CDC23 knockdown suppresses the proliferation, migration and invasion of liver cancer via the EMT process

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Abstract. Liver cancer (LC) is a malignant tumour that is associated with high mortality rates worldwide. Cell division cycle 23 (CDC23) acts as an oncogene in papillary thyroid cancer. In addition, epithelial-mesenchymal transition (EMT) is frequently involved in the malignant metastasis of various cancer types. Therefore, we hypothesized that CDC23 may regulate the malignant biological behaviours of LC cells through EMT. Proliferation, colony formation and Transwell assays, western blotting and xenograft experiments were performed. The results of the present study showed that CDC23 was highly expressed in LC cell lines. In addition, it was found via multiple *in vitro* assays that CDC23 knockdown reduced the proliferation, migration and invasion of LC cell lines. Finally, an *in vivo* study confirmed that CDC23 knockdown inhibited the growth of xenograft LC in nude mice. More importantly, the changes in the levels of EMT-related marker proteins were analysed in the sh-CDC23 group compared with the sh-NC group of cells and xenografts. E-cadherin was upregulated, and N-cadherin and vimentin were significantly downregulated after CDC23 silencing. Taken together, these results revealed that the knockdown of CDC23 inhibits the progression of LC by regulating EMT and that CDC23 may be a novel therapeutic target for LC.

Introduction

Liver cancer (LC) is a common malignancy worldwide (1,2). The most recent data from the National Cancer Centre in Beijing, China, show that LC is a common malignant tumour, and the mortality (17.1 per 100,000) and morbidity (18.3 per 100,000) rates associated with LC in China are higher compared with the global averages (mortality rate of 8 per 100,000 and morbidity rate of 10.1 per 100,000) in 2018 (3-5). Moreover, most patients with liver disease, such as hepatitis B or C-associated viral infections, and patients who abuse alcohol, which makes early diagnosis more difficult, are more likely to develop LC compared with other individuals (6). The pathogenesis of LC is not clear, and targeted treatments are lacking (7,8). Therefore, it is important to identify specific tumour markers to improve the treatment and prognosis of patients with LC.

Chromosomal instability is believed to be one of the causes of LC (9). Timely activation of the anaphase-promoting complex (APC) is considered to be an important factor for maintaining accurate chromosome segregation (10). APC is a polymeric protein complex that is composed of multiple tetratricopeptide repeat proteins: APC2, APC3, APC5, APC6, APC7, APC8 and APC11 (11). Previous studies have confirmed that abnormal regulation of APC-related tetratricopeptide repeat proteins may be involved in tumourigenesis (12-14), including LC (15,16).

Cell division cycle 23 (CDC23), which is also known as APC8, is an APC subunit that regulates mitosis by catalysing the formation of ubiquitin conjugates (17,18). Both the end of mitosis and the start of a new cell cycle depend on the ubiquitin-mediated degradation of key cell cycle proteins (19). CDC23 was identified in a genetic screen using *Saccharomyces cerevisiae*, and it was found that the products of CDC23 catalysis are required for ubiquitination (20). A study on papillary thyroid cancer (PTC) revealed that CDC23 was overexpressed in PTC tissue compared with normal tissue and that CDC23 exerts important biological effects on thyroid cancer cell proliferation and cell cycle progression (21). In addition, a previous study confirmed that inhibition of microRNA-204-3p by LINC00514 increased CDC23 expression and further led to PTC progression (22). Moreover, knockdown of CDC23 by small interfering RNA induced G_2/M arrest in breast cancer cells, although CDC23 has not been further studied in these cells (23).

Notably, although numerous studies on APC-related proteins have been carried out in cancer (12-14), studies on the role of CDC23 in LC are still lacking. Therefore, the main aim

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of the present study was to explore the role of CDC23 in LC using *in vitro* and *in vivo* experiments.

Materials and methods

Cell lines and culture. The cell lines used in the present study include the normal human liver cell line THLE-2 and LC cell lines (HepG2, Hep3B, Huh7, LM3), which were purchased from Shanghai Institutes (Shanghai Biowing Applied Biotechnology, Co., Ltd.). These cell lines were authenticated by short tandem repeat profiling. The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). The cell culture media were supplemented with 100 U/ml penicillin and streptomycin at 37˚C in 5% CO_2.

