CDCA attenuates LUAD pathogenesis progression via integrin $\alpha 5 \beta 1$ /FAK/p53 signalling pathway

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Research

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Abstract

Background

Lung cancer is the leading cause of cancer-associated death and includes non-small-cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC comprises approximately 80–85% of all lung cancers. Chenodeoxycholic acid (CDCA), one of the primary bile acids, has been reported to inhibit proliferation in carcinoma cells. To date, the role of CDCA in the migration, invasion and apoptosis of LUAD remains unknown.

Methods

Western blotting and quantitative real-time PCR were used to test the protein and mRNA expression levels in LUAD cell lines. Cell Counting Kit-8 (CCK-8) and clone formation assays were performed to evaluate the proliferation ability of different kinds of cells *in vitro*. Transwell assay were utilized to assess the motility of tumor cells. RNA-seq was performed to identify the transcriptional profile in A549 cells after treatment with CDCA. A xenograft model was established to study the effect of CDCA on LUAD growth *in vivo*. GraphPad Prism 8 software was used for statistical analysis, and a P value < 0.05 was considered statistically significant.

Results

CDCA can significantly inhibited the migration and invasion capabilities of LUAD cells. Furthermore, CDCA promoted the progression of apoptosis in LUAD cells. Mechanistically, we revealed that CDCA blocked the integrin $\alpha 5 \beta 1$ signalling pathway by inhibiting the expression of integrin $\alpha 5$ and $\beta 1$ subunits and p-FAK in LUAD cells. Furthermore, we found that CDCA increased the levels of p53, a downstream gene of the integrin $\alpha 5 \beta 1$ /FAK pathway.

Conclusion

Our findings indicated that CDCA attenuates LUAD pathogenesis via the integrin $\alpha 5 \beta 1$ /FAK/p53 axis in LUAD cells.

Background

Lung cancer is the leading cause of cancer-associated death, according to the latest epidemiological data, consisting of more than 50 histomorphological subtypes[1–3]. Over the past few decades, NSCLC and SCLC have been the most frequently used diagnostic terms for lung cancer, and NSCLC comprises approximately 80–85% of all lung cancers and is mainly divided into LUAD, approximately 40–50% of cases, and LUSC, approximately 20–30% of cases, comprising the predominant histological types[2, 3]. Clinically, most NSCLC patients are diagnosed at an advanced or metastatic stage, and the 5-year survival rate of NSCLC patients remains at 10% [4, 5]. Advances in the knowledge of pathways and the development of targeted drugs to disrupt the activities of the pathways in recent years have greatly

improved the curative effect and the quality of life for NSCLC patients[6, 7]. In LUAD, several targetable major pathways, including EGFR, integrin α V β 1/FAK and PI3K/AKT, have been identified, and the corresponding inhibitors have displayed clinical benefits[8–12]. Unfortunately, while targeted therapy with specific inhibitors in LUAD has provided disease control, tumours inevitably develop drug resistance[13]. Understanding resistance mechanisms and developing combinational therapies are essential for improving treatment outcomes.

Bile acids are natural detergents that allow lipids to be digested in the intestinal lumen, and they play a major role in the maintenance of bile flow and intestinal homeostasis. Depending on the nature of chemical structures, different bile acids exhibit distinct biological effects[14]. CDCA (Fig. 1), one of the primary bile acids, has been reported to induce apoptosis through oxidative stress in BCS-TC2 cells and hepatocellular carcinoma cells[15, 16]. Moreover, E.O. Im and colleagues demonstrated that CDCA treatment leads to proliferation arrest in breast cancer cells by upregulating the cyclin-dependent kinase inhibitor, p21, and downregulating the cell cycle-associated proteins, cyclin D1 and cyclin D3[17]. In contrast, CDCA inhibits EGF-induced Tam-resistant breast cancer growth by inducing the activities of farnesoid X receptor (FXR)[18].

