Silencing CDC25A inhibits the proliferation of liver cancer cells by downregulating IL-6 in vitro and in vivo

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Abstract. Cell division cycle 25A (CDC25A) is a core regulator of the cell cycle that has a dual-specific phosphatase activity, which is closely associated with the occurrence and development of a tumor, and is overexpressed in liver cancer. However, the molecular mechanism of CDC25A in the development of liver cancer remains unclear. The purpose of the present study was to further investigate the effect of CDC25A on cell proliferation in vitro and in vivo and to investigate whether an interaction exists between CDC25A and interleukin (IL)-6 in liver cancer. An Affymetrix human gene expression profiling chip screened differentially expressed genes in HepG2 cells with silenced CDC25A and the IL-6 signaling pathway was revealed to be significantly inhibited (P<0.05). In the present study, the effects of CDC25A on cell proliferation and migration were analyzed using cell cycle, MTT and Transwell assays. Reverse transcription-quantitative PCR, western blot and immunohistochemistry analyses confirmed that silencing the CDC25A gene downregulated the expression of IL-6 in HepG2 cells and the mRNA and protein expression of IL-1β, mitogen-activated protein kinase kinase kinase 14 (NIK) and nuclear factor-κB (NF-κB), which are regulatory molecules upstream of IL-6. In addition, silencing CDC25A by short hairpin RNA inhibited the development of liver cancer xenograft tumor types in nude mice, and decreased the expression of IL-1β, NIK, NF-κB and IL-6 in xenograft tumor types. In conclusion, silencing CDC25A significantly inhibited the proliferation of liver cancer cells in vitro and in vivo, potentially via an interaction with IL-6 through the downregulation of the IL-1β/NIK/NF-κB signaling axis.

Introduction

Liver cancer is a malignant tumor type that seriously endangers human life and health, and its morbidity and mortality rates continue to rise faster compared with those of other cancer types (1). China is a high-risk area for liver cancer, accounting for ~50% of new patients and liver cancer-associated mortalities globally each year (2). The occurrence and development of liver cancer are known to utilize a complex evolutionary process involving the accumulation of multiple factors, stages and gene variations (3). Identifying the genes that serve key functions in the development of liver cancer and the associated molecular mechanisms are prerequisites for the treatment of liver cancer. To identify the key genes affecting the development of liver cancer, a previous study proposed a research strategy based on the cross-species screening of early-stage disease, and CDC25A was revealed to be highly expressed in liver cancer (4).

As an important member of the CDC25 family, CDC25A is a bispecific protein phosphatase that hydrolyses tyrosine and serine/threonine residues for dephosphorylation (5). CDC25A activates the cyclin-cyclin-dependent kinase complex, which promotes the transition of the G1/S and G2/M phases and is an important target for DNA damage response (6,7). CDC25A, which has the characteristics of a proto-oncogene, is known to be highly expressed in various malignant tumor types, including lung cancer (8), breast cancer (9) and esophageal cancer (10), and is closely associated with poor patient prognosis. To further investigate the molecular mechanism of CDC25A in liver cancer, an Affymetrix human gene expression profiling chip was used to analyze the differentially expressed genes in HepG2 cells with the CDC25A gene silenced. Based on ingenuity pathway analysis, signaling pathways were revealed to be enriched among the genes with significant differential expression, and the interleukin (IL)-6 signaling pathway was revealed to be significantly inhibited.

IL-6 is a cytokine produced by various cells and serves important functions in information transmittance, immune cell activation and regulation, T and B cell activation, proliferation and differentiation and inflammatory responses (11). Previously, one study demonstrated that IL-6 is closely associated with the occurrence, development and metastasis of various malignant tumor types, which may be associated with its antiapoptotic and proangiogenic effects (12). IL-6 has been confirmed to affect liver cancer proliferation and serves an important function in the development and recurrence of...
liver cancer (13). IL-6 is a major inducer of signal transducer and activator of transcription 3 (STAT3) phosphorylation and activation. IL-6 binds to glycoprotein 130 and induces Janus kinase phosphorylation, which results in the phosphorylation of Tyr705 in STAT3, the promotion of STAT3 binding to DNA and the activation of certain genes downstream of STAT3 to promote the development of liver cancer (14,15).

