

High-intensity focused ultrasound enhances the cisplatin sensitivity to human non-small cell lung cancer *in vitro* and *in vivo*

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Primary research

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Abstract

Background

The incidence and mortality of non-small cell lung cancer (NSCLC) rank first among malignant tumors worldwide. Cisplatin (CDDP) is currently the first-line chemotherapy drug used in clinical practice. However, the underlying mechanism that NSCLC cells are resistant to CDDP has not been fully elucidated. Therefore, it is urgent to explore the exact function of chemotherapy resistance and improve the anti-tumor effect of CDDP treatment.

Method

We investigated the surviving fraction of H1299 and A549 cells treated with different High-intensity focused ultrasound (HIFU) and various CDDP concentrations. HIFU (400W/cm²) and CDDP (10 μM) were selected to probe the further function in H1299 and A549 cells. Flow cytometry, MTT, and colony formation assays were performed to evaluate the apoptosis and proliferative effect of HIFU and CDDP on NSCLC cells. Transwell assays were used to analyze the migration and invasion abilities of NSCLC cells with HIFU and CDDP treatments. Platinum (Pt) accumulation was further measured in H1299 and A549 cells with different treatments. Finally, An NSCLC patient-derived xenograft model was used to explore the effect of HIFU and CDDP on NSCLC tumor growth.

Results

HIFU combined with CDDP can markedly reduce the surviving rate and promote the apoptosis of NSCLC cells. Furthermore, co-treatment with HIFU and CDDP significantly inhibited the proliferation, colony formation, migration, and invasion of NSCLC cells compared to that with any single treatment. Moreover, the combined therapy can effectively promote Pt accumulation in NSCLC cells. Further functional analysis suggested that HIFU combined with CDDP can inhibit tumor growth in an NSCLC patient-derived xenograft model. Finally, the CDDP method effectively upregulated the expression level of apoptosis-related protein, cleaved-PARP, which could be further enhanced by the HIFU treatment.

Conclusion

Our results revealed that HIFU enhances the anti-NSCLC effect of CDDP both *in vitro* and *in vivo*, providing a promising combination therapy for clinical NSCLC treatment.

Introduction

The incidence and mortality of lung cancer rank first among all malignant tumors worldwide [1]. Lung cancer is mainly divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. NSCLC cells have relatively slow diffusion and metastasis compared with SCLC cells [2]. NSCLC patients account for about 80% of the total number of lung cancer patients. Studies have shown that at least 40% of lung cancer

patients are diagnosed as advanced stages; only 25% of diagnosed patients are still in stage I [3]. Chemotherapy is one of the main treatments for advanced and partial mid-term and post-operative patients. Although chemotherapy for lung cancer has made some progress, its 5-year survival rate (15%) has not increased significantly in the past 25 years [1, 3, 4]. Therefore, the tolerance of lung cancer cells to chemotherapy is the main cause for the low survival rate of lung cancer patients. Currently, known reasons related to the tumor chemotherapy drug resistance include the following items: 1) the uptake and elimination of chemotherapeutic drugs by tumor cells, which are mediated by proteins involved in transmembrane transport mechanisms, such as P-glycoprotein and multidrug resistance-associated proteins; 2) physiological activities such as apoptosis, proliferation and the abnormal intracellular environment in cells are also linked to inducing chemo-resistance [5].

Cisplatin (CDDP), firstly discovered in 1965, is a metal complex with anticancer activity [6]. CDDP is a non-specific cytotoxic drug regulating cancer cell cycle progression; the main mechanism of action is to cross-link with DNA *in vivo* and inhibit tumor cell replication and transcription, thus promoting tumor cell apoptosis; it is currently the first-line chemotherapy drug used in clinical practice [7]. The underlying mechanism that lung cancer cells are resistant to CDDP has not been fully elucidated. It is believed to be mainly related to the following factors: 1) the concentration of CDDP in the cells is reduced; 2) the activity changes of apoptosis pathways such as DNA damage repair and PI3K/ERK-regulated pathway [8]. Therefore, it is necessary to explore the exact function of chemotherapy resistance and improve the anti-tumor effect of CDDP.

High-intensity focused ultrasound (HIFU) is a non-invasive topical treatment of anti-tumor technology [9]. HIFU has several advantages, including better tissue penetration, good directionality, and good focusing performance. In clinical practice, this technique can focus the ultrasound energy on the target tissue to denature the target region protein without damaging the surrounding normal tissue [10]. Based on these advantages, HIFU is getting increasing attention for cancer therapy. Recently, HIFU has been widely used in benign and malignant tumors such as uterine fibroids, breast cancer, liver cancer, pancreatic cancer, bone tumors, and kidney cancer [11]. Previous studies have suggested that magnetic resonance-guided high-intensity focused ultrasound (MR-HIFU) has the great potential for localized heat-mediated drug delivery of temperature-sensitive liposomes (TSL), which affects drug uptake and distribution [12]. Therefore, we speculated that HIFU may also increase the sensitivity of tumor cells to CDDP.

In this study, we applied HIFU combined with CDDP to human NSCLC cells and patient-derived xenograft mice. The different effects of HIFU, CDDP, and their combination on NSCLC were well studied. In addition, we have explored the possible function mechanism of HIFU combined with CDDP in the treatment of NSCLC, which provides a promising therapeutic tool for future NSCLC treatment.

