Molecular Medicine

Secreted Frizzled Related Protein 1 Modulates Taxane Resistance of Human Lung Adenocarcinoma

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Taxanes, such as docetaxel and taxol, have been used as firstline chemotherapies in advanced lung adenocarcinoma (LAD), but limited responses to chemotherapy remain a major impediment in the clinic. Treatment with 5-azacytidine increases the sensitivity of SPC-A1/DTX cell line to taxanes. The results of DNA methylation microarray and cDNA array analysis indicate that DNA methylation contributes to the downregulation of secreted frizzled related protein 1 (SFRP1) in SPC-A1/DTX cells. Overexpression of SFRP1 reverses the chemoresistance of taxane-resistant LAD cell lines and enhances the *in vivo* sensitivity of taxane-resistant LAD cells to taxanes. Meanwhile, short hairpin RNA (shRNA)-mediated SFRP1 knockdown decreases the sensitivity of parental LAD cell lines to taxanes. Furthermore, FH535, a reversible Wnt signaling inhibitor, enhances the sensitivity of taxane-resistant LAD cells to taxanes. The level of SFRP1 in tumors of nonresponding patients is significantly lower than that in tumors of responders. Taken together, our results provide the direct evidence that SFRP1 is a clinically important determinant of taxanes resistance in human LAD cells, suggesting that SFRP1 might be a novel therapeutic target for the treatment of taxane-resistant LAD patients.

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INTRODUCTION

Lung cancer is the leading cause of cancer-related death around the world (1). As the most common type of lung cancer, lung adenocarcinoma (LAD) comprises 30% to 35% of primary lung tumors (2). Taxanes, such as docetaxel and taxol, are used as firstline therapeutic agents in advanced LAD and other solid tumors with genotoxic effects including inhibition of microtubule depolymerization and promotion of microtubule polymerization (3,4). However, chemoresistance has become the greatest obstacle in the treatment of LAD. Thus, a better understanding of the molecular mechanisms involved in taxanes resistance of LAD cells will be helpful to improve the outcome of taxanes chemotherapy.

Aberrant DNA methylation of the CpG islands plays an important role in the development of carcinogenesis by downregulating tumor suppressors (5,6). Emerging evidence shows that DNA methylation contributes to the acquired chemotherapy resistance (7). However, the correlation of DNA methylation with taxanes resistance of LAD is rarely reported. Previously, we established a docetaxel-resistant SPC-A1 cell line (SPC-A1/DTX) and confirmed that pretreatment with 5-azacytidine enhanced the sensitivity of SPC-A1/DTX cells to taxanes. Here, we performed DNA methylation microarray analysis and found that a total of 18 genes, including secreted frizzled related protein 1 (*SFRP1*), were hypermethylated in SPC-A1/DTX cell line compared with parental SPC-A1 cell line. SFRP1, a 35-kDa secreted glycoprotein, is well described as an extracellular glycoprotein to antagonize the Wnt/β-catenin signaling pathway (8,9). In addition, SFRP1 acts as a candidate tumor suppressor under the regulation of DNA methylation in a variety of tumors including LAD (10–12). We have reported that epigenetic inactivation of SFRP1 correlated with a poor prognosis of LAD patients (13). However, the association of SFRP1 with taxanes resistance in LAD cell lines needs to be elucidated.

In this study, we investigated the roles of SFRP1 in taxane-induced drug resistance, and showed that loss of SFRP1 mediated by aberrant promoter methylation resulted in reduction of sensitivity of LAD cells to taxanes and that restoration of SFRP1 expression could reverse the taxanes resistance of LAD cells both *in vitro* and *in vivo*.

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MATERIALS AND METHODS

Cell Culture and Treatment

The human LAD cell lines (SPC-A1 and A549) and taxol-resistant A549 cell line (A549/Taxol) were purchased from

Shanghai Institute of Cell Biology (Shanghai, China). The final concentration of taxol for A549/Taxol cell line was 200 μg/L. The docetaxel-resistant SPC-A1 cell line (SPC-A1/DTX) was established by continuous exposure to increasing concentration of docetaxel. The first selection concentration of docetaxel was 0.008 μg/L. After 14 months selection, docetaxel-resistant SPC-A1 cells were grown in the presence of 5 μg/L docetaxel. These cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS, Gibco [Thermo Fisher Scientific Inc., Waltham, MA, USA]), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere. FH535, G418, 5-azacytidine and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). LAD cell lines were seeded into 6-well plates at a density of 2×10^5 cells/well and treated with freshly prepared 5-azacytidine for 5 d (media changed every day).

DNA Methylation Microarray Analysis

Total DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). DNA methylation microarray using Illumina Infinium HumanMethylation450 BeadChip, which included more than 450,000 Methylation sites, was performed by Phalanx Biotech Group (Shanghai, China) and the acquired data was analyzed by SAM software. Differentially detected signals were generally accepted as true when the ratio of the $P < 0.05$ and were then selected for cluster analysis. To select multiple probes for an enriched genes test, candidate genes were chosen when the value of δ-β showed >0.7 in the methylation test compared with control samples. The microarray analysis was repeated at least three times.

DNA Extraction and Methylation-Specific Polymerase Chain Reaction (MSP)

Genomic DNA was extracted from cultured cells using QIAamp DNA Mini Kit (Qiagen). After quantification by spectrophotometer, 1 μg of genomic DNA was bisulphite-treated with EZ-DNA methylation Gold Kit (Zymo Research, Orange, CA, USA), and finally resuspended in 10 μL TE buffer. MSP primers were designed to match the sequencing region and are displayed in Supplementary Table 1. Simultaneous reactions for both unmethylated and methylated primers were performed for 35 cycles using the following conditions: 95°C for 30 sec, 58°C for 1 min and 72°C for 1 min using platinum Taq (Invitrogen [Thermo Fisher Scientific]). The PCR products were separated on 2% agarose gels.

Plasmids and Transfection

The expression plasmid of SFRP1 was a kind gift of Yoshitaka Sekido (Nagoya University, Nagoya, Japan). Short hairpin RNA (shRNA) targeting of SFRP1 was synthesized and subsequently cloned into the pSilencer4.1-CMVneo vector (Invitrogen [Thermo Fisher Scientific]). The sequence of shRNA is listed in Supplementary Table 1. The recombinant plasmids were named pSil/shSFRP1 and pSil/shcontrol, respectively. Cells were transfected using Lipofectamine 2000 (Invitrogen [Thermo Fisher Scientific]) according to the manufacturer's protocol. The shRNA transfected cell lines were named SPC-A1/shSFRP1, SPC-A1/shcontrol, A549/shSFRP1 and A549/shcontrol, respectively. After selection, SFRP1 stable transfectants were isolated and maintained in RPMI 1640 medium containing G418 (200 μg/L). The stably transfected cell lines were named SPC-A1/DTX/SFRP1, SPC-A1/DTX/control, A549/Taxol/SFRP1 and A549/Taxol/control, respectively.

RNA Isolation and Real-Time PCR

RNA was extracted using Trizol reagent (Invitrogen) and reversely transcribed into cDNA using a PrimeScript RT reagent Kit (Takara, Dalian, China) following the vendor's instructions. Quantitative real-time PCR was performed by PRISM 7900 Sequence Detection System (Applied Biosystems [Thermo Fisher Scientific]). GAPDH was amplified as endogenous control. The primers used for real-time PCR are listed in Supplementary Table 1.

Western Blotting

Equivalent amounts (60 μg protein/lane) of protein lysates were separated electrophoretically on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were incubated overnight at 4°C with primary antibodies to SFRP1 (1:250, Abcam, Cambridge, MA, USA), β-catenin (1:1000, bioWORLD, Dublin, OH, USA), p-GSK3β (1:1000, bioWORLD), GSK3β (1:1000, bioWORLD), cyclin D1 (1:1000, Cell Signaling Technology, Danvers, MA, USA) or c-myc (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following being probed with HRP-conjugated secondary antibody, the membrane was developed with ECL substrate (Cell Signaling Technology) according to the manufacturer's instructions.

Cell Viability Assay

Cells were cultured in 96-well plates with 3×10^3 cells/well and treated with various concentrations of drugs for 72 h. Then MTT was added and incubated at 37°C for 4 h. The resulting formazan crystals were solubilized in 100 μL dimethyl sulfoxide (DMSO) and absorbance at 490 nm was measured using a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA).

Flow Cytometric Analysis of Cell Cycle

After the treatments, cells were harvested, fixed in 70% ethanol at 4°C, and then were subjected to propidium iodide (PI)/RNase staining. Flow cytometric analysis was determined using a FACScan instrument and CellQuest software (BD Biosciences, San Jose, CA, USA).

Flow Cytometric Analysis of Apoptosis

Apoptotic rate was assessed by annexin V-FITC apoptosis detection kit (KeyGen Biotech, Nanjing, China) according to the manufacturer's protocol.

