Thermal cycling-hyperthermia sensitizes non-small cell lung cancer A549 cells to EGFR tyrosine kinase inhibitor erlotinib

GUAN-BO LIN[1,2*], WEI-TING CHEN[1,2*], YU-YI KUO[1,2], HSU-HSIANG LIU[2,3], YOU-MING CHEN[2,3], SHR-JENG LEU[4] and CHIH-YU CHAO[1-3]

[1]Department of Physics, Laboratory for Medical Physics and Biomedical Engineering, National Taiwan University, Taipei 106319, Taiwan, R.O.C.; [2]Molecular Imaging Center, National Taiwan University College of Medicine, Taipei 100233, Taiwan, R.O.C.; [3]Graduate Institute of Applied Physics, Biophysics Division, National Taiwan University, Taipei 106319, Taiwan, R.O.C.; [4]Department of Biotechnology and Laboratory Science in Medicine, National Yang Ming Chiao Tung University, Taipei 112304, Taiwan, R.O.C.

Received November 13, 2024; Accepted February 26, 2025

DOI: 10.3892/or.2025.8891

Abstract. Molecular targeted therapy has emerged as a mainstream treatment for non-small cell lung cancer (NSCLC), the most common type of lung cancer and the leading cause of cancer-related death in both men and women. Erlotinib (Erl), a targeted therapy inhibiting EGFR pathways, has shown notable response rate in NSCLC cells. However, limited efficacy of the treatment has been reported due to resistance among a proportion of patients with NSCLC. Therefore, sensitizers are required to potentiate the efficacy of Erl in NSCLC treatment. The present study proposed a novel thermal therapy, thermal cycling-hyperthermia (TC-HT), as a supplement to amplify the effects of Erl. It was demonstrated that TC-HT reduced the half-maximal inhibitory concentration of Erl to 0.5 μM and TC-HT sensitized A549 NSCLC cells to Erl via the downstream EGFR signaling cascades. Furthermore, the combination treatment of Erl and TC-HT induced G2/M cell cycle arrest and inhibition of cell proliferation and migration. In addition, by slightly raising the temperature of TC-HT, TC-HT treatment alone produced antineoplastic effects without damaging the normal IMR-90 lung cells. The method presented in this study may be applicable to other combination therapies and could potentially act as a starter for anticancer treatments, with fewer side effects.

Correspondence to: Dr Chih-Yu Chao, Department of Physics, Laboratory for Medical Physics and Biomedical Engineering, National Taiwan University, 1 Section 4, Roosevelt Road, Taipei 106319, Taiwan, R.O.C.
E-mail: cychao@phys.ntu.edu.tw

*Contributed equally

Key words: combination treatment, sensitizer, hyperthermia, non-small cell lung cancer, erlotinib

Introduction

As one of the most common types of cancer, lung cancer was the leading cause of cancer-related deaths worldwide according to the World Health Organization in 2020 (1). Non-small cell lung cancer (NSCLC) accounts for 85% of lung cancer-associated deaths (2). Available therapies for NSCLC treatment include surgery, chemotherapy, radiation and targeted therapy. In order to enhance anticancer effects, there is urgent need for combination treatments or new agents targeting one or more molecular pathways.

Targeted treatment, intended to inhibit cancer malignancies with a focus on critical molecules, has been regarded as a promising therapy to treat patients with NSCLC (3). Among various targeted therapies, anti-EGFR therapy has been the one with the most extensive application in the clinical treatment of NSCLC (4). EGFR is a receptor tyrosine kinase of the ErbB family which serves a key role in cell proliferation, survival, differentiation and migration (5). When activated by ligand binding, EGFR will autophosphorylate and trigger downstream signaling cascades, including MAPK and PI3K-Akt pathways (6), leading to cell cycle progression and inhibiting the activation of proapoptotic proteins. Therefore, several EGFR-inhibiting agents, such as monoclonal antibodies and tyrosine kinase inhibitors (TKIs) have been developed for anticancer treatments (7). Erlotinib (Erl), a first generation EGFR-TKI, has exhibited an improved survival rate in patients with NSCLC , prolonging the survival of patients with advanced NSCLC after chemotherapy (8). However, certain tumors will develop acquired resistance to Erl over time (9), and prolonged administration of high-dose Erl can lead to adverse effects, including rashes and diarrhea (10). Additionally, certain patients demonstrate intrinsic resistance to Erl, which further constrains its therapeutic effectiveness (11-13). Therefore, it is worthwhile to investigate the effect of combination treatment in potentiating the efficacy of Erl at a low dose, in place of chemotherapy drugs, such as cisplatin and pemetrexed, which have been applied as sensitizers to enhance the efficacy of Erl (14,15), with side effects such as hypertension and severe diarrhea (16).

On the other hand, hyperthermia has emerged as a significant therapeutic modality for cancer, recognized not only for its capability to induce direct cytotoxic effects on cancer cells, but also for its role in increasing tumor sensitivity when used in combination with other treatment modalities (17). Additionally, several studies have reported that hyperthermia may exert a synergistic effect when paired with chemotherapy or radiotherapy in cancer treatment (18-20). However, conventional hyperthermia techniques may result in adverse effects such as pain and discomfort, as well as non-specific thermal damage to adjacent healthy tissues, potentially leading to severe complications, such as pain, unpleasant sensations and burns (18,21). Therefore, modifying conventional hyperthermia and integrating it with pharmacological agents or therapies to enhance both anticancer efficacy and safety represents a promising strategy.

In the present study, a novel thermal therapy, thermal cycling hyperthermia (TC-HT), was proposed as a substitute for chemotherapy drugs or agents, to investigate whether TC-HT may be an effective sensitizer to amplify the anticancer effect of Erl. Given the safety and effectiveness of TC-HT reported in our previous studies (22,23), the aim of the present study was to investigate the role of TC-HT in effectively sensitizing A549 cells to Erl, thereby improving therapeutic outcomes.

Materials and methods

Cell culture. The human NSCLC cell lines A549 (cat. no. 60074) and H460 (cat. no. 60373) and the normal lung cell line IMR-90 (cat no. 60204) were purchased from the Bioresource Collection and Research Center of the Food Industry Research and Development Institute. Both A549 and IMR-90 cell lines were cultured in DMEM (HyClone; Cytiva), while the H460 cell line was cultured in RPMI-1640 medium (Corning, Inc.). All media were supplemented with 10% fetal bovine serum (HyClone; Cytiva) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.), and all cells were maintained in a humidified incubator with 5% CO_2 at 37˚C.

Drug treatment and TC-HT exposure. Erl, purchased from MedChemExpress, was dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) to a concentration of 5 mM as the stock solution and stored at -20˚C. A549 cells were seeded in 96-well plates at $3x10^3$ cells/well and incubated overnight at 37˚C before being treated with 0.5, 2.0, 8.0 or 10.0 μM of Erl, TC-HT or a combination treatment. For the combination treatment, TC-HT was applied for 1 h before Erl treatment. After single or combination treatment, the cells were maintained at 37˚C in the cell culture humidified incubator for an additional 24, 48 and 72 h for further analyses. The TC-HT parameters employed in this study were based on our previous study involving different cell types (22-25), consisting of a high temperature period for 3 min and a cooling period of 30 sec, and this protocol was repeated continuously for 10 cycles (Fig. 1A). A modified PCR system, which featured waterproof capabilities in the heating area, was utilized to conduct TC-HT (Fig. S1A). A minimal volume of water served as a thermal conduction medium, with cell culture dishes placed directly on the heating area, while the modified PCR apparatus regulated the heating and cooling processes. The actual temperatures sensed by the cells at the bottom of the well were measured by a needle thermocouple (Fig. S1B).

Cell viability assay and cell morphology. The viability of A549 cells was assessed by MTT assay (Sigma-Aldrich; Merck KGaA). After drug or TC-HT treatment, the medium was replaced by DMEM containing 0.5 mg/ml MTT and incubated for 4 h at 37˚C. The formazan crystals were dissolved using 10% SDS (Bioman Scientific Co., Ltd.) at room temperature overnight. Thereafter, the optical density of formazan solution was measured at 570 nm using the Multiskan GO spectrophotometer (Thermo Fisher Scientific, Inc.). Background absorbance at 690 nm was subtracted, and the final absorbance value was expressed as a percentage of the untreated controls to represent the cell viability. The cell morphology of A549 cells under different treatments was observed and imaged using the Zyla 5.5 sCMOS camera (Andor Technology Ltd.).

Synergy quotient (SQ) calculation for synergism. The SQ was calculated by subtracting the baseline values from all treatment groups and dividing the resulting net effect of the combination by the total of the individual effects as follows: (Erl + TC-HT)/(Erl) + (TC-HT).

Western blotting. After treatment with Erl and/or TC-HT, cells were washed with ice-cold PBS and lysed in RIPA lysis buffer (MilliporeSigma) on ice for 30 min. Cell lysates were clarified by centrifugation at 23,000 x g for 30 min at 4˚C and the protein concentration in the supernatant fraction was measured using the Bradford protein assay (cat. no. BRA222; BioShop Canada Inc.). Proteins (20-40 μg) were subjected to 10% SDS-PAGE and electrotransferred to polyvinylidene fluoride membranes (MilliporeSigma). After incubation in a blocking buffer containing 5% bovine serum albumin (BioShop Canada Inc.) in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature, the membranes were immunoblotted with diluted primary antibodies at 4˚C overnight. The specific primary antibodies against phosphorylated EGFR (p-EGFR; cat. no. 4407; Cell Signaling Technology, Inc.), poly ADP-ribose polymerase (PARP; cat. no. 9542; Cell Signaling Technology, Inc.), p-JNK (cat. no. 4668; Cell Signaling Technology, Inc.), p-Akt (cat. no. 4060; Cell Signaling Technology, Inc.), total Akt (t-Akt; cat. no. 9272; Cell Signaling Technology, Inc.), MutT homolog 1 (MTH1; cat. no. 43918; Cell Signaling Technology, Inc.), p-p38 (cat. no. GTX133460; GeneTex, Inc.), Cdc2 (cat. no. GTX108120; GeneTex, Inc.) and GAPDH (cat. no. GTX100118; GeneTex, Inc.) were used. After being washed with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. 111-035-003; Jackson ImmunoResearch Laboratories, Inc.). All the primary antibodies were diluted at a 1:1,000 concentration and the secondary antibodies were diluted at a 1:10,000 concentration, according to the manufacturer's instructions. The membranes were visualized with an enhanced chemiluminescence substrate (Advansta Inc.) and quantified using an Amersham Imager 600 imaging system (AI600; GE Healthcare).

