Combined effects of low-dose gambogic acid and NaI[131] in drug-resistant non-small cell lung cancer cells

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Abstract. Radioactive seed brachytherapy is a method for treating drug-resistant, late-stage non-small cell lung cancer (NSCLC). To elucidate the mechanism of low-dose gambogic acid (GA) and NaI[131] in drug-resistant NSCLC cells, the human NSCLC A549 cell line and the drug-resistant A549/cisplatin (DDP) and A549/Taxol cell lines were treated with NaI[131], low-dose GA or a combination of both in the present study; the control group of each cell line was treated with phosphate-buffered saline (PBS). Following treatment, cell proliferation, apoptosis and cell cycle analysis was performed. Apoptosis-related proteins, namely CDK1, cyclin B, mutant p53 (mtp53), heat shock protein 90 (HSP90), Bax and Bcl-2, and P-glycoprotein 1 (P-gp), which is known to confer resistance to chemotherapy, were detected using western blotting and immunofluorescence analysis. mRNA levels of p53 and HSP90 were measured using reverse transcription-quantitative PCR. Compared with the PBS control group, the A549, A549/DDP and A549/Taxol cells treated with NaI[131], GA or a combination of the drugs exhibited G_2/M arrest and increased percentages of total apoptotic cells, as well as significantly decreased protein levels of CDK1, cyclin B, mtp53, HSP90, Bcl-2 and P-gp, increased protein levels of Bax and decreased mRNA levels of p53 and HSP90. The changes in the combination group were the most evident and were significantly different from the other groups (P<0.001). In conclusion, low-dose GA may be a potential radionuclide sensitizer.

Introduction

Lung cancer (LC), the most common type of cancer in the world, is a global burden and a public health issue due to its high fatality rates. In the USA in 2018, it constituted ~13 and 14% of newly detected cancer cases in men and women, respectively (1). In China, the age-standardized incidence rate of LC was 36.71 per 100,000 individuals in 2014, with men exhibiting a higher incidence than women (2). Non-small cell LC (NSCLC) accounts for 85% of all LC cases, with an overall 5-year survival rate of 16%. Treatment mainly includes surgical resection, targeted therapy, chemotherapy, radiotherapy and immunotherapy, although use of personalized medicine has been speculated upon (3). It is estimated that 30-55% of patients with NSCLC experience relapse in spite of curative resection, leading to subsequent mortality (4).

Cancer chemotherapy resistance, be it innate or acquired, can be a hindrance during the treatment phase; it occurs due to a variety of reasons, including amplified drug target molecules, decreased drug accumulation and enhanced drug export, alterations in drug metabolism by modifying signalling transduction molecules, DNA damage and evasion of apoptosis (5). Avoidance of apoptosis by upregulation of anti-apoptotic proteins, such as Bcl-2, and inactivation of pro-apoptotic proteins, such as Bax, is commonly seen in cancer cells, leading to a lack of response to chemotherapy (6). Overexpression of P-glycoprotein 1 (P-gp), leading to the transport of anticancer drugs out of the cancer cells, has been shown to confer resistance to chemotherapy in NSCLC (7). While conventional radiotherapy and stereotactic body radiation therapy are used for inoperable cases, iodine-125 seed brachytherapy has been proven to be a safe and effective technique, with improved median overall survival time (16 vs. 10 months) and quality of life (8-10). It has been shown that the combination of iodine-125 brachytherapy and chemotherapy is better than chemotherapy alone, with improved therapeutic efficacy for NSCLC (11,12).

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Although ionizing radiation is commonly used for LC treatment, reports suggest that it might have secondary undesirable effects such as promoting cancer malignancy and increasing the incidence of cardiac events, among others (13,14). Therefore, it is desirable to find a safe radionuclide sensitizer to reduce the side effects of radiotherapy without influencing the antitumour efficacy in clinical practice.

Gambogic acid (GA), extracted from the gamboge resin of *Garcinia hanburyi*, has been used in traditional Chinese medicine to treat infections and tumours; it exerts antitumour, antiangiogenic and antimetastatic activities in several types of cancer, including LC (15-17). In addition, conventional doses do not influence the functions of other cells in the body (18,19). GA exerts its anticancer activities via numerous targets and signalling pathways, promoting apoptosis, inducing cell cycle arrest, inhibiting angiogenesis and activating lymphocytes (20). GA in low doses causes autophagy in mutant p53 (mtp53) degradation in cancer cells (21) and protein levels of mtp53 are decreased following GA exposure (22).