Method And Materials

Cell culture and treatment

H1299 and A549 cell lines were obtained from ATCC (Virginia, USA). H1299 cells were cultured with RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS). A549 cells were maintained with DMEM (Gibco, USA) supplemented with 10% FBS. Cells were grown in a humidified 37 °C incubator with 5% CO₂. Before treatment, cells were seeded at a density of 1 × 10⁶ cells in a 100-mm dish for 24 hours. Subsequently, the cells were processed using a high-intensity ultrasound focusing system (Beijing Yuande FEP-BY02 Ultrasonic Focusing Knife, China). System parameters were set as listed: 1) ultrasonic frequency = 1 MHz ± 10%; 2) input electric power = 0.5~2 kW; 3) focal intensity = 0 W/cm², 100 W/cm², 200 W/cm², 400 W/cm², 600 W/cm²; 4) fixed irradiation time = 5 s. CDDP was purchased from TargetMol (China).

Apoptosis assay

Annexin V-APC/7-AAD apoptosis detection kit (KGA1023-1026, KGI) was used to assess the cell apoptosis with FACSCalibur (BD Biosciences, USA) following the manufacturer's instruction. Cells were cultured in medium containing different CDDP concentrations (0 μM, 5 μM, 10 μM, 20 μM, 40 μM) for 24 hours and then irradiated with an ultrasonic wave of 400 W/cm² for 5 s. After treatment, cells were collected by centrifugation at 1500 rpm for 5 min and washed with 1× PBS three times, and then incubated with 5 μL of FITC-conjugated Annexin V and 5 μL of PI for 15 min at room temperature in the dark.

MTT

Cell proliferation was evaluated by an MTT Kit (Sigma-Aldrich, USA) following the manufacturer's protocol. Cells were divided into four groups: Con, CDDP (10 μM), HIFU (400 W/cm²), and HIFU (400 W/cm²) + CDDP (10 μM). The treated cell suspension was seeded into 96-well plates at a density of 4,000 cells/well. Cells were cultured for indicated times (24, 48, 72, or 96 hours). Then, 20 μL of MTT solution was added to each well and incubated for 4 hours. The culture medium was carefully removed in each well. 150 μL of dimethyl sulfoxide was added in per well, which was shaken at a low speed for 10 min on a shaker. After the crystals were thoroughly dissolved, the absorbance was measured at 595 nm wavelength with the microplate reader (Synergy H4 Hybrid Reader, BioTek, Winooski, USA). Each data point presents the mean ± s.d. of three independent experiments.

Colony formation

A total of 500 cells in different groups were seeded into 6-well plates. Cells with various treatments were cultured for about 14 days in a humidified 37 °C incubator with 5% CO₂, the colonies were fixed with 10% formaldehyde for 30 min and then stained with 0.5% crystal violet (Beyotime, China) for 30 min. The well was washed with 1× PBS and air-dried. After drying, the colonies were photographed by the microscope (Olympus, Tokyo, Japan).

Transwell assay

Transwell assay was performed for evaluating cell migration and invasion. After different treatments, 1×10^5 cells in 250 μL of serum-free medium were placed in the upper chamber (8.0 μm pore size; Corning, USA) with a porous membrane for migration assay, while the lower chamber was inserted into a 12-well plate filled with 500 μL medium supplemented with 10% FBS. After 24 hours of incubation at 37 °C, non-migrative cells were removed from the upper surface of the membrane with cotton swabs, and migrative cells on the lower membrane surface were fixed with 4% formaldehyde and stained with 0.1% crystal violet (Beyotime, China). Five random 200 \times visual fields per well were photographed and calculated under a Nikon Inverted Research Microscope Eclipse Ti microscope. Cell invasion assay was simultaneously conducted as above, except for the chambers with Matrigel solution (BD, USA).

Determination of cellular Pt accumulation

1×10^6 cells were seeded in 100-mm tissue culture dishes (Corning, USA). After 24 hours, cells were exposed to freshly dissolved CDDP (10 μM). After 1 hour of CDDP drug exposure, the medium was removed and the cells were washed with ice-cold 1 \times PBS, scraped and collected in double-distilled water. Samples were freeze-dried overnight and Pt was solubilized in HNO_3 following the standard procedures. Total Pt content was measured by flameless absorption spectroscopy (FAAS) (Model 3300, PerkinElmer, Norwalk, CT). Under these conditions, the detection limit was 10 mg/L. Pt levels were expressed as nmol Pt/ 10^6 cells, with cell number assessed by counting parallel cultures.

Western blot analysis

Cells in each group were collected and lysed in RIPA buffer (Beyotime, China). The protein concentration was determined by a BCA Protein Assay kit (Sangon Biotech, China). 30 μg protein in each group was separated for 10% SDS-PAGE. The proteins were transferred to a polyvinylidene fluoride membrane and blocked with 5% non-fat milk for 1 hour at 4 °C. The membranes were then incubated with primary antibodies, anti-GAPDH (Abcam, USA) (ab9485, 1:2500 dilution) and anti-cleaved-PARP (Abcam, USA) (ab32064, 1:1000 dilution) at 4 °C overnight. Anti-rabbit IgG was used as the secondary antibody for 1 hour at room temperature. The results were finally detected by ECL Chemiluminescence Kit (Thermo Fisher Scientific, USA). The bands were obtained by GeneGnome 5 (Synoptics Ltd., UK).

