Original Article

Cullin7 induces docetaxel resistance by regulating the protein level of the antiapoptotic protein Survivin in lung adenocarcinoma cells

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Background: Lung adenocarcinoma (LUAD) is the most common subtype of non-small cell lung cancer (NSCLC). Chemotherapy resistance is the main cause of chemotherapy failure. Cullin7 (Cul7) is highly expressed in LUAD and is associated with poor prognosis. Moreover, Cul7 is abnormally overexpressed in docetaxel-resistant LUAD cells. Therefore, further exploration of the role and molecular mechanism of Cul7 in LUAD docetaxel resistance is necessary.

Methods: We established docetaxel-resistant cell lines (A549DTX and H358DTX cell lines) by exposing cells to gradually increasing concentrations of docetaxel. Cell (A549, A549DTX, H358, and H358DTX cell lines) sensitivity to docetaxel was determined via a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. And then quantitative polymerase chain reaction (qPCR) and Western blotting were performed to measure the expression of Cul7 and Survivin in A549, A549DTX, H358, and H358DTX cell lines. Subsequently, we knocked down *Cul7* in docetaxel-resistant cells and overexpressed *Cul7* in parental cells via lentiviral transduction to further validate the correlation between *Cul7* and docetaxel resistance, while exploring the molecular mechanism of docetaxel resistance it caused. Immunofluorescence and immunohistochemical (IHC) staining were also used to evaluate the expression and cellular localization of Cul7. To confirm the effect of Cul7 expression on cell apoptosis, we used flow cytometry to detect the apoptosis rate of A549 and A549DTX cells with the same drug concentration.

Results: Cul7 was highly expressed in A549DTX and H358DTX cells. However, when *Cul7* expression was knocked down in A549DTX and H358DTX cells, cell sensitivity to docetaxel was significantly increased. In addition, we found that Cul7 was coexpressed with *Survivin*. Silencing *Survivin* reversed the docetaxel insensitivity caused by *Cul7* overexpression. High expression of Cul7 and Survivin in docetaxel-resistant

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LUAD cells inhibited the intrinsic apoptosis pathway and promoted cell proliferation. Therefore, the Cul7/Survivin axis may play a role in inducing LUAD docetaxel chemoresistance.

Conclusions: Cul7 and Survivin were both highly expressed in docetaxel-resistant LUAD cells. Our results suggest that Cul7 may inhibit apoptosis and promote the proliferation of LUAD cells by increasing the Survivin protein level, which in turn contributes to docetaxel chemoresistance in LUAD.

Keywords: Lung adenocarcinoma (LUAD); *Cullin*7 (*Cul*7); Survivin; apoptosis

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Introduction

Lung cancer is the malignancy with the highest mortality in men and women, with more than 350 people dying of lung cancer every day (1). Non-small cell lung cancer (NSCLC) accounts for nearly 85% of lung cancer cases, and lung adenocarcinoma (LUAD) is one of the most common pathological subtypes of NSCLC (2,3). Docetaxel exerts a significant antitumor effect as an important first-line and second-line drug. It is widely used in the treatment of NSCLC, but docetaxel resistance is common, which greatly affects its long-term efficacy. Studies have shown that defects in apoptosis contribute to docetaxel resistance, and changes in the levels of apoptosis-related proteins (Survivin, BCL-2, p53, etc.) are closely related to cell sensitivity to docetaxel (4,5). To improve the prognosis of patients with LUAD, obstacles to an effective treatment response must be identified and overcome.

Cullin7 (Cul7), an important member of the Cullin protein family, is the core scaffold protein of the Cullin E3 ligase complex, thereby participating in substrate selection and degradation of proteins (6). Cul7 plays an important role in cell transformation, cell cycle regulation, aging and apoptosis through its ubiquitin ligase activity (7). Studies have shown that *Cul*7 is highly expressed in breast cancer, lung cancer and hepatocellular carcinoma and is closely related to the occurrence and development of various malignant tumors (8-12). Abnormal expression of *Cul*7 can lead to disrupted microtubule dynamics, tissue mitosis and cytokinesis. Moreover, knocking down *Cul*7 can enhance the sensitivity of lung cancer cells to paclitaxel (13,14). Survivin is the substrate protein of the Cullin9 (Cul9) E3 ubiquitin ligase enzyme, and it can be ubiquitinated and degraded by Cul9. Moreover, Cul7 can inhibit Cul9-mediated ubiquitination and degradation of Survivin, and knocking down *Cul*7 reduces the level of the Survivin protein (15). Therefore, we hypothesized that *Cul*7 can lead to docetaxel resistance in LUAD by regulating the level of the Survivin protein.

In our present study, docetaxel-resistant LUAD cell lines (A549DTX and H358DTX) were established through drug selection screening, and we explored the mechanism by which docetaxel resistance develops in LUAD. We found that Cul7 may play an important role in LUAD docetaxel resistance. Meanwhile, Cul7 was coexpressed with Survivin and may promote the occurrence of docetaxel resistance in LUAD by increasing the protein level of Survivin. These results are helpful to clarify the molecular mechanism of docetaxel resistance in LUAD and provide a theoretical basis for the reversal of drug resistance and clinical treatment in LUAD. We present this article in accordance

Highlight box

Key findings

- Cullin7 (Cul7) may lead to docetaxel resistance in lung adenocarcinoma (LUAD) by increasing the Survivin protein level.

What is known and what is new?

- Cul7 is highly expressed in various tumors, including LUAD, and is associated with poor prognosis.
- In our study, Cul7 was abnormally overexpressed in docetaxel-resistant LUAD cells. Moreover, we further validated the correlation between Cul7 and docetaxel resistance in LUAD and identified the Cul7 target protein Survivin. High expression of Cul7 and Survivin in docetaxel-resistant LUAD cells inhibited the intrinsic apoptosis pathway and promoted cell proliferation.

What is the implication, and what should change now?

- These results indicate that Cul7 might inhibit the activation of the intrinsic apoptotic pathway and cell proliferation by upregulating the protein level of Survivin in LUAD docetaxel resistance. Cul7 may become a potential target for overcoming docetaxel resistance in LUAD and help guide new strategies for personalized clinical treatment with docetaxel.

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Methods

Cell culture and establishment of cell lines with acquired docetaxel resistance

LUAD cell lines (H1299, A549 and H358 cell lines) were purchased from the Typical Culture Preservation Commission Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were confirmed to be free of mycoplasma contamination. The A549DTX, H358DTX and H1299DTX cell lines were obtained from Professor Wang Luo, University of Michigan, USA. The parental and drug-resistant LUAD cell lines were cultured in RPMI 1640 medium (H1299, H1299DTX, H358 and H358DTX cells) or F12K medium (A549 and A549DTX cells) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin at 37 ℃ in a humidified atmosphere containing 5% CO_2. Docetaxel-resistant A549, H358 and H1299 cell lines were established by continuous exposure to increasing concentrations of docetaxel, and the cells were grown in the presence of a 5 nM final concentration of docetaxel (Invitrogen, USA, Cat: #114977-28-5).

