Clinical and Translational Oncology (2025) 27:2014–2023
https://doi.org/10.1007/s12094-024-03760-6

RESEARCH ARTICLE

Synergistic effects of combined hyperthermia and electric fields treatment in non-small cell lung-cancer (NSCLC) cell lines

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Received: 12 September 2024 / Accepted: 4 October 2024 / Published online: 22 October 2024

Abstract

Purpose Lung cancer remains a leading cause of cancer-related mortality, with non-small cell lung cancer (NSCLC) being particularly challenging due to poor survival rates, emphasizing the need for new treatments. This study examined the therapeutic effects of combining hyperthermia (HT) with tumor-treating electric fields (TTF) in NSCLC.

Methods Cells were exposed to four different conditions: hyperthermia at 42 °C for 30 min, electric fields at 150 kHz and 0.8 V/cm for 24 h, a combination of both treatments, or no treatment (control). Cell proliferation was measured using WST and colony-formation assays, while apoptosis, DNA damage, and repair protein levels were analyzed via Western blotting. Metastatic potential was evaluated with a transwell assay, and cell migration was assessed using the wound-healing assay.

Results The combination therapy significantly inhibited colony formation and reduced cell migration and invasion more effectively than individual treatments. The combined treatment also enhanced apoptosis, as indicated by increased cleaved-PARP and Annexin V levels. In addition, the DNA-damage marker γ-H2AX was elevated, while BRCA1, a protein involved in DNA repair, was significantly downregulated compared to the individual treatments.

Conclusions These results suggest that the enhanced anticancer effects of HT and TTF are due to increased DNA damage and suppression of DNA-repair mechanisms, highlighting the potential of this combination therapy for NSCLC treatment.

Keywords Non-small cell lung cancer (NSCLC) · Electric fields · Hyperthermia · Combination therapy

Introduction

More than 80% of patients with lung cancer are diagnosed with non-small cell lung cancer (NSCLC). Lung cancer patients have a 5-year relative survival rate of 28%, making lung cancer one of the leading causes of cancer-related death in both men and women worldwide. Approximately 350 people die from lung cancer every day, a number about 2.5-fold higher than the number of patients who die from colorectal cancer [1, 2]. This reflects the aggressive nature of lung cancer and the fact that many patients are diagnosed at an advanced stage of disease [3]. Immunotherapy and targeted therapy have been shown effective in the treatment of NSCLC, but these agents have both short- and long-term side effects [4]. Therefore, new treatments of lung cancer are required. Intermediate frequency (100–300 kHz) and low intensity (1–3 V/cm) AC electric fields have been shown effective in cancer treatment [5, 6], leading to the development of tumor-treating fields (TTF) therapy to treat solid tumors. Electric fields have been combined with chemotherapy and radiotherapy in vitro and in vivo to treat various types of cancer cells [7–10]. Recent clinical trial results showed that combining TTF with an immune-checkpoint inhibitor or docetaxel in patients with metastatic NSCLC significantly improved median overall survival when compared with standard systemic therapy alone (13.2 months vs. 9.9 months) [11]. However, when cancer cells are exposed to anticancer treatments, they may survive by altering the tumor microenvironment and exploiting immune-checkpoint pathways [12, 13]. Therefore, new combination therapies are needed to overcome cancer-cell resistance to treatment.Hyperthermia (HT), an adjuvant method for cancer

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treatment, has shown minimal cytotoxicity while significantly improving responses to radiotherapy and chemotherapy [14, 15]. The combination of HT with other treatments has been reported more effective than either alone in treating various types of cancer, including lung cancer [16, 17]. Moreover, the combination of TTF and HT has been shown to be more effective than either alone when treating pancreatic cancer- and glioblastoma-cell lines [18, 19], suggesting that these combinations have a multi-modal therapeutic effect on other cancer-cell lines. Therefore, this study aimed to evaluate the combined treatment effect of HT and TTF on NSCLC, assessing whether this combination had a synergistic anticancer effect when compared with monotherapy.

Materials and methods

Setting experiments for TTF and HT

An electric signal was generated using a function generator (AFG-2112, Good Will Instrument Co., Ltd), which was connected to a high-voltage amplifier (A303, A. A. Lab Systems Ltd). The electric field generated by the amplified electric signal was designed to be applied to the cells through wires attached to the dish. A pair of insulated wires were each fixed at 3-cm intervals on the bottom of the cell dish, with the cells attached between these wires. The intensity of electric fields was set to 0.8 V/cm and the frequency was set to 150 kHz. Cells were exposed to HT for 30 min in a water bath at a temperature that could be adjusted within 0.1 °C, with the water temperature measured using a thermocouple.

Cell culture

Human-derived NSCLC-cell lines, H460 and A549, were purchased from the Korea Cell Line Bank. The culture medium used RPMI1640 as a basal medium, containing 10% fetal bovine serum and 1% antibiotics. The incubator was set to 5% CO2 and 37 °C.