Tumor xenograft experiments

Animal experiments were formally approved by the Department of Ultrasound, the First Affiliated Hospital of Xinxiang Medical College Ethics Committee. Nude BALB/c male mice (5 weeks old) were used in this study. Sufficient water and food were given during the experiment. As previously reported[13], fresh lung cancer tissue from an NSCLC patient, approximately $2 \times 2 \times 2 \text{ mm}^3$ in size, was inoculated into the abdominal wall of one side of Nude BALB/c nude mice. The mice were divided into four groups with different groups: Con (n = 6), CDDP (2 mg Pt/kg), HIFU (1000 W/cm²), HIFU (1000 W/cm²) + CDDP (2 mg Pt/kg). Saline or CDDP was injected on days 0, 6, 12, 18, and 20, respectively. Mouse tumor volumes were measured weekly using a vernier caliper and recorded when the tumor volume reached 80 mm³. Tumor

volume was calculated using the formula: Tumor volume (mm^3) = (width) \times (height)²/2. After 20 days, mice were sacrificed for further analysis, and tumor weight and survival rate were recorded.

Statistical analysis

Statistical analysis was employed using SPSS software. The significance of difference between the two groups was calculated with Student's t-test; One-way ANOVA by Tukey's post hoc test was used to determine the significance among over two groups. *P* values less than 0.05 were considered significant (*, $P < 0.05$; **, $P < 0.01$). The error bars show the means \pm s.d. of three independent biological experiments.

Results

HIFU and CDDP treatment can effectively reduce NSCLC cell surviving rate

We first explored the surviving rate of H1299 and A549 cells with different HIFU treatments (0, 100, 200, 400, and 600 W/cm^2). Our results showed that high HIFU energy effectively reduced the surviving fraction of both NSCLC cell lines (Figure 1A). Notably, the surviving rate of both cell lines with 200, 400, or 600 W/cm^2 HIFU treatments was significantly lower than those in the control group ($P < 0.05$). Based on that, we selected 400 W/cm^2 as an optimized indicator for further studies. Moreover, we observed that 5 μM and 10 μM CDDP combined with 400 W/cm^2 HIFU treatment significantly decreased the NSCLC cell surviving rate compared with CDDP treatment alone. We thus selected 10 μM CDDP as an optimized index to perform further studies. Besides, we further studied the apoptosis of H1299 and A549 cells with different treatments, including the control group, HIFU (400 W/cm^2) group, CDDP (10 μM) group, and HIFU (400 W/cm^2) + CDDP (10 μM) group. The results suggested that the apoptosis rate of H1299 and A549 cells in HIFU (400 W/cm^2) + CDDP (10 μM) group was significantly higher than that in the other three groups (Figure 1C). These results revealed that HIFU and CDDP treatment could effectively reduce the cell survival of NSCLC.

HIFU combined with CDDP effectively inhibits NSCLC cell proliferation and colony formation

To further investigate the function of HIFU combined with CDDP in NSCLC, we assessed the cell proliferation of H1299 and A549 cells with different treatments. The MTT results revealed that HIFU, CDDP, and HIFU + CDDP treatments could significantly decrease the proliferation of NSCLC cells in a time-dependent manner (Figure 2A). More importantly, the cell proliferation of HIFU + CDDP treatment was significantly lower than that in the HIFU or CDDP group ($P < 0.01$) (Figure 2A). Similarly, colony formation assays indicated that the colony numbers of NSCLC cells in the co-treatment group were remarkably lower than those in the HIFU or CDDP group ($P < 0.01$) (Figure 2B). Collectively, HIFU + CDDP treatment was an effective approach to inhibit the proliferation and colony formation of NSCLC cells.

HIFU combined with CDDP effectively represses NSCLC cell migration and invasion

We next investigated the migration and invasion capabilities among the control, HIFU, CDDP, or HIFU + CDDP treated NSCLC cells group. The results revealed that HIFU, CDDP, and HIFU + CDDP treatments could significantly reduce the cell migration and invasion compared with that in the control group ($P < 0.01$) (Figure 3A and B). Furthermore, cell migration and invasion of the HIFU + CDDP co-treatment group was markedly lower than that in any single treatment group ($P < 0.01$) (Figure 3A and B). These results showed that HIFU combined with CDDP effectively represses NSCLC cell migration and invasion.

HIFU combined with CDDP can promote Pt accumulation

It has been reported that a reduction of Platinum (Pt) accumulation is positively associated with CDDP resistance in various cancers, including lung cancer, ovarian, and leukemia[14, 15]. Thus we also assessed the Pt accumulation in A549 and H1229 cells with different treatments. The result showed that Pt accumulation of both cell lines in the HIFU + CDDP co-treatment group was significantly higher than that in the CDDP group ($P < 0.01$) (Figure 4). These data suggested that HIFU treatment was beneficial to the Pt accumulation of CDDP in A549 and H1229 cells. Therefore, we concluded that HIFU treatment improves the clinical anti-tumor efficacy of CDDP.

