

## Original Article

# CDC37 promotes the proliferation and invasion of human hepatocellular carcinoma cells through AKT1 signaling pathway

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**Abstract:** The molecular co-chaperone CDC37 (co-chaperone cell division cycle 37) is a co-chaperone of HSP90. It has been shown to form complex with a variety of protein kinases including AKT1 and then stabilize them. Moreover, CDC37 is upregulated in human hepatocellular carcinoma cells. Here, we found that the expression levels of CDC37 were upregulated in human hepatocellular carcinoma cell lines with highly invasive potential. Overexpression of CDC37 in hepatocellular carcinoma cells could increase cell proliferation and invasion. Meanwhile, overexpression of CDC37 could increase the protein levels of AKT1. PINK1 (PTEN Induced Putative Kinase 1), a CDC37 target kinase, was also found to be upregulated by the overexpression of CDC37. Furthermore, overexpression of PINK1 could increase the activity of AKT1. Importantly, overexpression of CDC37 could not increase the cell proliferation and invasion of human hepatocellular carcinoma cells in the presence of AKT1 inhibitor A-674563. These results demonstrate that CDC37 promotes cell proliferation and invasion through stabilizing and activating AKT1. Thus, our findings support that CDC37 may be a useful therapeutic target in human hepatocellular carcinoma.

**Keywords:** CDC37, human hepatocellular carcinoma, proliferation, invasion, AKT1, PINK1

## Introduction

Human hepatocellular carcinoma (HCC) is one of the most common adult liver malignancy worldwide [1]. HCC is the sixth most prevalent cancer and the third most common cause of cancer mortality [2]. Thus, the understanding of the key molecular drivers of HCC is important to the identifications of treatments.

AKT1, which is aberrant activated in HCC, plays critical roles in the development of human cancer [3-5]. There are various factors contributing to activation of the AKT1 signal transduction pathway in human cancer. Overexpression or amplification of AKT1 was found in several types of cancers [6-8]. Aberrant activation of AKT1 was also found in human cancers, including breast carcinoma, lung carcinoma, ovarian carcinoma, prostate carcinoma and hepatocellular carcinoma [9-11]. Aberrant activated AKT1 can mediate cell proliferation, anti-apoptotic growth, invasion and metastasis by phosphorylating and activating the downstream kinases

[11]. For example, glycogen synthase kinase 3 Beta (GSK-3 $\beta$ ) can be phosphorylated by AKT1 to protect cyclin D1 from degradation [12, 13]. AKT1 also directly phosphorylates cyclin-dependent kinase inhibitor p27 to allow its cytoplasmic localization and degradation [14]. Moreover, the activity or stability of AKT1 can be regulated by other factors. AKT1 acetylation by p300/CBP-associated factor (PCAF) enhances the phosphorylation of AKT1 and promotes its activity [15, 16]. The molecular co-chaperone CDC37, a co-chaperone of HSP90, has been shown to form complex with AKT1 and stabilize it [17, 18].

CDC37 is upregulated in human hepatocellular carcinoma cells [19-21]. Inhibition of the expression of CDC37 could inhibit the cell cycle progression and cell growth of hepatocellular carcinoma cells [20, 21]. Here, we investigate whether CDC37 is involved in the regulation to the cell growth and invasion in human hepatocellular carcinoma cells.

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## Material and methods

### *Cell culture and reagents*

The human hepatocellular carcinoma cells lines HCC-LM3, HepG2, Hep3B, SMMC7721, Huh7 and the human embryonic kidney cells (HEK-293T) were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Scotland, UK), supplemented with 10% fetal calf serum (GIBCO, Scotland, UK) at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere. Selective AKT1 inhibitor A-674563 was from Santa Cruz Biotechnology (Santa Cruz, CA).

### *RNA isolation and real-time RT-PCR*

Total RNA were extracted from cells using Trizol (Invitrogen, California, USA) according to the manufacturer's protocol. SuperScript III Reverse Transcriptase (Invitrogen, California, USA) was used to perform the reverse transcription and cDNA synthesis. The qRT-PCR assays were performed to evaluate the expression of target genes and the primers were designed as follows: 5'-GGAGCAGAAACACAAG-ACCTTCG-3' (CDC37, sense); 5'-GACCAGGTAAT-TGGCTGTCTCC-3' (CDC37, antisense), 5'-GTCT-CCTCTGACTTCAACAGCG-3' (GAPDH, sense); 5'-ACCACCCTGTTGCTGTAGCCAA-3' (GAPDH, antisense), 5'-TGGACTACCTGCACTCGGAGAA-3' (AKT1, sense); 5'-GTGCCGCAAAGGTCTTCATGG-3' (AKT1, antisense). The mRNA expression levels were standardized to GAPDH mRNA by the  $\Delta\Delta C_t$  method. All experiments were performed at least three times.