Integrins, a series of heterodimeric cell-surface molecules that consist of α and β subunit chains, are the most important mediators of extracellular-intracellular signal transduction, and they are widely known to be involved in cancer survival, metastasis and invasion[10, 19, 20]. There are 18 α and 8 β subunits, including integrin α 5 β 1, a classic receptor for the transmission of extracellular signalling to intracellular signaling in mammals[21, 22]. Following the activation of integrin α 5 β 1, focal adhesion kinase (FAK), one of the main integrin-associated signalling and adaptor molecules, is autophosphorylated at tyrosine 397 (Y397), leading to the activation of multiple biological-associated processes and downstream signalling pathways[23–25]. FAK has been reported to be upregulated in several advanced-stage solid tumours and has been described to promote tumour progression[26–28]. Increasing evidence has revealed that FAK has an important role in regulating the proliferation of tumour cells by modulating cyclin D1, p27, MAPK and other cell cycle-related proteins[29, 30]. Chan et al. demonstrated that FAK protects MDCK cells from UV-induced apoptosis through activation of the PI3K/Akt signalling pathway[31]. Moreover, FAK inhibits apoptosis processes by interacting with death domain kinase receptor-interacting protein (RIP), a component of the death receptor complex in programmed cell death, and blocking the function of the death domain of RIP[32]. The p53 tumour suppressor, the most commonly mutated gene in cancers, has been reported to affect cell survival, apoptosis and proliferation arrest by modulating multiple regulatory signals[33–35]. Nevertheless, wild-type p53 is generally maintained in an inactive or a low state due to the interference of several molecules or primary degradation by MDM2[36]. Moreover, p53 has been reported to promote cell migration[37, 38], and FAK has been reported to physically interact with p53. In addition, the phosphorylation of FAK at Y397 has been reported to increase the activity of MDM2, which subsequently facilitates p53 ubiquitination[39]. In continuation of our previous study and the development of novel small molecule drugs for the therapy of LUAD[40], we elucidated the antitumour effects of CDCA in LUAD cells and the underlying mechanisms in the present study. In brief, we revealed

that CDCA inhibits EMT, migration and invasion or promotes apoptosis of LUAD cells through the integrin/FAK/p53 axis.

Methods

Chenodeoxycholic acid (CDCA)

Chenodeoxycholic acid (CDCA, purity greater than 98%) was obtained and purified from the bile of geese in our laboratory as described by Z. W. Yan [41], and it was identified by ^1H NMR and ^{13}C NMR.

Cell culture

The human lung cancer cell lines, A549 and H1650, were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 1% penicillin/streptomycin and 10% foetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.), at 37 °C in a 5% CO₂ atmosphere.

Cell viability assay

A549 and H1650 cells were seeded into 96-well plates at a density of 2×10^3 cells per well in 100 μl of medium with 10% FBS and cultured for 24 hours. Different concentrations of CDCA were then added to the medium for 24 hours followed by CCK8 treatment. The viability of cells was measured at 450 nm by a spectrophotometer (Bio–Rad, CA, USA).

Western blot assay

Total protein of A549 and H1650 cells was collected and extracted using RIPA lysis buffer (Jinkelong Biotechnology Co., Ltd., Beijing, China), containing protease inhibitor cocktail and a protein phosphatase inhibitor (Beyotime Institute of Biotechnology, Haimen, China), followed by centrifugation at 10,000 g for 10 min at 4 °C. Following electrophoresis, the samples were then transferred to PVDF membranes, and the membranes were incubated with rabbit anti-integrin $\alpha 5$, rabbit anti-integrin $\beta 1$, rabbit anti-FAK and rabbit anti-p-FAK primary antibodies (Cell Signaling Technology, Inc.) overnight at 4 °C. The membranes were then incubated with the indicated secondary antibodies (Santa Cruz Biotechnology, Inc., SC-2005) for 1 h at room temperature. Detection was performed using an electrochemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.). β -actin (Santa Cruz Biotechnology, Inc.) was used as an internal control.

RNA sequencing

After treatment with CDCA at a concentration of 0.4 mM or in total medium containing 1% DMSO for 24 h, cells were lysed with 1 ml of RNAiso Plus (TaKaRa, Osaka, Japan). RNA sequencing was performed on the Illumina HiSeqXTen sequencing platform (NovelBio Bio-Pharm Technology Co. Ltd., Shanghai). The dosing group and control group were evaluated in a 3:3 ratio.