HIFU combined with CDDP suppresses NSCLC progression *in vivo*

We further explored the anti-tumor effect of HIFU+CDDP *in vivo*. The results suggested that HIFU (400 W/cm²) + CDDP (10 μ M) treatment could effectively reduce tumor weight and volume compared with that in other groups (Figure 5A to C). Meanwhile, body weight and survival analysis indicated that co-treatment significantly promoted the mice body weight and survival period compared with that in the control group ($P < 0.05$) (Figure 5D and E). Besides, the western blot result demonstrated that the apoptosis-related protein expression of cleaved-PARP in the HIFU + CDDP or CDDP treatment group was upregulated relative to that in the control group and HIFU group (Figure 5F). Taken together, we indicated that CDDP promotes the cleaved-PARP expression, and this increased expression of the apoptotic-associated protein in the combined therapy may enhance the sensitivity of NSCLC cells to CDDP.

Discussion

In preclinical and clinical applications, the HIFU ablation of local tumors is very efficient. It was reported that HIFU combined with carmustine in the treatment of hamster optic canal cell tumors. The cure rate with HIFU combined chemotherapy group was 40%; while the cure rate with a single HIFU group was 29% [16]. Yang et al reported that HIFU combined with doxorubicin achieved a higher survival rate in a liver cancer model [17]. To reproduce the actual clinical situation, we set a 70% ablation volume to assess whether there are malignant cells in the tumor or marginal part to escape the damage of HIFU. Our results indicated a super-addictive effect when chemotherapy (CDDP) is combined with HIFU *in vitro* and *in vivo*. HIFU ablation of tumor volume by 70% largely delayed the NSCLC tumor growth in xenograft mice, indicating that HIFU can effectively inhibit lung cancer progression *in vivo*. Consistent with previous

studies, no significant difference in cancer cell survival could be detected between the single HIFU and chemotherapy group.

However, the synergistic mechanism of HIFU combined with chemotherapy is not clear. Previous studies suggested that this may be due to an increase in permeability of the cell membrane, which leads to better diffusion of chemotherapeutic drugs in the cells[18, 19]. In our study, HIFU combined with CDDP has a significant synergistic effect on NSCLC. Therefore, we speculate that it may be related to the following two reasons: First, CDDP can increase the cytotoxicity of cells before HIFU treatment, which is more conducive to HIFU destruction of tumor cells; second, tumor cells outside the target area of HIFU treatment can be degraded by diffused ultrasound. Furthermore, these cells affected by the spread of ultrasound have high sensitivity to CDDP chemotherapy, subsequently resulting in a super-synergistic effect.

Many studies have shown that apoptosis function have implicated in tumor resistance to cisplatin [20]. Our result showed that CDDP treatment effectively increases the apoptotic-associated protein expression (cleaved-PARP), which could be further enhanced by HIFU treatment. Therefore, HIFU treatment may be an effective approach to ameliorate the drug resistance of CDDP. The biological effects of HIFU after ablation of tumor tissue are mainly thermal, mechanical and cavitation effects, and vascular damage in target tissues, which can cut off the blood supply of the tumor, causing ischemia and hypoxia in the target tissue[21]. Thus, it indirectly leads to tumor tissue death. Besides, the tumor tissue after ablation can stimulate host immunity and increase the host's anti-tumor efficacy [22]. Another outstanding advantage of this technique is that the accumulation of thermal energy in the target zone can cause tissue boiling; the microbubbles in the irradiated area can form a "mirror" to prevent energy from entering the tissue below the boiling area [23]; thus this technique can effectively protect deep tissues.

Conclusion

In summary, we demonstrated that HIFU combined with CDDP effectively inhibits cell proliferation, migration and invasion but promotes cell apoptosis of NSCLC. Moreover, HIFU could promote Pt accumulation in NSCLC cells. This combined therapy also inhibits tumor growth of NSCLC *in vivo*. Our results provide a promising combined therapy for future NSCLC treatment.

Declarations

Acknowledgments

Not applicable.

Funding

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Availability of data and materials

The analyzed data and materials during this study are available from the corresponding author on reasonable request.

Authors' contributions

WL conceived the project and supervised the project. WL, FH, WH, BY, and ZW performed the biological experiments and analyzed data. WL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of the Department of Ultrasound, the First Affiliated Hospital of Xinxiang Medical College. Written informed consent was obtained from all patients and conducted in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