### *Western blot*

Cells were harvested, washed twice in PBS, and lysed in lysis buffer (protease inhibitors were added immediately before use) for 30 min on ice. The protein concentration was determined using BCA assay kit (Merck Millipore Bioscience, Germany). Then the samples were electrophoresed by 10% SDS-PAGE gel, and transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore Bioscience, Germany). The membranes were blocked with 5% BSA for 1 hour at room and then the primary antibodies were added for incubation overnight at 4°C. After being washed twice with TBST for 15 min, peroxidase conjugated secondary antibodies were incubated with the membrane for 30 min. Followed by washing twice with TBST for 15 min, the membranes were visualized by an enhanced chemilumi-

nescence system (ECL, Amersham Pharmacia Biotech). The primary antibodies: rabbit anti-human CDC37 (1:1000) and rabbit anti-human GAPDH antibody (1:5000) were from Abcam (Cambridge, MA, USA); Rabbit anti-human AKT1 (1:1000), mouse anti-human phospho-AKT1 (S473, 1:1000) and rabbit anti-human PINK1 (1:1000) were from Cell Signaling Technologies (Beverly, MA, USA). Goat anti-Mouse IgG peroxidase conjugated secondary antibodies (1:10000) and Goat anti-Rabbit IgG peroxidase conjugated secondary antibodies (1:10000) were from GeneTex (Irvine, CA, USA).

### *Lentiviral vector production and cell transduction*

The lentiviral expression vectors pBoBi-puro-CDC37 (NM\_007065) and pBoBi-puro-PINK1 (NM\_032409) were purchased from Genesent (Shanghai, China). Lentivirus plasmid vectors pLKO.1-puro with shRNA specific for CDC37 and non-target shRNA control were purchased from Sigma-Aldrich (St. Louis, MO, USA). The target sequences to CDC37 were: sh-a, GCC-CATTCAAGTCTCTGCTTT; sh-b, CCAGACAATCGT-CATGCAATT. The target sequences of non-target shRNA control: CCGGCAACAAGATGAAGAG. Lentiviral vectors were produced in HEK 293T cells with packaging plasmids. The recombinant viral supernatants were harvested from HEK 293T cells and then used to infect target cells in the presence of 8 µg/ml polybrene. After selected with puromycin-containing media for 72 hours, then the expression levels of target genes were analyzed by western blot.

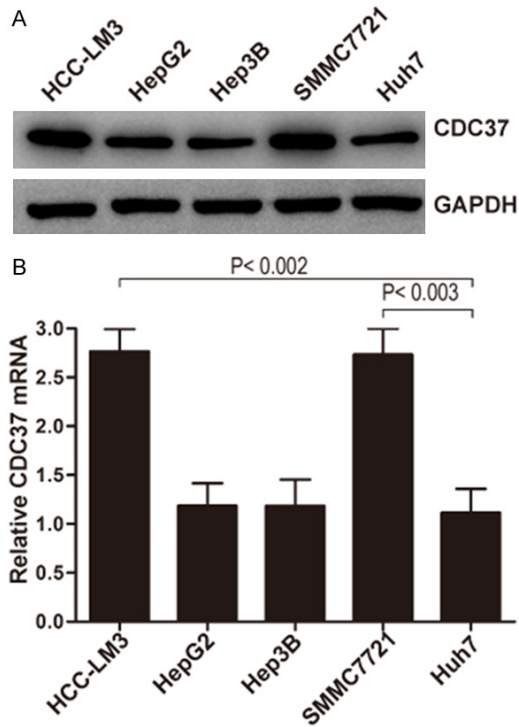
### *Plate colony formation assay*

The cells were seeded at a density of 2000 cells per well in 6-well plates. After 2 weeks, cells were washed with PBS, fixed in 10% methanol for 15 min, and stained with Giemsa for 20 min. Visualized colonies were then photographed and scored. Each plate colony formation experiment was repeated at least three times.

