Effects of Far-infrared Ray on Temozolomide-treated Glioma in Rats

JIN-CHERNG CHEN[1,2] and JUEN-HAUR HWANG[2,3,4]

[1]*Department of Neurosurgery, Dalin Tzu Chi Hospital,*
Buddhist Tzu Chi Medical Foundation, Chiayi, Taiwan, R.O.C.;
[2]*School of Medicine, Tzu Chi University, Hualien, Taiwan, R.O.C.;*
[3]*Department of Otolaryngology-Head and Neck Surgery,*
Dalin Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Chiayi, Taiwan, R.O.C.;
[4]*Department of Medical Research, China Medical University Hospital,*
China Medical University, Taichung, Taiwan, R.O.C.

Abstract. *Background/Aim: Malignant glioma is a rapidly progressive primary brain cancer. The aim of the study was to investigate the effect of far-infrared ray (FIR) on temozolomide (TMZ)-treated glioma in rats. Materials and Methods: Male, 8-week old, Fischer 344 inbred rats with glioma were randomly divided into three study groups (20 rats in each group). The control group received saline only once daily for 5 days. The TMZ group received TMZ (30 mg/kg) once daily for 5 days. The TMZ plus FIR group received TMZ (30 mg/kg) once daily for 5 days and infrared-c irradiation of 40 min twice daily for 4 weeks. The relative tumor fold and the expression of hypoxia-induced factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) were compared using one-way ANOVA at the end of study. Results: The relative tumor fold of the TMZ+FIR group was significantly higher compared to the control group, and was borderline higher compared to the TMZ group at Day 7. The relative tumor fold of TMZ+FIR group was significantly higher compared to the control group and the TMZ group at Days 14, 21 and 28. HIF-1α expression of TMZ+FIR group was borderline higher compared to the control group at Day 28. The VEGF expression of TMZ+FIR group was significantly higher compared to the control group and the TMZ group at Day 28. Conclusion: FIR might increase the growth of glioma under TMZ treatment in rats possibly via increasing VEGF expression, but not HIF-1α expression.*

Malignant glioma is the most common primary brain cancer in adults. Despite many efforts, treatment of glioblastoma multiforme (GBM) remains one of the most challenging tasks. Poor cancer location, opportunistic deletion of tumor suppressor genes, amplification and/or mutational hyper-activation of receptor tyrosine kinase receptors, defects in the apoptosis signaling machinery, rapid growth rate, and poor response to current treatment modalities, might account for the poor prognosis of malignant glioma (1, 2).

The status of oxygenation in the tumor microenvironment may also affect the prognosis of cancer patients. Hypoxia is a major regulator of tumor development and aggressiveness. The two principal mediators of hypoxia response, hypoxia-induced factor-1 and -2 (HIF-1 and HIF-2), are known to have different responses to hypoxia. Stromal HIF-1 and HIF-2 have been reported to have opposing roles in breast cancer progression (3), while intralesional hypoxia near areas of necrosis with high levels of HIF-1 has been associated with poor prognosis in breast cancer (3). The most hypoxic and immature GBM cells are resistant to temozolomide (TMZ) due to high expression of HIF-1α and O6-methylguanine-DNA-methyltransferase (MGMT) (4). A previous study has shown that intermittent hypoxia increases tumor growth in a mouse model of sleep apnea, and circulating vascular endothelial growth factor (VEGF) appears to be a crucial mediator of tumor growth (5). Alveolar hypoxia promotes murine lung tumor growth through a VEGFR-2/epidermal growth factor receptor (EGFR)-dependent mechanism (6). Similarly, angiogenesis

Correspondence to: Juen-Haur Hwang, Department of Otolaryngology-Head and Neck Surgery, Dalin Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, No. 2 Minsheng Road, Dalin, Chiayi, 62247, Taiwan, R.O.C. Tel: +886 52648000, Fax: +886 52648006, e-mail: g120796@tzuchi.com.tw

is a significant feature of GBM (7). Exosomes derived from GBM cells grown at hypoxic compared to normoxic conditions are potent inducers of angiogenesis both *ex vivo* and *in vitro* through a phenotypic modulation of endothelial cells (8). Additionally, EGF activation of EGF receptor expressed on glioma cells leads to enhanced secretion of VEGF by glioma cells (9). VEGF has, thus, become a new target in the treatment of GBM (7, 10).

