p21 promotes gemcitabine tolerance in A549 cells by inhibiting DNA damage and altering the cell cycle

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Abstract. Gemcitabine is one of the most widely used chemotherapy drugs for advanced malignant tumors, including non-small cell lung cancer. However, the clinical efficacy of gemcitabine is limited due to drug resistance. The aim of the present study was to investigate the role of p21 in gemcitabine-resistant A549 (A549/G$^+$) lung cancer cells. IC_{50} values were determined using a Cell Counting Kit-8 (CCK-8) assay. mRNA and protein expression levels of genes were measured by reverse transcription-quantitative PCR and western blotting, respectively. The cell cycle distribution and apoptosis rate were analyzed by flow cytometry. DNA damage in cells was evaluated by single-cell gel electrophoresis. The results of western blot analysis and the CCK-8 assay demonstrated that the expression of p21 was higher in A549/G$^+$ cells than in gemcitabine-sensitive cells. Knockdown of p21 expression in gemcitabine-resistant cells sensitized these cells to gemcitabine (with the IC_{50} decreasing from 84.2 to 26.7 μM). Cell cycle analysis revealed different changes in the cell cycle distribution in A549/G$^+$ cells treated with the same concentration of gemcitabine, and decreased expression of p21 was shown to promote G_1 arrest. The apoptosis assay and comet assay results revealed that decreased p21 expression resulted in accumulation of unrepaired DNA double-strand breaks (DSBs) and induction of apoptosis by gemcitabine. The present study demonstrated that knockout of p21 mRNA expression in A549/G$^+$ cells promotes apoptosis and DNA DSB accumulation, accompanied by G_1 arrest. These results indicated that p21 is involved in regulating the response of A549 cells to gemcitabine.

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Introduction

The lung cancer incidence rate (14%) and mortality rate (18%) remain high worldwide (1), posing an extreme threat to human health. Chemotherapy plays an irreplaceable role in the treatment of lung cancer. Most chemotherapeutic agents kill cells by interfering with DNA replication or inducing DNA damage, leading to apoptosis (2). Gemcitabine, a deoxycytidine analog, has been used as the first-line chemotherapy drug for lung cancer (3,4). The most important mechanism of gemcitabine is inhibition of DNA synthesis. Gemcitabine is not only a cell cycle-specific antimetabolic drug that acts mainly during the DNA synthesis (S) phase of the cell cycle but also blocks the G_1/S transition. These effects are detrimental to cell cycle progression. Therefore, gemcitabine can prevent DNA replication (5-7). Unfortunately, acquired resistance limits the efficacy of gemcitabine. Factors contributing to resistance include alterations in gemcitabine uptake and metabolism, gene regulation and apoptotic pathways (8,9). Therefore, studying the mechanisms of lung cancer drug resistance and reversing this resistance are urgent issues to be solved in the treatment of lung cancer.

p21, also called $p21^{Waf1/Cip1}$ or cyclin-dependent kinase inhibitor 1A (CDKN1A), plays an important role in regulating cell proliferation and maintaining genomic stability (10). It can induce G_1 arrest and cell senescence in response to various stimuli, including oncogene-induced proliferation (11,12). A number of studies have reported that p21 plays dual roles in maintaining the stability of the genome and regulating cell proliferation and drug resistance (11,13).

Previous studies have suggested that upregulation of p21 expression could increase the sensitivity of lung cancer cells to cisplatin. For instance, Wang *et al* showed that overexpression of p21 in drug-resistant cells blocked the

G_1/S transition. In addition, it reduced the expression of Bcl-2 and Bcl-XL while increasing the expression of Bax and Bak, which may promote apoptosis and reverse cisplatin resistance (14). Liu *et al* also revealed that upregulation of p21 expression induced cell cycle arrest in lung cancer cells, inhibited cell proliferation and increased the sensitivity of lung adenocarcinoma cells to cisplatin (15). However, some studies arrived at the opposite conclusions. In these studies, high expression of p21 was considered to promote drug resistance. For example, Zhao *et al* demonstrated that overexpression of p21 induced G_1 arrest and inhibited DNA synthesis. However, at the same time, DNA repair in these cells was enhanced, and the DNA damage induced by cisplatin was mitigated. These factors resulted in resistance to cisplatin (16). In addition, Guo *et al* revealed that the expression of p21 was increased in A549 cells under hypoxic conditions, resulting in cell cycle arrest at the G_1/G_0 phase boundary; however, cisplatin resistance in these A549 cells was simultaneously increased. One potential reason for this phenomenon is that cell cycle arrest led to an increase in the number of cells in a non-proliferative state and reduced the effect of cisplatin on these cells (17).

Given the mechanism by which gemcitabine inhibits cell proliferation and the role of p21 in the cell cycle, in the present study, gemcitabine-resistant A549 (A549/G^+) cells were established to confirm that p21 plays a critical role in the drug resistance of lung cancer cells by altering cell cycle progression and apoptosis. In addition, p21 knockdown was demonstrated to increase DNA damage and apoptosis induced by gemcitabine, providing a therapeutic strategy to overcome drug resistance.

