Effectiveness of tumor-treating fields to reduce the proliferation and migration of liposarcoma cell lines

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Abstract. Liposarcoma (LPS) is a rare type of soft tissue sarcoma that constitutes 20% of all sarcoma cases in adults. Effective therapeutic protocols for human LPS are not well-defined. Tumor-treating fields (TTFields) are a novel and upcoming field for antitumor therapy. TTFields combined with chemoradiotherapy have proven to be more effective than TTFields combined with radiotherapy or chemotherapy alone. The present study aimed to assess the effectiveness of TTFields in inhibiting cell proliferation and viability for the anticancer treatment of LPS. The present study used TTFields (frequency, 150 kHz; intensity, 1.0 V/cm) to treat two LPS cell lines (94T778 and SW872) and analyzed the antitumor effects. According to trypan blue and MTT assay results, TTFields markedly reduced the viability and proliferation of LPS cell lines along with the formation of colonies in three-dimensional culture. Based on the Transwell chamber assay, TTFields treatment also markedly reduced the migration of LPS cells. Furthermore, as shown by the higher activation of caspase-3 in the Caspase-3 activity assay and the results of the reactive oxygen species (ROS) assay, TTFields increased the formation of ROS in the cells and enhanced the proportion of apoptotic cells. The present study also investigated the inhibitory effect of TTFields in combination with doxorubicin (DOX) on the migratory capacity of tumor cells. The results demonstrated that TTFields treatment synergistically induced the ROS-induced apoptosis of LPS cancer cell lines and inhibited their migratory behavior. In conclusion, the present study demonstrated the potential of TTFields in improving the sensitivity of LPS cancer cells, which may lay the foundation for future clinical trials of this combination treatment strategy.

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Introduction

The most widely occurring soft-tissue sarcoma is human liposarcoma (LPS) as it constitutes 24-45% of all soft-tissue sarcomas (1,2). Effective therapeutic methods for treating this sarcoma are underdeveloped despite of its wide occurrence and that poses certain issues as metastatic diseases cannot be treated via surgery, radiotherapy, or chemotherapy (3). LPS has 3 main subcategories based on its histopathological manifestations: i) Well-differentiated (WDLPS) or de-differentiated liposarcomas (DDLPS), ii) myxoid or round cell LPS (MRC), and iii) pleomorphic LPS (4). For the majority of LPS cases (85-90%), a fusion of surgery as well as radiotherapy has been proven to be successful in hindering its reappearance at the surgical site (5). However, such outcomes differ based on the subtype of sarcoma. Radiation therapy is usually used before, after or even during the surgery to eliminate the malignant cells and to reduce their reoccurrence at the same site. The effectiveness of chemotherapy for curing liposarcoma is yet undefined, and, in the metastatic or unresectable setting, various liposarcomas are considered relatively chemotherapy-resistant and there is no consensus to warrant the use of systemic treatment currently in the adjuvant or neoadjuvant setting. However, it is used in certain scenarios when the patients are at a critical stage or when there is a high chance for reoccurrence of the tumor (6). Surgical resection also remains the definitive management, and the vast majority of extremity WDLS can be resected with negative margins, and their clinical behavior does not warrant the use of chemotherapy in either the adjuvant or neoadjuvant setting. Thus, due to therapeutic limitations, new treatment regimens and a better understanding of LPS are needed to address these drawbacks.

Tumor treating fields (TTFields) are an emerging field that offers a non-invasive anticancer therapy model. TTFields (also known as alternating electric field therapy) make use of transcutaneous delivery of alternating electric fields of low-intensity (1-3 V/cm) and intermediate-frequency (100-300 kHz), which apply biophysical forces on charged as well as polarizable

molecules called dipoles (7,8). The effectiveness of TTFields as anticancer therapy is affected by the duration of the treatment (for example, the application of TTFields for more than 18 h per day has been proven to improve the patient's survival), the intensity of the electrical field (where increased intensity is directly proportional to reducing tumor proliferation), and electrical field frequency (the application for which differs between the different cancers) (9). TTFields has been proven to hinder tumor growth and induce the elimination of tumor cells in murine and human cell models (10) via impeding the proper development of the mitotic spindle apparatus and the activation of the mitotic spindle checkpoint (7,11). This causes the blebbing of the plasma membrane and disturbs cell division, which would ultimately lead to the segregation of abnormal chromosomes, disrupts cell-division cycle, and the production of injured cell, subsequently leading to cell death or apoptosis (12-15). TTFields have been approved by the Food and Drug Administration (FDA) in the United States as a modality for monotherapy for newly diagnosed and recurrent GBM, according to the results of the EF-11 trial (16) and clinical trials of humans who are being treated for some other tumor types. Moreover, many preclinical studies (both lab and animal studies) that utilize TTFields are already in progress for various cancers, such as breast, cervical, stomach, and liver cancers, etc. (7,17-20). Some of these studies indicate that TTFields may have better effectivity with other anti-cancer therapies such as chemotherapy, immunotherapy, and radiation therapy, leading to a synergistic effect. For treating LPS, the main modality used as curative therapy is surgical resection. Moreover, large liposarcomas at an extreme stage or those occurring in the retroperitoneal area have a high local reoccurrence rate (15 and 75%) and a generally low survival rate in patients (21). In such cases, inculcating neo-adjuvant approaches like chemotherapy or radiotherapy, might be useful in improving the local control, although such advancements have been scarce in improving the survival rate for the disease in the last two decades (22,23).

