Am J Cancer Res 2022;12(6):2673-2685
www.ajcr.us /ISSN:2156-6976/ajcr0143426

Original Article

Combination therapy of Doxorubicin with TTFields and radiation: newer approaches to combat lung cancer

Won Seok Lee, Eun Ho Kim

Department of Biochemistry, School of Medicine, Daegu Catholic University, 33 17-gil, Duryugongwon-ro, Nam-gu, Daegu 427-724, Korea

Received April 9, 2022; Accepted May 17, 2022; Epub June 15, 2022; Published June 30, 2022

Abstract: Background: Tumor-treating fields (TTFields) have been used singly or with chemoradiation for treating glioblastoma and mesothelioma but not yet for lung cancer. Survival rates in lung cancer remain abysmal despite advances in early diagnosis and targeted therapies. Aims and objectives: We aimed to investigate the effectiveness of TTFields in inhibiting lung cancer growth and metastasis, as well as the therapeutic effectiveness of TTFields alongside radiation and chemosensitivity-enhancing agents in an in vitro model. Methods: We generated TTFields yielding 0-800 V sine-wave signals, 0.9 V/cm applied electric field intensity, and 150 kHz frequency. The human lung cancer cell lines A549 and H460 were used in this study. Cell viability, colony formation, cell death detection, and cell invasion assays were performed to assess the therapeutic effectiveness of TTFields; sensitization of lung cancer cells to TTFields by doxorubicin (DOX); and the combined effect of TTFields, DOX, and irradiation (IR). Results: Lung cancer cells showed a nearly 20% decrease in cell viability at 1 V/cm and 150 kHz. In A549 and H460 cells, TTFields increased apoptosis through increased cleaved caspase3, hindered cell migration and invasion, and improved chemosensitivity to DOX. The combination of DOX and TTFields showed better antitumor results than those of each individually. However, the DOX/TTFields/IR combination was most effective in reducing the viability and migration of lung cancer cells. Conclusion: TTFields as an adjuvant therapy offers probability for improving lung cancer patient outcomes.

Keywords: TTFields, Doxorubicin, lung cancer, apoptosis

Introduction

Lung cancer is one of the most widely occurring cancers in the world and its rate of occurrence has increased over the years [1, 2]. Despite recent developments of lung cancer treatments using targeted therapy, survival rates are still poor. About 79% of lung cancer patients develop metastases and the 5-year survival rate of patients with distant metastases is approximately 5% [3]. Thus, metastases are responsible for the increased mortality rates in lung cancer [4]. Because of the increased mortality rates even with early diagnosis and various treatment methods, it is critical to fully understand the molecular mechanisms of metastatic progression in lung cancer and to use that knowledge to develop improved therapeutic alternatives.

According to the 2011 National Comprehensive Cancer Network guidelines for lung cancer, radiation has a pivotal role in treating all stages of lung cancer, both as definitive treatment and palliative therapy [5-7]. It is also suggested that radiation oncology could be studied under multidisciplinary evaluation such that patients can take advantage of definitive local therapy, especially those with absolute or relative contraindications to surgery as determined by their thoracic surgeons [8]. Despite the development in recent years in the fields of radiation physics and biology, and in the facilities within radiotherapy, the effectiveness of radiation therapy remains unsatisfactory. Extensive research has been conducted on radiosensitization in order to sensitize tumor cells to radiation [9]. An ideal radiation sensitizer would significantly increase the efficacy of radiotherapy while having very little or no adverse side effects on normal tissues. For this purpose, antitumor drugs like doxorubicin are mainly utilized within cancer therapy [10].

Tumor Treating Fields (TTFields) are a recently developed and distinct antineoplastic therapy comprising of alternative electric fields that are low-intensity and of intermediate frequency. Data supporting their clinical effectiveness have been gathered since their FDA approval as a single-agent therapy for recurrent glioblastoma (GBM), as adjuvant therapy with standard chemoradiation for postoperative glioblastoma, and as therapy for mesothelioma [11-13]. Preclinical studies indicate two main effects of TTFields on tumor cells, prolonged mitosis and disrupted mitotic spindle assembly as well as cell membrane destruction close to the cleavage furrow during telophase (Cancers 2021, 13, 2283). TTFields appear to exert their effects on cells undergoing division, impairing the formation of the mitotic spindle and, through the dielectrophoretic effect during telophase/cytokinesis, compromising organelles and biomolecules via impairing chromosomal segregation as well as cell division [14, 15]. These processes trigger cancer cell death, thus inhibiting tumor cell growth. However, the effect of TTFields has not been researched further beyond GBM clinical trials. Therefore, we undertook this study to analyze the effects of TTFields in inhibiting lung cancer tumor growth and metastasis [16]. This study further evaluated the therapeutic effectiveness of combining TTFields with radiation and doxorubicin in *in vitro* models and explored its underlying mechanisms.

Materials and methods

Experimental setup of the electric fields

TTFields were 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 V to 800 V and resulted in an applied electric field intensity and frequency of 0.9 V/cm and 150 kHz, respectively [17]. We used 0.9 V/cm as the field intensity because of its use in clinical settings. For irradiation 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 lung cancer cell lines (H460 and A549) were purchased from ATCC (Manassas, VA, USA) and cultured in RPMI 1640 medium supplemented with 10% FBS, glutamine, HEPES and antibiotics at 37°C in a 5% CO_2 humidified incubator.

Cell viability assays

Cell viability was determined by trypan blue exclusion [18]. An equal volume of trypan blue reagent was added to a cell suspension, and the percentage of viable cells was evaluated by microscopy.

Colony-forming assays

TTFields were applied to cells 6 hour after proton exposure and the cells were then incubated for 48 hours [19]. After 14-20 days, colonies were stained with 0.4% crystal violet (Sigma, St. Louis, MO, USA). Experiments were performed in triplicate.