Quantitative real-time polymerase chain reaction (qRT–PCR)

For the detection of apoptosis-related gene expression in A549 and H1650 cells regulated by CDAC, we first extracted total RNA using TRIzol reagent (Invitrogen, CA, USA), and the RNA concentration was determined by a NANODROP 2000 (NanoDrop, Wilmington, DE, USA). Total RNA was reverse transcribed into cDNA using the PrimeScript RT Reagent Kit (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Quantitative real-time PCR (qRT–PCR) experiments were conducted using a SYBR Green Premix Ex Taq II kit (Takara, Japan) according to the manufacturer's instructions. The primers were synthesized by the Shanghai Biotechnology Company, and the corresponding sequences of the primers are shown in Table 1. Each group was composed of three duplicate wells. The relative mRNA expression levels were normalized to β -actin, and the $\Delta\Delta C_t$ method was applied to calculate the relative quantity of mRNA.

Cell apoptosis

For the detection of apoptosis induced by CDCA, an apoptosis kit (BD) and flow cytometry or fluorescence microscopy were used. In short, for flow cytometry detection, trypsin digestion was terminated by the addition of the original complete medium, and the harvested cells were centrifuged at 1000 g for 5 min. Then, 100 μ l of binding buffer containing 5 μ l of Annexin V-FITC and propidium iodide (PI) was added to the resuspended cells at room temperature for 15 min in the dark, and apoptosis was detected using flow cytometry (Thermo Fisher Scientific, Waltham, MA, USA) and analysed using FlowJo V10.0.7 (BD). For fluorescence microscopy detection, cells were washed twice with PBS, and diluted Hoechst 33342 and PI were added followed by incubation for 20 min at 4 °C. Imagers were then acquired by fluorescence microscopy.

Wound-healing assay

A wound-healing assay was performed to analyse cell migration. Briefly, A549 and H1650 cells (1.0×10^5) were seeded into 6-well plates and cultured to 100% confluence. Subsequently, the complete medium was replaced with serum-free RPMI-1640 medium, and cells were then incubated for 6 h at 37 °C. The confluent cell monolayer was then scratched with a sterile 100 μ l pipette tip and treated with CDCA. The wounds were visualized at 0 h and after incubation with CDCA for 12 hours and 24 hours using an inverted microscope, and images were acquired at 40 $\times$ magnification.

Transwell assay

A549 and H1650 cells were added to the upper chamber of the Transwell for the migration assay. For the invasion assay, the upper chamber was precoated with Matrigel. The lower chamber contained 800 μ l of medium with 20% FBS. Subsequently, cells were treated with CDAC for 24 hours. Cells in the lower chamber were fixed with methanol for 20 min and then stained with crystal violet. The stained cells were counted under an inverted microscope and imaged at 100 $\times$ magnification.

Immunofluorescence

A549 and H1650 cells were plated onto coverslips and treated with CDAC for 24 h. Cells were fixed with 5% paraformaldehyde solution and incubated with 1% Triton X-100 PBS solution for 20 min at room temperature. After washing with PBS, nonspecific binding sites were blocked with 5% nonfat milk in PBS for 1 hour at room temperature. Cells were then incubated with the anti-p-FAK primary antibody overnight at 4 °C. After washing, cells were incubated with secondary antibody solution (Alexa Fluor® 633 goat anti-rabbit) for 1 h in the dark at room temperature. Nuclei were stained with DAPI (1 µg/mL) for 15 min at room temperature. Images were then acquired using a Leica SP8 confocal microscope under standardized conditions.

Molecular docking

To simulate the molecular-level interaction between integrin $\alpha 5\beta 1$ and its potential inhibitor, CDCA, a molecular docking simulation was performed using the AutoDock-vina (version 1.1.2) program. The three-dimensional structure of integrin $\alpha 5\beta 1$ was downloaded from the Protein Data Bank (PDB ID: 4wk4), and the ligand structure of CDCA was constructed and energy-minimized by the Chemoffice package. Before docking simulation, crystal ligands and water molecules in integrin $\alpha 5\beta 1$ were removed by PyMOL 1.7, and AutoDockTools was used to prepare the pdbqt files of the protein and ligand for docking. The docking box was centred at the interface of the α - and β -subunits with the box dimension set to 32 Å in all directions. All other docking parameters were set as default, and the pose with the lowest affinity value was selected for further analysis.

