Am J Cancer Res 2020;10(10):3475-3486
www.ajcr.us /ISSN:2156-6976/ajcr0119711

Original Article

Sorafenib increases tumor treating fields-induced cell death in glioblastoma by inhibiting STAT3

Jeong-Yub Kim[1], Yunhui Jo[2], Hoon-Kyu Oh[3*], Eun Ho Kim[4*]

[1]Division of Radiation Biomedical Research, Korea Institute of Radiological and Medical Sciences, Seoul 01812, Republic of Korea; [2]Department of Bio-Convergence Engineering, Korea University, Seoul 02842, Republic of Korea; [3]Department of Pathology, School of Medicine, Daegu Catholic University, Nam-gu, Daegu, Republic of Korea; [4]Department of Biochemistry, School of Medicine, Daegu Catholic University, Nam-gu, Daegu, Republic of Korea. [*]Equal contributors.

Received August 6, 2020; Accepted September 22, 2020; Epub October 1, 2020; Published October 15, 2020

Abstract: A newly diagnosed or recurrent Glioblastoma multiforme (GBM) can be treated with Tumor-treating fields (TTFields), an emerging type of alternative electric field-based therapy using low-intensity electric fields. TTFields have a penchant to arrest mitosis, eventually leading to apoptosis. Therefore, it is regarded as a potential anticancer therapy. However, in this study, we confirmed the combined efficacy of sorafenib and TTFields to improve the treatment efficiency of malignant GBM. Experimentation revealed the ability of sorafenib to decrease the signal transducer and activator of transcription 3 (STAT3) and this inhibition increased the sensitivity of TTFields in preventing tumor expansion. It was found that both combinatorial as well as monotherapy aimed to inhibit or reduce the level of STAT3, but the extent was different and based upon the reaction conditions. This drug is also capable of arresting multiple kinase pathways along with STAT3-related proteins (Mcl-1 and Survivin). *STAT3* silencing can also be accomplished by RNA interference and can increase the TTFields-sensitizing effect of sorafenib. If the effects are reversed and gene regulating STAT3 is expressed more, it annihilates the effects of treatment. Moreover, sorafenib plus TTFields significantly inhibited xenograft tumor growth and combinatorial treatment reduced STAT3 expression more effectively in vivo. These in vitro and in vivo results indicate that sorafenib tends to sensitize GBM cells to TTFields-induced apoptosis by inhibiting STAT3.

Keywords: Tumor-treating fields, sorafenib, glioblastoma multiforme, STAT3

Introduction

Glioblastoma multiforme (GBM) is a rare but lethal primary brain malignancy in adults, with the average incidence in the USA being approximately 11,000 annually [1]. Owing to the infiltrative nature of the disease, its treatment is difficult and necessitates surgical resection, leading to a median survival time of only 3-6 months [2-4]. In the early 1960s, the Brain Tumor Study Group (later renamed the Brain Tumor Cooperative Group) succeeded in significantly extending the survival time of patients with GBM through the incorporation of adjuvant combined therapy to the treatment regimen [5-7]. Presently, combined therapy can result in a median survival time of approximately 1 year, whereas its combination with an oral alkylating agent like temozolomide can extend the survival period further to 14-16 months [8-10].

Tumor-treating fields (TTFields) is a new type of tumor therapy in which alternating electric fields of low intensity and intermediate frequency are transmitted using a non-interfering transducer placed locoregionally around the anatomical region of the tumor cells. A study demonstrated that TTFields were able to disrupt cell division in the cells of interest, while other preclinical manifestations concluded the anti-tumor effects of TTFields vary according to the type of malignancy involved in the experiment [11, 12]. It has been found that TTFields have made significant improvements to increase the life expectancy of GBM patients. After subsequent studies, the US Food and Drug Administration has given approval for the use of

this treatment for patients with newly diagnosed GBM or recurrent GBM after surgery, and is typically combined with radiation therapy and adjuvant temozolomide [13, 14].

With respect to the mechanism of action, TTFields can affect multiplying cells because they cast their effect on the charged macromolecules and other cell components, which are important constituents of mitosis. They accomplish this by causing structural changes to or the dislocation of these cellular components. Such dislocation of particular organelles or other components would subsequently alter the physiological functions of the cell and affect its division cycle, disrupting normal mitosis. The effects of TTFields on various cellular processes can be explained by dipole alignment and dielectrophoresis under the action of applied external stimulation [15, 16]. A molecule in a dipole state has negative and positive poles at its opposite ends that are generated by the movement, or alignment, of the electrons present in the molecule in a specific direction. In the presence of an external uniform alternating electrical field, the charged molecules inside the cell tend to align to the direction opposite to their charge and their normal pathways are disrupted, eventually inhibiting the cell division and its proliferation [17]. This is because the moving molecules are highly polar and are responsible for the completion of the cell cycle [18, 19].

Sorafenib (Nexavar), a potent inhibitor of multiple kinases, has been found to block tumor cell proliferation by targeting the Raf-mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) signaling pathway [20]. This drug has an anti-angiogenic effect by interfering with tyrosine kinases, such as vascular endothelial growth factor receptors (i.e., VEGFR2 and VEGFR3) and platelet-derived growth factor receptor beta (PDGFRβ) [20, 21]. A phase I open-label study deals with determining the optimal dose of sorafenib in combination with radiation therapy and temozolomide to determine the safe and maximum tolerated level of the drug. The trial was conducted on 17 patients with newly diagnosed high-grade gliomas. It has been shown that multiple pathways regulate the spread of malignant gliomas. These pathways, along with endothelial cells, play key roles in disease progression as well as resistance development against subsequent treatments. Resistance against treatment is usually associated with elevated levels of VEGF and its receptors and PDGFR activation [22-24]. Moreover, up to 30% of patients carry gene amplifications on band 4q12 on chromosome 4, which carries *VEGFR2*, *PDGFRα*, and *KIT* [21, 25, 26]. Concordantly, it was found that major mutations were found in MAPK tyrosine pathways in lieu of Ras or Raf kinases and it is responsible for poor prognosis and elevated apoptosis. It has been found that the majority of the kinase pathways are inhibited by sorafenib, indicating that it can serve as an effective anticancer treatment. Indeed, experimental and clinical trials have shown that sorafenib exhibits synergy with not only radiation therapy but also TTFields. Its combination with TTFields therapy results in the arrest of mitosis and a reduction in colony-forming units, thereby delaying Hepatocarcinoma (HCC) growth in animal models [27]. Additional studies have confirmed that sorafenib, temozolomide, and other cytotoxic agents can act synergistically to reduce the viability of tumor cells [28, 29]. Hence, we can use this combination strategy with TTFields to harness the highest advantage of the drug.

