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Apigenin Enhanced Antitumor Effect of Cisplatin in Lung Cancer via Inhibition of Cancer Stem Cells

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ABSTRACT

Cancer stem cell theory has been proposed to explain tumor heterogeneity and the carcinogenesis process. Highly tumorigenic lung cancer stem cells develop resistance to cisplatin (CDDP), a common chemotherapy drug. Herein, we attempted to clarify whether apigenin (API) can improve the antitumor efficiency of CDDP in lung cancer using cancer stem cells. Lung cancer stem cells were identified as CD 133 positive cancer cells in non-small cell lung cancer (NSCLC) A549, H1299 cells and CDDP-resistant NSCLC A549R cells. The cytotoxic effect of API was measured in CDDP-treated A549, H1299, and A549R cells. API repressed CD 133 positive cells and enhanced the antitumor effect of CDDP in A549, H1299, and A549R cells. The synergistic antitumor effect of API and CDDP was blocked by addition of the p53 inhibitor Pifithrin-α, and siRNA targeting the *p53* gene in A549R cells. Furthermore, API eliminates CDDP-induced CSC via p53, since A549R cells lacking p53 and Pifithrin-α addition derepressed the decrease in CD 133 positive cells after API treatment in CDDP-treated A549 and A549R cells. The findings indicate that API might eliminate cancer stem cells and enhance the antitumor effects of CDDP in NSCLC via p53.

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Introduction

Although important advances in the treatment of non-small cell lung cancer (NSCLC) have been achieved over the past 20 years, this type of lung cancer is still the most common cause of cancer-associated death worldwide (1). Platinum-based doublet therapy, such as cisplatin (cis-diamminedichloroplatinum [II]; CDDP) in combination with another cytotoxic therapy, has been the standard therapy for patients with advanced stage NSCLC (2). Cisplatin therapy improves absolute 5-year survival by 5.3% according to the Lung Adjuvant Cisplatin Evaluation program (3). However, many NSCLC patients are intrinsically resistant to cisplatin-based therapies, and more importantly, some acquire resistance to cisplatin, and tumors originally sensitive eventually develop cisplatin resistance (4, 5). Cancer stem cells (CSCs), one sub-population of cisplatin-resistant NSCLC cells, are believed to be responsible for tumor relapse, invasion, and ultimately disease dissemination through the acquisition of mesenchymal cell traits (6).

This new concept for CSCs, first introduced in the late 1990s, has gradually gained worldwide acceptance and now influences all approaches to cancer research and therapy. Based on the CSC concept, drug resistance is mostly caused by intrinsic or acquired resistance mechanisms of accumulating CSCs (7). $CD133^+$ cancer cells are characteristic of CSCs in NSCLC (8). Both $CD133^+$ lung adenocarcinoma cells (9) and glioblastoma cells (10) can develop resistance to CDDP. It is therefore critical to find ways to improve the sensitivity of CSCs to CDDP.

Apigenin (4, 5, 7-trihydroxy flavone; API) is a natural flavone present in common fruits and vegetables including parsley, celery, celeriac, and chamomile tea. It exerts strong anti-inflammatory and protective effects in collagen-induced arthritis (11). Moreover, antimetastatic effects have been reported in multiple cancers including breast, prostate, skin, lung and ovarian cancers (12–14). Additionally, API suppresses CSC-like properties in triple-negative breast cancer cells (15), and it represses hypoxia-induced CSC marker gene expression in a head and neck squamous

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cell carcinoma cell line (16). In NSCLC, API induces human lung cancer H460 cell death (17), and enhances the cytotoxic effects of CDDP (18). Thus, we are interested in whether API plays an anti-tumor role in the CDDP-induced increase in CSCs and acquired resistance in NSCLC.

The role of API in CDDP-induced CSCs in NSCLC was investigated, and API was found to repress upregulation of CD133 in CDDP-treated and CDDP-resistant NSCLC, and enhance the antitumor effect of CDDP in NSCLC. Moreover, p53, a tumor suppressor, was induced after API addition, resulting in repression of the CDDP-induced increase in CSCs and acquired resistance in NSCLC. Thus, API exerts synergistic anti-tumor effects in CDDP-resistant NSCLC via CSC and p53.