Molecular dynamics (MD) simulation

MD was performed using the sander module implemented in the Amber 18 suite with the ff14SB force field used for the protein system and the GAFF force field used for the ligand. Hydrogen atoms and sodium ions (to neutralize charge) were added to the protein with the leap utility. The simulation system was immersed in a truncated octahedral box full of TIP3P explicit water extended 10 Å outside the protein on all sides. The initial structure of the complex was treated as follows: (a) water molecules and counter ions were relaxed to minimize energy during 10,000 minimization steps (5,000 steepest descent steps, SD; and 5,000 conjugate-gradient steps, CG) with the protein and ligand restrained with a force constant of 500 kcal/mol•Å²; and (b) the whole system was then minimized without restraints during 10,000 minimization steps (5,000 SD and 5,000 CG). After energy minimization, the system was gradually heated in the NVT ensemble from 0 to 300 K over 50 ps using the Berendsen coupling algorithm. This procedure was followed by 50 ps of NPT simulations at 300 K and 1 atm pressure using the Langevin dynamics algorithm. After equilibration, a 10000 ps production MD simulation was performed. A time step of 2.0 fs was used, and coordinates of the system were saved every 20 ps. All processing and trajectory analyses were performed using CPPTRAJ or VMD programs.

In vivo tumour xenograft animal model

Female BALB/c nude mice (3–4 weeks old weighing 16–20 g) were obtained from the Experimental Animal Center of Soochow University. A total of 1.5×10^6 A549 cells were inoculated subcutaneously into the flanks, and the female mice were randomly divided into the following two groups (six mice per group): a DMSO control group and a CDCA group ($50 \text{ mg} \cdot \text{kg}^{-1}$). When the tumour weight reached nearly 100 mm^3 , DMSO or CDCA was administered to mice every 4 days via intraperitoneal injection. Tumour growth was evaluated by volume (V), which was calculated using the following formula: $V = L (\text{tumour length}) * W (\text{tumour width})^2 / 2$.

Statistical analysis

Results are presented as the mean $\pm$ SD. All statistical analyses were performed with GraphPad Prism 5.02 (GraphPad Software) and SPSS 16.0 software (SPSS, Inc.). The statistical significance of differences among multiple groups was evaluated using one-way ANOVA. Significant differences between two groups (parametric) were analysed using Student's t-test, and significant differences between two groups (non-parametric) were analysed by the Mann-Whitney U test. $P < 0.05$ was considered to indicate a statistically significant difference. All experiments were repeated three times independently.

Results

1. CDCA inhibits lung adenocarcinoma cell viability

To better understand the antitumour role of CDCA, we examined the effect of increasing doses of CDCA on lung adenocarcinoma cell viability by CCK-8 assay. As shown in Fig. 2A-C, CDCA significantly inhibited lung carcinoma cell viability in a concentration-dependent manner but not in bronchial epithelial BEAS-2B cells. Moreover, the half-maximal inhibitory concentration (IC₅₀) of CDCA was 1.0 mM in BEAS-2B cells, 0.35 mM in A549 cells and 0.36 mM in H1650 cells (Fig. 2D-F). Collectively, these results suggested the potential anti-LUAD function of CDCA.

2. CDCA promotes apoptosis in lung adenocarcinoma

To investigate the effect of CDCA on apoptosis, A549 and H1650 cells were treated with various concentrations of CDCA for 24 h, stained with Hoechst 33342/PI and imaged using a fluorescence microscope. The results showed significantly enhanced apoptosis percentages with increasing CDCA concentrations (Fig. 3A, B). To further explore the effects of CDCA on apoptosis, we performed flow cytometry analysis using Annexin V-FITC/PI staining. As shown in Fig 3C and D, a significantly increased number of apoptotic cells was detected following the increase in the concentration of CDCA (Fig. 3C, D). These results suggested the critical role of CDCA in inducing apoptosis and indicated the role of CDCA in the lethality of lung adenocarcinoma.

