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ORIGINAL RESEARCH

Differential Expression And Effects Of Peroxiredoxin-6 On Drug Resistance And Cancer Stem Cell-Like Properties In Non-Small Cell Lung Cancer

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Objective: Cancer stem-like cells (CSC) are thought to be involved in the cisplatin resistance of tumors. This study was designed to investigate the effect of PRDX6 on CSCs present in cisplatin-resistant non-small cell lung cancer (NSCLC) tumors.

Materials and methods: CD133+/ABCG2+ H1299 CSCs and A549 CSCs were isolated. The IC_{50} values for cisplatin in treatment of CSCs were detected using the CCK8 assay. Then the isolated cells were identified using CD133. Wnt/β-catenin expression was evaluated by Western blot assays. Specimens of tumor and adjacent para-carcinoma tissue were collected from 30 NSCLC patients and examined by immunohistochemistry (IHC), qRT-PCR, and Western blotting to determine and compare their levels of PRDX6 and CD133 expression. Finally, siRNA-mediated silencing of PRDX6 was employed with both types of CSCs to determine the impact of PRDX6 on CD133 enrichment by flow cytometry, cell viability, and sphere formation ability.

Results: High levels of PRDX6 and CD133 expression were detected in samples of tumor tissue from NSCLC patients, and expression of PRDX6 and CD13 presented a positive relationship. Increasing levels of cisplatin resistance and upregulated levels of PRDX6, ABCG2, Wnt, and β-catenin expression were detected in CD133+/ABCG2+ H1299 and A549 CSCs. Transfection with siRNA targeting PRDX6 changed these cellular characteristics by decreasing the levels of PRDX6, ABCG2, Wnt, and β-catenin expression. We further demonstrated that exogenous silencing of PRDX6 effectively inhibited the sphere formation ability of CSCs and re-sensitized them to cisplatin.

Conclusion: Our results strongly suggest that PRDX6 promotes cisplatin resistance in human lung cancer cells by promoting the stem-like properties of cancer cells. Our findings also suggest PRDX6 as a target for treating cisplatin resistant NSCLC.

Keywords: PRDX6, CSCs, cisplatin-resistance, NSCLC, cancer stem-like cell

Introduction

Non-small cell lung cancer (NSCLC) accounts for ~80% of all lung cancers and has a dismal 5-year patient survival rate of 15%. Furthermore, ~66% of NSCLC cancer patients initially present with stage IV disease.[1,2] In recent years, the 5-year survival rate of NSCLC patients has not substantially increased, and remains as low as 20%, especially among patients with stage III/IV disease.[3] Although new therapies have benefitted patients with predefined subclasses of carcinoma, cisplatin-based chemotherapy remains

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the standard treatment for NSCLC. However, cisplatin resistance to targeted therapy, which can result from multiple factors, is a major issue affecting the efficacy of NSCLC treatments.[4,5] Many previous studies revealed that a combination of factors, including accelerated drug clearance, activation of alternative proliferation signaling pathways, and suppression of apoptotic pathways, may be involved in cisplatin resistance. Recent studies have indicated that some unique populations of cells are capable of surviving tumor treatments, and those cells are designated as cancer stem cells (CSCs), due to their stem cell-like characteristics, self-renewal ability, and multi-potency.[6–8]

As a special population of undifferentiated cells that contribute to the pathogenesis and progression of tumors, CSCs have been found in a variety of cancers, including myeloid leukemia, glioblastoma, gastric, and epithelial cancers.[9,10] Due to their stem cell properties, CSCs have the capacity for multipotency, unlimited self-renewal, and proliferation with a natural tolerance to chemotherapy that result from their decreased cell cycling and enhanced expression of proteins associated with DNA repair and resistance to apoptosis.[11] Various alleged stem cell markers, selective for human stem cells and their counterparts in tumors, have been used to identify and isolate CSCs; these markers include CD133 (prominin-1), a five-transmembrane glycoprotein,[12] and ATP-binding cassette superfamily G member 2 (ABCG-2).[13] ABCG2 is always co-expressed with CD133, and is accepted as a drug resistance marker due to its ability to confer the side population's phenotype.[14,15] Accordingly, the identification of some oncogenic factors that result in a persistent activation of CSCs is essential for further elucidating NSCLC pathobiology, as well as for developing novel effective therapies.