Methods

Cell Culture and Treatment

The human primary NSCLC cell line A549 with p53 expression and metastatic NSCLC cell line H1299 lacking p53 expression were obtained from American Type Culture Collection (Manassas, VA, USA), and cultured in DMEM medium (Gibco) or RPMI1640 (Gibco) supplemented with 10% fetal calf serum, 2 mM glutamine (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml), and maintained at 37 °C with CO_2 in a humidified atmosphere.

CDDP-resistant A549 cells (A549R) was established by a stepwise protocol as previous reports (19). Briefly, CDDP (Sigma) was treated at IC_{50} for 72 h and then cells were allowed to recover for another 72 h. Therefore, A549 cell was continually exposure to escalating concentration of CDDP over the period of 6 mo. Finally, A549R cells were maintained in 10 μM CDDP.

Both A549 and A549R cells were treated with or without 10 μM CDDP for 24 h, and API (Sigma) were added 2 h before CDDP treatment at indicated concentration. Pifithrin-α was treated 2 h prior to API addition at 10 μM.

For H1299 cells, 20 μM CDDP was treated for 36 h, and API were added 2 h before CDDP treatment at indicated concentration.

MTT

All the cells were seeded into 96-well plates and incubated overnight for CDDP and API treatment as above. MTT analysis was used to identify the inhibition ratio as report (20). Briefly, cells were washed by PBS and inculcated in 0.5 mg/mL MTT solution at 37 °C for 2 h. DMSO was used to dissolve the residual cell layer, and the optical density was measured at 490 nm wavelength using a microplate reader (Thermo Scientific). Growth inhibition was calculated using the following equation:

Growth inhibition ratio (%) = (A492 control − A492 sample)/(A492 control − A492 blank) ×100

FACS

To measure the apoptosis, cells were labeled with 1 mL PI solution (50 mg/L) and Annexin V (FITC) (Beyotime) at dark place for 30 min, then cells were wash and suspended in PBS for analysis by FACScan flow cytometer (Becton Dickinson).

To measure the CSC cell amount, cells were staining by CD133/1 (AC133) phycoerythrin (PE)-labeled antibody or isotype control antibody (IgG1) (Miltenyi Biotec) in FACS buffer (0.5% BSA in PBS with EDTA) for 30 min on ice. Then cells were washed and suspended in FACS buffer for analysis by FACScan flow cytometer (Becton Dickinson).

Western Blot Analysis

All the cells were homogenized and lysed in lysis buffer (50 mM Hepes pH 7.4, 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/mL aprotinin and 10 μg/mL leupeptin) at 4 °C for 60 min. The supernatants were collected for Western blot analysis, and protein concentration was determined by the Folin assay. Equal amounts protein samples were run in SDS-PAGE and trans-bolting to PVDF membranes. Primary antibodies were purchased form SCBT, and horseradish perosidase (HRP)-conjugated secondary antibody were used, and the specific protein level was and visualized by using ECL as the HRP substrate. Image J was used to quantify the densitometry of each band.

siRNA Transfection

A549R cells were transfected with a mixture of 15 μg of double-stranded siRNA targeted to negative control, *P53* and 60 μL TranSmarter (Abmart) as manufacturer description. The transfected cells were maintained for 24 h, before treatment.

Statistical Analysis

Results are presented as the mean ± standard deviation (SD). Differences between groups were examined for statistical significance using Student's *t*-test.