Cell death detection assays

The treatment, harvesting, and staining of cells were done with a cellular death detection reagent in line with the manufacturer's instructions [20]. Multiskan EX (Thermo Fisher Scientific, Germany) was used to calibrate cell mortality at 450 nm.

ROS assays

Cells were grown and collected as specified by the manufacturer, and ROS was quantified at 450 nm through a Multiskan EX (Thermo Fisher Scientific, Germany) [21]. The fluorescent ROS indicator C2', 7'-dichlorodihydrofluorescein diacetate (H2DCFDA; 5 M; Molecular Probes) was used to visualize ROS in monocytes. FACS was used to identify fluorescent cells by engaging a FACSortTM flow cytometer and the CellQuestTM software (BD Biosciences).

Three-dimensional (3D) culture systems

Cells were plated in 96-well plates at 1×10^4 cells/well. Matrigel was used as a basement layer to pre-coat the 96-well plates in the 3D culture model where 40 ul of Matrigel was added in each well before incubation for 30 minutes at 37°C [22]. Cells were seeded on the gel in the best-fit culture medium and wells were imaged during a span of 10 days.

Analysis of transwell chambers

Transwell chambers assisted in measuring invasion *in vitro* in line with the manufacturer's recommendations [23]. Cells were cultured on the layers of transwell upper chamber at 4×10^5 cells/ml in 150 µl of milieu and treated with TTFields as needed for 24 hours. The upper chamber's medium contained no serum, and the bottom chamber's media included 10% FBS as a chemical allure. Imaging of cells that transferred through Matrigel-coated layers was done after 24 hours of incubation and cells were dyed with the crystal violet solution provided in the transwell invasion test kit (Chemicon, Millipore, Billerica, MA, USA).

Western blot analysis

Total proteins from liposarcoma cells were extracted in RIPA buffer (50 mM Tris-Cl, PH 7.4; 1% NP-40; 150 mM NaCl, and 1 mM EDTA) supplemented with protease inhibitors (1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 mM Na_3VO_4) and quantified using the Bradford method. Protein samples (30 µg) were separated by SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane [24]. After blocking non-specific antibody binding sites, the membrane was incubated overnight at 4°C with mouse monoclonal antibodies. After incubation with peroxidase-conjugated secondary antibodies at 37°C for 1 h, the protein bands were visualized using enhanced chemiluminescence reagent (GE Healthcare Biosciences, Pittsburgh, PA, USA) and detected using the Amersham Imager 680 (GE Healthcare Biosciences).

Flow cytometry

Cells were cultured and treat with radiation, TTFields or doxorubicin for 48 hr. Cells were washed with ice-cold PBS, trypsinized, and resuspended in ×1 binding buffer [10 mM HEPES/NaOH (PH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$] at 1×10^6 cells/ml. Aliquots (100 µl) of cell solution were mixed with 5 µl annexin V FITC (PharMingen) and 10 µl propidium iodide stock solution (50 µg/ml in PBS) by gentle vortexing, followed by 15 min incubation at room temperature in the dark. Buffer (400 µl ×1) was added to each sample and analyzed on a FACScan flow cytometer (FACS Canto2, BD biosciences, Franklin Lakes, NJ, USA). A minimum of 10,000 cells was counted for each sample, and data analysis was performed in CellQuest software (BD Biosciences).

Statistical analysis

Statistical significance was determined using the ANOVA statistical test followed by Prism 6 software (La Jolla, California, USA). Differences were considered significant if the *P*-value was less than 0.05 or 0.001. ($^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$).

Results

The effectiveness of TTFields in reducing the proliferation of lung cancer cells

To determine the optimal frequency for TTFields, we subjected A549 and H460 lung cancer cells to different conditions for a period 48 hours (**Figure 1A**). Both cell lines showed a frequency-dependent decrease in cell viability (approximately 20% at 1.0 V/cm, 150 kHz) up to 150 kHz and there was no significant difference between 150 kHz and 200 kHz. In order to evaluate the cytotoxicity induced by TTFields, a cell viability assay was done. It was observed that the application of TTFields for 48 hours largely decreased the proliferation of A549 and H460 cells as shown by trypan blue staining (**Figure 1B**) and MTT assays (**Figure 1C**). Furthermore, we noticed that colonies formed by TTFields-treated cells were smaller than those formed in untreated 3D cultures (**Figure 1D**). Additionally, the survival fraction showed a reduction in clonogenic efficiency of 42% in A549 cells and 46% in H460 cells after treatment (**Figure 1E**). Altogether, these findings show that TTFields have the capacity to limit the proliferation of lung cancer cells.

Improvement in apoptosis and metastatic inhibition of lung cancer cells by TTFields

A cell death detection kit was used to understand whether the apoptosis of lung cancer cells was triggered by TTFields. It was observed that exposure to TTFields for 72 hours considerably increased the number of cells undergoing apoptosis in both lung cancer cell lines (**Figure 2A**). To investigate whether TTFields initiate cellular apoptosis on lung cancer, we conducted apoptosis by annexin V and PI staining using Flow cytometry. The number of cells

