

COL3A1 Overexpression Associates with Poor Prognosis and Cisplatin Resistance in Lung Cancer

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Background: Collagen type III alpha 1 chain (COL3A1) is reported to mediate drug resistance in various cancers, and public database analysis indicated its overexpression in lung cancer.

Aims: To investigate the effects of COL3A1 on modulating cisplatin (DDP) resistance in lung carcinoma.

Study Design: A cell study.

Methods: Gene Expression Omnibus datasets were used to determine the differentially expressed genes between H460 and H460/DDP cell lines using bioinformatics analysis. COL3A1 expression and its clinical value in lung cancer prognosis were analyzed using GEPIA and UALCAN databases. Its roles in modulating the growth, viability, apoptosis, and drug resistance were also assessed in vitro.

Results: In H460/DDP cells, the COL3A1 was among the up-regulated genes compared to H460 cells. COL3A1 overexpression and its association with poor survival in patients with adenocarcinoma were detected by public database analysis. In A549 and H1299 cells, COL3A1 overexpression was associated with increased cell growth and clone formation but decreased cell apoptosis, whereas its reduced expression led to decreased cell growth and clone formation and increased cell apoptosis.

Conclusion: COL3A1 is upregulated in lung cancer cells with DDP resistance, and its downregulation sensitizes the cells to DDP.

INTRODUCTION

Lung cancer is a highly malignant type of cancer causing approximately one million death worldwide annually.¹ Although the standard chemotherapy drugs for patients with non-small cell lung cancer (NSCLC) ineligible for surgery are platinum-based, for instance, cisplatin (DDP), their efficacies are often limited due to drug resistance, leading to treatment failure and poor prognosis.^{2,3} Therefore, finding and identifying new biomarkers that can predict drug resistance is important to increase treatment outcomes.

Type III collagen alpha 1 (COL3A1) is a fibrous collagen encoding the precursor of type III collagen α -1 chain.⁴ Studies have reported that the collagen family, including COL3A1, is closely involved in carcinogenesis⁵⁻⁷ and drug resistance.⁸ Shi et al.⁹ found that METTL3 could downregulate COL3A1 level by promoting its m6A methylation, inhibiting triple-negative breast cancer (TNBC) metastasis. The upregulation of COL3A1 expression was shown to partly neutralize the effects of the miR-29 family in inhibiting

methotrexate (MTX) resistance in osteosarcoma, suggesting that COL3A1 overexpression could mediate the resistance of osteosarcoma cells to MTX.⁸ Moreover, COL3A1 expression was dramatically increased in drug-resistant ovarian cancer cells to several kinds of drugs, including DDP, MTX, paclitaxel (PAC), vincristine (VIN), doxorubicin (DOX), and topotecan (TOP), indicating that COL3A1 had important involvements in the drug resistance of ovarian cancer.¹⁰ Recently, Zhang et al.¹¹ compared COL3A1 expression between pan-cancer and normal tissues from public databases and observed that COL3A1 expression was highly upregulated in lung cancer, particularly lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), which was also related to advanced cancer stages and poor survival.

Despite vast research in this field, the implication of COL3A1 in DDP-resistant lung cancer is poorly understood. Thus, this study aimed to assess COL3A1 expression and its roles in lung cancer cells with DDP resistance via bioinformatics analysis and in vitro experiments.



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MATERIALS AND METHODS

Gene Expression Omnibus (GEO) datasets

The GSE21656 dataset was obtained from GEO (<http://www.ncbi.nlm.nih.gov/geo/>). It contained raw RNA transcriptome information on three non-resistant lung cancer (H460) and three resistant lung cancer (H460/DDP) cell lines. Similarly, GPL6244 was obtained and used for mRNA expression profiling analysis.

Differentially Expressed Genes (DEGs)

H460 and H460/DDP cell lines were screened for DEGs using R v4.0.2 software (<https://www.r-project.org/>).^{12,13} Background correction, standardization, and calculation of expression values were performed using the Affy, Impute, and limma packages of the R software. The limma package was used to normalize the median value of all samples, following which a robust multichip average was created, and the raw data were log-transformed. DEGs were identified based on thresholds of $p < 0.005$ and $|\log_2 \text{fold change}| > 1$. Pheatmap and ggplot2 were used to create a heat map and a volcano plot in R, respectively.¹⁴

Functional Enrichment

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed on Metascape (<https://metascape.org>) to identify intersecting DEGs¹⁵ using GO terms biological processes, cellular components, and molecular functions, with $p < 0.05$ denoting significance.

GEPIA and UALCAN Database

GEPIA (<http://gepia.cancer-pku.cn/index.html>) and UALCAN (<http://ualcan.path.uab.edu/>) databases were used to analyze COL3A1 expressions in LUAD and LUSC.