Materials and methods

Cell culture. A549 is a non-small cell lung cancer cell line and was purchased from the research facilities of Peking Union Medical College (Beijing, China). The A549/G^+ cell line was induced and established by the authors of the present study and continues to be maintained (18). Gemcitabine is produced by Eli Lilly and Company. All cells were cultured in DMEM (HyClone; Cytiva) supplemented with 10% fetal bovine serum (Sangon Biotech Co., Ltd.) and 1% penicillin/streptomycin in humidified air at 37˚C with 5% CO_2.

Antibodies and reagents. Anti-γH2AX (1:1,000; cat. no. 9718; Cell Signaling Technology, Inc.), anti-GAPDH (1:1,000; cat. no. AF0006; Beyotime Institute of Biotechnology), anti-P21 (1:1,000; cat. no. 2947; Cell Signaling Technology, Inc.), anti-cyclin D1 (1:1,000; cat. no. 55506), anti-cyclin A2 (1:1,000; cat. no. 4656), anti-cyclin E1 (1:1,000; cat. no. 4129), and anti-cleaved caspase-3 (1:1,000; cat. no. 9661; all from Cell Signaling Technology, Inc.) antibodies were used.

Cell survival and cytotoxicity assays. Cell proliferation *in vitro* was monitored by a Cell Counting Kit-8 (CCK-8) assay in 96-well plates. A total of $2x10^3$ cells in complete medium were seeded into each well, and 10 μl of CCK-8 reagent (cat. no. CK04-13; Dojindo Molecular Technologies, Inc.) was added for an additional 1 h of incubation at 37˚C. The plates were read and the absorbance was measured at 450 nm using a microplate reader (BioTek Synergy HTX; BioTek Instruments, Inc.).

Reverse transcription-quantitative PCR (qPCR) assay. Total RNA was isolated from cells with RNAiso Plus (Takara Bio, Inc.) and the quantity was measured by using NanoDrop equipment (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed using the Prime Script RT Master Mix (Takara Bio, Inc.) according to the manufacturer's instructions. The TB Green Premix Ex Taq™ II Kit (Takara Bio, Inc.) was used to detect relative mRNA expression using the PIKOREAL 96 Real-Time PCR System (Thermo Fisher Scientific, Inc.) with 40 cycles of PCR thermocycling at 95˚C for 15 sec and 60˚C for 60 sec, followed by 95˚C for 10 min. The primer sequences were as follows: GAPDH forward, 5'-CAGGAGGCATTG CTGATGAT-3' and reverse, 5'-GAAGGCTGGGGCTCA TTT-3'; p21 forward, 5'-GCCCGTGAGCGATGGAACTTC-3' and reverse, 5'-CCTGCCTCCTCCCAACTCATCC-3'. All the target genes were calculated using the $2^{-\Delta\Delta Cq}$ method (19) and normalized against the expression level of GAPDH.

Western blot analysis. Cells were lysed with RIPA buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) containing a protease and phosphatase inhibitor cocktail. Protein concentration was detected by the BCA method (Thermo Fisher Scientific, Inc.). SDS-PAGE gels (10%) (Beyotime Institute of Biotechnology) were used for protein electrophoresis with equal amounts of protein (30 μg). The separated proteins were transferred to PVDF membranes (MilliporeSigma) and blocked with 5% skimmed milk in TBS with 0.2% Tween-20 at room temperature for 2 h. The membranes were then incubated with the aforementioned antibodies at 4˚C overnight. On the second day, the membranes were washed and incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies at the dilution of 1:1,000 (cat. nos. A0208 and A0216; Beyotime Institute of Biotechnology) at room temperature for 2 h. Labeled proteins were detected by an enhanced chemiluminescence detection system (Azure Biosystems), and the protein band grayscale analysis was performed using ImageJ 1.51k software (National Institute of Health).

Cell cycle analyses. Harvested cells were washed with PBS, fixed with 70% ethanol at 4˚C for 2 h and then treated with 450 μl of RNase and 50 μl of propidium iodide (PI; cat. no. KGA512; Nanjing KeyGen Biotech Co., Ltd.) at room temperature for 15 min. The samples were analyzed by flow cytometry (FACS Canto™ II; BD Biosciences) and the data were analyzed using Flowjo.7.6 software (BD Biosciences).

Apoptosis assay. Cells were washed once in PBS and incubated with 5 μl of 7-AAD in 50 μl of binding buffer (cat. no. KGA1017; Nanjing KeyGen Biotech Co., Ltd.) for 15 min at 37˚C in a humidified atmosphere in the dark. Then, 450 μl of binding buffer and 1 μl of Annexin V-PE (cat. no. KGA1017; Nanjing KeyGen Biotech Co., Ltd.) were added at room temperature. The apoptosis rate was determined by flow cytometry (FACS Canto II; BD Biosciences) and the data were analyzed using Flowjo.7.6. software within 1 h of staining.

siRNA transfection. Cells were seeded at a density of $2x10^5$ cells per well in a six-well plate. On the next day, transfection of siRNA (50 nM) into cells was performed with Lipofectamine™ 3000 Transfection Reagent (cat. no. L3000015; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 24 or 48 h according to the manufacturer's instructions. siRNAs and the control siRNAs were purchased from Guangzhou Ribobio Co., Ltd. The sequence of the siRNA targeting *p21* was 5'-GAATGAGAGGTTCCT AAGA-3'; and the sequence of the control is not disclosed by Ribobio company; however, the product number was provided by the company (siN0000001-1-5).