The transcription factor signal transducer and activator of transcription 3 (STAT3), which plays a critical role in inflammation and tumor progression is activated by cytokines and growth factors, which are activated by tyrosine phosphorylation [30]. It was recently found that GBM cells are regulated by *STAT*. GBM cell lines pretreated with recombinant tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), upon subsequent action of sorafenib, restrained tumor advancement by reducing the level of phosphorylated STAT3 (p-STAT3). The drug was specified in a time- and dose-dependent manner and also subsequently reduced the expression of STAT3-related proteins (i.e., Mcl-1, survivin, and cyclin D1) [31]. Interestingly, the sorafenib-mediated inactivation of STAT3 that led to GBM inhibition was in fact a kinase-dependent process, which was further regulated by tyrosine phosphatases along with SH2-domain-containing cytosolic phosphatase-1 (SHP-1) [32].

Radiation therapy, TTFields therapy, and anticancer drugs, or their combination, has been

widely used in several malignancies. However, the clinical potentials and mechanisms of combining TTFields with a tyrosine kinase inhibitor drug (sorafenib) have not yet been explored for GBM. This study aims to focus on the preclinical study of sorafenib in a combinatorial treatment to curb malignant GBM. Here we have investigated the susceptibility of GBM both *in vivo* and *in vitro* upon the action of sorafenib with TTFields. The study elucidates the mechanism of its action in the context of STAT3 reduction. The inferences of this work can be used as clinical manifestations of application of this novel treatment in patients with unresectable or recurrent GBM.

Materials and methods

Experimental setup of electric fields

To generate the TTFields, PVC-insulated wires of 0.17 mm thickness with an outer diameter of 0.4 mm and a dielectric breakdown of 25 kV/mm purchased from Seoil Electric Wire Co., Ltd. (Gyeonggi, South Korea) was used and powered using an arbitrary function generator (AFG-2112, Good Will Instrument Co., Ltd., Taiwan). The generator was connected to a high-voltage amplifier bought from A303 (A. A. Lab Systems Ltd, Israel) which produced sine waves with a potential of 0 to 800 V [33]. To apply the electric field to the cell lines, a pair of insulated wires were attached to the bottom of each cell dish placed at a unit centimeter stretch between them [33]. For all experiments, the intensity and frequency of the applied electric fields were 0.9 V/cm and 150 kHz, respectively. We chose 0.9 V/cm because it is very close to the intensity currently used in clinical TTFields therapy.

Antibodies and chemicals

Sorafenibtosylate was purchased from Selleckchem (Houston, TX, USA). For *in vitro* experiments, a 10 mmol/L stock solution of sorafenib was prepared by dissolving the drug in dimethyl sulfoxide (DMSO). The stock solution was stored at 4°C. Antibodies for immunoblotting such as Mcl-1, β-actin, and ki-67 were purchased from Santa Cruz Biotechnology (San Diego, CA, USA). Other antibodies such as anti-STAT3, Survivin, cleaved PARP, and c-Myc were procured from Cell Signaling (Danvers, MA, USA).

Cell culture

The cell lines, U87 and U373, containing human GBM, were provided by the Korean Cell Line Bank (Seoul, South Korea). Both cell lines were grown and maintained in Dulbecco's Modified Eagle's medium (supplemented with 10% fetal bovine serum, glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], and antibiotics) at 37°C in a humidified incubator containing an atmosphere of 5% CO_2.

Colony formation assay

At 24 h after exposure of the GBM cells to the TTFields, the cells were treated with sorafenib (final concentration, 5 μmol/L) for 72 h. After 14-20 days, the colonies were stained with 0.4% crystal violet (Sigma, St. Louis, MO, USA). Various factors need to be considered to determine the number of viable cells. These include plating efficiency (PE) and the survival fraction. PE is defined as the portion of the cells which were seeded and eventually furnished into colonies; it is represented in the terms of percentage and varies according to the culture environment. The survival fraction, expressed as a function of condition, was calculated as follows: survival fraction = colonies counted/(cells seeded × PE/100).

Detection of apoptotic cells using annexin V staining

The cells treated with sorafenib were later exposed to the TTFields for duration of 48 h. The cells obtained from this treatment were then washed with ice-cold phosphate-buffered saline (PBS), treated with trypsin and resuspended in one times the buffer. This binding buffer has 10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, and 2.5 mM $CaCl_2$. These solutions were mixed to make the total density of the solution equal to 1×10^6 cells/mL. Then, 100 μL of the cell solution was mixed with 5 μL of annexin V FITC (PharMingen, San Diego, CA, USA) and 10 μL of propidium iodide stock solution (50 μg/mL in PBS) was added by slightly mixing to stain the plates. Following this, the whole setup was incubated for 15 min at an optimum temperature and in the dark to prevent any photosensitive or heat-sensitive reactions. After incubation, cells were collected and mixed with a buffer (400 μL, 1 ×) and counted using the Fluorescence-activated cell sorting

(FACS) technique. We used a FACScan device (Becton Dickinson, Franklin Lakes, NJ, USA). On average, 10,000 cells were recovered from each of the samples after the treatment. CellQuest software from BD Biosciences (Franklin Lakes, NJ, USA) was used for data analysis.

Trypan blue staining assay

Trypan Blue Staining Solution (ab233465, Cambridge, UK) is a vital stain that colors dead tissues or cells blue. Since cells are very selective, in a viable cell, the trypan blue will not pass through the membrane; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue color when visualized using a microscope.

Cell viability assay

To determine cell viability, U87 and U373 cell lines were incubated under appropriate conditions in a 96-well-plate at a density of 5,000 cells/well for 24 h. To determine the number of viable cells, a culture media with EZ-Cytox was mixed and incubated for another 4 h. Then a Multiskan EX (Thermo Scientific, Waltham, MA, USA) was used to measure cell viability at a wavelength of 450 nm.

STAT3 SiRNA transfections

The following human STAT3-specific siRNAs, synthesized by Bioneer (Bioneer, Seoul, South Korea), were used: sense CGUCAUUAGCAGAAUCUCA, antisense UGAGAUUCUGCUAAUGACG (STAT3 siRNA).

The siRNA duplexes were transfected into cells using Lipofectamine® RNAiMAX Reagent according to the manufacturer's guidelines. siRNA was transfected in the STAT3 gene using Stealth RNAi where a non-targeting siRNA (Bioneer) was employed as a control group. During this process, the transfections were performed again by using Lipofectamine® RNAiMAX and Opti-MEM reduced serum medium (Gibco® Life Technologies, Carlsbad, CA, USA), when 60-70% of the cells started to merge, to make the terminal value of 20 nmol/L of siRNAs. Then the cells were incubated in this culture for duration of 5 h after which the pH of the medium was altered to normal without the administration of antibiotics. Cell lines in this novel culture were allowed to rest and incubated for another 48 h. Samples were then collected and examined to note the transfection sequence which was repeated three additional times.