Survival Analysis

The influence of COL3A1 on the overall survival (OS) of patients with LUAD and LUSC was assessed with the Kaplan-Meier Plotter web tool (<https://kmplot.com/analysis/>).

Cell Culture

H1299, A549, and NCI-H1703 cells (lung cancer cell lines) and BEAS-2B (normal human lung cell line) were obtained from the American Type Culture Collection (VA, USA) and used for cell culture. PC-9 cells (BeNa Culture Collection, Beijing, China), another lung cancer cell line, were also bought. A549 was cultured in F-12K Medium. H1299 and NCI-H1703 in RPMI-1640 Medium (Thermo Fisher Scientific, MA, USA). PC-9 was cultured in a DMEM medium (Thermo Fisher Scientific) containing high glucose levels, 10% fetal bovine serum, and 1% (v/v) penicillin/streptomycin. The base medium for BEAS-2B cells can be obtained

from a BEBM kit (no. CC-3170, Lonza/Clonetics Corporation, Basel, Switzerland). All cells were kept at 37 °C in 5% CO₂ during cell culture.

Establishing DDP-Resistant Cells

Here, drug-resistant cell lines A549/DDP and H1299/DDP were obtained upon immersing A549 and H1299 cells in increasing concentration of DDP (Sigma-Aldrich, MO, USA) from 5 to 30 μM for 12 days and maintained in a complete culture medium containing 5 μM of DDP for the subsequent studies.¹⁶

Modification of Gene Expression

The scramble control (NC and shNC), lentiviral vector COL3A1 (catalog id: RC211766L3V), and COL3A1 shRNAs (shCOL3A1, catalog id: TL313797V) were bought from Origene (Beijing, China). GenePharma (Shanghai, China) synthesized the lentiviral vectors used to upregulate ZEB1 expression. Then, 5 μg/ml polybrene (Sigma-Aldrich) was used to infect the NC, COL3A1, shNC, and shCOL3A1 cells, after which they were immersed in 7 μg/mL puromycin for 2 weeks to obtain stable transfected cell lines.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was obtained using TRIzol (Invitrogen, USA), which were then converted into cDNA with the PrimeScript RT Master Mix kit (RR036A; Takara). Then, qRT-PCR was performed using a 2 x SYBR Green PCR Mastermix kit (Solarbio, Beijing, China) with the Applied Biosystems 7,500 Real-Time PCR System (Applied Biosystems, MA, USA). Table 1 shows the primers used in this study.

Western Blotting

RIPA (Solarbio, Beijing, China) and 1% protease inhibitor (Solarbio) were used to obtain total proteins. Then, 20 μg of the indicated proteins from each group were separated with 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA), blocked with 5% non-fat milk for 1 h at room temperature to prevent nonspecific bindings, followed by incubation with primary antibodies (anti-β-actin antibody, id:ab8226; dilution, 1:5000, Abcam, MA, USA; and anti-COL3A1 antibody, id: 30565; dilution, 1:2000, Cell Signaling Technology, MA, USA) at 4 °C for 15–18 h. Then, after incubation with HRP-conjugated secondary antibodies for 1 h at room temperature, ProBlot-48 (Tecan, Switzerland) was used to evaluate protein signaling following immersion in an ECL reagent (Millipore, USA), and ImageJ was used to view the proteins on the blots.

TABLE 1. Gene Primers Used in Quantitative Reverse-Transcription Polymerase Chain Reaction

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
COL3A1	TGCCCTACTGGTCCTCAGAA	AGGTATCCCAGCTGGACCTT
GAPDH	TGACAACTTGGTATCGTGGAGG	AGGCAGGGATGATGTTCTGGAGAG

Colony Formation

Approximately 0.5×10^3 cells (per well) with stable COL3A1 overexpression or downregulation were incubated on 6-well plates for 12–14 days at 37 °C, followed by staining with crystal violet for 10 min and counting the clone numbers using inverted microscopy.

Cell Counting Kit-8 (CCK-8) Assay

Approximately 4×10^3 cells (per well) with stable COL3A1 overexpression or downregulation were added onto 96-well plates and maintained for 4 h in a culture medium with 10% CCK-8 solution (Beyotime, Beijing, China) at 37 °C, after which their OD₄₅₀ values were detected using a spectrophotometer (Fisherbrand™ accuSkan™ GO UV/Vis, Thermo).

Cell Apoptosis Assessment

After cell transfection, cell apoptosis of the cell lines was determined with the FITC AnnexinV Apoptosis Detection Kit (BD Biosciences, NY, USA) following the provided manual, after which FlowJo was used to determine their apoptotic rates.