Thus, this study investigates the effectivity of TTFields on treating liposarcoma and their capability in hindering the proliferation and migration of tumor cells in preclinical study.

Materials and methods

Experimental setup of the electric fields. TTFields was generated using a pair of insulated wires connected to a functional generator and a high-voltage amplifier, which generated sine-wave signals ranging from 0 to 800 V and resulted in an applied electric field intensity and frequency of 0.9 V/cm and 150 kHz, respectively (14,24). We used 0.9 V/cm as the field intensity because of its use in clinical settings. For TTFields treatment, cells were plated in 100-mm dishes and incubated at 37˚C under humidified conditions and 5% CO_2 atmosphere until they reached 70-80% confluency.

Cell culture. Human liposarcoma SW872 (HTB-92-ATCC) and 94T778 (ATCC CRL-3044) cancer cells were purchased from the ATCC (Manassas, VA, USA) and cultured in RPMI 1640 medium (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.

Cell viability assay. To evaluate the effect of cell viability, it was determined by trypan blue exclusion assay (20). An equal volume of trypan blue reagent was added to a cell suspension, and the percentage of viable cells was evaluated using microscopy. Assays were performed in triplicate.

Water-soluble tetrazolium (WST-1) assay. For the cytotoxicity assay to evaluate the proliferation rate, liposarcoma cells were seeded in 96-well culture plastic plates at a density of $1x10^3$ cells per well. TTFields was added to the dishes and the cells were incubated for 48 h followed by application of the water-soluble tetrazolium (WST)-1 cytotoxicity assay reagent (Roche Diagnostics, Laval, Quebec, Canada: CAS No.150849-52-8) per the manufacturer's recommendations. Cell viability was assessed by determining the A450 nm of the cell culture media after adding WST-1 for 2 h. The results were reported as a percentage of the optical density of the untreated control cells, which was designated as 100% cell viability. Percentage of cytotoxicity was calculated as follows: (1-Aexp/Acontrol) x100; where Aexp and Acontrol are the absorbance values of the experimental drug-treated and control untreated cells, respectively.

Three-dimensional (3D) culture system. Human SW872 and 94T778 liposarcoma cells were seeded in 96-well plates at $1x10^4$ cells/well to inhibit the proliferation by TTFields. In the 3D culture model, 96-well plates were pre-coated with Matrigel as a basement membrane by adding 40 μl of Matrigel to each well followed by incubation at 37˚C for 30 min. Cells were plated onto the gel in an appropriate medium, and wells were photographed after a duration of 10 d.

Colony-forming assay. Liposarcoma cancer cells (500-1,000) were seeded into 6-well plates in triplicate and treated with TTFields (1.0 V/cm; 150 kHz), doxorubicin (Sigma-Aldrich, St. Louis, MO, USA) (5 μM) or both concurrently for 48 h to evaluate the proliferation after each treatment. After 14-20 d, colonies were fixed with 100% Methanol and stained with 0.4% crystal violet (Sigma, St Louis, MO, USA).

Cell death detection assay. To evaluate the cell death after TTFields treatment, cells were treated, harvested, and stained with Cell Death Detection ELISA kit (Roche Diagnostics GmbH: 11774425001) in accordance with the manufacturer's protocols (25). Cell death was then measured using Multiskan EX (Thermo Fisher Scientific, Germany) at 450 nm.

Caspase-3 activity assay. To evaluate the pNA light emission can be quantified using a spectrophotometer or the activity of caspase3 after TTFields treatment, Caspase-3 activity was analyzed in the SW872 and 94T778 cell lines 72 h after concurrent treatment with TTFields (1.0 V/cm; 150 kHz) and 5 μM doxorubicin using detection kits (Caspase-Glo 3/7 assay kit: G8091, Promega, Madison, WI, USA). The assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrates of DEVD-pNA (for caspase-3). The microtiter plate reader

at 405 nm. Comparison of the pNA absorbance of apoptotic and control samples allows the determination of the fold increase in caspase activity.

ROS assay. Liposarcoma cells were cultured, and harvested at the indicated times, according to the manufacturer's protocol using Cellular ROS Assay Kit (ab113851) to confirm the relationship between ROS production and the enhancement of TTFields-induced apoptosis, and ROS was then measured using Multiskan EX (Thermo Fisher Scientific, Germany) at 450 nm (26).