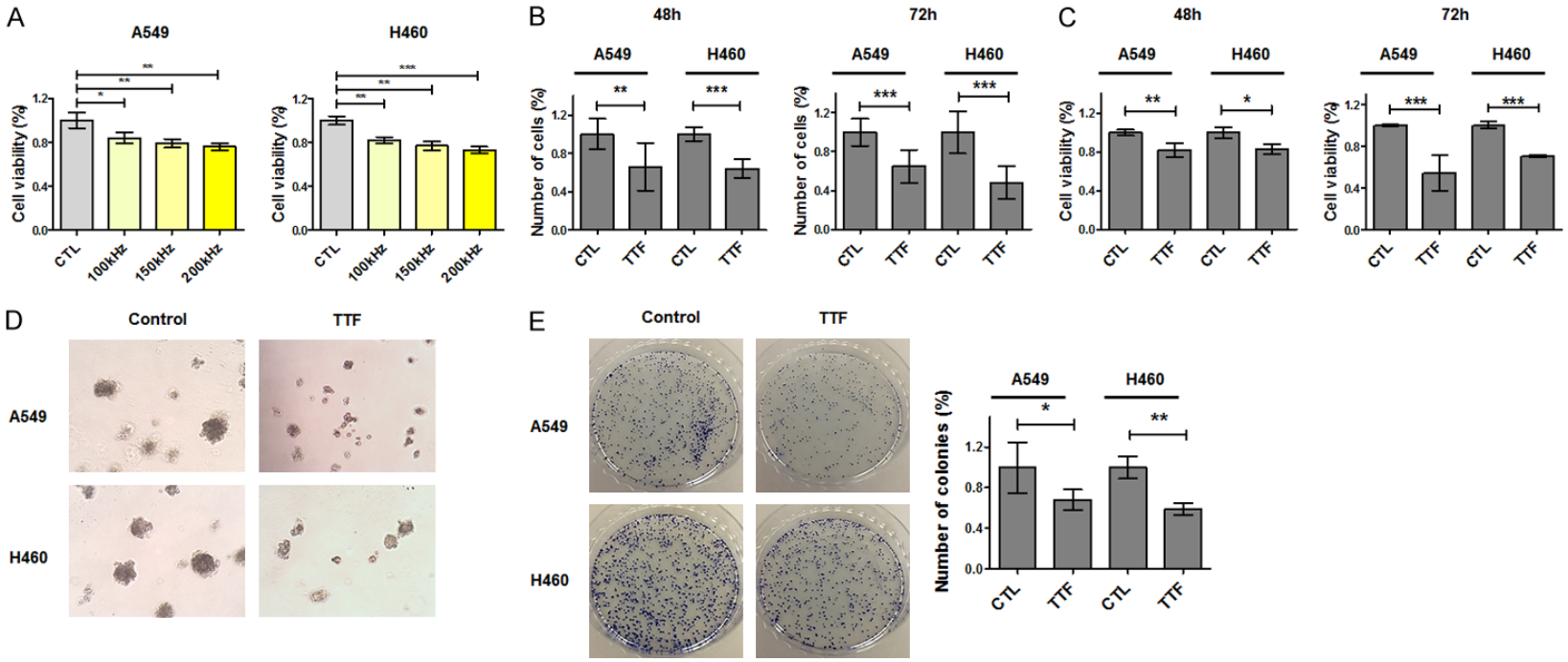

Figure 1. Effects of TTFields on the viability of lung cancer cells. (A-C) The lung cancer cell viability analysis according to frequency (A), cell counting by tryphan blue (B), and the MTT assay (C). *P<0.05, **P<0.01, ***P<0.001. (D) 3D colony cultures after the application of TTFields for 48 h. (E) The sensitivity of lung cancer cells treated with TTFields was measured via the colony forming assay. The survival fraction was determined as colonies counted/(cells seeded × plating efficiency/100). *P<0.05, **P<0.01.

apoptosis of lung cancer cells increased due to 48 hours of TTFields (**Figure 2B**). Furthermore, we analyzed whether the enhanced TTFields-induced cytotoxicity caused further activation of caspase-3, known as the chief mediator of cell death. Our results indicated increased caspase-3 activation in response to TTFields in comparison with the control group (**Figure 2C**). Moreover, ROS production was also caused by treating lung cancer cell lines with TTFields (**Figure 2D**), which suggests that TTFields treatment generated ROS that boosted intracellular caspase signaling that led to apoptosis. Next, we analyzed the effect of TTFields on the invasive and migratory capacities of lung cells using Matrigel chamber assays, and showed that treatment with TTFields considerably hindered cell migration in comparison with the control group (**Figure 2E**). Similarly, in the Matrigel invasion assay, TTFields treatment appeared effective at inhibiting the invasive behavior of both lung cancer cell lines, by 38% and 49% respectively (**Figure 2F**). In conclusion, we found TTFields treatment to considerably reduce tumor cell motility as well as invasiveness.

Sensitization of lung cancer cells to TTFields by doxorubicin

In order to analyze the effectiveness of doxorubicin (DOX) on lung tumor cells by the MTT assay, A549 and H460 cells were treated with various concentrations of DOX (**Figure 3A**). After 24 hours, it was observed that cell growth was inhibited, which was evident even in cells treated with less than 5 μg/ml of DOX ($P<0.05$). These data also showed the sensitivity of A549 and H460 cells to DOX, which was again concentration dependent. Moreover, the combination of DOX and TTFields showed better antitumor results compared to monotreatment based on trypan blue staining and MTT assays (**Figure 3B**, **3C**). Additionally, colonies in combination-treated cells were smaller than those in single-treated 3D cultures (**Figure 3D**). In the colony forming assay, it was also observed that the survival rate was synergistically lower with the combination of TTFields and DOX as compared to that of single treatment (**Figure 3E**).

Joint effect of TTFields and DOX for the apoptosis of lung cancer cells

To investigate whether doxorubicin and TTFields induce apoptosis, we analyzed apoptosis through Annexin V and propidium iodide staining and cell death detection kit. The two lung cancer cell lines were exposed to a combination of doxorubicin and TTFields for 48 hours and a considerable increase in the number of apoptotic cells were observed (**Figure 4A**). Apoptosis assay was performed using Flow cytometry to confirm that doxorubicin and TTFields collaboratively induce apoptosis. The number of apoptotic cells of lung cancer cells increased due to 48 hours of doxorubicin and TTFields (**Figure 4B**). We then focused on the activity of caspase3 to analyze if its increased stimulation could boost the cytotoxicity of the combination therapy. The results showed a noticeable boost in the activity of caspase3 with combination treatment as opposed to monotherapy with doxorubicin (**Figure 4C**). Apoptotic cell death also increased after combination treatment. Moreover, the relationship between ROS production and the boost of TTFields-induced apoptosis using doxorubicin was observed. We found that ROS production intensified more with combination treatment than with the monotreatment method (**Figure 4D**). This helps explain the increase in the apoptotic rate during the combination treatment. We then investigated the effectiveness of TTFields and DOX on the migratory abilities of lung cancer cells using Matrigel chamber assays, which indicated that treatment based on TTFields considerably inhibited cell migration compared to the control group (**Figure 4E**).