Our previous results showed that a combination of low-dose GA and NaI[131] was able to significantly inhibit cell proliferation, as well as induce cell apoptosis, in A549/DDP cells (23). Therefore, further study was required on the plausible cellular mechanisms related to the effect of the combination therapy on two drug-resistant LC cell lines. Additionally, exploration to see if the combination therapy conferred inhibition to therapy resistance in NSCLC, so as to achieve better therapeutic results, was required. The current *in vitro* study aimed to elucidate whether low-dose GA could sensitize NaI[131] to enhance its influence on cell cycle regulation, apoptosis and drug resistance in two NSCLC cell lines, namely A549/Taxol and A549/cisplatin (DDP) cells, that are resistant to Taxol and DDP, respectively.

Materials and methods

Cell culture. The human NSCLC A549 cell line was a gift from the Laboratory of Pathology at the School of Medicine of Southeast University (Nanjing, China). The DDP-resistant A549/DDP cell line and the Taxol-resistant cell line A549/Taxol were procured from Nanjing KeyGen Biotech Co., Ltd.. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Shanghai ExCell Biology, Inc.) and incubated in a humidified 5% CO_2 incubator at 37°C. When the cells reached a confluency of 80-90%, the original culture medium was discarded, the cells were treated with trypsin (0.25%) for 1-2 min, and the digestion was terminated by adding an equal volume of serum-containing culture medium. Cells were dispersed by gentle pipetting, and the cell suspension was transferred to a 15-ml centrifuge tube, after which the suspension was centrifuged at 200 x g at 4°C for 5 min. The supernatant was discarded, and 1-2 ml of culture medium was added to resuspend the cells. The cells were then transferred to a culture flask for continuous culture.

Experimental groups and drug intervention. A549, A549/DDP and A549/Taxol cells were harvested in the logarithmic phase of growth. A total of $5x10^4$ cells per well were seeded in 6-well plates and cultured at 37°C, with 5% CO_2, for 24 h and then divided into the NaI[131], GA, combination and control groups. The NaI[131] group was treated with 10.3 MBq NaI[131], the GA group with 2.9 μg/ml GA, and the combination group with 10.3 MBq NaI[131] and 0.3 μg/ml GA, as described previously (23). The control group was treated with PBS (2 ml per well). The cells were harvested after 48 h of drug treatment.

Cell cycle assay. Cells were fixed in 70% alcohol for 2 h. RNase A (100 μl) was added to the cells for 30 min, followed by the addition of 400 μl propidium iodide stain (Nanjing KeyGen Biotech Co., Ltd.) and incubation at 4°C in the dark for 30 min. Cellular DNA content was measured using a flow cytometer with fluorescence-activated cell sorting (FACS) (FACSCalibur; Becton, Dickinson and Company). The cell cycle profiles were analysed using FACSDiva software (version 7.0; Becton, Dickinson and Company).

Cell apoptosis assay. An Annexin V-APC/7-AAD cell apoptosis detection kit (cat. no. KGA1024; Nanjing KeyGen Biotech Co., Ltd.) was used for the detection of apoptosis according to the manufacturer's instructions. After drug treatment, cells were mixed with 500 μl binding buffer, 5 μl Annexin V-APC and 5 μl 7-AAD, and incubated at room temperature in the dark for 5-15 min. Cell apoptosis was measured using a FACSCalibur flow cytometer (Becton, Dickinson and Company). Data were analysed using FACSDiva software (version 7.0; Becton, Dickinson and Company).