Nuclear factor-κB (NF-κB), an important transcriptional regulator, participating in the bodily inflammatory response, the immune response, cell apoptosis, differentiation, regeneration and other important physiological and pathological processes. NF-κB is an important molecular marker linking the inflammatory response with tumors (16,17). Activated NF-κB is involved in cell proliferation, increases the expression of inflammation-associated factors, inhibits apoptosis and promotes the malignant transformation, invasion and metastasis of a tumor (18,19). One study confirmed that NF-κB serves an important function in the induction of IL-6 expression (20). Interestingly, CDC25A has been revealed to positively regulate NF-κB, inhibit apoptosis and promote tumor cell survival (21). Therefore, the present study hypothesized that CDC25A may regulate the expression of IL-6 through the NF-κB gene, further affecting the occurrence and development of liver cancer.

The present study investigated the molecular mechanism of CDC25A in the development of liver cancer, and the results may provide a novel theoretical basis for the treatment of liver cancer based on the utilization of CDC25A as a target.

Materials and methods

Cell culture. HepG2 cells (human liver cancer cell line) were purchased from the Shanghai Cell Biology Institute and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO₂ incubator.

Cell transfection. Lentiviral particles [LV-short hairpin RNA (shRNA)-CDC25A] for silencing the CDC25A gene and negative control empty lentiviral particles (LV-shRNA-NC) were obtained from Shanghai GeneChem Co., Ltd., and the lentiviral interference sequences were 5'-CATGCACCA CGAGGACTTT-3' and 5'-TTCTCCGAACGTGCAGT-3', respectively. The two lentiviruses contained a green fluorescent protein (GFP) marker. Cells were divided into the logarithmic growth phase were plated into 6-well plates at 1x10⁶ cells /ml in ice-cold PBS) were prepared, and then subsequently fixed with 70% ethanol at 4°C for 2 h. Subsequent to treatment with RNase A (10 µg/ml) for 30 min at 37°C, the cells were resuspended in 500 µl propidium iodide solution. Cell cycle distribution was analyzed by a FACS Calibur flow cytometer (BD Biosciences).

Gene chip screening. Gene chip screening was performed as described in our previous study (22).

Construction of the liver cancer xenograft model. A total of 30 female BALB/c nude mice (weighing 18-24 g and aged 4-6 weeks) were purchased from Beijing Vital River Laboratory Animal Technology co., Ltd. (Beijing, China). The study was ethically approved and supervised by the Ethics Committee of Guangxi Medical University Affiliated Tumor Hospital (approval no. LW2019045). The mice were housed in a specific pathogen-free animal room at 22-24°C with a humidity of 55-70% and a 12/12 h light/dark cycle at the Experimental Animal Center of Guangxi Medical University, and all animals were provided ad libitum access to standard laboratory feed and water. Their health and behavior were monitored every 5 days. The nude mice were randomly divided into the following three groups (n=10): The knockdown (KD), NC and control groups. The KD group was HepG2 cells transfected with LV-shRNA-CDC25A, the NC group was HepG2 cells transfected with LV-shRNA-NC, and the control group was untreated cells. All cells were resuspended at a concentration of 1x10⁷/ml, and cell suspensions (200 µl) were subcutaneously injected into the right axilla (0.5 cm) of the nude mice. The tumor volume was observed and recorded every 7 days. When the animals exhibited a loss
of appetite and inability to eat, clinical symptoms of severe loss of organ function or when the tumor was observed to be ulcerated, infected or necrotic, the animals were euthanized. Following 35 days, the nude mice were euthanized by cervical dislocation, then the breathing, corneal reflex and heartbeat were observed to determine euthanasia. The criteria for verifying animal mortality are no breathing, no heartbeat and no corneal reflex. The liver cancer xenograft tumors were then harvested. The tumor volume formula used was as follows: Volume (mm$^3$) = width$^2$ (mm$^2$) x length (mm)/2.