### *In vitro matrigel invasion assay*

Cells were added onto BD Falcon Cell Culture Inserts coated with Matrigel inside BD BioCoat Matrigel Invasion Chambers (Becton Dickinson, Bedford, MA, USA). After cultured at 37°C for 48 h, cells invaded through Matrigel to the bottom side of the inserts were stained with hematoxylin-eosin (H&E), photographed under microscope and quantified.

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**Figure 1.** The expression levels of CDC37 in human hepatocellular carcinoma cell lines. A. CDC37 and GAPDH protein expression levels were determined by western blot analysis in five human hepatocellular carcinoma cell lines as indicated. B. The mRNA expression levels of CDC37 in five human hepatocellular carcinoma cell lines were determined by real-time RT-PCR analysis. GAPDH was used as an internal quantitative control.

### Statistical analysis

Data are presented as means  $\pm$  SE of the indicated number of experiments, and statistical analyses were performed using Student's t test using GraphPad prism 5. A *P* value of  $< 0.05$  was considered statistically significant. The results were reproduced in three repeated experiments.

### Results

#### *CDC37 is overexpressed in human hepatocellular carcinoma cell lines with highly invasive potential*

Previous studies have showed that CDC37 is upregulated in several types of cancers, including human hepatocellular carcinoma cells [21-23]. Here, we examined the expression levels of CDC37 in five human hepatocellular carcinoma cell lines. The expression of CDC37 was

detected in all of these cell lines (**Figure 1A**). In addition, western blot analysis indicated that HCC-LM3 and SMMC7721 cells with highly invasive potential express higher levels of CDC37 compared to other lowly highly invasive cell lines (**Figure 1A**). We then performed real-time PCR to examine the expression levels of CDC37 mRNA. As shown in **Figure 1B**, the expression levels of CDC37 mRNA were consistent with the protein expression in the same human hepatocellular carcinoma cell lines. These results indicated that CDC37 was abundantly overexpressed in human hepatocellular carcinoma cell lines, especially in the highly invasive cell lines.

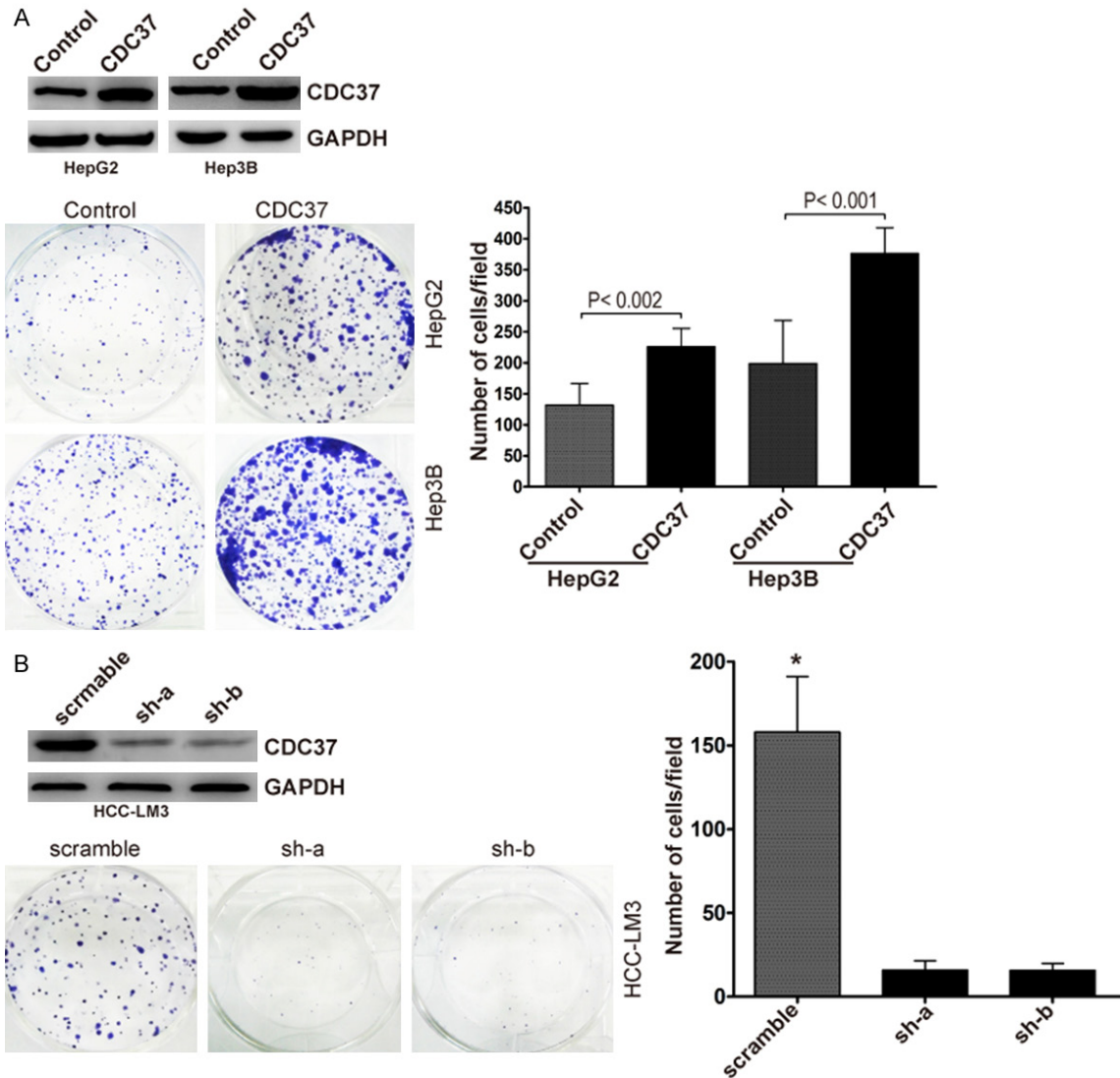
#### *CDC37 promotes the proliferation of human hepatocellular carcinoma cells*