Cytotoxic effects of the combined treatment method using DOX and TTFields after irradiation on lung cancer cell lines

Figure 5A and **5B** show the results from cell viability analysis of A549 and H460 lung cancer cells cultured with 5 mM DOX and TTFields after irradiation for 24 and 48 hours, respectively. Significantly reduced viabilities of cells were observed for IR/TTFields/DOX treatment in both lines. Clonogenic survival of lung cancer cells after the combined treatment with doxorubicin and TTFields after irradiation is shown in **Figure 5A** and **5B**, respectively. Additionally, the colonies formed by mono-treated 3D cultures were larger than those formed upon combinatorial treatment (**Figure 5C**). IR/TTFields/DOX treatment groups showed the smallest size of spheres. The survival fraction (SF) of A549 and H460 cells, treated with radiation as

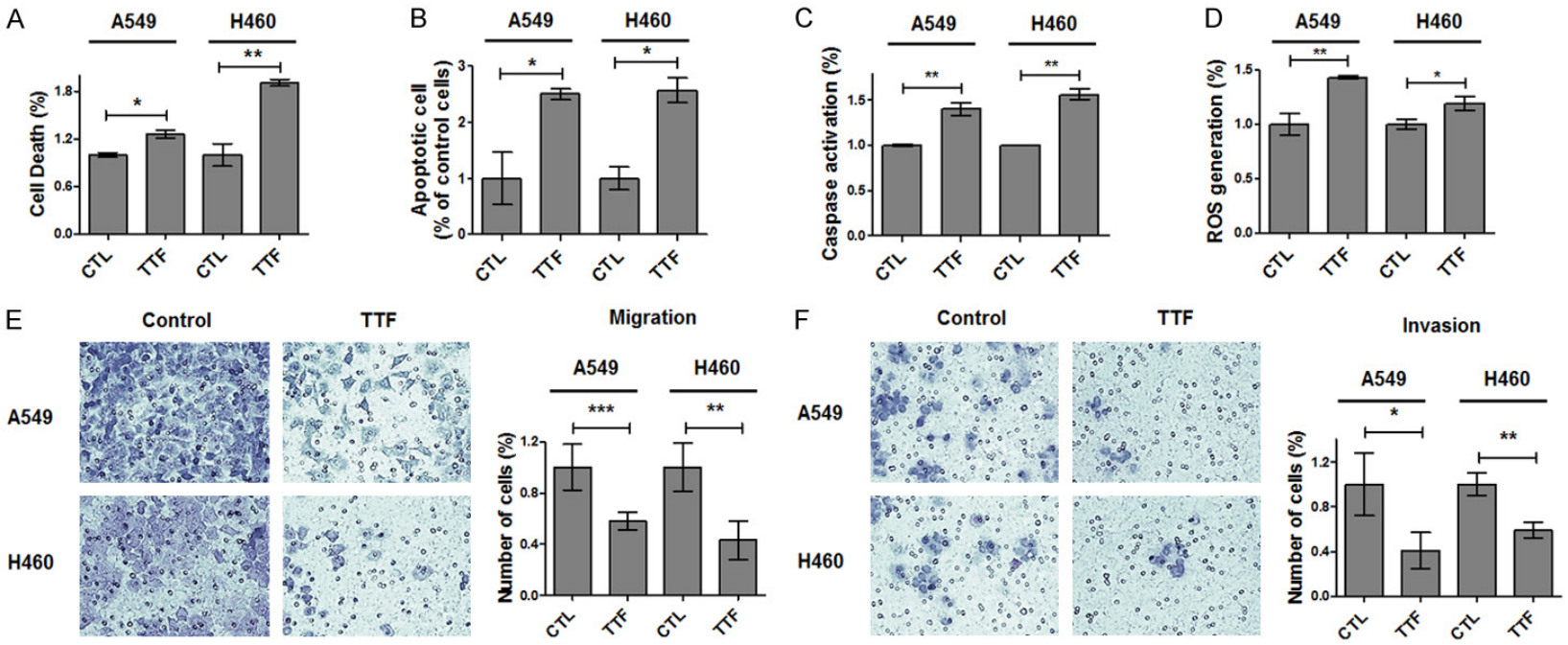

Figure 2. Effects of TTFields on the apoptosis of lung cancer cells. A. Analysis of cell death in lung cancer cells 72 h after treatment with TTFields by the cell death ELISA kit. Data were collected using a Multiskan EX at 405 nm. *P<0.05, **P<0.01. B. Cells were exposed to TTFields for 48 h and FACS analysis was performed. *P<0.05, **P<0.01, ***P<0.001. C. Analysis of caspase activity in lung cancer cells 72 h after treatment with TTFields by the caspase 3/7 detection kit. **P<0.01. D. Analysis of ROS in the two lung cancer cell lines 72 h after treatment with TTFields by the ROS detection kit. *P<0.05, **P<0.01. E, F. Tumor cell migration and invasion after 24 h of TTFields treatment was examined using Transwell chamber assays. Representative microscopy images 400×. The number of invading tumor cells that penetrated through the Matrigel was counted in five high-intensity fields. *P<0.05, **P<0.01, ***P<0.001.

