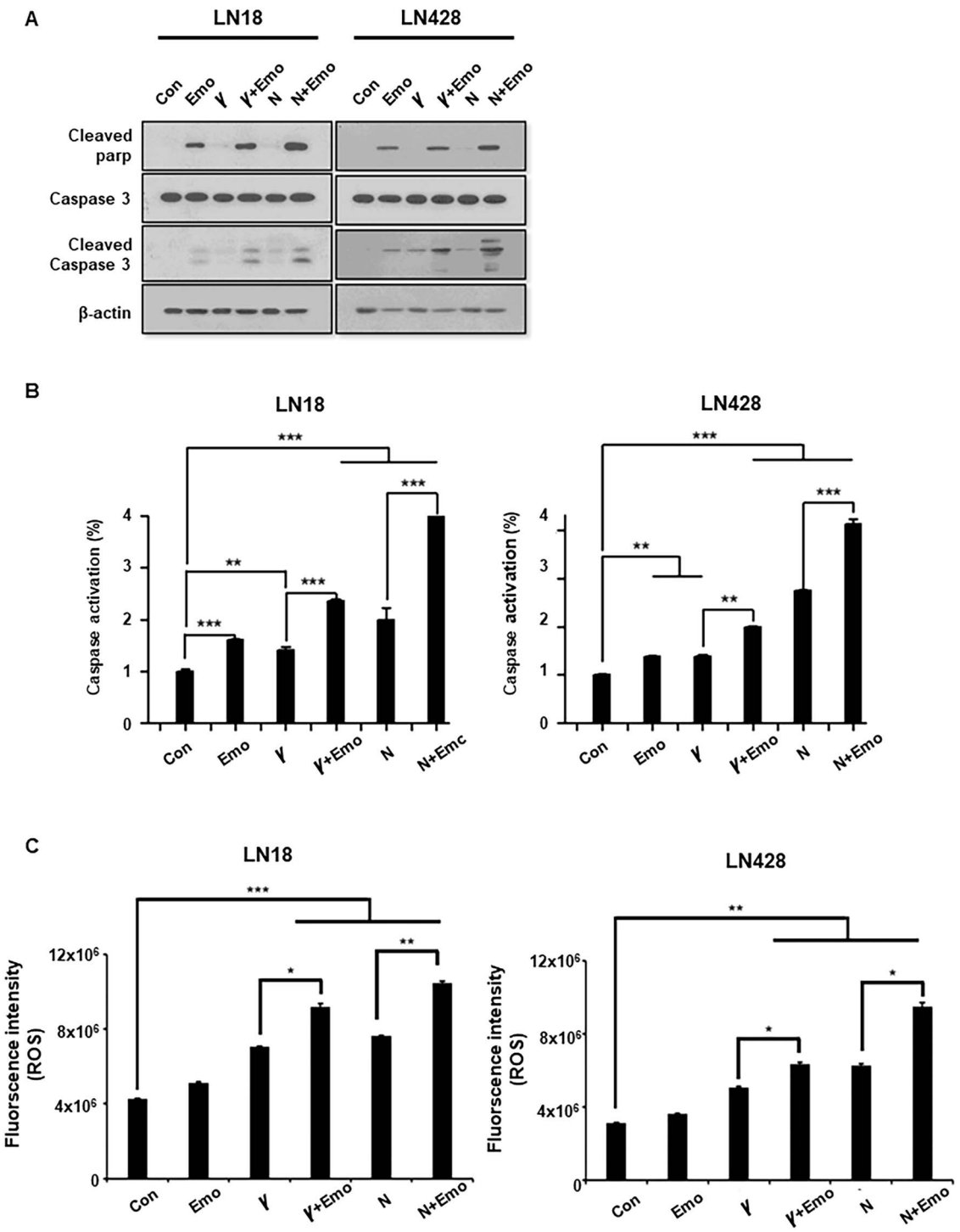

Fig. 3. Effects of emodin and neutron on ROS in GBM cells. (A) Cell lysates (30 μg) were immunoblotted (IB) with indicated antibodies. (B, C) Analysis of caspase3 activation and ROS generation in LN18 and LN428 cell line 24 h after treatment with emodin, neutron (6 GyE) or combination by a caspase 3 assay kit and ROS detection kit. Values are the means ± SD from three experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

[21]. Inhibition of lung metastasis of human breast cancer in a mouse xenograft model by emodin was found to be through the downregulation of MMP-2, MMP-9, uPA, and uPAR expression as well as decreased activity of p38 and ERK [22].