Plasmid transfection. Cells were seeded 1 day before transfection. Empty vector GV658 was used as the control group and p21 expression recombinant plasmid GV658-p21 was purchased from Shanghai GeneChem Co., Ltd. Plasmid (2 μg) was transfected into cells using Lipofectamine 3000 Transfection Reagent at room temperature (cat. no. L3000015; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 24 h or 48 h according to the manufacturer's instructions, and then 24 h or 48 h later, the transfected cells were used for the following experiments.

Comet assay. A neutral comet assay was performed to evaluate DNA double-strand break (DSB) damage. Collected cells were washed with PBS. The first layer of agarose was prepared, 75 μl of 1% normal melting point agarose (Biofroxx; neoFroxx) was added to the slides, which were quickly covered with a coverslip and placed at 4˚C for 10 min in the dark. Subsequently, 75 μl of 0.7% low melting point (LMP)-agarose (Beijing Solarbio Science & Technology Co., Ltd.) and 10 μl of the cell suspension were mixed, quickly added to the first layer of gel and covered with a coverslip. After the second layer of agarose had solidified, 75 μl of 0.7% LMP-agarose was added to make the third agarose layer. After this agarose layer had solidified, the slides were immersed in ice-cold lysis solution for 2 h at 4˚C in the dark, and electrophoresis was then performed in a horizontal electrophoresis apparatus [BG-subMIDI(V); Beijing Baygene Biotech Co., Ltd.]. Following electrophoresis, the slides were immersed in neutralization buffer for 10 min at room temperature. PI was pipetted onto each sample, and the slides were incubated for 10 min at room temperature in the dark. Images were acquired with an Olympus microscope connected to a charge-coupled device (CCD) camera (final magnification, x200). The tail moment was calculated using Comet Assay Software Project 4 (CASP 4; Perspective Instruments, Ltd.) as an indicator of DNA damage.

Statistical analysis. Data are presented as the mean ± standard deviation from three independent assays. One-way analysis of variance (ANOVA) was used to compare the sample mean and pairwise comparisons between multiple groups (followed by Dunnett's if all comparisons were vs. a single control or Tukey/Bonferroni if the groups were compared with each other). Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, Inc.; Dotmatics). P-values of <0.05 were considered to indicate statistically significant differences.

Results

p21 is markedly upregulated in A549/G$^+$ cells and p21 upregulation potentially induces gemcitabine resistance in A549 cells. In a previous study conducted by the authors (20), it was observed that miR-17-5p and let-7i-5p overexpression in A549/G$^+$ cells could increase their sensitivity to gemcitabine, while the expression of p21 protein in these cells was decreased. To investigate whether the expression level of p21 is related to the gemcitabine tolerance of A549 cells, the mRNA and protein expression levels of p21 in A549 and A549/G$^+$ cells were verified by reverse transcription-quantitative PCR and western blotting, respectively. Compared with that in A549 cells (Fig. 1A and B), the p21 expression level in A549/G$^+$ cells were increased (P<0.001). p21 plays a dual role in tumors (13,21,22). Therefore, it was hypothesized that the high level of p21 expression in A549/G$^+$ cells contributed to the acquisition of drug resistance, and this hypothesis was later verified. p21 was overexpressed in A549 cells, and A549/G$^+$ cells were transfected with siRNA-p21 (Fig. 1C and D). Subsequently, a CCK-8 assay was performed to measure the IC_{50} value of gemcitabine. The results (Fig. 1E and F) revealed that knockdown of p21 expression in A549/G$^+$ cells resulted in a decrease in the IC_{50} value of gemcitabine from 84.2 to 26.7 μM (P<0.05); by contrast, overexpression of p21 expression in A549 cells increased the IC_{50} value from 15.4 to 40.1 μM (P<0.001). Thus, p21 knockdown sensitized A549 cells to gemcitabine. These results indicated that p21 upregulation in A549/G$^+$ cells may contribute to gemcitabine resistance.

Treatment with 10 µM gemcitabine increases the percentage of A549 cells in the G_0/G_1 phase and reduces the percentage of A549/G$^+$ cells in the G_1 and S phase. Gemcitabine is a potent replication inhibitor, but whether A549 cell tolerance to gemcitabine is associated with the cell cycle remains unclear. Subsequently, the two aforementioned cell lines were treated with the same concentration of gemcitabine, and flow cytometry was performed to assess the cell cycle distribution. As illustrated in Fig. 2A, in A549 cells, treatment with 10 μM gemcitabine resulted in a slight decrease in the percentage of cells in the S phase (Fig. 2B; 16.62% vs. untreated 26.38%) and an increase in the percentage of cells in the G_0/G_1 phase (Fig. 2B; 78.70% vs. untreated 57.55%). However, when A549/G$^+$ cells were treated with 10 μM gemcitabine, there was a decrease in the percentage of cells in the G_1 phase (Fig. 2C; 50.35% vs. untreated 66.29%), accompanied by an increase in the percentage of cells in the S phase (Fig. 2C; 31.08% vs. untreated 21.61%) and a lesser increase in the percentage of cells in the G_2/M phase (Fig. 2C; 19.96% vs. untreated 10.24%).