Lentiviral infection. GV493 Lentivirus expressing short hairpin (sh)RNA targeting CDC23 (sh-CDC23) and negative control (NC) lentivirus (sh-NC) were designed and constructed by Shanghai Jikai Gene Chemical Technology Co., Ltd. The sh-CDC23 target sequence was 5'-CCAGTGTTACATCAA ATAT-3', while the sh-NC target sequence was 5'-TTCTCC GAACGTGTCACGT-3'. HepG2 and LM3 cells were transduced with these lentiviruses for 24 h (MOI=20). Subsequently, the medium was replaced with fresh complete culture medium supplemented with 5 μg/ml puromycin for 5 days, and the cells were harvested for subsequent experiments.

Western blotting. All cells and tissues were lysed in RIPA lysis buffer (Applygen Technologies, Inc.) and centrifuged at 4˚C for 30 min (300 x g) to obtain the lysates. The specific experimental steps were described in our previous study (24). The protein concentration was determined by the bicinchoninic acid (BCA) method. Proteins (50 μg/lane) were separated on 10% gels using SDS-PAGE and transferred to PVDF membranes. The following primary antibodies were used: Anti-CDC23 (cat. no. ab177148; 1:1,000), anti-E-cadherin (cat. no. ab76055; 1:1,000), anti-N-cadherin (cat. no. ab18203; 1:1,000), anti-vimentin (cat. no. ab8978; 1:1,000) (all from Abcam) and anti-β-actin (cat. no. CL488-66009; 1:2,000; Proteintech Group, Inc.). Antibodies were incubated with the PVDF membranes overnight at 4˚C. The Horseradish peroxidase-conjugated secondary antibodies (1:8,000; cat. nos. sc-2357 and sc-2005; Santa Cruz Biotechnology, Inc.) were incubated with the PVDF membranes at room temperature for 1 h. The target protein was detected with a Pierce enhanced chemiluminescence detection reagent (Thermo Fisher Scientific, Inc.).

Cell proliferation assay. Cell proliferation was determined using Cell Counting Kit-8 (CCK-8; Sigma-Adrich; Merck KGaA) assays according to the manufacturer's instructions. For the CCK-8 assays, $1x10^4$ HepG2 and LM3 cells/well were seeded in 96-well plates. After 24, 48, 72 and 96 h, a total of 10 μl CCK-8 solution was added to each well. The plates were incubated at 37˚C for 2 h, and the absorbance values at 450 nm were measured using a microplate reader (Thermo Fisher Scientific, Inc.).

Colony formation assay. Cells in the logarithmic growth phase from the sh-CDC23 group and sh-NC group were digested with EDTA + 0.25% trypsin at 37˚C for 3 min, and dissolved into single-cell suspensions in DMEM culture media supplemented with 10% FBS. A total of $2x10^2$ cells were inoculated into in 6-well plates, and the cells were cultured in 2 ml complete media. The cells were placed in an incubator and cultured at 37˚C with 5% CO_2 and saturated humidity for 14 days. Cell growth was observed during culture, and the culture was stopped when macroscopic colonies appeared in the culture dish. The supernatants were discarded, and the cells were carefully washed twice with PBS. The cells were fixed with 5 ml 100% methanol for 15 min at room temperature and then stained using 1 ml 0.1% crystal violet for 15 min at room temperature. Each group was run in triplicate, and the number of colonies formed (>50 cells) was manually calculated.

Transwell assays. HepG2 and LM3 cells ($2.0x10^5$/ml) in DMEM without serum were added to the upper chambers of a Transwell plate (24-well, 8.0-μm pores) (BD Biosciences). DMEM supplemented with 10% FBS was added to the lower chambers. For the invasion assays, a total of 50 μl Matrigel (BD Biosciences) was used to precoat the membrane surface before addition of the cells at 37˚C for 4 h. After incubation at 37˚C for 48 h, the non-invaded cells were removed, and the invaded cells were stained with a 0.1% crystal violet solution at 37˚C for 10 min. The cells were observed under an inverted fluorescence microscope.