Mitochondrial membrane potential (MMP) measurement. After treatment with Erl and/or TC-HT, cells were washed and resuspended with PBS followed by staining with 20 nM

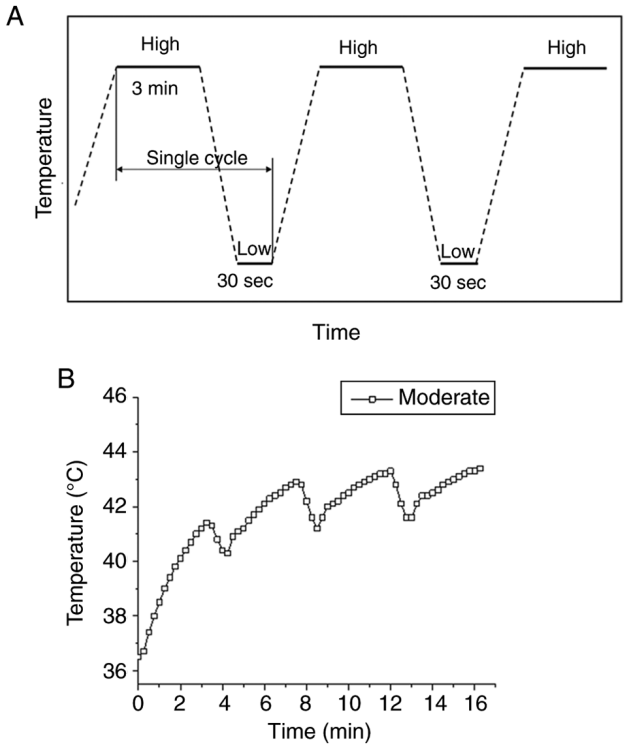

Figure 1. *In vitro*-applied TC-HT. (A) Schematic representation of the high and low temperatures and their duration settings during TC-HT treatment. (B) Actual temperature with moderate temperature TC-HT setting in the culture well measured every 15 sec using a needle thermocouple. TC-HT, thermal cycling-hyperthermia.

3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$; Enzo Life Sciences, Inc.) for 30 min at 37˚C in the dark. The fraction of mitochondrial depolarization in cells was indicated by a decrease in fluorescence intensity measured using a flow cytometer (FACSVerse; BD Biosciences) and data were analyzed using FlowJo (version 7.6.1; FlowJo LLC).

Wound healing assay. A549 cells were seeded and cultured as a confluent monolayer in 35 mm Petri dishes. Wounds were made by scratching straight lines across the cell monolayer using a 10 μl pipette tip. Non-adherent cells and debris were removed by gently rinsing the cells with PBS (HyClone; Cytiva). After PBS was discarded and replaced with fresh medium, the cells were treated with either Erl and/or TC-HT. Each wound was observed and imaged using the Zyla 5.5 sCMOS camera (Andor Technology Ltd.) at 0 h and 24 h post treatment. The distances between wound edges were measured and analyzed using ImageJ (version 1.51j8; National Institutes of Health).

Colony formation assay. A549 cells were seeded at a density of 300 cells/dish in 35 mm Petri dishes overnight for cell adherence. Subsequently, cells were treated with Erl and/or TC-HT. The cell medium was replaced 24 h after treatment, and the cells were cultured for additional 10 days, with fresh medium replaced every 3 days during the culture period. The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature and stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA) at room temperature for 15 min for visualization and cell counting. A colony was defined as a cluster consisting of ≥50 cells. The colonies were imaged using a light microscope and manually counted, and the number of colonies in each group was normalized to the control group.

Cell cycle analysis. After 24 h of treatment with 10 μM Erl and/or TC-HT, the cells were harvested, rinsed with PBS and fixed with 70% ethanol at 4˚C for 30 min before staining. The cells were stained for 30 min in the dark at room temperature with propidium iodide and ribonuclease A (Gibco; Thermo Fisher Scientific, Inc.). Subsequently, the stained cells were subject to the cell cycle analysis using a flow cytometer (FACSVerse; BD Biosciences), and data were analyzed using FlowJo (version 7.6.1; FlowJo LLC).

Statistical analysis. Results were expressed as the mean ± standard deviation (n=3). Statistical analyses were performed using OriginPro 2015 (version 92E; OriginLab Corporation). Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TC-HT for in vitro application. The thermal cycling treatment was applied with a modified PCR machine as previously described (22,23). The schematic temperature and duration settings are shown in Fig. 1A, where the temperature was increased to the desired high temperature setting and maintained for 3 min, followed by a natural cooling period for 30 sec. In practice, the heating device was switched off in the cooling process, and the low temperature setting was chosen to be 37˚C to mimic human body temperature. A single cycle containing a high temperature and a low temperature period was repeated 10 times. Fig. 1B shows the actual temperature in the culture well measured by a thermocouple at moderate temperature TC-HT setting. The cycling temperature came to an equilibrium state after the third heating period, thereafter the temperature cycling between 41.5-43.0˚C for moderate temperature TC-HT (Fig. 1B). In the current study, the moderate temperature TC-HT treatment (41.5-43.0˚C) was adopted in the subsequent experiments to study the synergistic anticancer effect of Erl and TC-HT in A549 cancer cells.

TC-HT potentiates the anticancer efficacy of Erl in A549 NSCLC cells. To determine the effect of the combination of Erl and TC-HT treatment on human NSCLC cell viability, A549 cells were treated with different concentrations of Erl with or without TC-HT. At 72 h post treatment, cell viability was assessed using an MTT assay. Erl exhibited a significant antineoplastic effect in a concentration-dependent manner (Fig. 2A). The IC_{50} of Erl was ~10 μM in A549 cells after 72 h treatment. Compared with treatment with Erl alone, TC-HT therapy sensitized A549 cells to Erl and reduced the cell viability to ~30% of the control group (Fig. 2A). It is noteworthy that the combination of Erl and TC-HT demonstrated synergistic ability compared with Erl monotherapy, which reduced the IC_{50} to 0.5 μM. To determine the synergistic effect of TC-HT and Erl, SQ calculations were conducted,

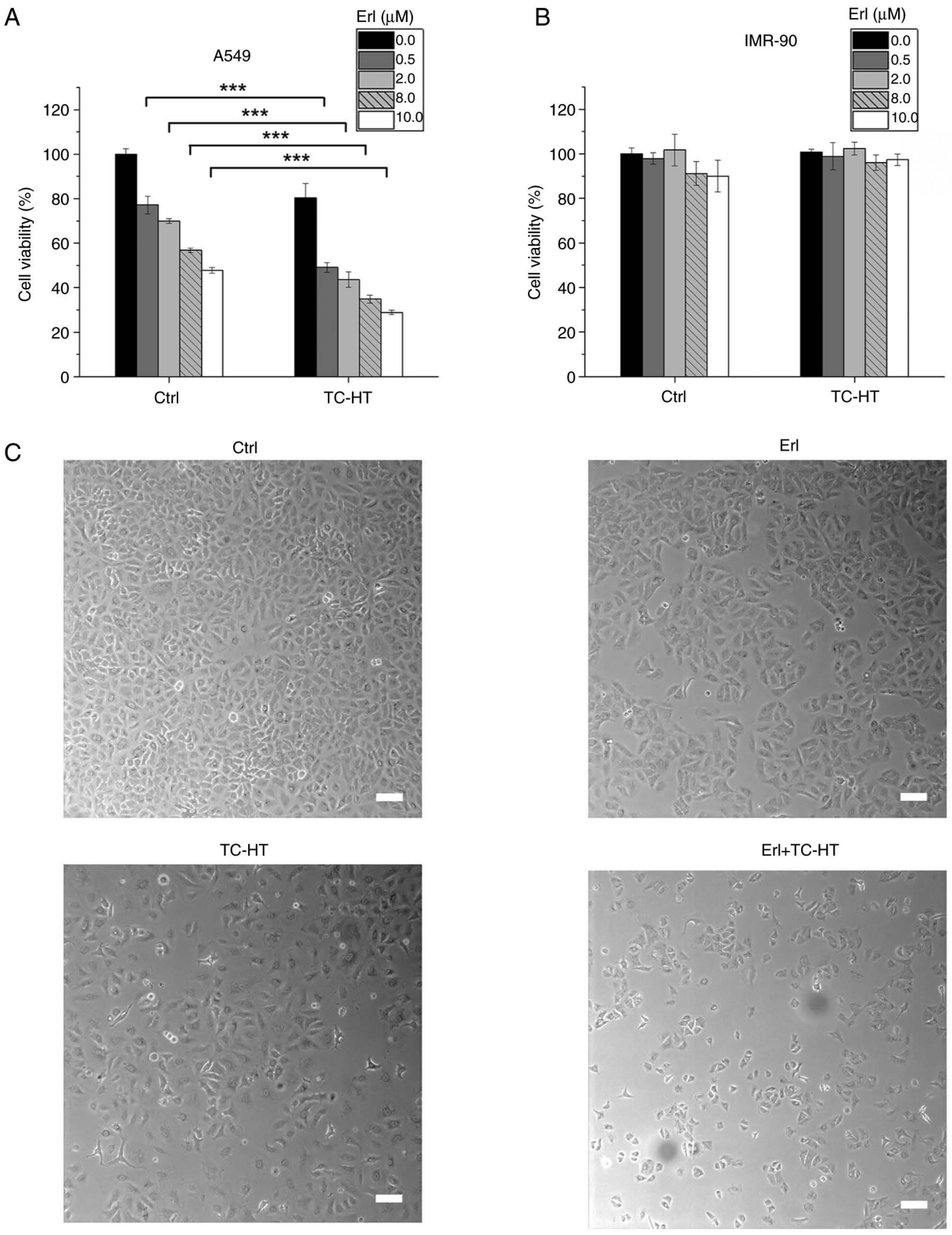

Figure 2. Effect of Erl or TC-HT or in combination on the cell viability and morphological changes of A549 cells at 72 h. (A) MTT viability assay of A549 non-small cell lung cancer cells treated with different concentrations of Erl or in combination with moderate temperature TC-HT treatment (41.5-43.0˚C). (B) MTT viability assay of IMR-90 normal lung cells treated with various concentrations of Erl or in combination with moderate temperature TC-HT treatment (41.5-43.0˚C). (C) Representative images of morphological changes of A549 cells under a light microscope after treatment with 10 μM Erl, moderate temperature TC-HT treatment (41.5-43.0˚C) or in combination. Scale bar, 100 μm. Data are shown as mean ± standard deviation (n=3). Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test. ***P<0.001. Erl, erlotinib; TC-HT, thermal cycling-hyperthermia; ctrl, control.

where an SQ value >1.0 indicated a synergistic effect (25-27). The SQ calculations of cell viability indicated a synergistic effect when TC-HT was combined with Erl at concentrations of 0.5-8.0 μM (Table SI). The highest SQ value was observed with the combination treatment using 0.5 μM Erl, suggesting that the synergistic effects at lower concentrations of Erl