Plasmid transfections

Various plasmids as carriers were also devised as vectors to transfer the recombinant DNA using standard DNA recombination cloning strategy. Human STAT3 cDNA (wild-type) was purchased from OriGene (Rockville, MD, USA). The wild-type form of STAT3 was generated using formal (CTGGCCTTTGGTGTTGAAAT), reverse (AAGGCACCCACAGAAACAAC). The plasmids were transfected to the targets by using Mirus 2020 Reagent as per the manufacturer's guidelines.

Tumor xenografts in nude mice

A single-cell suspension (2×10^6 cells) was subcutaneously injected into the flank of 5-week-old BALB/c nude mice (Nara Biotech, Seoul, South Korea). When the tumor reached a minimal volume of 100 to 200 mm^3, a 1 V/cm TTF, 30 mg/kg sorafenib (3 times a week) or combination treatment was started and continued for seven days. Tumor volumes were determined using the formula $(L \times l^2)/2$ by measuring tumor length (L) and width (l) with a caliper. All animal work was carried out per our institute policies while abiding by the guidelines of the Institutional Animal Care and Use Committee of KIRAMS (kirams2019-0073, 28/06/2019).

Positron emission tomography and computed tomography

A Siemens Inveon positron emission tomography (PET) scanner was used for PET imaging [34]. Experimental mice were first warmed using a heating pad. Then, 200 μCi of [^{18}F]-FDG was administered into the tail vein and the mice were given 2% isoflurane in the presence of 100% oxygen (Forane solution; ChoongWae Pharma, Seoul, South Korea). Next, X-ray computed tomography (CT) was performed to obtain the anatomical imaging of mice from various angles starting from 180° and 360° projection, using the Inveon system. Experimental mice were exposed to the rays for 200 ms and the scanning lasted around 504 s. The X-ray CT images were then reconstructed, which altered the pixel size to 109.69 μm × 109.69

μm. After 30 min of transfection and CT imaging, PET imaging was done for the next 15 min. The emission list-mode PET data were sorted into three-dimensional sinograms and then reconstructed using the two-dimensional ordered subset expectation maximization (OSEM2D) method. The final image had a pixel dimension of 0.38 mm × 0.38 mm × 0.79 mm. All relevant corrections, such as normalization, dead-time correction, and random correction, were performed for all datasets. X-ray CT data were used to delineate the region of interest (ROI). The PET and CT image data were co-registered using Inveon Research Workplace (version 2.0; Siemens Medical Solutions). The maximum pixel values for the ROI on the PET imaging were measured and converted to radioactivity counts per minute (cpm) values.

Western blotting

After the treatment of the GBM cells with sorafenib, they were treated with TTFields. The cells were then lysed with RIPA buffer and then the total proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After the separation of proteins, they were transferred to a nitrocellulose membrane and subsequently blocked with 1% (v/v) non-fat dried milk prepared in Tris-buffered saline containing 0.05% Tween-20 and incubated with the respective antibodies. Primary antibodies were diluted at 1:1,000 and secondary antibodies at 1:5,000. The immunoreactive protein bands were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, UK) and scanned.

Immunohistochemistry

Immunohistochemical assays were performed by resecting the breast sections over a paraffin section of 4 μm thickness which was then mounted over coated glass slides to determine proteins. After this, the administered antigens were retrieved from the plating and the local endogenous peroxidases and nonspecific protein binding sites were inhibited. The slide sections were incubated with the primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies.

Statistical analysis

All data are expressed as mean ± standard deviation values. Statistical differences between groups were assessed using Student's *t*-test (two-tailed) and ANOVA analysis. *P*-values were interpreted as follows; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Results

Sorafenib increased TTFields-induced apoptosis in GBM cells

The purpose of this study was to better understand, analyze, and evaluate the action of sorafenib in sensitizing GBM cell lines to TTFields-induced apoptosis by examining the apoptotic effects of the drug. Toward this, two GBM cell lines were administered doses of sorafenib and examined using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. From the observations, we can conclude an enhanced sensitivity in both U373 and U87 cell lines where the tumor progression was limited within 48 h of application of the treatment with ≥ 5 μg/mL sorafenib ($P < 0.05$) (**Figure 1A**). Similar observations were also reported after the application of TTFields to the same cell lines where a 20% reduction in cell sustainability was found at 0.9 V/cm [27]. A greater growth inhibitory effect was observed due to the combined action of sorafenib and TTFields, thereby highlighting the better antitumor effect of the combinatorial therapy relative to monotherapy on the U373 and U87 cell lines. This was further verified by both the cell counting assay (**Figure 1B**) and colony formation assays (**Figure 1C**). To know whether the combination treatment is successful or not, we stained the cell lines with annexin V and propidium iodide for FACS analysis. The observations showed a remarkable alleviation in the severity of GBM of the treated cell lines within 48 h of exposure while elevating apoptosis (**Figure 1D**). The serial doses of sorafenib treatments were applied to the GBM cell lines to determine the combined dosage of TTFields and sorafenib with better anti-GBM efficacy (**Figure 1E**). The above experiment points to the fact that sorafenib tends to make the GBM cells prone to the action of TTFields *in vitro*.