To examine the role of CDC37 in human hepatocellular carcinoma cells, stable CDC37 overexpressing HepG2 or Hep3B cells were established using lentiviral expression system. Western blot analysis showed that the expression levels of CDC37 protein were dramatically increased in CDC37 stably overexpressing cells compared to the empty vector controls (**Figure 2A**). We then investigated the function of CDC37 to the proliferation of human hepatocellular carcinoma cells. Our findings showed that stably overexpressing CDC37 in HepG2 or Hep3B cells significantly promoted the cell proliferation compared to the empty vector controls (**Figure 2A**). Moreover, stable CDC37 knock-down HCC-LM3 cells were also established using lentivirus-mediated RNA interference. Western blot analysis showed that the expression levels of CDC37 protein were dramatically decreased in CDC37 stably knock-down HCC-LM3 cells compared to the scramble controls (**Figure 2B**). In CDC37 stably knock-down HCC-LM3 cells, cell proliferation is greatly reduced compared to the scramble controls (**Figure 2B**). Taken together, these results indicated that CDC37 was essential to the proliferation of human hepatocellular carcinoma cells.

#### *CDC37 promotes the cell invasion and the activity of AKT1 of human hepatocellular carcinoma cells*

To investigate whether CDC37 affects the cell invasion of human hepatocellular carcinoma cells, we performed cell invasion assay to assess the invasive potential between CDC37