3. CDCA suppresses the EMT, migration and invasion in LUAD

To investigate the role of CDCA in the progression of LUAD cell epithelial-mesenchymal transition (EMT), migration and invasion, we treated A549 and H1650 cells with CDCA. Subsequently, the mesenchymal markers, N-cadherin and Snail, as well as the epithelial marker, E-cadherin, were analysed. As shown in Fig. 4A, CDCA treatment significantly decreased the expression of mesenchymal markers (N-cadherin and Snail) but significantly increased the expression of the typical epithelial marker, E-cadherin. Subsequently, a wound-healing assay was performed to investigate whether CDCA affects migration in LUAD cells. The results showed that CDCA significantly inhibited the migration of A549 and H1650 cells (Fig. 4B). The results from the Transwell assay further demonstrated the decreased migration ability of A549 and H1650 cells after CDCA treatment (Fig. 4C). Furthermore, the invasion assay showed that CDCA treatment inhibited the invasive ability of A549 and H1650 cells (Fig. 4D). Collectively, these results demonstrated that CDCA suppresses the EMT, migration and invasion of LUAD cells.

4. CDCA inhibits the focal adhesion pathway

To determine how CDCA exerts its anti-NSCLC function, RNA-Seq was performed to identify the transcriptional profile in A549 cells after treatment with CDCA, and the analysis was performed using bioinformatics methods. In total, 3370 genes were identified [false discovery rate (FDR) <0.05 and absolute fold change (FC absolute) >1], among which 1494 genes were upregulated and 1876 genes were downregulated (Fig. 5A, B). KEGG pathway enrichment analysis was then performed for these genes, which indicated that CDCA negatively regulated the focal adhesion pathway (Fig. 5C).

Integrins have been reported to play an important role in the activation of the focal adhesion pathway. Therefore, we performed molecular docking studies between integrin $\alpha 5 \beta 1$ and CDCA. The molecular docking results showed that the ligand bound at a shadow groove between the two subunit interfaces of integrin (Fig. 5D) with a binding energy of -8.2 kcal/mol. In addition, the binding site, which was a half-open pocket with hydrophilicity on one side and hydrophobicity on the opposite side, was exposed to the bulk solvent. The binding geometry was also further analysed (Fig. 5E). The carboxyl oxygen of the CDCA ligand formed hydrogen bonds at the following locations: backbone N and side chain hydroxyl of Ser134; backbone N and side chain amide N of Asn224; and side chain hydroxyl of Ser132. All these residues were located on chain B of integrin. In addition, ligand carboxyl groups also formed electrostatic interactions with Mg ions in the binding site. Furthermore, the hydrophobic rings in the ligand formed hydrophobic packing at the surrounding residues, including Leu225, Trp157 and Tyr133. This interaction may also contribute to the stable binding of ligands. Moreover, the trajectory during 50–1100 ns was used for the binding free energy calculation using the MM-GBSA method. As shown in Table 2, the binding free energy between the ligand and the protein was -25.44 kcal/mol, indicating good binding between the ligand and protein.

5. CDCA suppresses the expression of p-FAK by inhibiting integrin $\alpha 5$

We performed a western blot assay to detect the expression of integrin $\alpha 5 \beta 1$ after cells were treated with CDCA. The expression of integrin $\alpha 5$, integrin $\beta 1$ and p-FAK was significantly decreased in A549 cells treated with 0.3 mM and 0.4 mM CDCA compared to the untreated control. Compared to A549 cells, the

expression of these proteins was significantly decreased in H1650 cells (Fig. 6A). To further verify the function of CDCA, we performed an immunofluorescence assay with an anti-p-FAK antibody in A549 cells (Fig. 6B). The results showed that as the CDCA concentration increased, p-FAK was significantly downregulated in A549 cells. Moreover, the same results were observed in H1650 cells. Taken together, these results demonstrated the antagonistic role of CDCA in the integrin $\alpha 5\beta 1$ signalling pathway. In addition, the effects of CDCA on A549 and H1650 cells were reversed after knockdown of integrin $\alpha 5$ according to the CCK-8 assay (Fig. 6C). Western blotting showed that the protein expression of p-FAK was also reversed after knockdown of integrin $\alpha 5$ (Fig. 6D).