Taken together, it is reasonable to expect that interventions affecting glioma oxygenation status and/or VEGF expression might affect the prognosis of glioma in some aspects. Infrared-C (IR-C) radiation (usually referred to as far-infrared ray, FIR) is non-ionizing radiation with wavelengths of 3-1,000 μm in the infrared band as defined by the International Commission on Illumination (11). In the IR-C band, the FIR of 4-14 μm is also called "growth rays" due to its several advantageous effects in several organisms (12, 13). IR-C radiation can promote blood circulation, reduce oxidative stress and relieve pain, either by thermal or through non-thermal regulations in animals or humans (14, 15). Also, sleep disorders, high sympathetic tone and hypertension can be released by IR-C treatment (16).

Despite all the benefits, heat and FIR are not totally harmless on living tissues. FIR might be involved in both photo-aging and photo-carcinogenesis (17). With some contradictory biological effects, the impact of FIR on glioma still remains unknown. In this study, therefore, we aimed to evaluate the effects of applying FIR in TMZ-treated glioma in rats.

Materials and Methods

Cell line and cell culture. RT2 was kindly provided by National Taiwan University Hospital (Taipei, Taiwan, ROC). Cells were maintained in Dulbecco's Modified Eagle Medium (Gibco, Berlin, Germany) that was supplemented with L-glutamine, streptomycin, penicillin (Gibco, Berlin, Germany) and 10% fetal calf serum (Gibco, Berlin, Germany). All cells were cultured at 37°C in a 5% CO_2 incubator.

Animal care and study. The study protocol was approved by the Institutional Animal Care and Use Committee of the Dalin Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Chiayi, Taiwan, R.O.C. (1031003-2).

Male, 8-week old, Fischer 344 inbred rat were purchased from the National Laboratory Animal Center (Taipei, Taiwan, ROC). Food and water were provided *ad libitum* to the animals in standard cages. The rats were maintained in a daily cycle of 12-h period of light and darkness. Animals were monitored for morbidity or signs of distress throughout the study. Humane endpoints for these studies were based on weight loss (>10-15%) and clinical observations for behavior (decreased activity, hunched posture, shivering, labored breathing, moribund). We did not observe any adverse events during our studies, including signs of illness or mortality prior to the experimental endpoint.
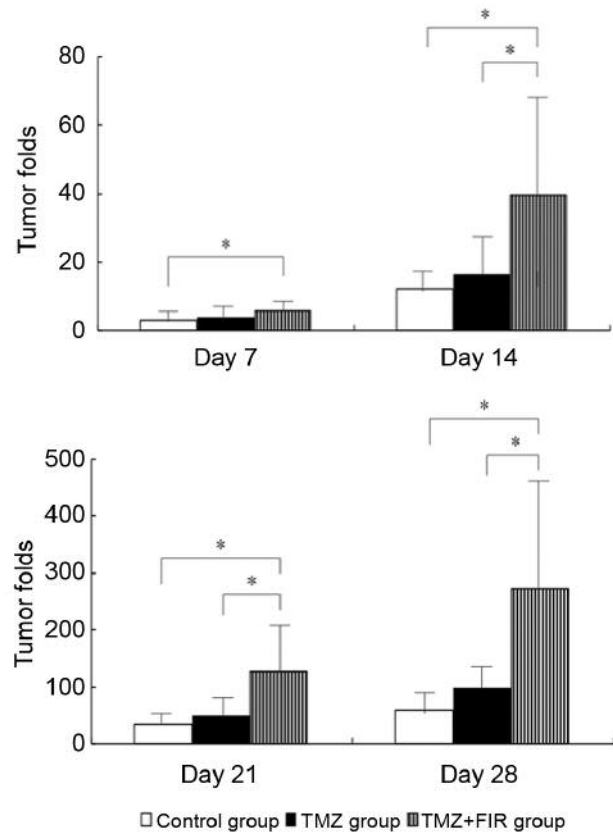