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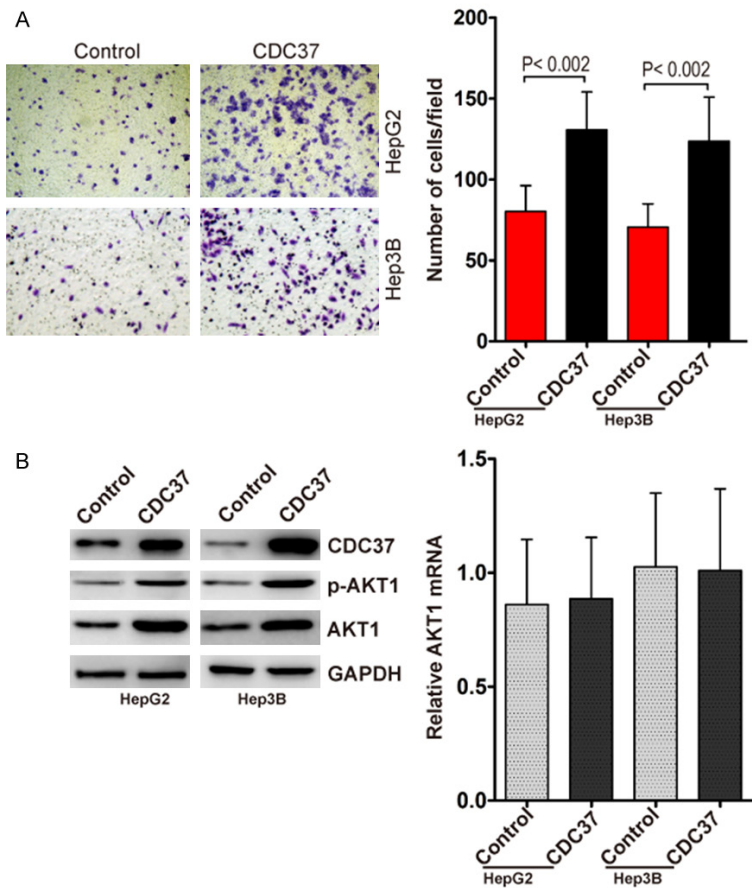
**Figure 2.** CDC37 promotes the proliferation of human hepatocellular carcinoma cells. A. CDC37 protein levels in vector control and CDC37 stably over-expressed HepG2 or Hep3B cells by western blot analysis (upper panel). GAPDH was used as an internal quantitative control. Representative photographs of colony formation in vector control and CDC37 stably over-expressed HepG2 or Hep3B cells (down panel). Representative data are shown as the mean  $\pm$  SD. n=3; P < 0.002 or P < 0.001, vs. each control group. B. CDC37 protein levels in scramble control and CDC37 stably knock-down (sh-a/sh-b) HCC-LM3 cells by western blot analysis (upper panel). GAPDH was used as an internal quantitative control. Representative photographs of colony formation in scramble control and CDC37 stably knock-down HCC-LM3 cells (down panel). Representative data are shown as the mean  $\pm$  SD. n=3; \*P < 0.0001, vs. each knock-down group.

stably overexpressing cells and the empty vector controls. As shown in **Figure 3A**, overexpression of CDC37 promoted the cell invasion in HepG2 or Hep3B cells compared to their empty vector controls. CDC37 has been shown to form complex with AKT1 and then stabilize it [21]. Moreover, active AKT1 was reported to enhance cell invasion and migration in several types of cancer cells [24-26]. We then assessed

the effect of CDC37 on the activity of AKT1. AKT1 is activated by phosphorylation at Thr308 and Ser473 [27]. The phosphorylation at Ser473 of AKT1 can represent the active form of AKT1 [28]. We then detected the change of the levels of AKT1 total and phosphorylated protein at Ser473 after the overexpression of CDC37 in in HepG2 or Hep3B cells. As shown in **Figure 3B**, overexpression of CDC37 resulted



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**Figure 3.** CDC37 promotes the cell invasion and the activity of AKT1. A. In-vitro cells in invasion assay were stained and counted in vector control and CDC37 stably over-expressed HepG2 or Hep3B cells.  $P < 0.002$  compared with the control group. B. CDC37, AKT1, p-AKT1 (phosphorylation at Ser473) and GAPDH expression levels were determined by western blot analysis in vector control and CDC37 stably over-expressed HepG2 or Hep3B cells (left panel). The mRNA expression levels of AKT1 in vector control and CDC37 stably over-expressed HepG2 or Hep3B cells were determined by real-time RT-PCR analysis (right panel). GAPDH was used as an internal quantitative control.

in an increase in the levels of phosphorylated AKT1 protein at Ser473 and total AKT1 proteins. Moreover, the expression levels of AKT1 mRNA were not changed by the overexpression of CDC37 (**Figure 3B**). Our results revealed that overexpression of CDC37 could promote the invasion in human hepatocellular carcinoma cells. Therefore, we hypothesized that the increase of cell invasion by overexpression of CDC37 might be due to the increased protein levels and, hence, the levels of active AKT1.