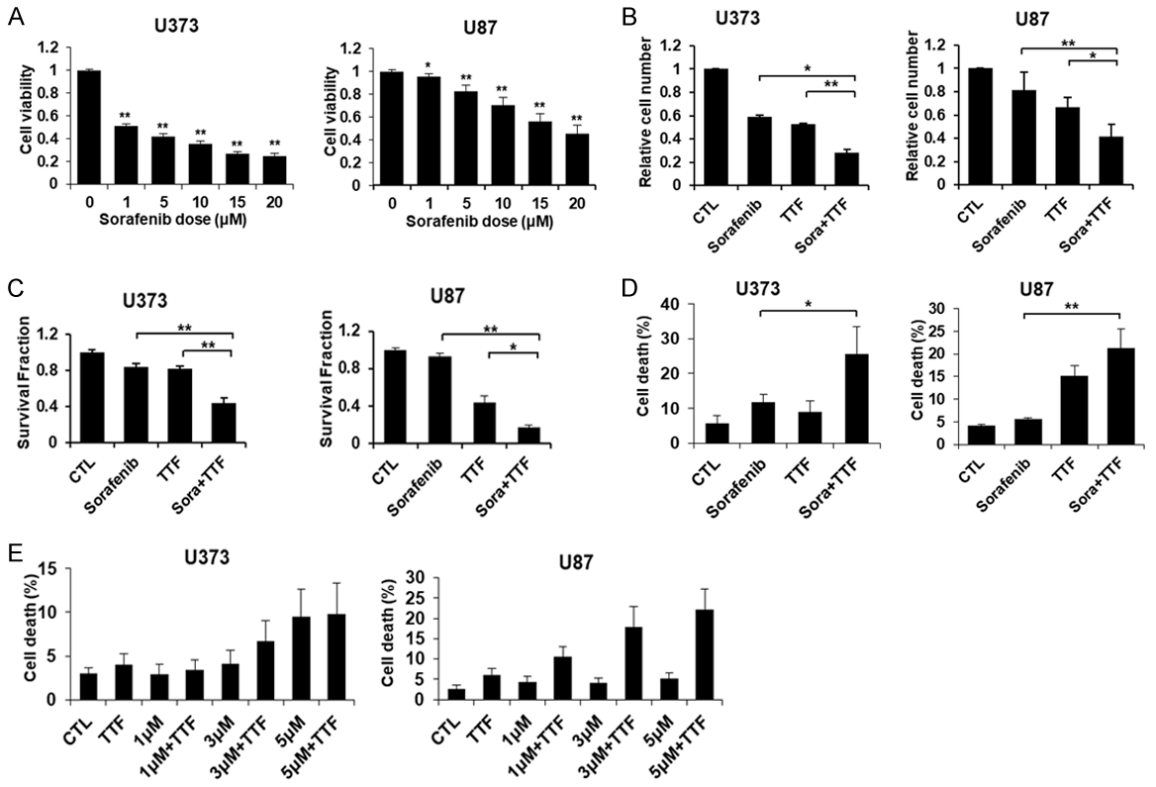

Figure 1. Sorafenib enhances TTFields-induced apoptosis in GBM cells. A. Sorafenib inhibited the viability of GBM cells in a dose-dependent manner. The viability of U373 and U87 cells treated with the indicated doses of sorafenib was found by staining with 0.4% trypan blue; $*P < 0.05$; $**P < 0.01$. B. It was found that the cell lines administered with a combination treatment showed the least viable cell count in comparison to monotherapy using either TTFields or Sorafenib. The growth rate is as assessed using cell counting assay; $*P < 0.05$; $**P < 0.01$. C. The sensitivity of the two chosen cell lines was inferred from the degree of survival fraction using a colony-forming assay; $*P < 0.05$; $**P < 0.01$. D. U373 and U87 cell lines were administered 5 μmol/L of sorafenib and/or TTFields for 48 h for annexin V/propidium iodide staining; $*P < 0.05$; $**P < 0.01$. E. The combined TTFields plus sorafenib treatment induced GBM cell apoptosis in a dose-dependent manner.

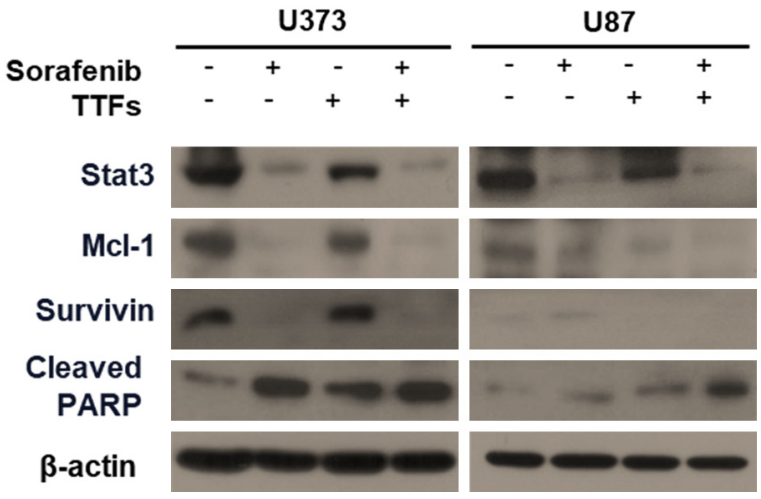

Figure 2. STAT3 downregulation causes the sensitization of TTFields. This downregulation of STAT3 is inhibited by further action of Sorafenib. Cells were pretreated with TTFields were treated with sorafenib for 24 h. Cell lysates were then prepared for western blotting for investigating the proteins of interest.

Combined sorafenib plus TTFields treatment was associated with lower STAT3 levels in GBM cells

A previous study had suggested that STAT3 might play a role in mediating cancer cell resistance to TRAIL [31]. Therefore, we examined the expression of STAT3-regulated proteins to investigate the possible molecular targets involved in the sorafenib-mediated TTFields sensitization of GBM cells to apoptosis. As shown in **Figure 2**, the downregulation of STAT3 and its related proteins (Mcl-1 and Survivin) was evident in both cell lines. The cells were then