**Hematoxylin and eosin (H&E) staining.** Xenograft tumors were fixed with 4% paraformaldehyde at room temperature for 24 h, embedded in paraffin and serially sectioned at a thickness of 5 µm. The sections were dewaxed and stained with H&E using a staining kit (cat. no. c0105; Beyotime Institute of Biotechnology), and visualized using a phase contrast microscope (Olympus Corporation) at a magnification of x200.

**RNA extraction and RT-qPCR.** Total RNA was extracted from the liver cancer cells and xenograft tumors using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), and cDNA was synthesized using the PrimeScript RT Reagent kit (Takara Bio, Inc.), according to the manufacturer's protocol. qPCR was performed with a qTOWER® PCR instrument (Analytik Jena AG) and SYBR® Premix Ex Taq™ (Takara Bio, Inc.), according to the manufacturer's protocol. The reaction conditions were as follows: Predenaturation at 95°C for 30 sec, denaturation at 95°C for 5 sec and annealing at 60°C for 30 sec for a total of 40 cycles. The results obtained were analyzed using the 2$^{-ΔΔCq}$ method (23). The primers used were as follows: CDC25A forward, 5'-TTCCCTCTTT TTACACCCAGCT-3' and reverse, 5'-TGGTTTGTCAAG GTTGTGATTC-3'; IL-6 forward, 5'-ACTCAACCTTTC AGAAGCAGATT-3' and reverse, 5'-CCATCTTTGGAA GTTTCAGGTTG-3'; IL-1β forward, 5'-GCCAGTGAATG ATGGCTTATT-3' and reverse, 5'-AGGACCTCTTACCT GTTTAGG-3'; NIK forward, 5'-AGGAAGAGCAGGCGCCGA CTG-3' and reverse, 5'-TGCCCTGGAGCCTTCTTTG-3'; NF-κB forward, 5'-CCCCAGACCTTGTAGAAAGG-3' and reverse, 5'-GGATTTCCAGGGTCTGGAAC-3'; GAPDH forward, 5'-TGACTTCAACAGCGACACCCA-3' and reverse, 5'-CACCCTTGTGTCGTAGCAAAA-3'.

**Western blot analysis.** The cells and xenograft tumors were lysed with RIPA-phenylmethylsulfonyl fluoride buffer (cat. no. P0013K; Beyotime Institute of Biotechnology) to extract the total protein, and the protein concentration was determined using the BCA method. Protein buffer was added to the samples and denatured at 100°C. Each well was loaded with 50 µg proteins. Then, the samples were electrophoresed on a 10% SDS-PAGE gel at 60V for 150 min, transferred to a 0.22-µm-pore size polyvinylidene difluoride (PVDF) membrane, and blocked with 5% skimmed milk at room temperature for 2 h. The membranes were washed and incubated while shaking overnight at 4°C with anti-IL-1β (1:5,000; rabbit; cat. no. ab20485; Abcam), anti-IL-6 (1:1,000; rabbit; cat. no. ab235706; Abcam), anti-IL-1β (1:1,500; rabbit; cat. no. ab2105; Abcam), anti-NIK (1:1,000; rabbit; cat. no. ab15583; Abcam), anti-NF-κB (1:1,000; rabbit; cat. no. ab8227; Abcam) and anti-GAPDH (1:1,000; mouse; cat. no. ab8245) antibodies. Subsequent to washing, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1,000; goat anti-rabbit or anti-mouse antibody; cat. nos. A0208 and A0216, respectively; Beyotime Institute of Biotechnology) for 1 h at room temperature. The PVDF membranes were scanned with an enhanced chemiluminescence detection system ChemiDoc MP (Bio-Rad Laboratories, Inc.) and the densitometry was performed using Image Lab software (version 3.0; Bio-Rad Laboratories, Inc.).