### CDC37 increases the stability of PINK1 kinase

PTEN-induced kinase 1 (PINK1) was reported to increase the phosphorylation of AKT1 at Ser473 and promote the activity [29]. Moreover,

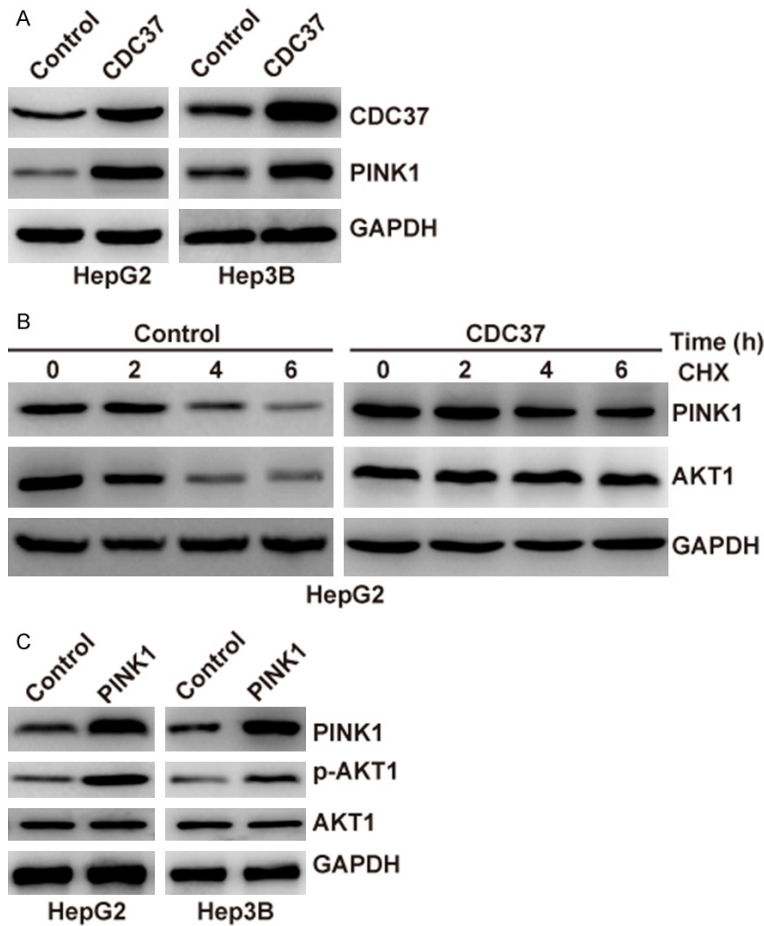
the stability of PINK1 protein was enhanced by the direct interaction with CDC37 [30]. Here, we found that overexpression of CDC37 increase the protein levels of PINK1 in HepG2 or Hep3B cells (**Figure 4A**). Furthermore, we performed cycloheximide (CHX) chase experiments in vector control and CDC37 stably overexpressing HepG2 cells to determine endogenous PINK1 protein stability. In the vector control HepG2 cells, we found endogenous PINK1 destabilized at 4 h after the treatment with CHX. At 6 h, the protein levels of endogenous PINK1 continued to decrease (**Figure 4B**). In CDC37 stably overexpressing HepG2 cells, endogenous PINK1 was found to be slightly destabilized at 4 h. At 6 h, the protein levels of endogenous PINK1 did not change compared to the levels at 4 h (**Figure 4B**). The stability of AKT1 protein was found to be consistent with PINK1 protein in CHX chase experiments. Additionally, overexpression of PINK1 in HepG2 or Hep3B cells was found to increase the phosphorylation of AKT1 at Ser473. These findings suggest that overexpression of

CDC37 could stabilize AKT1 and activate AKT1 by stabilizing PINK1.

### Inhibition of AKT1 attenuates CDC37 induced cell proliferation and invasion

In order to test the hypothesis that enhanced cell proliferation and invasion induced by overexpression of CDC37 might be due to the increased activity of AKT1, selective AKT1 inhibitor A-674563 was used. In HepG2 or Hep3B cells, CDC37 overexpression induced upregulation of phosphorylation of AKT1 at Ser473 was inhibited (**Figure 5A**). Moreover, we performed cell invasion assay to assess whether A-674563 affect the cell invasion in CDC37 stably overexpressing human hepatocellular carcinoma cells.

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**Figure 4.** CDC37 increases the stability of PINK1 kinase. A. CDC37, PINK1 and GAPDH expression levels were determined by western blot analysis in vector control and CDC37 stably over-expressed HepG2 or Hep3B cells. B. The vector control and CDC37 stably over-expressed HepG2 cells were treated with 50  $\mu\text{g}/\text{mL}$  of cycloheximide (CHX) for indicated times. Endogenous PINK1, AKT1 and GAPDH protein levels were determined by western blot analysis. C. PINK1, p-AKT1, AKT1 and GAPDH expression levels were determined by western blot analysis in vector control and PINK1 stably over-expressed HepG2 or Hep3B cells.

nomia cells. As shown in **Figure 5B**, AKT1 inhibitor A-674563 significantly inhibited the increase of invasion induced by the overexpression of CDC37. In addition, enhanced cell proliferation by the overexpression of CDC37 was also inhibited by A-674563 (**Figure 5C**). These results indicate that increased cell proliferation and invasion induced by the overexpression of CDC37 was mediated by AKT1 signaling pathway.