Briefly, cells were resuspended in 0.5 mL binding buffer supplemented with 5 μL annexin V and 5 μL PI, and incubated for 15 min at 37°C in the dark. The apoptotic rate was detected by flow cytometry.

Colony Formation Assay

Cells were seeded in 6-well plates at a density of 500 cells per well. After 14 d, the colonies were fixed with 70% ethanol and stained with 0.1% crystal violet. Then the number of colonies larger than 1 mm was manually counted. These experiments were repeated at least three times.

Luciferase Assay

SPC-A1/DTX cells (4×10^4 cells/well) were seeded into 24-well plates and transfected with pTOPflash (or pFOPflash), pcDNA3.1/SFRP1 (or pcDNA3.1), mutated S33A β-catenin and pRL-SV40. After 48 h of transfection, cells were assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to manufacturer's instructions. Each assay was determined from triplicate wells and the experiment was repeated at least three times.

Xenograft Transplantation

Male BALB/c nude mice at 5 to 6 wks of age were purchased from the Animal Laboratory Unit of Jinling Hospital. SPC-A1/DTX/control, SPC-A1/DTX/SFRP1, A549/Taxol/control or A549/Taxol/SFRP1 (2.0×10^6) cells were suspended in 100 μL PBS and injected subcutaneously into the right side of the posterior flank with 10 mice per group. Tumor volumes were calculated as described previously (14). When the average tumor size reached about 50 mm^3, mice were treated with docetaxel or taxol through intraperitoneal injection at a dose of 1 mg/kg, one dose every other day with three doses total. After 17 d, all mice were killed and tumor tissues were used to perform hematoxylin and eosin (H&E) staining and immunostaining analysis for proliferating cell nuclear antigen (PCNA). All the animal experiments were approved by the Institute Animal Care and Use Committee of Jingling Hospital.

Clinical Samples

Tumor tissues of 33 patients who had undergone a complete resection for early NSCLC and received chemotherapy were obtained in Jinling Hospital during March 2006 and September 2008 according to protocols approved by the Ethics Committee of Jinling Hospital. Patients were selected by these criteria: patients who suffered from primary LAD; a histological diagnosis of LAD with at least one measurable lesion; postoperative chemotherapy comprised either with docetaxel 75 mg/m^2 and cisplatin 100 mg/m^2 or docetaxel 75 mg/m^2 and carboplatin AUC 6 mg/mL/min administered for all patients, given every three wks for a maximum of five cycles. Tumor samples were divided into complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD) groups according to the patient's responses assessed by computerized tomographic (CT) imaging and the Response Evaluation Criteria in Solid tumors (RECIST).

Immunohistochemistry (IHC)

Tumor tissues were paraffin-embedded, formalin-fixed and immunostained for SFRP1 (1:100; Abcam) or PCNA (1:100; bioWORLd) using standard immunohistochemistry procedures and positive tumor cytoplasm was scored as described previously (15).

Statistical Analysis

Data were presented as mean ± SEM. Comparisons between groups were carried out by Student *t* test and values of $P < 0.05$ were considered statistically significant. The probability of survival was plotted by the Kaplan-Meier method and compared by the log-rank test. All the statistical differences were analyzed by SPSS12.0 statistical analytical software (SPSS, Chicago, IL, USA).

All supplementary materials are available online at www.molmed.org.

RESULTS

Hypermethylation of the CpG Islands Contributes to Downregulation of SFRP1 in SPC-A1/DTX Cell Line

The SPC-A1/DTX cell line has been established from parental SPC-A1 cell line after the selection by sequential, pulsed exposure to increasing concentrations of drugs. The IC_{50} value of the SPC-A1/DTX cell line for docetaxel was increased significantly compared with that of the parental SPC-A1 cell line (87.34 ± 3.30 versus 6.48 ± 0.54 μg/L, $P < 0.01$, Figure 1A). Interestingly, the IC_{50} values of SPC-A1/DTX and SPC-A1 cells for taxol were 10.80 ± 1.79 and 1.67 ± 0.23 μg/L, respectively, suggesting that the SPC-A1/DTX cell line acquired cross-resistance to taxol (Figure 1A). As shown in Figure 1B, pretreatment of 5-azacytidine significantly decreased the IC_{50} value of SPC-A1/DTX cells for docetaxel or taxol by 65.4% or 52.6%, respectively ($P < 0.01$). In addition, treatment of 5-azacytidine could inhibit the proliferating ability and induce apoptosis enhancement in the SPC-A1/DTX cell line ($P < 0.01$, Figures 1C, D), suggesting that DNA methylation might play a critical role in the formation of chemoresistant phenotype of LAD cells.

To investigate the roles of DNA methylation in chemoresistance of LAD cells, DNA methylation microarray assays were performed to analyze the different status of methylation between SPC-A1/DTX and the parental SPC-A1 cell line. As shown in Supplementary Table 2, 18 hypermethylated genes (δ-β >0.7) including *SFRP1* were identified. The results of cDNA microarray analysis also indicated that a total of 2332 genes were differentially expressed (more than two-fold change) and *SFRP1* were significantly downregulated in the SPC-A1/DTX cell line (about 24.15-fold change, Supplementary Table 3). To confirm the results of microarray analysis, real-time PCR and Western blotting were performed. Compared with parental SPC-A1 cells, the mRNA and protein levels of SFRP1 were decreased markedly in the SPC-A1/DTX cell line (Figure 2A).

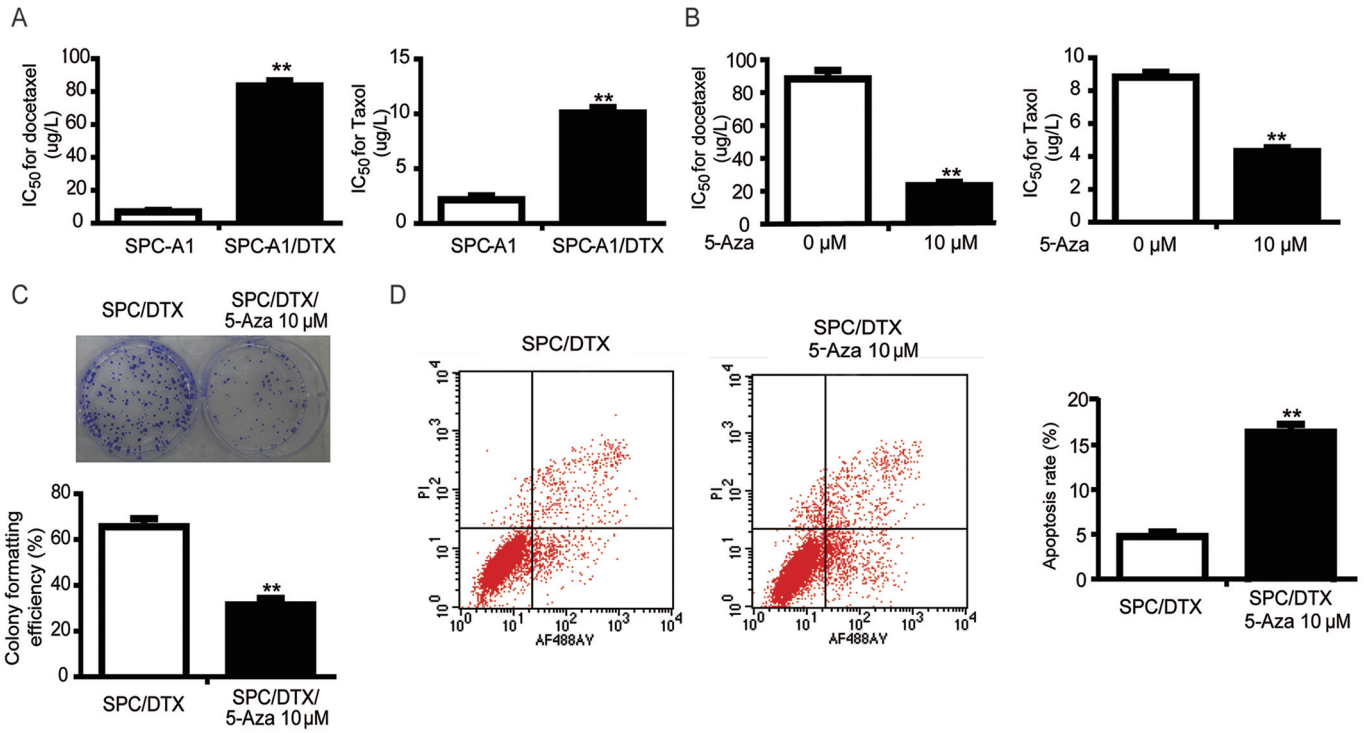

Figure 1. Docetaxel resistance in SPC-A1/DTX cells acquired cross-resistance to taxol and 5-Azacytidine and enhanced the sensitivity of SPC-A1/DTX cells to docetaxel and taxol. (A) The IC_{50} values of docetaxel or paclitaxel for the SPC-A1 and SPC-A1/DTX cell lines were determined using MTT assay. (B) The IC_{50} values of the SPC-A1/DTX cell line to docetaxel or taxol after 5-azacytidine treatment. (C) The proliferating ability of the SPC-A1/DTX cell line was determined by colony formation assay after 5-azacytidine treatment. (D) The apoptotic rate was determined by flow cytometric analysis in the SPC-A1/DTX cell lines after 5-azacytidine treatment. $^{**}P < 0.01$.