Immunofluorescence (IF). Cells were air-dried on a glass slide and fixed in 4% paraformaldehyde at room temperature for 30 min. Slides were rinsed three times in PBS, followed by blocking with 50-100 μl goat serum (1:10; cat. no. C0265; Beyotime Institute of Biotechnology) and incubation at room temperature for 20 min. Next, 50-100 μl primary antibodies, namely, anti-mtp53 (1:200; cat. no. ab32509; Abcam) or anti-heat shock protein 90 (HSP90) (1:200; sc-69703; Santa Cruz Biotechnology, Inc.), was added and incubated at 37°C for 2 h under humidified conditions. Slides were rinsed three times in PBS, followed by addition of 50-100 μl horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibody (1:200; cat. no. KGAA35 or KGAA37; Nanjing KeyGen Biotech Co., Ltd.) and incubated at 37°C for 1 h at room temperature. After rinsing the slides three times in PBS, 50-100 μl DAPI staining solution (Nanjing KeyGen Biotech Co., Ltd.) was added and incubation occurred at room temperature for 5 min in the dark. Sections were mounted using anti-fade mounting medium (Sangon Biotech, Co., Ltd.). Protein expression in cells was observed under a fluorescence microscope (BX61; Olympus Corporation). Images of five areas that exhibited strong signals were captured and then analysed using ImageJ software (version 1.52; National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA synthesis was performed using a RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Specific mRNA quantification was performed by PCR using the Thunderbird SYBR® qPCR mix (cat. no. QPS-201; Toyobo Life Science) in an ABI
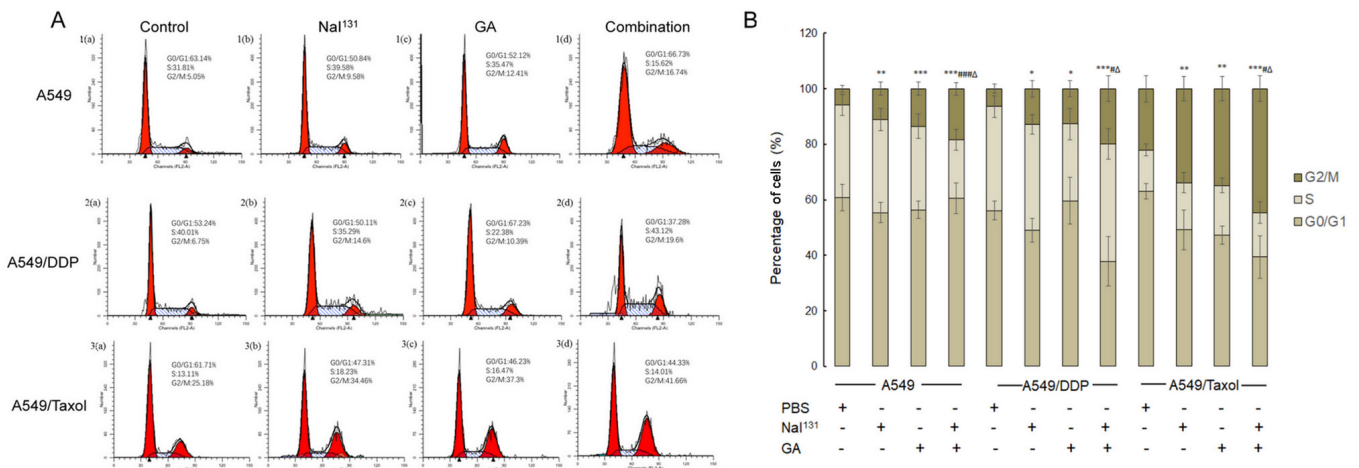
Figure 1. Analysis of stages in the cell cycle. (A) A549, A549/DDP and A549/Taxol cells were treated with PBS (control), NaI[131], GA or the combination of both, for 48 h, and the stages of the cell cycle in each group were detected: The first peak represents the G_0/G_1 phase, the second peak represents the G_2/M phase and the plateau represents the S phase. (B) Graphical representation of the results. * refers to comparisons with the control group; # refers to comparisons with the NaI[131] group and Δ refers to comparisons with the GA group. *,#,ΔP<0.05; **P<0.01; and ***,###P<0.001. DDP, cisplatin; GA, gambogic acid; PBS, phosphate-buffered saline.

Step One Plus Real Time-PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the following reaction conditions: Holding stage at 95°C for 5 min; cycling stage at 95°C for l5 sec, 6°C for 20 sec and 72°C for 40 sec, for a total of 40 cycles; and melting curve stage at 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec. The $2^{-\Delta\Delta Cq}$ method (24) was used to calculate the relative mRNA expression of the target genes. The primers used were as follows: p53 forward, 5'-CCGCAG TCAGATCCTAGCG-3' and reverse, 5'AATCATCCATTG CTTGGGACG-3'; HSP90 forward, 5'-CATAACGATGAT GAGCAGTACGC-3' and reverse, 5'-GACCCATAGGTTCAC CTGTGT-3'; and GAPDH forward, 5'-CATCTTCTTTTG CGTCGCCA-3' and reverse, 5'-TTAAAAGCAGCCCTGGTG ACC-3'. The experiment was repeated 3 times.

Western blot analysis. Total cellular protein was extracted using RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology), and the protein concentration was determined using the bicinchoninic acid method. Proteins (20 µg per lane) separated on a 12% gel by SDS-PAGE were transferred onto a nitrocellulose membrane and blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 h at room temperature, followed by addition of primary antibodies as follows: Anti-CDK1 (1:300; cat. no. ab131450), anti-cyclin B (1:300; cat. no. ab172317), anti-Bcl-2 (1:300; cat. no. ab692), anti-Bax (1:300; cat. no. ab77566) (all Abcam) or anti-P-gp (1:300; cat. no. PB0162; Wuhan Boster Biological Technology, Ltd.). Incubation occurred overnight at 4°C. The following day, the membrane was washed three times with 5% TBST for 10 min each. HRP-labelled goat anti-mouse IgG secondary antibody (1:5,000; cat. no. KGAA35; Nanjing KeyGen Biotech Co., Ltd.) was added, and the membrane was incubated at room temperature for 30 min. After the membrane was washed 3 times with 5% TBST for 10 min, protein bands were visualized using the ECL Chemiluminescence kit (cat. no. KGP116; Nanjing KeyGen Biotech Co., Ltd.). Gel-Pro32 software (Media Cybernetics) was used to analyse the band intensities.