Cell-viability assay

Cells were seeded into 96-well plates at a density of 2,000–5,000 cells per well and cultured for 24 h. The medium was replaced with culture medium containing WST-8 reagent (QM2500, BIOMAX), and the cells were incubated in a CO2 chamber at 37 °C for 0.5 to 4 h. Cell viability was detected using a microplate reader (PHOmo, Autobio Labtec Instruments) at 450 nm. For direct cell counting, cells were stained with trypan blue, and unstained cells were counted under a microscope using a hemocytometer (DHC-N01-5, Incyto).

Colony formation assay

Cells were cultured for 10 days after exposure to TTF and/or HT. Grown colonies were visualized by staining with 0.4% crystal violet solution. Plating efficiency (PE) was defined as the percentage of plated cells forming colonies, and the survival rate was calculated as follows; the number of created colonies/(the number of plated cells × PE/100).

Flow cytometry

After harvesting, Cells were pretreated with propidium iodide (PI) and annexin V, according to the manufacturer's method. These cells were subsequently fractionated using a BD FACSVerse flow cytometer. At least 10,000 cells were analyzed for each sample.

Invasion and migration assay

Metastasis assays were measured using transwell chamber dishes. Briefly, 200 μL of serum-free RPMI1640 containing 4,000 cells was plated on the membrane of the upper dish of a Matrigel or gelatin-pretreated Transwell. RPMI1640 (with FBS) medium was placed in the lower chamber. After culturing the cells for 48 h, the cells that had passed through the membrane were identified. The cells were stained with crystal violet solution and photographed using a microscope.

Wound-healing assay (migration assay)

NSCLC-cell lines were seeded in a very high density of about 250,000 cells/well in a 12-well plate and attached. The next day, a physical scratch was created in the center of the cell layer using a pipette tip. Afterward, we observed under a microscope how the cells filled in the scratched area.

Western blotting

Control and experimental cells were harvested simultaneously and lysed in RIPA buffer. Proteins were separated by size using SDS-page gel electrophoresis and then transferred to PVDF membrane. After transfer, the membranes were blocked for 1 h at room temperature using 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T). The membranes were then incubated with the appropriate primary antibodies diluted in blocking buffer at 4 °C overnight. Following primary antibody incubation, membranes were washed three times in TBS-T and incubated with secondary antibodies, diluted in blocking buffer, for 1 h at room temperature. The membranes were washed again and developed using an enhanced chemiluminescence

(ECL) detection reagent. Protein bands were visualized and captured using a digital imaging system such as the iBright CL750 (ThermoFisher Scientific).

Statistical analysis

Means were compared using Student's t test, with p values less than 0.05 considered statistically significant.

Results

Effects of TTF or HT on lung *cancer* cell lines

Before testing the combination of TTF and HT, the effects of each treatment on lung-cancer cell lines were evaluated. The frequency of TTF was set at 150 kHz, which was shown to have a limited effect on cell proliferation. Compared to untreated cells, the number of cells treated decreased with treatment time (Fig. 1A). The effect of TTF intensity on cell viability was assessed by applying TTF for 48 h. A TTF intensity of 0.8 V/cm was found to reduce lung-cancer cell viability by up to 50% (Fig. 1B). The effects of HT alone were evaluated by treating two NSCLC-cell lines at temperatures ranging from 39 °C to 43 °C for 15, 30, and 60 min. After treatment, the cells were cultured for 1 to 3 days, and cell viability was determined relative to the control group. Both cell lines showed significant reductions in viability at 43 °C, with these reductions depending on the duration of exposure to HT but being independent of the post-HT incubation period (Fig. 1C).

Combined effects of HT + TTF on lung-*cancer* cell lines

Based on the previous results, NSCLC were heat-treated at 43 °C for 30 min, and TTF was applied to the cells at an intensity of 0.8 V/cm for 24 h. The untreated control group was compared with experimental groups that received either treatment alone or both treatments together (Fig. 2A). The number of cells observed after combined HT and TTF was lower than the number after either treatment alone (Fig. 2B). In addition, As a result of measuring cell viability using the WST reagent, it was confirmed that the combination of TTF and HT inhibited cell growth more effectively than either treatment alone (Fig. 2C). The proliferation of NSCLC was performed through clonal formation analysis after treatment. Similarly, the inhibitory effect on proliferation was found to be stronger in the group receiving the combined HT and TTF treatment (Fig. 2D).

