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Original Article

The combination of tumor treating fields and hyperthermia has synergistic therapeutic effects in glioblastoma cells by downregulating STAT3

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Abstract: Glioblastoma multiforme (GBM), the most common type of brain tumor, is a very aggressive and treatment-refractory cancer, with a 5-year survival rate of approximately 5%. Hyperthermia (HT) and tumor treating fields (TTF) therapy have been used to treat cancer, either alone or in combination with other treatment methods. Both treatments have been reported to increase the efficacy of other treatment techniques and to improve patient prognosis. The present study evaluated the therapeutic effects of combining HT and TTF on GBM cell lines. Cells were subjected to HT, TTF, HT+TTF, or neither treatment, followed by comparisons of cell proliferation, apoptosis, migration and invasiveness. Clonogenic assays showed that the two treatments had a synergistic effect. The levels of cleaved PARP and cleaved caspase-3 were higher and apoptosis was increased in cells treated with HT+TTF than in cells treated with HT or TTF alone. In addition, HT+TTF showed greater inhibition of GBM cell migration and invasiveness and greater downregulation of STAT3 than either HT or TTF alone. The stronger anticancer effect of HT+TTF suggested that this combination treatment can increase the survival rate of patients with difficult-to-treat cancers such as GBM.

Keywords: Tumor treating fields, hyperthermia, combined therapy, glioblastoma, cancer

Introduction

Glioblastoma multiforme (GBM), the most common type of central nervous system tumor, is a very aggressive and treatment-refractory cancer, with more than 50% of patients dying within one year after diagnosis and a 5-year survival rate of approximately 5% [1, 2]. A newly developed treatment technique, called tumor treating fields (TTF), uses alternating electric fields with a frequency of 100-300 kHz and an intensity <3 V/cm and has been approved by the U.S. Food and Drug Administration (FDA) to treat patients with GBM [3-5]. This treatment technique has been reported to prevent spindle formation during cell division, and dielectrophoresis has been found to inhibit cell division when cleavage furrows are formed [3, 6, 7]. A phase 3 clinical trial in patients with GBM showed that the combination of TTF and temozolomide (TMZ) prolonged both progression-free survival (PFS) and overall survival (OS) [4]. In addition, TTF, which involves attaching an electrode to the patient's skin near the tumor and applying an alternating electric field, is non-invasive and has fewer side effects than surgery or chemotherapy [7, 8]. At present, TTF is regarded as a secondary treatment supplementing other modalities, such as anticancer drugs, rather than being considered a primary treatment mode.

Hyperthermia therapy (HT) is a treatment that kills cancer cells by heating and has been reported to have therapeutic effects on various types of cancer, including melanoma and tumors of the head and neck, breast, brain, bladder, cervix, rectum, lung, and esophagus [9, 10]. This treatment is used alone or in combination with chemotherapy or radiation thera-

py [9-11]. The combination of HT and radiation therapy has been reported to have survival benefits in patients with GBM [12]. In addition, HT was found to inhibit proliferation and induce apoptosis in glioma cells [13-15], suggesting that HT may contribute to the treatment of patients with GBM.

Signal transducer and activator of transcription 3 (STAT3) is a latent cytoplasmic transcription factor shown to be constitutively activated in a variety of cancers, including hematological malignancies and solid tumors [16, 17]. This aberrant activation of STAT3 appears to inhibit apoptosis in cancer cells and to induce cell proliferation, invasion, metastasis, and angiogenesis [17]. Targeting the STAT3 signaling pathway may therefore be an efficient therapeutic approach for a variety of cancers. HT has been reported to inhibit proliferation and induce apoptosis of rat glioma cells through STAT3 signaling [18]. In addition, TTF treatment was found to downregulate STAT3 in GBM both in vitro and in vivo [19]. Therefore, the purpose of this study was to evaluate the synergistic effect of the combination of HT and TTF in glioma cells and to determine whether these agents target the STAT3 signaling pathway.