Knockdown of p21 promotes gemcitabine-induced G_1 arrest. Considering the role of p21 in the cell cycle, it was speculated that the different changes in the cell cycle in gemcitabine-resistant cells are involved in the difference in p21 expression. To test this hypothesis, A549/G$^+$ cells were transfected with siRNA-p21 to knockdown p21 expression prior to gemcitabine treatment for 24 h and cell cycle analysis was performed using flow cytometry. The reason

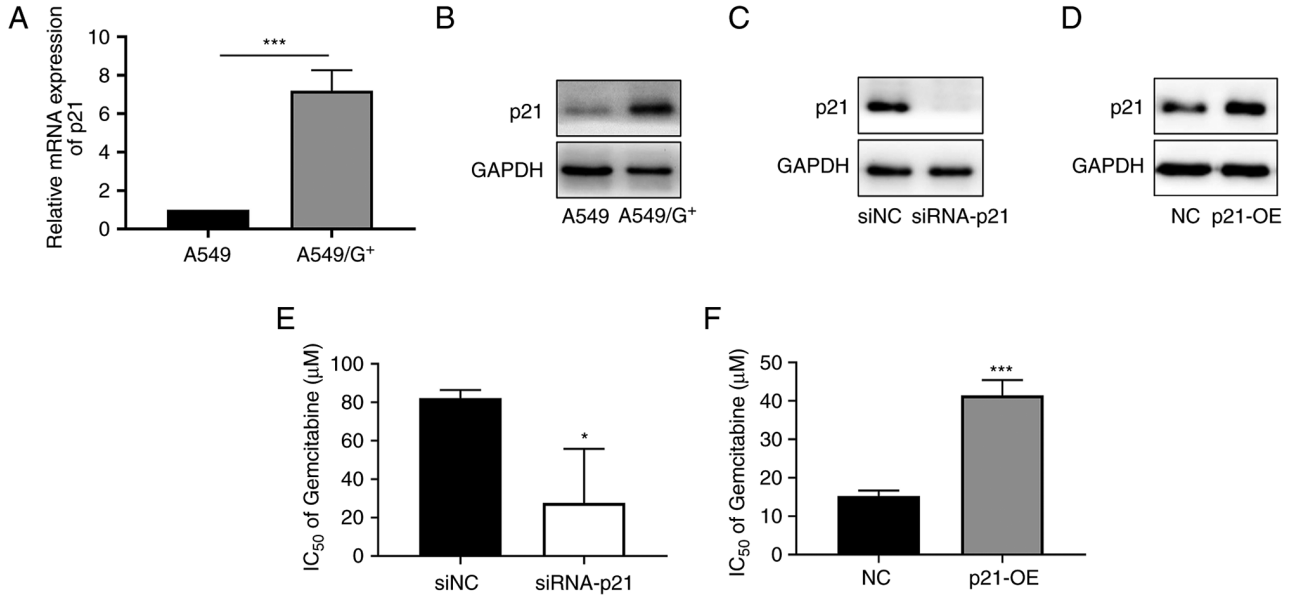

Figure 1. p21 is markedly upregulated in gemcitabine-resistant A549 cells. (A) p21 mRNA and (B) protein expression levels in A549/G$^+$ and parental A549 cells. (C) A549 cells were transfected with siRNA, and western blotting was used to verify the interference efficiency of p21. (D) A549/G$^+$ cells were transfected with plasmids, and western blotting was used to verify the efficiency of p21 overexpression. (E) After p21-siRNA or siNC was successfully transfected into A549/G$^+$ cells, the cells were treated with different concentrations of gemcitabine (1, 5, 10, 25, 50, 100, 500 and 1,000 μM), and a CCK8 proliferation assay was performed to detect the IC_{50}. (F) Overexpression of p21 in A549 cells was assessed after treatment with different concentrations of gemcitabine (1, 5, 10, 25, 50, 100, 500 and 1,000 μM), and then a CCK8 proliferation assay was performed to detect the IC_{50}. siRNA, small interfering RNA; NC, negative control; OE, overexpressed; A549/G$^+$, gemcitabine-resistant A549; CCK-8, Cell Counting Kit 8. *P<0.05 and ***P<0.001 vs. respective controls.