To investigate whether DNA methylation contributed to downregulation of SFRP1, MSP analysis was performed and the CpG islands in the promoter of SFRP1 gene was incompletely methylated in SPC-A1 cells, whereas SPC-A1/DTX cells were more densely methylated than parental cells (Figure 2B). In addition, treatment with different concentrations of 5-azacytidine could significantly increase the expression of SFRP1 in the SPC-A1/DTX cell line at both mRNA and protein levels (Figure 2C). Moreover, the mRNA and protein levels of SFRP1 were decreased in a dose- and time-dependent manner after exposure to docetaxel in SPC-A1 cells (Figures 2D, E). To determine the role of SFRP1 on taxanes resistance in LAD cells, another taxane-resistant A549 cell line, A549/Taxol, was used. As shown in Supplementary Figure 1A, the IC_{50} value of A549/Taxol cells for taxol was increased significantly compared with that of parental A549 cells (7.28 ± 0.56 versus 0.18 ± 0.37 μg/mL, $P < 0.01$) and A549/Taxol cells showed cross-resistance to docetaxel compared with A549 cells (6.19 ± 0.36 versus 0.27 ± 0.02 μg/mL, $P < 0.01$). The mRNA and protein levels of SFRP1 were decreased significantly in A549/taxol cells compared with parental A549 cells (Supplementary Figure 1B). Furthermore, the mRNA and protein levels of SFRP1 were decreased in a dose- and time-dependent manner after exposure to Taxol in A549 cells (Supplementary Figures 1C, D). These results indicated that DNA methylation might contribute to the downregulation of SFRP1 in taxane-resistant LAD cells.

SFRP1 Restoration Increases the Sensitivity of Taxane-Resistant LAD Cell Lines to Taxanes

To investigate whether SFRP1 affected the sensitivity of LAD cell lines for taxanes, pcDNA/SFRP1 was stably transfected into SPC-A1/DTX and A549/Taxol cell line, which was confirmed by Western blotting (Figure 3A). Compared with SPC-A1/DTX/control cells, the IC_{50} values of SPC-A1/DTX/SFRP1 cell line for docetaxel and taxol were decreased significantly by 52.9% and 47.4%, respectively (Figure 3B). Meanwhile, compared with A549/Taxol/control cells, the IC_{50} values of A549/Taxol/SFRP1 cell line for taxol and docetaxel were decreased significantly by 45.7% and 44.1%, respectively (Figure 3C). In addition, colony formation assay showed that the proliferating ability of the SPC-A1/DTX/SFRP1 cell line was significantly suppressed compared with SPC-A1/DTX/control cells, which indicated the proliferation inhibitory function of SFRP1 *in vitro* (Figure 3D). Similar results were obtained in A549/Taxol/SFRP1 cells com-

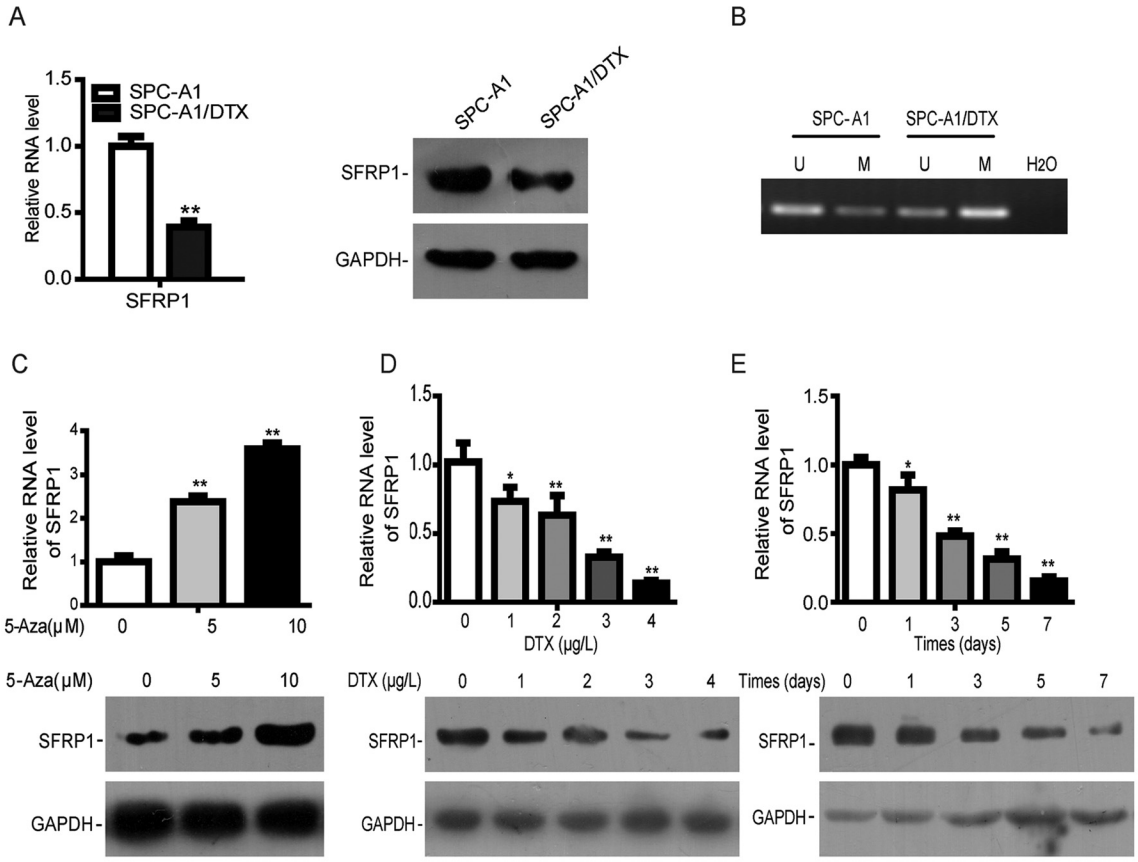

Figure 2. SFRP1 was downregulated significantly in the SPC-A1/DTX cell line and the methylation status of SFRP1 promoter was significantly increased in the SPC-A1/DTX cell line compared with the parental SPC-A1 cell line. (A) The mRNA and protein levels of SFRP1 in the SPC-A1 and SPC-A1/DTX cell lines were determined by real-time PCR and Western blot. (B) The methylation status of SFRP1 CpG island in the SPC-A1/DTX cell line compared with the parental SPC-A1 cell line was determined by MSP analysis. Water blank was used as a negative control. (C) After treatment with 5 or 10 μmol/L 5-azacytidine for 72 h, the mRNA and protein levels of SFRP1 were determined by real-time PCR and Western blotting in SPC-A1/DTX cells. (D) Real-time PCR and Western blotting were performed to detect the expression of SFRP1 mRNA and protein levels in SPC-A1 cells treated with various concentrations of DTX (0, 1, 2, 3 and 4 μg/L) for 24 h. (E) Real-time PCR and Western blotting were performed to detect the expression of SFRP1 mRNA and protein in SPC-A1 cells treated with DTX (1.0) μg/L) for various time points (0, 1, 3, 5 and 7 d). *$P < 0.05$, **$P < 0.01$.

pared with A549/Taxol/control cells (Figure 3E). Furthermore, overexpression of SFRP1 in both SPC-A1/DTX/SFRP1 and A549/Taxol/SFRP1 cell lines triggered the accumulation of cells at the G1-phase and decreased the cells at S-phase (Figures 3F, G) and significantly increase the apoptotic rate of SPC-A1/DTX (4.01 ± 1.33 % versus 8.27 ± 0.87 %) and A549/Taxol cells (3.24 ± 0.87 % versus 6.83 ± 1.56 %, $P < 0.01$, Figures 3H, I). Therefore, restoration of SFRP1 could reverse the chemoresistance of taxane-resistant LAD cells for docetaxel and taxol by inducing a G1-phase accumulation and apoptosis enhancement.