Figure 1. *Relative tumor folds each week in the three groups: i) control, ii) TMZ groupd, and iii) TMZ+FIR. The relative tumor fold of TMZ+FIR group was significantly higher compared to the control group, and was borderline higher compared to the TMZ group on Day 7 (p=0.013 and 0.086, respectively). The relative tumor fold of TMZ+FIR group was significantly higher compared to the control group and the TMZ group on Day 14 (p<0.001 and 0.001, respectively), on Day 21 (p<0.001 for both comparisons), and Day 28 (p<0.001 for both comparisons). TMZ: Temozolomide; FIR: far-infrared ray.*

Development of subcutaneous tumors. RT2 cells (1×10^7 cells in 500 μl per injection site) were suspended in phosphate-buffered saline (PBS). Rat were anesthetized by continuous flow of 3% isofluorane (2.5% LPM oxygen), and RT2 glioma cells were implanted into the subcutaneous space of right flank in Fischer 344 rats. Animals were observed for 45 to 60 min following tumor implantation, until they fully recovered.

Tumor growth was monitored using a digital caliper (H&H 161-506, Taiwan, ROC) every week. Tumor volume was estimated according to the following formula: Volume=0.5*(d1*d2*d3), in which d1, d2 and d3 are the three dimensions of the tumor, respectively (18).

Grouping and treatments. Two weeks later, rats with tumor volume greater than 62.5 mm³ were randomly and equally divided into three study groups (20 rats in each group). Rats were treated by oral instillation of saline or TMZ, and/or IR-C irradiation (Far
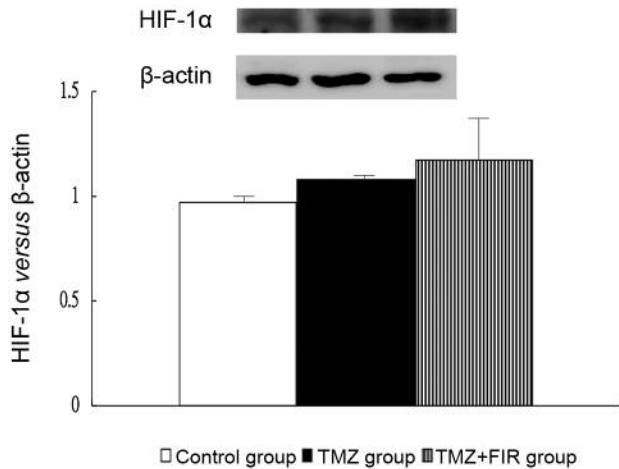
Figure 2. *Results of western blotting of HIF-1α in the three groups: i) control, ii) TMZ groupd, and iii) TMZ + FIR at the end of the study. The HIF-1α expression of TMZ+FIR group was borderline higher compared to the control group on Day 28. The HIF-1α expression of TMZ group was not significantly higher compared to the control group on Day 28. TMZ: Temozolomide; FIR: far-infrared ray.*
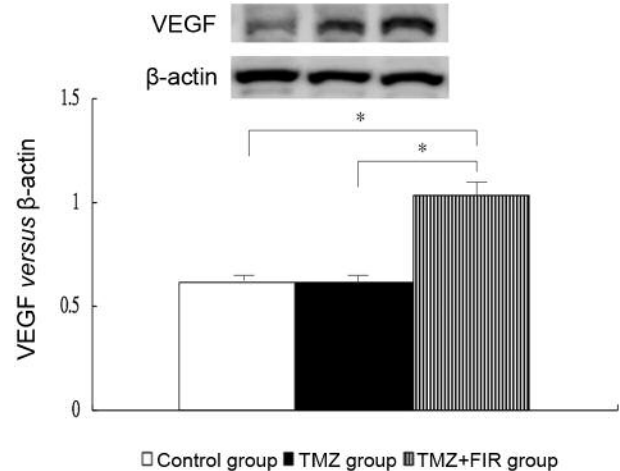