Transwell chamber assay. The migratory ability of liposarcoma cells was measured using Transwell chambers (Corning Costar, Cambridge, MA, USA) according to the manufacturer's protocol and reference (27). Briefly, cells were seeded onto the membrane of the upper chamber of the Transwell at a concentration of $4x10^5$ cells/ml in 150 μl of medium and were left untreated or treated with TTFields for 24 h. The medium in the upper chamber was serum-free, whereas the medium in the lower chamber contained 10% (v/v) FBS as a source of chemo-attractants. Cells that passed through the Matrigel®/gelatin-coated membrane were stained with Cell Stain Solution containing crystal violet supplied in the Transwell chamber assay (Chemicon, Millipore, Billerica, MA, USA) and photographed after a 24-h incubation period.

Statistical analysis. Statistical significance was determined using one-way ANOVA and Tukey's post hoc test. Values represent the mean of three experimental repeats ± SD. Data analysis was performed using the GraphPad Prism 6 software (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of TTFields on the proliferation of liposarcoma cancer cell lines. To determine the optimal TTFields voltage and frequency, SW872 and 94T778 cells were subjected to various conditions (Voltage, 0, 1.0, 1.2 and 1.5 V/cm; frequency, 0, 100, 150, and 200 kHz) for 48 h (Fig. 1A). The two liposarcoma cancer cell lines exhibited a voltage-dependent reduction in cell viability (~20% at 1.0 V/cm; 150 kHz). As a result of processing the frequency of various conditions, the viability of the cell was the most reduced at 150 kHz, the condition used in general various cancer types (28-30). As shown in Fig. 1B and C showed at first, we indicated that TTFields restricted the proliferation of cells as well as their viability *in vitro*, utilizing a trypan blue exclusion and WST-1 assays within a time-dependent way in SW872 and 94T778 cells. Moreover, cell colonies in untreated 3D cultures were larger in comparison to those formed by TTFields-treated cells (Fig. 1D). Colony forming assays were incorporated for understanding similar effects *in vitro* (Fig. 1E). Collectively, these findings suggest that TTFields can inhibit the proliferation of LPS.

Apoptosis and migration on liposarcoma is amplified by TTFields. To observe the effect of TTFields inducing apoptosis on LPS, we analyzed early apoptosis using a cell death detection kit. In LPS cell lines, it was noticed that a 72-h TTFields exposure considerably increased the amount of cells undergoing apoptosis (Fig. 2A). Subsequently, we studied whether TTFields enhanced cytotoxicity was caused due to an increased activation of caspase, leading to increased apoptotic cell death. An increase in the activation of caspase-3 in response to TTFields treatment was analyzed in comparison to the control group (Fig. 2B). ROS are small molecule metabolites of oxygen that tend to participate in redox reactions because of their high reactivity (31). A link was observed between the production of ROS and the enhancement of TTFields induced apoptosis. The production of ROS was synergistically caused by TTFields for treating liposarcoma cancer cell lines (Fig. 2C) and that ROS created by the TT Fields treatment increases intracellular caspase signaling and, consequently, apoptosis. Next, the effects of TTFields on liposarcoma cells' migratory capacity was evaluated using Matrigel chamber assays, which demonstrated that treatment using TTFields majorly impeded the cell migration compared to the control group (Fig. 2D).

Doxorubicin sensitizes LPS to TTFields. Doxorubicin was the most common regimen as 1st line therapy for soft-tissue sarcomas (32). To investigate the effect and mechanism of enhancing the therapeutic efficacy of Doxorubicin and TTFields combined treatment for LPS, we first confirmed the cell viability. To analyze the effect of DOX on LPS cells via WST-1 assay, SW872 and 94T778 cells were treated with different quantities of DOX for understanding the effect of DOX on LPS (Fig. 3A). After 48 h, an inhibition of cell growth was observed with it being statistically relevant in cells that were treated with ≥5 μg/ml DOX ($P<0.05$). Moreover, the data showed that SW872 and 94T778 cells were sensitive to DOX and were dependent on their concentration. The treatment using the combination of DOX and TTFields produced significantly higher antitumor effects on SW872 and 94T778 cells compared to either treatment being done alone through the use of trypan blue cell viability and WST-1 assays (Fig. 3B and C). In addition, formation of tumor colonies in combination-treated cells were smaller than single-treated 3D cultures (Fig. 3D). In the colony forming assay, survival fraction values were reduced in the combination containing TTFields and DOX compared with that of single treatment on liposarcoma (Fig. 3E).