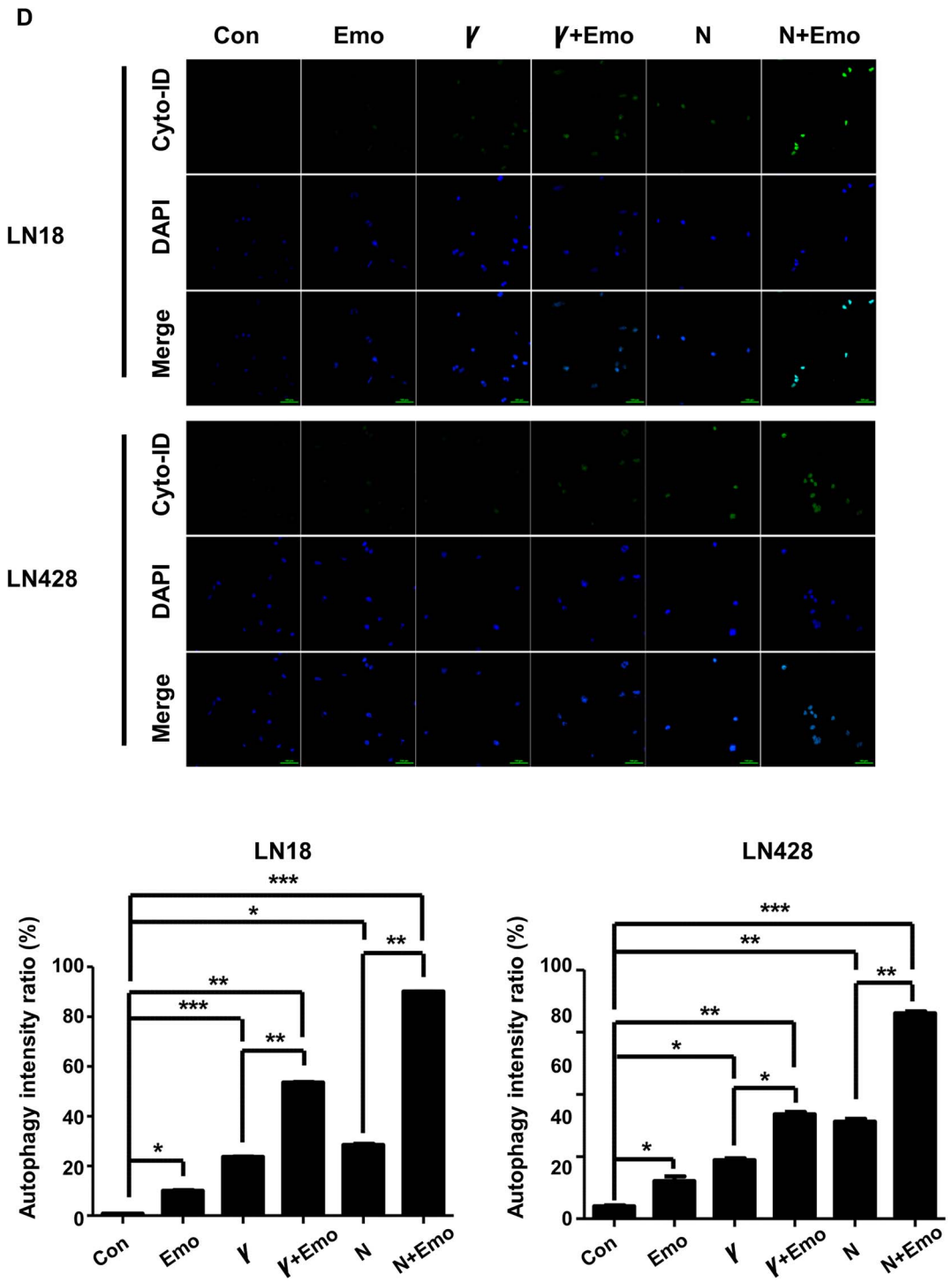

Fig. 4. Effects of emodin and radiation on autophagic cell death in GBM cells. (A) Cyto-ID staining of LN18 and LN428 cells with and without neutron beam (6 GyE) or with and without emodin treatment. Values are the means ± SD from three experiments; * *P* < 0.05, ** *P* < 0.01, * *P* < 0.001.**