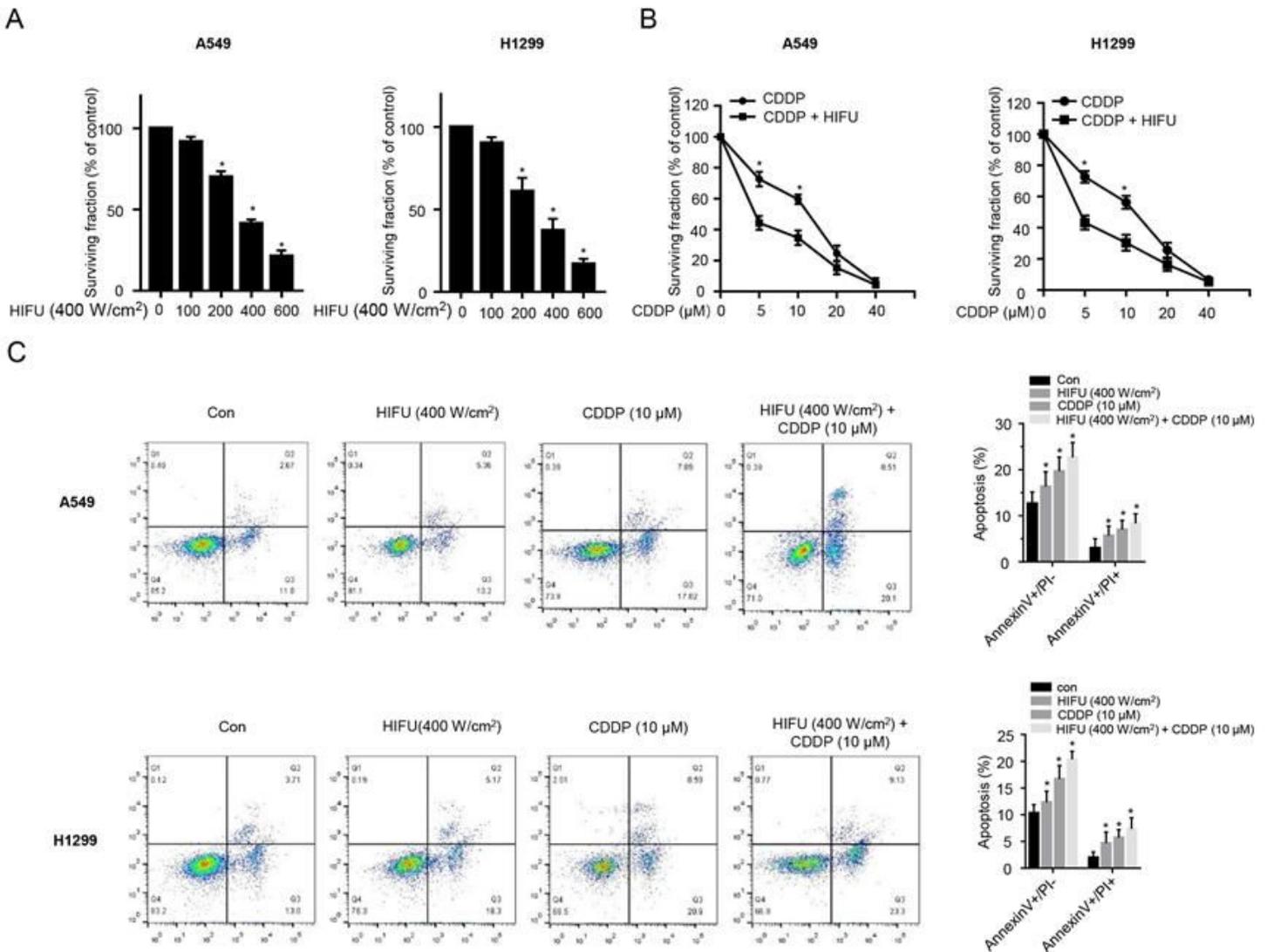


Figure 1

Surviving and apoptosis analysis of H1299 and A549 cells with HIFU and CDDP treatments. (A) Surviving fraction analysis of H1299 and A549 cells with HIFU treatments. (B) Surviving fraction analysis of H1299 and A549 cells with CDDP or HIFU + CDDP treatments. (C) Cell apoptosis analysis of A549 cells with four different treatments: Control, CDDP (10 µM), HIFU (400 W/cm²), and HIFU (400 W/cm²) + CDDP (10 µM). All data are representative of three independent experiments and expressed as mean ± s.d.; *P < 0.05.