Effects of combined HT + TTF treatment on metastasis

Vimentin plays an essential role in the metastatic process, and its increased expression enhances the motility, invasiveness, and survival of cancer cells, thereby promoting metastasis. Therefore, vimentin is a crucial molecular target in cancer treatment. Because TTF has been reported to inhibit cancer-cell metastasis, the effects of combined TTF and HT on the motility and invasiveness of H460 and A549 cells were evaluated. Western blotting revealed that the expression of vimentin was significantly lower in both cell lines following treatment with HT + TTF than following HT or TTF alone (Fig. 3A). Invasion assays were performed using transwell with matrigel. The results showed that combined treatment with HT and TTF significantly inhibited cell invasiveness compared to the effect of either treatment alone (Fig. 3B). Similarly, in cell-motility assays using transwell plates, the combination therapy exhibited a stronger inhibitory effect on cell migration compared to monotherapy (Fig. 3C). Scratch assays also showed that cell migration was more inhibited by combined HT and TTF than by either HT and TTF alone (Fig. 3D).

*Effects of combined HT + TTF treatment on apoptosis*Hyperthermia is known to induce apoptosis in various cancer-cell lines through the intrinsic pathway, and TTF has also been reported to induce apoptosis in NSCLC cells in vitro and in vivo. It is suggested that a greater apoptotic effect can be expected when HT and TTF are combined than when each treatment is used alone.

Cell apoptosis was evaluated by analyzing the levels of expression of cleaved PARP, a marker of apoptosis, in H460 and A549 cells using Western blotting. Marker expression was higher in the combination treatment group than in the monotherapy group (Fig. 4A). These findings were confirmed by analyzing the levels of expression of another apoptotic marker, annexin V, using flow cytometry. Again, the combination of HT and TTF was found to increase apoptosis in H460 and A549 NSCLC cells to a greater extent than either HT or TTF alone (Fig. 4B).

Reduction in DNA damage repair mechanisms with combined HT + TTF treatment

When DNA double-strand breaks (DSBs) occur, H2AX is phosphorylated to form γH2AX, which acts as a signal at the damage site to recruit various DNA-damage-repair proteins, including BRCA1. Although HT was found to increase the expression of p-H2AX, this increase was more pronounced in cells receiving both HT and TTF treatments. Moreover, the combined HT and TTF treatment reduced the expression of BRCA1 to a greater extent than either HT or TTF alone (Fig. 5).