Statistical Analysis

The study experiments were independently repeated three times. IBM SPSS version 21.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses. Student t-tests and one-way analysis of variance with Tukey's test were employed. Significance was set at $P < 0.05$.

RESULTS

DEGs Associated with DDP Resistance Based on the GEO Database

DEGs between H460 and H460/DDP cells were identified using the transcriptome data of GEO. Figure 1a was the correction histogram of the removing batch. Principal component analysis (PCA) showed that the two groups could be well differentiated (Figure 1b). In total, 71 upregulated genes and 107 downregulated genes were identified in H460/DDP cells compared with H460 cells, as shown in the volcano plot (Figure 1c) and heatmap (Figure 1d). Functional enrichment analysis identified 178 DEGs mainly enriched in T-helper 1 cell cytokine production, blood vessel morphogenesis, and transmembrane receptor protein tyrosine kinase signaling pathway in both the GO (Figure 2a) and KEGG (Figure 2b) analyses.

COL3A1 Overexpression was Associated with Poor Lung Cancer Survival

COL3A1 was identified as an upregulated gene in H460/DDP cells based on the GEO database, and accumulated evidence demonstrated its key roles in drug resistance in several cancers.^{8,10} Thus, we focused on COL3A1 and explored its expression and clinical values in the prognosis of lung cancer using public databases. The UALCAN and GEPIA databases showed that COL3A1 was overexpressed in LUAD and LUSC (Figure 3a, 3b).

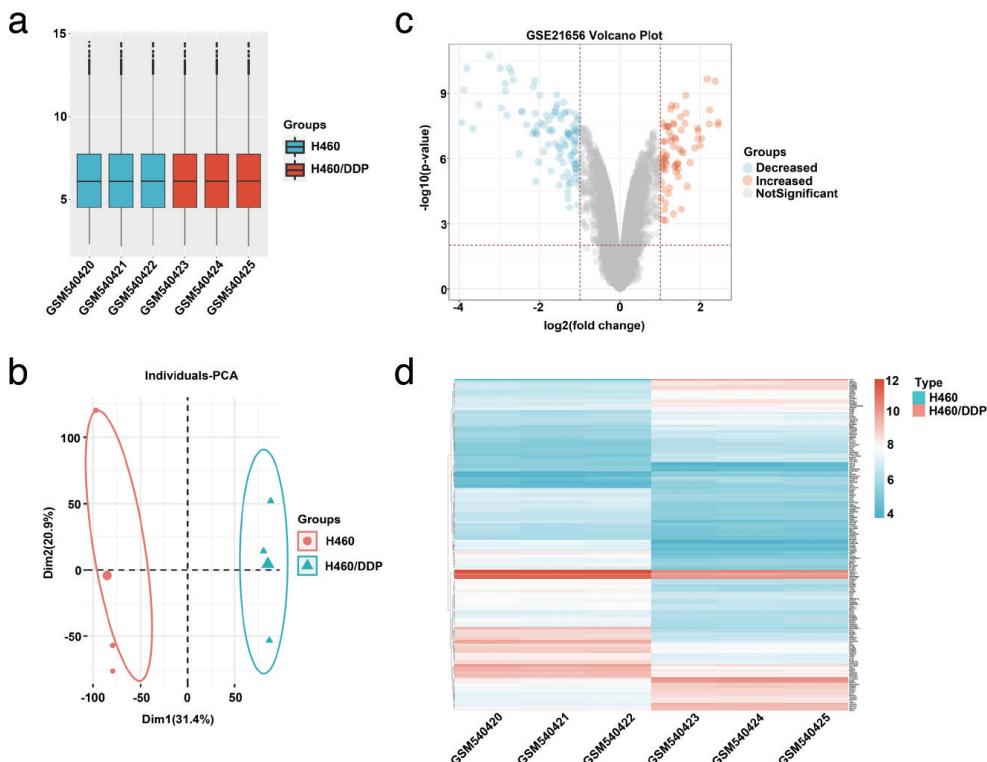


FIG. 1. Identification of differentially expressed genes (DEGs) associated with DDP resistance in lung cancer. (a) Correction histogram of removing a batch of the H460 group and the H460/DDP group. (b) PCA of the H460 group and H460/DDP group. DEGs in the volcano plot (1c) and heatmap (1d).

Survival analyses demonstrated the association of higher COL3A1 expression with lower OS rate in LUAD and LUSC (Figure 3c). These results demonstrated that COL3A1 overexpression significantly correlated with poor lung cancer survival.