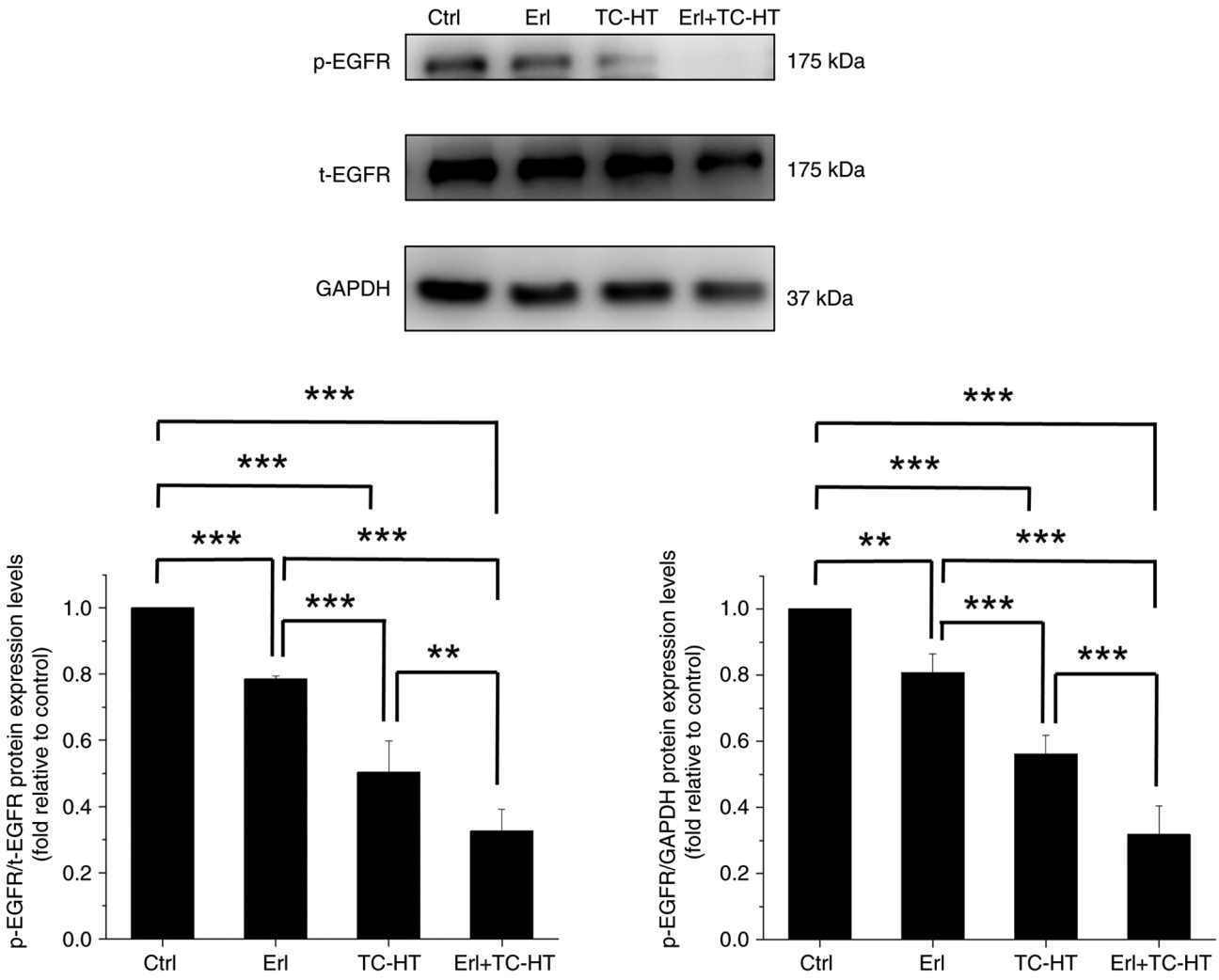

Figure 3. Effect of Erl combined with TC-HT on p-EGFR protein expression in A549 cells. Western blotting of p-EGFR protein expression in A549 cells treated with 10 μM Erl, moderate temperature TC-HT, and the combination treatment. The expression levels of p-EGFR were normalized to GAPDH. Each relative expression level was compared with the control and represented as fold relative to the control. Data are shown as mean ± standard deviation (n=3). Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test. **P<0.01, ***P<0.001. Erl, erlotinib; TC-HT, thermal cycling-hyperthermia; p, phosphorylated; t, total; ctrl, control.

were more pronounced compared with those at higher concentrations. To enhance understanding of the temporal effects on cell viability, the viability of A549 cells was evaluated at 24 and 48 h (Fig. S2A). Additionally, the investigation was expanded to include the H460, another NSCLC cell line, assessing the impact of the combination of Erl and TC-HT on NSCLC (Fig. S2B). These results demonstrated that the combination of Erl and TC-HT led to a significant time- and dose-dependent decrease in cell viability in both A549 and H460 cell lines. To verify the effect of this combined treatment in normal cells, the normal lung cell line IMR-90 was used and the same treatment conditions were applied. The cell viability of IMR-90 normal lung cells decreased to ~90% when treated with 8 or 10 μM Erl, while it remained unaffected by doses <2 μM Erl and/or TC-HT (Fig. 2B). Additionally, Erl and TC-HT combination treatment resulted in notable morphological changes in A549 cells, including shrinkage and fragmentation of cells as well as a decreased number of cells (Fig. 2C). These result suggested that TC-HT may serve as a sensitizer with minimal observed adverse effects to increase the anticancer effect of targeted therapy drugs (22,23).

Effect of Erl combined with TC-HT on EGFR protein expression in A549 cells. The signaling pathways involved in the anticancer mechanism of Erl and TC-HT combination treatment were investigated. Despite the application of targeted therapy agents aimed at inhibiting EGFR activity, limited efficacy remains a challenge in certain NSCLC cells with intrinsic resistance to Erl (11-13). *In vitro* studies have identified A549 cells as resistant to the EGFR-TKI Erl (15,28,29). Consequently, it is imperative to explore innovative strategies to augment the anticancer effects of Erl (14,30-32). In the present study, Erl alone caused a marked decrease in EGFR phosphorylation compared with the control cells (Fig. 3). Moreover, while TC-HT alone was capable of significantly reducing p-EGFR expression compared with the control cells, the combination of Erl and TC-HT resulted in a more substantial inhibitory effect on p-EGFR expression compared with the single treatment and control cells. Additionally, a comparable

decrease in p-EGFR expression was also observed in the combination of low-dose Erl and TC-HT, compared with both low-dose Erl alone and the control cells (Fig. S3). Although this combination was less effective in inhibiting p-EGFR compared with higher concentrations of Erl in conjunction with TC-HT, the lower doses of Erl still exhibited significant inhibitory effects when combined with TC-HT. These results suggested that TC-HT may modulate the resistance of A549 cells to Erl.

Effect of Erl combined with TC-HT on EGFR signaling pathways in A549 cells. In the present study, the expression levels of JNK and Akt proteins (33) were examined in A549 cells. JNK, an EGFR downstream protein belonging to the MAPK family, is responsive to stress stimuli and heat shock, with its phosphorylation capable of altering the activities of several proteins that reside in mitochondria or act in the nucleus to trigger apoptosis (34). TC-HT treatment significantly increased the expression levels of the p-JNK protein compared with the control or Erl-treated A549 cells (Fig. 4A). Furthermore, it was demonstrated that the treatment combining Erl and TC-HT was more effective in increasing p-JNK protein expression levels compared with TC-HT alone. On the other hand, another EGFR downstream protein, Akt, is involved in cellular survival pathways by inhibiting the apoptotic process (35). Once activated by phosphorylation, Akt actively regulates several transcription factors facilitating the expression levels of survival proteins. It was shown that the protein expression levels of p-Akt in Erl-treated A549 cells were significantly lower than that compared with untreated cells, while the inhibitory effect on phosphorylation of Akt was further enhanced upon TC-HT treatment and was more pronounced in the combination Erl and TC-HT treatment group (Fig. 4B). Meanwhile, t-Akt exhibited a trend similar to that of the p-Akt protein, suggesting that decrease in the phosphorylation of Akt by these treatments was caused by a reduction in the amount of Akt present, leading to a weakened survival signal. Moreover, it has been reported that both JNK and Akt signaling pathways are important regulators in influencing mitochondrial function. In addition, mitochondria-dependent apoptosis is associated with a loss in MMP, causing the release of cytochrome *c* and other apoptotic factors (36). To confirm that the apoptosis caused by Erl and TC-HT could be related to mitochondrial disruption, MMP was assessed with DiOC6(3) fluorescence staining using flow cytometry. It was confirmed that Erl and TC-HT individually had no effect on MMP level compared with the control, but the combination treatment caused significant MMP depolarization in A549 cells, indicating mitochondrial dysfunction in their apoptosis mechanism (Fig. S4).

The injured mitochondria release cytochrome *c* into the cytoplasm, cleaving caspase 9 and thus activating caspase 3 downstream (37), which enters further the nucleus and cleaved PARP. It should be noted that PARP serves an important role in mitochondria-mediated apoptosis, in addition to being a key enzyme for DNA repair. In apoptosis, the PARP protein is typically cleaved and inactivated, thereby suppressing DNA repair and causing programmed cell death (38). To understand the apoptosis mechanism in A549 cells triggered by the combination Erl and TC-HT treatment, the present study evaluated the expression levels of apoptosis-related proteins using western blotting. It was shown that the ratio of cleaved PARP to full length PARP in TC-HT-treated cells was significantly higher compared with that in Erl-treated cells (Fig. 4C). Moreover, it was demonstrated that the ratio of cleaved PARP to full length PARP in combination Erl and TC-HT treatment was significantly higher compared with that in the TC-HT treatment alone, indicating that the combination treatment enhanced apoptosis of A549 cells via the mitochondrial pathway. Furthermore, the MTH1 protein has received increasing attention in cancer treatment, due to its key role in DNA repair (39). High expression level of MTH1 is deemed to be a sign of NSCLC metastasis (40). Therefore, the present study examined MTH1 protein expression levels via western blotting. MTH1 protein expression levels were found to significantly decrease in Erl-treated cells compared with controls, while the inhibitive effect on MTH1 protein expression levels was significantly higher in the TC-HT treatment group, which significantly increased further in the combination Erl and TC-HT treatment group (Fig. 4D). These results suggested that the combination of Erl and TC-HT may prevent MTH1-related DNA repair and induce the death of cancer cells via apoptosis.