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA, Cat: #15596018) according to the manufacturer's instructions. Reverse transcription of mRNA was performed using a RevertAid RT Kit (Invitrogen, Cat: #K1691). qPCR analysis of cellular mRNA was performed with an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems, Cat: #4368577) according to the manufacturer's instructions. The following primer sequences were used: (I) *Cul7*, forward sequence 5'-AGCAAAAGGATATACCAGGAG-3' and reverse sequence 5'-TCCGTCTCTTCTCCAAGTTC-3'; (II) *Survivin*, forward sequence 5'-CAGATTTGAATCGCGGGACCC-3' and reverse sequence 5'-CCAAGTCTGGCTCGTTCTCAG-3'; and (III) *GAPDH* forward sequence 5'-AGCCACATCGCTCAGACA-3' and reverse sequence 5'-GCCCAATACGACCAAATCC-3'.

Western blot analysis

Cells were lysed in radio-immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, USA, Cat: #V900854) containing Phosphatase Inhibitor 2 (Sigma-Aldrich, USA, Cat: #P5726), Phosphatase Inhibitor 3 (Sigma-Aldrich, Cat: #P0044), a protein inhibitor cocktail (Sigma-Aldrich, Cat: #P8340) and 100× PMSF (Sigma-Aldrich, Cat: #329-98-6) at a ratio of 100:1:1:1:1. The protein concentration was measured with a BCA assay kit (Beyotime, China, Cat: #P0010) and a Multiskan GO Spectrum spectrophotometer (Thermo Fisher Scientific, USA). Fifty micrograms of protein lysate was added to each well for protein separation by 8–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were separated on the basis of their molecular weight and then transferred onto 0.22-μm-thick Immuno-Blot polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were then blocked with 5% evaporated milk in TBST for 1.5 h at room temperature and subsequently incubated with primary antibodies (anti-Cul7, Sigma-Aldrich, c1743, 1:2,000; anti-Survivin, Cell Signaling Technology, USA, 2808, 1:1,000; anti-BCL-2, Cell Signaling Technology, 15071, 1:1,000; anti-Bax, Cell Signaling Technology, 2774, 1:1,000; anti-Caspase3, Cell Signaling Technology, 9662, 1:1,000; anti-cleaved-Caspase3, Cell Signaling Technology, 9664, 1:1,000; anti-p21, Cell Signaling Technology, 2947, 1:1,000; anti-p27, Cell Signaling Technology, 3686, 1:1,000; anti-CyclinD1, Cell Signaling Technology, 2978, 1:1,000; anti-β-actin, Sigma-Aldrich, A1978, 1:2,000) at 4 ℃ overnight. Then, the membranes were incubated with a secondary antibody [anti-rabbit IgG, horseradish peroxidase (HRP)-linked, Cell Signaling Technology, 7074, 1:10,000; or anti-mouse IgG, HRP-linked, Cell Signaling Technology, 7076, 1:10,000] for 1 h at room temperature after washing three times with TBST. β-actin was used as the protein loading control. The membranes were then incubated with chemiluminescence solutions for 3 min. Protein bands on the membranes were visualized using a MiniChemi chemiluminescence imaging system (Beijing Sage Creation Science Co., Ltd., China) or developed on X-ray films by using an automatic X-ray film processor (Kodak Corporation, X-OMAT2000, USA).

Immunofluorescence staining

To determine cellular localization and measure the expression of Cul7, cells were seeded onto round adherent coverslips and then fixed with 4% paraformaldehyde for 20 min at room temperature. For better immunofluorescence staining, cells were permeabilized with 0.1% Triton X-100

for 20 min at room temperature. Then, the slides were blocked with 0.5% BSA blocking solution at 37 ℃ for 30 min. The cells were further incubated with a primary antibody (1:500, Sigma-Aldrich, Cat: #HPA030095) at 4 ℃ overnight. Next, the cells were incubated with a secondary antibody (1:300, anti-rabbit Alexa Fluor 488, Thermo Fisher Scientific, Cat: #A11008) for 1 h at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 1 μg/mL; Sigma-Aldrich, Cat: #D9542) by incubation for 5 min at room temperature in the dark. The slides were covered with Fluoromount medium consisting of sulfate and 10% glycerol at a ratio of 1:3 and then imaged as soon as possible under a fluorescence microscope in the dark.

Lentiviral transduction, gene silencing and siRNA interference

The lentivirus-based human *Cul*7 shRNA construct (5'-GCACATGTTGAGTAGTCCTGATTAT-3') and negative control (NC) shRNA construct (5'-TTCTCCGAACGTGTCACGT-3') were ordered from Gemma Biotechnology Co., Ltd. (Shanghai, China). To obtain cells with stable knockdown of *Cul*7 and NC cells, cell clones were selected using G418 reagent (Gibco, USA, Cat: #11811023) for at least 1 week. The pcDNA3 mammalian expression vector containing the full-length human *Cul*7 cDNA sequence was a generous gift from Dr. Y. Xiong (Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, USA). LUAD cell lines (A549 and H1299) were transfected using Lipofectamine 2000 reagent (Invitrogen, USA, Cat: #11668019). LUAD cell lines stably overexpressing *Cul*7 were screened with G418 reagent (Gibco, USA, Cat: #11811023). The overexpression efficiency of Cul7 was evaluated by qPCR and Western blotting.

Survivin-targeting small interfering RNA *(siRNA-Survivin)* constructs were synthesized by and purchased from Gemma Technology Co., Ltd. The *siRNA-Survivin* sequences were as follows: (I) *Survivin-semilaevis-233*, sense 5'-CCACUGUCCCAGUGAGAAUTT-3' and antisense 5'-AUUCUCACUGGGACAGUGGTT-3'; (II) *Survivin-semilaevis-303*, sense 5'-GAACCAGACGAUGACCCAUTT-3' and antisense 5'-AUGGGUCAUCGUCUGGUUCTT-3'; and (III) *Survivin-semilaevis-488*, sense 5'-CCACAUCUUAGAGAGUCUATT-3' and antisense 5'-UAGACUCUCUAAGAUGUGGTT-3'. H1299-HACul7 cells were seeded into 6-well plates (2×10^5 cells/well) and transfected according to the manufacturer's method. Cellular RNA and protein were extracted after transfection with *siRNA-Survivin* for 24 and 48 h. The interference efficiency was verified by qPCR and Western blotting.

Cell proliferation assay

Cells were processed into single-cell suspensions (2×10^4 cells/mL) and seeded into 96-well plates. When the cells had completely adhered to the wells, the medium was replaced with fresh medium containing various concentrations of docetaxel. After 24, 48 and 72 h of treatment, cell viability was determined after incubation with MTS reagent (Promega, Cat: #3581) for 2 h. The absorbance was measured at 490 nm using a Multiskan GO Spectrum spectrophotometer (Thermo Fisher Scientific). The data were plotted using GraphPad Prism 5 software (GraphPad Software, USA).