Peroxiredoxins (PRDXs) comprise a newly discovered class of non-selenium-dependent peroxidase proteins that are widely distributed in various organisms.[16] PRDX a type of antioxidant enzyme, is thought to catalyze redox reactions and maintain the balance of hydrogen peroxide in cells.[17] Presently, PRDX1-PRDX6 have been found to contain a 1-Cys PRDX group and 2-Cys PRDX group. PRDX1-5 belongs to the 2-Cys group, and PRDX6 belongs to the 1-Cys PRDX group.[18,19] Besides helping to protect cells against oxidative stress (OS), PRDX6 uniquely possesses calcium independent phospholipase A2 (PLA2) activity and glutathione peroxidase activity that can help to prevent oxidative stress.[20] Moreover, numerous studies have proven that PRDX6 plays essential roles in tumor maintenance and cell survival by protecting cells from OS-induced apoptosis.[21,22] Recent studies have also confirmed that PRDX6 can attenuate cisplatin-induced apoptosis.[23] In contrast, silencing of PRDX6 expression was shown to result in peroxide-induced cell death.[24] Meanwhile, PRDX6 was also shown to promote the invasion and metastasis of lung cancer cells by activating the Akt pathway.[25] However, the role played by PRDX6 in NSCLC, and its mechanism of action, remain unclear.

In this study, we examined the levels of PRDX6 and CD133 expression in NSCLC cells and tissues, the correlation between cisplatin resistance and PRDX6 expression, and further demonstrated the effect of PRDX6 on CSC maintenance in NSCLC. Our results suggest that downregulation of PRDX6 expression might be a potential biomarker and represent a strategy for treating NSCLC patients.

Materials And Methods

Patient Characteristics, Clinical Features, And Tissue Harvest

Between December 2016 and December 2017, this study enrolled a total of 60 pathologically diagnosed NSCLC patients, including 30 patients with resistance to cisplatin. No patient had a radiological record of disease development. The protocol for this study was approved by the Ethics Committee of Nanchong Central Hospital, and a signed Informed Consent form was obtained from each participant prior to study enrollment. Tumor tissues and adjacent tissues were harvested and immediately stored at −80°C or fixed in 4% paraformaldehyde for use in further experiments. The study was performed in accordance with the ethical principles of the Declaration of Helsinki.

Immunohistochemistry Analysis (IHC)

IHC methods were used to detect PRDX6 in tumor and para-carcinoma tissues. The tissues were fixed overnight in 4% paraformaldehyde, and then embedded in paraffin and sliced into 5 μm sections with a microtome (Cat. #HM325, Thermo, USA). After deparaffinization and hydration, the sections were incubated overnight at 4°C with a primary antibody against PRDX6 (Abcam, Cambridge, UK). The immunostained tissue sections were then photographed 3 times at ×400 magnification.

Cell Culture And Treatment

A549 and H1299 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A549 cells were cultured for ~24 h in high glucose-DMEM medium (Hyclone, Logan, Utah, USA); H1299

cells were cultured for 24 h in Roswell Park Memorial Institute (RMPI-1640) medium (Hyclone). All media contained 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (P1400, Solarbio, Beijing, China). Both A549 and H1299 cells were grown in a humidified atmosphere with 5% CO_2 at 37°C.

Following dilution into single cell suspensions and being seeded into the wells of different plates (1 x 10^5 cells/mL), the cells were transfected with or without PRDX6-siRNA (1 nM, Genepharma, Shanghai) for 72 h, with the use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Subsequently, the cellular proteins were harvested at specified times. Cells transfected with PRDX6-siRNA-NC served as control cells.

Immunomagnetic Separation (IMS)

Cells were rinsed in PBS and resuspended at a density of 1 × 10^7 cells/mL. Next, 300 mL of cell suspension was incubated with FcR blocking reagent (100 mL) and CD133-PE antibody (100 mL) for 30 min at 4°C in the dark. An immunoglobulin G-PE antibody served as a negative control. The cell suspension (500 mL) was rinsed twice with PBS and used to determine the percentage of CD133+ cells by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). The CD133-positive (CD133+) and CD133-negative (CD133−) cells were re-suspended in serum-free RMPI media. Flow cytometry was used to determine the percentages of CD133+ cell subpopulations prior to and following the separation process.

Immunofluorescence Staining

After separation, cell spheres were plated onto coverslips in RPMI 1640 medium containing 10% FBS and let sit for ~ 24 h before any further use. After 48 h of transfection, the SW620 cells were fixed with 4% PFA for 20 min, and then incubated with 0.3% Triton X-100 for 10 min at room temperature. Next, the treated cells were blocked with 5% goat serum for 30 min at 37°C, and then incubated overnight with the anti-F-actin IgG (1:2000, Biosensis, Australia) at 4°C; after which, they were incubated with goat anti-IgG conjugated to Cy3 (dilution, 1:400; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at 37°C. Finally, DAPI (1:1000; Sigma-Aldrich, Inc., MO, USA) was used to counterstain the nuclei, and the cells were observed and photographed under an inverted fluorescence microscope (Olympus). Cells in the negative control group were incubated with PBS rather than the primary antibody.

Cell Proliferation Assay

Cells were plated into individual wells and treated with different concentrations of cis-platinum for 24 h. Next, 100 uL of CCK8 solution (Dojindo, Japan) was pipetted into each well and incubated at 37°C for an additional 1 h. The absorbance of each well was measured at 450 nm with a microplate reader.