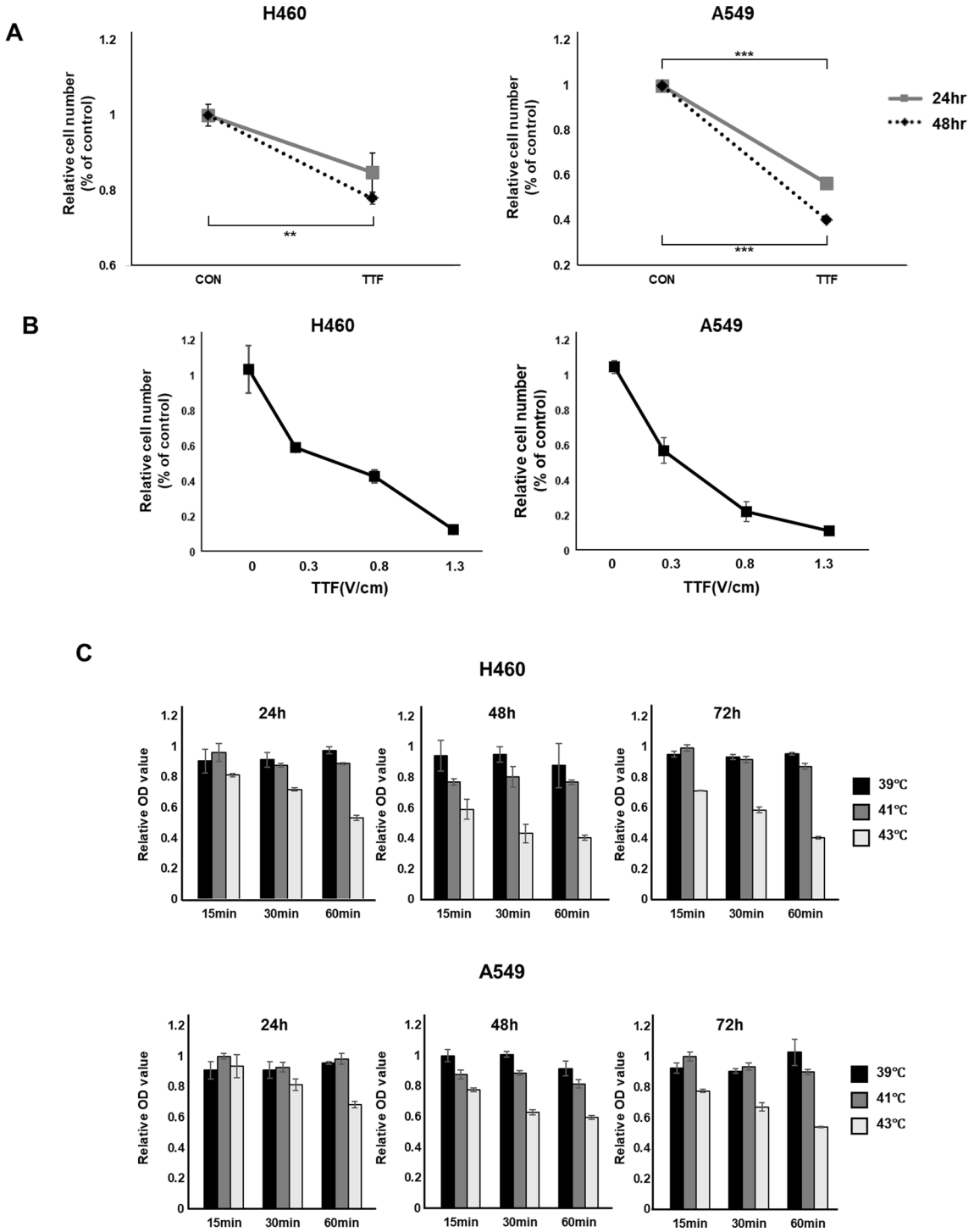

Fig. 1 **A**, **B** TTF reduced the viability of lung-cancer cells with effects depending on both the treatment duration and intensity. **A** H460 and A549 lung-cancer cells were subjected to TTF for 24 or 48 h, with cell viability measured using MTT assays. **B** Cells were subjected to 0.3, 0.8 and 1.3 V/cm TTF, and the numbers of cells counted after 0.4% Trypan Blue staining. **C** HT reduced lung-cancer cell survival in a manner dependent on both temperature and duration. Cells were heated to 39 °C, 41 °C, or 43 °C for 15, 30 or 60 min and incubated at 37 °C for 24, 48 or 72 h, with cell viability measured using MTT assays. Each value represents the mean ± SD of three experiments. *, $P < 0.01$