Figure 3. *Results of western blotting of VEGF in the three groups: i) control, ii) TMZ groupd, and iii) TMZ+FIR at the end of the study. The VEGF expression of TMZ+FIR group was significantly higher compared to the control group and the TMZ group on Day 28 (p=0.001 for both comparisons). The VEGF expression of TMZ group was not significantly higher compared to the control group on Day 28. TMZ: Temozolomide; FIR: far-infrared ray.*

Infrared Physiatric Unit TY-101F, WS FAR IR MEDICAL TECHNOLOGY CO., LTD). The control group received only saline once daily for 5 days. The TMZ group received TMZ (30 mg/kg) once daily for 5 days. The TMZ plus FIR group received TMZ (30 mg/kg) once daily for 5 days and IR-C irradiation of 40 min twice daily for 4 weeks.

The relative tumor fold was calculated by the tumor volume measured each week compared to that at the start of this study (18).

Western blotting analysis. All rats were sacrificed by inhalation of 5% isoflurane followed by decapitation at the end of the study (4 weeks after start of this study and 6 weeks following the injection of RT2 cells). Tumor masses were collected and snap-frozen in liquid nitrogen and were stored at −80˚C until analysis.

Sections of the tumor masses were washed in PBS, and broken into small tumor pieces using scissors in 0.2 ml T-PER tissue protein extraction reagent (T-PER™ Tissue Protein Extraction Reagent, Thermo Fisher Scientific Inc., USA), according to the manufacturer's instructions.

The protein concentration was determined using the BCA protein quantitative method (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific Inc., USA). After SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) protein electrophoresis, proteins were transferred to a PVDF (polyvinylidene fluoride) membrane. Then, 1× Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5% skim milk was added to block the membrane for 1 hour. Membranes were washed three times in TBS-T for ten min each. Subsequently, primary antibodies of HIF-1α (1:1,000, Abcam plc, UK), VEGF (1:5,000, Merck Millipore, USA) and β-actin (1:5,000, Sigma-Aldrich, USA), were diluted in TBS-T containing 5% non-fat dry milk, were added to the membrane and were incubated overnight at 4˚C. Membranes were washed three

times in TBS-T. Subsequently, horseradish peroxidase (HPR)-conjugated secondary antibodies in TBS-T with 5% skim milk were added to the membrane and were incubated for 1 hour. After incubation, Membranes were washed with TBS-T. The film was finally developed using enhanced chemiluminescence (ECL) (WesternBright ECL HRP substrate, Advansta Inc, USA). Membranes were visualized and analysed with an Odyssey® Fc Imaging System and Image Studio™ Software and organization and with Empiria Studio™ Software for analysis (LI-COR Biosciences, USA). The internal control was β-actin.

Statistical analysis. The relative tumor folds, and protein expression of HIF-1α and VEGF relative to that of β-actin, were compared between the three groups using one-way analysis of variance (ANOVA) with a *post-hoc* Bonferroni correction. All analyses were performed using the commercial software Stata (StataCorp LLC, College Station, TX, USA), and *p*-Values<0.05 were considered statistically significant.

Results

The general condition and body weight in all three groups were similar before and after the experiment. No animal in the three groups expired or experiences adverse effects during any of the experimental procedures (20 rats survived in each group).

Figure 1 shows the relative tumor folds during each week in the three groups. The relative tumor fold of TMZ+FIR group was significantly higher compared to the control group, and borderline higher compared to the TMZ group

at Day 7 (one-way ANOVA, $p=0.0117$ and *post-hoc* Bonferroni correction, $p=0.013$ and 0.086, respectively). The relative tumor fold of TMZ+FIR group was significantly higher compared to the control group and the TMZ group at Day 14 (one-way ANOVA, $p<0.0001$ and *post-hoc* Bonferroni correction, $p<0.001$ and 0.001, respectively), at Day 21 (one-way ANOVA, $p<0.0001$ and *post-hoc* Bonferroni correction, $p<0.001$ and <0.001, respectively), and at Day 28 (one-way ANOVA, $p<0.0001$ and *post-hoc* Bonferroni correction, $p<0.001$ and <0.001, respectively).