Real-Time Reverse Transcription-PCR (qRT-PCR)

Total cellular RNA was isolated with an RNA Isolation Kit and UNIQ-10 column (Sangon, Shanghai, China). Next, 1 μg of total RNA was reverse transcribed in a 20 μL volume of reaction solution with the use of a GoScriptTM Reverse Transcription Kit (Promega, Madison, WI, USA).

A 2 μL aliquot of cDNA was subjected to qRT-PCR performed with a TaKaRa Ex Taq RT-PCR Version 2.1 kit (TaKaRa, Shiga, Japan). The relative levels of gene expression were normalized to those for GAPDH and analyzed using the $2^{-\Delta\Delta Ct}$ method. The PCR primers used for PRDX6, CD133, ABCG-2, and GAPDH were as follows: PRDX6: F: 5′-AAGCTAGCGCCCTAGCGCGATCGCGAGG-3′, R: 5′-AAACGCGATCGATATCGACCCCATCGAC-3′; CD133: F: 5′-GGCGCCTATAGCTAGCTAGCGCGAT-3′, R: 5′-CGCGATCGATGATATCGCGCATA-3′; GAPDH: F: 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, R: 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′.

Western Blot Assay

A total protein isolation kit (Solarbio, Beijing, China) was used to extract the total proteins from tissues and cells. The protein concentration of each sample was estimated with a BCA protein assay kit (P0012, Beyotime, Shanghai, China). After separation by 10% SDS-PAGE electrophoresis, the target proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA, USA), which were subsequently blocked with 5% non-fat milk for 2 h. The membranes were then incubated with primary antibodies overnight at 4°C, and then treated with a secondary antibody (peroxidase-conjugated) for 2 h. Finally, the immunostained proteins were visualized with X-ray film (Fujifilm). The primary antibodies included anti-PRDX6 (1:10000; Santa Cruz Biotechnology, Dallas, TX, USA), anti-CD133 (1:10,000; Abcam, Cambridge, UK), anti-ABCG-2 (1:4000; Abcam), anti-Wnt (1:1500; Abcam), anti-β-catenin (1:2000; Abcam), and anti-GAPDH (1:10,000; Santa Cruz Biotechnology).

Sphere Formation Assay

After 7 days of culture, spheres were seeded into 6-well plates and cultured in a serum-free medium supplemented with EGF (20 ng/mL, Sigma-Aldrich), 2% B27 (Invitrogen), and basic fibroblast growth factor (20 ng/mL, bFGF; BD).

Data Analysis

All statistical analyses were performed using SPSS Statistics for Windows, Version 17.0 (SPSS, Inc., Chicago, IL, USA), and results are expressed as the mean ± SD. Differences between groups were analyzed by using one-way ANOVA or student's *t*-test. A P-value < 0.05 was considered statistically significant.

Results

CD133+ And ABCG2+ Subpopulations Presented Resistance To DDP

Prior to immunomagnetic selection by flow cytometry, our results showed that CD133+/ABCG2+ cells comprised 0.22% ± 0.03% of the H1299 cells and 0.10% ± 0.04%) of the A549 cells. After cell subpopulation enrichment, 74.89% ± 5.35% of the cells were CD133+/ABCG2+ in H1299-CSC, while 0.22 ± 0.03% were CD133 of cells in H1299. Similarly, 81.36 ± 3.02% of cells with CD133 +/ABCG2+ in A549-SCS, while 0.10 ± 0.04% in A549 cell line (Figure 1A and B).

As shown in Figure 1C and D, for both the H1299 cell line and A549 cell line, the cisplatin IC_{50} values for the enriched H1299 and A549 stem-like cells were increased when compared to values for the non-enriched human lung cancer cells, suggesting that the enriched stem-like cells had a higher potential for cisplatin resistance (Figure 1E).

PRDX6 Expression Was Positively Related To CD133 In NSCLC

Upregulation Of PRDX6 And CD133 In NSCLC Patients

Immunofluorescence studies were performed to demonstrate the expression of PRDX6 and CD133 in A549 (Figure 2A) and H1299 (Figure 2B) cells, and both cell types showed more intense staining after enrichment. We also found that PRDX6 and ABCG2 expression, as well as the expression of several important signaling pathway proteins such as Wnt and β-catenin, were induced after immunomagnetic selection and the enrichment of human lung cancer cells (Figure 2C).

To investigate the PRDX6 expression in patients' tissues, immunohistochemical assay was performed. Results of immunohistochemical assays showed that PRDX6 was expressed at higher levels in the tumor tissues than in the adjacent tissues (Figure 2D). Next, qRT-PCR analyses were performed to validate findings from the IHC assays. The qRT-PCR analyses suggested that both PRDX6 and CD133 mRNA levels were up-regulated in the tumor tissues when compared with those in the adjacent tissues (Figure 2E, left and middle panel). Interesting, we found that CD133, a stem cell marker, was positively related to PRDX6 (Figure 2E, right panel). Furthermore, similar results were obtained by Western blot analyses (Figure 2F).