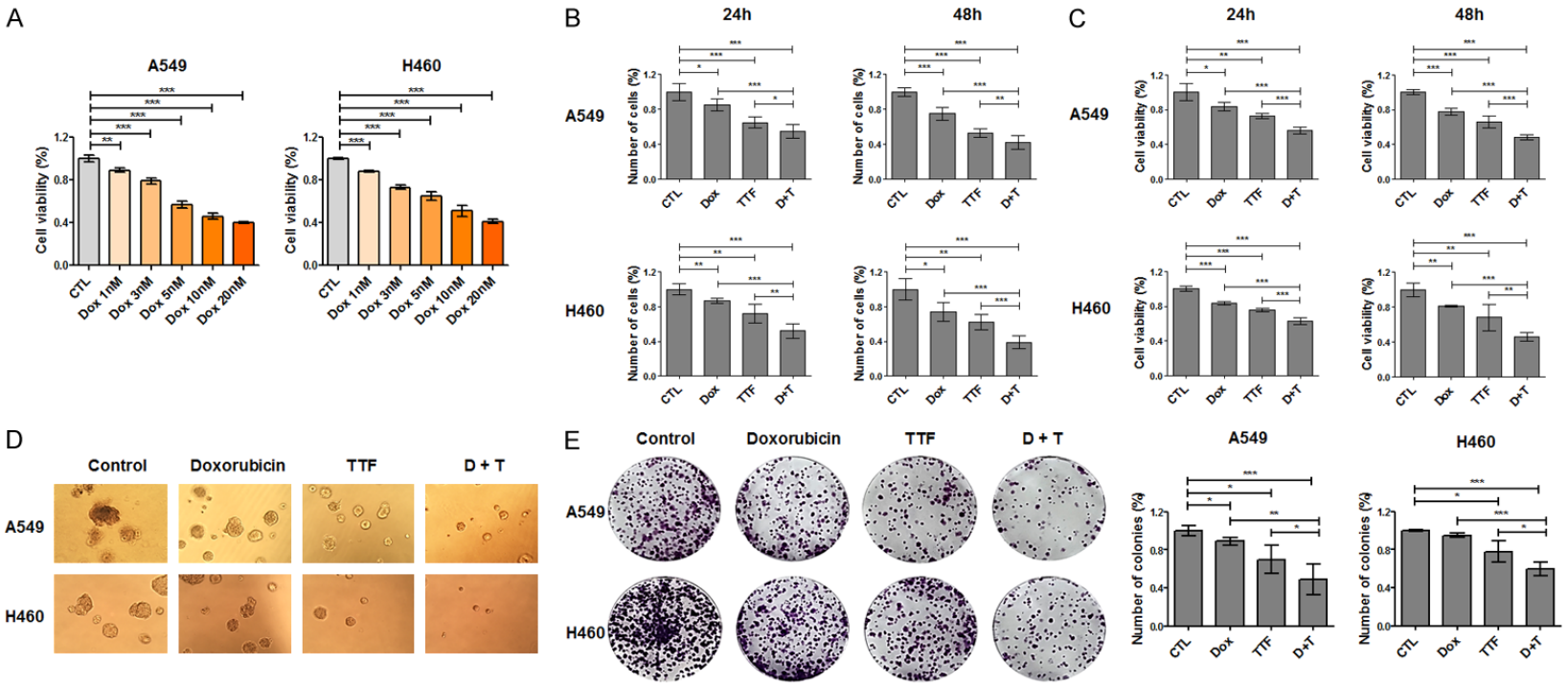

Figure 3. TTFields sensitize lung cancer cells to doxorubicin. (A) To evaluate the effect of doxorubicin by the MTT assay, lung cancer cells were treated with different concentrations of doxorubicin for 24 h. **P<0.01, ***P<0.001. Lung cancer cells were treated with TTFields alone, doxorubicin alone, or both for 24 h and 48 h, and cell viability was determined using the trypan blue (B) or MTT assay (C). *P<0.05, **P<0.01, ***P<0.001. (D) 3D colony culture and colony forming assays after the application of TTFields alone, doxorubincin alone, or both for 48 h. *P<0.05, **P<0.01, ***P<0.001. (E) The sensitivity of lung cancer cells treated with TTFields and doxorubicin was measured via the colony forming assay.