Figure 2 shows the results of western blotting of HIF-1α in the three groups at the end of study. The HIF-1α expression in the TMZ+FIR group was borderline higher compared to the control group at Day 28 (one-way ANOVA, $p=0.0821$ and *post-hoc* Bonferroni correction, $p=0.096$), while for the TMZ group HIF-1 expression was not significantly higher compared to the control group at Day 28 (*post-hoc* Bonferroni correction, $p=0.474$).

Figure 3 shows the results of the western blotting of VEGF in the three groups at the end of study. The VEGF expression in the TMZ+FIR group was significantly higher compared to the control and the TMZ groups at Day 28 (one-way ANOVA, $p=0.0006$ and *post-hoc* Bonferroni correction, $p=0.001$ and $p=0.001$, respectively), however, the VEGF expression in the TMZ group was not significantly higher compared to the control group at Day 28 (*post-hoc* Bonferroni correction, $p=1.000$).

Discussion

This experimental study shows that FIR might increase the growth of glioma under TMZ treatment and that this might be associated with increasing VEGF expression, but not with HIF-1α expression. In other words, FIR was harmful in glioma with TMZ treatment possibly via increasing VEGF expression in the lesions.

Rapid cellular proliferation, hypervascularity, cerebral edema, and focal necrosis are key histopathologic features of GBM (9). The most hypoxic and immature GBM cells are resistant to TMZ due to the high expression of HIF-1α and MGMT (4). An intratumoral hypoxic gradient drives stem cell distribution and MGMT expression in glioblastoma (19). Conversely, down-modulating the HIF-1α/MGMT axis might increase GBM responsiveness to chemotherapy (4). Also, glioblastoma stem-like cells secrete the pro-angiogenic VEGF-A factor in extracellular vesicles, which carry essential information that can adapt the microenvironment to the tumor's needs, inducing tumour-associated angiogenesis (20). VEGF can increase cell motility, but it does not affect cell proliferation in glioma cell lines (10). Overexpression of VEGF isoforms have been shown to drive vascularization, oxygenation, and growth but not progression to GBM in a human model of gliomagenesis (21).

Studies on phototherapy and/or irradiation in glioma are limited and diverse. Irradiation has been shown to increase cell motility, but it does not affect cell proliferation in glioma cell lines (10). Radiation-induced VEGF can also increase cell motility of glioma *in vitro* (22). On the contrary, photothermal ablation therapy can significantly prolong the survival of tumor-bearing mice in an orthotopic mouse xenograft model of glioma (23). Near-infrared light can trigger release of Paclitaxel from biodegradable microspheres and enhance its antitumoral activity via a photothermal effect (24). PEGylated gold nanorods and near-infrared stimulation have been shown to trigger release of doxorubicin from thermosensitive liposomes in a mouse tumor model of human glioblastoma (25), while nanoshell-mediated photothermal therapy with a near infrared laser, has improved survival in a murine glioma model (26). Collectively, it seems that phototherapy and/or irradiation itself may be harmful for cancer control, but it could enhance the effects of chemotherapy on glioma.

Concerning FIR therapy, previous studies have shown that it could increase temperature in the body tissues, elevate motility of body fluids (27), and exert a nitric oxide (NO)-related biological effect to increase skin microcirculation in rats (27, 28). In addition, FIR may reduce the growth of some cancer cells *in vitro* with a low level of heat shock protein 70 (29). However, FIR might also be involved in both photo-aging and photo-carcinogenesis (17).

In our study we found that FIR can enhance the growth of glioma when using TMZ therapy possibly *via* increased VEGF expression. These contradictory results might be due to different cancer types, protocols of IR-C irradiation, and/or combinations of other therapy. Even though there are groups claiming that FIR may be helpful for glioma control (30) clinicians should recommend the use of FIR for glioma treatment with caution. We suggest a large-scale clinical trial on this issue in the future.

Conflicts of Interest

Authors' Contributions

JCC prepared the manuscript and coordinated the study. JCC carried out the experiments. Both JCC and JHH were involved in designing the experiments. All authors read and approved the final manuscript.

Acknowledgements

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