Combined effect of TTFields and DOX on apoptosis and migration of liposarcoma cells. To investigate the capacity of Doxorubicin and TTFields in inducing apoptosis, we analyzed early apoptosis using cell death detection kit. In the two liposarcoma cell lines, it was noticed that an exposure of 72 h to Doxorubicin and TTFields exhibited a remarkable increase in the amount of early apoptotic cells (Fig. 4A). Such observations underline an increase in the action of caspase3 in the combined treatment method compared to Doxorubicin used alone on LPS. (Fig. 4B). ROS production was induced more strongly under combined treatment compared to that under mono treatments (Fig. 4C) and this can explain the increase in apoptotic rate when combination treatment is used. Next, we analyzed the effects of TTFields and DOX on the migratory capacities of LPS cells using Matrigel chamber assays, which indicated that combined treatment considerably reduced cell migration in comparison with the single group on LPS (Fig. 4D).

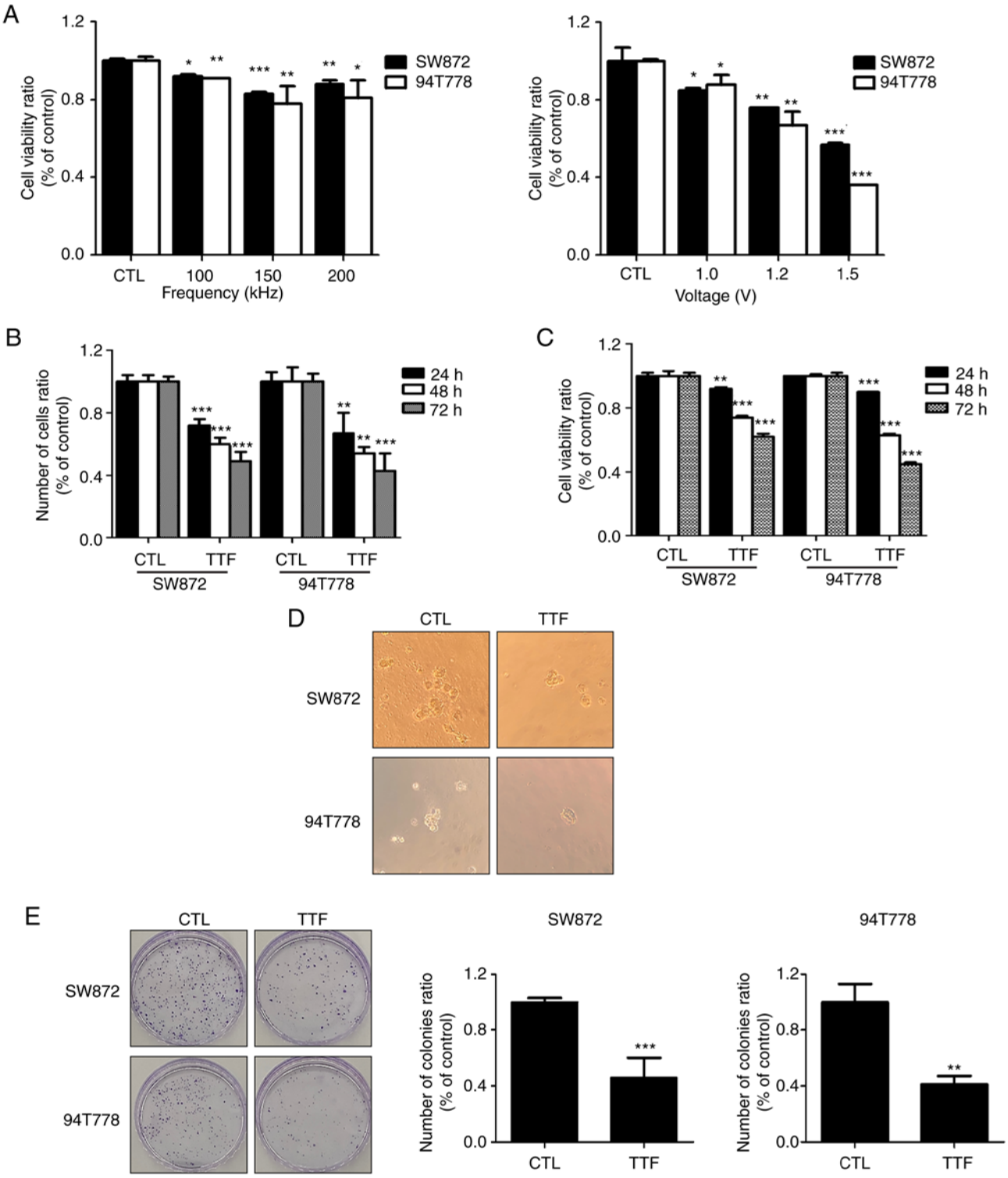

Figure 1. Effect of TTFields on the viability of liposarcoma cells. (A) The analysis of liposarcoma cancer cell viability analysis according to the frequency and the voltage. *P<0.05, **P<0.01, ***P<0.001 vs. CTL. The proliferation rate was detected by (B) cell counting and (C) WST-1 assay. **P<0.01, ***P<0.001 vs. CTL. (D) 3D colony culture (magnification, x400). (E) The sensitivity of liposarcoma cells treated with TTFields was measured via a colony formation assay. **P<0.01, ***P<0.001 vs. CTL. CTL, control; TTF, tumor-treating fields.