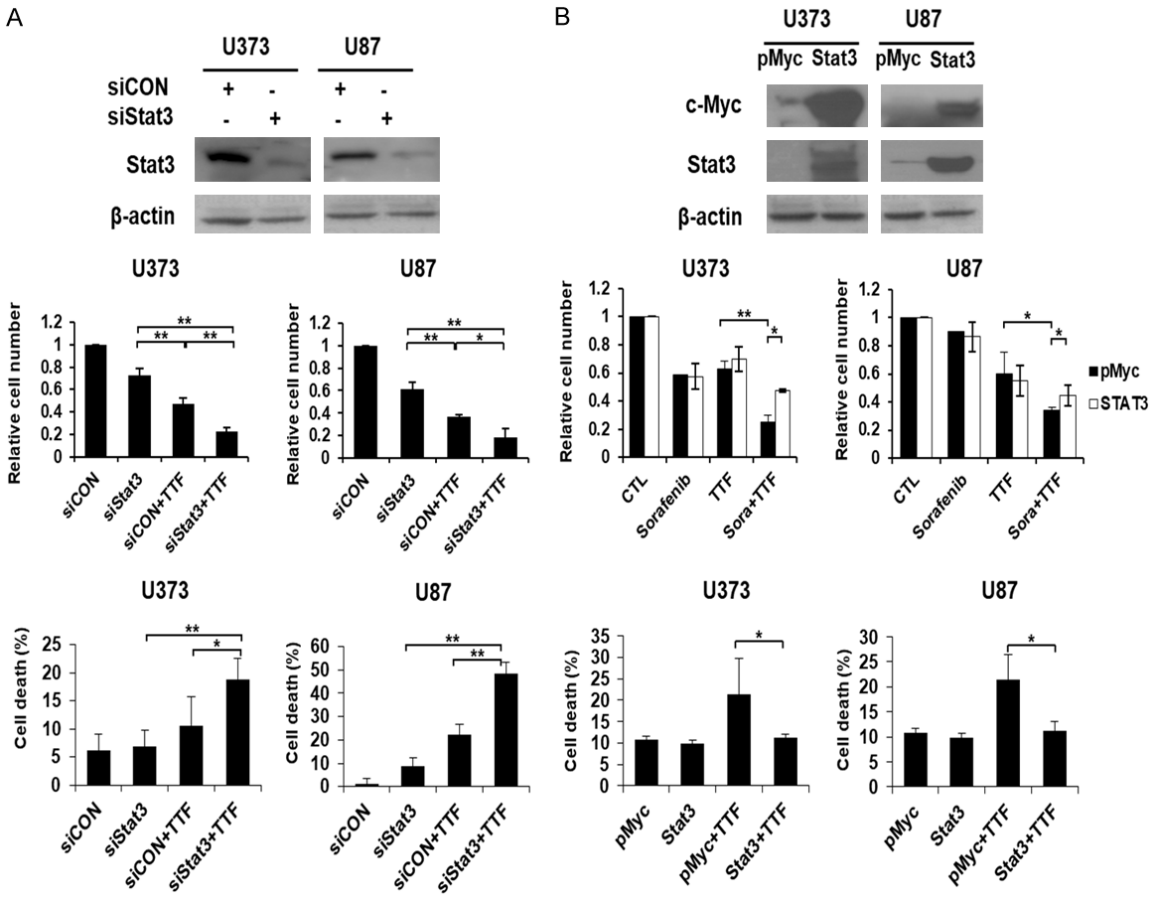

Figure 3. Validation of *STAT3* targeting *in v*itro. A. STAT3 silencing via siRNA increased the sensitivity of the GBM cells to the TTFields. Cells were transfected with either scrambled (control) or STAT3 siRNAs for 48 h and then to TTFields for 24 h. The proliferation rate was measured using trypan blue cell counting and the cell death rate was measured by FACS; *P < 0.05; **P < 0.01. B. The number of live cells was measured using cell counting. GBM cells transiently expressing Myc-STAT3 (on right) were pretreated with TTFields for 24 h, and then with sorafenib for another 24 h. The proliferation rate was measured by trypan blue cell counting and the cell death rate was measured by FACS analysis; *P < 0.05; **P < 0.01.

further co-treated with sorafenib plus TTFields, which also decreased the total STAT3 levels. The decrease in STAT3 was associated with the poly (ADP-ribose) polymerase (PARP) gene. These results provided evidence that apoptosis was induced in cells exposed to sorafenib plus TTFields (**Figure 2**). Therefore, we can infer that sorafenib sensitizes the cells to TTFields and inhibits STAT3, showing the promising role of STAT3 for enabling cell sensitization to TTFields.

Manipulation of the STAT3 expression altered the cell sensitivity to TTFields

To validate the role of STAT3 in the TTFields-sensitizing effect of sorafenib, the *STAT3* gene was either knocked down or overexpressed in GBM cells. For this purpose, the cells were transfected with either scrambled (control) or *STAT3* siRNAs for 24 h and then exposed to TTFields. The gene knockdown resulted in enhanced apoptotic cell death, with a decrease in the relative cell number (**Figure 3A**), indicating that the silencing of *STAT3* had significantly improved the sensitivity of the GBM cells to the TTFields. This strongly indicated that by targeting the STAT3 signaling pathway, the sensitivity of the GBM cells toward TTFields can be altered. Subsequently, we generated cells transiently expressing *STAT3* to investigate the sensitizing effect of sorafenib in the presence of the transcription factor. Ectopically expressed *STAT3* significantly abolished the combined effects of sorafenib plus TTFields on apoptosis

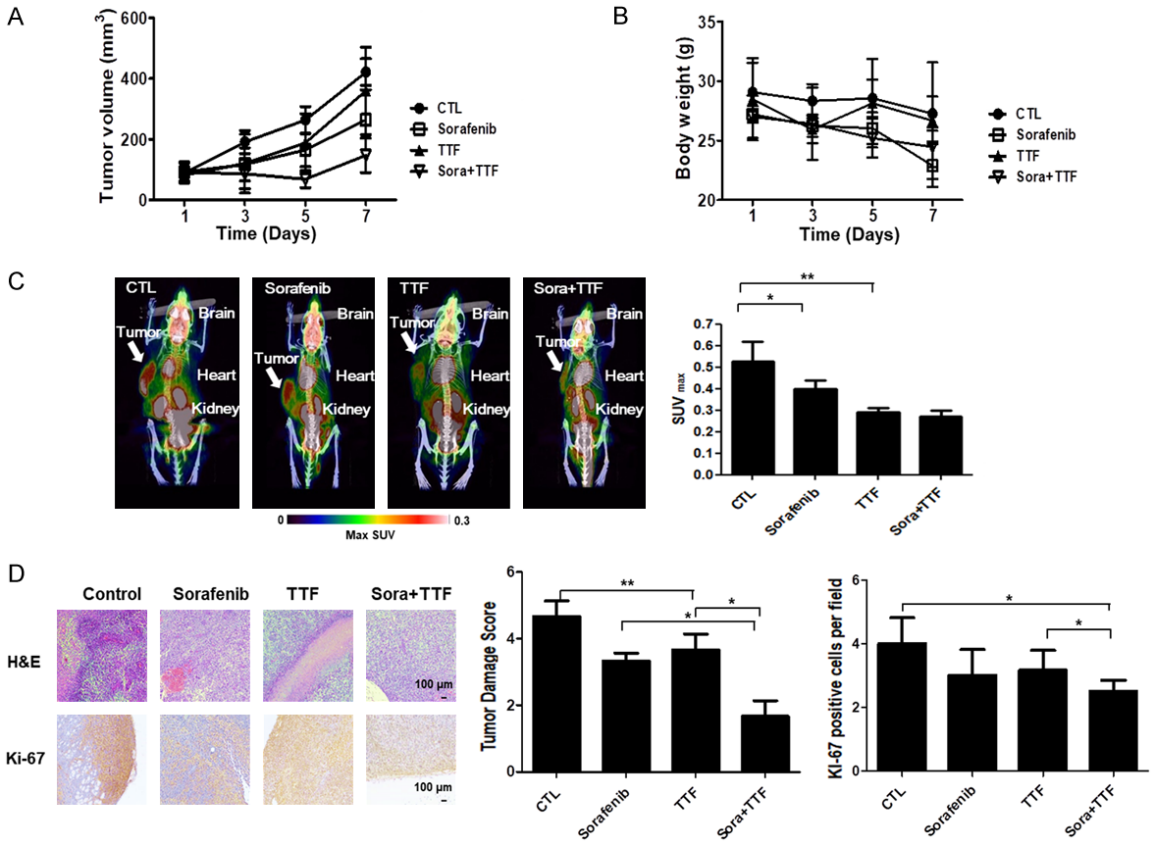

Figure 4. TTFields-sensitizing effect of sorafenib in *in vivo* models of GBM. A. Nude mice were injected with U373 cells lines and then treated with either a monotherapy of TTFields or sorafenib, or a combination of the two. Tumor volumes were measured by: volume = (length × width^2 × 3.14)/6 (n = 5). Tumors were excised and weighed at the end of the experiment (7 days). B. The body weights of the mice were not very different for sorafenib-, TTFields-, and combinatorial therapy-treated groups. C. PET and CT imaging of U373 tumor-bearing mice after fluorine-18-fluorodeoxyglucose ([^{18}F]-FDG) injection and subsequent anesthesia. The [^{18}F]-FDG radioactivity is presented as the maximal standardized uptake value (mean ± standard deviation); *P < 0.05; **P < 0.01. D. Hematoxylin & eosin (H&E) staining and immunohistochemistry for Ki-67 expression; *P < 0.05, **P < 0.01.