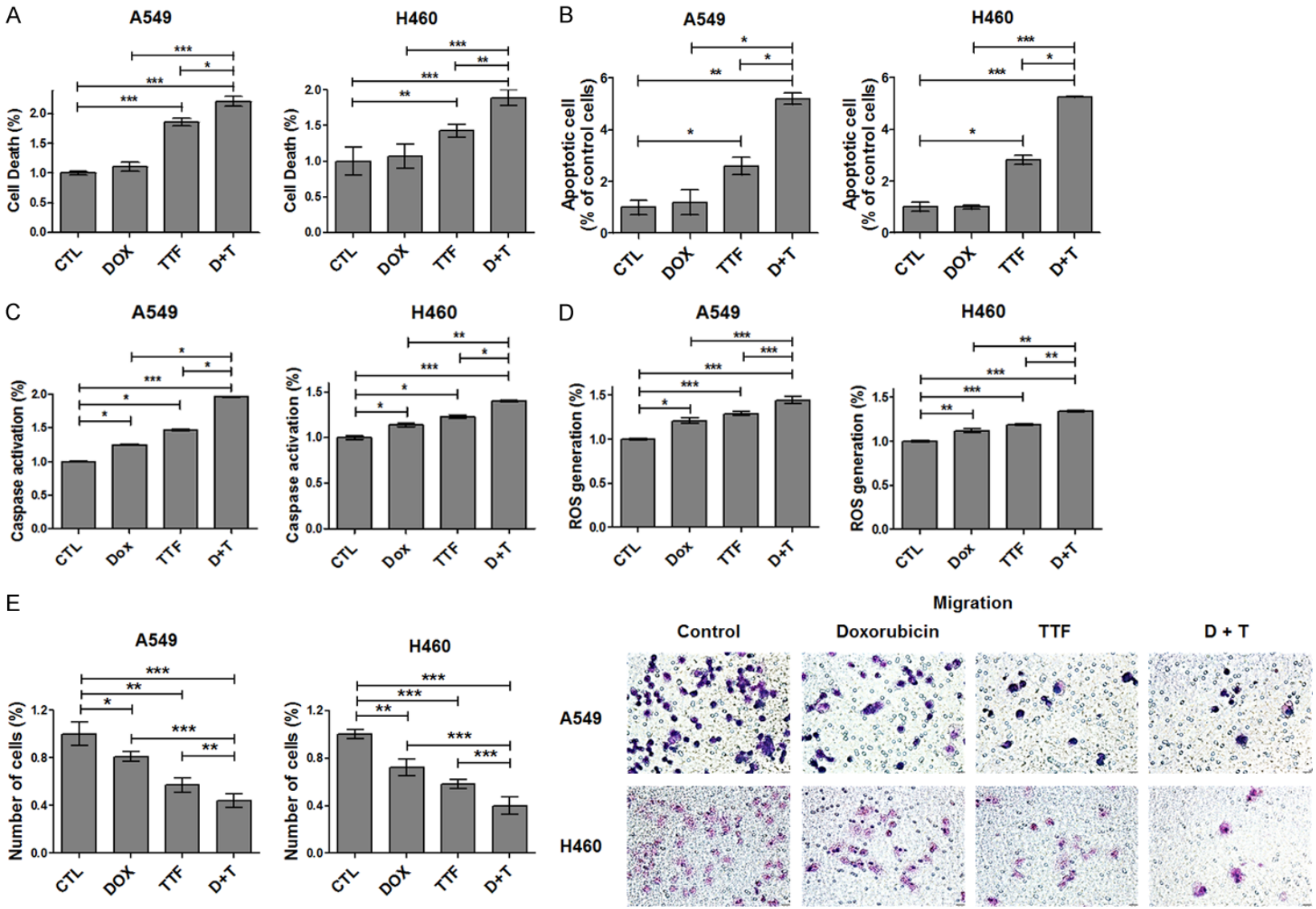

Figure 4. TTFields promote apoptosis in lung cancer cells in response to doxorubicin. A. Analysis of cell deathin lung cancer cells 72 h after treatment with TTFields by the cell death ELISA kit. Doxorubicin was added for 48 h to 72 h. Data were collected using a Multiskan EX at 405 nm. *P<0.05, **P<0.01. ***P<0.001. B. Cells were exposed to doxorubicin and TTFields for 48 h and FACS analysis was performed. *P<0.05, **P<0.01, ***P<0.001. C. Analysis of caspase activities in lung cancer cells 72 h after treatment with TTFields by the caspase 3/7 detection kit. Doxorubicin was added for 48 h to 72 h. *P<0.05, **P<0.01. ***P<0.001. D. Analysis

of ROS in the two lung cancer cell lines 72 h after treatment with TTFields by the ROS detection kit. Doxorubicin was added for 48 h to 72 h. *P<0.05, **P<0.01, ***P<0.001. E. Tumor cell migration after 24 h of TTFields and doxorubicin treatment was examined using Transwell chamber assays. Representative microscopy images 400×. *P<0.05, **P<0.01, ***P<0.001.