Animal studies. A total of 10 male BALB/c nude mice (weight, 16-18 g; age, 4 weeks) were obtained from Hunan Slack Jingda Experimental Animal Co., Ltd. [production permit no. SCXK (XIANG) 2013-0004]. The mice were randomly divided into two groups (sh-NC and sh-CDC23 groups; n=5 mice/group) and subcutaneously injected with $2x10^6$ LM3 cells. The mice were monitored weekly (25,26) and the tumour volume (formula: $L \times S^2 \times 0.5$, where L and S represent the maximum and minimum diameter of the tumour, respectively) was assessed. After 4 weeks, the mice were anesthetized with isoflurane (3-6%), followed by cervical dislocation to ensure death. All details of the housing conditions were described in our previous study (27). The present study was approved by the Ethics Committee of The First Affiliated Hospital of Nanchang University (Nanchang, China; approval no. 202112020). All experiments were conducted in accordance with Nanchang University and Canadian Council on Animal Care (CCAC) ethical guidelines. CCAC guidelines were used to define humane endpoints, including a tumor not exceeding 10% of the animal's body weight, a tumor location that does not affect normal functions or cause pain, weight loss of >20%, ulceration or infection of the growth site, metastases and self-mutilation.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software; Dotmatics) and SPSS 18 (SPSS, Inc.). The data are presented as the mean ± standard deviation of three independent experiments. Unpaired Student's t-test was used to assess differences between two groups. One-way ANOVA followed by Dunnett's multiple comparisons test was utilized to analyse the differences between multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

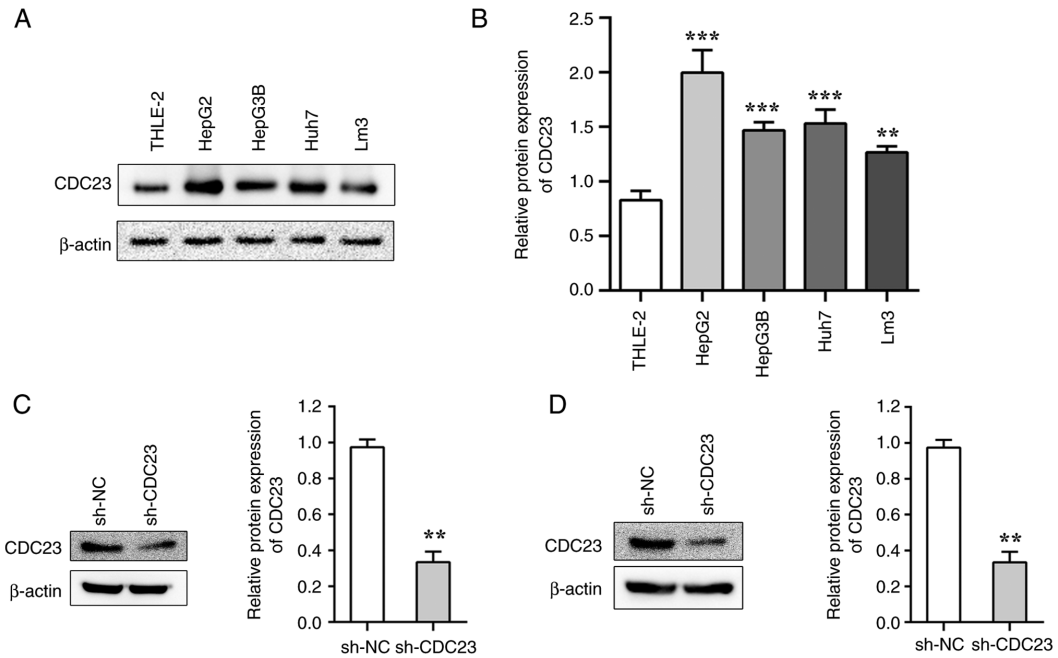

Figure 1. CDC23 protein expression level in human LC cell lines. (A) Western blot assay and (B) bar graph showing the protein expression levels of CDC23 in LC cell lines and THLE-2 normal liver cell line. After transduction, the protein expression levels of CDC23 were significantly decreased in (C) LM3 and (D) HepG2 cells. **P<0.01 and ***P<0.001 vs. THLE-2 or sh-NC. CDC23, cell division cycle 23; LC, liver cancer; sh, short hairpin; NC, negative control.