COL3A1 Promoted Cancer Cell Growth

The results showed significant overexpression of COL3A1 in A549, H1299, PC-9, and NCI-H1703 than in BEAS-2B cells (Figure 4a, 4b). Then, we explored the role of COL3A1 in lung cancer development in A549 and H1299 cells in vitro because they showed a median expression level of COL3A1 in comparison with that of PC-9 and NCI-H1703 cell lines. We found that the transfection of COL3A1 increased COL3A1 expression, whereas shCOL3A1 decreased COL3A1 expression in the cells (Figure 4c). A significant increase in cell growth (Figure 4d) and clone formation ability (Figure 4e) and a significant decrease in cell apoptosis (Figure 4f) were observed in COL3A1 overexpressed A549 and H1299 cells, whereas the downregulation of COL3A1 demonstrated opposite results. These findings indicate that COL3A1 served as an oncogene in lung cancer.

COL3A1 Decreased Lung Cancer Cell Sensitivity to DDP

Further, A549/DDP and H1299/DDP cells were constructed to explore the influence of COL3A1 on modulating lung cancer

resistance to DDP. Our results showed that both resistant cell lines had significantly greater IC₅₀ values than the non-resistant A549 and H1299 cells (Figure 5a), whereas opposite results were observed upon silencing COL3A1 in A549/DDP and H1299/DDP cells (Figure 5b) and their ability to form cell clones (Figure 5c) but increased their apoptosis (Figure 5d). These results revealed that COL3A1 made lung cancer cells more resistant to DDP.

DISCUSSION

DDP can improve the survival of patients with NSCLC, but the occurrence and development of drug resistance significantly decrease its therapeutic effects.^{17,18} Thus, we explored the significance of COL3A1 in lung cancer resistance to DDP. Our results showed that COL3A1 was overexpressed in DDP-resistant lung cancer H460 cells, whereas COL3A1 downregulation sensitized lung cancer cells to DDP.

The close implication of the collagen family in tumorigenesis has been well documented.¹⁹⁻²⁴ For example, collagen prolyl 3-hydroxylation (P3H) was shown to have low expression in lung cancer, whereas its overexpression suppressed cancer cell growth, migration, and invasion, and it had pro-apoptotic roles in lung cancer.²⁵ As expected, COL3A1 has vital roles in carcinogenesis, where it serves as an oncogene.⁸⁻¹⁰ Zhang et al.²⁶ reported a