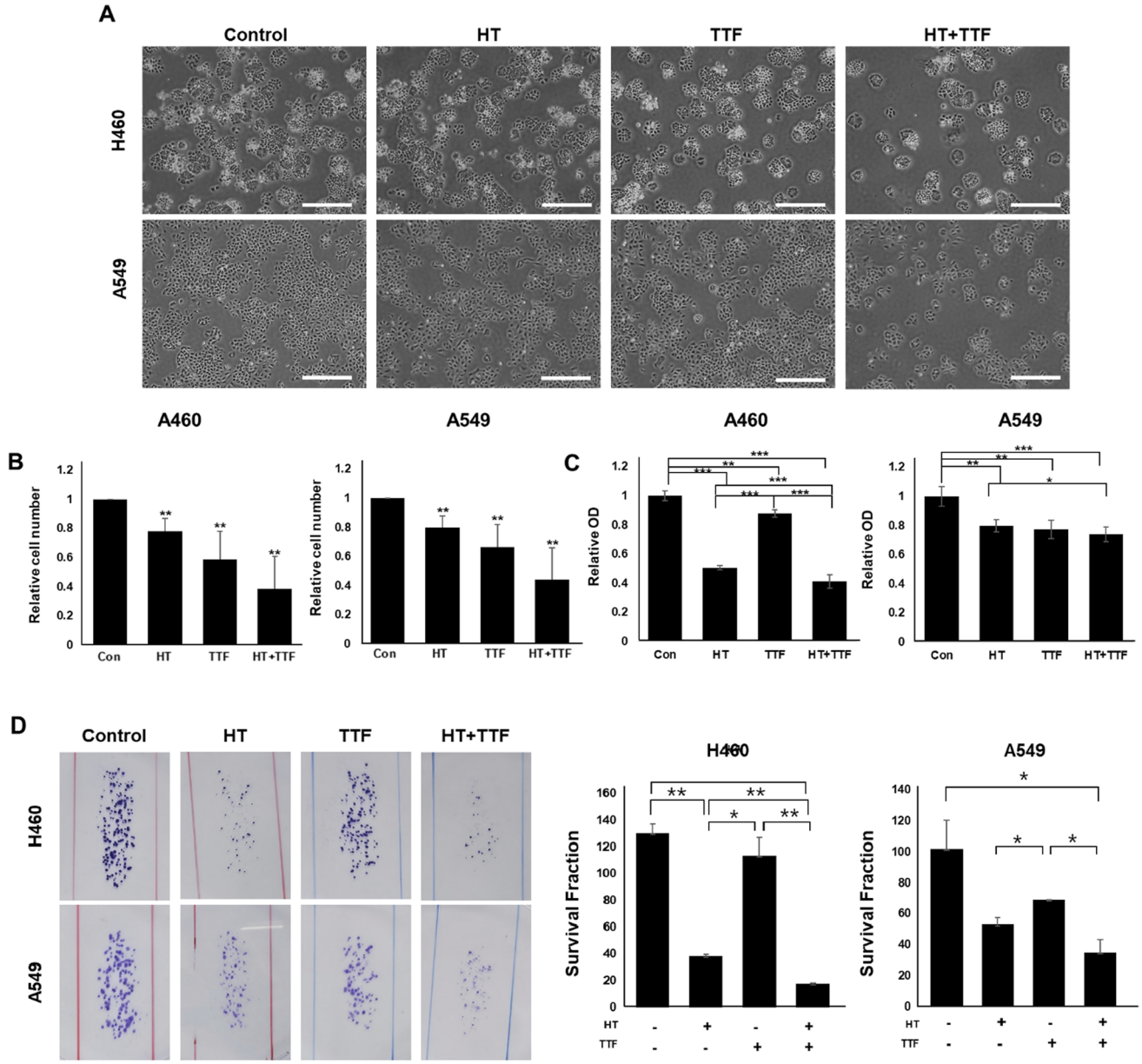

Fig. 2 Effects of HT + TTF or HT and TTF alone. Morphology (**A**), (**B**) cell counts after culture, **C** MTT-assay results, and **D** survival in a clonogenic assay of H460 and A549 lung-cancer cells. Cells were exposed to TTF (0.8 V/cm and 150 kHz) or left untreated, and then immediately heated to 43 °C for 30 min. Each value represents the mean ± SD of three experiments. *, $P < 0.01$. Scale bar; 250 μm (**A**)

Discussion

NSCLC accounts for about 80% to 85% of all lung cancers, with a 5-year survival rate of approximately 63% when detected at an early stage, but only 7% when detected at advanced stages [20]. Hence, there is a need to develop NSCLC treatments that enhance survival rates without causing additional systemic toxicity. HT has been shown effective in treating various types of cancer, including lung cancer, and has been reported to boost the efficacy of other cancer treatments [21]. TTF also exhibit activity against various types of cancer cells, both alone and in combination with other therapeutic methods [8]. Notably, both treatments influence DNA damage and repair mechanisms, contributing to cancer-cell death [22, 23]. This study is the first to demonstrate that the combination of HT and TTF is more effective than each treatment alone in NSCLC cells. The synergistic combination of HT and TTF resulted in enhanced inhibition of cancer-cell growth and metastatic potential. Furthermore, the analysis of protein expression patterns revealed that combined HT and TTF had a greater effect than either alone on genes involved in DNA-damage and -repair in cancer