Although the anticancer effect of emodin has been reported in numerous studies, its effect on enhancing the high LET radiosensitivity of cancer cells has not been investigated to date. Because of limitations in available facilities, radiation sensitivity enhancement by the drug was confirmed using a neutron beam as the high LET radiation, which has a relative biological effectiveness (RBE) similar to that of the carbon ion

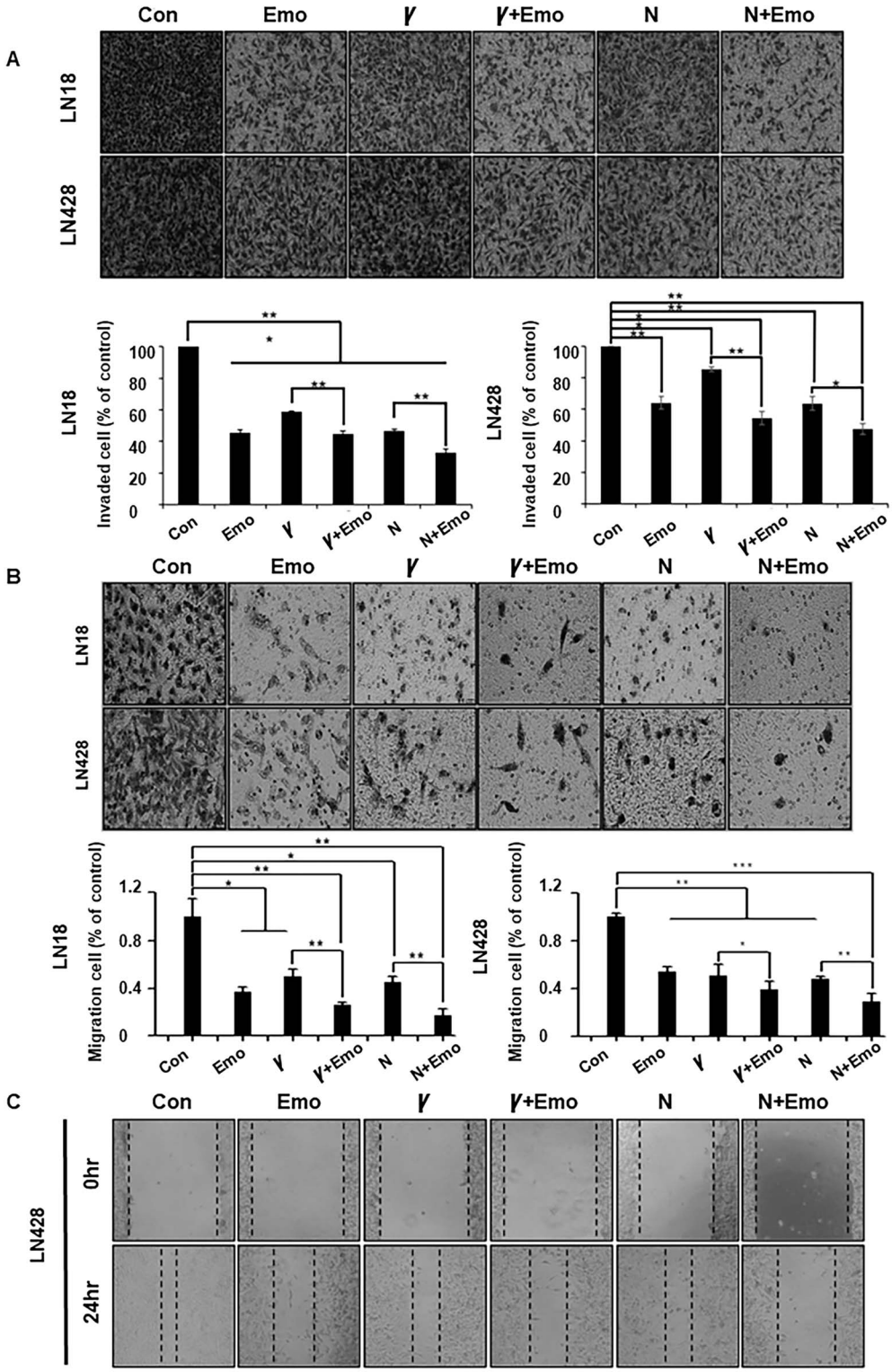

Fig. 5. Effect of combinatorial treatment with neutron beam and emodin on the invasiveness of glioblastoma cells. (A, B) Tumor cell invasion and migration after 24 hr treatment were examined by transwell chamber assays. The number of invading tumor cells that penetrated through the Matrigel and gelatin was counted using 5 high-intensity fields. Values represent the means of 3 experiments ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (C) GBM cells were treated with neutron (6 GyE) for 6 hr emodin treatment and then incubated for 24 h. Then, the cells were scraped with yellow pipette tips for the scratch assay.

beam mainly used in clinical practice [23]. The present study investigated the effect of emodin on the high LET radiosensitivity of GBM cells *in vitro* and examined its underlying mechanisms for radiosensitivity. In this study, we found that emodin significantly inhibits GBM cell proliferation in dose-dependent and time-dependent manners. This result is consistent with previous reports on inhibitory effects of emodin treatment in various human cancer cells such as hepatoma cells [24], skin cancers [25], gastric cancers [26], leukemic cells [27] and breast cancer cells [28]. Our study also demonstrated that the IC_{50} at 24 hours of emodin treatment in LN18 and LN428 cells was much higher as compared to other human cancer cells such as lung squamous cancers [29], liver cancers [24] and tongue squamous cancers [30]. It could be suggested that emodin may exert a cell-dependent type effect which is less sensitive against LN428 cells than other cells. Hence, it can be deduced that neutron radiation is a good type of radiation that can be used alongside emodin to slow down the brain cancer progression identified as gliomas. As a point of departure, this study has huge significance in the sense that it provides limelight for further research in this area because of the current demands in saving lives lost through various forms of cancer. In addition, emodin has several properties revolving around antiviral effects, antibacterial, antifungal, antiparasitic and antioxidant effects which imply that its application might be wider than expected hence the need to widen the scope of research in