Combination treatment of Erl and TC-HT causes G2/M cell cycle arrest in A549 cells. To further evaluate the anticancer effects of the combination of Erl and TC-HT treatment on human NSCLC, the cell cycle progression in A549 cells was examined by flow cytometry. Treatment with moderate TC-HT led to a significant increased in cell cycle arrest at the G2/M phase (22.95±0.56%) compared with the group treated with Erl (13.06±1.16%) and the untreated cells (12.66±1.18%; Fig. 5A and B). Meanwhile, the combination of Erl and moderate TC-HT caused significant accumulation of cells in the G2/M phase (33.77±2.31%) compared with the single treatments and the untreated cells, with a concomitant reduction of cells in the G0/G1 phase. Besides, no significant differences in the S phase were observed among the various treatments tested. Next, relevant proteins involved in the G2/M progression were examined to investigate the mechanism of the combined treatment effect on cell cycle distribution. Cdc2 is a core regulator in the cell cycle and it binds to the cyclin B complex guiding G2/M cell cycle transition (41,42). It has been previously reported that the inhibition of Cdc2 expression resulted in G2/M cell cycle arrest (43-45). In the present study, the protein expression levels of Cdc2 were reduced significantly upon treatment with the combination of Erl and TC-HT compared with the single Erl and TC-HT treatments and the control group (Fig. 5C). Meanwhile, as a well-known MAPK member, p38 serves an important role in cell cycle regulation, and numerous studies have reported that activation of the p38 pathway can induce G2/M cell cycle arrest via Cdc2 suppression in human NSCLC cells (46-48). Consistently, it was shown that the protein expression levels of p-p38 were significantly increased by the combination treatment of Erl and moderate TC-HT (Fig. 5D). Taken together, these results suggested that the combined treatment could synergistically induce cell cycle arrest in the G2/M phase by increasing the activation of p38 while reducing Cdc2 expression in cells, thereby suppressing cell cycle progression in human lung cancer A549 cells.

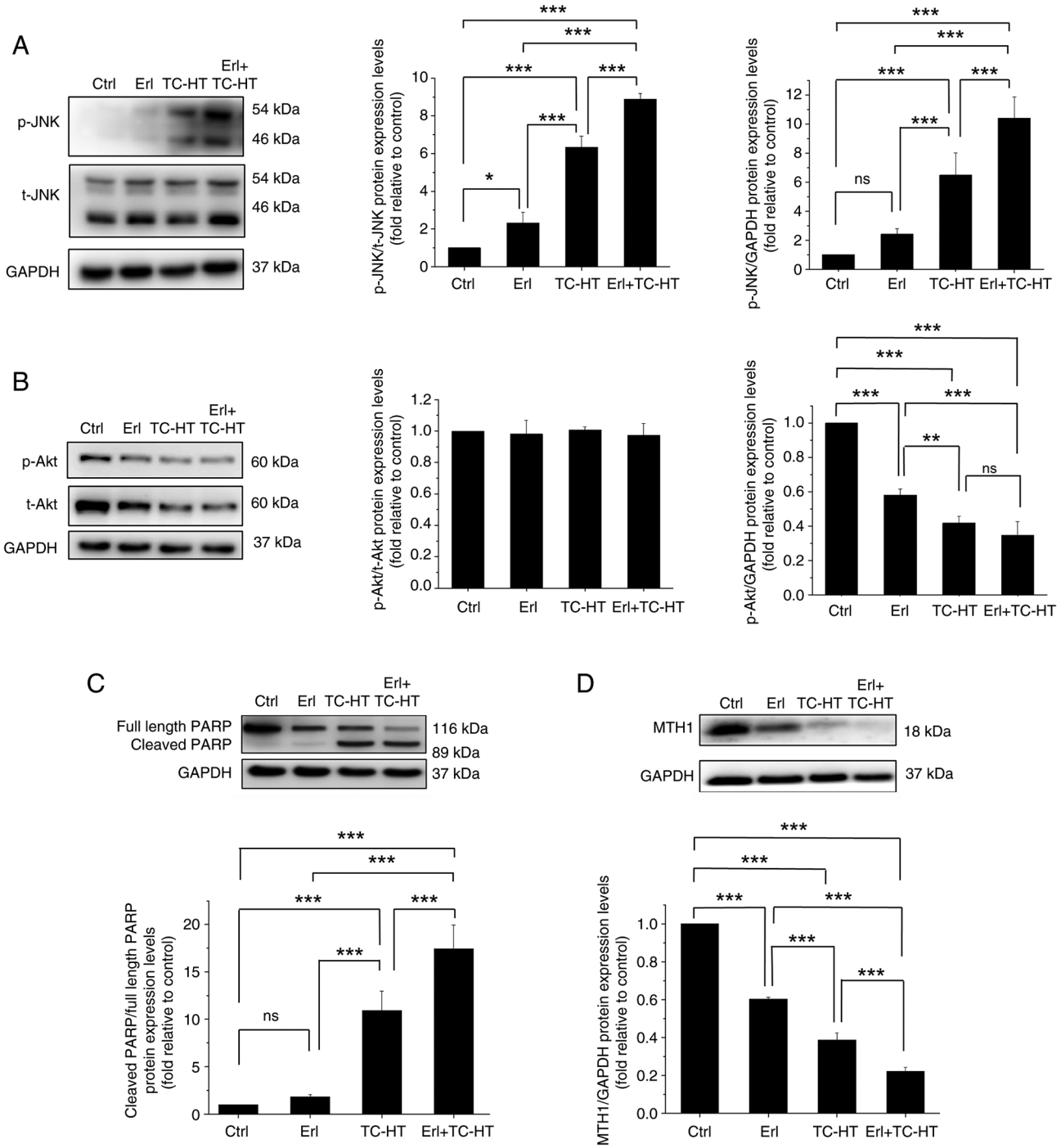

Figure 4. Effect of Erl combined with TC-HT treatment on survival- and apoptosis-related protein expression in A549 cells. The anticancer experiments were conducted in A549 cancer cells treated with 10 μM Erl, moderate temperature TC-HT and the combination treatment. Western blotting of (A) p-JNK, (B) p-Akt and t-Akt, (C) cleaved PARP and full-length PARP (D) MTH1. The protein expression levels of p-JNK, p-Akt, t-Akt and MTH1 were normalized to GAPDH, and cleaved PARP was normalized to full-length PARP. Each relative expression level was compared with the control and represented as fold relative to the control. Data are shown as mean ± standard deviation (n=3). Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test. *P<0.05, **P<0.01, ***P<0.001. Erl, erlotinib; TC-HT, thermal cycling-hyperthermia; p, phosphorylated; t, total; PARP, poly ADP-ribose polymerase; MTH1, MutT homolog 1; ctrl, control; ns, not significant.

Anti-proliferation and anti-migration effects of the combination treatment (Erl + moderate temperature TC-HT) in A549 NSCLC cells. Cancer mortality is associated with cancer recurrence and metastasis, both of which are common clinical issues in NSCLC (49,50). Therefore, it is important to reduce the proliferation and migration ability of cancer cells. To further confirm the anti-proliferative and anti-migration activities of the Erl and TC-HT combination treatment in NSCLC, wound healing (Fig. 6A) and colony formation assays (Fig. 6B) were performed using A549 cells. Erl-treated cells showed a significantly decreased migration capacity compared with the control cells, while cells treated with TC-HT alone exhibited a significant reduction in migration compared with single Erl treatment and the control

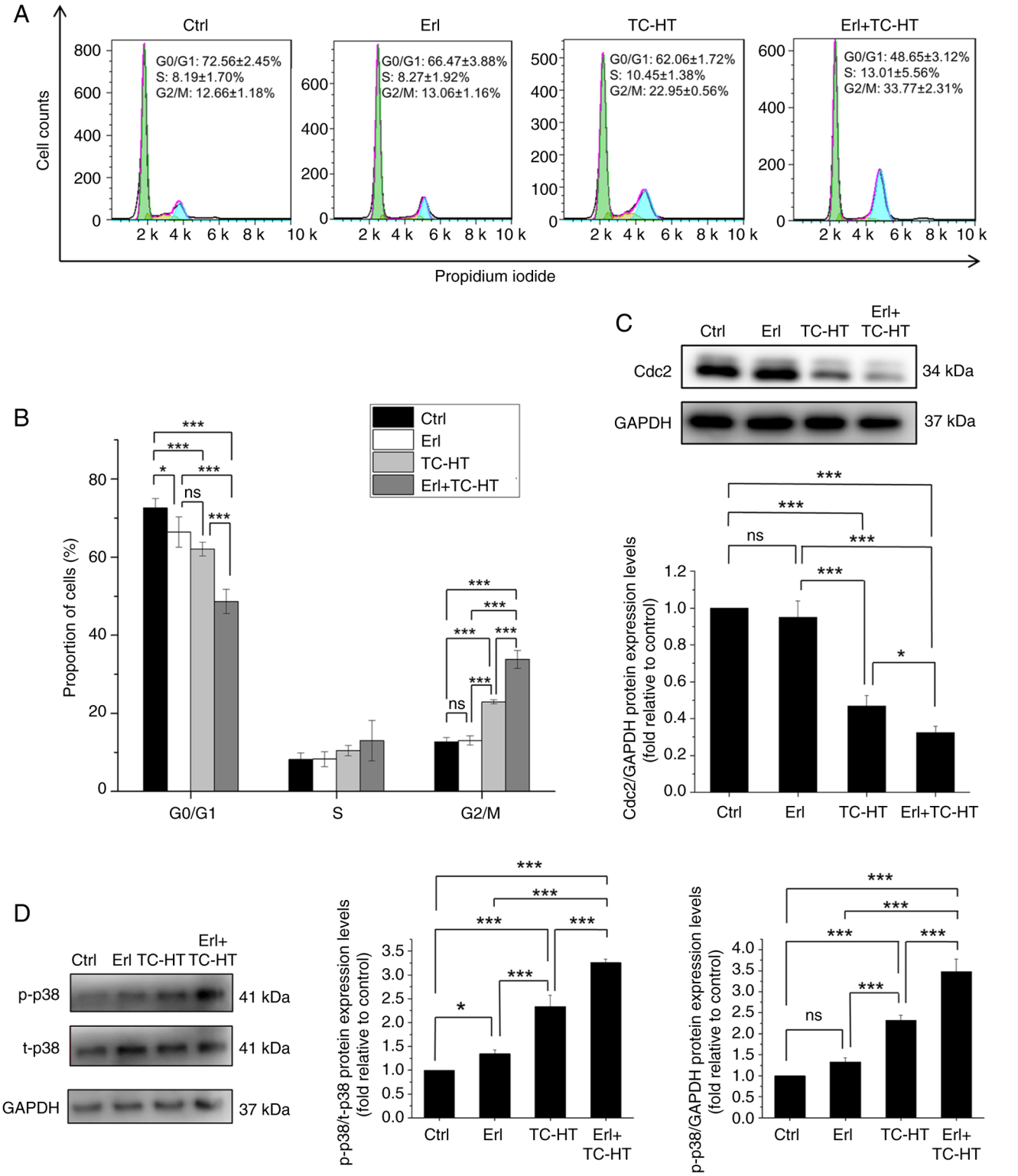

Figure 5. Effect of Erl or TC-HT or in combination on the G2/M cell cycle arrest and protein expression levels of Cdc2 and p-p38 in A549 cells. Cell cycle and protein expression level analysis were conducted in A549 cancer cells treated with 10 μM Erl, moderate temperature TC-HT (41.5-43.0˚C) and the combination treatment. (A) Flow cytometry analysis on cellular DNA content profiles of each group of A549 cells. (B) Proportion of A549 cells in G0/G1, S and G2/M phases of the indicated group. Western botting of (C) Cdc2 and (D) p-p38 protein expression. The protein expression levels of p-p38 and Cdc2 were normalized to GAPDH. Each relative expression level was compared with the control and represented as fold relative to the control. Data are shown as mean ± standard deviation (n=3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test. *P<0.05, ***P<0.001. Erl, erlotinib; TC-HT, thermal cycling-hyperthermia; p, phosphorylated; t, total; ctrl, control; ns, not significant..