ShRNA-Mediated SFRP1 Knockdown Leads to Decreased Sensitivity of Parental LAD Cell Lines to Taxanes

The effectiveness of shRNA targeting SFRP1 was confirmed by Western blotting (Figure 4A). As shown in Figure 4B, compared with the SPC-A1/shcontrol cell line, the IC_{50} values of the SPC-A1/shSFRP1 cell line for docetaxel and taxol were increased by 69.6% and 78.3%, respectively ($P < 0.01$). Likewise, compared with the A549/shcontrol cell line, the IC_{50} values of the A549/shSFRP1 cell line for docetaxel and taxol were increased by 67.2% and 57.5%, respectively ($P < 0.01$, Figure 4C). Moreover, it was shown that inhibition of SFRP1 could increase the proliferation ability of SPC-A1 and A549 cells ($P < 0.01$, Figures 4D, E). Compared with the SPC-A1/shcontrol and A549/shcontrol cell lines, the percentage of G1-phase cells was decreased and the percentage of S-phase cells was increased in SPC-A1/shSFRP1 and A549/shSFRP1 cells ($P < 0.01$, Figures 4F, G). However, compared with SPC-A1/shcontrol or

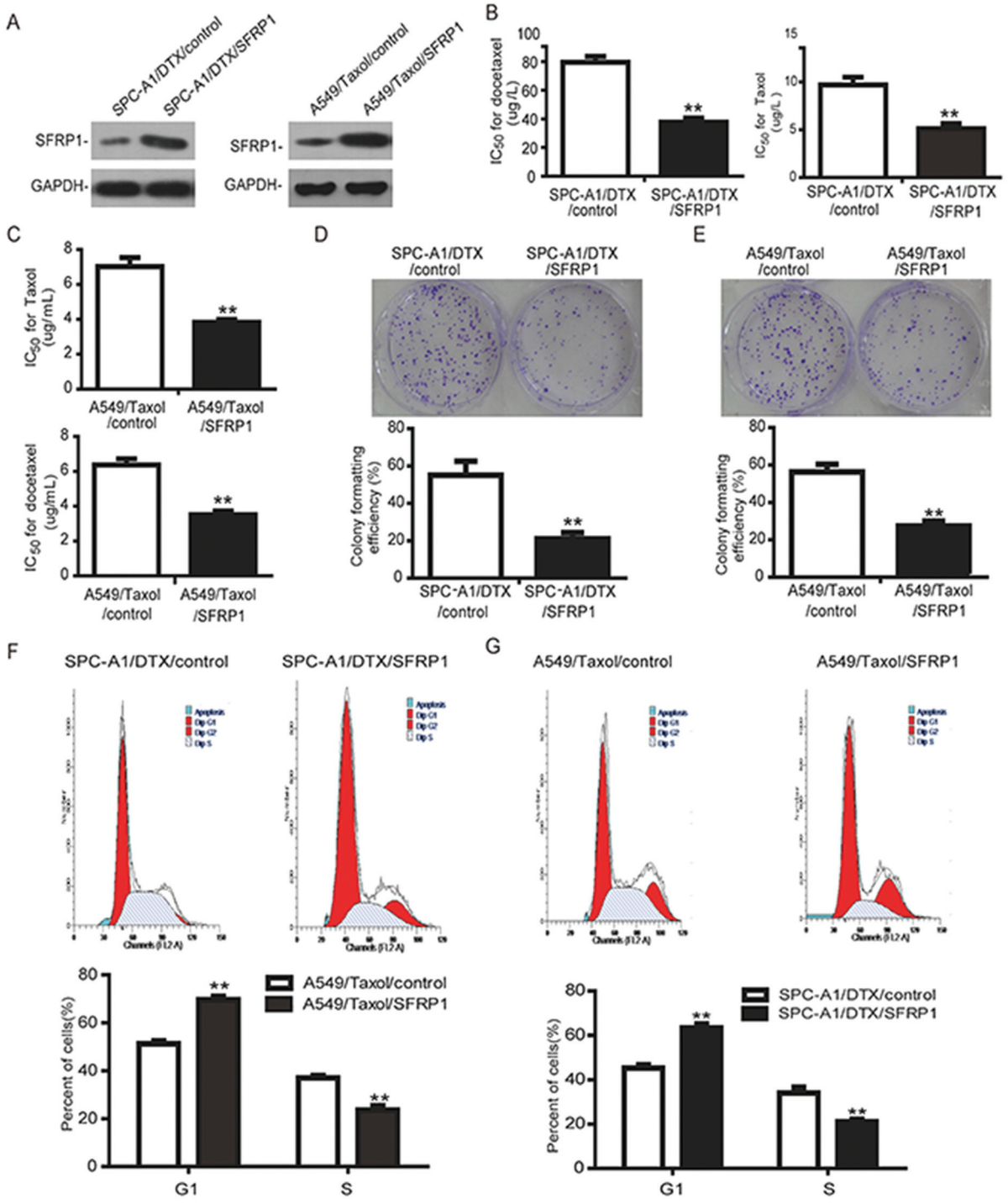

Figure 3. Overexpression of SFRP1 increased chemosensitivity of the SPC-A1/DTX or A549/Taxol cell line to docetaxel and taxol *in vitro*. (A) The protein level of SFRP1 was determined in the SPC-A1/DTX/SFRP1 and A549/Taxol/SFRP1 cell lines. The IC_{50} values of DTX and Taxol for the SPC-A1/DTX/SFRP1 (B) or A549/Taxol/SFRP1 (C) cell line were determined by MTT assay. The proliferating ability of the SPC-A1/DTX/SFRP1 (D) or A549/Taxol/SFRP1 (E) cell line was determined by colony formation assay. Cell cycle of the SPC-A1/DTX/SFRP1 (F) or A549/Taxol/SFRP1 (G) cell lines was determined by flow cytometric analysis. The apoptotic rate of the SPC-A1/DTX/SFRP1 (H) or A549/Taxol/SFRP1 (I) cell line was determined by flow cytometric analysis. $^{**}P < 0.01$.

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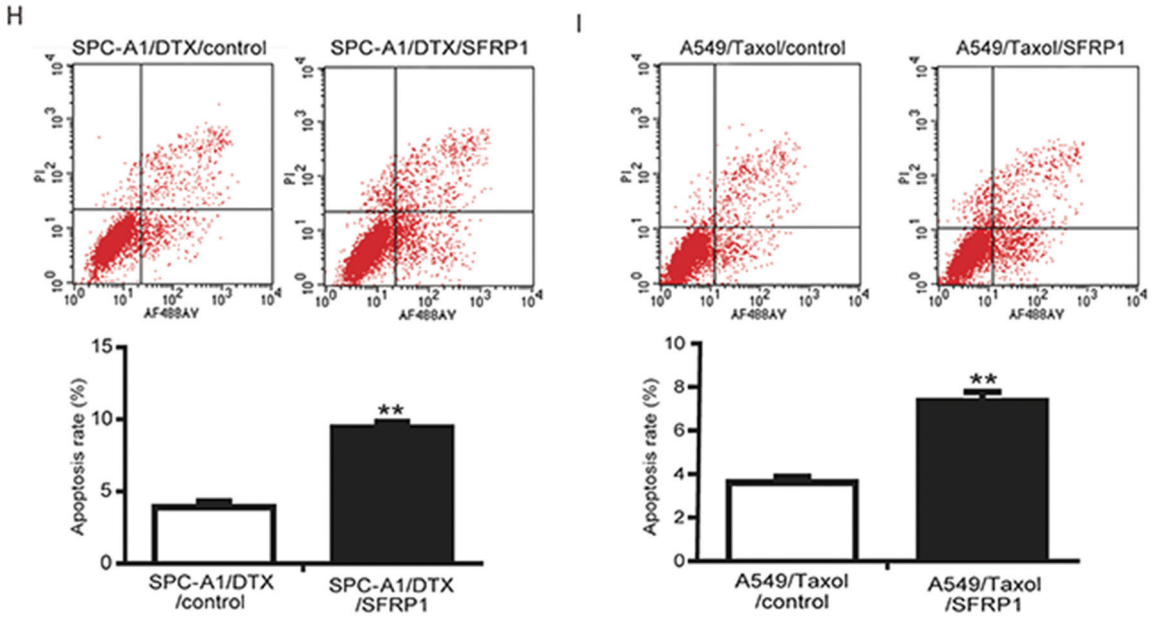

Figure 3. ***Continued.***

A549/shcontrol cells, there was no difference in apoptotic rate in SPC-A1/shSFRP1 or A549/shSFRP1 cells (data not shown). Thus, downregulation of SFRP1 could result in decreased sensitivity of parental LAD cells to taxanes.

Overexpression of SFRP1 Reverses the Chemoresistance of Taxane-Resistant LAD Cell Lines *In Vivo*

To further explore the role of SFRP1 on the *in vivo* chemosensitivity of LAD cells, stably transfected LAD cell lines (SPC-A1/DTX/control, SPC-A1/DTX/SFRP1, A549/Taxol/control and A549/Taxol/SFRP1 cells) were subcutaneously inoculated into nude mice. About 10 d after implantation, all the mice developed tumors and tumor volumes were measured. Following the treatment of docetaxel, the average tumor size was decreased significantly in the SPC-A1/DTX/SFRP1 group compared with that in the SPC-A1/DTX/control group (212.5 ± 13.45 versus 61.7 ± 8.37 mm^3, $P < 0.01$, Figures 5A, B). In addition, immunostaining analysis showed that PCNA-positive cells were markedly decreased in tumors of SPC-A1/DTX/SFRP1 groups (Figure 5C). Furthermore, overexpression of SFRP1 increased the chemosensitivity of A549/Taxol cell lines as demonstrated by tumor size analysis and PCNA staining after the treatment of taxol (Figures 5D–F). These results indicated that overexpression of SFRP1 could significantly enhance the *in vivo* response of LAD cells to taxanes.