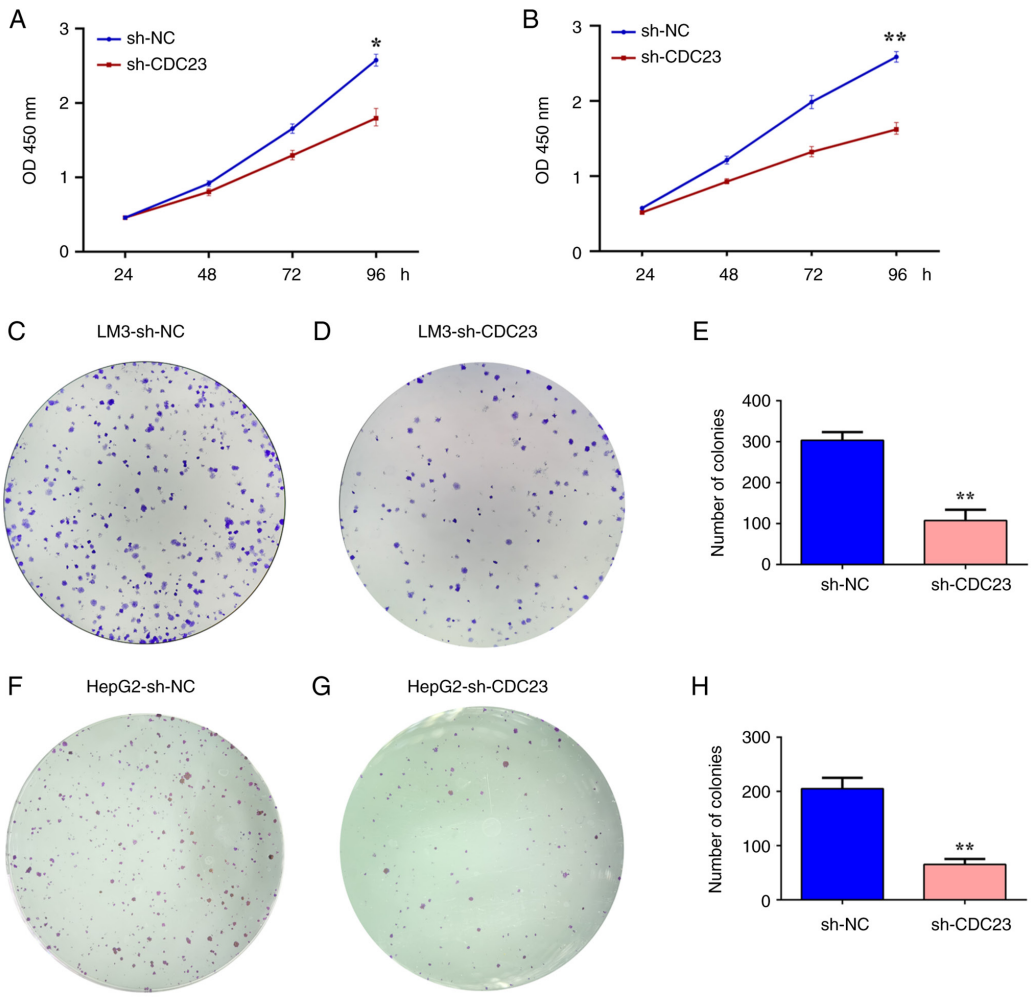

Figure 2. Effect of CDC23 knockdown on the proliferation of liver cancer cells. The proliferation of (A) LM3 and (B) HepG2 cells was assessed using Cell Counting Kit-8 assays. The proliferation of (C-E) LM3 and (F-H) HepG2 was assessed using colony formation assays. *P<0.05 and **P<0.01 vs. sh-NC. CDC23, cell division cycle 23; NC, negative control; sh, short hairpin; OD, optical density.