**Immunohistochemistry.** The sections at a thickness of 5 µm were dewaxed, immersed in sodium citrate buffer, incubated at 100°C for 2 min in an autoclave, and then rinsed 3 times with PBS to retrieve the antigens. Then, the sections were placed in 3% H$_2$O$_2$ and incubated at room temperature for 25 min in the dark to block the endogenous peroxide. The sections were sequentially incubated overnight with rabbit anti-human CDC25A (1:2,000; cat. no. GB11283; Wuhan Servicebio Technology Co., Ltd.) and rabbit anti-human IL-6 (1:600; cat. no. GB11117; Wuhan Servicebio Technology Co., Ltd.) antibodies at 4°C, and then with a goat anti-rabbit immunoglobulin G solution (1:200; cat. no. G23303; Wuhan Servicebio Technology Co., Ltd.) for 50 min at room temperature. The immunocomplexes were visualized using 3,3'-diaminobenzidine and a light microscope (Olympus Corporation) at a magnification of x200. Two blinded pathologists independently assessed all specimens. The staining intensity was scored as follows: 0 (negative); 1 (weak); 2 (moderate); and 3 (strong). The positive range scores were defined as follows: 0 (0-20%); 1 (21-50%); 2 (51-80%) and 3 (81-100%). The final score was obtained by multiplying the intensity score and the positive range score, and a score ≥4 was regarded as high expression.

**Statistical analysis.** Analyses were performed using SPSS 19.0 statistical software (IBM Corp.). Differences among the CDC25A-shRNA, CDC25A-NC and control groups or among the KD, NC and control groups were analyzed using one-way analysis of variance followed by Student-Newman-Keuls. Correlational analysis was performed with Pearson's correlation coefficient. Measurement data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Efficiency of lentiviral infection in HepG2 cells.** The expression of GFP was detected after 48 h. Following transfection, the mRNA and protein expression levels of CDC25A in the CDC25A-shRNA group were significantly lower compared with those in the CDC25A-NC and control groups (P<0.05; Fig. 1A and B).

**Effect of silencing CDC25A on liver cancer cell growth.** The effect of silencing CDC25A on the growth of HepG2 cells was confirmed by an MTT assay, revealing that the proliferation of HepG2 cells was significantly decreased in LV-shRNA-CDC25A transfected cells compared with the CDC25A-NC and control groups (P<0.05; Fig. 1C).
Effect of silencing CDC25A on liver cancer cells migration. Migration experiments revealed that significantly fewer cells passed through the membrane in the CDC25A-shRNA group compared with the CDC25A-NC and control groups (P<0.05; Fig. 1D), and no significant difference was observed between the CDC25A-NC and control groups.

Effect of silencing CDC25A on liver cancer cell cycle. As presented in Fig. 2A and B, the percentage of cells in the G1 phase was significantly increased, accompanied by a significant decrease in S and G2 phases in the CDC25A-shRNA group in comparison with the CDC25A-NC and control groups (P<0.05). RT-qPCR analysis revealed that the mRNA expression levels of IL-1β, NIK and NF-xB in the CDC25A-shRNA group were significantly lower compared with those in the CDC25A-NC and control groups (P<0.05). These results suggest that CDC25A may affect the expression of IL-6 in HepG2 cells.

Effect of silencing CDC25A on IL6 expression in HepG2 cells. The differentially expressed genes in HepG2 cells in which the CDC25A gene was silenced mainly involved interferon signaling, the phosphoinositide-3-kinase/protein kinase B signaling pathway, the IL-6 signaling pathway, the P53 signaling pathway and the cell cycle pathway (Fig. 3A), and the IL-6 signaling pathway exhibited the most significant inhibition. RT-qPCR analysis revealed that the mRNA expression levels of IL-1β, NIK and NF-xB in the CDC25A-shRNA group were significantly lower compared with those in the CDC25A-NC and control groups (P<0.05). These results indicate that silencing the CDC25A gene may inhibit the growth of liver cancer xenografts.