6. CDCA regulates p53-mediated apoptosis-related gene expression

It has been reported that activated FAK is physically associated with p53 and inhibits apoptosis progression. Moreover, FAK increases the transformation of p53 through MDM2-mediated p53 ubiquitination[42]. To investigate whether the CDCA-mediated decrease in FAK affects p53 and the expression of its related genes, we evaluated the expression of p53 and GADD45, a downstream gene of p53. The results showed that CDCA significantly induced the expression of p53 and GADD45 at both the RNA and protein levels (Fig. 7A-C). Furthermore, we detected the levels of p21, P21^{as}, MCL-1, and Bax, which are genes associated with p53 upregulation, and we also analysed the expression of IGFBP3 and BCL-2, which are genes associated with p53 downregulation. Fig. 7D-I shows that the expression of p21, P21^{as}, MCL-1 and Bax was significantly increased but that the expression of IGFBP3 and BCL-2 was significantly decreased in A549 and H1650 cells after treatment with CDCA. These results indicated that CDCA induces apoptosis by mediating p53-regulated gene expression.

7. CDCA attenuates tumour growth in a murine xenograft model

To further assess the effect of CDCA in vivo, A549 cells were injected into BALB/c athymic mice. When the tumour volume reached 100 mm³, CDCA was injected intraperitoneally every 4 days at a concentration of 50 mg/kg. Compared to the control group, the increase in tumour volume and weight was inhibited in the CDCA treatment group (Fig. 8A-D). After 4 weeks of treatment, the mice were sacrificed, and the xenograft tumours were removed. H&E staining confirmed the presence of tumour cells (Fig. 8E). Western blotting was performed to evaluate the protein expression of integrin $\alpha 5$, FAK and p-FAK (Fig. 8F). The data indicated that CDCA decreased the protein levels of integrin $\alpha 5$ and p-FAK. These results were consistent with the in vitro results and confirmed that CDCA inhibits tumour growth by attenuating the expression of integrin $\alpha 5$ and p-FAK in vivo.

Taken together, these results indicated that CDCA modulates the EMT, migration, invasion and apoptosis of LUAD cells via the integrin $\alpha 5\beta 1$ /FAK/p53 axis.

Discussion

Multiple roles of CDCA have been reported in a series of carcinomas[15, 43]. In prostate cancer cells, CDCA modulates the cell cycle and apoptosis by suppressing Cdk2 and cyclin E-dependent kinase

activities without changing their expression[44]. In colon adenocarcinoma, CDCA induces apoptosis through oxidative stress with increased ROS generation by activating plasma membrane enzymes[15]. In colon carcinoma HCT-116 and HT-29 cells, CDCA induces cell detachment and apoptotic nuclear morphology in detached cells as observed by DAPI staining[45]. Binding motifs of bile acids in the BRCA1, oestrogen receptor 1 and oestrogen receptor 2 genes have been identified by chromatin immunoprecipitation sequencing[46]. Based on these findings, we hypothesized that CDCA, one of the most abundant metabolites of bile acids, may be involved in the mechanism of apoptosis in LUAD cells. The present study revealed that CDCA promotes apoptotic progression by increasing p53 expression via the integrin $\alpha 5\beta 1$ signalling pathway in LUAD cells.

In the present study, we showed that CDCA was an effective inhibitor of integrin $\alpha 5\beta 1$. Integrin $\alpha 5\beta 1$ has been demonstrated to play an important role in multiple types of cancers. Tenascin-C promotes ewing sarcoma tumour progression by targeting MALAT1 through integrin $\alpha 5\beta 1$ -mediated YAP activation[47]. Periostin promotes the expression of integrin $\alpha 5\beta 1$ via pAKT in autophagy-mediated EMT and migration in colorectal cancer cells[48]. Another study has shown that suppression of integrin $\alpha 5$ expression in glioblastoma cells reverses the resistance of cells to EGFR-TKIs[49]. ATN-161, an effective $\alpha 5\beta 1$ integrin inhibitor, has been reported to inhibit tumour growth and metastasis in melanoma, breast cancer and lung cancer[50–52]. Integrin $\alpha 5\beta 1$ is an effective target in cancers, and CDCA is a new drug candidate for use in LUAD therapy.