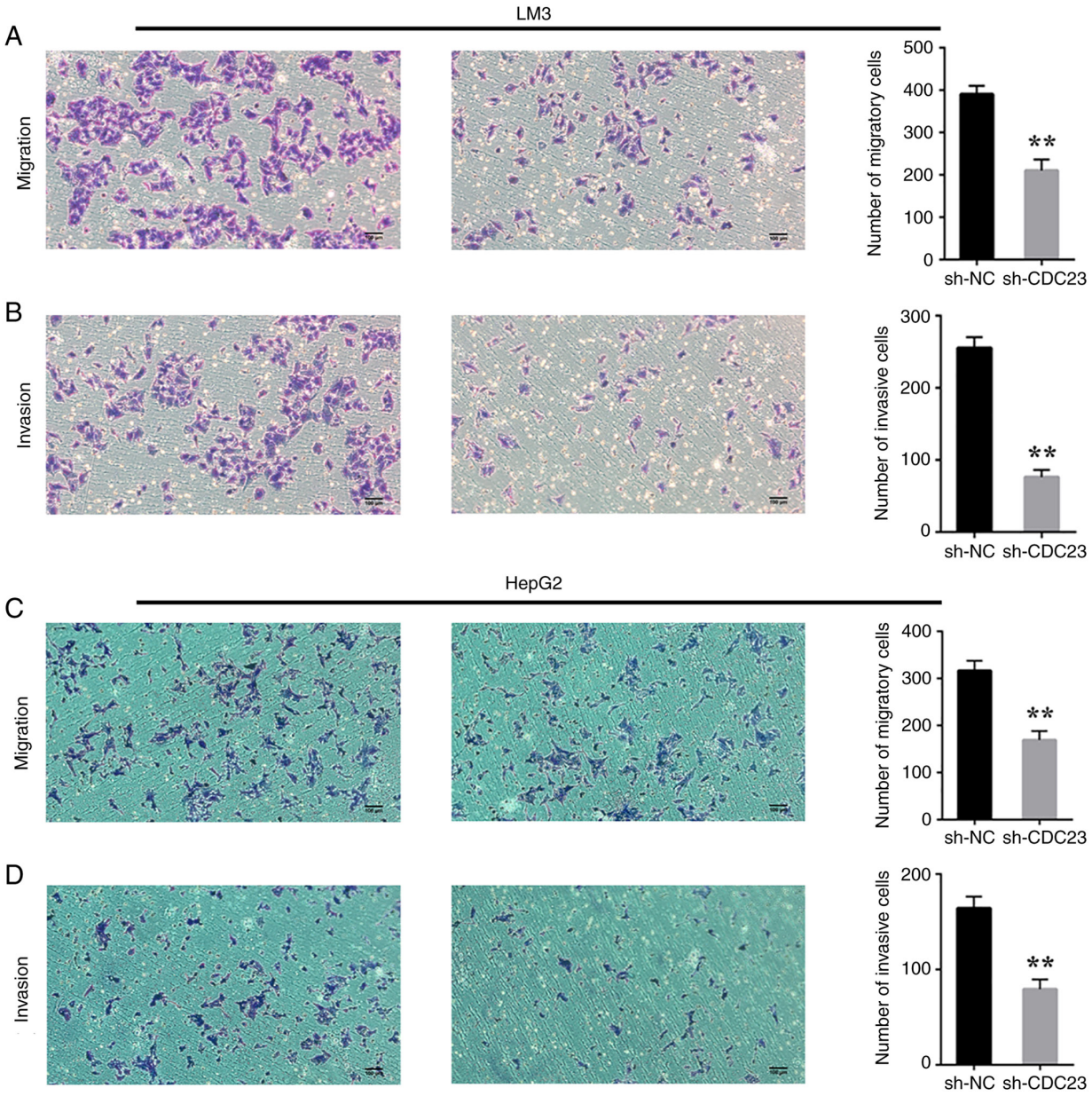

Figure 3. Effect of CDC23 knockdown on the migration and invasion of liver cancer cells. The migration and invasion of (A and B) LM3 and (C and D) HepG2 cells were measured using Transwell assay (magnification, x100; scale bar, 100 μm). **P<0.01 vs. sh-NC. CDC23, cell division cycle 23; NC, negative control; sh, short hairpin.

Results

CDC23 is highly expressed in LC cell lines. The results showed that the protein expression levels of CDC23 were significantly higher in LC cell lines compared with that in the normal liver cell line THLE-2 (Fig. 1A and B). Among the LC cell lines, CDC23 was highly expressed in LM3 and HepG2. Therefore, the LM3 and HepG2 cell lines were selected for further experiments. After transduction with sh-CDC23, the protein expression levels of CDC23 were significantly decreased in LM3 and HepG2 cells compared with the sh-NC group (Fig. 1C and D).

Knockdown of CDC23 inhibits the proliferation and colony formation of LM3 and HepG2 cells. The experimental results of the present study showed that CDC23 was highly expressed in LC cell lines, and we hypothesized that CDC23 may promote the progression of LC. CCK-8 and colony formation assays were used to assess the effects of CDC23 on the proliferation and colony formation ability of LC cell lines. CCK-8 assays showed that knockdown of CDC23 significantly suppressed the proliferation of LM3 and HepG2 cells (Fig. 2A and B). Colony formation experiments also revealed that knockdown of CDC23 significantly suppressed the clonogenic ability of LM3 and HepG2 cells (Fig. 2C-H).