Immunohistochemical (IHC) staining

Paraffin embedded tissue sections were obtained from the patients with LUAD from the Affiliated Hospital of Hebei University between 2020 to 2021. Tissue sections were conventionally processed by dewaxing and clearing with xylene and an alcohol gradient, and then, heat-induced antigen repair procedures were performed. IHC staining was performed according to the instructions for the IHC detection kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Cat: #PV-9000). After washing three times with PBS, the slides were incubated with primary antibodies (anti-Cul7, 1:200, Sigma-Aldrich, Cat: #HPA030095; anti-Survivin, 1:500, Cell Signaling Technology, Cat: #2808) for 3–4 h at 37 ℃. Subsequently, the slides were incubated with an HRP-conjugated secondary antibody for 20 min at 37 ℃. After washing three times with phosphate-buffered saline (PBS), a DAB kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Cat: #ZLI-9017) was used for staining, which was conducted by incubation in the dark. Images showing IHC staining were assessed by two pathologists who were blinded to the tumor and clinical information. Images of Cul7 and Survivin IHC staining were analyzed using Image-Pro Plus software (Media Cybernetics Image Technology Co., Ltd., USA) and assessed on the basis of the mean staining intensity (IOD SUM/area). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of the Affiliated Hospital of Hebei University (No. HDFY-

LL-2020-136) and informed consent was taken from all individual participants.

Flow cytometry

For apoptosis analysis, cells were treated with 5 nM docetaxel for 48 h and then stained with a FITC Annexin V apoptosis detection kit (BD Biosciences, USA, Cat: #556547). A cell suspension of 10^6 cells/mL was prepared with 1× binding buffer, and 100 µL of the cell suspension was removed for staining with 5 µL of FITC annexin V and 5 µL of propidium iodide (PI) by incubation for 15 min in the dark at room temperature. After adding 400 µL of binding buffer, apoptotic cells were detected with a BD Accuri C6Plus flow cytometer (Becton, Dickinson and Company, USA).

Statistical analysis

All data are presented as the mean ± standard error values and were analyzed with SPSS 19.0 statistical software (IBM Corporation, USA). Differences were analyzed for significance by two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) depending on the number of groups. A P value of less than 0.05 was considered to indicate significance. All experiments were repeated independently 3 times.

Results

Cul7 is upregulated in docetaxel-resistant LUAD cells

Stable docetaxel-resistant LUAD cell lines (A549DTX and H358DTX) were established in our laboratory. The MTS assay data showed that the sensitivity of the A549DTX and H358DTX cells to docetaxel was significantly decreased compared with that of the parental cells (A549 and H358) (*Figure 1A,1B*), and the half maximal inhibitory concentration (IC_{50}) values were significantly increased compared with those in the parental cells (A549 and H358) (A549 *vs.* A549DTX: 3.58±0.67 *vs.* 34.38±7.23, P=0.017; H358 *vs.* H358DTX: 8.72±1.21 *vs.* 62.27±7.49, P=0.005) (*Figure 1C,1D*). The drug resistance of A549DTX and H358DTX cells was increased by 10- and 7-fold, respectively. The drug resistance of H1299DTX cell was not stable, but the IC_{50} values was increased by 6-fold at least compared to the parent cell H1299 (H1299 *vs.* H1299DTX: 9.2±1.41 *vs.* 104.51±73.27, P=0.087) (Figure S1A,S1B). Moreover, we found that the mRNA and protein expression levels of Cul7 were significantly increased in A549DTX, H358DTX and H1299DTX cells (*Figure 1E,1F*, Figure S1C,S1D). Furthermore, we obtained the same results in the immunofluorescence experiment and found that the nuclear localization of Cul7 was significantly increased (*Figure 1G*). These results suggest that upregulated Cul7 expression might be associated with docetaxel chemosensitivity in LUAD cells.

Cul7 reduces the sensitivity of LUAD cells to docetaxel

To determine whether *Cul7* is associated with docetaxel resistance, we knocked down *Cul7* expression in A549DTX and H358DTX cells. The results of qPCR and Western blot analyses showed that Cul7-knockdown cells (A549DTX-shCul7 and H358DTX-shCul7) were successfully established (*Figure 2A,2B*). Subsequently, the MTS assay data showed that the sensitivity of A549DTX-shCul7 and H358DTX-shCul7 cells to docetaxel was significantly increased compared with that of the A549DTX-shNeg and H358DTX-shNeg cells (*Figure 2C,2D*) and that the IC_{50} values were significantly decreased (A549DTX-shNeg *vs.* A549DTX-shCul7: 32.238±4.58 *vs.* 9.951±0.96, P=0.011; H358DTX-shNeg *vs.* H358DTX-shCul7: 72.271±5.78 *vs.* 14.247±0.15, P=0.003) (*Figure 2E,2F*) compared with those in A549DTX-shNeg and H358DTX-shNeg cells. Moreover, the protein expression level of Cul7 was increased in a dose-dependent manner when A549 and H1299 cells were exposed to docetaxel (*Figure 2G*). To further determine the correlation between Cul7 and docetaxel chemoresistance, we overexpressed *Cul7* in A549 and H1299 cells. The mRNA and protein expression of Cul7 was clearly increased in A549-HACul7 and H1299-HACul7 cells compared with A549-pCDNA3 and H1299-pCDNA3 cells, as shown by qPCR and western blotting (*Figure 3A,3B*). Moreover, the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay data showed that the sensitivity of A549-HACul7 and H1299-HACul7 cells to docetaxel was significantly decreased compared with that of A549-pCDNA3 and H1299-pCDNA3 cells (*Figure 3C,3D*) and that the IC_{50} values were significantly increased (A549-pCDNA3 *vs.* A549-HACul7: 6.32±1.3 *vs.* 19.64±1.4, P<0.001; H1299-pCDNA3 *vs.* H1299-HACul7: 14.76±2.34 *vs.* 29.73±3.12, P=0.003) (*Figure 3E,3F*) compared with those in A549-pCDNA3 and H358-pCDNA3 cells. These results indicate that the expression level of Cul7 affects the sensitivity of LUAD cells to docetaxel, indicating a close relationship between Cul7 expression and drug resistance.