cells (Fig. 6C). Furthermore, the Erl and TC-HT combination treatment caused a more significant decrease in A549 cell migration compared with that of the single treatments. Compared with the control group, the wound closure percentage in the Erl-treated group and the TC-HT-treated group was 83.8 and 59.2%, respectively, while the wound

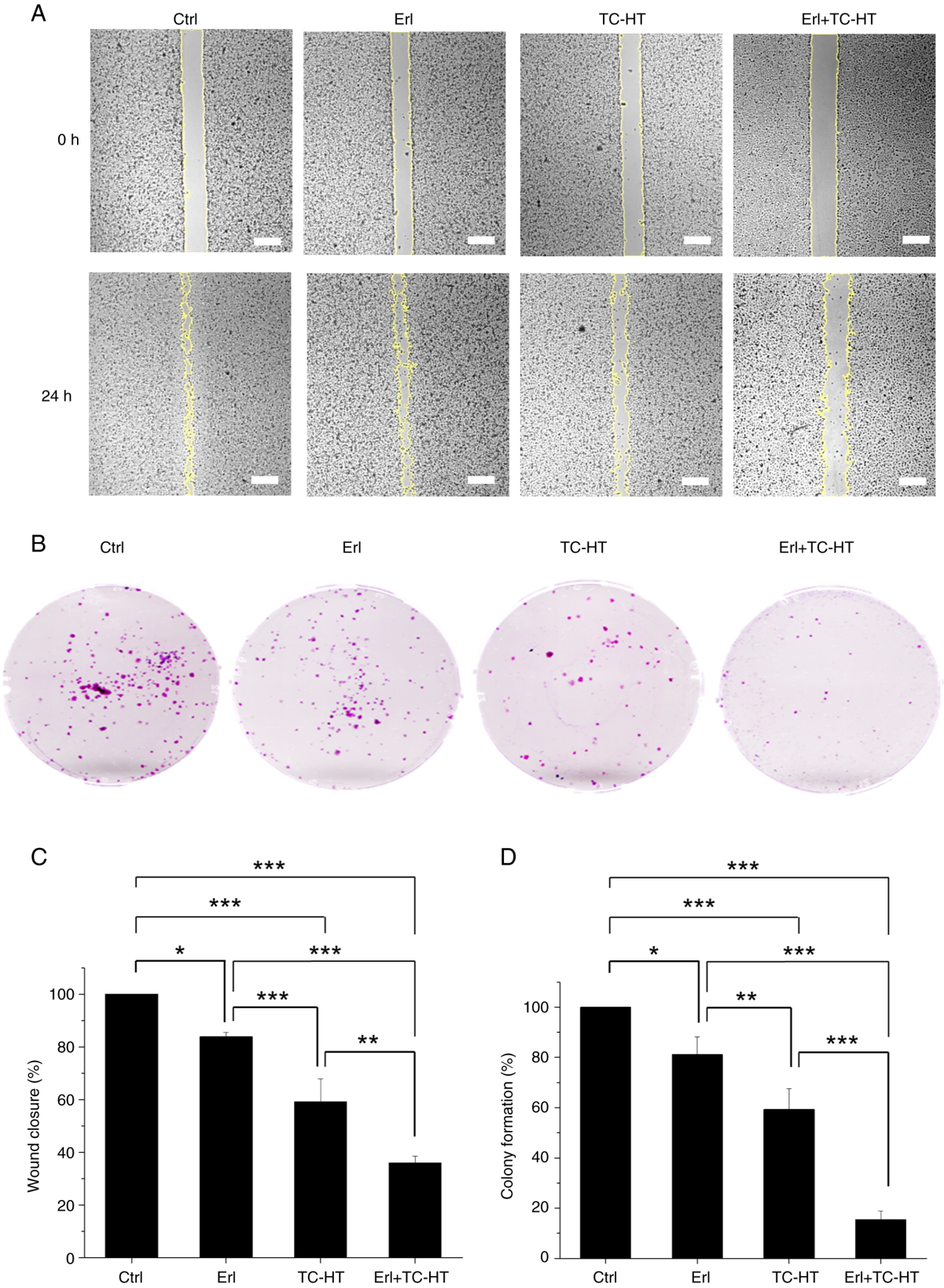

Figure 6. Effect of Erl or TC-HT or in combination on the inhibition of A549 cell colony formation and migration. (A) Wound healing assay to determine the effect of Erl, TC-HT or combination treatment on the migration ability of A549 cells. After scratch gaps were made, A549 cells were treated with 10 μM Erl, moderate temperature TC-HT or the combination treatment. Yellow lines indicate wound edges detected by ImageJ (version 1.51j8; National Institutes of Health). Magnification, x40. (B) Colony formation assay for A549 cells treated with 10 μM Erl, moderate temperature TC-HT or the combination treatment. (C) Wound closure rate for A549 cells was determined as the percentage of the area closed after 24 h from the initial wound area, and each group was normalized to the control group and expressed as a fraction of 100. The areas of cell-free gaps were measured and quantified using ImageJ (version 1.51j8; National Institutes of Health). (D) Colony formation rate for A549 cells at 10 days after treatment with 10 μM Erl, moderate temperature TC-HT or the combination treatment. Each group was normalized to the control group and represented as a percentage. The colony counting was performed using ImageJ (version 1.51j8; National Institutes of Health). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test. *P<0.05, **P<0.01, ***P<0.001. Erl, erlotinib; TC-HT, thermal cycling-hyperthermia; ctrl, control.

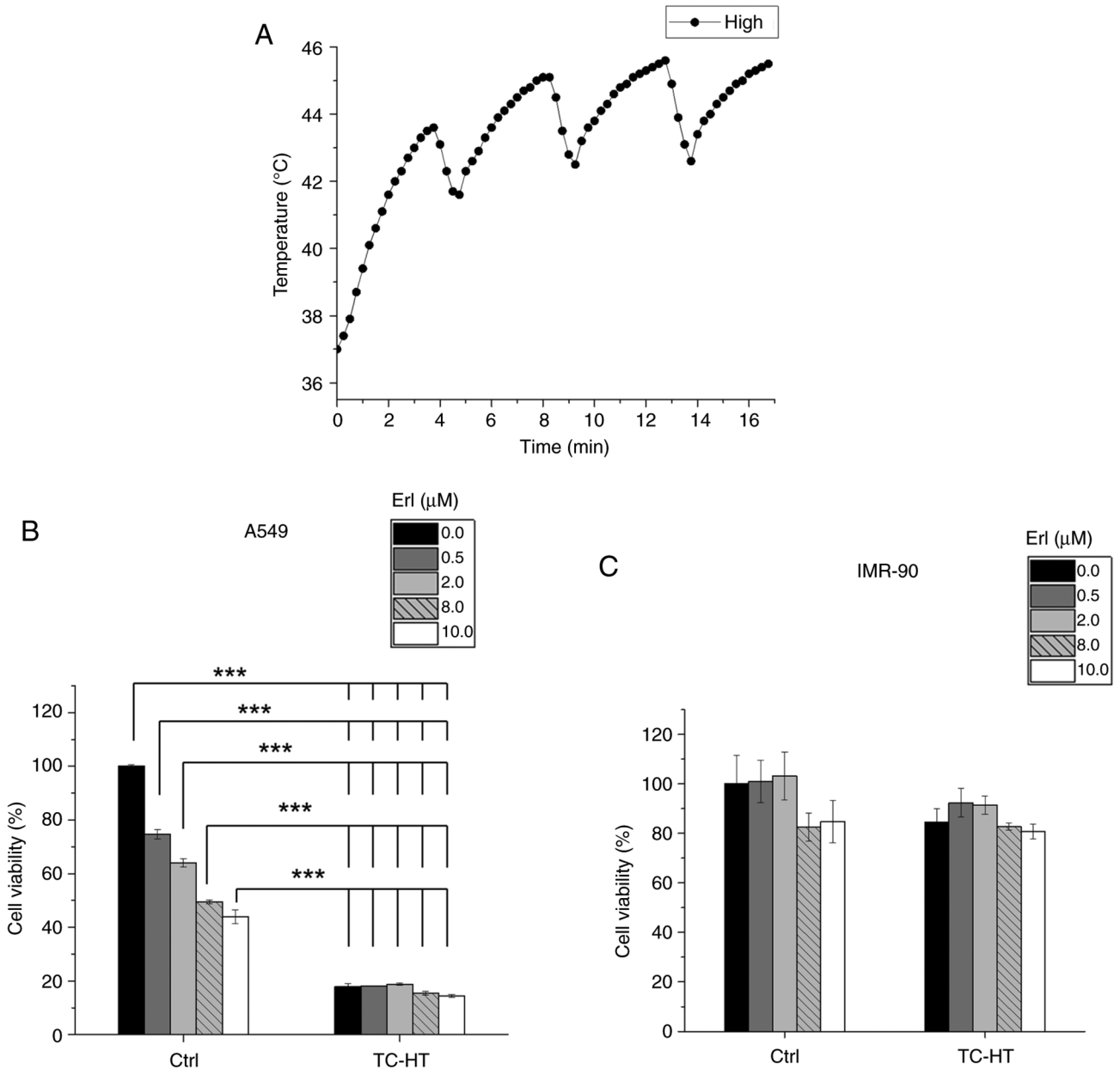

Figure 7. Effect of high temperature TC-HT or Erl or a combination treatment on cell viability. (A) Actual temperature with high temperature TC-HT setting in the culture well measured every 15 sec using a needle thermocouple. (B) MTT viability assay of A549 non-small cell lung cancer cells treated with different concentrations of Erl or combination with high temperature TC-HT treatment (42.5-45.6˚C). (C) MTT viability assay of IMR-90 normal lung cells treated with various concentrations of Erl or in combination with high temperature TC-HT treatment (42.5-45.6˚C). Data are shown as mean ± standard deviation (n=3). Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test. ***P<0.001. Erl, erlotinib; TC-HT, thermal cycling-hyperthermia; ctrl, control.

closure percentage in the combined treatment group was only 35.9%. The results indicated that the migration ability of A549 cells was significantly suppressed after the application of the Erl and TC-HT combination treatment. On the other hand, it was demonstrated that colony formation ability of A549 cells treated with either Erl or TC-HT was significantly decreased compared with the control cells, whereas the colony formation ability of cells treated with the combination treatment was further significantly reduced compared with that of the single treatments (Fig. 6D). Overall, the combination treatment was shown to impede A549 cell proliferation and migration, indicating the potential anticancer efficacy of the Erl and TC-HT combination treatment.