Results

Effects of TTF or HT alone

Before combining TTF and HT, the efficacy of each treatment alone was evaluated. The ability of TTF to inhibit cell proliferation was evaluated in U373 and A172 GBM cell lines. The therapeutic effects of TTF have been reported to depend on several conditions, including the intensity, frequency, and duration of the alternating electric fields [3, 20]. The duration of TTF treatment has been shown to be proportional to tumor growth inhibition [20], although increased treatment time was also shown to reduce treatment efficiency. Therefore, to provide more efficient TTF treatment, especially when combined with HT, U373 and A172 cells were treated with TTF for 2 h/day for 2 or 3 days. Relative to control, untreated cells, the numbers of cells decreased with increasing treatment time (**Figure 1A**). When the treatment time was fixed at 2 h/day for 2 days, an increase in electric field intensity from 0 to 1.1 V/cm resulted in a gradual reduction in GBM cell growth (**Figure 1B**).

To assess the effects of HT alone, both GBM cell lines were incubated at temperatures of 39 to 43°C for 15 or 30 min, followed by incubation at 37°C for 2 or 3 days, and the numbers of cells relative to the control group were calculated. The numbers of U373 and A172 GBM cells decreased with increasing temperature and heating time (**Figure 1C**).

Effects of combined treatment

Although HT is used alone to treat cancer, it is mainly used in combination with other treatments, such as chemotherapy or radiation therapy. TTF has also been found to prolong survival in cancer patients when combined with chemotherapy [4, 21-23]. To evaluate the effect of this combination, U373 and A172 GBM cells were treated with HT (41°C for 30 min/day), TTF (1.1 V/cm for 2 h/day), both, or neither, and the numbers of cells were measured. The numbers of cells were lower after treatment with HT plus TTF than with either alone (**Figure 2A**). In addition, the MTT assay also showed that TTF+HT inhibited the growth of GBM cells more than either TTF or HT alone (**Figure 2B**). The proliferation of GBM cells after treatment was assessed by clonogenic assays, which found that combined treatment with TTF and HT inhibited the proliferation of both GBM cell lines more than TTF or HT alone, suggesting that TTF sensitizes cells to HT treatment (**Figure 2C**).

Effects of HT and TTF on apoptosis

HT has been reported to induce apoptosis in many types of cancer by activating the intrinsic apoptotic pathway [24]. In addition, TTF has been shown to induce apoptosis in cancers, both in vitro and in vivo [25, 26], suggesting that the induction of apoptosis by HT+TTF may be greater than that by either alone. To assess apoptosis after HT and/or TTF treatment, the protein levels of the apoptosis markers cleaved PARP and cleaved caspase 3 in U373 and A172 GBM cells were analyzed by western blotting. The expression of these apoptotic markers was greater in cells treated with both HT and TTF than in cells treated with HT or TTF alone (**Figure 3A**). To confirm that HT+TTF increased apoptosis, the expression of annexin V, another marker of apoptosis, was analyzed by flow cytometry. Although both HT and TTF alone increased apoptosis in U373 and A172