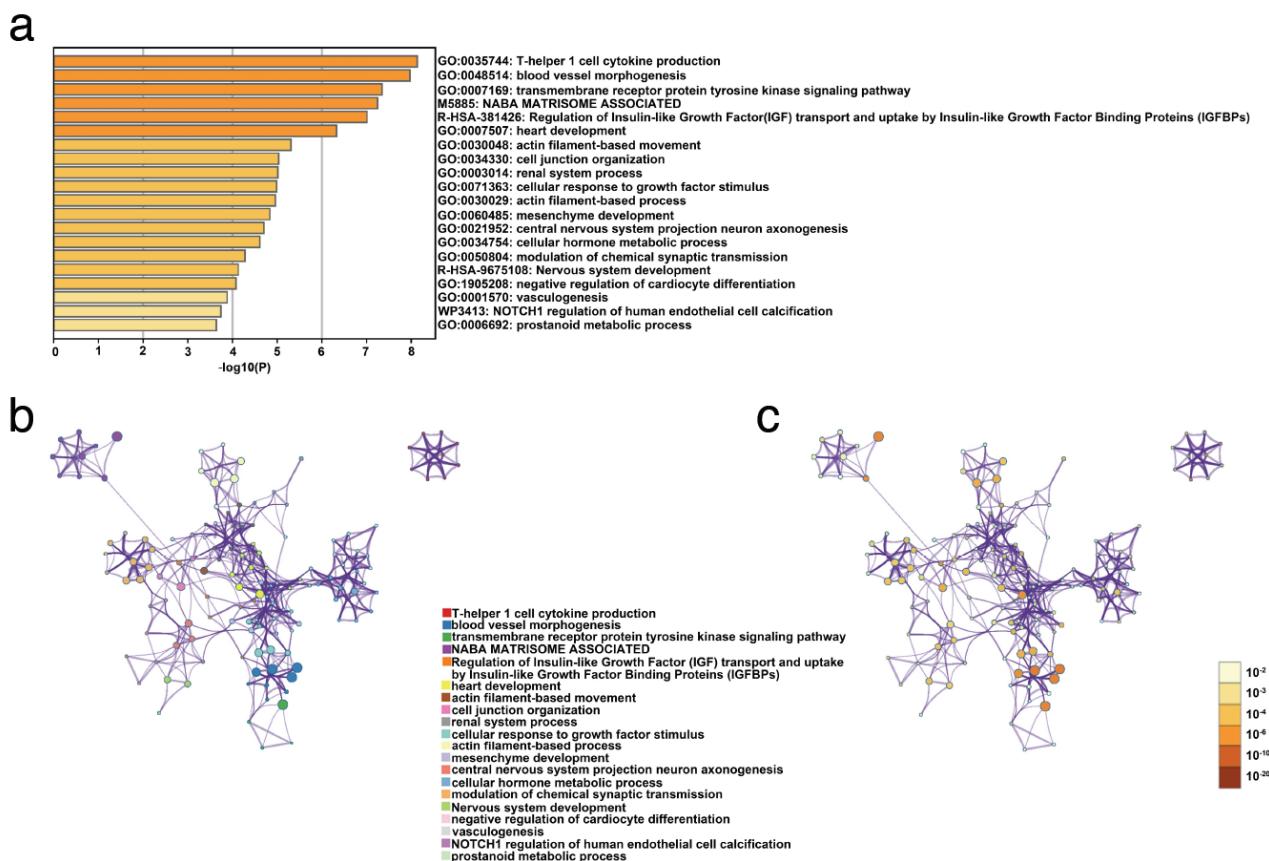


FIG. 2. Function analysis of hub genes ($p < 0.05$). (a-c) Functions of differentially expressed genes mainly enriched in the regulation of T cell chemotaxis, peptide ligand-binding receptors, and chemokine receptors bind chemokine signaling.

significant upregulation of COL3A1 expression in esophageal cancer and showed its association with advanced clinical cancer stages. In addition, COL3A1 was overexpressed in TNBC tissues and cells and was linked to a lower OS rate.²⁷ Further, COL3A1 silencing caused significant inhibitions in *in vitro* cell proliferation, invasion, migration, and immune escape and *in vivo* tumor growth in xenograft mice, suggesting that COL3A1 has an oncogenic role in TNBC. COL3A1 was overexpressed in breast cancer tissues compared with controls, and its expression was further upregulated by radiation,²⁸ indicating that COL3A1 might be implicated in regulating the radiation resistance of breast cancer. Zhou et al.²⁹ reported a significantly higher COL3A1 expression in esophageal squamous cell carcinoma (ESCC) cells than in their normal counterparts and showed that it was an independent factor associated with poor OS in univariate and multivariable analyses. Besides, COL3A1 downregulation and overexpression respectively inhibited and promoted cancer cell proliferation, migration, and invasion *in vitro* and cancer growth *in vivo*, indicating the oncogenic role of COL3A1 in ESCC. Su et al.³⁰ reported that COL3A1 overexpression could neutralize the suppressive effects of let-7d on renal cancer cell proliferation, motility, and PBMC recruitment, indicating that COL3A1 also served as an oncogene in renal cell carcinoma. Consistently, our results demonstrated COL3A1 overexpression in lung cancer cells, accelerated lung cancer cell growth and restrained apoptosis, and contributed to poor OS in lung cancer patients. Taken together,

these results confirmed that COL3A1 acted as an oncogene in lung cancer.

We also observed a significantly higher COL3A1 expression in H460/DDP cells than in its lung cancer non-resistant H460 cells, suggesting that COL3A1 regulated lung cancer sensitivity to DDP. As expected, silencing COL3A1 increased the sensitivity of A549/DDP and H1299/DDP to DDP, confirming that increased COL3A1 expression made the cancer cells more prone to DDP resistance.

Previously, COL3A1 was reported to participate in modulating the drug resistance of cancers (10, 31). COL3A1 expression was markedly elevated in DDP, PAC, DOX, TOP, VIN, and MTX-resistant ovarian cancer cell lines.¹⁰ COL3A1 upregulation partly neutralized the effects of the miR-29 family on inhibiting MTX resistance in osteosarcoma.⁸ miR-let-7b increased the sensitivity of ovarian cancer cells to Taxol by downregulating COL3A1 expression.³¹ Altogether, these studies revealed a chemoresistance-promoting role of COL3A1 in cancers, which was also confirmed in lung carcinoma. For instance, Peng et al.³² showed that collagen promoted cancer resistance to anti-PD-1/PD-L1 by promoting CD8+ T cells exhaustion. Yamazaki et al.³³ reported that collagen type I induced cancer cell resistance to EGFR-TKI in lung carcinoma. Further, collagen IV was reported to inhibit DDP-induced apoptosis in lung cancer.³⁴ Here, we demonstrated for the first time that COL3A1, a member of the collagen family, promoted DDP resistance in lung cancer.