PRDX6 Silencing Inhibited The Stem-Like Properties Of Lung Cancer Cells

Knockdown of PRDX6 with siRNA was used to examine the role of PRDX6 in cisplatin resistance. After measured by flow cytometry, results showed CD133+/ABCG2+cells accounted for 40.46% ± 5.04% of total H1299 cells in NC and 16.07% ± 2.41% of total A549 cells after PRDX6 knockdown. Besides, that was 64.98 ± 4.88% and 36.47 ± 4.59%, respectively (Figure 3A and B).

Meanwhile, sphere formation assays were conducted to examine the role of PRDX6 in promoting cellular self-renewal, which is a critical trait of CSCs. The sphere formation efficiency of A549 CSCs was suppressed by siRNA, as indicated by a decrease in both spheroid diameters (Figure 3C). Forced depletion of PRDX6 dramatically also reduced the numbers of H1299 CSCs (Figure 3C).

Western blot assays also showed the effect of PRDX6 silencing. As shown in Figure 3D, the levels of PRDX6, ABCG2, and some important signaling pathway proteins such as Wnt and β-catenin were decreased in human lung CSCs transfected with PRDX6 siRNA.

Knockdown Of PRDX6 Suppressed Cisplatin Resistance In H1299 And A549 Cancer Stem-Like Cells

As shown in Figure 4, the cisplatin IC_{50} values for H1229 and A549 stem-like cells were significantly reduced when the cells were transfected with stable PRDX6 siRNA, suggesting that cisplatin resistance was inhibited by knockdown of PRDX6.