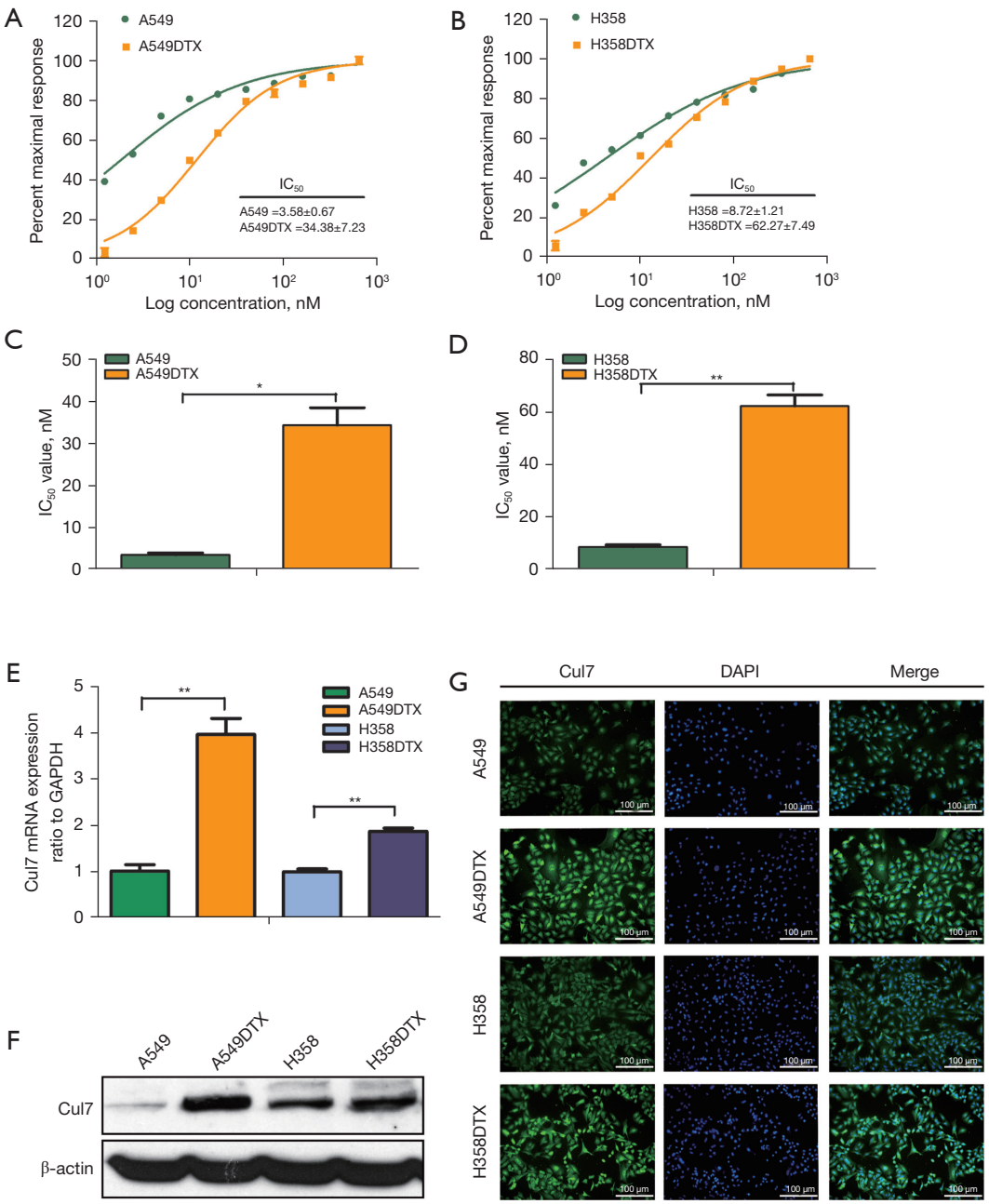

Figure 1 Docetaxel-resistant lung adenocarcinoma cell lines show decreased docetaxel sensitivity and increased Cul7 expression. (A,B) A549, A549DTX, H358, and H358DTX cells were seeded into 96-well plates at 2×10^3 cells/well, and medium containing various docetaxel concentrations (0, 1.25, 2.5, 5, 10, 20, 40, 80, 160, 320, 640 nM) was added. The cells were cultured for 72 h. Cell viability was evaluated by the MTS method, and the data were analyzed by GraphPad Prism 5 software. Data are presented as the mean ± standard error. (C,D) The IC_{50} values obtained via the MTS method in three independent experiments are presented in the form of a histogram. (E,F) The mRNA and protein expression of Cul7 in A549, A549DTX, H358 and H358DTX cells was measured by qPCR and Western blotting. (G) The expression and localization of Cul7 were evaluated by an immunofluorescence assay. *, P<0.05; **, P<0.01. IC_{50}, half maximal inhibitory concentration: Cul7, Cullin7; DAPI, 4',6-diamidino-2-phenylindole; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; qPCR, quantitative polymerase chain reaction.