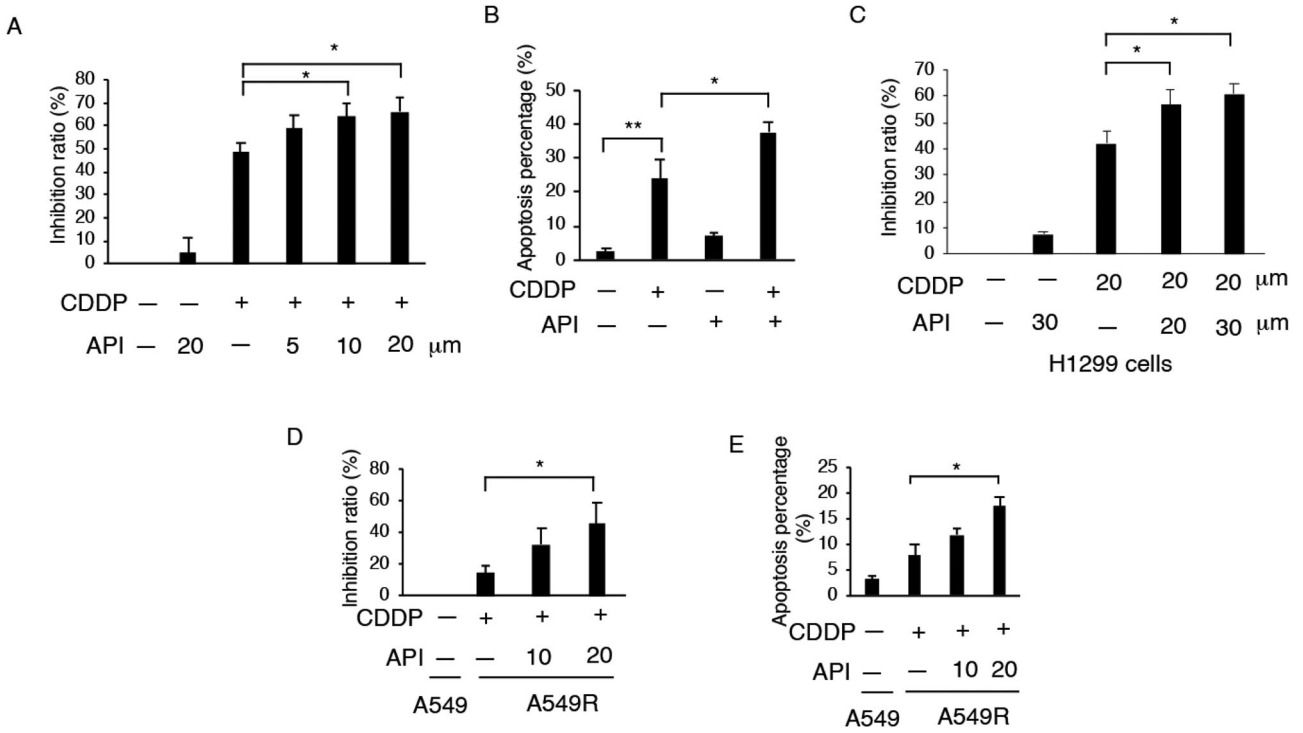

Figure 1. API eliminates acquired CDDP resistance in A549 cells. API enhanced the cytotoxicity effect of CDDP in A549 cells (A and B) and H1299 cells (C). (D and E) API eliminated acquired CDDP resistance in A549 cells. A549 (A and B) and A549R (D and E) cells were treated with or without 10 μM CDDP and or API at indicated concentrations for 24 h. (A and D) The inhibition ratio was measured by MTT assay. (B and D) Apoptotic cell percentage was quantified by FACS assay with Annexin V-PI staining. (C) H1299 cells were treated with or without 20 μM CDDP and or API at indicated concentrations for 36 h. Results are expressed as means ± SD; $n = 3$. $^{*}P < .05$; $^{**}P < .01$.

Results

API Eliminates Acquired CDDP Resistance in A549 Cells

To understand the synergistic anti-tumor effect of API in CDDP-treated NSCLC, the A549 cell line (a typical NSCLC models) were employed, and the inhibition ratio was quantified by MTT assay. The CDDP-induced inhibition ratio was further significantly increased from 58.8% to 64.07% and 65.9% after API addition at 10 and 20 μM, respectively, which alone did not induce A549 cell death (Figure 1A). The apoptotic cell population was quantified by FACS with Annexin V (AV) and propidium iodide (PI) staining. The apoptotic (AV positive) cell population was increased to 23.9% after CDDP addition, and API further increased the number of apoptotic cells to 37.4% (Figure 1B). This indicates that API could enhance the cytotoxic effect of CDDP in NSCLC.

Another p53-null NSCLC cell line, H1299 cell, was used to confirm the cytotoxic effect of API and CDDP. A relative higher dose (20 μM) of CDDP could induce 41.1% cells death after 36 h treatment, and 30 and 40 μM API addition could significantly enhance the cytotoxic effect of API to 56.7% and 60.7%, respectively (Figure 1C). This indicated that the synergistic anti-tumor effect of API and CDDP is partly dependent on p53, since higher stringency of API and CDDP are needed to induce the H1299 cells death.