Statistical analysis. Statistical analyses of the data were performed using SPSS software (version 22.0; IBM Corp.). All data are presented as the mean ± SD. Comparisons among multiple groups were performed by one-way analysis of variance, followed by Bonferroni's post hoc test. P<0.05 was used to indicate a statistically significant difference.

Results

Analysis of stages in the cell cycle. First, the combination treatment was assessed with regard to any effect it may have on the cell cycle, since our previous study showed that treatment of A549/DDP cells with low-dose GA together with NaI[131] was able to significantly inhibit cell proliferation (23). NaI[131], GA and a combination of both were used to treat A549, A549/DDP and A549/Taxol cells, while the control group was treated with PBS. The cell cycle conditions in all groups were observed after 48 h (Fig. 1A and B). The data showed that in the A549, A549/DDP and A549/Taxol cells, the percentage of cells in the G_2/M phase was considerably increased in the NaI[131], GA and combination groups compared with that in the control group. The increase in the combination group was significantly higher than that in the NaI[131] and GA groups (P<0.001). Moreover, the highest percentage of cells in the G_2/M phase was found in A549/Taxol cells. Additionally, the percentage of cells among these groups in the $G_0/G1$ and S phases was not significantly different. These results suggested that G_2/M arrest occurred in the cells in all the groups, but was most pronounced in the combination group.

Analysis of cell apoptosis. An Annexin V-APC/7-AAD assay was used to revalidate the effect of low-dose GA combined with NaI[131] on cell apoptosis. NaI[131] and GA both induced apoptosis in the A549, A549/DDP and A549/Taxol cells. The results after 48 h of incubation are shown in Fig. 2A and B. Compared with that in the control group, the percentage of total apoptotic cells in the NaI[131], GA and combination groups increased significantly, while the percentage of apoptotic cells
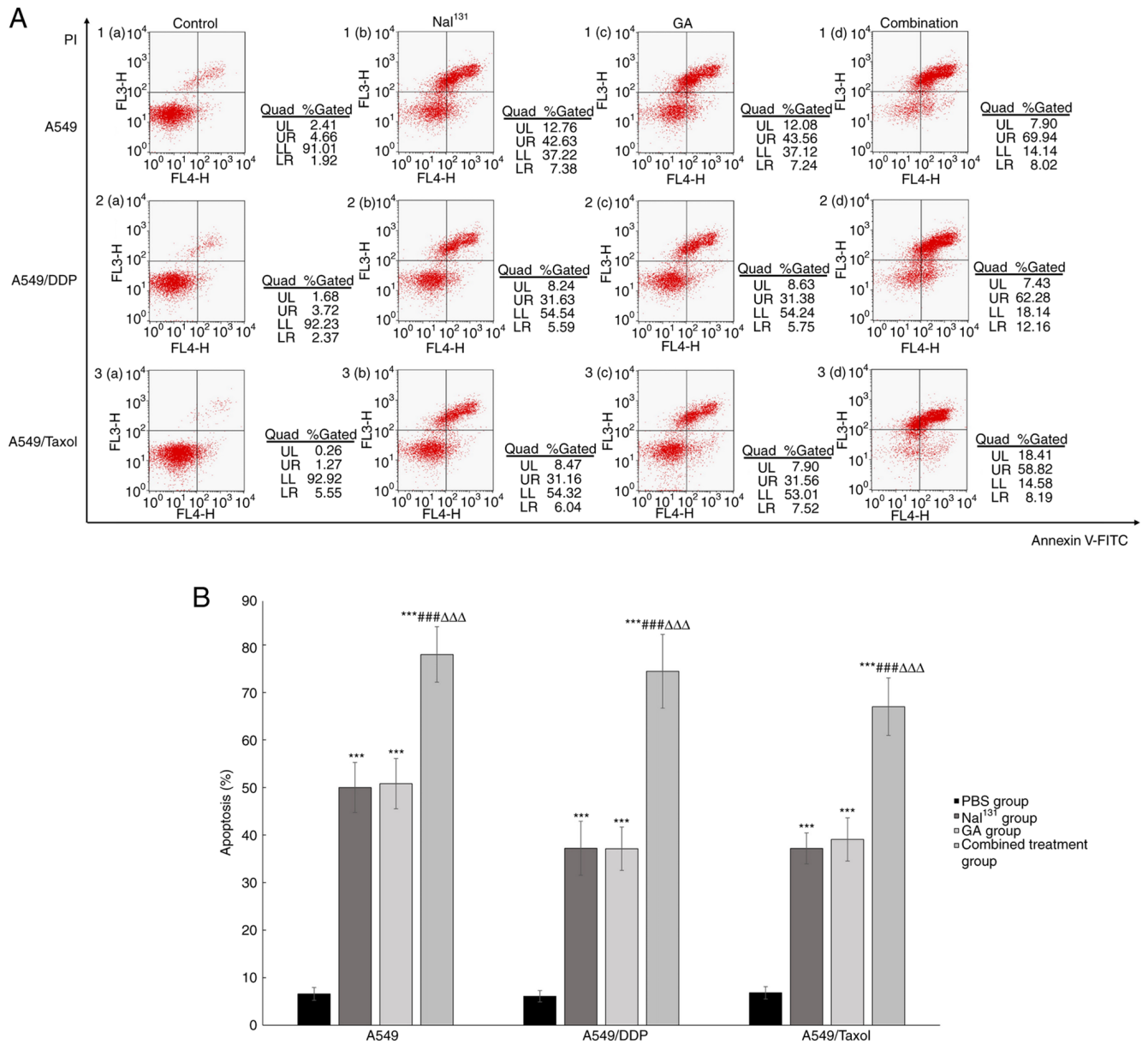
Figure 2. Analysis of cell apoptosis. (A) A549, A549/DDP and A549/Taxol cells were incubated with PBS (control), NaI[131], GA or the combination of both, for 48 h, and the percentages of cells at all stages of apoptosis were detected by flow cytometry. The upper right quadrant suggests the number of apoptotic cells in the late stage, while the lower right quadrant suggests the number of apoptotic cells in the early stage. (B) Graphical representation of the results. * refers to comparisons with the control group; # refers to comparisons with the NaI[131] group and Δ refers to comparisons with the GA group. ***,###,ΔΔΔP<0.001. DDP, cisplatin; GA, gambogic acid; PBS, phosphate-buffered saline.