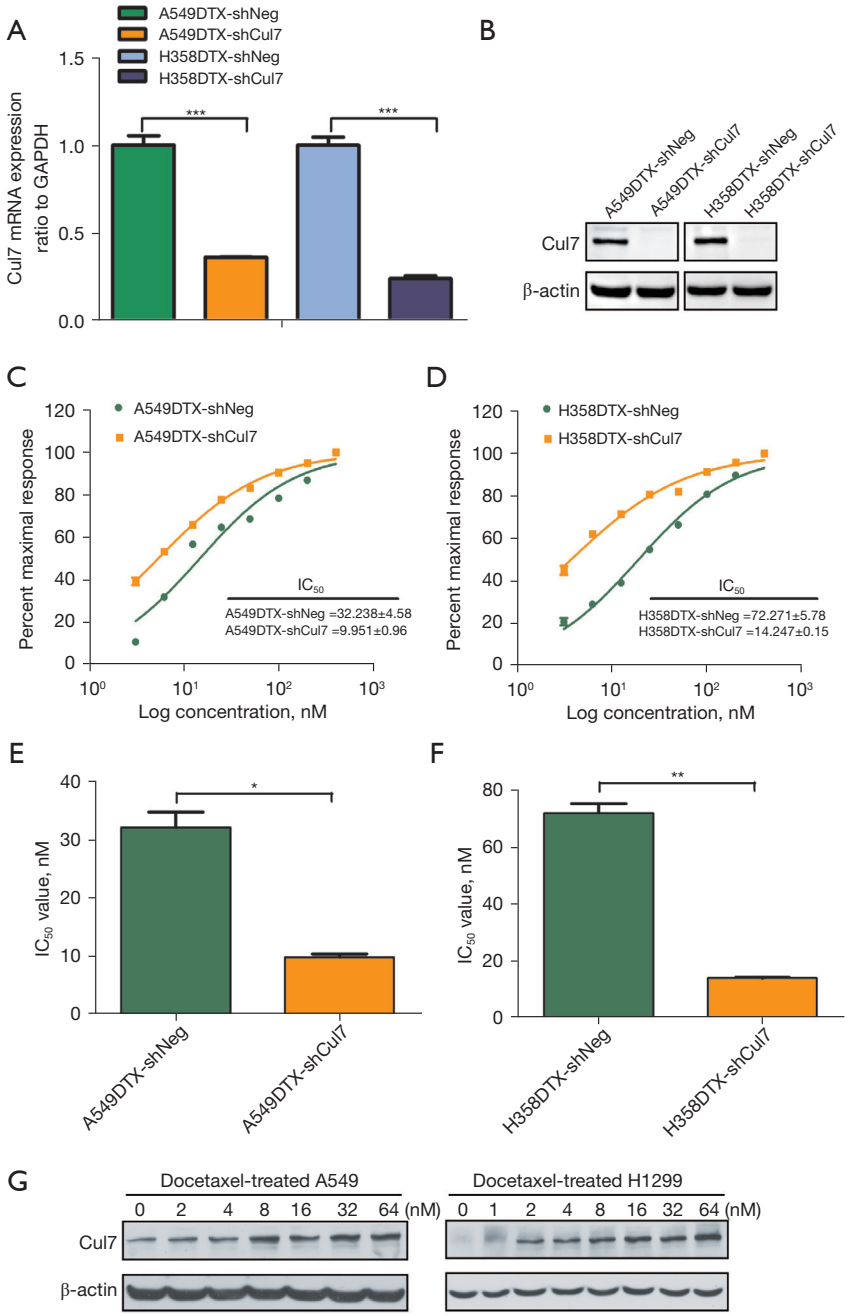

Figure 2 Downregulation of Cul7 expression promotes increased docetaxel sensitivity. (A,B) Cul7 was knocked down in A549DTX and H358DTX cells, as verified by qPCR and Western blotting. (C,D) The viability of A549DTX-shNeg, A549DTX-shCul7, H358DTX-shNeg and H358DTX-shCul7 cells treated with various docetaxel concentrations (0, 3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 nM) was determined by the MTS method, and the data were analyzed by GraphPad Prism 5 software. Data are presented as the mean ± standard error. (E,F) The IC_{50} values obtained via the MTS assay in three independent repeated experiments are presented in the form of a histogram. (G) A549 and H1299 cells were treated with various docetaxel concentrations (0, 2, 4, 8, 16, 32, and 64 nM) for 48 h, and protein levels were measured by western blotting. *, P<0.05, **, P<0.01, ***, P<0.001. Cul7, Cullin7; IC_{50}, half maximal inhibitory concentration; qPCR, quantitative polymerase chain reaction; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

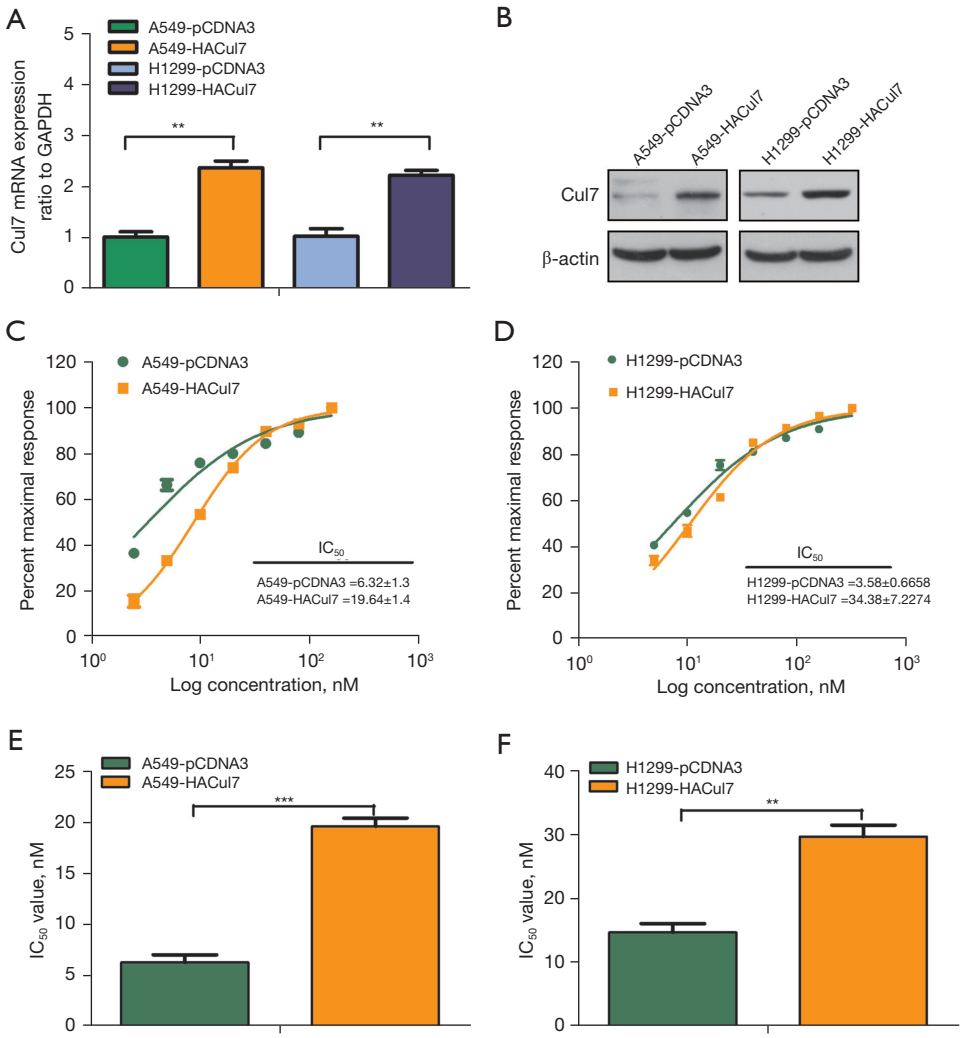

Figure 3 High expression of Cul7 resulted in decreased docetaxel sensitivity in LUAD cell lines. (A,B) *Cul7* was overexpressed in A549 and H1299 cells, as verified by qPCR and Western blotting. (C,D) The viability of A549-pCDNA3, A549-HACul7, H1299-pCDNA3 and H1299-HACul7 cells treated with various docetaxel concentrations (0, 5, 10, 20, 40, 80, 160, and 320 nM) was evaluated by the MTS method, and the data were analyzed by GraphPad Prism 5 software. Data are presented as the mean ± standard error. (E,F) The IC_{50} values obtained via the MTS assay in three independent repeated experiments are presented in the form of a histogram. **, P<0.01; ***, P<0.001. Cul7, Cullin7; IC_{50}, half maximal inhibitory concentration; LUAD, lung adenocarcinoma; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

Cul7 decreases LUAD cell sensitivity to docetaxel by upregulating the protein level of the antiapoptotic protein Survivin