Moreover, to clarify the role of API in acquired CDDP resistance in NSCLC, CDDP-resistant A549 cells (A549R) were established by a stepwise protocol as described in the "Materials and Methods" section. In A549R, the cytotoxic effect of CDDP was dramatically reduced; only 14.6% cell death was observed in CDDP-treated A549R cells, while 20 μM API addition significantly increased the inhibition ratio to 45.2% (Figure 1D). Increased apoptosis was also observed in API- and CDDP-treated A549R cells, compared with CDDP-treated A549 cells (Figure 1E). Thus, API could eliminate acquired CDDP resistance in NSCLS.

API Eliminates CDDP-Induced CSC in NSCLC

CSC cells are reported to contribute to acquired CDDP resistance in cancer cells. We therefore performed FACS analysis and CD133 staining to quantify the number of CSCs in NSCLC (8). Firstly, CDDP was found to increase $CD133^{+}$ A549 cells from 0.2% to 1.1%, and API significantly repressed this CDDP-

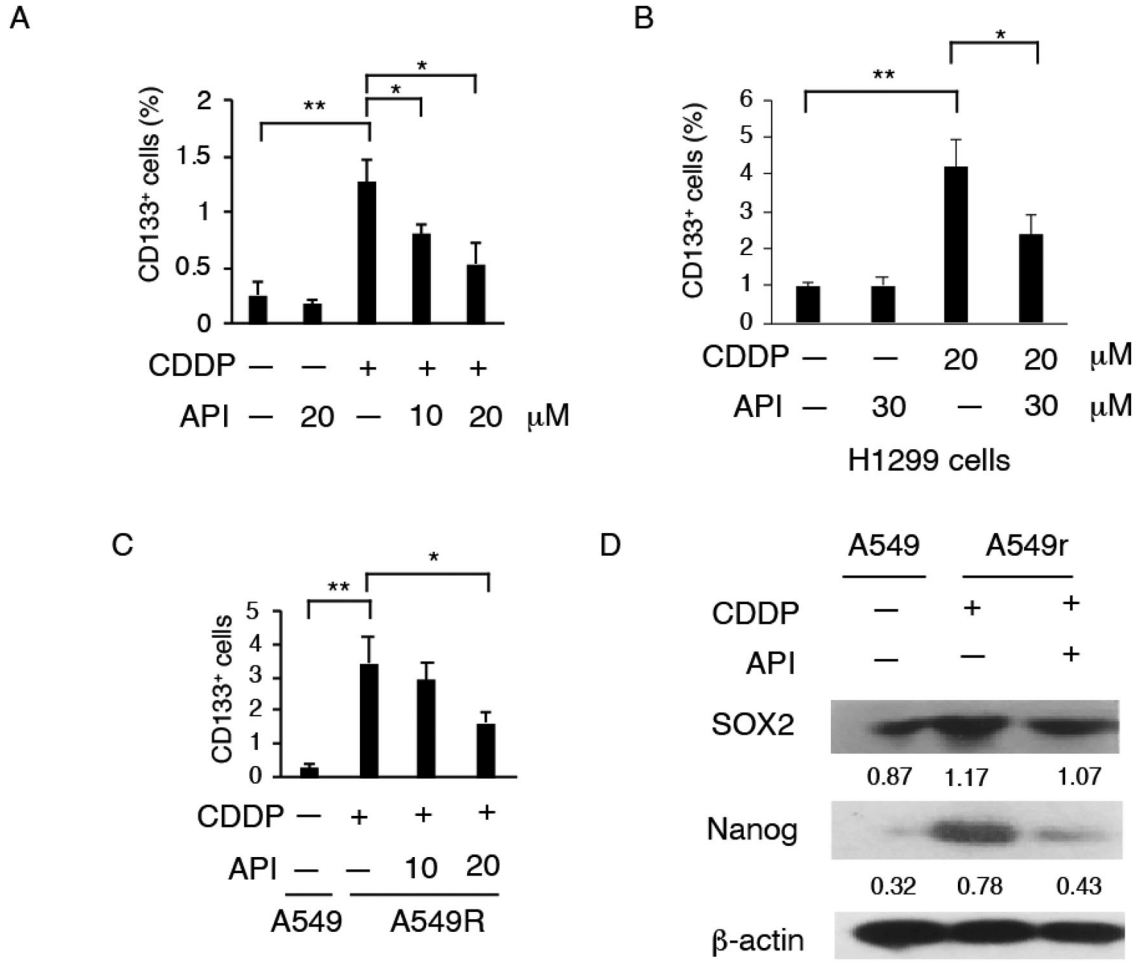

Figure 2. API eliminates CDDP-induced CSC in A549 cells. API reduced CDDP-induced increased CD133$^+$ cell population in A549 (A), H1299 (B), and A549R cells (C). Cells were treated by CDDP and API as above. Then the cells were staining by control IgG1 and CD133-PE antibodies, and analyzed by FACS. The percentages of CD133$^+$ cells are expressed as means ± SD; $n = 3$. $^*P < .05$; $^{**}P < .01$. (D) API reduced the expression of CSC markers in A549R cells. Western blot was used to analyze the expression of SOX2 and Nanog in A549 and A549R cells after CDDP and API addition. The relative densitometry ratios to β-actin are shown.