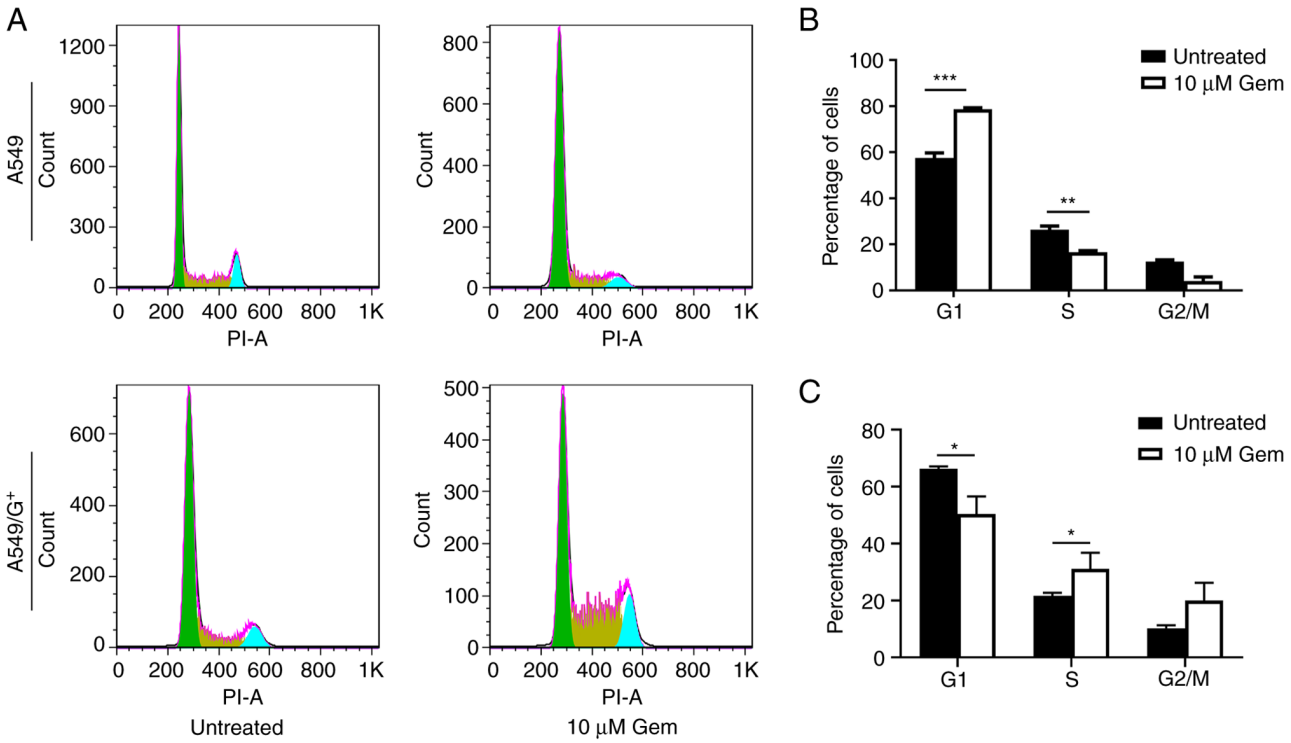

Figure 2. Changes in the cell cycle distribution of two cell lines following treatment with the same concentration of gemcitabine. (A) A549 and A549/G$^+$ cells were treated with 10.0 μM gemcitabine for 24 h and assessed by PI staining and flow cytometry. (B) A549 cell cycle changes with 10.0 μM gemcitabine. (C) A549/G$^+$ cell cycle changes with 10.0 μM gemcitabine. A549/G$^+$, gemcitabine-resistant A549; Gem, gemcitabine. *P<0.05, **P<0.01 and ***P<0.001 vs. respective controls.

why sensitive cells were not selected is that the expression level of p21 in the sensitive cells is already extremely low. If siRNA is used to interfere with its expression, the knockdown effect is not significant. Therefore, drug-resistant cells were selected for interference experiments. The results revealed that the expression of p21 in A549/G$^+$ cells was decreased,