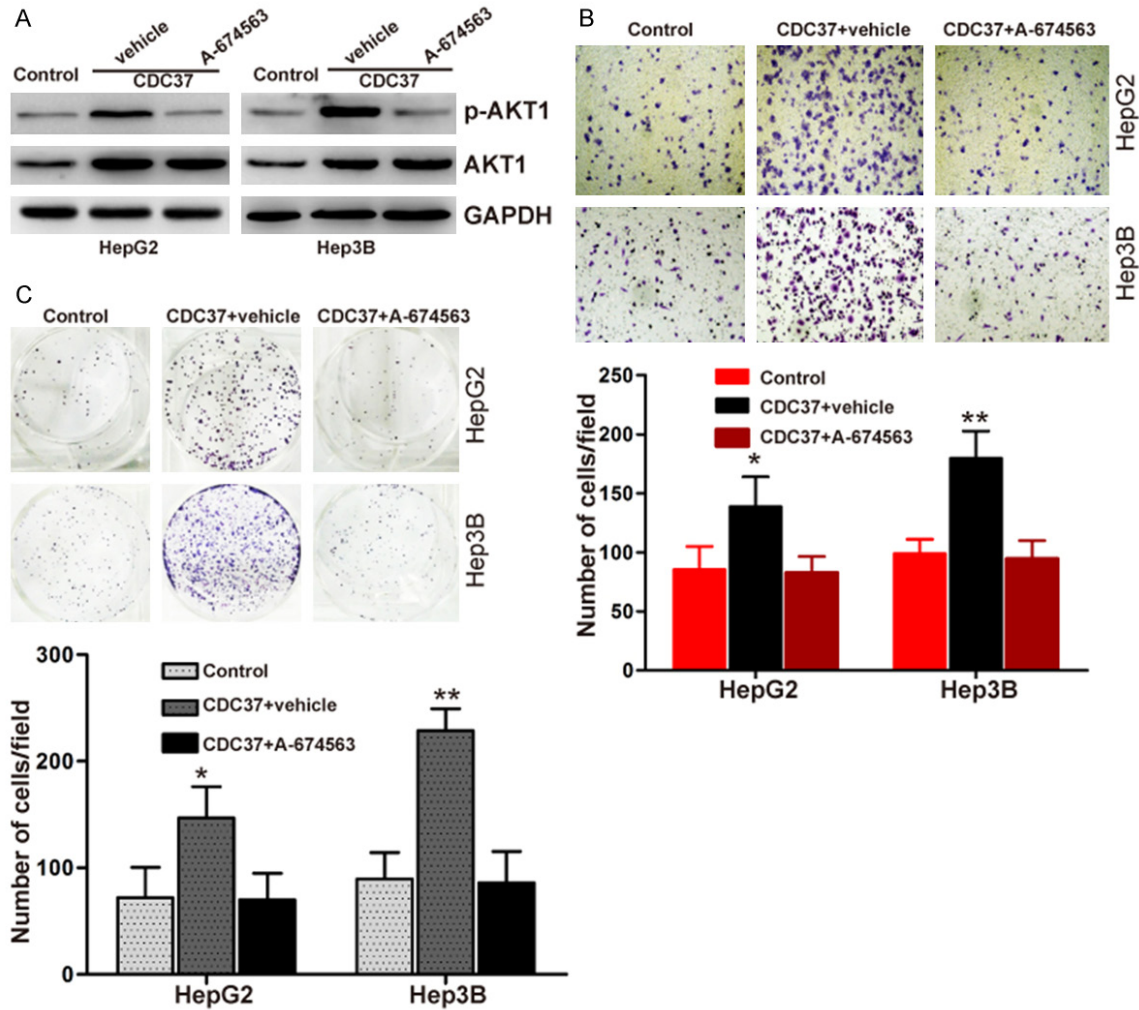
### Discussion

In this study, we demonstrate that expression levels of CDC37 were upregulated in human

hepatocellular carcinoma cell lines with highly invasive potential. Overexpression of CDC37 promotes the cell proliferation and invasion of human hepatocellular carcinoma cells. Meanwhile, overexpression of CDC37 increases the levels and the activity of AKT1, which could enhance cell invasion and migration in several types of cancer cells. Importantly, a selective AKT1 inhibitor A-674563 could attenuate CDC37 induced cell proliferation and invasion of human hepatocellular carcinoma cells. These results suggest that increased cell proliferation and invasion induced by the overexpression of CDC37 was mediated by AKT1 signaling pathway.