CDC25A affects liver cancer growth by targeting IL-6 through the IL-1β/NIK/NF-xB signaling axis. The expression levels of CDC25A and IL6 pathway-associated molecules...
(including IL-$\beta$, NIK, NF-$\kappa$B and IL-6) in the liver cancer xenografts were evaluated by RT-qPCR, western blot analysis and immunohistochemistry. As presented in Fig. 5A and B, the mRNA and protein expression levels of CDC25A and IL-6 were significantly reduced in the KD group compared with the NC or control groups (P<0.05). Further immunohistochemistry analysis revealed that the CDC25A and IL-6 proteins were strongly expressed in the NC and control groups, but their expression levels were lower in the KD group (Fig. 5C). A significant positive correlation was observed between the expression levels of CDC25A and IL-6 proteins in the xenograft tissue samples (R=0.669, P<0.05; data not shown). The mRNA and protein expression levels of IL-$\beta$, NIK and NF-$\kappa$B in the KD group were significantly lower compared with those in the NC or control group (P<0.05; Fig. 6A-C). These results indicate that CDC25A overexpression in liver cancer may positively regulate IL-6 expression by regulating the IL-$\beta$/NIK/NF-$\kappa$B signaling axis, thereby promoting the growth of liver cancer.

**Discussion**

CDC25A is a bispecific protein phosphatase consisting of 524 amino acid residues that contains an N-terminal regulatory domain and a C-terminal catalytic domain (24). CDC25A has been revealed to promote cell cycle progression, and the overexpression of CDC25A causes abnormal cell cycle regulation and results in tumorigenesis (25). In addition, previous studies have revealed that CDC25A serves key functions in apoptosis, cell metabolism and tumor cell metastasis (4,26). Overexpressed CDC25A may interact with tumor-associated factors including mitogen-activated protein kinase kinase kinase 5, NF-$\kappa$B, STAT3, NIMA related kinase 11, pyruvate kinase M1/2 and forkhead box O1 to promote tumor progression (27-29). CDC25A is overexpressed in liver cancer (30), which is positively associated with clinicopathological parameters including portal vein thrombosis, extrahepatic metastasis and tumor differentiation in patients with liver cancer (31).

In the present study, lentiviral-mediated shRNA transfection was used to silence the expression of CDC25A in HepG2 cells, and the treated cells were inoculated subcutaneously into nude mice. Silencing the CDC25A gene significantly inhibited the growth of the xenograft tumors (P<0.05), indicating that CDC25A is a key gene in the development of liver cancer. Xu et al (32) produced a study that used CDC25A antisense to inhibit CDC25A in liver cancer cells, and revealed that cell growth, invasion and cell cycle were inhibited, which was consistent with the results of the present study.

IL-6 is a multifunctional cytokine that has been associated with multiple tumor types, including breast cancer (33), lung cancer (34) and ovarian cancer (35). IL-6 may promote the development of tumor types by mediating various signaling pathways, with the induction of STAT3 phosphorylation being the most common mechanism. The IL-6/STAT3 signaling transduction pathway is critical for the development and progression of malignant tumor types (36). IL-6-mediated STAT3 activation may substantially upregulate the expression of a number of genes associated with tumor cell proliferation, apoptosis (BCL2 like 1, MCL1 apoptosis regulator, BCL2 family member, survivin and P53), the hypoxia response, metastasis and angiogenesis and may downregulate the
expression of proapoptotic genes (37-39); this activation also serves a key function in the proliferation, apoptosis, invasion and metastasis of liver cancer cells (40,41). The present screened the differentially expressed genes in HepG2 cells with silenced CDC25A using a gene chip and revealed that the IL-6 signaling pathway was the most significantly inhibited pathway. The expression of IL-6 in HepG2 cells was confirmed to be significantly decreased subsequent to silencing the CDC25A gene (P<0.05). The same results were obtained in vivo, and a significant positive correlation was observed between CDC25A and IL-6 expression in xenograft tumors (P<0.05). These results suggest that CDC25A may promote the development of liver cancer by regulating the expression of IL-6. Subsequently, the present study investigated how CDC25A regulates IL-6.

NF-κB is well known to inhibit apoptosis and promote tumor cell survival via various mechanisms. NF-κB, an important transcription factor that links inflammation and tumorigenesis, regulates the expression of various proinflammatory cytokines, including IL-6, in cancer cells and promotes
tumor cell proliferation (42). Targeting the inhibition of NF-κB may downregulate the expression of IL-6 (43).