In a follow-up study, we found that Cul7 and the antiapoptotic protein Survivin were simultaneously highly expressed in A549DTX, H358DTX and H1299DTX cells (*Figure 4A*, Figure S1E). We also found that *Cul7* silencing reduced Survivin protein level and that *Cul7* overexpression increased Survivin protein level (*Figure 4B*). However, neither knockdown nor overexpression of *Cul7* caused a change in the *Survivin* mRNA expression level (*Figure 4C,4D*). These results suggested that Cul7 may regulate the protein level of Survivin. Instantaneous Acute silencing of *Survivin* reversed the docetaxel insensitivity caused by high *Cul7* expression in H1299-HACul7 cells (*Figure 4E-4G*). Chemosensitivity was significantly increased, as determined by comparing the IC_{50} values in

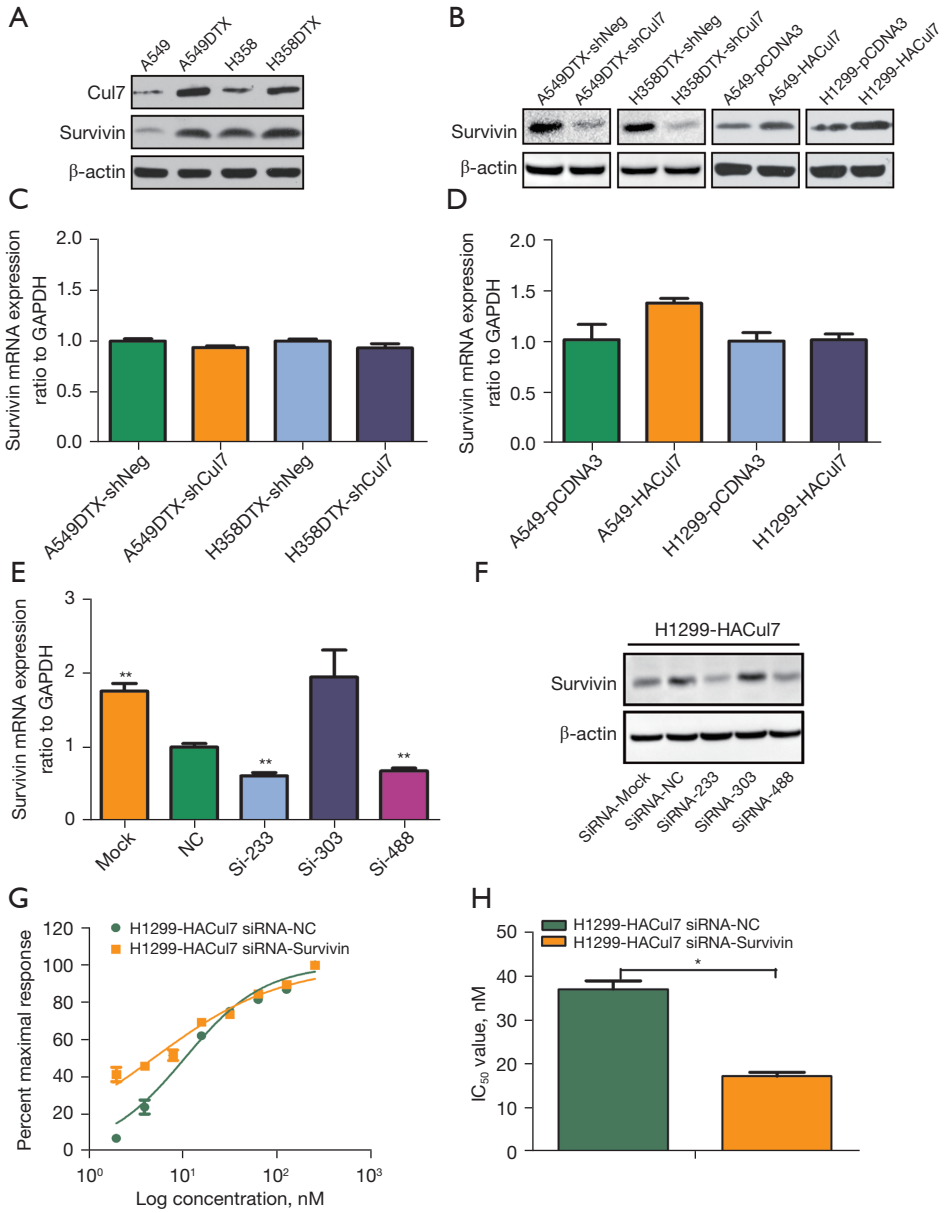

Figure 4 Cul7 regulates the protein level of Survivin to influence the sensitivity of LUAD cells to docetaxel. (A) Western blotting was performed to measure the protein expression of Cul7 and Survivin in A549, A549DTX, H358 and H358DTX cells. (B) Western blotting was performed to measure the protein level of *Survivin* in A549DTX-shNeg, A549DTX-shCul7, H358DTX-shNeg, H358DTX-shCul7, A549-pCDNA3, A549-HACul7, H1299-pCDNA3 and H1299-HACul7 cells. (C,D) qPCR was performed to measure the mRNA expression of Survivin in A549DTX-shNeg, A549DTX-shCul7, H358DTX-shNeg, H358DTX-shCul7, A549-pCDNA3, A549-HACul7, H1299-pCDNA3 and H1299-HACul7 cells. (E,F) *Survivin* expression was acutely knocked down in H1299-HACul7 cells, as verified by qPCR and Western blotting and siRNA233-Survivin small interfering RNA was selected for subsequent experiments. (G) The viability of H1299-HACul7 siRNA-NC and H1299-HACul7 siRNA-*Survivin* cells treated with various docetaxel concentrations (0, 2, 4, 8, 16, 32, 64, 128, and 256 nM) was evaluated by the MTS method, and the data were analyzed by GraphPad Prism 5 software. (H) The IC_{50} values obtained via the MTS method in three independent repeated experiments are presented in the form of a histogram. *, P<0.05; **, P<0.01. Cul7, Cullin7; IC_{50}, half maximal inhibitory concentration; LUAD, lung adenocarcinoma; qPCR, quantitative polymerase chain reaction; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