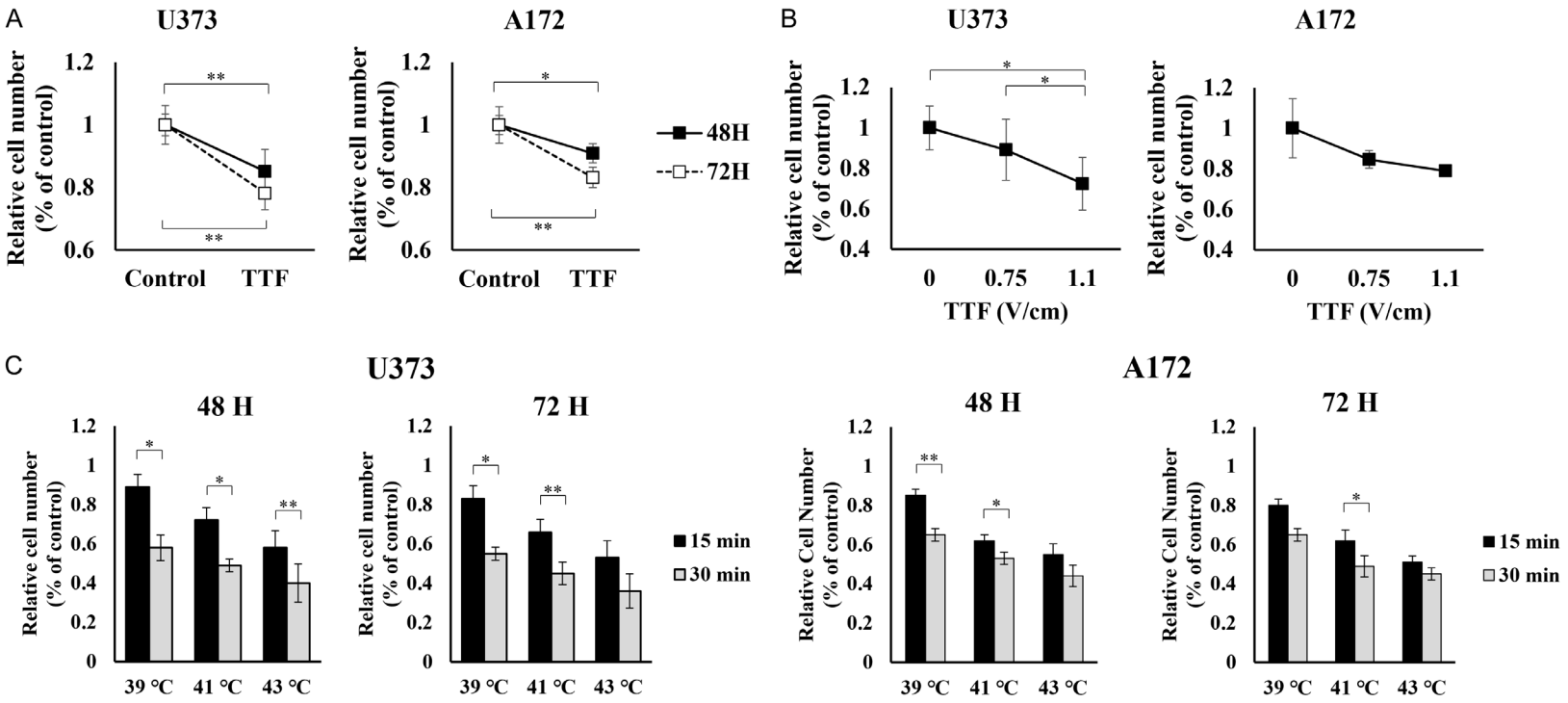
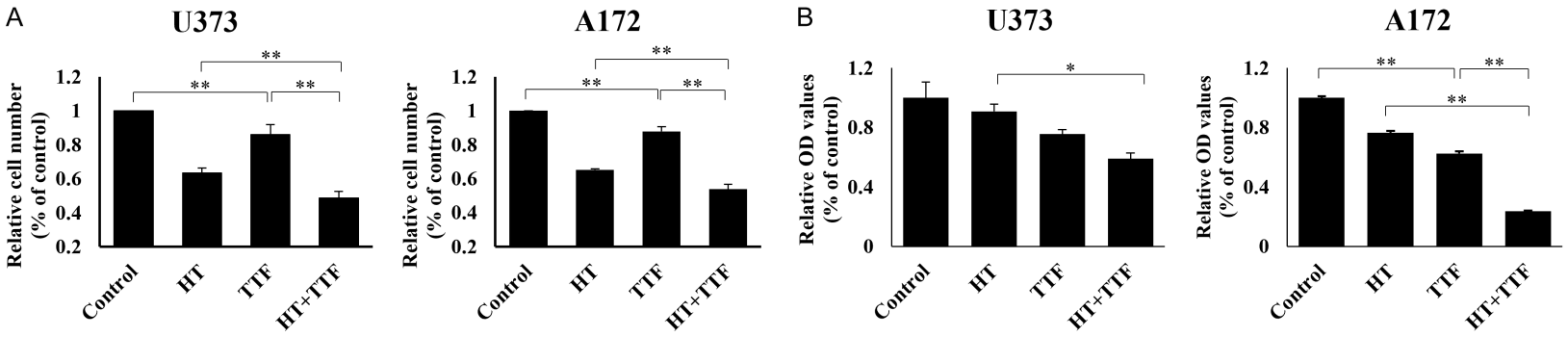