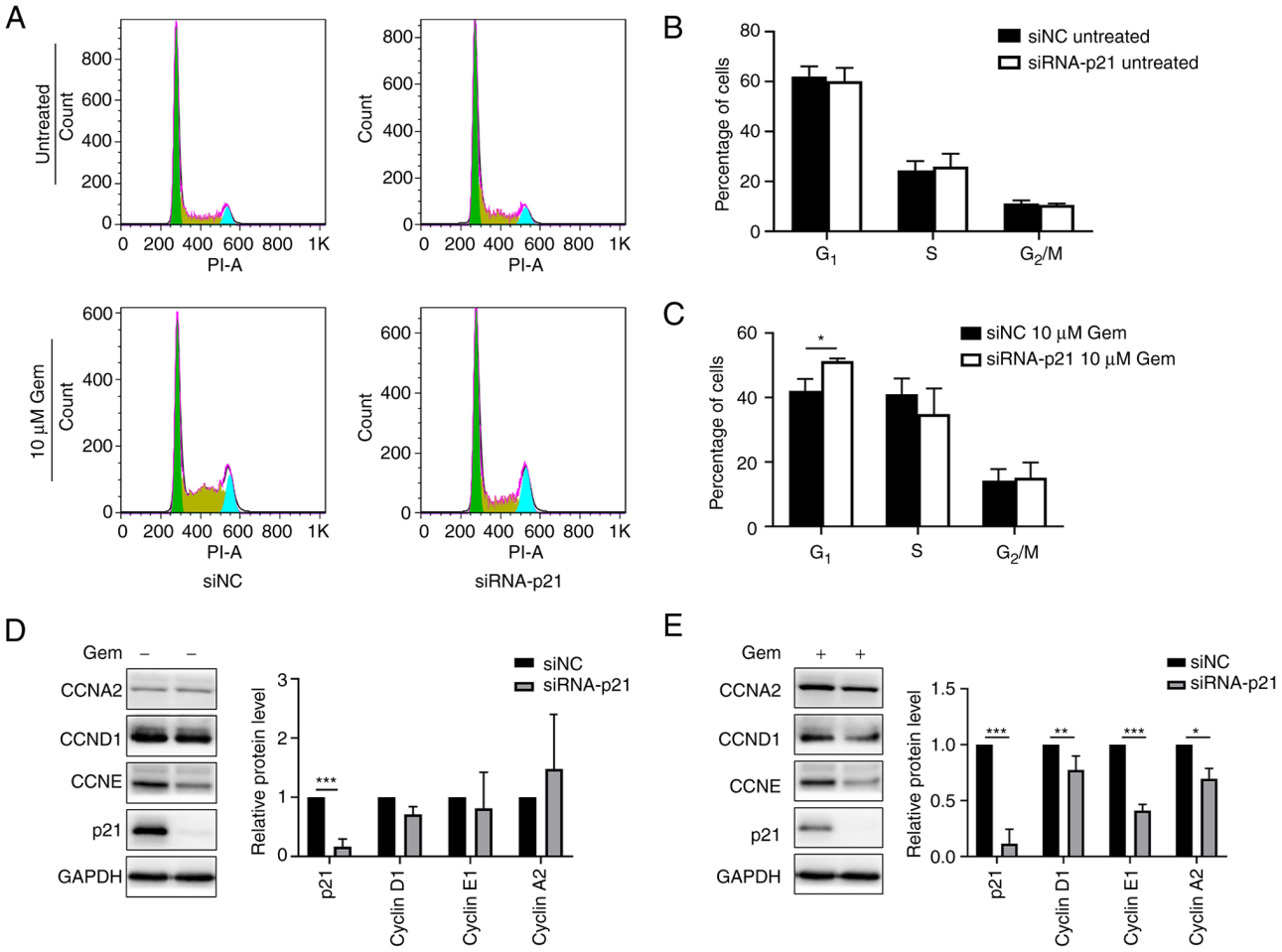

Figure 3. Knockdown of p21 promotes gemcitabine-induced G_1 arrest. (A) A549/G$^+$ cells were transfected with siNC or siRNA-p21. They were then treated with 10.0 μM gemcitabine for 24 h. The cell cycle distribution was detected by PI staining and is shown in the bar graph as percentages of cells. (B) Cell cycle changes in the untreated siRNA-p21 group. (C) Cell cycle changes in the siRNA-p21 group treated with 10.0 μM gemcitabine. (D) Western blotting detection of cell cycle-related proteins (CCNA2, CCNE1 and CCND1) in siNC (or siRNA-p21)-transfected A549/G$^+$ cells. GAPDH protein was used as an internal control. (E) Western blotting detection of cell cycle-related proteins (CCNA2, CCNE1 and CCND1) in siNC (or siRNA-p21)-transfected A549/G$^+$ cells combined with gemcitabine (10 μM). siRNA, small interfering RNA; NC, negative control; Gem, gemcitabine. *P<0.05, **P<0.01 and ***P<0.001 vs. respective controls.

but there was no significant change in the cell cycle distribution in the absence of gemcitabine (Fig. 3A and B). However, the percentage of G_1-phase cells was significantly increased after gemcitabine treatment (Fig. 3C; 51.14% vs. untreated 42.14%). Furthermore, as revealed in Fig. 3D, the expression levels of cyclin E1, cyclin A2 and cyclin D1, which are related to the G_1 and S phases, did not change significantly when p21 expression was knocked down in A549/G$^+$ cells in the absence of gemcitabine. However, as revealed in Fig. 3E, following treatment with gemcitabine, cyclin E1, cyclin A2, and cyclin D1 expression was reduced in the siRNA-p21 group. The aforementioned results indicated that p21 expression in A549/G$^+$ cells is involved in modulating the expression of cell cycle regulators and that a reduction in p21 expression could promote gemcitabine-induced G_1 arrest. Moreover, similar results demonstrated that an increase in the number of G_1-phase cells is related to apoptosis (23). Considering these observations, it was assumed that the failure of gemcitabine to induce G_1 arrest may be an important cause of gemcitabine resistance and that the reduction in p21 expression could inhibit progression through the G_1 phase.

Knocked down p21 expression results in accumulation of unrepaired DSBs and induction of apoptosis by gemcitabine, which enhances sensitivity to gemcitabine. Gemcitabine, a nucleoside analog, can be incorporated into replicating DNA, resulting in partial chain termination and stalling of replication forks, which can cause DNA DSB damage and apoptosis to kill tumor cells (7,24). To assess the difference in gemcitabine-induced DNA DSB damage between A549 cells and A549/G$^+$ cells, the two cell lines were treated with the same concentration of gemcitabine. As depicted in Fig. 4A, the level of the DSB indicator γ-H2AX was increased by gemcitabine treatment, but its change trend was opposite that of p21 expression. The γ-H2AX expression level in A549 cells was higher than that in A549/G$^+$ cells (P<0.05), suggesting that gemcitabine-induced DNA DSB damage was more severe in A549 cells than in A549/G$^+$ cells. In addition, it was hypothesized that upregulation of p21 in drug-resistant cells reduced gemcitabine-induced DSB formation and apoptosis. Therefore, p21 was overexpressed in A549 cells and siRNA-p21 was transfected into A549/G$^+$ cells, and these cells were then treated with gemcitabine to evaluate the level of γ-H2AX. As revealed in Fig. 4B, the apoptosis activity, as indicated by caspase-3