the future. An ideal radiosensitizer enhances the radiosensitivity of tumor cells and is harmless to or protects normal tissue. Previous studies demonstrated that emodin has no toxic effects on normal cells [31]. However, whether or not it protects normal tissue remains to be determined in this study. We plan to investigate the combined effect of boron neutron capture therapy using a thermal or epi-thermal neutron beam as well as a fast neutron beam from the viewpoint of treatment of brain tumor and reduction in damage to normal brain tissue. The exposure of cells to the combination of two cytotoxic modalities, emodin and neutron beam among radiation, demonstrated an increase in the cell death of GBM cells. This indicated that emodin may be an effective high LET radiosensitizer and a potential therapeutic agent for the inhibition of tumor cell proliferation and metastatic potential. The combination effect with drugs is smaller for fast neutrons and carbon-ions, which are high LET radiations, than for X-rays and gamma-rays, which are low LET radiations. In the case of high-LET radiation, the treatment alone has great efficacy; therefore, the rate of increase in radiation sensitivity when combined with drugs may be less than that of low-LET radiation. The sensitivity enhancement ratios of carboplatin were reported as 1.41 and 1.21 for X-ray and for carbon-ion beam irradiations, respectively, which was significantly higher for X-rays than for carbon-ion beams [32]. However, the sensitivity enhancement ratio of paclitaxel was 1.29 and 1.22 for X-ray carbon-ion beam irradiation, respectively, which was not significantly different. Metformin also enhanced the effects of ionizing radiation, including Co-60 γ-ray and 13 and 70 keV/μm carbon ion beams to induce clonogenic cell death, apoptosis, γH2AX formation, and inhibition of cell migration in HCC cells [33]. Thus, several studies have found a drug-dependent difference in the degree of increase in radiation sensitivity. Further *in vivo* studies are required to clarify the anticancer effect of emodin and its mechanisms with the intention of clinical application in detail. And emodin has not been widely used as an oncotherapeutic agent due to its poor bioavailability, high cytotoxicity at high concentrations and the occurrence of drug resistance. Thus, the search for an approach that could synergistically increase its bioactivity at lower concentrations with radiation is needed.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ETHICAL STATEMENT

Our study did not require ethical board approval because it did not involve human or animal trials.

ACKNOWLEDGMENTS

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