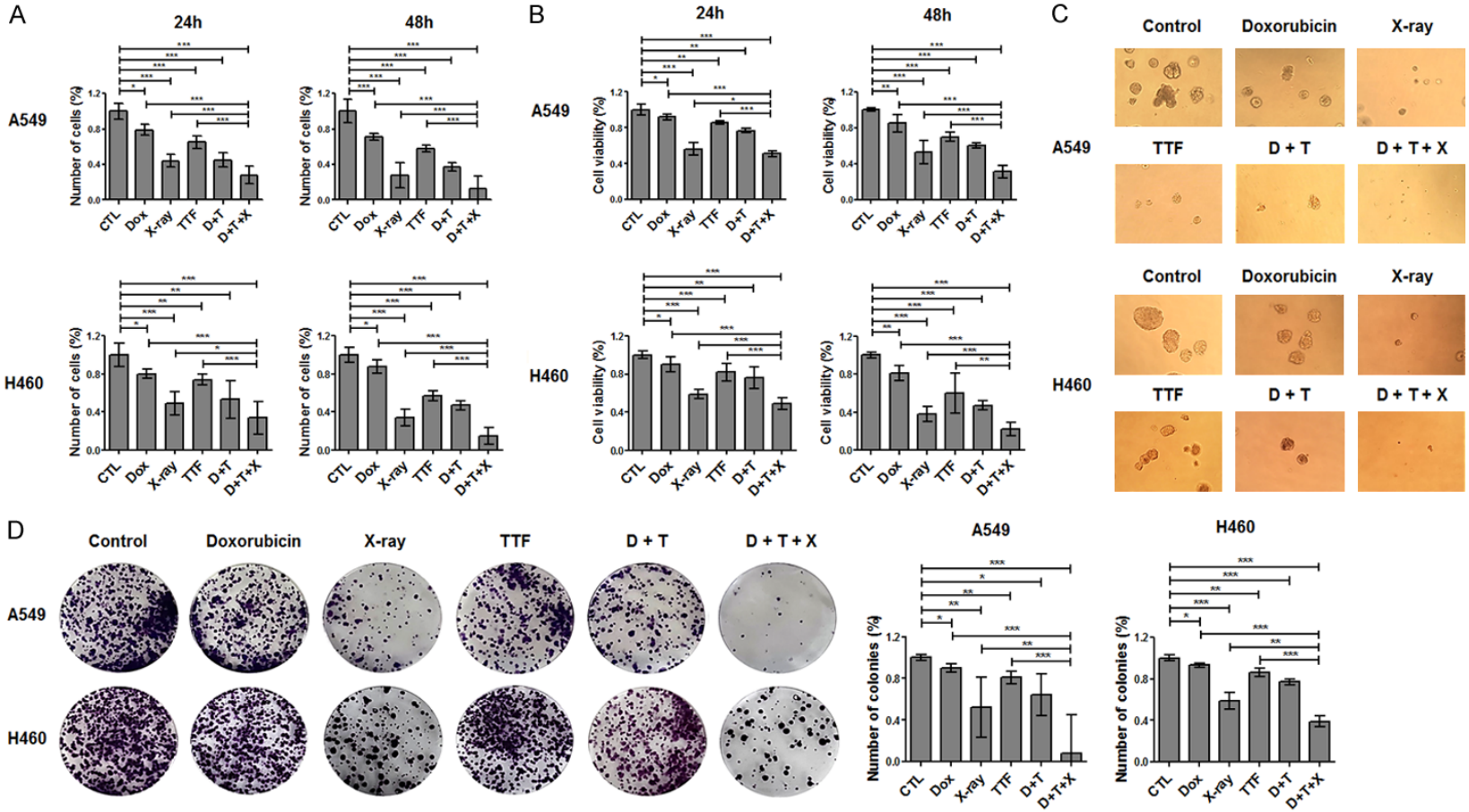

Figure 5. Effects of combinatorial treatment with TTFields, doxorubicin, and X-ray on the cytotoxicity of lung cancer cells. (A, B) Lung cancer cells were treated with TTFields and doxorubicin for 24 and 48 h, respectively, followed by X-ray irradiation. The analysis of lung cancer cell viability was done using cell counting by tryphan blue (A) and the MTT assay (B). *P<0.05, **P<0.01, ***P<0.001. (C) 3D colony culture and colony forming assays after the application of TTFields, doxorubincin, or both together for 48 h, followed by X-ray. (D) The sensitivity of lung cancer cells treated with TTFields and doxorubicin followed by X-ray was measured via the colony forming assay. *P<0.05, **P<0.01, ***P<0.001.

well as TTFields/DOX, was compared to that of irradiation alone. Moreover, the decreased percentage of SF (DOX + TTFields/DOX + TTFields + IR) was calculated as 87.50% and 49.35% for A549 and H460 cell lines, respectively (**Figure 5D**).

Effects of combined DOX and TTFields treatments after irradiation on inducing apoptosis

The results on apoptosis induced via treatment and the potential for metastasis as indicated by cell death detection assays, caspase activity assays, ROS detection, and transwell chamber assays are shown in **Figure 6A**, **6C**, **6D**, **6F** for A549 and H460 lung cancer cell lines. Our results showed that the number of apoptotic cells of lung cancer increased due to 48 hours of doxorubicin, TTFields, and X-ray irradiation compared to that of dual treatment (**Figure 6B**). The combination therapy induced caspase3 and PARP expression as compared to treatment with radiation alone, thus substantiating the role of doxorubicin, TTFields combined therapy in enhancing cellular apoptosis. Doxorubicin and TTFields combined X-ray irradiation produced increased caspase3 and PARP activation rates compared with the untreated group or dual treatment (**Figure 6E**).

Discussion

For lung cancer, the 5-year survival rate in stage 1B is 73%, and even if surgery, chemotherapy, and radiation treatment are all performed, the 5-year survival rate in stage 3A is only 41% [25]. The main cause of this low survival rate is the recurrence of lung cancer [25]. Due to these characteristics, adjuvant therapy is performed for locally advanced cancer [26]. These anti-cancer treatment also have severe side effects due to their effects on normal cells and limitations such as difficulties acting on the dormant state [27]. Since radiation therapy is a localized treatment, it does not have significant effects on removing tumor cells that float in the whole body [27]. Due to these limitations, TTFields have emerged and are an FDA-approved technology for recurrent glioma and newly diagnosed glioblastoma, and mesothelioma, and a phase III lung cancer trial (LUNAR trial) is also in progress [28]. Certain conventional anticancer therapies such as chemotherapy and radiotherapy are promising in the treatment of cancers [29], which can improve therapeutic benefits, reduce side effects, and overcome the resistance to chemotherapy drugs or radiation [30]. Our results showed that TTFields enhanced the cancer cells' chemosensitivity to DNA damage agents such as doxorubicin. We also demonstrated that TTFields combined with doxorubicin and radiation produced synergistic tumor inhibition in lung cancer.

TTFields have not been approved by the FDA for treating lung cancer, pending the results of the ongoing LUNAR trial for stage 4 patients [31]. Based on the positive clinical data such as EF-14 date and STELLAR date, it is thought that advanced lung cancer will play an additional role as adjuvant therapy in addition to anti-cancer radiation. The optimal frequency for antimitotic effect varies by cancer type, and can be adjusted for maximal anticancer effect [32]. In addition, unlike systemic chemotherapy, the delivery of TTFields can be locally directed, minimizing the risk of systematic adverse effects [32]. TTFields have been demonstrated to have minimal toxicity that is confined to the skin in *in vivo* models and multiple clinical trials [33]. This may enable TTFields to be combined with other anticancer treatments for greater efficacy without increased toxicity [32]. Such combination therapy involving TTFields requires further evaluation. Theoretically, maintenance TTFields therapy may also serve as a bridge between chemotherapy while the patient recovers from chemotherapy-related toxicities. Locoregional delivery and the low toxicity profile of TTFields highlight the potential to achieve tumor control and response in critical organs without the dose-limiting toxicity seen with other regional therapies [32]. Preclinical studies have demonstrated possible potentiation of immune system responses against the tumors following the application of TTFields [32]. Such systemic effects will need to be further evaluated through preclinical and clinical investigations. Ongoing phase III studies of TTFields include secondary endpoints, which assess local versus systemic effects in patients receiving TTFields compared with control patients [13].