Discussion

As a common soft sarcoma issue, liposarcoma is observed in approximately 20% of overall sarcomas in adults (33-35). Because soft-tissue sarcomas constitute a heterogeneous group of rare tumors, management by an experienced multidisciplinary team of specialists is needed the standard of care from the time of diagnosis. Similar to numerous other sarcoma subtypes, there remains a paucity of treatment options for locally advanced or metastatic liposarcoma. Currently, only doxorubicin (36), trabectedin (37) and eribulin (38) have Phase III data to support their efficacy in advanced soft tissue

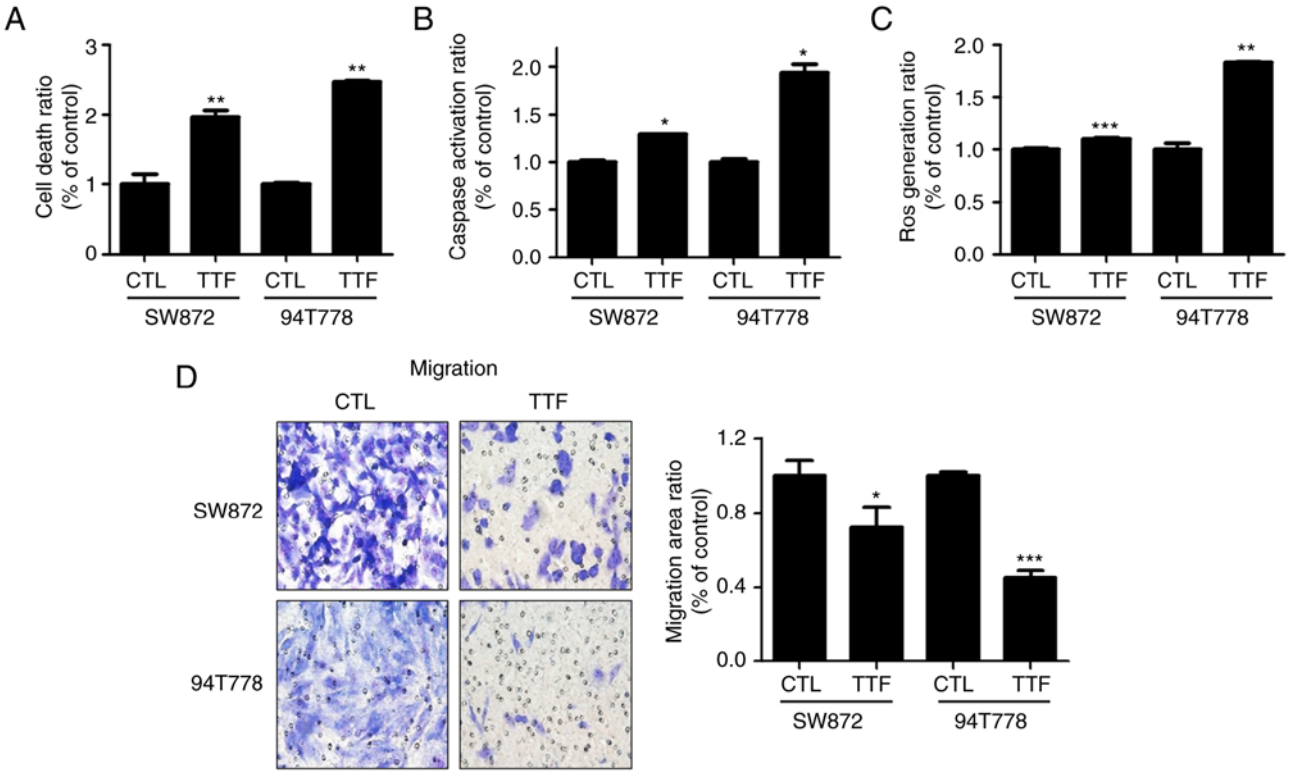

Figure 2. Effect of TTFields on the apoptosis of liposarcoma cells. (A) Analysis of cell death in two liposarcoma cell lines 72 h after treatment with TTFields by cell death detection kit. **P<0.01 vs. CTL. (B) Analysis of caspase activity in two liposarcoma cell lines 72 h after treatment with TTFields by caspase ELISA. Data were collected using a Multiskan EX at 405 nm. *P<0.05 vs. CTL. (C) Analysis of ROS in two liposarcoma cell lines 72 h after treatment with TTFields by ROS detection kit. **P<0.01, ***P<0.001 vs. CTL. (D) Tumor cell migration after 24-h TTFields (1.0V/cm, 150kHz) treatment was examined by Transwell chamber assays (magnification, x400). The number of migratory tumor cells that penetrated through the gelatin was counted using five high-intensity fields. *P<0.05, ***P<0.001 vs. CTL. CTL, control; TTF, tumor-treating fields.

sarcomas, including liposarcoma. Several emerging systemic therapeutic agents from a range of different classes have shown promise in Phase II clinical trials to date, including tyrosine kinase inhibitors (39-41) CDK inhibitors (42), mTOR inhibitors (43), thiazolidinediones (44), and Selinexor (41). Several other agents from the same classes as these agents, as well as cabazitaxel (45) and the role of immunotherapy in liposarcoma are currently under investigation in Phase II clinical trials (46). Further work in Phase III randomized clinical trials is required to explore the efficacy of these newer treatments in the management of liposarcomas, including further biomarker-led studies to investigate additional targets for treatment.