was almost identical in the NaI[131] and GA groups, with 40-50% cell death. Cell apoptosis was most evident in the combination group (P<0.001); late-stage apoptotic activity was highest in this group.

Expression levels of P-gp and proteins related to cell cycle control and apoptosis. To better understand the effect of combination treatment on drug-resistant NSCLC cells, levels of proteins related to the cell cycle, apoptosis and drug resistance, namely CDK1, cyclin B, Bcl-2, Bax and P-gp, were measured using western blotting (Fig. 3A). The expression levels of CDK1, cyclin B, Bcl-2 and P-gp were decreased in the NaI[131], GA and combination groups compared with those in the control group. The expression levels were the lowest in the combination group in comparison to all other groups

(P<0.001) (Fig. 3B-D and F). By contrast, the expression levels of Bax were elevated in all the groups compared with that in the control, but the increase was most evident in the combination group (P<0.001) (Fig. 3E).

Expression of mRNA and protein levels of p53 and HSP90. The present study observed the expression and distribution of mtp53 protein in cells by IF, and indirectly indicated whether mtp53 is consistent with its protein expression by detecting the level of total p53 mRNA. The study also assessed whether the changes in the cell cycle and apoptosis brought about by the combination treatment on drug-resistant NSCLC cells was influenced by p53. Results of RT-qPCR and IF demonstrated that the expression levels of p53 decreased in all the treatment groups compared with those in the control (Fig. 4A-C).
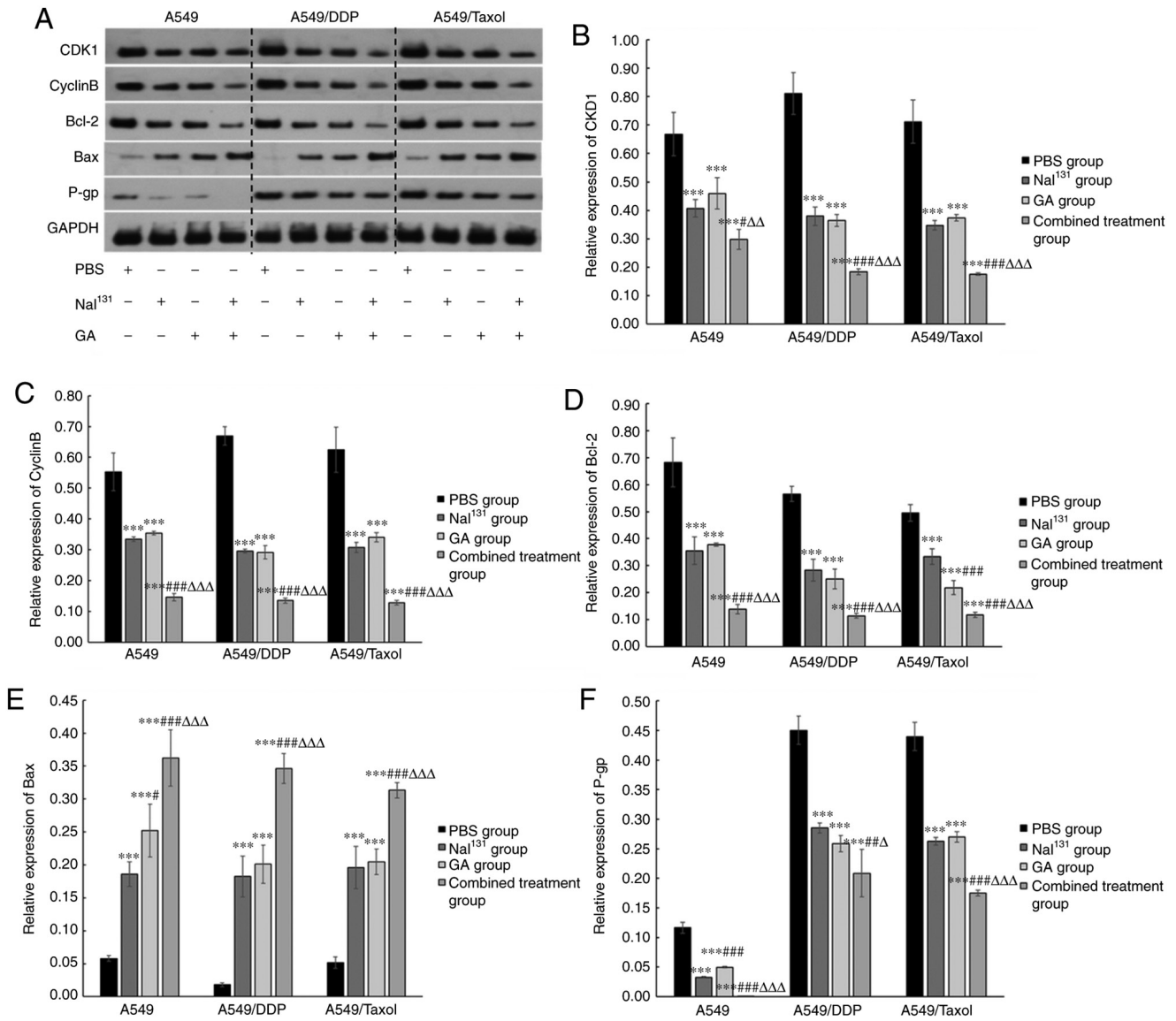