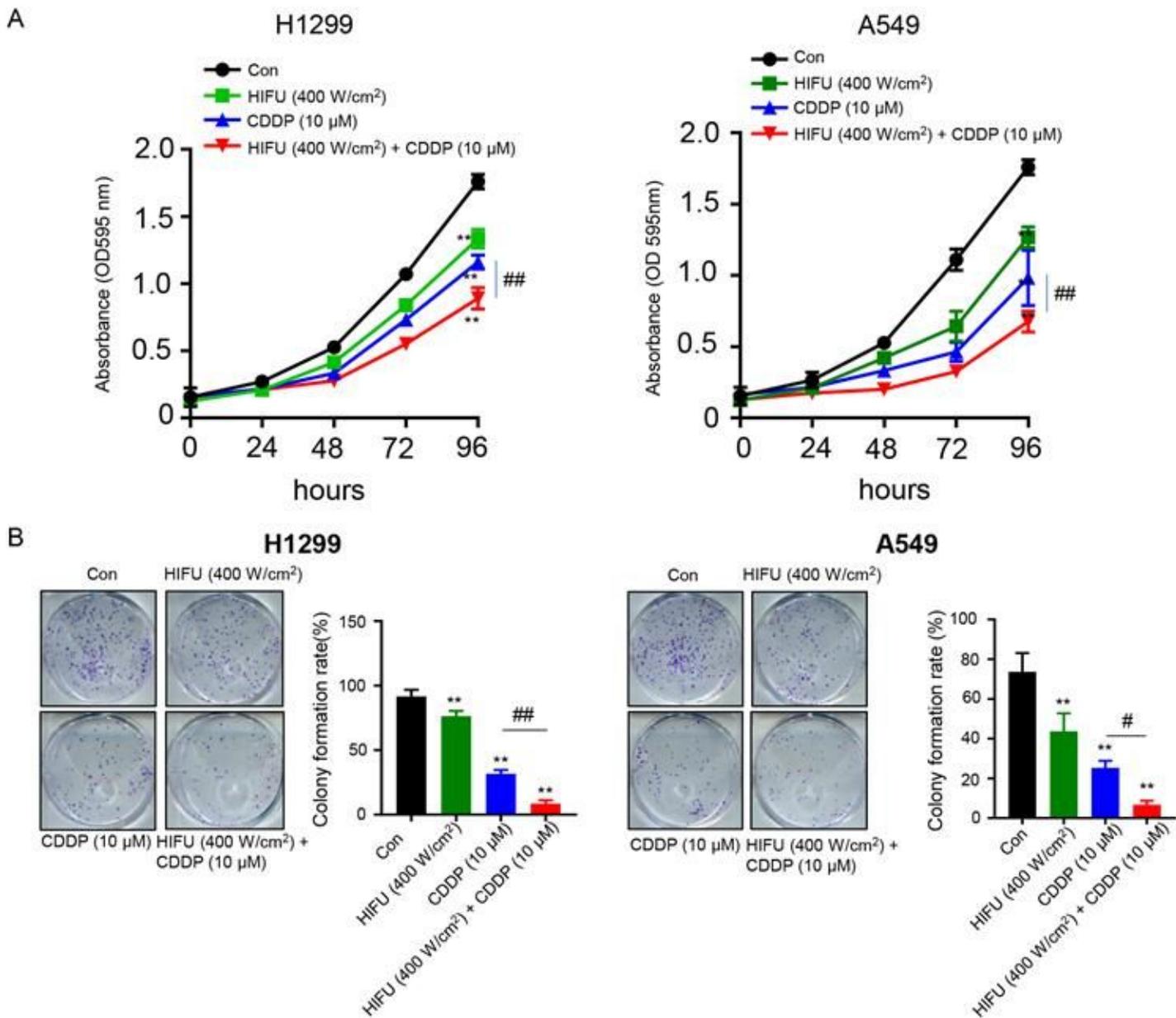


Figure 2

MTT and colony formation analysis of H1299 and A549 cells with HIFU and CDDP treatments. (A) MTT absorbance values of H1299 and A549 cells with four different treatments at 0, 24, 48, 72, or 96 hours. (B) Colony formation of H1299 and A549 cells with four different treatments: Control, CDDP (10 µM), HIFU (400 W/cm²), and HIFU (400 W/cm²) + CDDP (10 µM). All data are representative of three independent experiments and expressed as mean ± s.d.; **P < 0.01, # P < 0.05, ## P < 0.01.