Overexpression of SFRP1 Inactivates the Wnt Signaling Pathway in Taxane-Resistant LAD Cell Lines

Emerging evidence has shown that SFRP1 acts as a Wnt signaling antagonist and exerts inhibitory effects on the Wnt signaling pathway. We first tested whether the Wnt signaling pathway was activated in SPC-A1/DTX and A549/axol cells. Compared with the parental cell lines, both the phosphorylation of GSK3β at serine-9 and expression of β-catenin were increased in both SPC-A1/DTX and A549/Taxol cell lines. In addition, the mRNA and protein levels of c-myc and cyclin D1, two downstream targets of β-catenin, were also increased in both taxane-resistant LAD cell lines (Figures 6A, B). We then determined whether restoration of SFRP1 could inactivate the Wnt signaling pathway. As expected, upregulation of SFRP1 could decrease the p-GSK3β, β-catenin, cyclin D1 and c-myc (Figures 6C, D). Meanwhile, overexpression of SFRP1 could significantly suppress the transcriptional activity of β-catenin, even in the presence of pcDNA-S33Y β-catenin ($P < 0.01$, Figure 6E). These results indicated that upregulation of SFRP1 could inactivate the Wnt signaling pathway in taxane-resistant LAD cell lines.

To further confirm the role of Wnt signaling pathway in the acquired resistance to taxanes, taxane-resistant LAD cells were treated with a reversible Wnt pathway inhibitor, FH535. Although FH535 was dissolved in DMSO, preliminary experiments indicated that the solvent showed no cytotoxicity in SPC-A1/DTX and A549/Taxol cell lines at the concentrations used (data not shown). As shown in Figures 7A and B, FH535 could inhibit the mRNA and and protein expression levels of β-catenin, cyclin D1 and c-myc in taxane-resistant LAD cell lines. In addition, the IC_{50} values of SPC-A1/DTX cell line for docetaxel and taxol were decreased significantly after FH535 treatment ($P < 0.01$, Figure 7C). Similarly, the IC_{50} values of A549/Taxol cell line for docetaxel and taxol were decreased signifi-

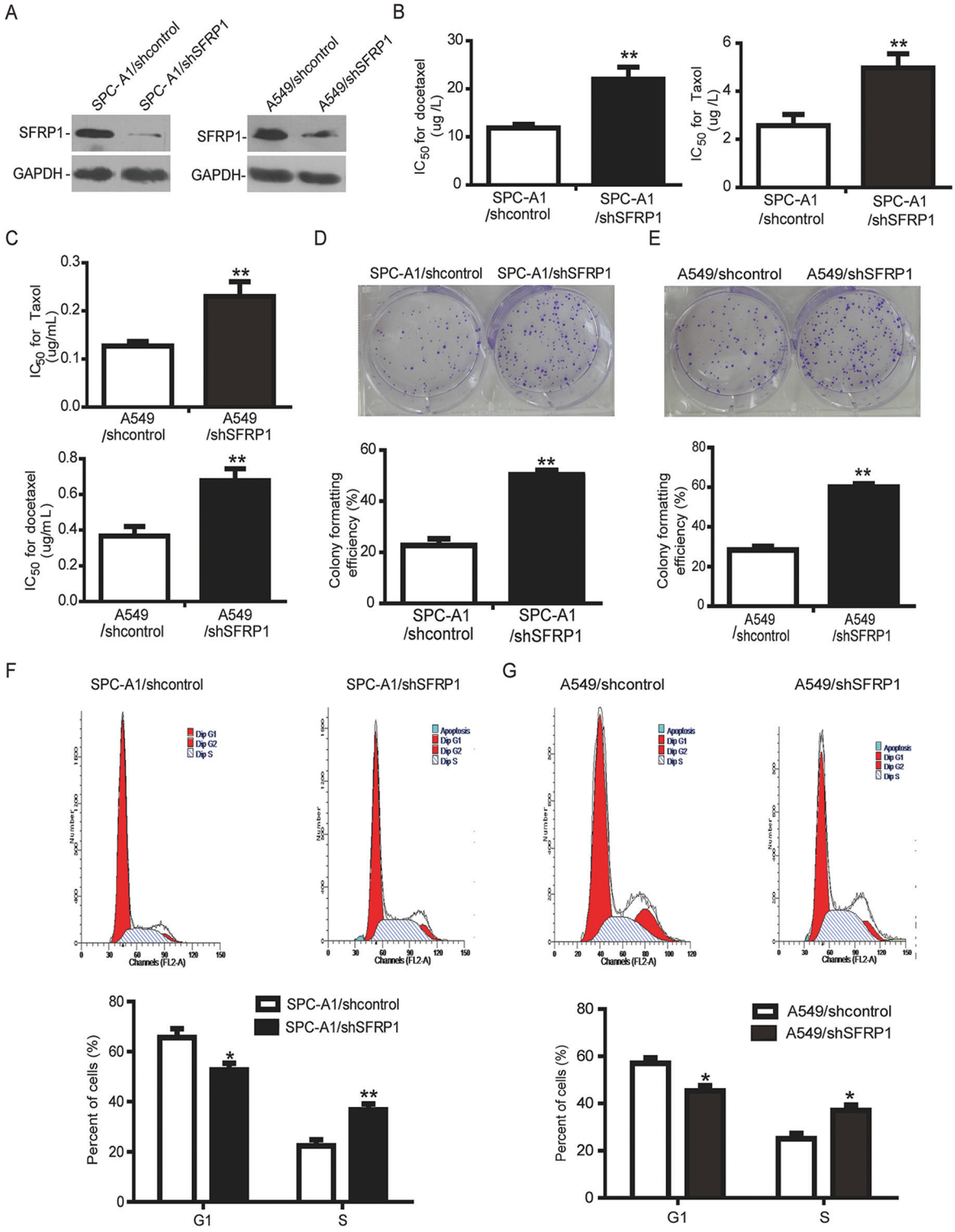

Figure 4. Knockdown of SFRP1 decreased chemosensitivity of the SPC-A1 or A549 cell line to docetaxel and taxol *in vitro*. (A) The protein level of SFRP1 was detected in the SPC-A1/shSFRP1 and A549/shSFRP1 cell lines. The IC_{50} values of docetaxel or paclitaxel for SPC-A1/shSFRP1 (B) or A549/shSFRP1 (C) were determined by MTT assay. The proliferating ability of the SPC-A1/shSFRP1 (D) or A549/shSFRP1 cell line (E) was detected by colony formation assay. The cell cycle of the SPC-A1/shSFRP1 (F) or A549/shSFRP1 (G) cell line was determined by flow cytometric analysis. $^{*}P < 0.05$, $^{**}P < 0.01$.