(**Figure 3B**). Together, these results demonstrate the importance of STAT3 inhibition in mediating the combined effects of sorafenib plus TTFields.

Combinatorial therapy with sorafenib plus TTFields reduced xenograft tumors in nude mice

The relevance and potential clinical implications of combinatorial therapy with sorafenib plus TTFields were assessed *in vivo* using xenograft tumors derived from the U373 cell line. Tumor-bearing mice were treated for 4 days with TTFields (1 fraction per day) followed by either the vehicle (DMSO) or oral sorafenib (dosage of 5 mg·kg^{-1}·day^{-1}; either alone or in combination) for the duration of this animal study. Necroscopy (data not shown) revealed that all the animals tolerated the treatments well, with no observable signs of toxicity or gross pathological abnormalities. Tumor growth was significantly suppressed by the 1-week combinatorial treatment with sorafenib plus TTFields compared with that observed in the untreated control tumors (**Figure 4A**). There were little visible signs of toxicity due to any treatment in mice, as demonstrated by the lack of differences in body weights (**Figure 4B**). As shown in **Figure 4C**, low uptake of [fluorine-18]-fluorodeoxyglucose ([^{18}F]-FDG) was observed in tumors in mice treated with TTFields plus sorafenib as compared with that in mice receiving monotherapy with either agent. The maximum standard uptake value was 0.53 ± 0.09 in the control group, 0.40 ± 0.04 in the sorafenib-treated group, 0.29 ± 0.02 in the

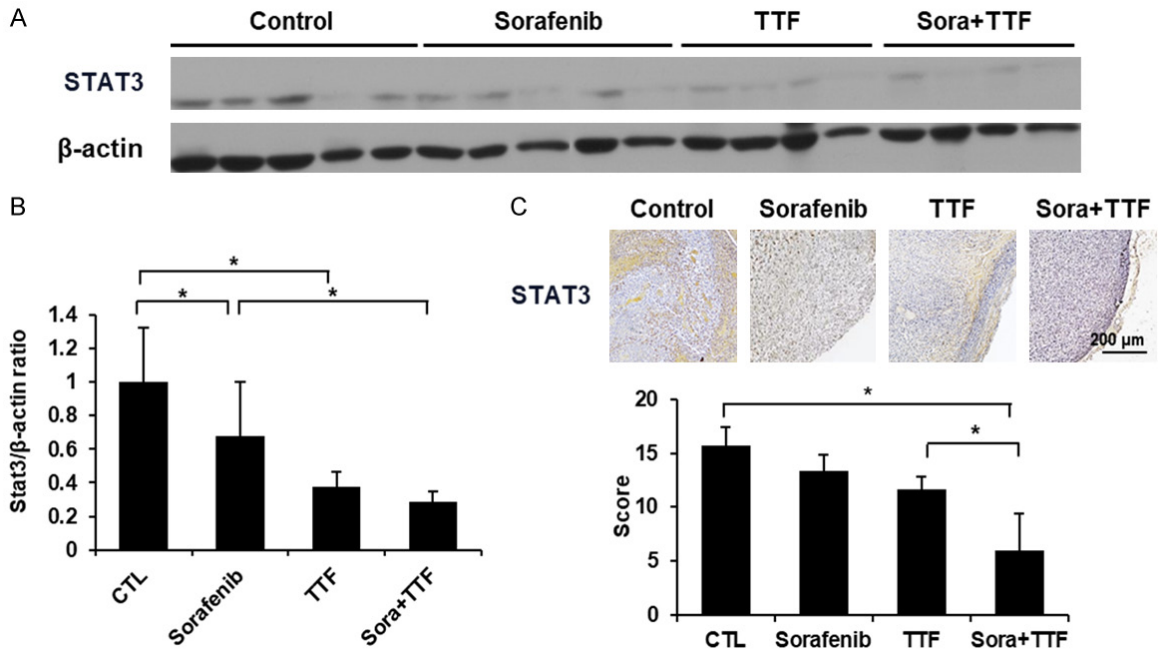

Figure 5. Validation of the *in vivo* target of STAT3. A. Western blotting for STAT3 expression in U373 tumors. B. The expression of STAT3 was normalized. Values are the means ± standard deviation; **P* < 0.05. C. Immunohistochemistry for STAT3 expression level in the xenografts; **P* < 0.05.

TTFields-treated group, and 0.27 ± 0.03 in the combinatorial therapy-treated group (**Figure 4C**). In xenograft tumor tissue, combination treatment decreased the number of Ki67-positive cells which are proliferation markers (**Figure 4D**). Based on treatment, we can conclude that monotherapy with sorafenib or TTFields did not show significantly different results and had only a mild effect on tumor cell reduction. By contrast, the sorafenib plus TTFields combination treatment exhibited better antitumor effects *in vivo*.

Combinatorial therapy with sorafenib plus TTFields inhibited tumor growth through the suppression of the STAT3 axis in vivo

The effects of the monotherapy and combinatorial therapy on STAT3 expression *in vivo* were also investigated by western blotting (**Figure 5A** and **5B**). Additionally, xenografts from mice subjected to different treatments were stained for STAT*3* expression using immunohistochemistry (**Figure 5C**). Collectively, the results verified that combinatorial therapy with sorafenib plus TTFields reduced STAT3 expression more effectively than monotherapy with either of the agents. Therefore, the antitumor effects of combinatorial therapy have been definitively verified *in vivo*.

Discussion

The lack of restorative therapeutic solutions is posing a great threat to patients with unresectable advanced GBM. Currently, surgery followed by radiation therapy with concurrent and adjuvant temozolomide represents the standard of care for newly diagnosed GBM [35]. However, in the treatment of all carcinomas, TTFields is currently being introduced as the fourth new protocol after surgery, chemotherapy, and radiation therapy. The study demonstrates the effect of combination therapy towards increased the survival rate and the extent of tumor suppression. Hence, acting as a potential curative strategy for GBM patients [27-29, 36], our present study not only verified the antitumor effects of combinatorial therapy with sorafenib plus TTFields, but also highlighted the mechanism of action of this treatment via inhibiting tyrosine kinases and phosphorylation of STAT3. In this experiment, GBM cells were treated with TTFields followed by sorafenib treatment and the effective inhibition of GBM growth *in vitro* and *in vivo* was inferred using this strategy. This conclusion can help establish a procedure with better anti-GBM efficacy to target the *STAT3* transcription. These results suggested that TTFields are not only effective but are also clinically relevant. With

pacing technology, refinements are in progress with an aim to avoid damage to the TTFields-sensitive healthy organs in the vicinity of the tumor cells, considering the same, substantial doses of TTFields are delivered to the cancerous cells.