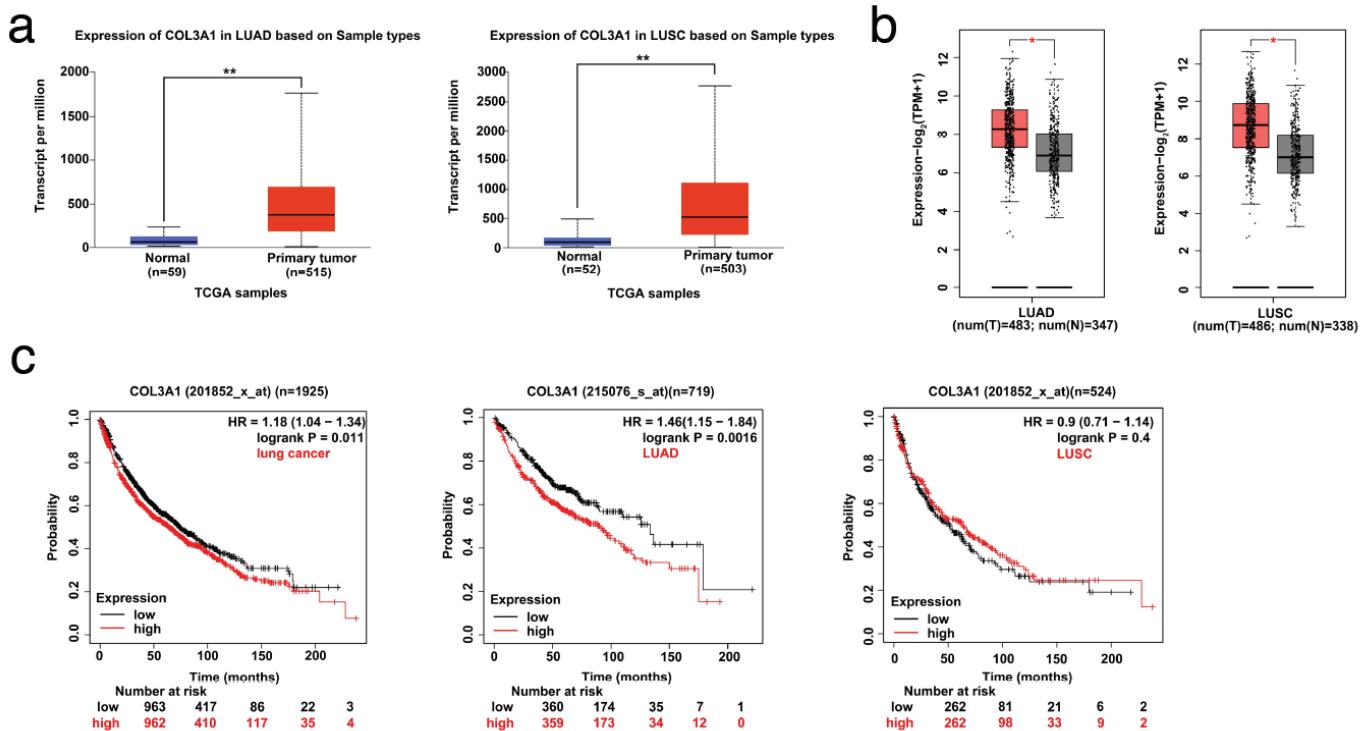


FIG. 3. COL3A1 expression is increased in lung cancer and associated with poor prognosis. Expression levels of COL3A1 in LUAD and LUSC assessed using the UALCAN (a) and GEPIA (b) databases. (c) Associations between COL3A1 expression levels and OS of patients with lung cancer, LUAD, and LUSC using kmplot.