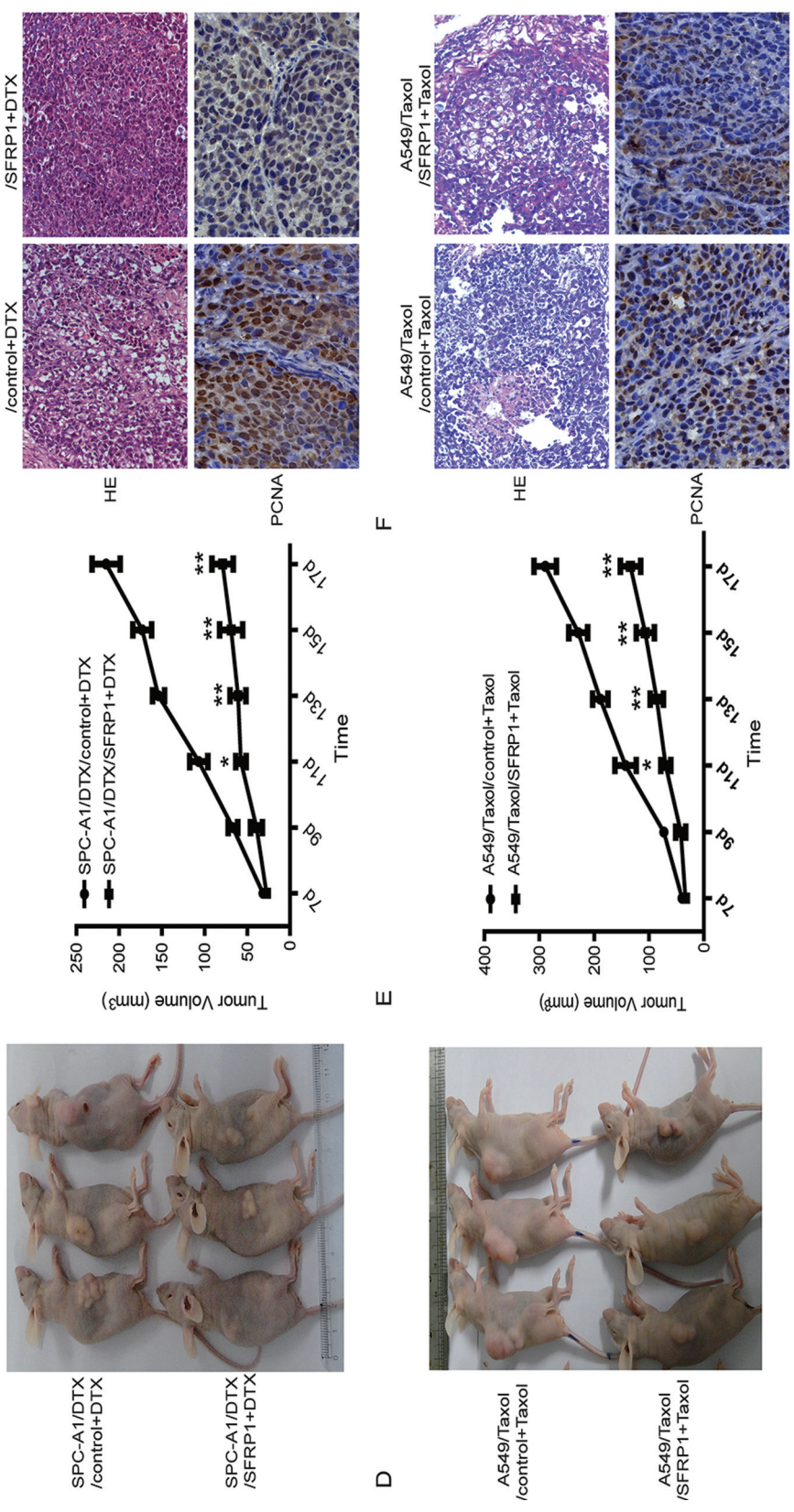

Figure 5. SFRP1 restoration enhanced the *in vivo* response of SPC-A1/DTX or A549/Taxol cells to docetaxel or taxol. (A) Representative photographs of tumors formed after treatment with docetaxel for 17 d in the SPC-A1/DTX/SFRP1 cell line subcutaneously transplanted nude mice. (B) Growth curves of tumors derived from the SPC-A1/DTX/SFRP1 cell line compared with the SPC-A1/DTX/control cell line. (C) H&E (upper) and PCNA (lower)-stained sections of the SPC-A1/DTX/SFRP1 cell line transplanted tumors. (D) Representative photographs of tumors formed after treatment with Taxol for 17 d in the A549/Taxol/SFRP1 cell line subcutaneously transplanted nude mice. (E) Growth curves of tumors derived from the A549/Taxol/SFRP1 cell line compared with the A549/Taxol/control cell line. $^{*}P < 0.05$, $^{**}P < 0.01$. (F) H&E (upper) and PCNA (lower)-stained sections of the A549/Taxol/SFRP1 transplanted tumors. n = 10 mice per group.

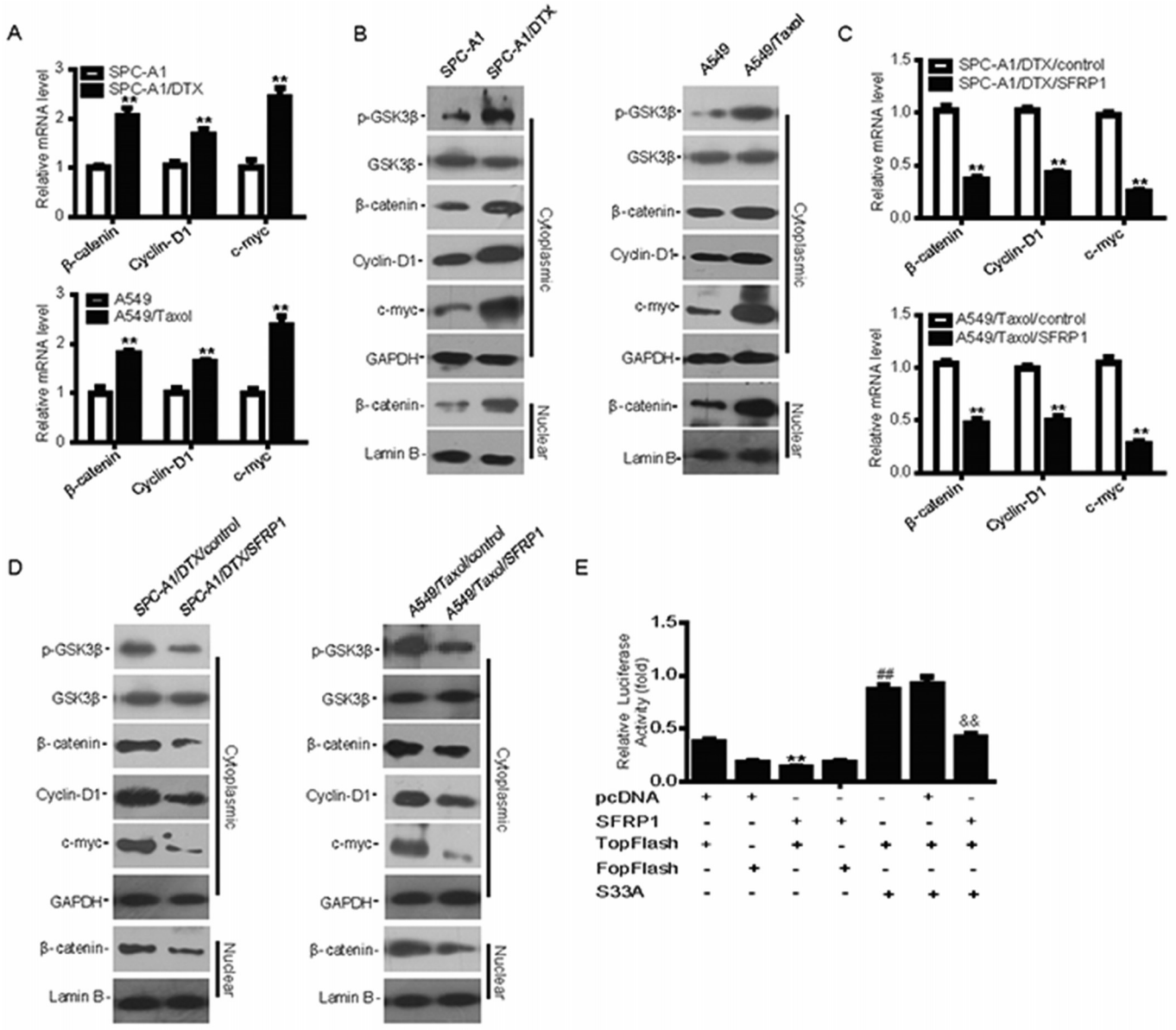

Figure 6. SFRP1 restoration suppressed the Wnt pathway in the SPC-A1/DTX and A549/Taxol cell lines. (A) The expression of β-catenin, cyclin D1 and c-myc was determined by real-time PCR in SPC-A1/DTX or A549/Taxol cells (**$P < 0.01$). (B) The expression of p-GSK3β, β-catenin (cytoplasmic and nuclear), cyclinD-1 and c-myc in the SPC-A1/DTX or A549/Taxol cell line was detected by Western blotting. (C) The mRNA expression of β-catenin, cyclin D1 and c-myc was determined by real-time PCR in the SPC-A1/DTX/SFRP1 and A549/Taxol/SFRP1 cell lines (*$P < 0.01$). (D) The expression of p-GSK3β, β-catenin, (cytoplasmic and nuclear), cyclinD-1 and c-myc in SPC-A1/DTX/SFRP1 and A549/Taxol/SFRP1 cells was detected by Western blotting. (E) Effects of SFRP1 on the transcriptional activity of β-catenin (**$P < 0.01$ versus pcDNA + Topflash, $^{\&\&}P < 0.01$ versus pcDNA + Topflash + S33Y).

cantly after FH535 treatment ($P < 0.01$, Figure 7D). Moreover, following treatment with FH535, the proliferation ability was inhibited significantly ($P < 0.01$, Figures 7E, F) and G1 arrested populations were also increased significantly in both SPC-A1/DTX cells and A549/Taxol cells ($P < 0.01$, Figures 7G, H). Likewise, the apoptotic rates were enhanced significantly by FH535 treatment in taxane-resistant LAD cells ($P < 0.01$, Figures 7I, J). Thus, FH535, a revisable Wnt signaling inhibitor, could reverse the chemoresistance of taxane-resistant LAD cell lines.