induced increase in CD133$^+$ A549 cells in a dose-dependent manner (Figure 2A). In p53-null H1299 cells, the CD133$^+$ H1299 cells population is 0.97%; 20 μM CDDP could significantly increase the CD133$^+$ H1299 cells to 4%; and a relative higher dose of API (30 μM) significantly repressed the CD133$^+$ H1299 cells population to 2.4% (Figure 2B). This indicating that CDDP is easier to induce CSCs generation in p53-deficent cells. Additionally, the proportion of CD133$^+$ cells was increased 10-fold in A549R cells compared with untreated A549 cells, and API addition significantly repressed the increase in CD133$^+$ A549 cells (Figure 2C).

Furthermore, Nanog and SOX2, CSC biomarkers, were analyzed by western blotting, and expressions of them were clearly increased in A549R cells compared with A549 cells, while API addition repressed this upregulation in A549R cells (Figure 2D). This suggests that API eliminates CDDP-induced CSCs in CDDP-resistant NSCLC.

API Exerts a Synergistic Antitumor Effect in CDDP-Resistant A549 Cells via p53

Since p53 is reported to regulate CSCs (21), we herein used the p53 inhibitor Pifithrin-α (PFT-α) to clarify whether the synergistic antitumor effect of API in CDDP-resistant A549 cells is dependent on p53. In A549R cells, the API-induced increase in cell death was significantly repressed by PFT-α addition (Figure 3A). Additionally, siRNA targeting p53 was used to silence p53 expression in A549R cells, and p53 protein abundance was decreased according to western blotting (Figure 3B). The apoptotic cell population was quantified by FACS in sip53-treated A549R cells, and the API-induced increase in apoptosis observed in