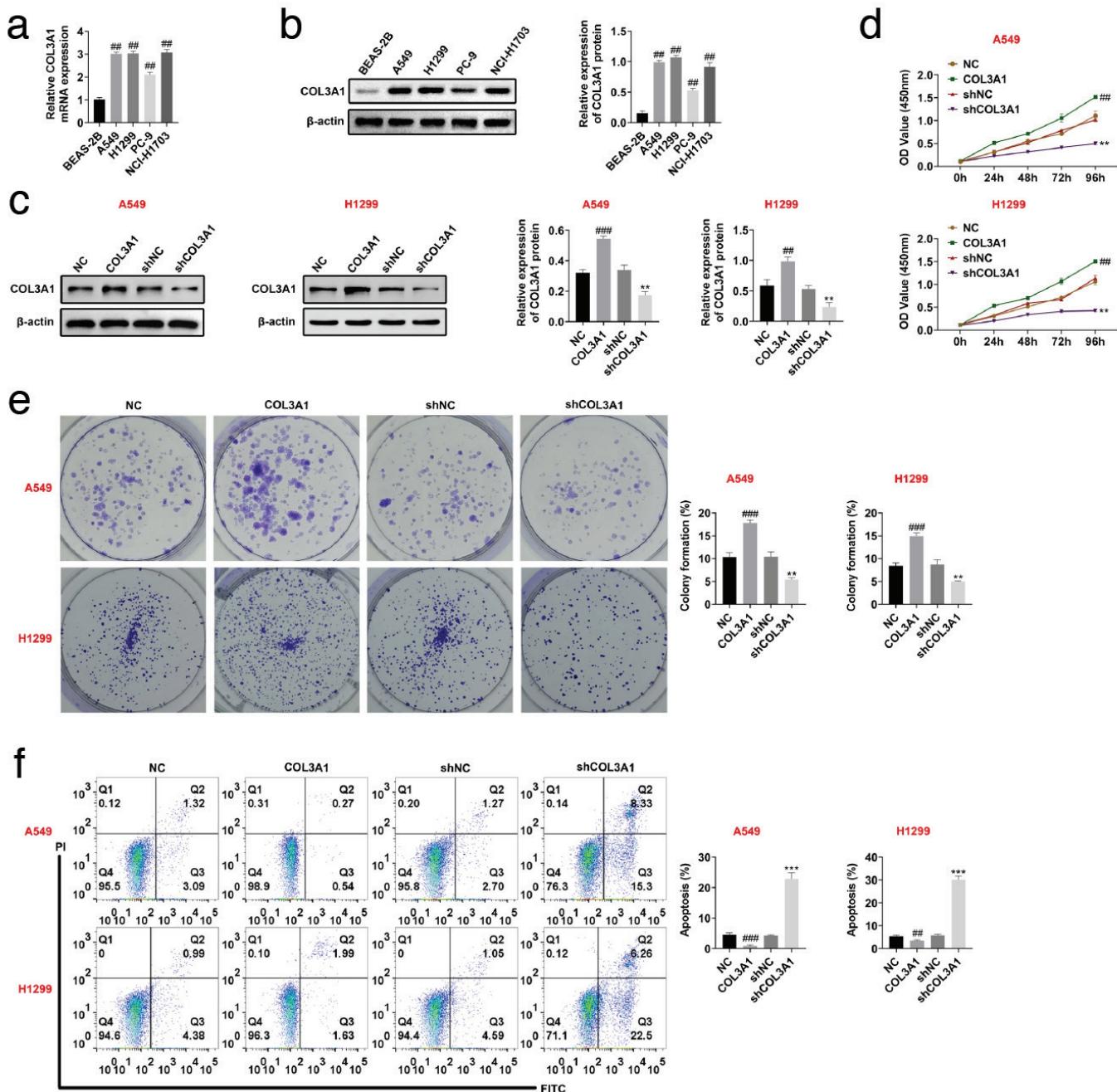


FIG. 4. COL3A1 promotes cell viability and inhibits cell apoptosis in lung cancer. The mRNA and protein levels of COL3A1 in BEAS-2B, A549, H1299, PC-9, and NCI-H1703 cells detected using qRT-PCR (a) and Western blotting (b) ($^{##}P < 0.01$). (c) Transfected efficiencies of shCOL3A1 and COL3A1 detected by Western blotting in A549 and H1299 cells ($^{##}P < 0.01$, $^{###}P < 0.001$, vs. NC group; $^{**}P < 0.01$, vs. shNC group). (d) COL3A1 effect on cell growth using CCK-8 assay ($^{##}P < 0.01$ vs. NC group; $^{**}P < 0.01$ vs. shNC group). (e) COL3A1 effect on cell viability using colony formation assay ($^{##}P < 0.01$, $^{###}P < 0.001$ vs. NC group; $^{**}P < 0.01$, $^{***}P < 0.001$ vs. shNC group). (f) Effects of COL3A1 on cell apoptosis on flow cytometry assay ($^{##}P < 0.01$, $^{###}P < 0.001$ vs. NC group; $^{**}P < 0.01$, $^{***}P < 0.001$ vs. shNC group).