Inflammatory cytokines, including IL-1, IL-6 and IL-8, may activate the STAT3/NF-κB pathway in tumor cells, and these pathways stimulate further cytokine production, resulting in a positive feedback loop (44). IL-1β is an inflammatory factor that is involved in the NF-κB cell signaling pathway. IL-1β binds its receptor, IL-1 receptor, and exerts

| Gene symbol | Gene title                                      | Fold change | Regulation | P-value |
|-------------|------------------------------------------------|-------------|------------|---------|
| IL-6        | Interleukin-6                                   | -5.769      | Down       | <0.001  |
| IL-1β       | Interleukin-1β                                  | -1.666      | Down       | <0.001  |
| NIK         | Mitogen-activated protein kinase kinase kinase 4 | -1.547      | Down       | <0.001  |
| NF-κB       | Nuclear factor-κB                               | -1.743      | Down       | <0.001  |

Figure 4. Effect of CDC25A downregulation on liver cancer xenograft growth. (A) Xenograft mice were sacrificed by cervical dislocation on day 35. (B) Xenograft tumors were harvested on day 35. (C) Xenograft tumor growth curve. (D) Weights of the xenograft tumors. (E) Representative haematoxylin and eosin staining images of the xenograft tumors (magnification, x200). *P<0.05 vs. NC or control groups. CDC25A, cell division cycle 25A; KD, knockdown; NC, negative control.
Figure 5. Association between CDC25A and IL-6. (A) mRNA and (B) protein expression levels of CDC25A and IL-6 were examined in xenograft tumors. *P<0.05 vs. NC or control groups. (C) Representative immunohistochemistry images of CDC25A and IL-6 expression in xenograft tumors (magnification, x200). CDC25A, cell division cycle 25A; IL, interleukin; KD, knockdown; NC, negative control.

Figure 6. CDC25A affects the protein and mRNA expression levels of the IL-1β/NIK/NF-κB signaling axis in xenograft tumors. (A) Quantification of the relative expression levels of IL-1β, NIK and NF-κB mRNA in the KD, NC and control groups. (B) Representative western blot images and (C) quantification of the relative protein expression levels of IL-1β, NIK and NF-κB in three groups. *P<0.05 vs. NC or control groups. CDC25A, cell division cycle 25A; IL, interleukin; NIK, mitogen-activated protein kinase kinase kinase 4; NF-κB, nuclear factor-κB; KD, knockdown; NC, negative control.
its effects on the NF-κB kinase NIK, which activates the IkB kinase complex and activates NF-κB (45,46). IL-1β may be induced by NF-κB activation, while IL-1β activates an autocrine signal loop that upregulates the NF-κB signal (47,48). The activated NF-κB then further promotes the expression of downstream targets IL-6 and IL-8 (49). This positive feedback loop promotes angiogenesis, tumor growth and metastasis. Gene chip analysis revealed that the IL-1β/NIK/NF-κB/IL-6 signaling axis was significantly downregulated in HepG2 cells subsequent to knocking out the CDC25A gene. The present study confirmed that the expression levels of IL-6, IL-1β, NIK and NF-κB were consistent with the chip results, and these results were also confirmed in vivo. Subsequent to silencing the CDC25A gene, the expression levels of IL-1β, NIK and NF-κB in the xenograft tumors were significantly decreased (P<0.05). The present study confirmed that CDC25A may regulate the expression of IL-6 by regulating the IL-1β/NIK/NF-κB signaling axis, thereby promoting the growth of liver cancer.

In summary, the present study revealed that CDC25A may positively regulate IL-6 through the IL-1β/NIK/NF-κB signaling axis, thereby promoting the proliferation and growth of liver cancer cells, and these results provide novel insight into the function and mechanism of CDC25A in liver cancer.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
JC designed and conducted the experiments. SC designed and conducted the experiments, analyzed the data and drafted the manuscript. YT designed the experiments and revised the manuscript critically for important intellectual content. CY, KL and XH contributed to the analysis and interpretation of data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was ethically approved by the Affiliated Tumor Hospital of Guangxi Medical University Ethics Committees (Guangxi, China).

Patient consent for publication
Not applicable.

Competing interests
The authors declare they have no competing interests.

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