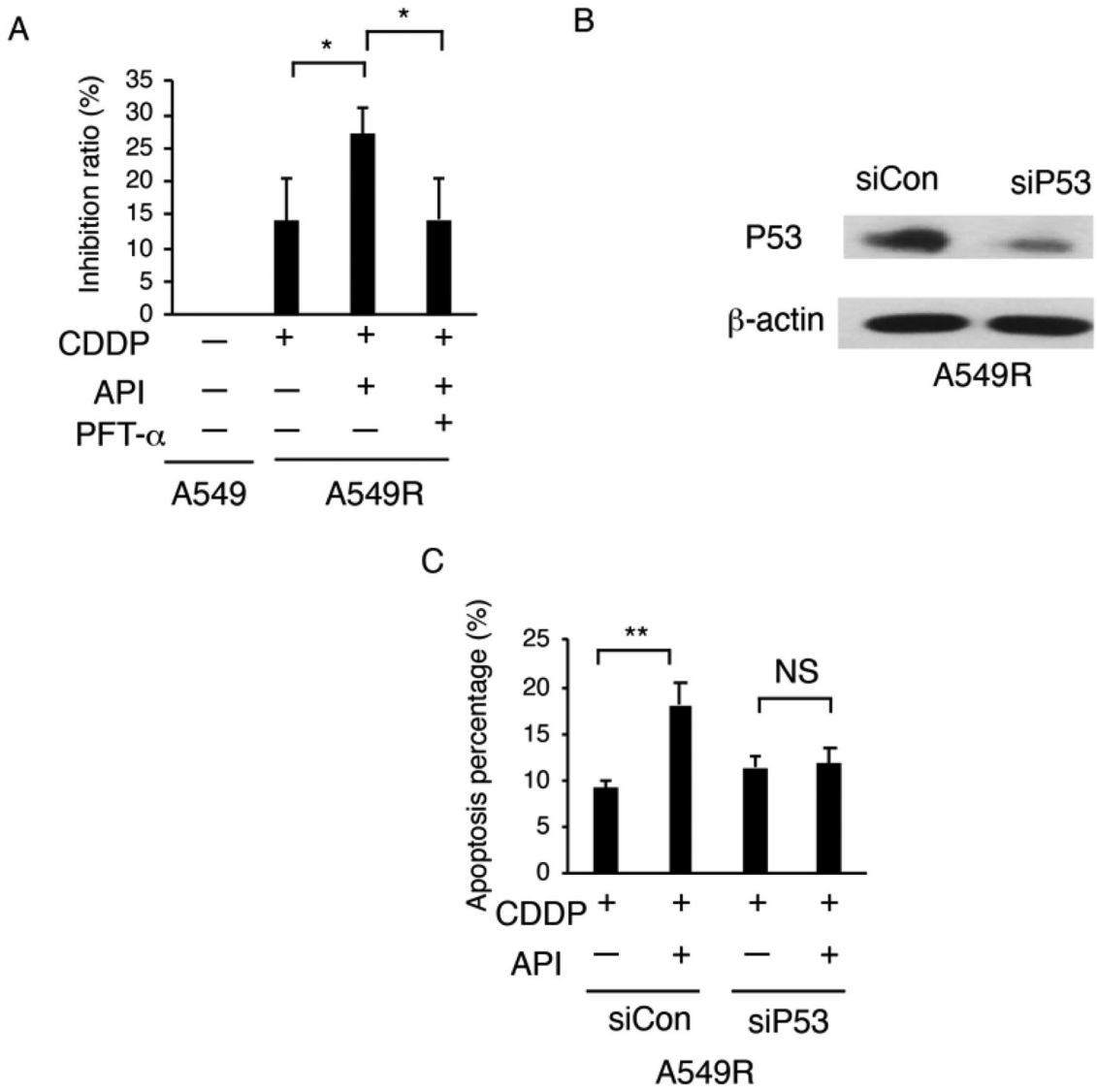

Figure 3. API exerts a synergistic antitumor effect in CDDP-resistant A549 cells via p53. (A) PFT-α repressed the synergistic anti-tumor effect in A549R cells. A549 and A549R cells were treated by PFT-α, CDDP, and API as above. The inhibition ratio was measured by MTT assay. Results are expressed as means ± SD; $n = 3$. $^{*}P < .05$. (B) Western blot analysis of P53 in A549R cells with siRNA against negative control gene (siCon) and P53 (siP53). (C) The synergistic anti-tumor effect is dependent on P53 in A549R cells. A549R cells were treated with siRNA against negative control gene (siCon) and P53 (siP53). Apoptotic cell percentage was quantified by FACS assay with Annexin V-PI staining after CDDP and API addition. Results are expressed as means ± SD; $n = 3$. $^{**}P < .01$; NS, not significant.

A549R cells did not occur in sip53-treated A549 cells (Figure 3C). Thus, API appears to eliminate acquired CDDP resistance in NSCLS via p53.

API Eliminates CDDP-Induced CSC via P53

Next, we investigated whether API eliminates CDDP-induced CSCs via p53 using FACS analysis with CD133 staining in A549 and A549R cells. In CDDP- and API-treated A549 and A549R cells, PFT-α could significantly derepress the decrease in the $CD133^{+}$ cell population (Figure 4A). Consistently, the API-induced decrease in $CD133^{+}$ cells was not observed in p53-silenced A549R cells (Figure 4B). Additionally, p53 expression was found to be induced in CDDP-treated A549R cells after API addition (Figure 4C). Meanwhile, expression of CSC markers SOX2 and Nanog was not altered after API addition in p53-silenced A549R cells (Figure 4C) Thus, API represses CDDP-induced CSCs in a p53-dependent manner.