Figure 1. A. TTF inhibited GBM cell viability in a time- and intensity-dependent manner. Cell counts using 0.4% Trypan Blue staining confirmed the time dependence of TTF effects for 48 and 72 hours. B. Cell counts using 0.4% Trypan Blue staining confirmed the intensity dependence of TTF effects at 0.75 and 1.1 V/cm. C. HT inhibited GBM cell viability in a temperature- and time-dependent manner. Cells were incubated for 48 or 72 min after 15 or 30 min of heating to 39, 41 and 43 °C, respectively, and the effects were confirmed by cell counts. The values represent the means ± SD for 3 experiments. *, P<0.05; **, P<0.01.



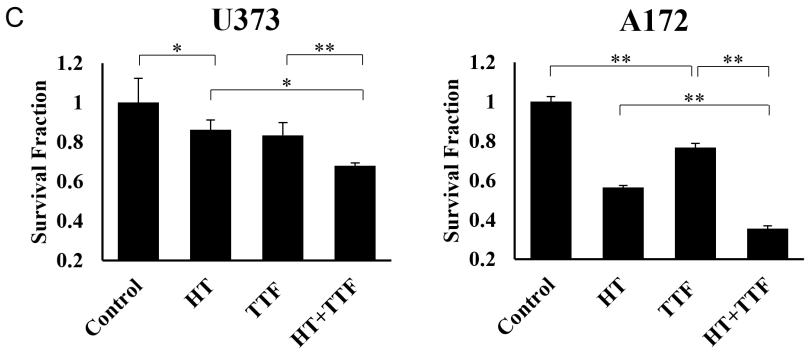


Figure 2. The viability of cells treated with a combination of HT and TTF was significantly lower than that of cells treated with HT or TTF alone. Effects of HT, TTF, or both on (A) cell counts and (B) MTT assay after treatment. (C) Effects of HT, TTF, or both on the survival of U373 and A172 GBM cells in a clonogenic assay. Cells were treated with or without TTF (1.1 V/cm and 150 kHz) and immediately heated at 41°C for 30 mins. The values represent the means ± SD for 3 experiments. *, P<0.05; **, P<0.01.