Discussion

The present study demonstrated the synergistic anticancer effect of the combination of Erl, an EGFR-TKI, and TC-HT on A549 NSCLC cells. EGFR is a receptor tyrosine kinase critical for the initiation and development of malignant tumors via the MAPK and PI3K/Akt pathways (6). Although EGFR-TKIs, such as Erl, can target and inhibit the EGFR pathways (7), their clinical benefit has been limited, due to the resistance to TKIs among a proportion of patients with NSCLC (9). In certain NSCLC tumors, the development of acquired resistance to Erl occurs following prolonged treatment periods (9). Additionally, the effectiveness of Erl is constrained by the intrinsic resistance

present in specific patient populations (11-13), underscoring the need to improve the sensitivity of these patients to Erl. Amid the efforts to combat the resistance to EGFR-TKIs (51), combination treatment has been deemed as a promising approach to overcome the resistance problem (52). Several studies have investigated the effect of combining Erl and other anticancer agents such as cisplatin, monoclonal antibodies, aspirin and capsaicin (14,53-55), aiming to enhance the therapeutic effect of this treatment. However, the combined use of multiple drugs in clinical applications is often hampered by unpredictable drug interactions or side effects. Therefore, alternate methods are required to enhance the anticancer effect of these drugs by combining physical stimulation with the use of a drug treatment. It has been previously reported that mild hyperthermia, when combined with other therapeutic approaches, has resulted in enhanced anticancer effects in various clinical trials, with treatment durations ranging from 10-90 min (56). Additionally, studies focusing on NSCLC have implemented hyperthermia therapies with durations of ~30 min (57-59). In accordance with these established research protocols and recognizing that an appropriate duration of thermal exposure may reduce damage to normal cells, the present study demonstrated the efficacy of TC-HT physical stimulation in augmenting the anticancer effects of Erl. Compared with Erl or TC-HT mono treatment, the combination treatment produced an increased inhibitory effect on A549 and H460 NSCLC cells, without damaging the IMR-90 normal lung cells, thereby circumventing drug interaction issues and potentially minimizing the risk of side effects by reducing the required dosage of Erl. To elucidate the molecular mechanisms underlying the anticancer effect of the combination treatment on A549 NSCLC cells, the expression levels of certain proteins in the apoptotic pathway were evaluated. It has been previously reported that the relative expression levels of phosphorylated proteins normalized to an internal control are associated with cellular survival or apoptosis (60-63). The focus of the present study was to elucidate the relative changes in signaling intensity and, to the best of our knowledge, the present study represented the first report into the enhanced apoptosis resulting from the combination of TC-HT and Erl in A549 cells. It was demonstrated that TC-HT significantly potentiated the efficacy of Erl in inhibiting the phosphorylation of EGFR, which amplified the inhibitory effect of Erl on A549 NSCLC cells. Subsequently, the expression levels of JNK and Akt proteins were investigated in the EGFR downstream pathway. The activation of JNK is involved in the induction of apoptosis (34), while the activation of Akt inhibits apoptosis (35). Additionally, both JNK and Akt proteins serve a crucial role in the regulation of mitochondrial function (36), and thus investigating the activation status of these proteins contributes to a better understanding of cellular apoptotic tendency. The results of the current study demonstrated that the combination of TC-HT and Erl increased p-JNK expression levels while decreasing p-Akt expression levels. Concurrently, the combination treatment of Erl and TC-HT significantly increased MMP depolarization in A549 cells, indicating that apoptosis induced by Erl and TC-HT may be partly associated with mitochondrial apoptosis pathways. In addition, the cleavage of the PARP protein and the downregulation of MTH1 expression have been reported to contribute to the initiation apoptosis in cancer cells (38,39). The present study demonstrated that a significant increase in the expression level of cleaved PARP was accompanied by a decrease in MTH1 expression levels. These findings suggested that the combination treatment of Erl and TC-HT effectively enhanced the apoptosis of A549 NSCLC cells.

Cell cycle dysregulation, a common feature of cancer, could be ameliorated through the modulation of cell cycle regulatory proteins, offering the potential to attenuate the uncontrolled proliferation of cancer cells (44,46-48). The present study demonstrated that the combination treatment of Erl and TC-HT resulted in G2/M phase arrest. The Cdc2 protein is considered to be a core regulator involved in the G2/M cell cycle transition (41,42). Significant downregulation of Cdc2 expression levels was demonstrated in the Erl and TC-HT combination treatment group. Furthermore, the expression levels of p38 were investigated, as previous studies in A549 NSCLC cells demonstrated that increased p38 phosphorylation resulted in a reduction of the level of Cdc2 protein expression, eventually leading to G2/M cell cycle arrest (46-48). In the current study, it was also demonstrated that the combined treatment of Erl and TC-HT significantly increased the protein expression levels of p-p38. These results indicated that the combination treatment of Erl and TC-HT effectively reduced the occurrence of mitosis in A549 NSCLC cells, inhibiting of cancer cell proliferation. It has been previously reported that Erl treatment alone has a mild or negligible effect on G2/M arrest, while combining Erl with other approaches, such as protein kinase C-β inhibitor enzastaurin and small molecule drug a-tocopheryl succinate hold promise for improving its efficacy (64,65). Similarly, the present study also demonstrated a negligible influence of Erl on G2/M arrest when Erl was administered alone, whereas a notable increase in G2/M arrest was observed when Erl was combined with TC-HT. It is noteworthy that TC-HT, as a method of physical stimulation, offers a novel method to avoid potential drug-drug interactions, meriting further investigation in anticancer applications.

Inhibiting cancer cell proliferation, recurrence and migration is an important part of cancer treatment, and high rates of recurrence and metastasis are concerns for patients with NSCLC (49,50). The present study demonstrated that the combined treatment of Erl and TC-HT reduced the viability of A549 NSCLC cells and induced G2/M cell cycle arrest. Additionally, the proliferation and migration of A549 cancer cells under the combined treatment of Erl and TC-HT was investigated. These results demonstrated significant findings concerning the migratory behavior of A549 cells under different treatments. It was shown that single Erl or TC-HT treatment reduced the migration area of treated cells in comparison with control cells. Notably, the combination treatment of Erl and TC-HT was more effective in inhibiting A549 NSCLC cell migration compared with either the single Erl or TC-HT treatment. Moreover, A549 NSCLC cells treated with Erl or TC-HT displayed reduced colony formation, whereas the combination treatment of Erl and TC-HT exerted an increased inhibitory effect on A549 cells compared with the single treatments. Collectively, these findings highlighted the combined treatment with Erl and TC-HT as a promising anticancer strategy as it significantly impeded both A549 NSCLC cell migration and colony formation.

HT has long been a promising cancer treatment method, to be applied alone or in combination with other conventional therapies (66). However, this treatment can cause cellular damage, due to an overdose of HT, a practical problem which can now be overcome by TC-HT due to the ability to control the thermal dosage applied, as the intermittent cooling process can avoid excessive thermal dosage accumulation and subsequent cytotoxic cell damage. Excessive thermal dosage may induce mitochondrial damage and oxidative stress not only in cancer cells, but also in normal cells (67). However, a previous study reported that cancer cells have higher levels of oxidative stress and thus they are more sensitive to thermal stress (68). Additionally, cancer cells are more thermosensitive compared with normal cells (69). In the current study, the results demonstrated that moderate temperature TC-HT treatment (41.5-43.0˚C) alone inhibited the viability of A549 NSCLC cells, reducing their viability to 80% compared with the control group, without damaging IMR-90 normal lung cells. To further examine the effect of TC-HT alone on cancer cells at a higher temperature, the temperature setting of TC-HT application was raised to the range of a high temperature TC-HT treatment (42.5-45.6˚C). It was demonstrated that the high temperature TC-HT treatment alone resulted in significant inhibition in the viability of A549 NSCLC cells, with their viability dropping to only 17.9% of that of the control group. It is noteworthy that under high temperature TC-HT treatment, the additional incorporation of Erl in the range of 0-10 μM did not cause a significant additional decrease in cell viability. Moreover, the TC-HT treatment at high temperature alone did not have an effect on the viability of IMR-90 normal lung cells, which remained at 80% of that of the control group, even in the case of combined treatment with 10 μM Erl. Additionally, a similar effect was observed in H460 NSCLC cells (Fig. S5). These results suggested that TC-HT may be a potential future anticancer treatment for NSCLC and reduce the current reliance on drug treatments. However, further research to explore its efficacy is required.

Hypoxia is also a common feature of NSCLC and can contribute to drug resistance (70). Hypoxic cancer cells exhibit an increased susceptibility to hyperthermia in both *in vitro* and *in vivo* models (71-74). However, certain challenges remain, as hypoxic conditions can lead to elevated expression of heat shock factor 1 (HSF1) and heat shock proteins (HSPs), which may confer protective effects (75,76). Additionally, HT has been associated with the upregulation of HSF1 and HSP expression, further enhancing thermotolerance in cancer cells (77,78). Studies have reported that EGFR-TKIs can mitigate hypoxia (70,79,80), underscoring the potential of the Erl and TC-HT treatment combination. Furthermore, it has been reported that the concurrent application of HT with HSF1 and HSP inhibitors may represent a promising anticancer strategy (78). Therefore, exploring the combination of HSF1 and HSP inhibitors, or other EGFR-TKIs, with TC-HT could significantly enhance therapeutic outcomes for anticancer treatment and merits further investigation. In practice, non-contact thermal modalities, such as radiofrequency (RF) and focused ultrasound (FUS), present promising localized heating techniques for cancer treatment. These approaches may be applicable for the treatment of NSCLC (20,81-83). Specifically, RF and multi-focal FUS can effectively regulate the heating area and temperature used, achieving precise thermal dissipation while maintaining the desired temperature. Although additional research is necessary to substantiate these possibilities, these features render these techniques suitable for the implementation of TC-HT in anticancer treatments.

In conclusion, the present study reported that TC-HT, a novel thermal treatment, is capable of sensitizing A549 cells to the EGFR-TKI Erl. The anticancer effect of the combination of TC-HT and Erl occurred through the downstream of EGFR signaling cascades, including the MAPK and PI3K-Akt pathways. These results showed that TC-HT could enhance the anticancer efficacy of Erl on A549 cells, thereby reducing Erl drug dosage and associated side effects. Furthermore, TC-HT may have the potential to be used as a drug-free cancer treatment method by raising the high temperature of TC-HT in its application. These findings highlighted the potential for TC-HT in combination therapy with other chemotherapy or targeted therapy drugs, and further studies are needed to examine specific TC-HT parameters in treating different types of cancers.