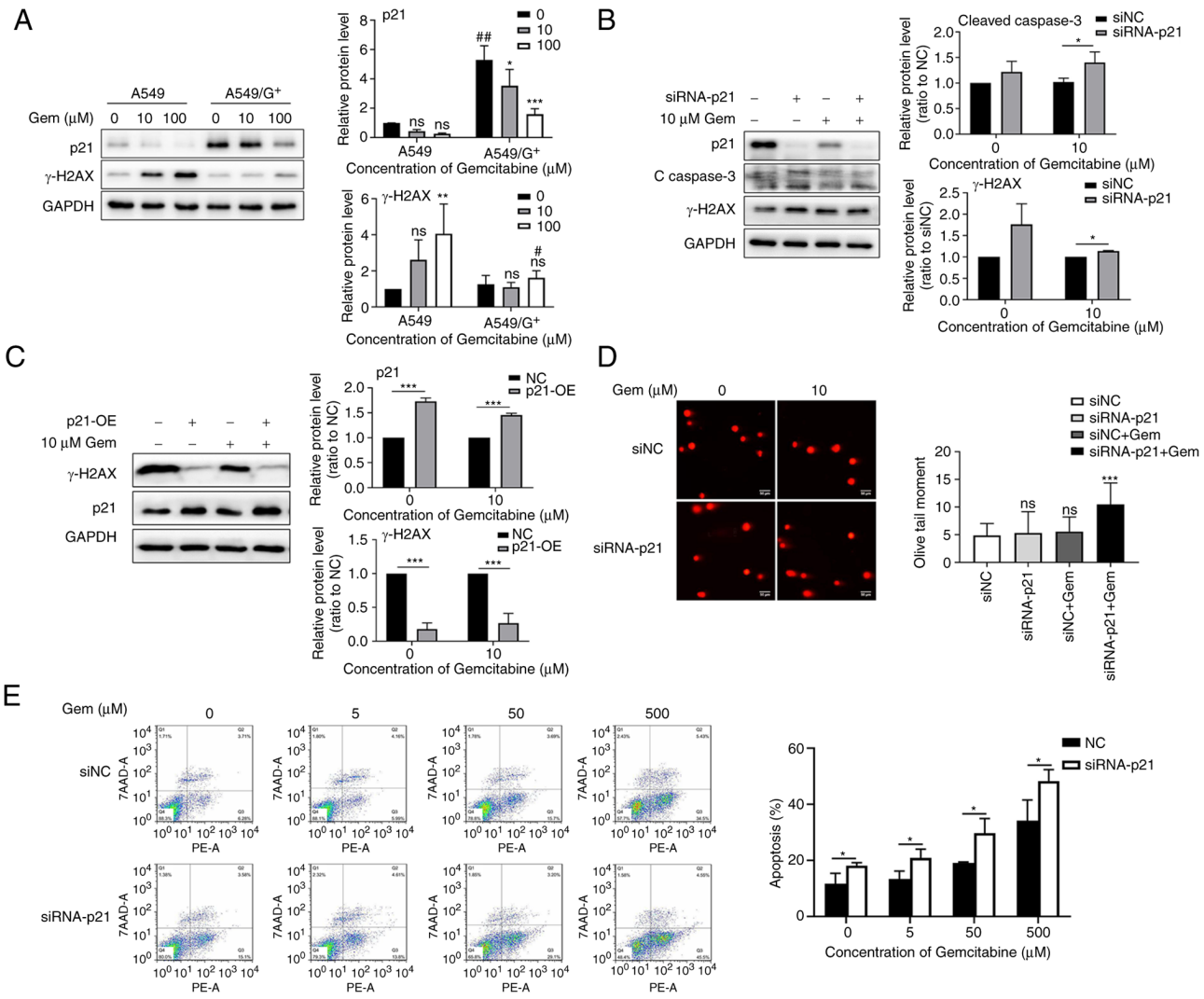

Figure 4. Knockdown of p21 expression results in accumulation of unrepaired DSBs and induction of apoptosis by gemcitabine. (A) Western blotting detection of DNA double-strand replication-related protein (γ-H2AX) in A549 cells and A549/G$^+$ cells coupled with various concentrations of gemcitabine (0, 10 and 100 μM). (B) Western blotting detection of apoptosis and DNA double-strand replication-related protein (γ-H2AX and cleaved caspase-3) in siNC- or siRNA-p21-transfected A549/G$^+$ cells treated with different concentrations of gemcitabine (0 and 10 μM). (C) Western blotting detection of DNA double-strand damage was observed with p21 overexpression. (D) Representative images of the comet assay in A549/G$^+$ cells treated with siRNA-p21 (or siNC) combined with gemcitabine. (E) Flow cytometric analysis of apoptosis in siNC- or siRNA-p21-transfected A549/G$^+$ cells treated with various concentrations of gemcitabine (0.0, 5.0, 50 and 500 μM). Gem, gemcitabine; A549/G$^+$, gemcitabine-resistant A549; siRNA, small interfering RNA; NC, negative control; OE, overexpressed. *P<0.05, **P<0.01 and ***P<0.001 vs. respective controls, $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the same drug concentration in A549 cells.

cleavage, was upregulated, as was the level of γ-H2AX, in the siRNA-p21 group compared with the siNC group. However, overexpression of p21 reduced gemcitabine-induced DNA damage (Fig. 4C). Furthermore, to confirm the role of p21 in gemcitabine-induced DSB formation and apoptosis, a comet assay was performed to detect DSBs and flow cytometry was conducted to determine the apoptosis rate after A549/G$^+$ cells were treated with siRNA-p21 and gemcitabine. Consistent with the western blot results, the decrease in p21 expression in A549/G$^+$ cells promoted gemcitabine-induced DNA damage and apoptosis (Fig. 4D and E).