Against this backdrop, TTFields represents a noninvasive and novel therapeutic solution to the treatment of liposarcoma based on our results. Recently, tumor cocktail therapy has become a popular concept for cancer treatment and according to preclinical work, because it mainly acts through the combination of a variety of drugs to inhibit tumor growth at multiple, such as combining nano- or immunotherapy drugs to target the abnormal tumor microenvironment (TME) and prevent immune escape or cancer cell growth to the greatest extent (47). In a broad sense, we described as a combination of multiple therapeutic regimens, on LPS as like TTFields and DOX. With advancements in research, TTFields combined with chemoradiotherapy is being considered as a more effective approach than radiotherapy and chemotherapy alone, and this has been confirmed in many clinical trials (48). Currently, many other existing therapies are becoming more effective when combined with TTFields. in combination with an immune checkpoint inhibitor, TTFields are capable of functioning in a synergistic way with some cytotoxic agents on various cancer types. However, the long-term efficacy of this therapy that involves TTFields needs additional assessment for setting on LPS.

The results of this study revealed that TTFields inhibited cell proliferation and cell viability with approximately 20% viability inhibition *in vitro* in a time-dependent manner in liposarcoma cells. Our results also indicate that TTFields has an inhibitory effect on migratory abilities through TTFields combined with doxorubicin. Moreover, TTFields treatment synergistically induced ROS production in liposarcoma cancer cell lines, thereby suggesting that the TTFields-generated ROS boosts intracellular caspase signaling and apoptosis on LPS with other cancer types (49-51).

To broaden the therapeutic application, we previously published a paper describing how these similar processes are used to treat glioma, lung cancer, osteosarcoma, hepatocarcinoma, and colon cancers (49-52). These cancers were considered for our study because there is a need to investigate treatment options for cancers that are rare in addition to cancer types for which radiation therapy is currently limited. We first performed a TTFields therapy trial on liposarcoma with this intention. These clinical trials will make it easier to understand how TTFields fit into treatment plans and determine whether it is feasible to expand the availability of TTFields to treat more types of