Overexpression or aberrant activation of AKT1 was found in several types of cancers, including breast carcinoma, lung carcinoma, ovarian carcinoma, prostate carcinoma and hepatocellular carcinoma [11]. Aberrant activated AKT1 can mediate cell proliferation [31], anti-apoptotic growth [32], invasion and metastasis [33] by phosphorylating and activating the downstream kinases. For example, Glycogen Synthase Kinase 3 Beta (GSK-3 $\beta$ ) [34], cyclin-dependent kinase inhibitor p27 [14], B-Cell CLL/Lymphoma 10 (BCL10) [35], SMAD Family Member 3 (SMAD3) and BCL2-Associated Agonist of Cell Death (BAD) can be phosphorylated by AKT1 [36, 37]. Importantly, AKT activation was necessary for the migration and invasion for the tumor cells [38]. It was reported that AKT1 together with the Inositol Polyphosphate 5-Phosphatase PIPP regulates AKT1-dependent breast cancer cell migration and invasion [39]. Thus, understanding the regulatory mechanisms of AKT1 activity and expression is necessary for developing effective AKT1-targeting therapeutic strategies.

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**Figure 5.** The effect of AKT1 inhibitor to the regulation of CDC37 induced cell proliferation and invasion. A. The vector control and CDC37 stably over-expressed HepG2/Hep3B cells treated with vehicle (DMSO) or 50 nM of A-674563 as indicated. AKT1, p-AKT1 and GAPDH expression levels were determined by western blot analysis. B. Invasive cells in invasion assay were stained and counted in vector control and CDC37 stably over-expressed HepG2/Hep3B cells treated with vehicle (DMSO) or A-674563. \* $P < 0.003$ , \*\* $P < 0.002$  compared with other group. C. The representative photographs of colony formation in vector control and CDC37 stably over-expressed HepG2/Hep3B cells treated with vehicle (DMSO) or A-674563. Representative data are shown as the mean  $\pm$  SD.  $n=3$ ; \* $P < 0.002$ , \*\* $P < 0.001$  compared with other group.

CDC37 is upregulated in human hepatocellular carcinoma cells [21]. Inhibition of the expression of CDC37 could inhibit the cell cycle progression and cell growth of hepatocellular carcinoma cells [18, 21]. Here, we found that the expression of CDC37 was detected in several hepatocellular carcinoma cell lines. CDC37 was also found overexpressed in hepatocellular carcinoma cell lines with highly invasive potential. Stable overexpression of CDC37 could promote the cell proliferation and invasion in human hepatocellular carcinoma cells. Stable knock-down of CDC37 greatly reduced the prolifera-

tion compared to the scramble controls in human hepatocellular carcinoma cells. These results indicated that CDC37 was essential to the proliferation of human hepatocellular carcinoma cells. Moreover, stable overexpression of CDC37 was found to promote the cell invasion of human hepatocellular carcinoma cells. Meanwhile, an increase of the activity of AKT1 was also found in CDC37 stably overexpressing human hepatocellular carcinoma cells. This imply that the increase of cell invasion by over-expression of CDC37 might be due to the increased activity of AKT1.



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PINK1 was reported to increase the phosphorylation of AKT1 at Ser473 and promote the activity of AKT1 [29]. The data presented herein indicate that overexpression of PINK1 can increase the phosphorylation of AKT1 at Ser473. Moreover, the stability of PINK1 or AKT1 protein was enhanced by the overexpression of CDC37. In addition, selective AKT1 inhibitor A-674563 could attenuate CDC37 induced cell proliferation and invasion of human hepatocellular carcinoma cells. These results suggest that increased cell proliferation and invasion induced by the overexpression of CDC37 was mediated by AKT1 signaling pathway.

### Disclosure of conflict of interest

None.

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