SFRP1 is a Candidate Predictor of Taxane-Resistant Lung Adenocarcinoma Tissues

To investigate the correlation between SFRP1 expression and the response of LAD patients with taxane-based adjuvant chemotherapy, a total of 33 clinical tumor

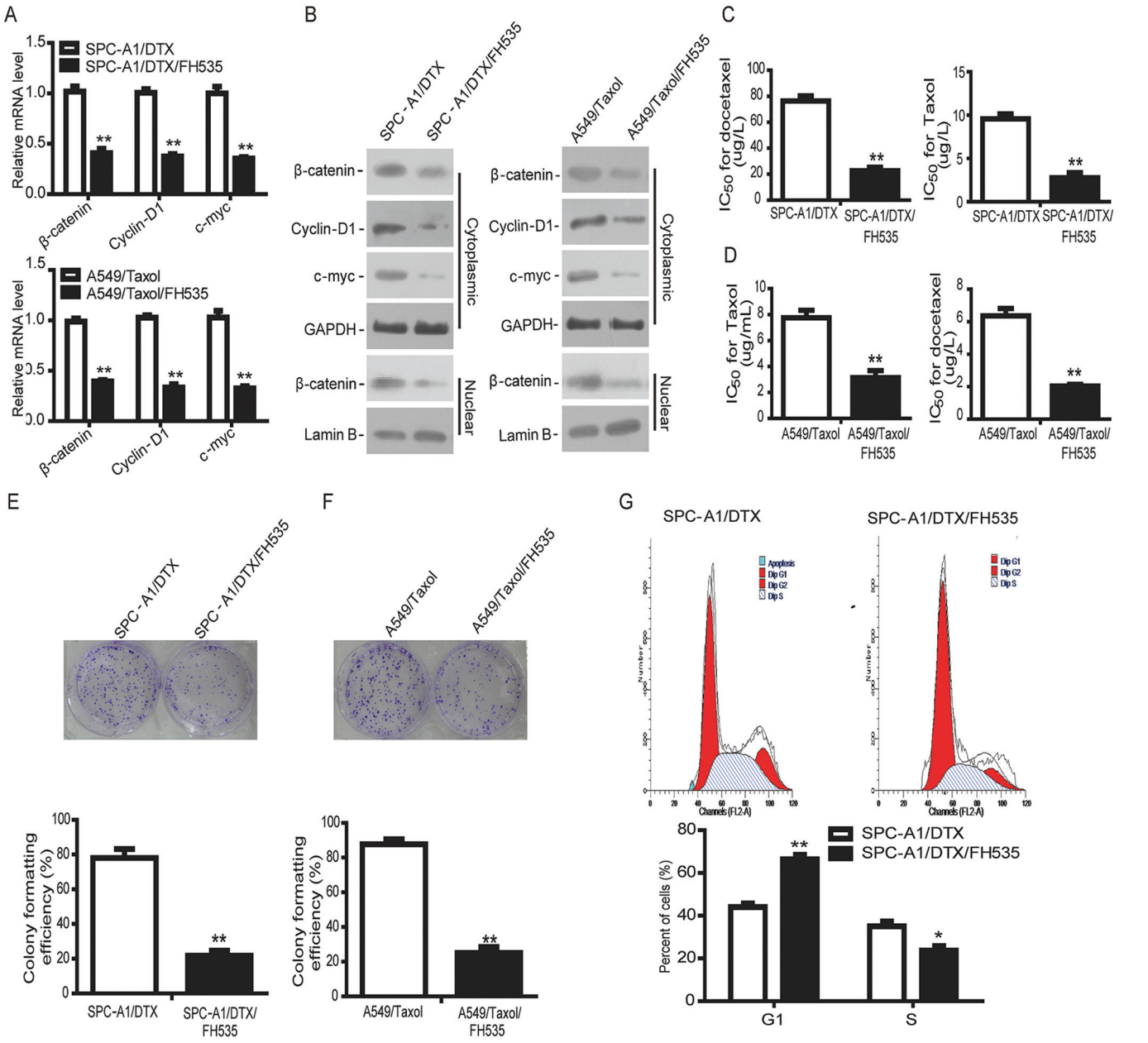

Figure 7. FH535 increased the chemosensitivity of the SPC-A1/DTX or A549/Taxol cell line to docetaxel and taxol. (A) The SPC-A1/DTX or A549/Taxol cell line was treated with 10 μmol/L or 20 μmol/L FH535 for 72 h, respectively. The expression of β-catenin, cyclin D1 and c-myc was determined by real-time PCR. (B) The expression of β-catenin (cytoplasmic and nuclear), cyclin D1 and c-myc was determined by Western blotting. MTT assay indicated the IC_{50} values of SPC-A1/DTX (C) or A549/Taxol cells (D) to docetaxel and taxol after treatment with FH535. The proliferating ability of the SPC-A1/DTX (E) or A549/Taxol cell line (F) treated with FH535 was detected by colony formatting assay. Cell cycle of SPC-A1/DTX (G) or A549/Taxol (H) cell line treated with FH535 was determined by flow cytometric analysis. The apoptotic rate of the SPC-A1/DTX (I) or A549/Taxol (J) cell line treated with FH535 was determined by flow cytometric analysis. $^{**}P < 0.01$.

Continued on next page

tissue samples were collected from patients with advanced LAD and divided into responding (CR + PR) and nonresponding (SD + PD) groups according to the response of patients to taxane-based adjuvant chemotherapy. The results of the immunohistochemistry assay showed that SFRP1 was mainly located in the cytoplasm of tumor cells and was nega-

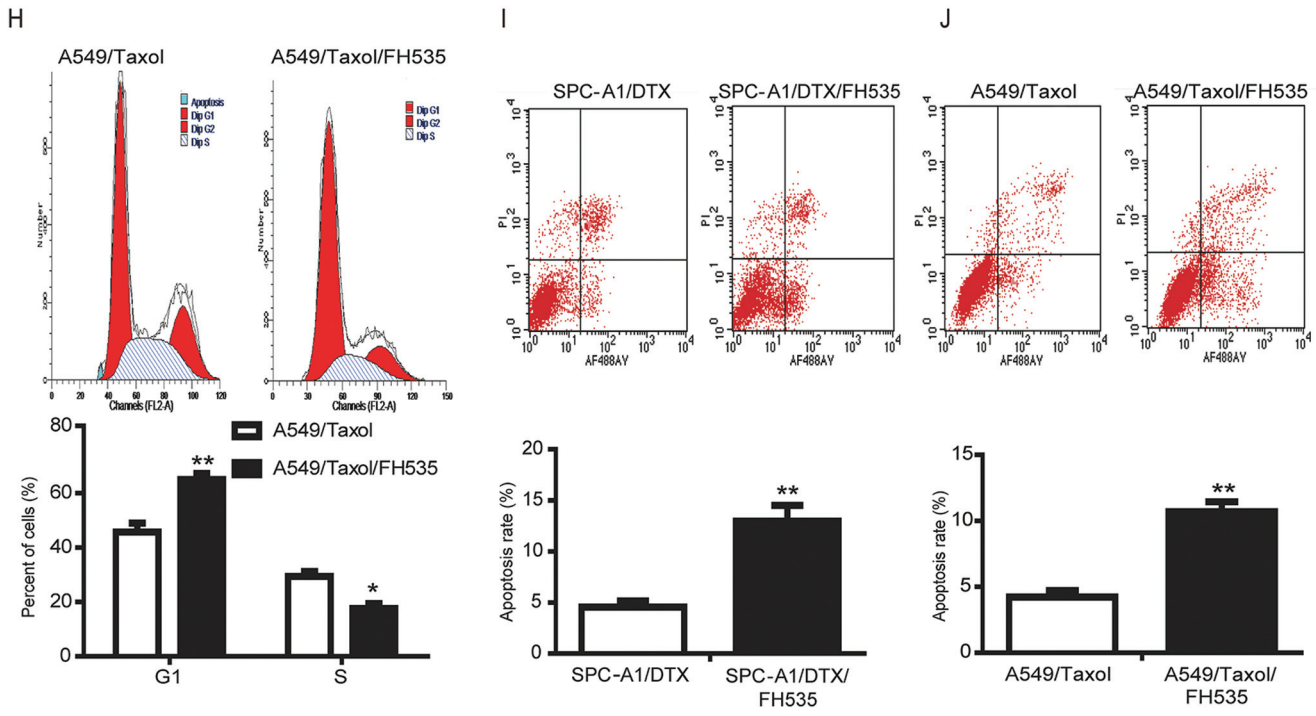

Figure 7. ***Continued.***

tively expressed in 5 of the 33 cases and positively expressed in 28 of the 33 cases. Among these 28 positive cases, it was of mild intensity (1+) in nine patients, of moderate intensity (2+) in 11 patients and of strong intensity in eight patients. Corresponding with immunostaining analysis, an overall stronger staining for SFRP1 was observed in the responding tumors, whereas weaker staining of SFRP1 was observed in nonresponding tumors ($P < 0.05$, Figure 8A). Further, Kaplan-Meier analysis demonstrated that the difference in the SFRP1 expression pattern within tumor samples may be attributed to the different disease-free survival (DFS) of patients. LAD patients with high SFRP1 expression had a more prolonged progression free survival than those with low SFRP1 expression ($P < 0.05$, Figure 8B). Thus, it suggested that SFRP1 contributed to the chemoresistance of taxane-based adjuvant chemotherapy.