Discussion

As a tumor suppressor, p53 inhibits cellular proliferation by inducing cell cycle arrest and apoptosis in response to cellular stresses (22). In this work, API

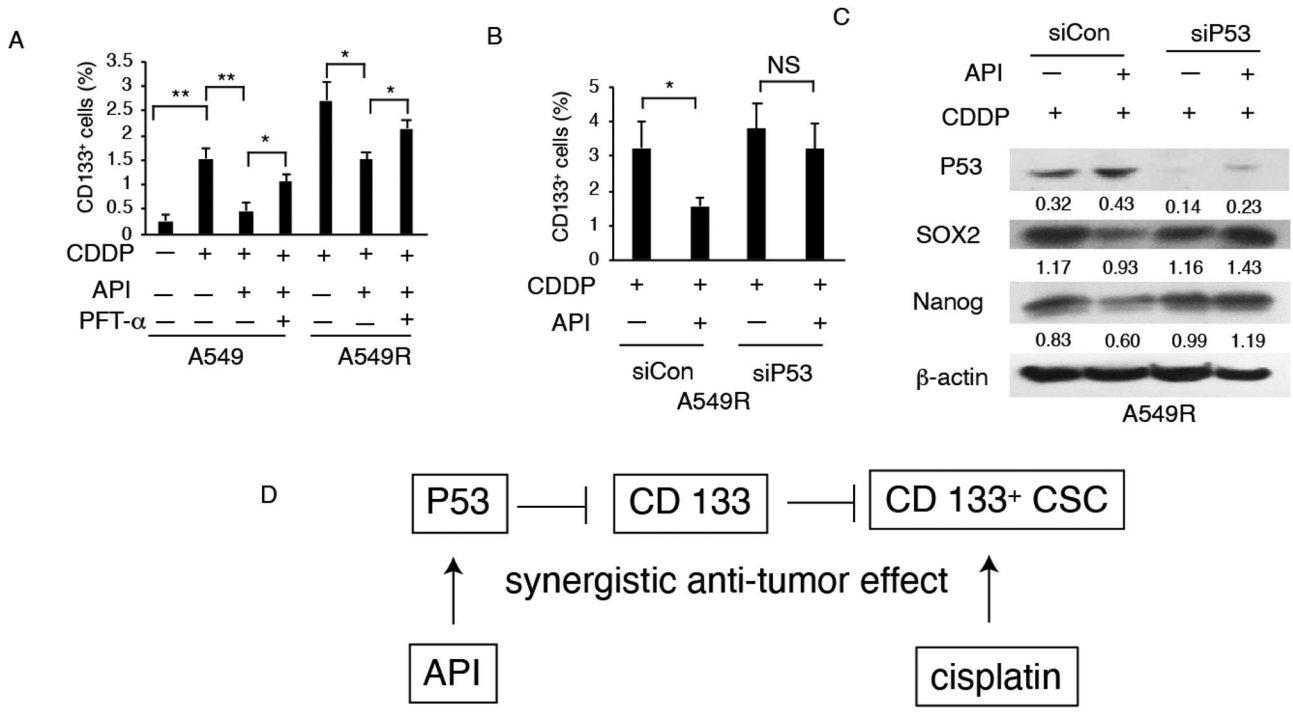

Figure 4. API eliminates CDDP-induced CSC via P53. (A) A549 and A549R cells were treated by CDDP, API, and PFT-α as above. (B) A549R cells with siRNA against negative control gene (siCon) and P53 (siP53) were treated with CDDP and API as above. Then the cells were staining by control IgG1 and CD133-PE antibodies, and analyzed by FACS. The percentages of $CD133^+$ cells are expressed as means ± SD; $n = 3$. $^*P < .05$; $^{**}P < .01$. (C) Western blot was used to analyze the expression of SOX2, Nanog and P53 in A549R cells with siRNA against negative control gene (siCon) and P53 (siP53) after CDDP and API addition. The relative densitometry ratios to β-actin are shown. (D) Proposed pharmacological mechanism of API in CDDP-treated NSCLC.

could enhance CDDP-induced apoptosis in p53-expressed A549 cells, and a relative stronger condition of API and CDDP is need in p53-null H1299 cells to induce similar scale of cell death as A549 cells, which indicating that the synergistic anti-tumor function of API and CDDP in NSCLC is dependent on p53. In CDDP-resistant NSCLC, p53 siRNA could block API-induced increased apoptosis and increased p53 expression was observed after API addition. This indicated that API eliminates acquired CDDP resistance in NSCLC via up-regulation p53.