Acknowledgements

The authors would like to acknowledge the service provided by the Research Core Facilities 3 Laboratory of the Department of Medical Research at National Taiwan University hospital for the use of the flow cytometry system.

Funding

The present study was supported by research grants from the Ministry of Science and Technology (grant nos. MOST 110-2112-M-002-004 and MOST 109-2112-M-002-004 to CYC) and the National Science and Technology Council (grant no. NSTC 112-2112-M-002-033 to CYC) of the Republic of China.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CYC conceived and supervised the study. GBL, WTC and CYC designed the study. GBL and WTC performed the experiments and collected the data. GBL and WTC confirmed the authenticity of all the raw data. GBL, WTC, YYK, HHL, YMC, SJL and CYC contributed to data analysis. GBL, WTC and CYC wrote the manuscript. All authors approved the final version of the 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.

References

1. World Health Organization: WHO report on cancer: Setting priorities, investing wisely and providing care for all. World Health Organization, 2020.
2. Balani C, Goss G and Blumenschein G Jr: Recent clinical developments and rationale for combining targeted agents in non-small cell lung cancer (NSCLC). Cancer Treat Rev 38: 174-184, 2012.
3. Imyanitov EN, Iyevleva AG and Levchenko EV: Molecular testing and targeted therapy for non-small cell lung cancer: Current status and perspectives. Crit Rev Oncol Hemat 157: 103194, 2021.
4. Wieduwilt MJ and Moasser MM: The epidermal growth factor receptor family: Biology driving targeted therapeutics. Cell Mol Life Sci 65: 1566-1584, 2008.
5. Yewale C, Baradia D, Vhora I, Patil S and Misra A: Epidermal growth factor receptor targeting in cancer: A review of trends and strategies. Biomaterials 34: 8690-8707, 2013.
6. Wee P and Wang Z: Epidermal growth factor receptor cell proliferation signaling pathways. Cancers (Basel) 29: 52, 2017.
7. Ciardiello F, De Vita F, Orditura M and Tortora G: The role of EGFR inhibitors in nonsmall cell lung cancer. Curr Opin Oncol 16: 130-135, 2004.
8. Gridelli C, Maione P, Bareschino MA, Schettino C, Sacco PC, Ambrosio R, Barbato V, Falanga M and Rossi A: Erlotinib in the treatment of non-small cell lung cancer: Current status and future developments. Anticancer Res 30: 1301-1310, 2010.
9. Lin Y, Wang X and Jin H: EGFR-TKI resistance in NSCLC patients: Mechanisms and strategies. Am J Cancer Res 4: 411-435, 2014.
10. Melosky B: Supportive care treatments for toxicities of anti-EGFR and other targeted agents. Curr Oncol 19: 59-63, 2012.
11. Zhu CQ, da Cunha Santos G, Ding K, Sakurada A, Cutz JC, Liu N, Zhang T, Marrano P, Whitehead M, Squire JA, *et al*: Role of KRAS and EGFR as biomarkers of response to erlotinib in National Cancer Institute of Canada Clinical Trials Group Study BR.21. J Clin Oncol 26: 4268-4275, 2008.
12. Calvo E and Baselga J: Ethnic differences in response to epidermal growth factor receptor tyrosine kinase inhibitors. J Clin Oncol 24: 2158-2163, 2006.
13. Garassino MC, Martelli O, Broggini M, Farina G, Veronese S, Rulli E, Bianchi F, Bettini A, Longo F, Moscetti L, *et al*: Erlotinib versus docetaxel as second-line treatment of patients with advanced non-small-cell lung cancer and wild-type EGFR tumours (TAILOR): A randomised controlled trial. Lancet Oncol 14: 981-988, 2013.
14. Raimbourg J, Joalland MP, Cabart M, de Plater L, Bouquet F, Savina A, Decaudin D, Bennouna J, Vallette FM and Lalier L: Sensitization of EGFR wild-type non-small cell lung cancer cells to EGFR-tyrosine kinase inhibitor erlotinib. Mol Cancer Ther 16: 1634-1644, 2017.
15. Li T, Ling YH, Goldman ID and Perez-Soler R: Schedule-dependent cytotoxic synergism of pemetrexed and erlotinib in human non-small cell lung cancer cells. Clin Cancer Res 13: 3413-3422, 2007.
16. Almanric K, Marceau N, Cantin A and Bertin É: Risk factors for nephrotoxicity associated with cisplatin. Can J Hosp Pharm 70: 99-106, 2017.
17. Oei AL, Vriend LE, Crezee J, Franken NA and Krawczyk PM: Effects of hyperthermia on DNA repair pathways: One treatment to inhibit them all. Radiat Oncol 10: 165, 2015.
18. Kaur P, Hurwitz MD, Krishnan S and Asea A: Combined hyperthermia and radiotherapy for the treatment of cancer. Cancers (Basel) 3: 3799-3823, 2011.
19. Kwon S, Jung S and Baek SH: Combination therapy of radiation and hyperthermia, focusing on the synergistic Anti-cancer effects and research trends. Antioxidants 12: 924, 2023.
20. Yang WH, Xie J, Lai ZY, Yang MD, Zhang GH, Li Y, Mu JB and Xu J: Radiofrequency deep hyperthermia combined with chemotherapy in the treatment of advanced Non-small cell lung cancer. Chin Med J 132: 922-927, 2019.
21. Beik J, Abed Z, Ghoreishi FS, Hosseini-Nami S, Mehrzadi S, Shakeri-Zadeh A and Kamrava SK: Nanotechnology in hyperthermia cancer therapy: From fundamental principles to advanced applications. J Control Release 235: 205-221, 2016.
22. Chen WT, Sun YK, Lu CH and Chao CY: Thermal cycling as a novel thermal therapy to synergistically enhance the anticancer effect of propolis on PANC-1 cells. Int J Oncol 55: 617-628, 2019.
23. Lu CH, Chen WT, Hsieh CH, Kuo YY and Chao CY: Thermal cycling-hyperthermia in combination with polyphenols, epigallocatechin gallate and chlorogenic acid, exerts synergistic anticancer effect against human pancreatic cancer PANC-1 cells. PLoS One 14: e0217676, 2019.
24. Kuo YY, Chen WT, Lin GB, Lu CH and Chao CY: Study on the effect of a triple cancer treatment of propolis, thermal cycling-hyperthermia, and low-intensity ultrasound on PANC-1 cells. Aging 15: 7496-7512, 2023.
25. Lu CH, Kuo YY, Lin GB, Chen WT and Chao CY: Application of non-invasive low-intensity pulsed electric field with thermal cycling-hyperthermia for synergistically enhanced anticancer effect of chlorogenic acid on PANC-1 cells. PLoS One 15: e0222126, 2020.
26. Hsieh CH, Lu CH, Chen WT, Ma BL and Chao CY: Application of non-invasive low strength pulsed electric field to EGCG treatment synergistically enhanced the inhibition effect on PANC-1 cells. PLoS One 12: e0188885, 2017.
27. Ruttanapattanakul J, Wikan N, Potikanond S and Nimlamool W: Combination of pinocembrin and epidermal growth factor enhances the proliferation and survival of human keratinocytes. Int J Mol Sci 24: 12450, 2023.
28. Zou Y, Ling YH, Sironi J, Schwartz EL, Perez-Soler R and Piperdi B. The autophagy inhibitor chloroquine overcomes the innate resistance of Wild-type EGFR Non-small-cell lung cancer cells to erlotinib. J Thorac Oncol 8: 693-702, 2013.
29. Otahal A, Aydemir D, Tomasich E and Minichsdorfer C: Delineation of cell death mechanisms induced by synergistic effects of statins and erlotinib in non-small cell lung cancer cell (NSCLC) lines. Sci Rep 10: 959, 2020.
30. Li YL, Hu X, Li QY, Wang F, Zhang B, Ding K, Tan BQ, Lin NM and Zhang C: Shikonin sensitizes wild type EGFR NSCLC cells to erlotinib and gefitinib therapy. Mol Med Rep 18: 3882-3890, 2018.
31. Howe GA, Xiao B, Zhao H, Al-Zahrani KN, Hasim MS, Villeneuve J, Sekhon HS, Goss GD, Sabourin LA, Dimitroulakos J and Addison CL: Focal adhesion kinase inhibitors in combination with erlotinib demonstrate enhanced Anti-tumor activity in Non-small cell lung cancer. PLoS One 11: e0150567, 2016.
32. Greve G, Schiffmann I, Pfeifer D, Pantic M, Schüler J and Lübbert M: The pan-HDAC inhibitor panobinostat acts as a sensitizer for erlotinib activity in EGFR-mutated and -wildtype non-small cell lung cancer cells. BMC Cancer 15: 947, 2015.
33. Atalay G, Cardoso F, Awada A and Piccart MJ: Novel therapeutic strategies targeting the epidermal growth factor receptor (EGFR) family and its downstream effectors in breast cancer. Ann Oncol 14: 1346-1363, 2003.
34. Takeuchi K and Ito F: EGF receptor in relation to tumor development: Molecular basis of responsiveness of cancer cells to EGFR-targeting tyrosine kinase inhibitors. FEBS J 277: 316-326, 2010.
35. Akca H, Tani M, Hishida T, Matsumoto S and Yokota J: Activation of the AKT and STAT3 pathways and prolonged survival by a mutant EGFR in human lung cancer cells. Lung Cancer 54: 25-33, 2006.
36. Matsuyama S and Reed JC: Mitochondria-dependent apoptosis and cellular pH regulation. Cell Death Differ 7: 1155-1165, 2000.
37. Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E and Boise LH: Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol 14: 32, 2013.
38. Yi M, Dong B, Qin S, Chu Q, Wu K and Luo S: Advances and perspectives of PARP inhibitors. Exp Hematol Oncol 8: 44573, 2019.
39. Gad H, Koolmeister T, Jemth AS, Eshtad S, Jacques SA, Ström CE, Svensson LM, Schultz N, Lundbäck T, Einarsdottir BO, *et al*: MTH1 inhibition eradicates cancer by preventing sanitation of the dNTP pool. Nature 508: 215-221, 2014.
40. Li DN, Yang CC, Li J, Ou Yang QG, Zeng LT, Fan GQ, Liu TH, Tian XY, Wang JJ, Zhang H, *et al*: The high expression of MTH1 and NUDT5 promotes tumor metastasis and indicates a poor prognosis in patients with non-small-cell lung cancer. Biochim Biophys Acta Mol Cell Res 1868: 118895, 2021.
41. Dorée M and Hunt T: From Cdc2 to Cdk1: When did the cell cycle kinase join its cyclin partner? J Cell Sci 115: 2461-2464, 2002.
42. Lim S and Kaldis P: Cdks, cyclins and CKIs: Roles beyond cell cycle regulation. Development 140: 3079-3093, 2013.