Figure 3. Protein expression levels of P-gp and associations with cell cycle control and apoptosis. After treatment of A549, A549/DDP and A549/Taxol cells with NaI[131], GA or the combination of both, for 48 h, the expression of intracellular CDK1, cyclin B, Bcl-2, Bax and P-gp protein in all groups was detected. (A) Western blots showing expression levels of CDK1, cyclin B, Bcl-2, Bax and P-gp protein following treatment. Graphical representation of the results for (B) CDK1, (C) cyclin B, (D) Bcl-2, (E) Bax and (F) P-gp. * refers to comparisons with the control group; # refers to comparisons with the NaI[131] group and Δ refers to comparisons with the GA group. #,ΔP<0.05; ##,ΔΔP<0.01; and ***,###,ΔΔΔP<0.001. DDP, cisplatin; GA, gambogic acid; PBS, phosphate-buffered saline; P-gp, P-glycoprotein.

The lowest signal was detected in the combination group (P<0.05). While the protein expression level of mtp53 was similar in A549 and A549/DDP cells treated with NaI[131] or GA, it decreased slightly in A549/Taxol cells treated with GA compared with that in control and NaI[131]-treated cells (P<0.05; Fig. 4C).

Additionally, the expression of HSP90 was assessed in drug-resistant NSCLC treated with the combination of drugs. mRNA expression of HSP90 (Fig. 4D) was significantly decreased in the combination group compared with that in the individual treatment groups with either NaI[131] or GA (P<0.05). IF results were in line with the RT-qPCR results, in that the protein expression levels of HSP90 (Fig. 4E and F) were decreased in all the groups compared with that in the control. The lowest signal was detected in the combination group (P<0.05). The relative expression levels of HSP90 remained

very similar in A549, A549/DDP and A549/Taxol cells treated with NaI[131] and GA. These results indicated that the effect of NaI[131] combined with GA on drug-resistant NSCLC cells may be associated with p53 and HSP90.