FAK has been widely proposed as a potential therapeutic target in lung cancer[53]. In lung surgical specimens, phosphorylated FAK is correlated with poor patient prognosis, and FAK can be regulated by integrin $\alpha 3\beta 1$, so that promotes NSCLC proliferation, migration, and invasion[54]. A variety of FAK kinase inhibitors have shown good efficacy in cancer treatment. The CFAK-C4 inhibitor disrupts the interaction between FAK and VEGFR3, which is related to cell survival, and this inhibitor is currently being evaluated as a therapy for pancreatic cancer[55, 56], another FAK inhibitor VS6063 can increase sensitivity to EGFR-TKIs in PC9 gefitinib-resistant cells[57]. In the present study, we demonstrated that CDCA induced apoptosis in LUAD by blocking the activity of the integrin $\alpha 5\beta 1$ pathway, which resulted in a significant decrease in FAK and p-FAK, suggesting that CDCA may be a potential therapeutic target of FAK.

The p53 tumour suppressor has been reported to induce cell survival, migration, apoptosis and proliferation arrest in many types of cancer cells by modulating multiple regulatory signals[33, 34]. Nevertheless, due to the interference of several molecules or primary degradation by MDM2, p53 usually remains inactive or in a low state in epithelial cells, and mutation of p53 leads to sustained proliferation and malignant transformation of cells[36, 58]. FAK has been reported to modulate the activity of p53 by physically interacting with p53 or regulating expression[42]. In the present study, we found that CDCA significantly upregulated the expression of p53 and apoptosis-associated genes. The present study also confirmed that CDCA induced apoptosis via the FAK/p53 axis.

Conclusion

In conclusion, we investigated the crucial role of CDCA in the apoptotic process in LUAD cells. We confirmed the mechanism by which CDCA modulates apoptosis progression through the integrin $\alpha 5 \beta 1$ /FAK/p53 axis, suggesting the potential role of CDCA as an anticancer drug for LUAD patients.

Declarations

Acknowledgments

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Author contributions

DS, YYZ and WJZ performed the experiments. YL, and JJZ analyzed data and help conduct partial experiment. YYZ wrote the paper. ZYL, ZWY and JAH supervising the study.

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Availability of data and materials

The data used during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The research is not involved with human participants, human data or human tissues.

The animal experiment was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Competing interests

Not applicable.

Consent for publication

The authors have no conflicts of interest.

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Tables

Table 1 Primers for RT–qPCR

Name	Sequence (5'-3')
P53	F: GAGGTTGGCTCTGACTGTACC R: TCCGTCCCAGTAGATTACCAC
P21	F: CGATGGAACCTTCGACTTTGTCA R: GCACAAGGGTACAAGACAGTG
GADD45	F: GAGAGCAGAAGACCGAAAGGA R: CACAACACCACGTTATCGGG
BAX	F: CCCGAGAGGTCTTTTTCCGAG R: CCAGCCCATGATGGTTCTGAT
BCL-2	F: GGTGGGGTCATGTGTGTGG R: CGGTTCAAGTACTCAGTCATCC
IGFBP3	F: AGAGCACAGATACCCAGAACT R: GGTGATTCAGTGTGTCTTCCATT
P2xm	F: GTCCGTCCCCTGGCTAAC R: CTGGCCTGTTTTTACACCGTG
MCL1	F: TGCTTCGGAACTGGACATCA R: TAGCCACAAAGGCACCAAAAG

F: forward R: reverse

Table 2 Binding free energy analysis of CDCA binding to integrin $\alpha 5\beta 1$

Energy Component	Binding free energy [kcal/mol]
VDWAALS	-35.9
EEL	396.5
EGB	-381.1
ESURF	-4.9
DELTA G gas	360.5
DELTA G solv	-385.9
DELTA TOTAL	-25.4

Figures

Figure 1

Chemical structure of CDCA.