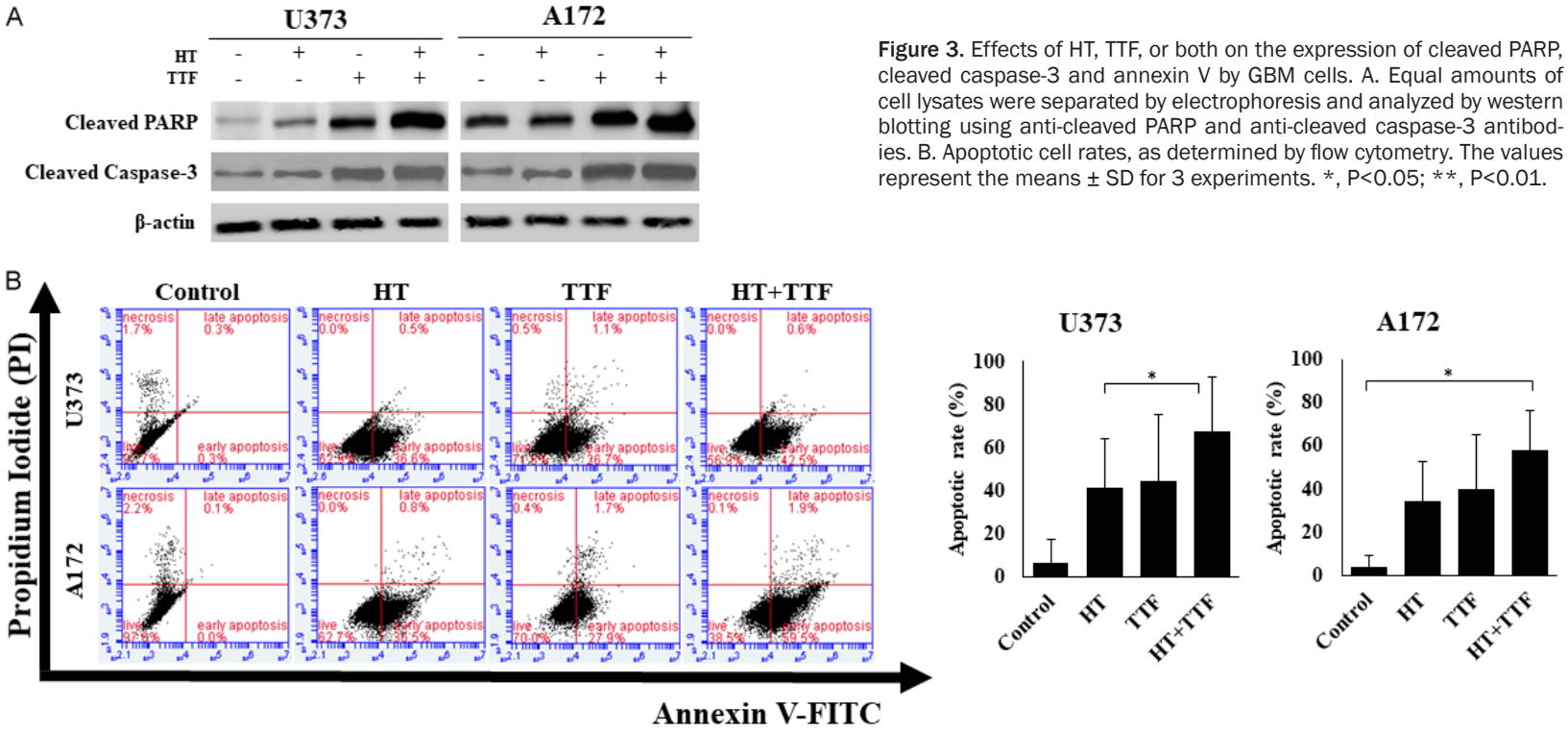


Figure 3. Effects of HT, TTF, or both on the expression of cleaved PARP, cleaved caspase-3 and annexin V by GBM cells. A. Equal amounts of cell lysates were separated by electrophoresis and analyzed by western blotting using anti-cleaved PARP and anti-cleaved caspase-3 antibodies. B. Apoptotic cell rates, as determined by flow cytometry. The values represent the means ± SD for 3 experiments. *, P<0.05; **, P<0.01.

GBM cells, the increase was higher in cells treated with both HT and TTF (**Figure 3B**).

Effects of HT and TTF on metastasis

Because metastases are responsible for approximately 10% of cancer-related deaths [27], preventing metastasis can have a profound effect on the survival rate of cancer patients. To metastasize, tumors must go through several steps, including migration and invasion [28]. As TTF has been reported to inhibit metastasis [29], the effects of TTF and HT on the migration and invasiveness of U373 and A172 cells were evaluated. Matrigel invasion assays showed that HT+TTF inhibited the invasiveness of U373 and A172 cells more than either HT or TTF alone (**Figure 4A**). Transwell chamber assays showed that the combination of HT and TTF inhibited cell migration more than HT or TTF alone (**Figure 4B**). Scratch assays also demonstrated that HT and TTF each inhibited cell migration, with the greatest inhibition shown by the combination of HT and TTF (**Figure 4C**).

Tumor metastasis requires cancer cells to dissociate from the surrounding extracellular matrix (ECM) and to become mobile and migrate from the primary site, a process requiring epithelial-mesenchymal transition (EMT) [30]. The effects of HT and/or TTF on EMT in GBM cells were assessed by assaying the expression of vimentin, a mesenchymal marker. Western blotting showed that treatment of both cell lines with HT+TTF significantly downregulated the expression of vimentin compared with HT or TTF alone (**Figure 4D**). Furthermore, when the expression level of STAT3 was evaluated, it was demonstrated that HT+TTF induced the downregulation of STAT3 and phosphorylated STAT3 (**Figure 4D**). The results of the combined treatment of HT and TTF, which inhibited tumor proliferation, induced apoptosis, and interfered with migration, invasion, and EMT, indicate that these therapeutic effects are due to the upstream signal, STAT3.