43. Chang CC, Heller JD, Kuo J and Huang RC: Tetra-O-methyl nordihydroguaiaretic acid induces growth arrest and cellular apoptosis by inhibiting Cdc2 and survivin expression. Proc Natl Acad USA Sci 101: 13239-13244, 2004.
44. Senju M, Sueoka N, Sato A, Iwanaga K, Sakao Y, Tomimitsu S, Tominaga M, Irie K, Hayashi S and Sueoka E: Hsp90 inhibitors cause G2/M arrest associated with the reduction of Cdc25C and Cdc2 in lung cancer cell lines. J Cancer Res Clin Oncol 132: 150-158, 2006.
45. Yoshida M, Matsui Y, Iizuka A and Ikarashi Y: G2-phase arrest through p21(WAF1/Cip1) induction and cdc2 repression by gnidimacrin in human hepatoma HLE cells. Anticancer Res 29: 1349-1354, 2009.
46. Su JC, Lin KL, Chien CM, Lu CM, Chen YL, Chang LS and Lin SR: Novel indoloquinoline derivative, IQDMA, induces G(2)/M phase arrest and apoptosis in A549 cells through JNK/p38 MAPK signaling activation. Life Sci 85: 505-516, 2009.
47. Pai JT, Hsu MW, Leu YL, Chang KT and Weng MS: Induction of G2/M cell cycle arrest via p38/p21Waf1/Cip1-dependent signaling pathway activation by bavachinin in non-small-cell lung cancer cells. Molecules 26: 5161, 2021.
48. Luo YH, Wang C, Xu WT, Zhang Y, Zhang T, Xue H, Li YN, Fu ZR, Wang Y and Jin CH: 18β-Glycyrrhetinic acid has Anti-cancer effects via inducing apoptosis and G2/M cell cycle arrest, and inhibiting migration of A549 lung cancer cells. Onco Targets Ther 14: 5131-5144, 2021.
49. Tamura T, Kurishima K, Nakazawa K, Kagohashi K, Ishikawa H, Satoh H and Hizawa N: Specific organ metastases and survival in metastatic non-small-cell lung cancer. Mol Clin Oncol 3: 217-221, 2015.
50. Uramoto H and Tanaka F: Recurrence after surgery in patients with NSCLC. Transl Lung Cancer Res 4: 242, 2014.
51. Passaro A, Jänne PA, Mok T and Peters S: Overcoming therapy resistance in EGFR-mutant lung cancer. Nat Cancer 2: 377-391, 2021.
52. Tong CW, Wu WK, Loong HH, Cho WC and To KK: Drug combination approach to overcome resistance to EGFR tyrosine kinase inhibitors in lung cancer. Cancer Lett 405: 100-110, 2017.
53. Cavazzoni A, Alfieri RR, Cretella D, Saccani F, Ampollini L, Galetti M, Quaini F, Graiani G, Madeddu D, Mozzoni P, *et al*: Combined use of anti-ErbB monoclonal antibodies and erlotinib enhances antibody-dependent cellular cytotoxicity of wild-type erlotinib-sensitive NSCLC cell lines. Mol Cancer 11: 91, 2012.
54. Hu X, Wu LW, Weng X, Lin NM and Zhang C: Synergistic anti-tumor activity of aspirin and erlotinib: Inhibition of p38 enhanced aspirin plus Erlotinib-induced suppression of metastasis and promoted cancer cell apoptosis. Oncol Lett 16: 2715-2724, 2018.
55. Chen JC, Ko JC, Yen TC, Chen TY, Lin YC, Ma PF and Lin YW: Capsaicin enhances erlotinib-induced cytotoxicity via AKT inactivation and excision repair cross-complementary 1 (ERCC1) down-regulation in human lung cancer cells. Toxicol Res 8: 459-470, 2019.
56. Bing C, Cheng B, Staruch RM, Nofiele J, Wodzak Staruch M, Szczepanski D, Farrow-Gillespie A, Yang A, Laetsch TW and Chopra R: Breath-hold MR-HIFU hyperthermia: Phantom and in vivo feasibility. Int J Hyperthermia 36: 1084-1097, 2019.
57. Sadhukha T, Wiedmann TS and Panyam J: Inhalable magnetic nanoparticles for targeted hyperthermia in lung cancer therapy. Biomaterials 34: 5163-5171, 2013.
58. Park J and Baek SH: Combination therapy with cinnamaldehyde and hyperthermia induces apoptosis of A549 Non-small cell lung carcinoma cells via regulation of reactive oxygen species and mitogen-Activated protein kinase family. Int J Mol Sci 21: 6229, 2020.
59. Heo J, Jo Y and Yoon M: Synergistic effects of combined hyperthermia and electric fields treatment in non-small cell lung-cancer (NSCLC) cell lines. Clin Transl Oncol: Oct 22, 2024 (Epub ahead of print). doi: 10.1007/s12094-024-03760-6.
60. Cheng H, An SJ, Dong S, Zhang YF, Zhang XC, Chen ZH, Jian-Su and Wu YL: Molecular mechanism of the schedule-dependent synergistic interaction in EGFR-mutant non-small cell lung cancer cell lines treated with paclitaxel and gefitinib. J Hematol Oncol 4: 5, 2011.
61. Volman Y, Hefetz R, Galun E and Rachmilewitz J: DNA damage alters EGFR signaling and reprograms cellular response via Mre-11. Sci Rep 12: 5760, 2022.
62. Feng YB, Chen L, Chen FX, Yang Y, Chen GH, Zhou ZH and Xu CF: Immunopotentiation effects of apigenin on NK cell proliferation and killing pancreatic cancer cells. Int J Immunopathol Pharmacol 37: 3946320231161174, 2023.
63. Xu J, Jiao W, Wu DB, Yu JH, Liu LJ, Zhang MY and Chen GX: Yishen Tongbi decoction attenuates inflammation and bone destruction in rheumatoid arthritis by regulating JAK/STAT3/SOCS3 pathway. Front Immunol 15: 1381802, 2024.
64. Steen NV, Potze L, Giovannetti E, Cavazzoni A, Ruijtenbeek R, Rolfo C, Pauwels P and Peters GJ: Molecular mechanism underlying the pharmacological interactions of the protein kinase C-β inhibitor enzastaurin and erlotinib in non-small cell lung cancer cells. Am J Cancer Res 7: 816-830, 2017.
65. Cheng F, Peng X, Meng G, Pu Y, Luo K and He B: Poly(ester-thioether) microspheres co-loaded with erlotinib and α-tocopheryl succinate for combinational therapy of non-small cell lung cancer. J Mater Chem B 8: 1728-1738, 2020.
66. Wust P, Hildebrandt B, Sreenivasa G, Rau B, Gellermann J, Riess H, Felix R and Schlag PM: Hyperthermia in combined treatment of cancer. Lancet Oncol 3: 487-497, 2002.
67. Belhadj Slimen I, Najar T, Ghram A, Dabbebi H, Ben Mrad M and Abdrabbah M: Reactive oxygen species, heat stress and oxidative-induced mitochondrial damage. A review. Int J Hyperthermia 30: 513-523, 2014.
68. Panieri E and Santoro M: ROS homeostasis and metabolism: A dangerous liason in cancer cells. Cell Death Dis 7: e2253, 2016.
69. Van der Zee J: Heating the patient: A promising approach? Ann Oncol 13: 1173-1184, 2002.
70. Salem A, Asselin MC, Reymen B, Jackson A, Lambin P, West CML, O'Connor JPB and Faivre-Finn C: Targeting hypoxia to improve non-Small cell lung cancer outcome. J Natl Cancer Inst 110: 14-30, 2018.
71. Gerweck LE, Nygaard TG and Burlett M: Response of cells to hyperthermia under acute and chronic hypoxic conditions. Cancer Res 39: 966-972, 1979.
72. Bicher HI, Hetzel FW, Sandhu TS, Frinak S, Vaupel P, O'Hara MD and O'Brien T: Effects of hyperthermia on normal and tumor microenvironment. Radiology 137: 523-530, 1980.
73. Overgaard J: Effect of hyperthermia on the hypoxic fraction in an experimental mammary carcinoma in vivo. Br J Radiol 54: 245-249, 1981.
74. Elming PB, Sørensen BS, Oei AL, Franken NAP, Crezee J, Overgaard J and Horsman MR: Hyperthermia: The optimal treatment to overcome radiation resistant hypoxia. Cancers 11: 60, 2019.
75. Kabakov AE and Yakimova AO: Hypoxia-induced cancer cell responses driving radioresistance of hypoxic tumors: Approaches to targeting and radiosensitizing. Cancers 13: 1102, 2021.
76. Hu C, Yang J, Qi Z, Wu H, Wang B, Zou F, Mei H, Liu J, Wang W and Liu Q: Heat shock proteins: Biological functions, pathological roles, and therapeutic opportunities. MedComm 3: e161, 2022.
77. Ahmed K, Zaidi SF, Mati-Ur-Rehman, Rehman R and Kondo T: Hyperthermia and protein homeostasis: Cytoprotection and cell death. J Therm Biol 91: 102615, 2020.
78. Scutigliani EM, Liang Y, Crezee H, Kanaar R and Krawczyk PM: Modulating the heat stress response to improve Hyperthermia-Based anticancer treatments. Cancers 13: 1243, 2021.
79. Karar J and Maity A: Modulating the tumor microenvironment to increase radiation responsiveness. Cancer Biol Ther 8: 1994-2001, 2009.
80. Nijkamp MM, Span PN, Bussink J and Kaanders JH: Interaction of EGFR with the tumour microenvironment: Implications for radiation treatment. Radiother Oncol 108: 17-23, 2013.
81. Zhang T, Chen L, Zhang S, Xu Y, Fan Y and Zhang L: Effects of high-intensity focused ultrasound on Cisplatin-resistant human lung adenocarcinoma in vitro and in vivo. Acta Biochim Biophys Sin 49: 1092-1098, 2017.
82. Qin Y, Sun Y, Liu Y, Luo Y and Zhu J: Pilot study of radio-frequency hyperthermia in combination with gefitinib in gefitinib-effective patients with advanced NSCLC. Thorac Cancer 7: 422-427, 2016.
83. Sekins KM, Leeper DB, Hoffman JK, Keilman GW, Ziskin MC, Wolfson MR and Shaffer TH: Feasibility of lung cancer hyperthermia using breathable perfluorochemical (PFC) liquids. Part II: Ultrasound hyperthermia. Int J Hyperthermia 20: 278-299, 2004.