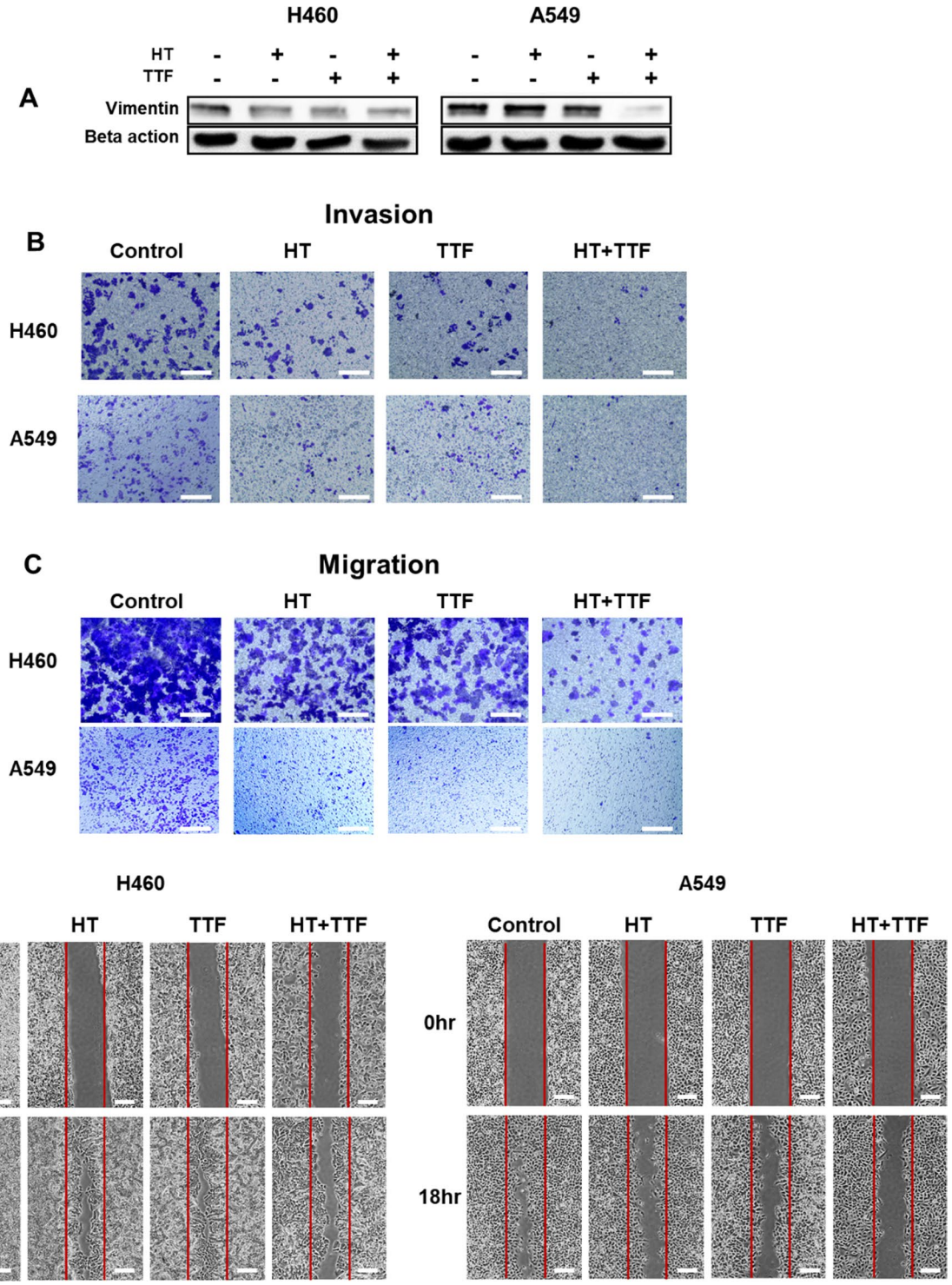

Fig. 3 Effect of combined HT and TTF on the invasion and migration of lung-cancer cells. **A** Western blotting was performed to confirm the expression level of vimentin using lysate from H460 and A549 cells. Expression levels were normalized to the intensities of actin bands in the same samples (n = 3, mean ± SD). **B** Evaluation of tumor-cell invasiveness using Transwell chamber assays. **C**, **D** Evaluation of tumor-cell migration cells using Transwell chamber assays (**C**) and scratch assays (**D**). Scale bars; 250 μm (**B**, **C**) and 100 μm (**D**)