Discussion

The present study investigated the efficacy of using GA as a radionuclide sensitizer to NaI[131] in an *in vitro* system. As an extension to our previous study (23) showing that GA below the conventional dose can enhance the apoptosis-promoting function of NaI[131] in A549/DDP cells, the current study revealed that compared with the control, in the combination group of all three NSCLC lines (A549, A549/DDP and A549/Taxol) there was a significant increase in the percentage of cells in the G2/M phase and an increase in the number of apoptotic cells,
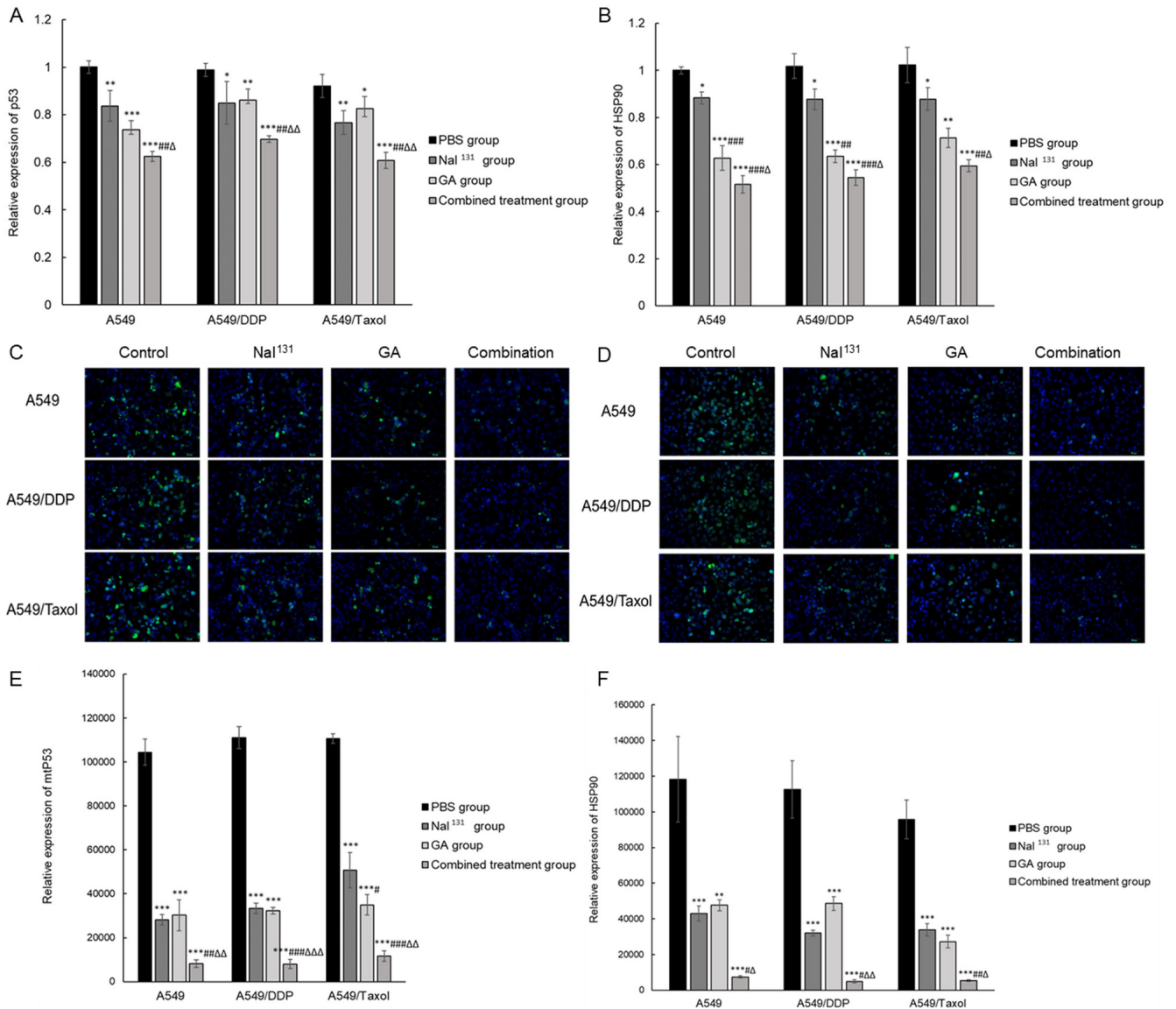
Figure 4. Expression of mRNA and protein levels of p53 and HSP90. Relative mRNA levels of (A) p53 and (B) HSP90 in all the groups were detected by reverse transcription-quantitative PCR after treatment of A549, A549/DDP and A549/Taxol cells with NaI[131], GA, or a combination of both. Protein expression of (C) mtp53 and (D) HSP90 in all groups was detected using IF after treatment of A549, A549/DDP and A549/Taxol cells with NaI[131], GA or a combination of both (x200 magnification). Graphical representation of the results of IF for (E) mtp53 and (F) HSP90. * refers to comparisons with the control group; # refers to comparisons with the NaI[131] group and Δ refers to comparisons with the GA group. *,#,ΔP<0.05; **,##,ΔΔP<0.01; and ***,###,ΔΔΔP<0.001. DDP, cisplatin; GA, gambogic acid; PBS, phosphate-buffered saline; mtp53, mutant p53; HSP90, heat shock protein 90; IF, immunofluorescence.

as well as a decrease in the levels of protein of CDK1, cyclin B, Bcl-2, P-gp, mtp53 and HSP90, and a significant increase in the protein levels of Bax. Studies have indicated that GA may induce cell cycle arrest at the G_1/S or G_2/M phases via various methods in different tumour cells. For example, Wang and Yuan (25), and Yang *et al* (26) reported that GA functioned in the upregulation of endogenous reactive oxygen species (ROS) levels in SKOV3 and RPMI-8226 cells. ROS leads to ATM-Chk2-mediated G_2/M arrest. ROS also promote cell cycle arrest by direct actions on the Cdc25 family of protein phosphatases (27).