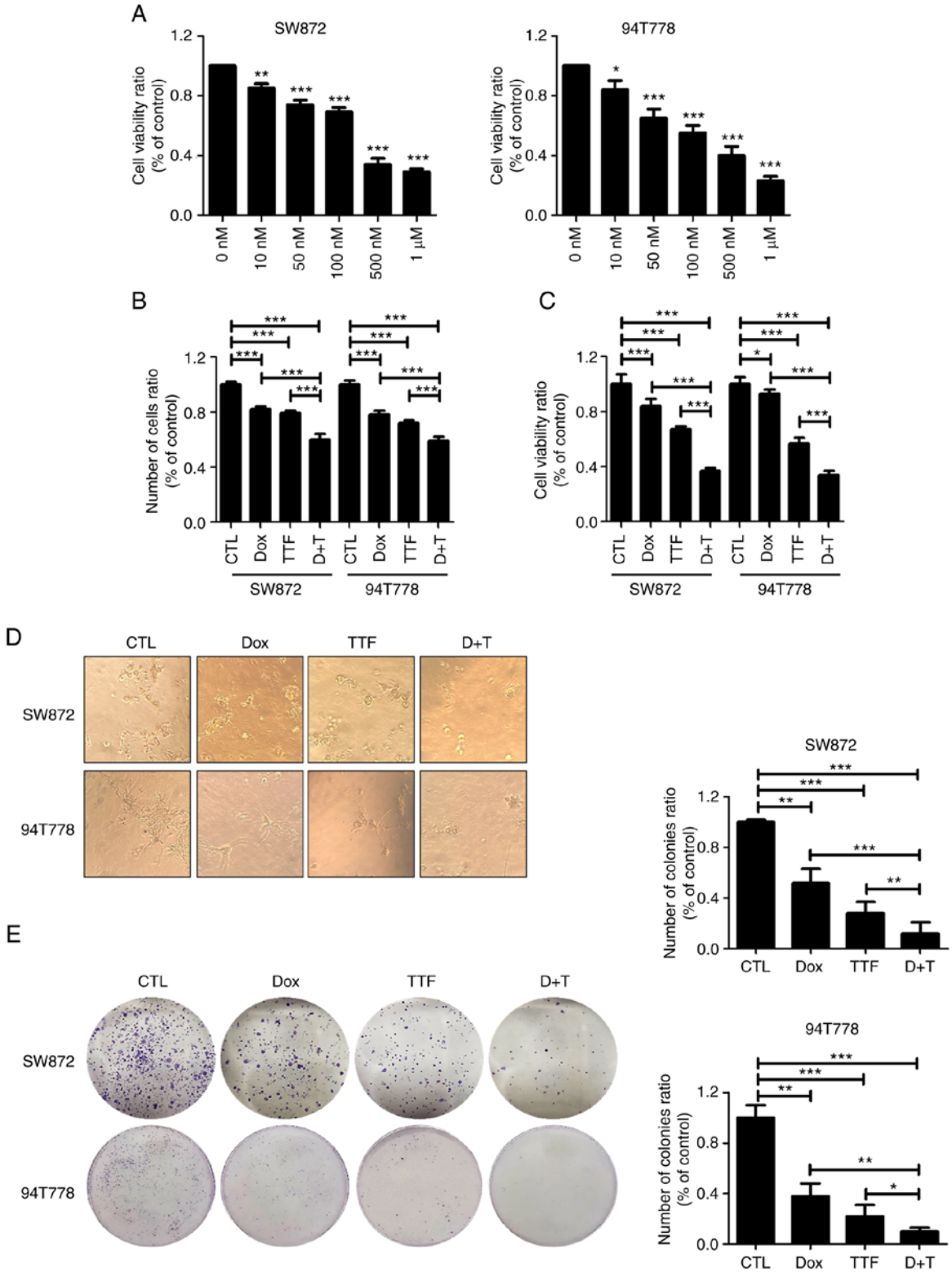

Figure 3. TTFields combined with Doxorubicin inhibit cell proliferation in liposarcoma. (A) Analysis of WST-1 assay in two liposarcoma cell lines 48 h after each treatment with TTFields by cell detection kit. *P<0.05, **P<0.01, ***P<0.001 vs. CTL. (B) Liposarcoma cells were treated with TTFields, doxorubicin, or combined treatment for 48 h, and the cell viability was determined by trypan blue exclusion assay. ***P<0.001. (C) WST-1 assay. *P<0.05, ***P<0.001. Values represent the means of three experiments. (D) 3D culture assay (magnification, x400). (E) The sensitivity of liposarcoma cells treated with TTFields was measured via a colony formation assay. *P<0.05, **P<0.01, ***P<0.001. CTL, control; TTF, tumor-treating fields; DOX, doxorubicin; D+T, doxorubicin combined with tumor-treating fields.

cancer in the future. According to numerous publications, TTFields cause disruptions in a wide range of biological activities, including autophagy, DNA repair, permeability, cell migration, and immune responses, in addition to their apoptotic effects (1,9,10,13,27,53-58). According to these reports, TTFields induce autophagy by blocking the