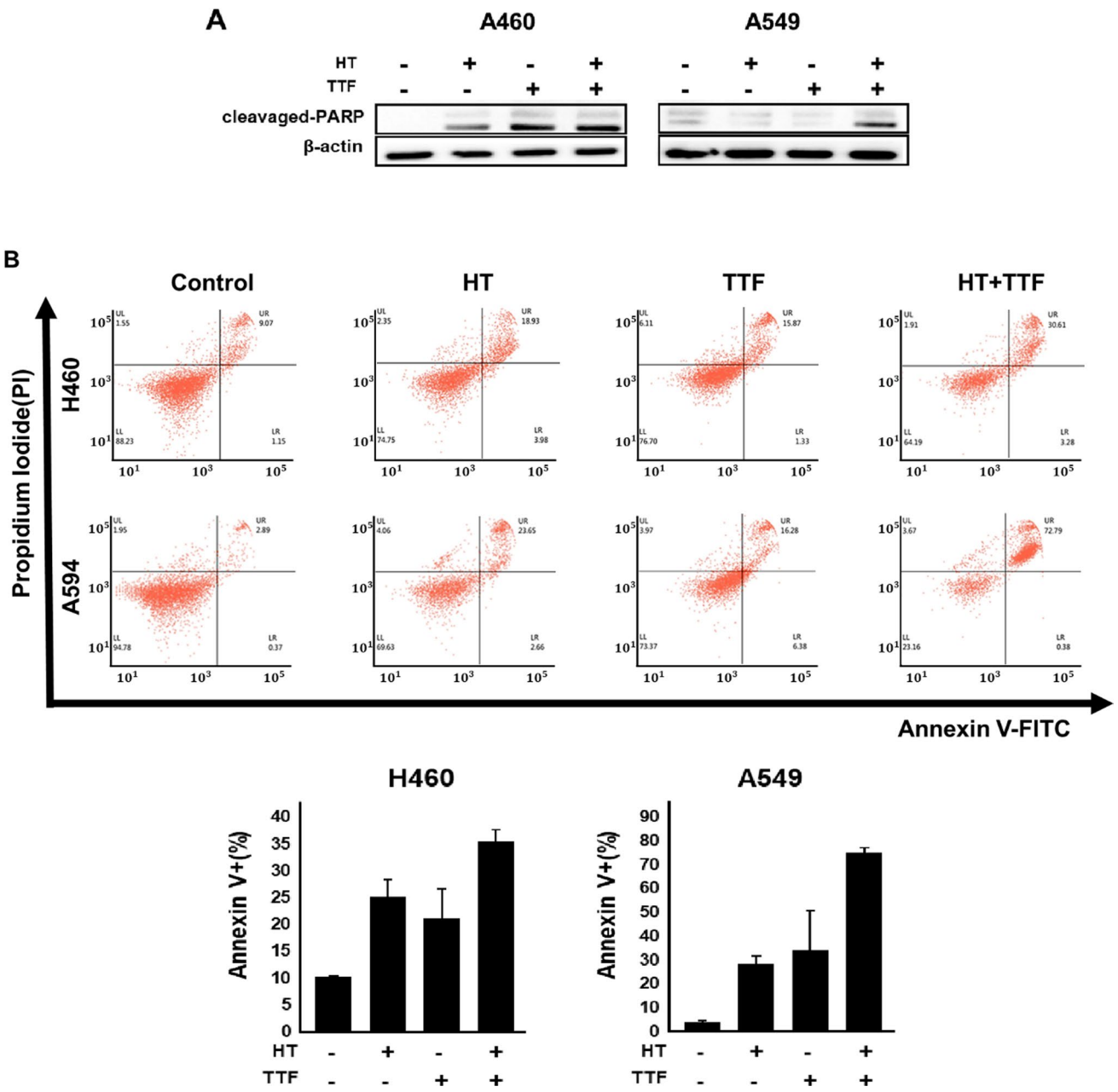

Fig. 4 Effects of HT and TTF on lung-cancer cell apoptosis. **A** The expression level of cleaved-PARP1 in NSCLC-cell lines was confirmed by immunoblotting and normalized to the intensity of the actin band (n = 3, mean ± SD). **B** H460 and A549 cells were subjected to HT (43 °C for 30 min), TTF, or both for 24 h prior to Annexin V/PI staining. Each bar represents the mean ± SEM of band intensity from independent experiments

cells. H2AX, PARP1, and BRCA1 all play crucial roles in the DNA-damage-repair process and interact with each other to form a complex damage-repair mechanism. These interactions are essential for maintaining effective DNA-repair and -damage response. The formation and removal of γ-H2AX foci are critical in the cellular DNA-repair process and have significant implications in cancer research and treatment, including in vivo measurements of radiation and evaluation of the efficacy of chemotherapy agents [24]. γ-H2AX recruits DNA-repair proteins (e.g., 53BP1, RAD51, and BRCA1) and functions as a novel component of the Fanconi anemia (FA)/BRCA pathway, resolving replication fork collapse and preventing chromosomal instability [25]. Poly (ADP-ribose) polymerase (PARP) is involved in DNA-damage repair, promotes poly(ADP-ribose) synthesis, and modifies nuclear proteins. During apoptosis, PARP1 is cleaved by caspase-3 into 89-kD and 24-kD fragments, disrupting DNA-repair and -promoting apoptosis. Thus,