Based on the aforementioned results, it was deduced that the reduction in p21 expression in A549/G$^+$ cells promoted gemcitabine-induced accumulation of DNA damage and apoptosis, which may sensitize cells to gemcitabine. Consistent with the findings of the present study, it was reported that p21 contributed to the ability of Langerhans cells to resist the detrimental effects of ionizing radiation by the rapid repair of DSBs and inhibition of apoptosis (25).

Discussion

p21 is downstream of the tumor suppressor p53 and mediates cell cycle arrest upon DNA damage (26). p21 has always been considered a tumor suppressor gene. However, an increasing number of studies have revealed that p21 plays two roles in the progression of cancer and in radio- and chemoresistance. Numerous studies have shown that high expression of p21 plays a primary role in promoting drug resistance in lung cancer or increasing A549 cell survival by protecting cells against cytotoxic anticancer agents (23,27). However, a small number of studies have indicated that high expression of p21 can increase the resistance of lung cancer cells to gemcitabine. Similarly, previous research conducted by the authors revealed

that downregulation of miR-17-5p in A549/G$^+$ cells can directly or through RRM2 increase p21 mRNA and protein expression (20).

It is generally considered that the role of p21 in tumors depends mainly on its localization and expression status in cells. Nuclear p21 can inhibit cell proliferation and promote apoptosis, while cytoplasmic p21 has carcinogenic and antiapoptotic effects. For example, a number of studies have reported that cytoplasmic p21 is involved in the chemoresistance of ovarian cancer cells (28), testicular embryonal tumors (29). It plays an important role in protecting cells against apoptosis and promoting tumor invasion, migration, and drug resistance (30,31).

Consistent with the findings of the present study, Ikeda *et al* revealed that the p21 expression level in A549/G$^+$ cells were increased. Their findings suggested that overexpression of p21 may be a molecular marker associated with gemcitabine resistance in the A549 cell line but did not elucidate the underlying mechanism (32). In the present study, upon treatment with the same concentration of gemcitabine, the cell cycle in the gemcitabine-sensitive and A549/G$^+$ cell lines was differentially altered. Gemcitabine successfully induced G_1 arrest in A549 but not in A549/G$^+$ cells. However, p21 knockout and treatment with gemcitabine increased the proportion of G1-phase cells among A549/G$^+$ cells. This finding was consistent with the study conducted by Gaben *et al*, which showed that inhibition of the expression of p21 could inhibit G_1 phase progression and was related to the antiproliferative effects of rapamycin in BP-A31 cells (33). Similarly, a recent study demonstrated that in RAL cells treated with the cisplatin-pemetrexed combination, knockdown of p21 could increase the number of apoptotic cells and induce G_1 arrest. These results suggested that p21 is involved in regulating the response of RAL cells under treatment with the combination of cisplatin and pemetrexed (23).

In addition to alterations in the cell cycle distribution, significant increases in the number of apoptotic cells, apparent G_1 arrest and accumulation of DNA DSB damage in CDKN1A-knockdown A549/G$^+$ cells were observed. These findings indicated that p21 is involved in modulating the response of A549 cells to gemcitabine and demonstrated that p21 may be a target for reversing drug resistance in lung cancer.

Of note, the reason why the authors of the present study used the A549 cell line for their present research is that it focuses on chemoresistance, and the chemical sensitivity and drug resistance characteristics of the A549 cell line are very obvious. In particular, A549 cells are more sensitive to doxycycline, gemcitabine, and other chemotherapy drugs but relatively resistant to paclitaxel, vincristine, and platinum compounds. Therefore, the A549 cell line is widely used in the screening and evaluation of chemotherapy drugs for lung cancer, in research on cellular signal transduction in lung cancer, and in antitumor immunotherapy research (34).

In several tumors, increased p21 expression mediates drug resistance and promotes cell survival (35,36). Reducing the expression of p21 may be a solution to reverse drug resistance. Recently, a novel p21 inhibitor, UC2288, was demonstrated to decrease p21 mRNA expression independent of p53 and attenuate p21 protein expression with a minimal effect on p21 protein stability (37). Yan *et al* discovered that inhibiting p21 in QGP-1 and NCI-H727 cells by treatment with UC2288, further enhanced the cytotoxicity of artesunate, implying that p21 may confer resistance to artesunate on these two cell lines (38). In addition, p21 inhibitor-induced death in cells with high p21 expression suggests that p21 suppression could be a therapeutic strategy for patients with clear cell carcinoma (39). Although the present research has certain limitations, such as only conducting experiments on a few cell lines and lacking evidence from *in vivo* experiments, it still suggests that p21 may be a new marker of drug resistance, and that p21 suppression could be a therapeutic strategy for patients with cancer.

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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 upon reasonable request.

Author contributions

ALL and YJL contributed to the conceptualization and supervision of the study. TF and XM performed the experiments and confirm the authenticity of all the raw data. TF, XM, SLD, ZYK, XCW, HHY and WXW contributed to acquiring and analyzing the data. TF and XM contributed to the writing of the original draft. TF, XM, ALL and YJL contributed to the manuscript review and editing. YJL contributed to the funding acquisition. All authors 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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