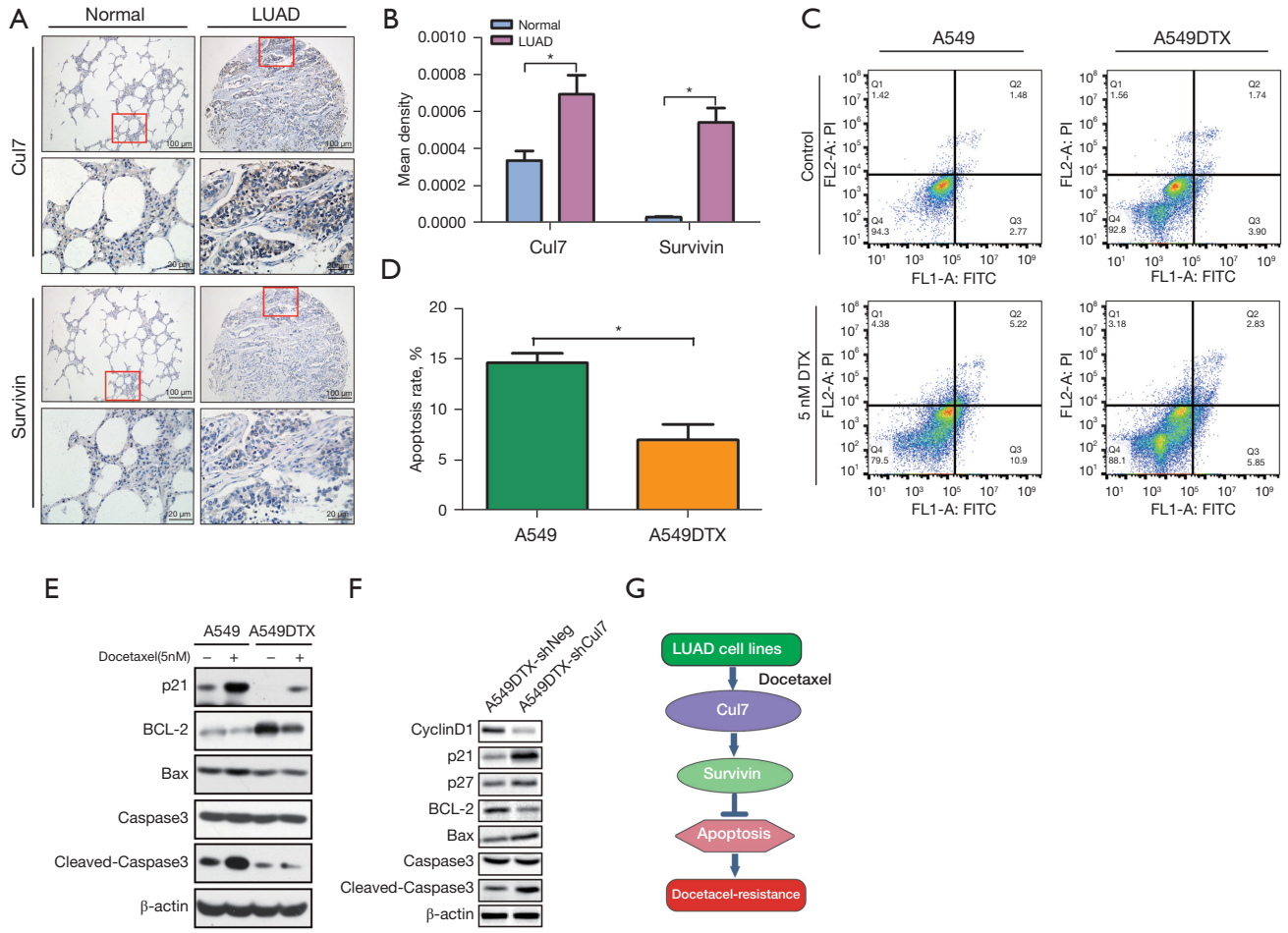

Figure 5 Apoptosis is inhibited in docetaxel-resistant LUAD cells via the Cul7/Survivin axis. (A,B) IHC staining was performed to evaluate the protein expression of Cul7 and Survivin in normal and LUAD tissues, and the mean staining intensity values were determined with Image-Pro Plus software. (C,D) Flow cytometry was performed to determine the apoptosis rates of A549 and A549DTX cells treated with docetaxel (0, 5 nM), and the apoptosis rate data were analyzed by GraphPad Prism 5 software and presented in the form of a histogram. (E) Western blot analysis of apoptosis-related proteins in A549 and A549DTX cells treated with docetaxel (0, 5 nM). (F) Western blot analysis of cell cycle- and apoptosis-related proteins in A549DTX-shNeg and A549DTX-shCul7 cells. (G) Diagram showing the docetaxel resistance acquisition mechanism in LUAD. The red box represents the position of the tissue slice corresponding to the enlarged image; *, P<0.05. LUAD, lung adenocarcinoma; Cul7, Cullin7; IHC, immunohistochemical.

H1299-pCDNA3 siRNA-NC and siRNA-Survivin cells (37.1±2.67 *vs.* 17.24±1.28, P=0.029) (*Figure 4H*). Therefore, we inferred that Cul7 might affect the docetaxel sensitivity of LUAD cells by regulating the protein level of Survivin.

Cul7 promotes cell proliferation and inhibits apoptosis via Survivin in docetaxel-resistant LUAD cells

Cul7 was coexpressed with Survivin in LUAD cells. The IHC staining results showed that Cul7 and Survivin were also coexpressed in LUAD tissues (*Figure 5A*). The mean staining intensity values representing the expression of Cul7 and Survivin were higher in LUAD tissues than in normal lung tissues (Cul7: P=0.036 and Survivin: P=0.022) (*Figure 5B*). The flow cytometry data also showed that the apoptosis rate of A549DTX cells was significantly lower than that of A549 cells treated with the same docetaxel concentration (5 nM) (*Figure 5C,5D*) (P=0.014). Meanwhile, there was no significant upregulation of Bax and Cleaved-Caspase3 protein expression was observed in A549 DTX

cells stimulated with docetaxel (5 nM) comparing to A549 cells (*Figure 5E*). These results suggested that Cul7 may inhibit apoptosis by increasing the level of the antiapoptotic protein Survivin in docetaxel-resistant LUAD cells. Moreover, the protein expression data showed that the level of the antiapoptotic protein BCL-2 was increased and the levels of the apoptotic proteins Bax and Cleaved-Caspase3 were decreased in A549DTX cells comparing to A549 cells (*Figure 5E*), suggesting inhibition of the intrinsic apoptotic pathway in A549DTX cells. The level of the proliferation-related protein p21 was decreased in A549DTX cells (*Figure 5E*). In addition, we verified changes in cell proliferation- and apoptosis pathway-related protein levels in A549DTX-shNeg and A549DTX-shCul7 cells. When Cul7 was knocked down in A549DTX cells, the protein levels of CyclinD1 and BCL-2 were decreased, and the protein levels of p21, p27, Bax and Cleaved-Caspase3 were increased (*Figure 5F*), suggesting cell-cycle arrest and activation of the intrinsic apoptotic pathway. As shown in the schematic in *Figure 5G*, Cul7 may promote cell proliferation and inhibit apoptosis by increasing the level of the antiapoptotic protein Survivin, leading to docetaxel resistance in LUAD cells.