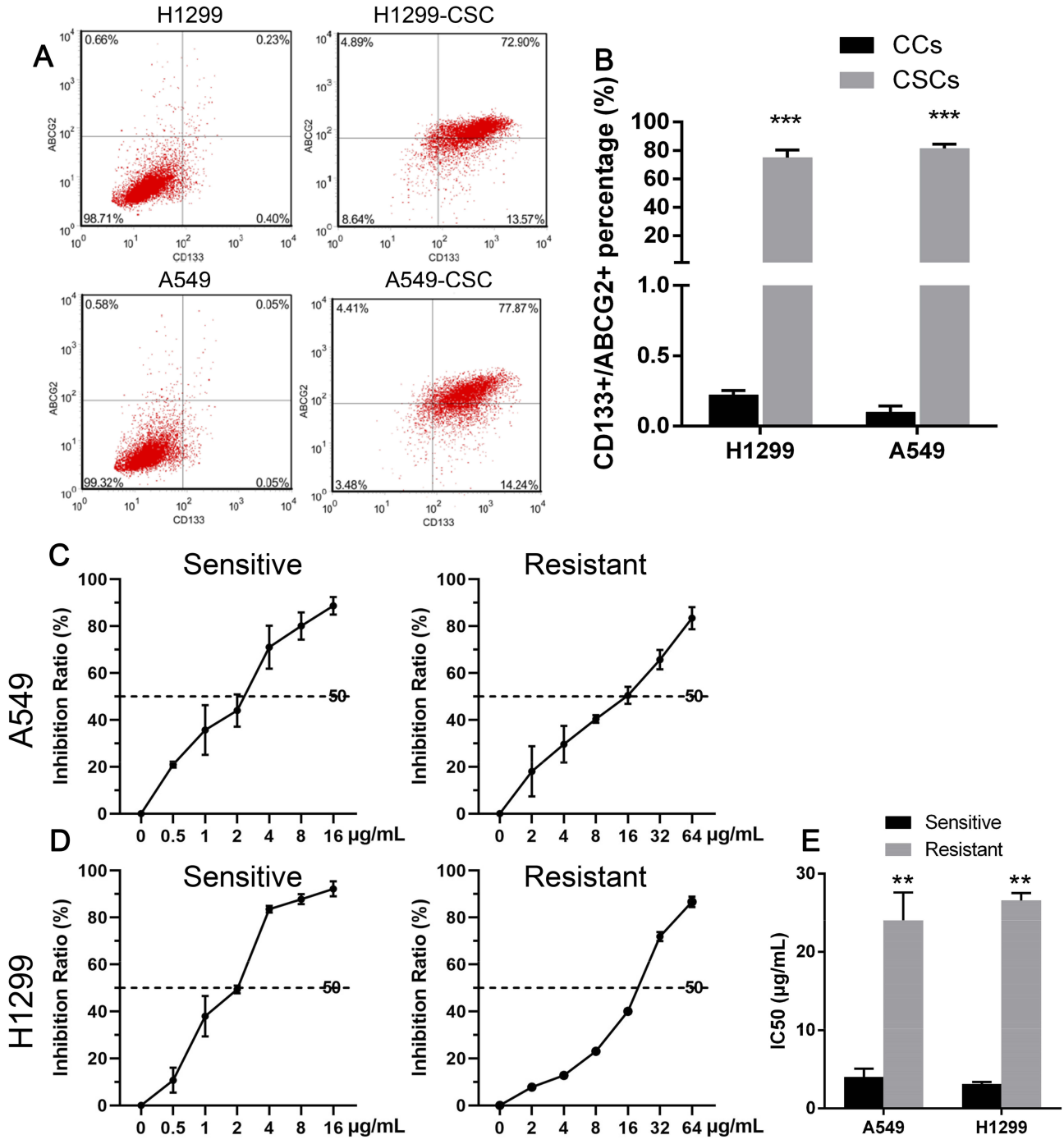

Figure 1 CD133+/ABCG2+ subpopulations presented resistance to DDP. (**A–B**) Representative flow cytometry graphs and the percentages of CD133+/ABCG2+ subpopulations among H1299 cells and A549 cells before and after immunomagnetic selection. (**C–E**) inhibitive rate of A549 (**C**) and H1299 (**D**) to cisplatin. ***P <0.001.

Discussion

Although significant advances have been made in using surgery, external radiation, and interventional radiology in the treatment of patients with NSCLC, the survival rate of those patients, and especially patients with stage III/IV disease, has plateaued in recent years. One reason for that plateau is the resistance of tumors to chemotherapeutic drugs.[26] Therefore, it is important to increase the sensitivity of cancer cells to chemotherapy. Our results suggest that NSCLC patients with cisplatin-resistance might have increased levels of PRDX6, as well as more cancer stem-like cells with positive markers, which is consistent with previous research. We also found