Discussion

GBM is the most aggressive malignant brain tumor; patients diagnosed with GBM have a 5-year overall survival rate of approximately 5% [31]. National Comprehensive Cancer Network (NCCN) guidelines recommend maximal safe resection, followed by treatment with TMZ plus radiotherapy or TMZ plus TTF [32]. TTF has been shown to increase the median OS of patients with GBM. For example, a phase 3 clinical trial reported that the median OS was longer in patients treated with TTF plus TMZ (20.9 months) than in patients treated with TMZ alone (16.0 months) [8]. More effective therapies are needed, including both new agents and the optimization of existing treatments. To our knowledge, this study is the first to show that the combination of TTF and HT was more effective than either alone in the treatment of GBM cells.

HT, which has been shown to be effective in treating a wide variety of cancers, including GBM, increases the therapeutic effects of other cancer treatment modalities [33, 34]. For example, HT has been found to increase the radiation sensitivity of radiation-resistant solid tumors by increasing oxygen delivery to hypoxic regions [35]. In addition, combinations of HT with radiation therapy and immunotherapy have been reported to inhibit the growth of cancers refractory to radiation therapy and immunotherapy [36]. The present study showed that HT can also enhance the therapeutic effect of TTF, a treatment using an alternating electric field. Similar to HT, TTF has been reported to be applicable to a wide range of tumor types, such as GBM [8], non-small-cell lung cancer [37-39], pancreatic cancer [25, 40], ovarian cancer [41], mesothelioma [42], liver cancer [43] and gastric cancer [44], suggesting that TTF may synergize with other treatment modalities in other types of cancer.

Metastasis is a major factor involved in worsening the prognosis of cancer patients, with prevention of metastasis being an important goal in cancer treatment [45]. GBMs rarely metastasize outside the central nervous system (CNS), perhaps due to the short survival time of GBM patients and the inability of cells to cross the blood-brain barrier (BBB) [46, 47]. However, GBM has been reported to metastasize through systemic lymphatic vessels [48]. Prolongation of OS in patients with GBM would therefore include inhibition of metastasis. The present study found that the combination of TTF and HT inhibited some of the steps required for tumor metastasis, such as cell invasion and migration, by targeting STAT3 signaling in GBM cells. Taken together, the results of the present study suggest that the combination of TTF and