To achieve this objective, we need to shift the focus towards a novel and more effective curative target strategy for GBM; thereby, considering the combination therapy. Upon experimentation, it has been found that STAT3 inhibition promotes better action to prevent tumor expression by elevating apoptosis. This STAT3 inhibition can be achieved by a multi-kinase drug inhibitor labeled as sorafenib. Our results have shown that combinatorial treatment with sorafenib plus TTFields increased GBM cell apoptosis and inactivated STAT3 in the cells. STAT3 inhibition increases the action of PARP genes; hence blocking carcinogens by alkylating them. This kinase drug inhibits tyrosine phosphorylation in the Ras, Raf or MAPK and VEGFR pathways. Whereas the previous literature reported it being another aspect, the latter showed its inhibition in hepatocellular carcinoma via kinase-independent SHP-1-mediated STAT3 inactivation [32].

This study concludes that the sensitization action is solely STAT3 dependent as a slight modification in its expressing gene showed considerable effects. These modifications altered the potent proteins present on the STAT3 such as Mc-1, cyclin D1, and Survivin. If both the kinase and non-kinase inhibition activities of sorafenib are taken into consideration, we can see that they would preferentially increase the drug's potential to act synergistically with TTFields to affect multiple operations and intracellular signaling pathways [37]. Thus, the targeting of molecules involved in these processes by sorafenib administration would sensitize GBM synergistically to the apoptotic effects of TTFields. Furthermore, as the sensitizing effect of sorafenib in GBM treatment is well characterized, it is important to compare its sensitizing effect and underlying mechanism of TTFields and the combined efficacy of sorafenib and temozolomide, known as standard treatment for GBM to enhance the efficacy and safety of these forms of GBM treatment. This combination therapy of TTFields and sorafenib will be the novel strategy acting as an advantage for the patients suffering from unresectable GBM.

Acknowledgements

The work was supported by a grant from the National Research Foundation of Korea (NRF) (No. NRF-2019M2A2B4095150) and through a grant from the Research Institute of Medical Science, Catholic University of Daegu (2020).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Eun Ho Kim, Department of Biochemistry, School of Medicine, Daegu Catholic University, 33 17-gil, Duryugongwon-ro, Nam-gu, Daegu, Republic of Korea. Tel: +82-53-650-4480; E-mail: eh140149@cu.ac.kr; Dr. Hoon-Kyu Oh, Department of Pathology, School of Medicine, Daegu Catholic University, 33 17-gil, Duryugongwon-ro, Nam-gu, Daegu, Republic of Korea. Tel: +82-53-650-4156; E-mail: ap510@cu.ac.kr

References

[1] Ostrom QT, Gittleman H, Fulop J, Liu M, Blanda R, Kromer C, Wolinsky Y, Kruchko C and Barnholtz-Sloan JS. CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2008-2012. Neuro Oncol 2015; 17: iv1-iv62.

[2] Bennett H and Godlee RJ. Excision of a tumour from the brain. Lancet 1884; 124: 1090-1091.

[3] Davis FG, Freels S, Grutsch J, Barlas S and Brem S. Survival rates in patients with primary malignant brain tumors stratified by patient age and tumor histological type: an analysis based on Surveillance, Epidemiology, and End Results (SEER) data, 1973-1991. J Neurosurg 1998; 88: 1-10.

[4] Walker MD, Alexander E Jr, Hunt WE, Leventhal CM, Mahaley MS Jr, Mealey J, Norrell HA, Owens G, Ransohoff J, Wilson CB and Gehan EA. Evaluation of mithramycin in the treatment of anaplastic gliomas. J Neurosurg 1976; 44: 655-667.

[5] Walker MD, Alexander E Jr, Hunt WE, MacCarty CS, Mahaley MS Jr, Mealey J Jr, Norrell HA, Owens G, Ransohoff J, Wilson CB, Gehan EA and Strike TA. Evaluation of BCNU and/or radiotherapy in the treatment of anaplastic gliomas. A cooperative clinical trial. J Neurosurg 1978; 49: 333-343.

[6] Walker MD, Green SB, Byar DP, Alexander E Jr, Batzdorf U, Brooks WH, Hunt WE, MacCarty CS, Mahaley MS Jr, Mealey J Jr, Owens G, Ransohoff J 2nd, Robertson JT, Shapiro WR, Smith KR Jr, Wilson CB and Strike TA. Randomized comparisons of radiotherapy and nitro-