A crucial function of the p53 gene is induction of cell cycle arrest and promotion of cell apoptosis and DNA repair (28). The cell cycle-related regulatory proteins CDK1 and cyclin B are the major regulatory proteins expressed in cells in the

S phase and during entry into the G_2/M phase (29). p53 can cause G_2/M phase arrest through downregulation of cyclin B/CDK1 expression (30). mtp53 can drive mitosis in tumour cells and promote tumour cell passage through the M phase via various pathways to cause tumour cell proliferation (31). Since γ-radiation can be persistently released after radioactive seed implantation in the tumour body, these cells actively proliferating in the G_2 and M phases are more sensitive to this radiation, causing cyclin B1-mediated G_2/M arrest and an elevated Bax/Bcl2 ratio (32). GA acting on tumour cells can arrest cancer cells at the G_1/S (33) or G2/M (34) phase. It has been shown that p53 has a crucial role in the induction of cell cycle arrest and the promotion of cell apoptosis and DNA repair (28). GA in low doses was reported to cause autophagy in mtp53 degradation in cancer cells (21). The present

experimental results showed that, in A549, A549/DDP and A549/Taxol cells, GA could decrease the levels of cyclin B, CDK1 and mtp53 in all the groups, and increase the number of cells in the G_2/M phase, indicating that GA could cause G_2/M arrest in these cells. In the combination group, the decrease in CDK1 and cyclin B levels was the highest, suggesting that low-dose GA may contribute to an increase in the number of cells in the radiation-sensitive stage by influencing cell cycle proteins and mtp53 to increase the lethal effect of NaI[131] on tumour cells.

The mtp53 protein is upregulated in a number of drug-resistant tumour cell lines, such as the human COLO 320DM (homozygous R248W p53) and MIA PaCa-2 cell lines, and is thus involved in tumour drug resistance (35,36). The mtp53 protein can selectively upregulate the expression of multiple drug resistance gene 1 (mdr1) (37). P-gp encoded by mdr1 can promote cellular excretion of chemotherapeutic drugs to produce drug resistance (6). The present study results showed that, in the combination group, levels of mtp53 and P-gp in all the cell types decreased, indicating that the two may have common drug-resistance pathways. Moreover, GA has been shown to downregulate mtp53 at the post-transcription level (22). The present study results concur with our previous study results (23) and demonstrate that the combinatorial effect of GA and NaI[131] may possibly assist in overcoming drug resistance, leading to a better response to chemotherapy. In normal cells, wild-type p53 protein is subject to ubiquitination and degradation mediated by mouse double minute 2 (MDM2) (30). However, mtp53 protein cannot directly activate the MDM2 ubiquitination degradation pathway, but can bind to HSP90. HSP90 and its associated chaperones can stabilize mtp53 protein to prevent its degradation, whereas inhibition of the function of HSP90 decreases mtp53 protein levels (38,39). GA has been shown to prevent Hsp90/mtp53 complex formation (22). A previous study showed that Hsp90 inhibitors can enhance radiosensitivity by inhibiting the binding of Hsp90 with client proteins (40). However, the effect of radionuclide on mtP53 in tumour cells has not been reported. The present study showed that, after intervention by NaI[131] and GA, the expression of HSP90 and p53 at the mRNA and protein levels decreased in all the cell types, suggesting that these two types of intervention could decrease the cellular expression of HSP90 and mtp53. In addition, the decrease was more evident in the combination group, indicating that low-dose GA combined with NaI[131] could significantly decrease HSP90 and mtp53 levels in drug-resistant cells, likely due to the ability of GA to destabilize and degrade mtp53 (22).

In conclusion, the present study illustrates that GA could be a favourable radionuclide sensitizer and that a combination of GA along with NaI[131] may have advantages in enhancing the effects of NaI[131] on tumour cells by way of governing cell cycle stages, promoting apoptosis and reducing drug-resistance, all of which are necessary cellular factors for cancer treatment in clinical settings. Follow-up animal experiments should be performed to verify this conclusion.

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Availability of data and materials

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

Authors' contributions

JH and MD confirm the authenticity of all the raw data. JH and MD were reponsible for conception and design. The collection and assembly of data was performed by YW, YX and SHH. The data analysis and interpretation was completed by XLZ, PSC and SFY. All authors have read and 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.

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