DISCUSSION

In the present study, we showed that DNA methylation mediated the downregulation of SFRP1 in docetaxel-resistant SPC-A1 cells and ectopic expression of SFRP1 could enhance the *in vitro* and *in vivo* sensitivity of taxane-resistant LAD cell lines to taxanes by inactivating the Wnt signaling pathway. In addition, the expression of SFRP1 in advanced LAD might contribute to the response of patients to taxane-based chemotherapy.

Tumor cells acquire resistance to taxanes through various mechanisms including alteration in tubulin dynamics, differences in β-tubulin isotype expression and upregulation of members of the ATP binding cassette transporters (ABC transporter family) in cancer cells (16,17). To better understand the molecular mechanisms involved in drug resistance of the LAD, SPC-A1/DTX and A549/Taxol cell lines were established in our lab. Previously, we demonstrated that these taxane-resistant LAD cells displayed morphological and physiological differences compared with parental LAD cells (18). Increasing evidence also indicated that epigenetic events including DNA methylation and posttranscriptional regulation played an important role in chemoresistance during cancer treatment (19,20). We have identified the miRNA expression profile involved in the development of docetaxel resistance in LAD by miRNA microarray (14,21,22). DNA methylation also plays critical roles in the development of chemoresistance by downregulating tumor suppressors, apoptosis mediators and DNA repair enzymes (23,24). Recently, the association of DNA methylation with the sensitivity of tumor cells to taxanes also was reported. The promoter methylation of Ras association domain family 1A (RASSF1A) and transforming growth factor, β-induced (TGFβI) modulated the efficacy of taxane-based chemotherapy in breast cancer and ovarian cancer, respectively (25,26). In this study, we observed that 5-azacytidine could enhance the sensitivity of LAD cell lines to taxanes, implying that DNA methylation might play an important role in taxanes resistance of LAD cells. By DNA methylation and cDNA microarray assays, we found that, due to promoter methylation, SFRP1 was downregulated

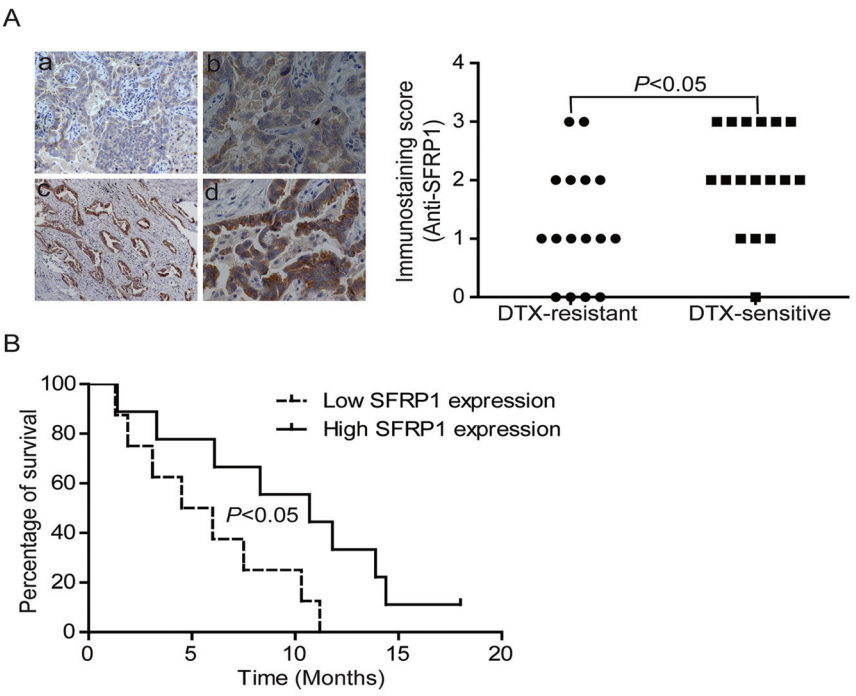

Figure 8. SFRP1 expression was downregulated in docetaxel nonresponding tumors from lung adenocarcinoma patients. (A) SFRP1 was detected by immunostaining analysis and scores of SFRP1 immunostaining was calculated in human lung adenocarcinoma tissues. Tumor tissues were obtained from 17 responding and 16 nonresponding patients. a. Negative staining (200×); b. negative staining (400×); c. positive staining (200×); d. positive staining (400×). (B) Kaplan-Meier survival curve indicated the different DFS of lung adenocarcinoma patients according to the level of SFRP1 protein expression in tumor tissues. The *P* value was determined with the log-rank test.

significantly in SPC-A1/DTX cells compared with SPC-A1 cells. Moreover, MSP analysis showed that the methylation status of *SFRP1* gene in SPC-A1/DTX cells was significantly higher than that in parental SPC-A1 cells.

SFRPs, a family of five secreted glycoproteins, acted as extracellular signaling molecules to antagonize the Wnt signaling pathway (27). The correlation between the expression of the SFRP family and chemosensitivity of cancer cells remains rarely reported (28,29). As a novel member of SFRP family, SFRP1 functions as a candidate tumor suppressor in several human malignancies (11,12,30). Accumulating evidence shows that SFRP1 exerts inhibitory effects on tumor cell growth, angiogenesis and invasion (31–33). SFRP1 was decreased significantly in frizzled7 (FZD7)-resistant Wilms tumor, and exogenous administration of SFRP1 could sensitize resistant cells to FZD7 antibody (34). Combinatorial treatment of renal cell carcinoma cell lines with decitabine and romidepsin induced the reexpression of SFRP1 (35). Herein, we showed that ectopic expression of SFRP1 could restore the sensitivity of taxane-resistant LAD cells for taxanes by inducing apoptosis enhancement and G1 phase arrest, while shRNA-mediated SFRP1 downregulation contributed to taxanes resistance in parental LAD cells *in vitro*. Moreover, overexpression of SFRP1 could inhibit the *in vivo* growth of taxane-resistant LAD cells combined with taxanes treatment.

Next, we explored the underlying molecular mechanisms of SFRP1-induced chemosensitivity enhancement of LAD cells. The Wnt/β-catenin pathway plays a critical role during development, such as controlling the proliferation, fate, specification, polarity and migration of cells (36,37). Sustained activation of the Wnt/β-catenin pathway has been demonstrated to promote tumor survival and metastasis (38,39). Moreover, emerging evidence indicates that Wnt/β-catenin pathway might be a mediator of chemoresistance (40,41). In our studies, we found that SFRP1 restoration could inhibit the expression of β-catenin and phosphorylated GSK3β, which finally downregulated the expression of downstream targets, cyclin D1 and c-myc. It has been reported that cyclin D1 was implicated in the pathogenesis of many cancers by modulating the G1/S restriction point of cell cycle and c-myc also was reported to be closely associated with the apoptosis in cancer cells (42). Therefore, we proposed that SFRP1 might act as a tumor suppressor to reverse the taxanes resistance of LAD cells by inactivating the Wnt signaling pathway. Accumulating evidence has indicated that chemoresistance was correlated with the process of epithelial–mesenchymal transition (EMT) and activation of the Wnt pathway could induce EMT in numerous models (43–46). Our previous study showed that SPC-A1/DTX cells showed EMT characteristics including elongated fibroblastoid shape, the switch of EMT marker proteins, and enhanced migratory and invasive potential (47). Herein, we demonstrated that restoration of SFRP1 could not only reverse the phenotype of EMT, but also inhibit the motility and invasiveness of the SPC-A1/DTX cell line (Supplementary Figure 2). Simultaneously, we analyzed the association of SFRP1 expression with the responses of LAD patients to taxane-based adjuvant chemotherapy. By immunohistochemistry, the patients with high SFRP1 expression had a more prolonged progression-free survival than those with low SFRP1 expression, suggesting that the level of SFRP1 in tumor tissues might contribute to the sensitivity of LAD to taxane-based chemotherapy.

Taken together, this study is the first to provide evidence that downregula-

tion of SFRP1 might contribute to the taxanes resistance of human LAD cells by activating the Wnt signaling. In addition, hypermethylation of SFRP1 may be used as a predictor of response of LAD patients to taxane-based chemotherapy. However, this study still has several limits. First, only two taxane-resistant cell lines were used and further experiments should be performed on some other taxane-resistant LAD cell lines. Second, the tissue sample number is small in the present study and further investigation of a large population will be helpful to strengthen the significance of this study.

CONCLUSION

We demonstrate that DNA methylation induces the downregulation of SFRP1 in taxane-resistant LAD cells, which contributes to taxanes resistance by activating the Wnt signaling. In addition, SFRP1 is a candidate predictor of taxane-resistant lung adenocarcinoma tissues in LAD patients to taxane-based chemotherapy. These results suggest that SFRP1 might be a potential target for the treatment of taxane-resistant LAD patients.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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