Figure 2

CDCA inhibits lung adenocarcinoma cell viability. (A-C) Cell viability was determined by CCK-8 assay following treatment with or without CDCA at the indicated concentration in A549 cells, H1650 cells and

bronchial epithelial BEAS-2B cells. (D-F) The IC₅₀ of CDCA in each cell line was calculated by the cell viability value. N.S: no significance, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3

CDCA promotes apoptosis in lung adenocarcinoma. (A, B) A549 and H1650 cells were washed twice with PBS, and diluted Hoechst 33342 and PI were added followed by incubation for 20 min at 4 °C. Images were acquired using fluorescence microscopy. (C, D) CDCA-treated A549 and H1650 cells were digested with trypsin, and complete medium was then added to terminate the reaction. Binding buffer containing

Annexin V-FITC and PI was added to the resuspended cells followed by incubation at room temperature for 15 min in the dark, and apoptosis was then detected by flow cytometry. The right histogram panel shows the statistics of the percentage of apoptotic cells in each group. *P < 0.05, ***P < 0.001.

Figure 4

CDCA suppresses the EMT, migration and invasion in LUAD. (A) A549 (left) and H1650 (right) cells were treated with CDCA for 24 hours. Protein levels of E-cadherin, N-cadherin and Snail were analysed using western blotting. (B) A wound-healing assay was used to analyse the effects of CDCA on A549 and H1650 cell migration. Following 24 hours of treatment with CDCA, the wound gap was visualized under a microscope. The black dotted line represents the wound edge. (C, D) A549 or H1650 cells were allowed to

migrate through a Transwell (8- μ m pore) with or without CDCA treatment for 24 hours. Migrated or invasive cells were stained and counted in at least three microscopic fields. Representative images (upper) and differences in migratory cell numbers (bottom) were compared between CDCA-treated and untreated cells. ***P < 0.001.

Figure 5

CDCA inhibits focal adhesion pathway. (A-C) Heat map of differentially expressed mRNAs in the treatment and control groups. A total of 3370 genes were identified [false discovery rate (FDR) <0.05 and absolute fold change (FC absolute) >1], among which 1494 genes were upregulated and 1876 genes were downregulated. KEGG pathway enrichment analysis was then performed using these genes. (D) Optimal conformation of molecular docking between integrin $\alpha 5 \beta 1$ and CDCA. (E) Interaction of CDCA with the active site residue of integrin $\alpha 5 \beta 1$.

Figure 6

CDCA suppresses the expression of FAK by inhibiting integrin $\alpha 5$. (A) A549 and H1650 cells were treated with different concentrations of CDCA for 24 hours. The protein expression of integrin $\alpha 5$, FAK and p-FAK was evaluated by western blot analysis. (B) Immunofluorescence staining of p-FAK after A549 and H1650 cells were treated with or without CDCA. (C) The cell viability of control or integrin $\alpha 5$ -deficient A549 and H1650 cells after treatment with CDCA was determined by CCK-8 assay. (D) Protein expression of integrin $\alpha 5$, FAK and p-FAK.

Figure 7

CDCA regulates p53-mediated apoptosis-related gene expression. (A-C) mRNA and protein levels were analysed by qRT-PCR and western blot analyses in CDCA-treated A549 and H1650 cells. (D-I) p21, P2xm, MCL1, BAX, IGFBP3 and BCL-2 levels were analysed by qRT-PCR in A549 and H1650 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 8

CDCA attenuates tumour growth in a murine xenograft model. (A) Diagram of the in vivo experimental process. (B) A549 cell xenografts in nude mice ($n = 6$) at the experimental endpoint. CDCA at a concentration of $50 \text{ mg} \cdot \text{kg}^{-1}$ was injected intraperitoneally every four days, and tumours were harvested and photographed as shown. (C) Each tumour was weighed. (D) Tumour growth curves in mice ($n = 6$ in each group) inoculated with the indicated cells on the indicated days. (E) H&E staining confirmed the presence of tumour cells. (F) Integrin $\alpha 5$, FAK and p-FAK protein expression in tumours was measured by western blot analysis. $**P < 0.01$, $***P < 0.001$.