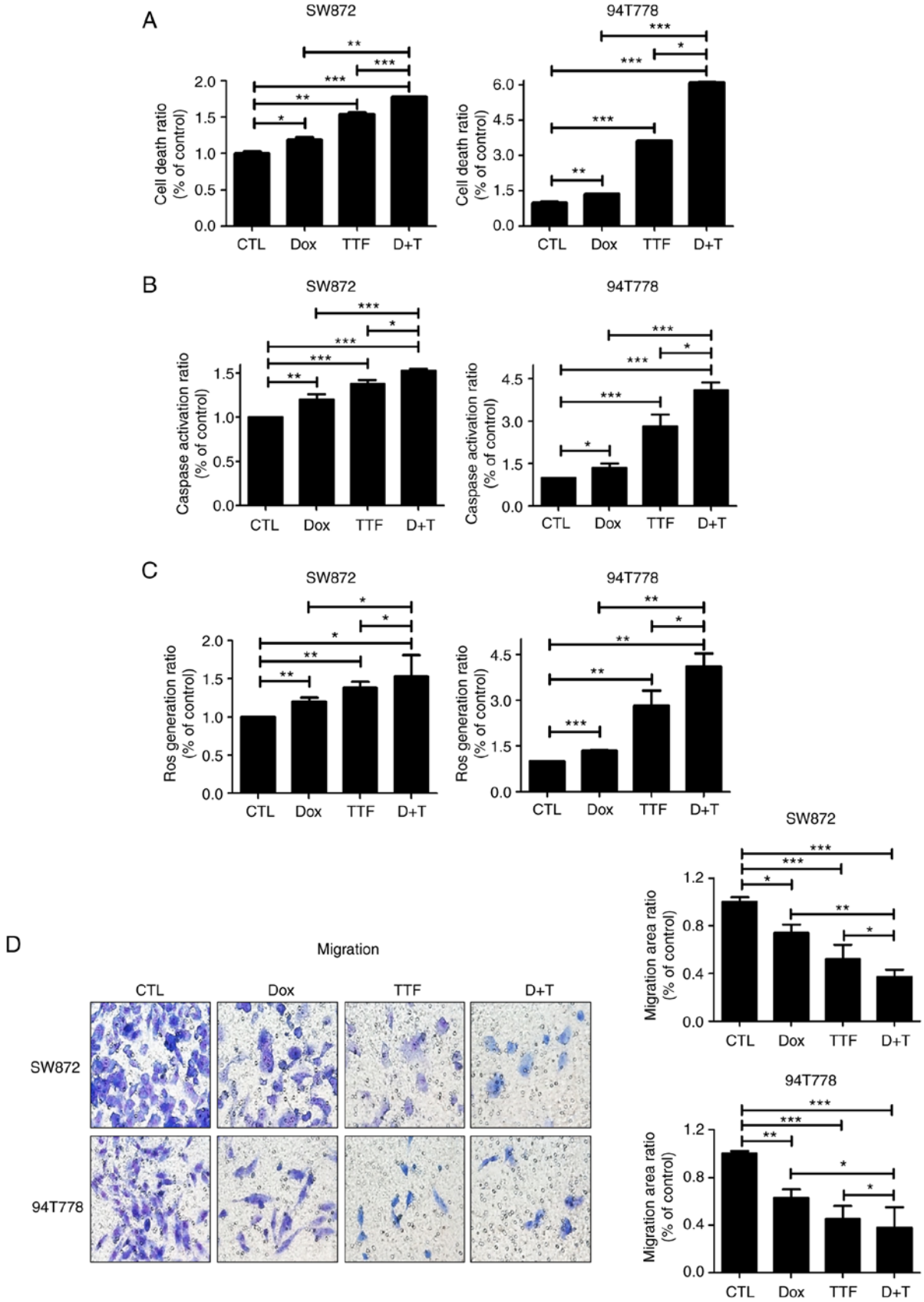

Figure 4. TTFields combined with Doxorubicin enhance cell death and inhibits the migration on liposarcoma. (A) Analysis of cell death in two liposarcoma cell lines 72 h after concurrent treatment with TTFields (1.0 V/cm, 150 kHz) and doxorubicin (5 μM) using a cell death detection kit. *P<0.05, **P<0.01, ***P<0.001. (B) Analysis of caspase activity in the two liposarcoma cell lines 72 h after treatment with TTFields and doxorubicin by caspase ELISA. Data were obtained using a Multiskan EX reader at 405 nm. *P<0.05, **P<0.01, ***P<0.001. (C) Analysis of ROS generation in two liposarcoma cell lines 6 h after treatment with TTFields (1.0 V/cm, 150 kHz) by Cellular ROS Assay Kit. *P<0.05, **P<0.01, ***P<0.001. (D) Tumor cell migration after 24-h TTFields, doxorubicin, or combined treatment examined by Transwell chamber assays. The number of migratory tumor cells that penetrated through the gelatin was counted using five high-intensity fields (magnification, x400). *P<0.05, **P<0.01, ***P<0.001. CTL, control; TTF, tumor-treating fields; DOX, doxorubicin; D+T, doxorubicin combined with tumor-treating fields.

Akt2/miR29b axis in glioblastoma cells (56) and these delay DNA damage repair following radiation treatment of glioma cells (54). And TTFields increase membrane permeability in GBM cells (57) and also induce immunogenic cell death when combined with anti-PD-1 therapy (58). Although there have been reports of many similarities between the biological mechanisms of TTFields, there have also been reports that the function of p53 is unclear. Numerous references, including my study, state that exposure to TTFields causes apoptosis through both p53-independent and p53-dependent mechanisms (14,59,60). We need to research into p53's impact using TTFields on liposarcoma.

Overall, our results show that TTFields is an effective therapeutic approach for liposarcoma; radiation or doxorubicin would be the TTFields-sensitizer based on our results demonstrated the effectiveness of TTFields as a sensitizer of 5-FU on colon cancers (61). Patient outcome enhancements have stagnated despite the emergence of revolutionary regimens that comprise traditional cytotoxic chemotherapy to treat liposarcoma over the past few decades. There is a need for optimizing clinical trials of TTFields-based tumor treatments via preclinical testing using patient samples or *in vivo* models and the application of electric fields alone or in combination with drugs.

In summary, TTFields has been found to curtail cell migration and proliferation of liposarcoma. These findings provide a molecular basis for the use of chemotherapeutic drugs as TTFields sensitizers to treat liposarcoma. The identification of TTFields seems to be key for the optimization of therapeutic strategies for liposarcoma and must a be a focus of future studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WSL participated in experiments, formal analysis and used GraphPad Prism 6 software. YJJ was involved in methodology and investigation. AHC and YHB participated in experiments. YBK and SMY were involved in formal analysis. EHK designed the project and wrote the manuscript. WSL and EHK revised the manuscript. EHK and WSL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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