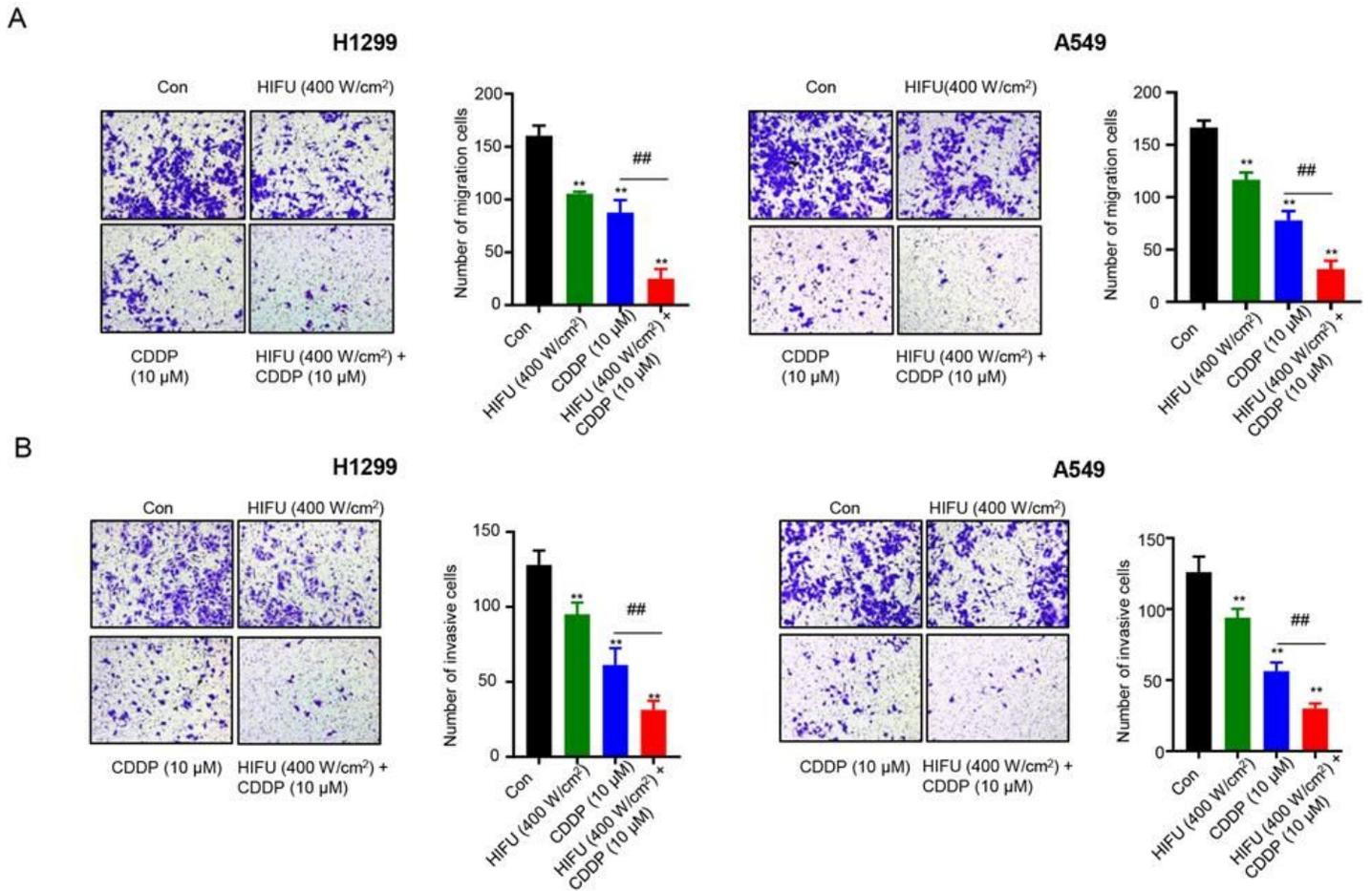


Figure 3

cell migration and invasion analysis of H1299 and A549 cells with HIFU and CDDP treatments. (A) migration analysis of H1299 and A549 cells with four different treatments: Control, CDDP (10 µM), HIFU (400 W/cm²), and HIFU (400 W/cm²) + CDDP (10 µM). (B) Invasion analysis of H1299 and A549 cells with four different treatments. All data are representative of three independent experiments and expressed as mean ± s.d.; **P < 0.01, ## P < 0.01.

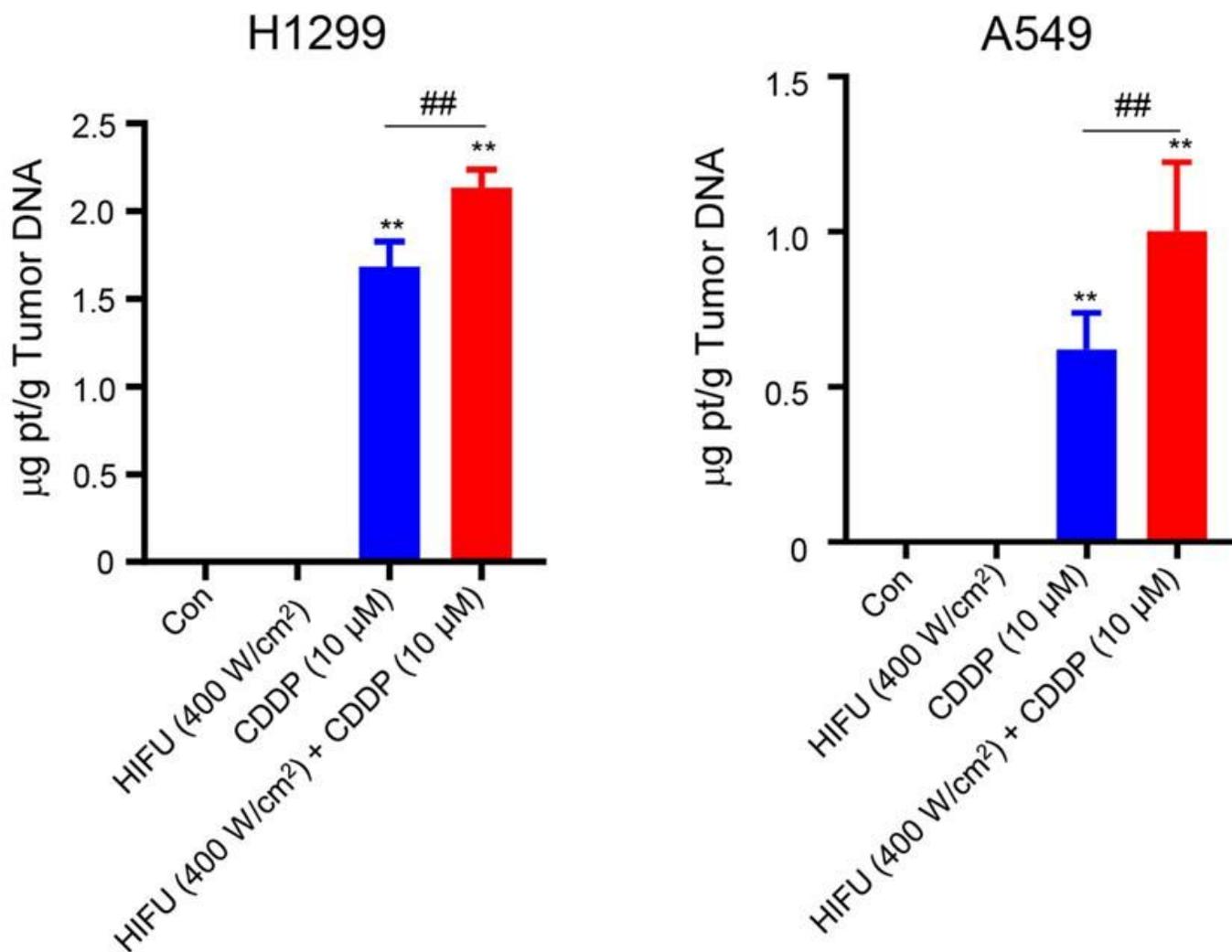


Figure 4

Pt accumulation analysis of H1299 and A549 cells with HIFU and CDDP treatments. Pt accumulation analysis of H1299 and A549 cells with four different treatments: Control, CDDP (10 µM), HIFU (400 W/cm²), and HIFU (400 W/cm²) + CDDP (10 µM). All data are representative of three independent experiments and expressed as mean ± s.d.; **P < 0.01, ## P < 0.01.