Discussion

In this study, we found that Cul7 was abnormally and highly expressed in A549DTX, H358DTX and H1299DTX cells. To determine the correlation between Cul7 and docetaxel resistance, we knocked down *Cul7* in docetaxel-resistant cells (A549DTX and H358DTX) and overexpressed Cul7 in the corresponding parental cells (A549 and H1299). The results suggested that Cul7 expression was positively correlated with insensitivity to docetaxel chemotherapy in LUAD cells. Next, we found that Survivin and Cul7 were simultaneously overexpressed in A549DTX, H358DTX and H1299DTX cells and that Survivin protein level was downregulated or upregulated with knockdown and overexpression, respectively, of Cul7. Subsequently, inhibiting *Survivin* expression reversed the docetaxel resistance caused by the high expression of *Cul7* in H1299-HACul7 cell. Moreover, the results of flow cytometry and Western blotting confirmed our hypothesis that the Cul7/Survivin axis promotes the insensitivity of LUAD cells to docetaxel by inhibiting the activation of the intrinsic apoptotic pathway based on experimental results from A549 and A549DTX. These results provide insight suggesting that the Cul7/Survivin axis may be an effective target for LUAD treatment.

Cul7 is a member of the Cullin protein family and functions as a scaffold protein for E3 ubiquitin ligases, which interact with the small ring finger protein ROC1 (also called RBX1). It has been reported that 3-M syndrome-related mutant genes (*Cul7*, *OBSL1*, *CCDC8*) and *FBXW8* can form the 3-M complex, affecting joints. The 3-M complex pathway and the downstream molecule *Cul9* play important roles in maintaining microtubule and genome integrity in cells (14,16). *Cul7* knockdown does not affect the localization of the microtubule organization center (MTOC), whose disruption leads mainly to abnormalities in microtubule dynamics and blockade of mitosis and cytokinesis. Mutation of genes encoding the 3-M complex proteins makes cells sensitive to microtubule damage. In addition, knocking down *Cul7* can significantly enhance the sensitivity of lung cancer cells (NCI-H1155) to paclitaxel (14). In addition, a study has shown that Cul7 can induce epithelial-mesenchymal transition in human choriocarcinoma cells (17). Meanwhile, epithelial-mesenchymal transition is an important mechanism leading to docetaxel resistance in NSCLC (18). Another study showed that Cul7 is highly expressed in NSCLC and that high Cul7 expression is positively correlated with poor prognosis (10). These results suggest that Cul7 plays an important role in the dynamic stability of microtubules and may be related to docetaxel resistance. Our results showed that Cul7 was highly expressed in docetaxel-resistant LUAD cells and caused docetaxel resistance. Therefore, our inference was supported.

Survivin is a member of the inhibitor of apoptosis protein (IAP) family, whose members play important roles in apoptosis and the cell cycle (19). Overexpression of Survivin mRNA and protein is found in a variety of malignancies and is associated with tumor initiation, invasion, poor prognosis and treatment resistance (20,21). Notably, Survivin small molecule inhibitors (YM155 and LQZ-7F) can synergize with docetaxel to increase docetaxel efficacy in various cancers (5,22). However, it is not clear whether Survivin can induce docetaxel resistance in LUAD cells. *Cul9* is highly structurally homologous to *Cul7* and can directly bind to *p53*. *Cul9* is a tumor suppressor gene, and *Cul9*-knockout mice spontaneously develop multiple organ tumors (23). Studies have shown that *Cul9* is a downstream molecule in 3-M complex signaling pathways (15,23). Survivin is a substrate protein of the Cul9 E3 ligase and can be ubiquitinated and marked for degradation by Cul9. Cul7 can inhibit Cul9-mediated ubiquitination and degradation of Survivin. Moreover, knocking down *Cul7* decreases the

level of the Survivin protein. Overexpression of *Survivin* reverses the abnormalities in microtubule dynamics and mitotic dysfunction caused by *Cul7* knockdown (15). Considering these results, we can identify the regulatory effect of *Survivin* on microtubules and determine that Survivin may be a key molecule downstream of Cul7. In our study, we confirmed that Cul7 regulates the protein level of Survivin, inducing docetaxel resistance in LUAD cells. This finding suggests that the Cul7-Survivin pathway may play an important role in maintaining microtubule stability, genome integrity and docetaxel resistance. However, the targeted mechanism by which Cul7 regulates Survivin in LUAD docetaxel resistance is still unclear, and further exploration in subsequent studies is needed.

To date, Cul7 and Survivin have been shown to play important roles in tumor development by inhibiting apoptosis and promoting proliferation. The data show that Cul7 inhibits apoptosis and promotes cell proliferation in a p53-dependent manner (24-26). However, there is a lack of experimental evidence to indicate that Cul7 induces p53 degradation through direct polyubiquitination of p53. Therefore, the specific reason for this phenomenon is not clear. Cul7 is overexpressed in breast cancer, lung cancer and hepatocellular carcinoma cell lines. When *Cul7* is silenced, the expression of CyclinD1, p27 and p21 is increased, leading to inhibited cell proliferation (8,9,11,27). It is worth mentioning that some studies have proven that Survivin is negatively regulated by wild-type p53 and induces apoptosis in a p53-dependent manner (19,28-30). In addition, we found that the upregulation and downregulation of Cul7 expression could lead to the corresponding changes of Survivin protein level in wild-type p53 A549 cell or in p53-null H358 and H1299 cells. Therefore, it is not clear whether Cul7 can lead to the accumulation of Survivin by inhibiting the expression of p53, and the specific molecular mechanism remains unknown and is worthy of further exploration. As Survivin is a key antiapoptotic molecule, its overexpression can simultaneously inhibit the intrinsic and extrinsic apoptotic pathways (31). It can partially inhibit the expression of Bax and specifically bind to the termini of the cell death executor proteases caspase-3 and caspase-7 to inhibit their activity and confer resistance to apoptosis induced by specific stimuli (32). In addition, robust activation of caspase-3 and an increase in the apoptosis rate can be observed in cancer cells when Survivin expression is downregulated or inhibited (33-36). In our study, when cells were stimulated with docetaxel (5 nM), BCL-2 was upregulated and the protein levels of p21, Bax, and Cleaved-Caspase3 were decreased in A549DTX cells, in contrast to A549 cells. Consistent with these results, we knocked down Cul7 expression, which led to increases in the protein levels of p21, p27, Bax, and cleaved caspase-3 and downregulation of cyclin D1 and BCL-2 expression. Therefore, we speculated that Cul7 might inhibit the activation of the intrinsic apoptotic pathway and cell proliferation by upregulating the protein level of Survivin in LUAD docetaxel resistance. These results further expand our understanding of a novel mechanism of docetaxel resistance in LUAD and help guide new strategies for personalized clinical treatment with docetaxel.

Conclusions

In conclusion, our study demonstrated that Cul7 might play a critical role in LUAD docetaxel resistance, potentially by upregulating Survivin protein level to inhibit the activation of the intrinsic apoptotic pathway and cell proliferation, promoting the development of drug resistance. Understanding the precise role of Cul7 in docetaxel resistance in LUAD would contribute to guiding novel LUAD treatment strategies to improve patient prognosis.

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Footnote

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uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-1110/coif). XL is from GZ Runsheng CytoMed Technology Co. Ltd. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of the Affiliated Hospital of Hebei University (No. HDFY-LL-2020-136) and informed consent was taken from all individual participants.

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