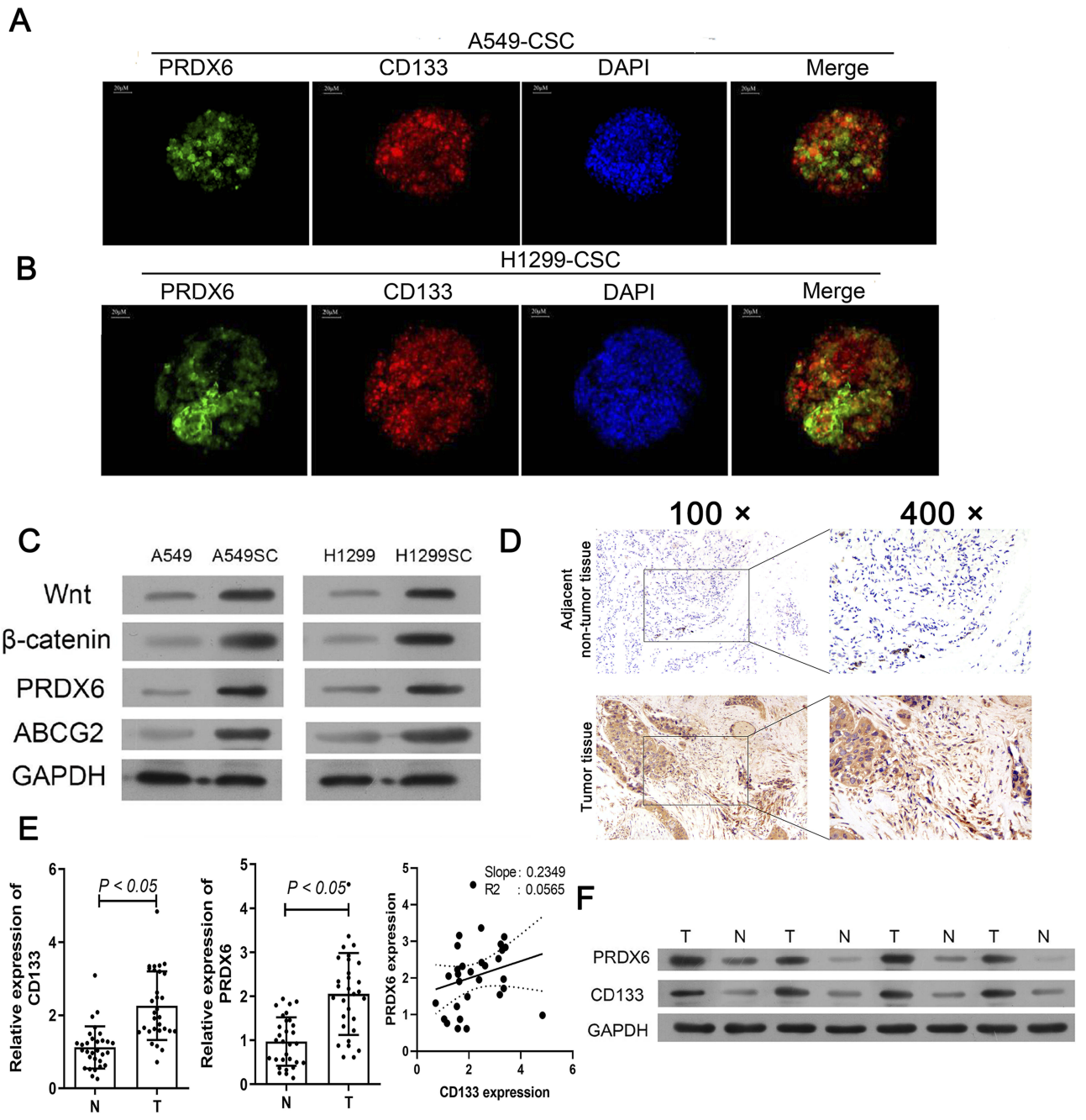

Figure 2 PRDX6 expression was positively related to CD133 in cells and clinical tissues. (**A–B**) Representative images showing immunofluorescent staining of A549 CSCs (**A**) and H1299 CSCs (**B**) for the presence of PRDX6 (green) and CD133 (red), with DAPI (blue) counterstaining; bar: 15 μm. (**C**) Levels of PRDX6, ABCG2, Wnt, and β-catenin proteins in human lung CSCs as detected by Western blotting, when compared to those in human lung cancer cells. ***Indicates $P < 0.001$ vs lung cancer cells. (**D**) PRDX6 levels in NSCLC patients were detected by IHC. (**E**) left panel: CD133 expression in clinical tissues. Middle panel: PRDX6 expression in clinical tissues. Right panel: positively relationship between CD133 and PRDX6 in clinical tissues. (**F**) expression of PRDX6 and CD133 in clinical tumor tissues (T) and para-carcinoma tissues (N).

that the CSCs might increase their tolerance to chemotherapy by activating the Wnt signaling pathway and possess an enhanced sphere formation ability that can be reversed by PRDX6 silencing. These findings imply that silencing of PRDX6 can be used as a new therapeutic strategy for the clinical management of cisplatin-resistant NSCLC. They also suggest PRDX6 as a specific pharmacological target and reducing PRDX6 levels as a potential method of therapeutic intervention.

Ever since the discovery of a subpopulation of CSCs in solid tumors with enhanced tumorigenicity and chemoresistance, CSCs have been considered as a cause of treatment