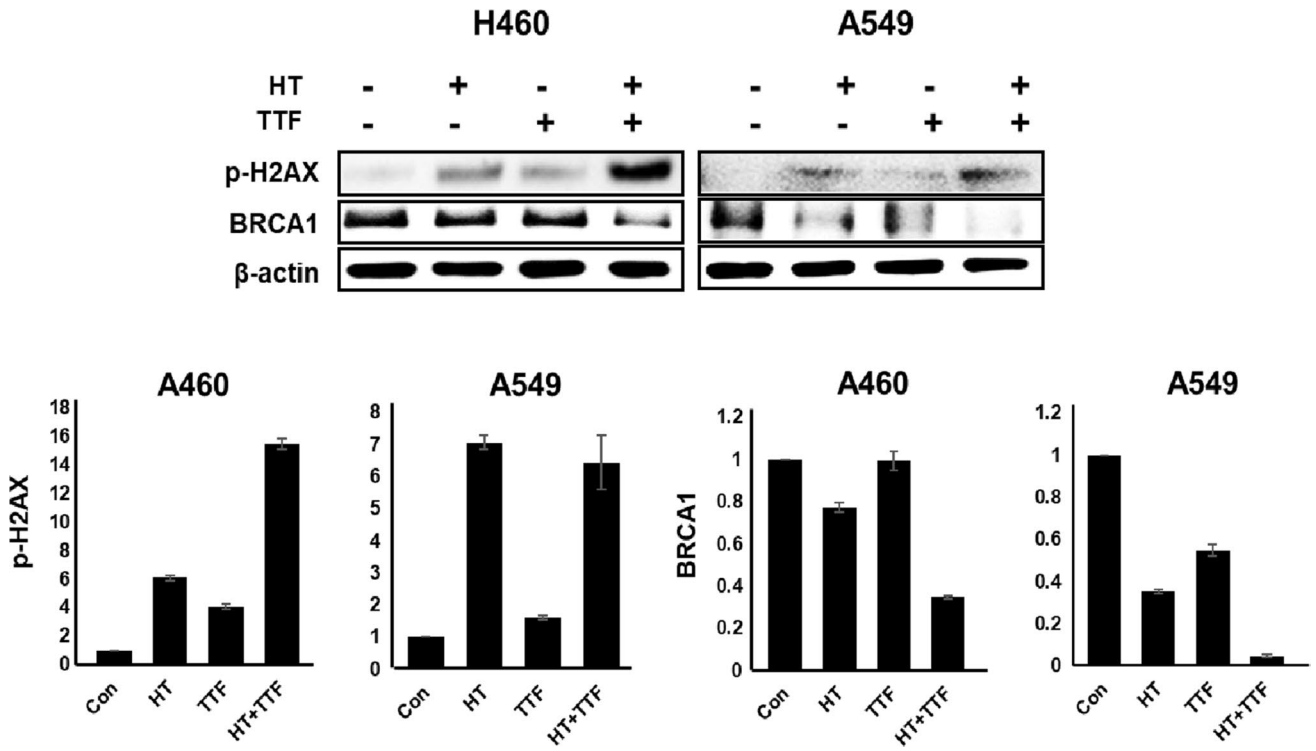

Fig. 5 Impact of HT, TTF, or their combination on patterns of expression of genes related to DNA damage in lung-cancer cells. **A** Equal amounts of lysates of H460 and A549 cells were fractionated through electrophoresis and examined through Western blotting with p-BRCA1 and p-H2AX antibodies. Band levels were measured and normalized to actin intensities (n = 3, mean ± SD). Each bar represents the mean ± SEM band intensity from independent experiments

cleaved PARP1 is regarded as a marker of apoptosis [26]. Western blot analysis demonstrated that the level of expression of cleaved-PARP protein was higher in cells exposed to both HT and TTF than in cells treated with either treatment individually. Moreover, the level of phosphorylated H2AX, a marker of DNA damage, was significantly higher in cells exposed to HT and the combined treatment of HT and TTF. The reduction in BRCA1 expression was more pronounced in cells exposed to both HT and TTF compared to those receiving either HT or TTF alone. Our results indicate that, although HT and TTF induced DNA damage in cancer cells, the repair mechanisms were insufficiently activated, leading to genetic instability. This phenomenon is analogous to the synthetic lethality strategy, in which the concurrent impairment of two genes results in cell death, whereas the impairment of either gene alone does not [27]. PARP inhibitors such as olaparib and niraparib are effective in treating cancers with BRCA1/2 mutations, and their mechanism of action targets DNA-repair pathways [28]. These findings suggest that the addition of PARP inhibitors to the co-treatment with HT and TTF may be more effective than the latter combination alone. Furthermore, because this study analyzed only a subset of genes related to DNA-repair pathways, future studies should explore a broader spectrum of gene expression profiles. This may result in a greater understanding of the effects of DNA-damage and -repair mechanisms on cancer-cell death.

The primary objective of this study was to evaluate the antitumor effects of HT and TTF on NSCLC cells in a controlled in vitro environment using well-established cell lines. By minimizing experimental variables, we were able to clearly delineate the effects of these physical treatments and elucidate their underlying mechanisms. However, to comprehensively assess the therapeutic potential of this combination, further in vivo studies and clinical trials are warranted. Further studies could focus on enhancing the antitumor efficacy by combining HT and TTF with PARP inhibitors. Moreover, integrating patient-derived samples in these studies would provide critical insights into the clinical relevance of the findings, thereby facilitating the translation of preclinical research into clinical practice.

Conclusion

The present research is the first to demonstrate that the combination of HT and TTF is more effective than either alone in NSCLC-cell lines. The synergistic administration of HT

and TTF significantly improved the suppression of NSCLC-cell growth and metastasis compared with either alone. The combination of HT and TTF showed a synergistic effect that increases DNA damage and impairs repair mechanisms in cancer cells. These results suggest that combined treatment with HT and TTF may slow the aggressive progression of NSCLC.

Acknowledgements This work was supported by National Research Foundation of Korea (NRF) grants funded by the Korean government (MSIT) (Grant Nos. NRF-2021R1A2C2008695 and NRF-2022R1A2C1010337). This work was also supported by a Korea Medical Device Development Fund grant funded by the Korean Government (the Ministry of Science and ICT, the Ministry of Trade, Industry and Energy, the Ministry of Health & Welfare, and the Ministry of Food and Drug Safety) (Project No.1711196423, RS-2023-00254868). Additionally, this research was financially supported by the Ministry of Trade, Industry and Energy (MOTIE) and the Korea Institute for Advancement of Technology (KIAT) through the International Cooperative R&D program (Project No. P0019304).

Data availability The data are available with restricted access due to privacy concerns. Interested researchers can request access by contacting contact information or method of data request.

Declarations

Conflict of interest The authors have no conflicts of interest to disclose.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study formal consent is not required.

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