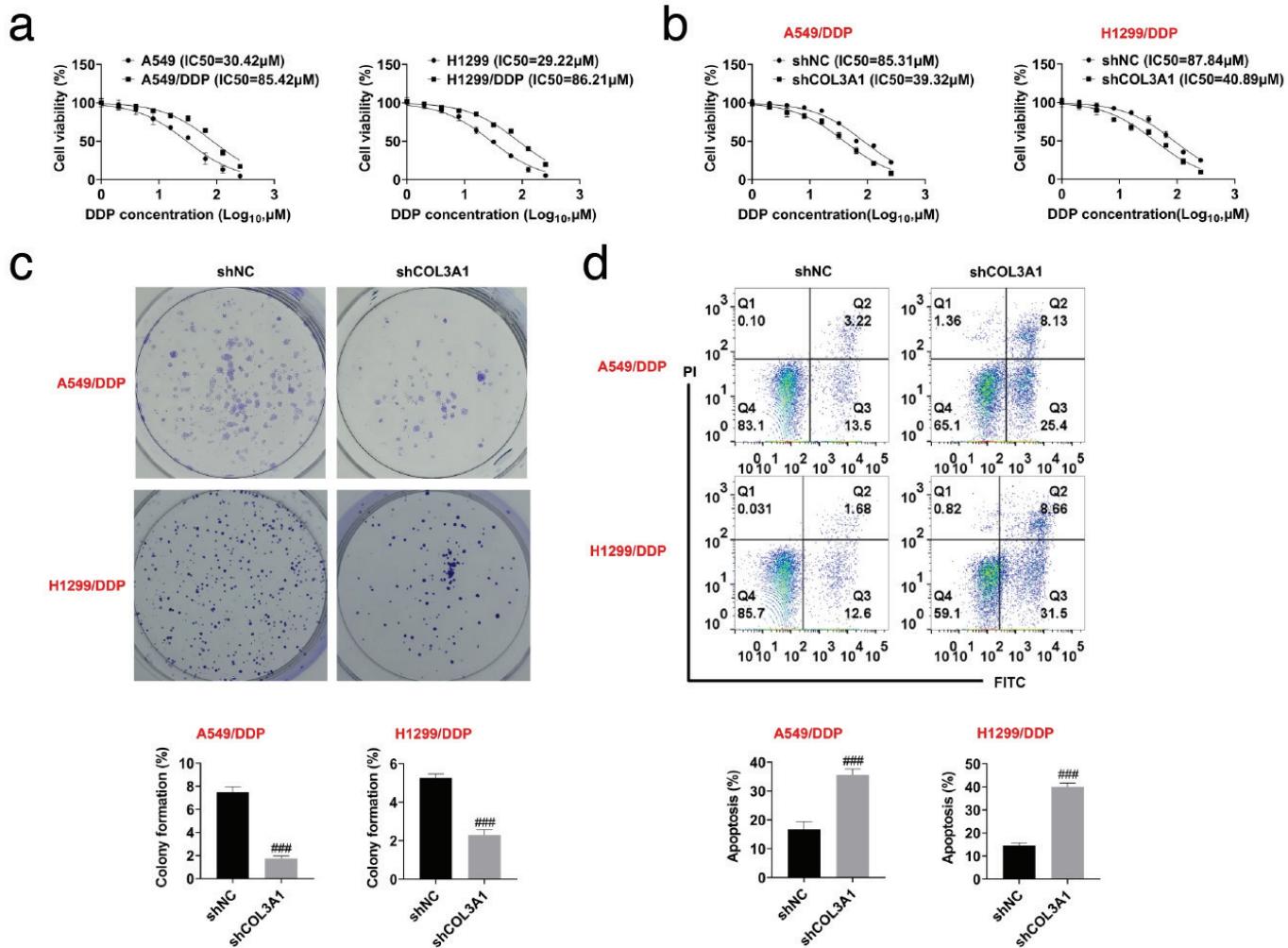


FIG. 5. COL3A1 promotes DDP resistance in lung cancer. (a) IC₅₀ of A549/DDP and H1299/DDP cells to DDP assessed using the CCK-8 assay. (b) Effects of COL3A1 downregulation on the IC₅₀ of A549/DDP and H1299/DDP to DDP assessed by the CCK-8 assay. (c) Effects of COL3A1 downregulation on the clone formation ability of A549/DDP and H1299/DDP to DDP assessed by the clone formation assay (### $P < 0.001$). (D) Effects of COL3A1 downregulation on the apoptosis of A549/DDP and H1299/DDP to DDP assessed by the flow cytometry assay (### $P < 0.001$).

This study has two main limitations. Although we demonstrated that COL3A1 could be an oncogene and promote drug resistance in lung cancer, the underlying mechanisms were not explored. In addition, the obtained study results were not validated *in vivo*.

In conclusion, this study revealed a significant increase in COL3A1 expression in lung cancer cells with DDP resistance and that COL3A1 downregulation decreased cell resistance to DDP. Taken together, targeting COL3A1 could potentially overcome DDP resistance in lung cancer.

Ethics Committee Approval: As this study is a cell culture and public database analysis, it does not require ethical approval.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Design- L.W., Y.S.; Data Collection or Processing- L.W., Y.S., Z.G.; Analysis or Interpretation- Z.G.; Writing- H.L.

Conflict of Interest: No conflict of interest was declared by the authors.

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