A previous In Vivo study showed that oral administration of API reduced the volume of tumors and induced the complete abolishment of distant organ metastasis in transgenic adenocarcinoma in a mouse prostate model (23). API also reduces malignant mesothelioma growth In Vivo, and upregulates p53 expression (24). Consistently, API treatment is reported to increase CDDP-induced DNA damage and apoptosis in HeLa, A549 MCF-7 and HCT 116 tumor cells in a p53-dependent manner, and caspase activation was also observed in CDDP- and API-treated tumor cells (18). An exogenous increase in intracellular p53 protein abundance was found to augment the effect of CDDP in NSCLC (25), and inhibition of protein lysine methyltransferase SMYD2 can sensitize CDDP to resistant cells in NSCLC by activating the p53 pathway (26). Thus, API might be a good candidate chemical for NSCLC therapy, especially when combination with CDDP.

p53 expression is activated by many cellular stresses, including oxidative stress and DNA-damaging agents via the JNK pathway (27). API-induced cellular stresses, including oxidative stress, can induce JNK phosphorylation and activate the JNK pathway, which further regulates cellular apoptosis, and upregulates pro-apoptotic genes and downregulates anti-apoptotic genes through the transactivation of several transcription factors, including c-JUN and p53 in NSCLC (28, 29). Thus, API might induce p53 expression in NSCLC via the JNK pathway.

On the other hand, a relative higher dose of API could enhance CDDP-induced p53-null H1299 cells death, and API also repressed the CDDP-induced CSCs population in H1299 cells which suggested that the synergistic anti-tumor function of API and CDDP in higher doses is independent on p53.Besides apoptosis, autophagy, type 2 cell death (30), could be induced by API in various cancer cells (31). The role of autophagy in cell survival or death is dependent on the stimulations and the dose of drug. Moreover, autophagy is reported to inhibit the CSCs population and enhance

the NSCLC cell death (32). Therefore, higher dose of API may induce autophagic cell death and repress autophagy-dependent CSCs generation in NSCLC.

P53 inhibitor and p53 siRNA derepressed the API-induced decreased of CSCs in CDDP-treated A549 cells. And higher level of CSCs is found in H1299, compare with A549 cells after CDDP and API addition. This suggested that API-induced p53 expression may inhibit the CDDP-induced CSCs generation in NSCLC. CSCs are a small population of cells within a tumor (0.001–0.1%) capable of self-renewal that can undergo differentiation to generate the phenotypic heterogeneity observed in tumors (33). CSCs are also known to have a high migratory capacity, enabling their spread from the primary tumor to secondary sites (34). Common chemotherapeutic agents targeting the proliferating cells promote CSC accumulation which further induce cancer relapse because CSC could establish higher invasiveness and chemoresistance (35). p53 is reported to represses the expression of more than 20 target genes that may contribute to maintenance of the pool of tumor-initiating cells, including Nanog, thereby allowing the CSC pool to expand (36), and CD44 is a commonly used marker of CSCs (37). Furthermore, $CD133^{+}$ CSCs were found to be repressed by activating p53 after API addition in NSCLC. Interestingly, binding of p53 to the CD133 promoter subsequently suppresses CD133 expression by reducing histone H3 acetylation, and CD133 depletion suppresses tumor cell proliferation, as well as expression of core stemness transcription factors including Nanog and SOX2 (21). Thus, we believe that API-induced p53-dependent CSC reduction may play an important role in eliminating acquired CDDP resistance in NSCLC.

Nanog and SOX2 are key pluripotent transcription factors which contribute to the reprograming of somatic cells into an embryonic stem cells-like state (38). In CSCs, up-regulation of Nanog and SOX2 modulates signaling pathways to inhibit apoptosis (39), and induces tumor-initiating and drug-resistant properties (40). Overexpression of Nanog and SOX2 significantly enhanced the proliferation, drug resistance, migration, and invasion abilities of gastric cancer cells (41). This suggested that API-induced decreased CSCs and key pluripotent transcription factors Nanog and SOX2 are one of key anti-tumor mechanisms in NSCLC.

Conclusion

API was found to eliminate acquired CDDP resistance and decrease the proportion of CSCs in a p53-dependent manner in NSCLC. An API-induced decrease in the CSC population could contribute to enhance the antitumor effect of CDDP via p53 (Figure 4D). Thus, API might be a good candidate for NSCLC therapy, especially when combined with CDDP.

Author Contribution

Study design: Li Y., Chen X.; Data collection: Bai J.; Statistical analysis: Yang B., Chen L.; Data interpretation: He W., Jiang X. Manuscript preparation: Xia S., Li X., Funds collection: Li Y.

Disclosure Statement

No potential conflict of interest was reported by the author(s).

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