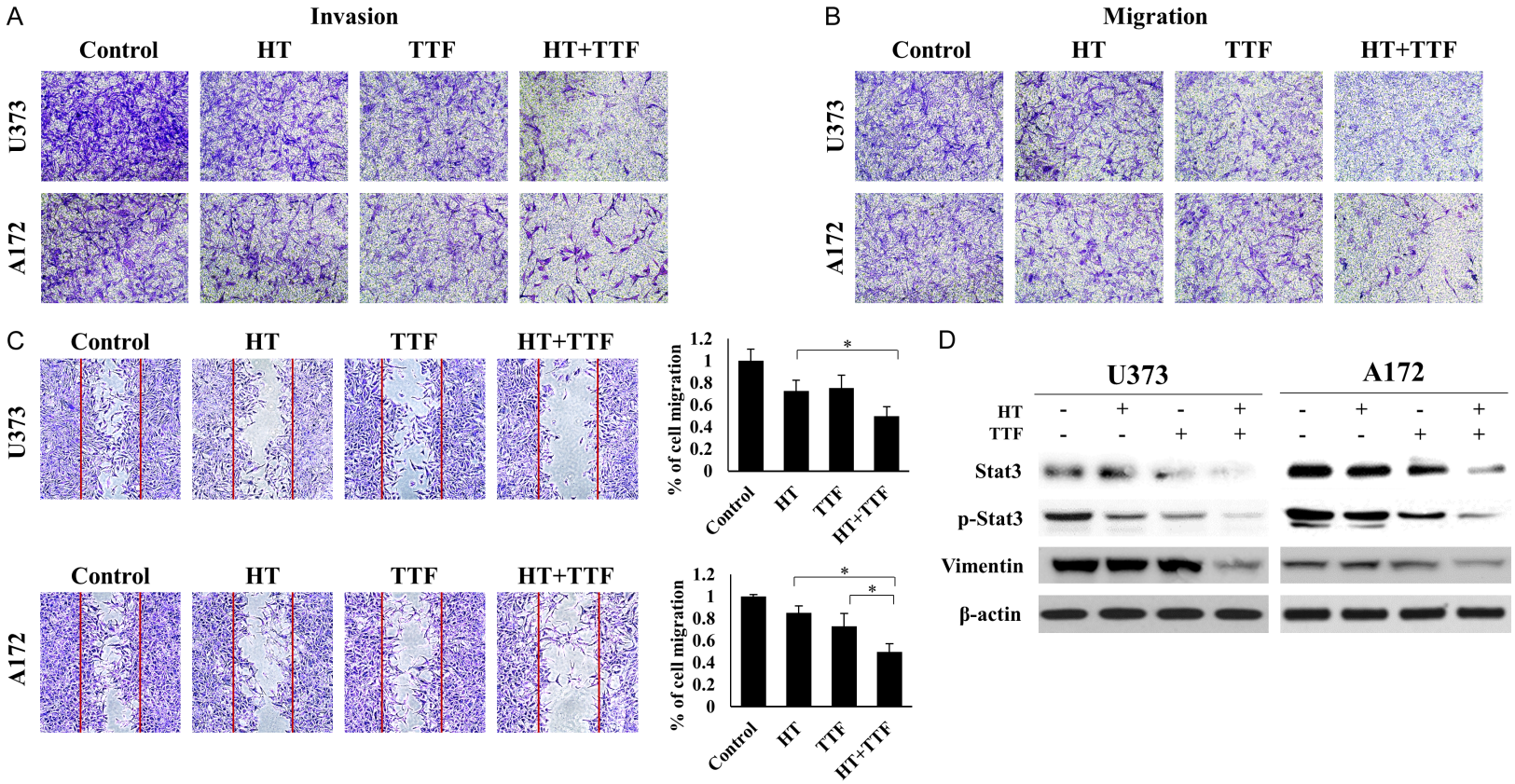


Figure 4. Effect of HT, TTF, or both on the invasion and migration of GBM cells. (A) Tumor cell invasion was assessed using the Matrigel invasion assay. (B, C) Tumor cell migration was assessed using the (B) Transwell chamber assay and (C) scratch assay. The values represent the means ± SD for 3 experiments. *, $p<0.05$; **, $p<0.01$. (D) Equal amounts of cell lysates were separated by electrophoresis and analyzed by western blotting using anti-STAT3, anti-phospho-STAT3 and anti-vimentin antibodies.

HT may improve OS in patients diagnosed with GBM.

The present study is the first to assess the in vitro effects of TTF and HT on GBM cells. Moreover, the ability of TTF and HT to inhibit cell proliferation and metastasis suggests that these methods act by downregulating STAT3. STAT3 is constitutively activated in several types of cancer and affects cell proliferation, differentiation, apoptosis, and metastasis [49]. In addition, STAT3 affects the tumor microenvironment, and activation of STAT3 has been shown to recruit immune cells and negatively modulate the activity of their immune-stimulating molecules [16, 50]. Therefore, STAT3 inhibitors, such as HT plus TTF, can enhance anticancer activity by reducing the immunosuppressive response.

The present study, however, did not investigate the mechanisms of action of TTF and HT, other than STAT3, indicating a need for additional in-depth studies. In addition, it will be necessary to evaluate whether HT+TTF treatment can be applied to other types of cancer with a high risk of metastasis. Furthermore, to more definitively demonstrate the synergistic effect, studies using animal models should be conducted to evaluate the in vivo activity of TTF and HT combination therapy.

In conclusion, this study showed that the combination of HT and TTF had synergistic therapeutic effects on GBM cells. Combination treatment was more effective than either modality alone in inducing cell apoptosis and inhibiting cell migration and invasion, suggesting that HT+TTF may improve the prognosis of patients with GBM. Because HT and TTF are currently used to treat patients and are not new treatment methods, demonstration of the in vivo effectiveness of HT+TTF treatment in patients may result in their more rapid clinical use.