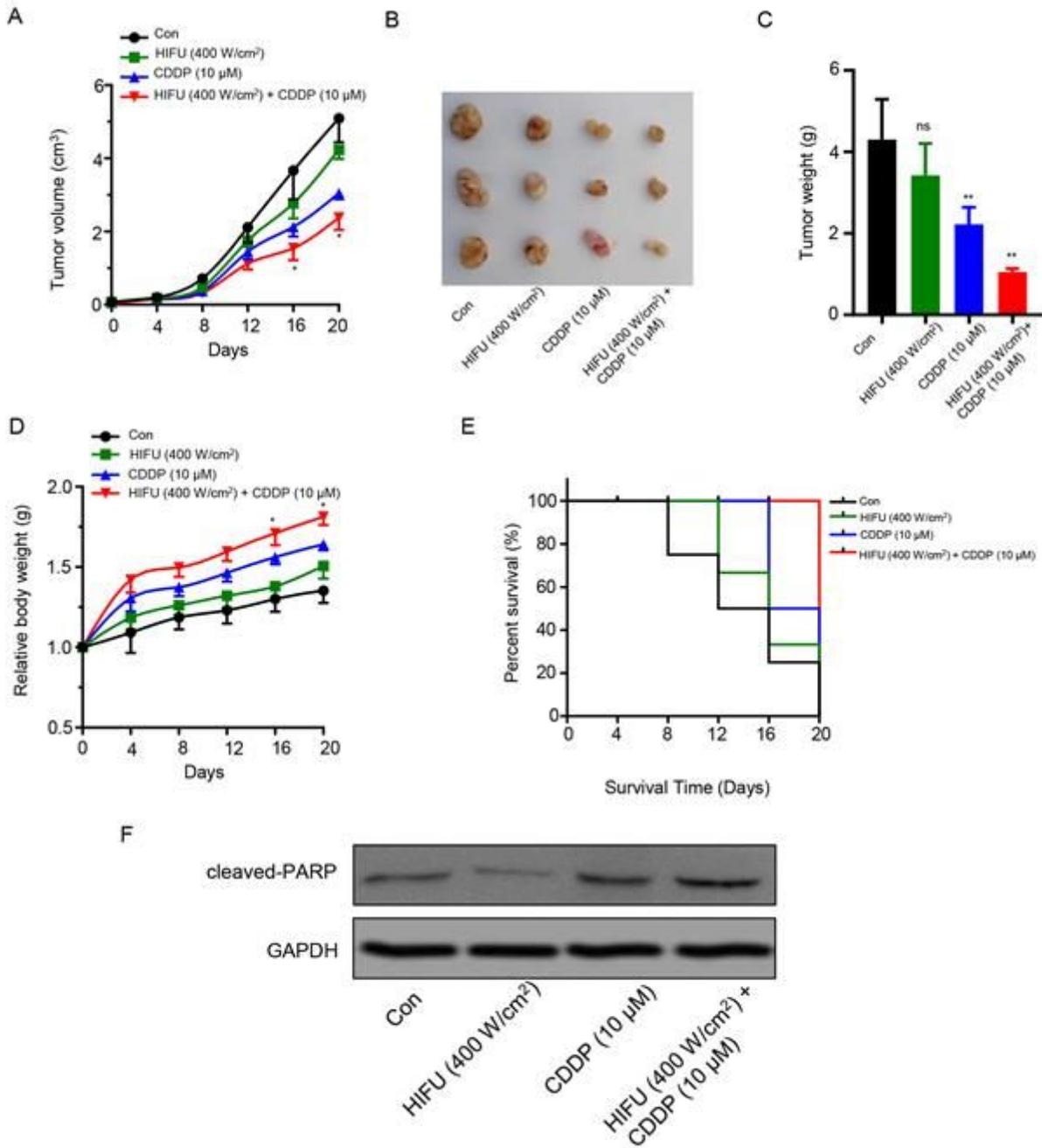


Figure 5

HIFU combined with CDDP suppresses NSCLC progression in vivo. (A and B) The tumor volume and representative white images of nude mice xenografts with NSCLC patients tumors with four different treatments: Control, CDDP (10 µM), HIFU (400 W/cm²), and HIFU (400 W/cm²) + CDDP (10 µM). (C) Tumor weight of nude mice xenografts with four different treatments. (D) Relative body weight analysis of nude mice with four different treatments. (E) Survival analysis of the mice with four different treatments. (F) Western blot analysis of apoptosis-related protein expression (cleaved-PARP) in four different treatments. *P < 0.05, **P < 0.01.