soureas for the treatment of malignant glioma after surgery. N Engl J Med 1980; 303: 1323-1329.
[7] Walker MD, Strike TA and Sheline GE. An analysis of dose-effect relationship in the radiotherapy of malignant gliomas. Int J Radiat Oncol Biol Phys 1979; 5: 1725-1731.
[8] Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, Hau P, Brandes AA, Gijtenbeek J, Marosi C, Vecht CJ, Mokhtari K, Wesseling P, Villa S, Eisenhauer E, Gorlia T, Weller M, Lacombe D, Cairncross JG and Mirimanoff RO; European Organisation for Research and Treatment of Cancer Brain Tumour and Radiation Oncology Groups; National Cancer Institute of Canada Clinical Trials Group. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 2009; 10: 459-466.
[9] Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E and Mirimanoff RO; European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005; 352: 987-996.
[10] Gilbert MR, Wang M, Aldape KD, Stupp R, Hegi ME, Jaeckle KA, Armstrong TS, Wefel JS, Won M, Blumenthal DT, Mahajan A, Schultz CJ, Erridge S, Baumert B, Hopkins KI, Tzuk-Shina T, Brown PD, Chakravarti A, Curran WJ Jr and Mehta MP. Dose-dense temozolomide for newly diagnosed glioblastoma: a randomized phase III clinical trial. J Clin Oncol 2013; 31: 4085-4091.
[11] Kirson ED, Gurvich Z, Schneiderman R, Dekel E, Itzhaki A, Wasserman Y, Schatzberger R and Palti Y. Disruption of cancer cell replication by alternating electric fields. Cancer Res 2004; 64: 3288-3295.
[12] Kirson ED, Dbaly V, Tovarys F, Vymazal J, Soustiel JF, Itzhaki A, Mordechovich D, Steinberg-Shapira S, Gurvich Z, Schneiderman R, Wasserman Y, Salzberg M, Ryffel B, Goldsher D, Dekel E and Palti Y. Alternating electric fields arrest cell proliferation in animal tumor models and human brain tumors. Proc Natl Acad Sci U S A 2007; 104: 10152-10157.
[13] Stupp R, Taillibert S, Kanner AA, Kesari S, Steinberg DM, Toms SA, Taylor LP, Lieberman F, Silvani A, Fink KL, Barnett GH, Zhu JJ, Henson JW, Engelhard HH, Chen TC, Tran DD, Sroubek J, Tran ND, Hottinger AF, Landolfi J, Desai R, Caroli M, Kew Y, Honnorat J, Idbaih A, Kirson ED, Weinberg U, Palti Y, Hegi ME and Ram Z. Maintenance therapy with tumor-treating fields plus temozolomide vs temozolomide alone for glioblastoma: a randomized clinical trial. JAMA 2015; 314: 2535-2543.
[14] U.S. Food and Drug Administration. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpma/pma.cfm?id=P100034.
[15] Gonzalez CF and Remcho VT. Harnessing dielectric forces for separations of cells, fine particles and macromolecules. J Chromatogr A 2005; 1079: 59-68.
[16] Schomerus H, van Bemmel KJ and Beenakker CW. Localization-induced coherent backscattering effect in wave dynamics. Phys Rev E Stat Nonlin Soft Matter Phys 2001; 63: 026605.
[17] Griffiths DJ. Introduction to electrodynamics. 3rd edition. Upper Saddle River, Prentice Hall; 1999.
[18] Davies AM, Weinberg U and Palti Y. Tumor treating fields: a new frontier in cancer therapy. Ann N Y Acad Sci 2013; 1291: 86-95.
[19] Gera N, Yang A, Holtzman TS, Lee SX, Wong ET and Swanson KD. Tumor treating fields perturb the localization of septins and cause aberrant mitotic exit. PLoS One 2015; 10: e0125269.
[20] Wilhelm S, Carter C, Lynch M, Lowinger T, Dumas J, Smith RA, Schwartz B, Simantov R and Kelley S. Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. Nat Rev Drug Discov 2006; 5: 835-844.
[21] Puputti M, Tynninen O, Sihto H, Blom T, Maenpaa H, Isola J, Paetau A, Joensuu H and Nupponen NN. Amplification of KIT, PDGFRA, VEGFR2, and EGFR in gliomas. Mol Cancer Res 2006; 4: 927-934.
[22] Hermanson M, Funa K, Hartman M, Claesson-Welsh L, Heldin CH, Westermark B and Nistér M. Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. Cancer Res 1992; 52: 3213-3219.
[23] Shih AH, Dai C, Hu X, Rosenblum MK, Koutcher JA and Holland EC. Dose-dependent effects of platelet-derived growth factor-B on glial tumorigenesis. Cancer Res 2004; 64: 4783-4789.
[24] Martinho O, Longatto-Filho A, Lambros MB, Martins A, Pinheiro C, Silva A, Pardal F, Amorim J, Mackay A, Milanezi F, Tamber N, Fenwick K,

Ashworth A, Reis-Filho JS, Lopes JM and Reis RM. Expression, mutation and copy number analysis of platelet-derived growth factor receptor A (PDGFRA) and its ligand PDGFA in gliomas. Br J Cancer 2009; 101: 973-982.

[25] Joensuu H, Puputti M, Sihto H, Tynninen O and Nupponen NN. Amplification of genes encoding KIT, PDGFRalpha and VEGFR2 receptor tyrosine kinases is frequent in glioblastoma multiforme. J Pathol 2005; 207: 224-231.

[26] Holtkamp N, Ziegenhagen N, Malzer E, Hartmann C, Giese A and von Deimling A. Characterization of the amplicon on chromosomal segment 4q12 in glioblastoma multiforme. Neuro Oncol 2007; 9: 291-297.

[27] Jo Y, Kim EH, Sai S, Kim JS, Cho JM, Kim H, Baek JH, Kim JY, Hwang SG and Yoon M. Functional biological activity of sorafenib as a tumor-treating field sensitizer for glioblastoma therapy. Int J Mol Sci 2018; 19: 3684.

[28] Grosu A, Gkika E, Brunner TB, Thimme R and Weinberg U. Phase II HEPANOVA trial of tumor treating fields concomitant with sorafenib for advanced hepatocellular carcinoma. J Clin Oncol 2013; 85: 321-328.

[29] Dal Lago L, D'Hondt V and Awada A. Selected combination therapy with sorafenib: a review of clinical data and perspectives in advanced solid tumors. Oncologist 2008; 13: 845-858.

[30] Aggarwal BB, Kunnumakkara AB, Harikumar KB, Gupta SR, Tharakan ST, Koca C, Dey S and Sung B. Signal transducer and activator of transcription-3, inflammation, and cancer. Ann N Y Acad Sci 2009; 1171: 59-76.

[31] Chen KF, Tai WT, Liu TH, Huang HP, Lin YC, Shiau CW, Li PK, Chen PJ and Cheng AL. Sorafenib overcomes TRAIL resistance of hepatocellular carcinoma cells through the inhibition of STAT3. Clin Cancer Res 2010; 16: 5189-5199.

[32] Tai WT, Cheng AL, Shiau CW, Huang HP, Huang JW, Chen PJ and Chen KF. Signal transducer and activator of transcription 3 is a major kinase-independent target of sorafenib in hepatocellular carcinoma. J Hepatol 2011; 55: 1041-1048.

[33] Jeong H, Sung J, Oh SI, Jeong S, Koh EK, Hong S and Yoon M. Inhibition of brain tumor cell proliferation by alternating electric fields. Appl Phys Lett 2014; 105: 203703.

[34] Constantinescu CC and Mukherjee J. Performance evaluation of an Inveon PET preclinical scanner. Phys Med Biol 2009; 54: 2885-2899.

[35] Hsieh CH, Jeng KS, Lin CC, Chen CK, Liu CY, Lin CP, Tai HC, Wang CH, Shueng PW and Chen YJ. Combination of sorafenib and intensity modulated radiotherapy for unresectable hepatocellular carcinoma. Clin Drug Investig 2009; 29: 65-71.

[36] Zhao JD, Liu J, Ren ZG, Gu K, Zhou ZH, Li WT, Chen Z, Xu ZY, Liu LM and Jiang GL. Maintenance of sorafenib following combined therapy of three-dimensional conformal radiation therapy/intensity-modulated radiation therapy and transcatheter arterial chemoembolization in patients with locally advanced hepatocellular carcinoma: a phase I/II study. Radiat Oncol 2010; 5: 12.

[37] Suen AW, Galoforo S, Marples B, McGonagle M, Downing L, Martinez AA, Robertson JM and Wilson GD. Sorafenib and radiation: a promising combination in colorectal cancer. Int J Radiat Oncol Biol Phys 2010; 78: 213-220.