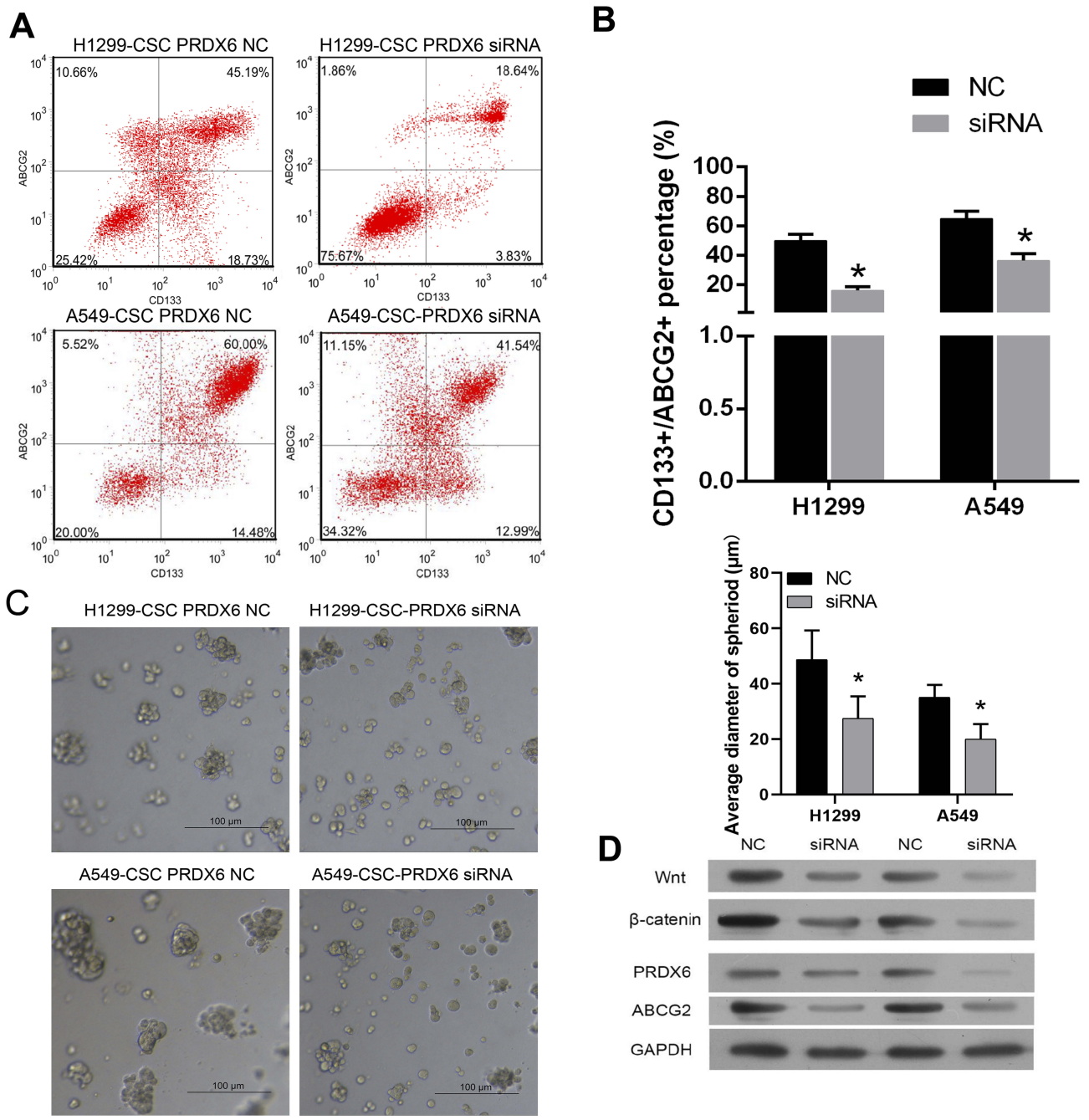

Figure 3 Effect of PRDX6 knockdown on the stem-like properties of lung cancer stem-like cells. (**A–B**) Representative graphs showing the results of immunomagnetic selection by flow cytometry and the percentages of CD133+/ABCG2+ subpopulations among H1299 cells and A549 cells before and after RNAi. (**C**) PRDX6 depletion reduced the stem-like properties of lung cancer cells in vitro. Representative images and quantification of the spheres formed by the indicated H1299 cells and A549 cells. (**D**) The levels of PRDX6, ABCG2, Wnt, and β-catenin proteins after siRNA transfection for 48 h, when compared with those in the negative control group. *Indicates $P < 0.05$ vs data from CSCs treated with the siRNA-NC and assayed at same time point.

failure and tumor recurrence after chemotherapy. Ovarian CSCs can survive cisplatin treatment due to enhanced translesion DNA synthesis (TLS) resulting from Pol η-mediated enhancement of miR-93 expression.[27] In breast cancer tumors, a distinct CSC population that potentially causes chemoresistance has been identified and implicated in the clonal evolution and expansion of cancer stem-like cells.[28,29] Although CSCs have been studied in other diseases, further research needs to be conducted on the biology of lung CSCs. There is also a need to identify new therapeutic targets for specifically eradicating that cell population. Recently, scientists generated and characterized a panel of cisplatin-resistant NSCLC cell lines