Our findings showed that the combination of DOX/TTFields/IR has the most impact in reducing the viability of lung cancer cells. The reduction was 40% without irradiation, 47% for irradiation, and finally 69% for IR in the presence of DOX/TTFields. These results indicate that

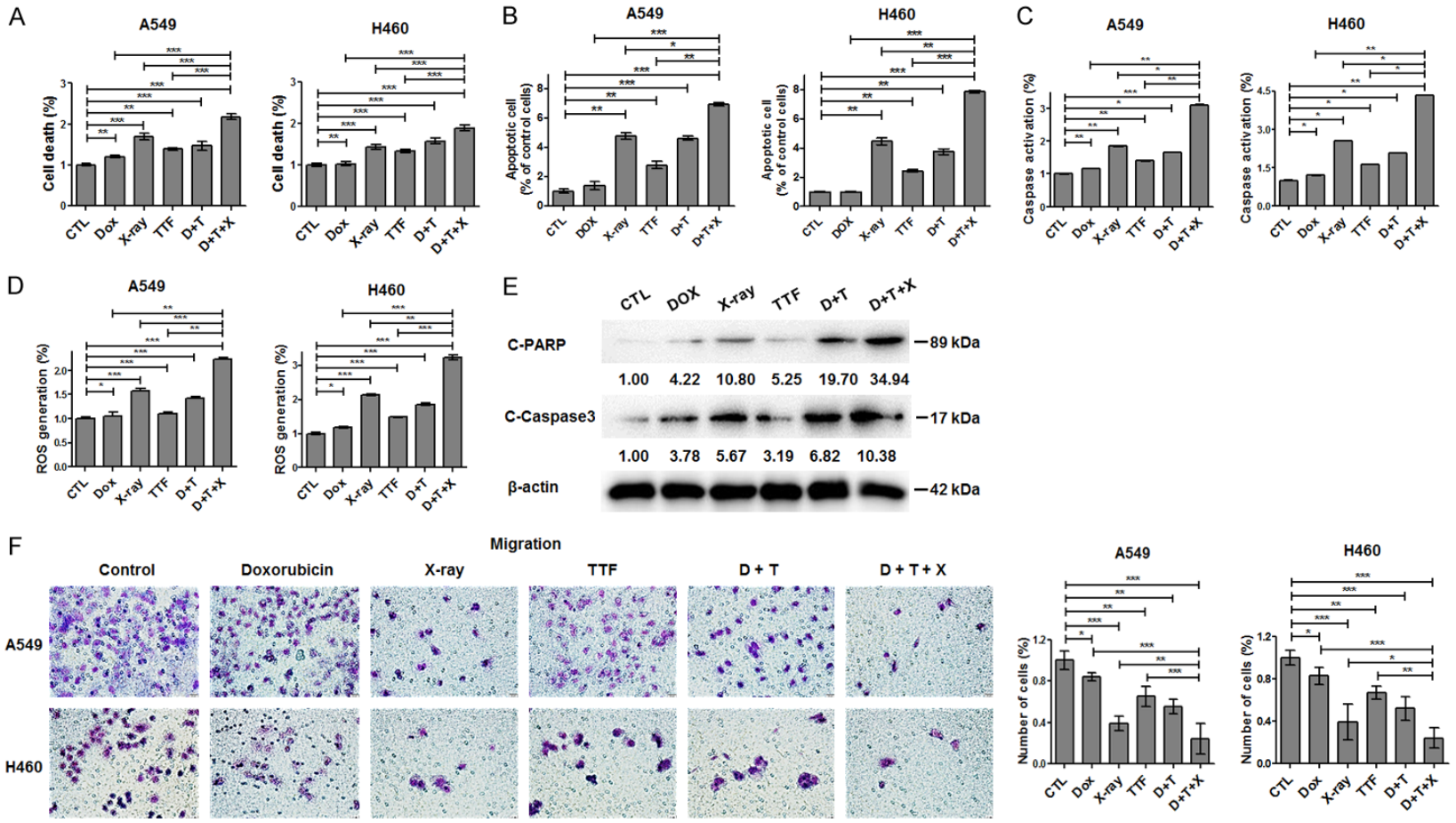

Figure 6. Effects of combinatorial treatment with TTFields, doxorubicin, and X-ray on the apoptosis of lung cancer cells. A. Analysis of cell deathin lung cancer cells 72 h after treatment with TTFields by the cell death ELISA kit. Doxorubicin was added for 48 h to 72 h followed by X-ray. Data were collected using a Multiskan EX at 405 nm. *P<0.05, **P<0.01. ***P<0.001. B. Cells were exposed to doxorubicin, TTFields and X-ray for 48 h and FACS analysis was performed. *P<0.05, **P<0.01, ***P<0.001. C. Analysis of caspase activities in lung cancer cells 72 h after treatment with TTFields by caspase 3/7 detection kit. Doxorubicin was added for 48 h to 72 h followed by X-ray. *P<0.05, **P<0.01. ***P<0.001. D. Analysis of ROS in the two lung cancer cell lines 72 h after treatment with TTFields by the ROS detection kit. Doxorubicin was added for 48 h to 72 h followed by X-ray. *P<0.05, **P<0.01, ***P<0.001. E. The indicated antibodies were used for western blot of lung cancer cell lysates treated with doxorubicin, TTFields, X-ray irradiation or a combination. F. Tumor cell migration after 24 h TTFields treatment was examined using Transwell chamber assays. Doxorubicin was added for 24 h followed by X-ray. Representative microscopy images 400×. *P<0.05, **P<0.01, ***P<0.001.

DOX/TTFields, when combined with radiotherapy, inhibit lung cancer cell growth, which may be due to growth arrest or cell death. Further studies using animal models and normal toxicity are imperative to examine the roles of TTFields in lung cancer. Further research will shed light on the underlying mechanisms regulating the antitumor effect of TTFields, and in turn contribute to the development of improved therapeutic strategies for lung cancer that will improve patient outcomes.

Acknowledgements

This study was supported by the Bumsuck Academic Research Fund in 2020.

Disclosure of conflict of interest

None.

Address correspondence to: Eun Ho Kim, Department of Biochemistry, School of Medicine, Daegu Catholic University, 33, 17-gil, Duryugongwon-ro, Nam-gu, Daegu 427-724, Korea. Tel: 82536504480; E-mail: eh140149@cu.ac.kr

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