Materials & methods

Experimental setup for TTF and HT

TTF was generated with a pair of insulated wires (Seoil Electric Wire Co., Ltd.; outer diameter, 0.4 mm; polyvinyl chloride insulation thickness, 0.17 mm; dielectric breakdown, 25 kV/mm) connected to a function generator (AFG-2112, Good Will Instrument Co., Ltd., Taiwan) and a high-voltage amplifier (A303, A. A. Lab Systems Ltd., Israel) that generated sine-wave signals ranging from 0-800 V [51]. To apply TTF to cell lines, a pair of insulated wires was attached to the bottom of each cell dish, 3 cm from each other. An electric field was applied for 2 h/day for 2 days at an intensity of 0.75-1.1 V/cm and a frequency of 150 kHz.

HT was performed using a water bath whose temperature was controlled with an accuracy of 0.1°C, and the temperature of the medium was confirmed by measuring it with a thermocouple. It took tens of seconds for the medium in the dishes in a 37°C incubator to increase to the set temperature (39-43°C) in the water bath, depending on the treatment conditions. After 30-60 minutes of HT, when the dishes were transferred back to the incubator, the temperature of the medium dropped to 37°C within minutes (39°C: 1 m 25 s, 41°C: 3 m 25 s, and 43°C: 6 m 38 s).

Antibodies and chemicals

Antibodies against cleaved poly (ADP-ribose) polymerase (PARP), cleaved caspase 3, STAT3, phospho-STAT3, vimentin and beta actin were obtained from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

Human glioblastoma U373 and A172 cell lines were purchased from the Korean Cell Line Bank (Seoul, South Korea) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), glutamine, HEPES, and antibiotics at 37°C in a humidified incubator with an atmosphere containing 5% CO_2.

Cell viability assay

Cells were seeded at a density of 5,000 cells per well in a 96-well plate and incubated for 24 h. For quantification of cell viability, an equal volume of culture medium containing EZ-Cytox reagent (EZ3000, Daeillab Service) was added to the cells. The cell viability was then measured using a microplate reader (PHOmo, autobio labtec instruments) at 450 nm.

Colony formation assay

Cells were subjected to TTF and/or HT and incubated for 14-20 d; the resulting colonies were stained with 0.4% crystal violet (Sigma, St.

Louis, MO, USA). The plating efficiency (PE) was defined as the percentage of seeded cells that formed colonies under specific culture conditions. The surviving fraction, expressed as a function of irradiation, was calculated as colonies counted/(cells seeded × PE/100).

Flow cytometry

Cells were stained for propidium iodide (PI) and annexin V in accordance with the manufacturer's protocol and fractionated on a FACSAria flow cytometer (BD). A minimum of 10,000 cells were counted for each sample.

Western blotting

After treatment, GBM cells were lysed with RIPA buffer, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. The membranes were blocked with 1% (v/v) nonfat dried milk in Tris-buffered saline with 0.05% Tween 20 and incubated with the appropriate antibodies. Primary antibodies were diluted 1:1000, and secondary antibodies were diluted 1:5000. Immunoreactive protein bands were visualized using a Fluorchem E system.

Invasion/migration assay

Invasiveness was measured in vitro using Transwell chambers in accordance with the manufacturer's protocol. Briefly, 4×10^5 cells/mL in 150 μL of DMEM were seeded onto the membrane of the upper chamber of the Transwell and treated with TTF, HT, a combination of both, or neither. The medium in the upper chamber was serum-free, whereas the medium in the lower chamber contained 10% FBS as a source of chemoattractant. Cells that passed through the Matrigel or gelatin-coated membrane were stained with Cell Stain solution containing Crystal Violet, supplied with the Transwell chamber assay (Chemicon, Millipore, GA, USA), and photographed after incubation for 24 h.

Wound-healing scratch assay

Human GBM cells were seeded onto 6-well plates (Corning) at 2.5×10^4 cells/well in 3 ml medium supplemented with 10% FBS. After 2 days, monolayers were disrupted mechanically using a sterile 200 μl pipette tip. The assay was performed in duplicate. Wells were photographed after 24 h. Cells were then stained with 0.2% crystal violet. Cell migration was monitored using a Nikon Eclipse Ti microscope with a DS-Fi1 camera, and cells were counted using ImageJ software (United States National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Means were compared using Student's t tests, with differences considered significant at a P value <0.05 or <0.01.

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Disclosure of conflict of interest

None.

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