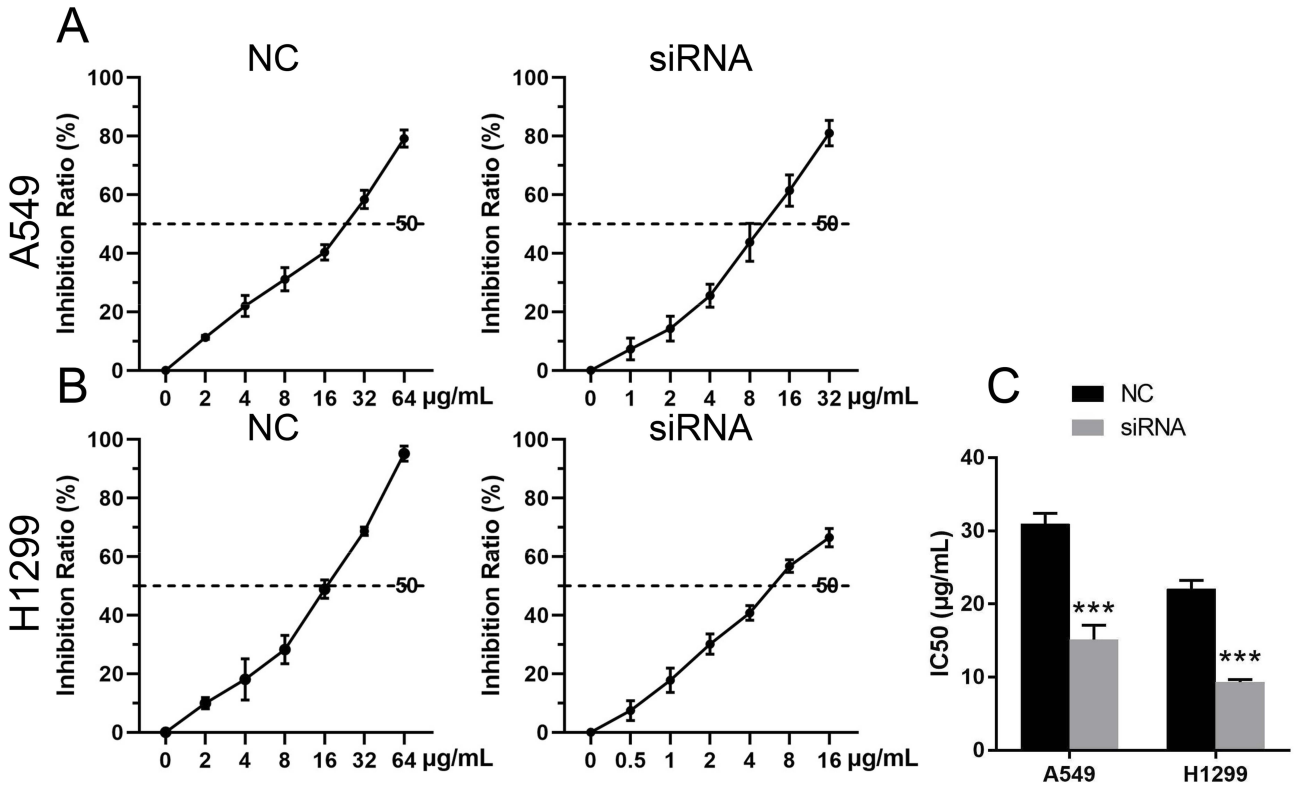

Figure 4 Effect of PRDX6 silencing on the viability of lung cancer stem-like cells. Transfected A549 cells (**A**) and H1299 cells (**B**) were treated with cisplatin at different concentrations, and the IC_{50} of cisplatin was measured and analyzed (**C**). *** indicates $p < 0.001$ vs the negative control.

with a putative stem-like signature for use as a valuable research tool.[30] Our study identified the protective effect of silencing PRDX6 in NSCLC CSCs in a cisplatin-resistance scenario in vitro, based on previous observations.

Several studies have found a link between the activation of PRDX family members and chemotherapy resistance. Overexpression of PRDX6 has been shown to promote lung tumor development by activating the JAK2/STAT3 pathway[31] and increasing glutathione peroxidase[31] and iPLA2 activity via upregulation of the AP-1 and JNK pathways.[31,32] Moreover, it was found that the development of drug resistance was accompanied by a significant increase in PRDX6 expression in various cisplatin-resistant sublines, including human erythroleukemia K652 cells,[33] human breast carcinoma MCF-7 cells,[34] and human ovarian carcinoma SKOV-3 cells,[23] further confirming the important contribution of redox-dependent mechanisms to the development of cisplatin resistance. Consistent with those findings, we have provided evidence that genetic silencing of PRDX6 or its enzymatic activity renders CSCs sensitive to chemical anti-cancer treatments and abrogates tumor cell sphere formation, laying the foundation for a promising therapeutic strategy. Endogenous and overexpressed PRDX6 was shown to reduce oxidative stress in cancer stem cells, as reflected by lower levels of oxidized phospholipids. This effect led to an accelerated malignant progression of existing tumors.[35] ABCG2, which is highly expressed in CSC populations, exports Hoechst-33342 and certain cytotoxic drugs from the interior regions cells, as detected by a side population (SP) analysis.[36] When combined with our results, this suggests that a positive correlation might be found between high rates of ABCG2/PRDX6 positivity and high populations of CSCs.

One of the main reasons for the unreasonable use of chemotherapy and occurrence of medicamentous adverse reactions is that CSCs are continually produced under conditions of disease progression and stabilization. Studies have suggested that disorders of highly conserved developmental pathways, including the mutant Wnt/β-catenin pathway, might regulate self-renewal in embryonic and adult stem cells. This regulation could promote CSC proliferation, metastasis, and chemoresistance.[37,38] Furthermore, recent studies have shown that the Wnt/β-catenin pathway might participate in regulating stem cells. For example, proliferation nuclear antigen-associated factor (PAF) has the ability to promote self-renewal and heterogeneity.[39] Consistent with our study results, aberrant activation of PRDX6 might be

involved in dysregulation of the Wnt/β-catenin pathway in NSCLC, and associated with the maintenance of CSCs.

In this study, we identified two different stem-like cell lines with a high tolerance to cisplatin and proved that PRDX6 could accelerate cisplatin resistance in human lung cancer cells by enhancing stem-like properties. Our results suggest a potential new therapeutic strategy of targeting PRDX6 during NSCLC treatment.

Ethics Approval And Informed Consent

Informed consent was obtained from all subjects who participated in the study. The study was performed in accordance with the ethical principles of the Declaration of Helsinki.

Availability Of Data And Materials

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

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Disclosure

All authors declare that they have no conflict of interest in this work.

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