CDC23 knockdown reduces the migratory and invasive abilities of LC cells. Considering the effect of CDC23 on the proliferation and colony formation of LC cells, it was examined whether CDC23 also affects the invasion and migration of these cells. A Transwell assay was carried out a to verify this hypothesis. As expected, the assay results showed that

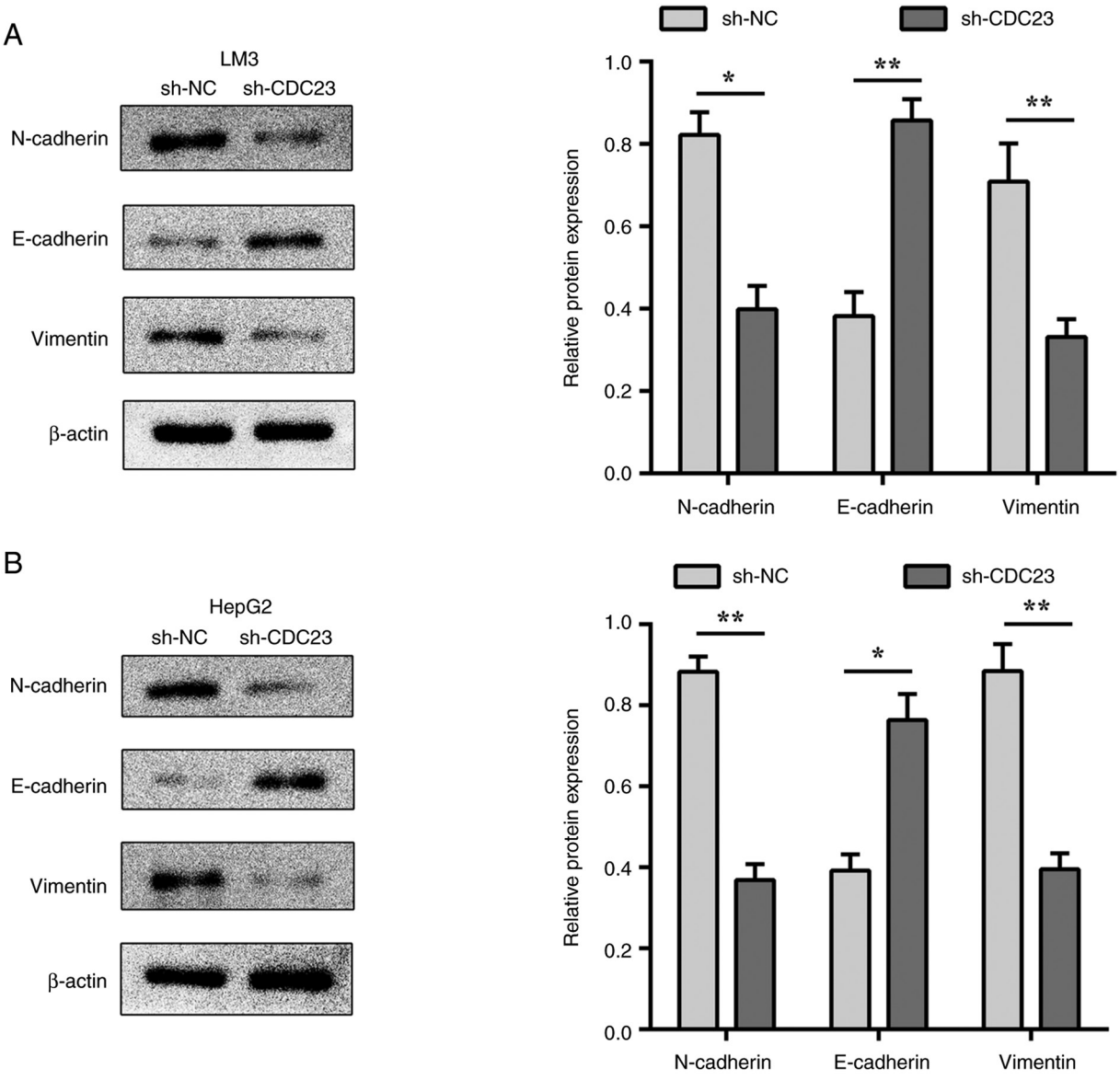

Figure 4. Effects of CDC23 knockdown on the protein expression levels of the epithelial-mesenchymal transition markers E-cadherin, N-cadherin and vimentin in (A) LM3 and (B) HepG2 cells. *P<0.05; **P<0.01. CDC23, cell division cycle 23; NC, negative control; sh, short hairpin.

knockdown of CDC23 significantly inhibited the migratory and invasive abilities of LM3 (Fig. 3A and B) and HepG2 cells (Fig. 3C and D).

Effect of silencing CDC23 on the expression of epithelial-mesenchymal transition (EMT)-related molecules. EMT plays a vital role in cancer cell metastasis (28). N-cadherin, vimentin and E-cadherin are key regulatory molecules of EMT (29). The results of the present study indicated that the knockdown of CDC23 significantly increased E-cadherin expression in LM3 and HepG2 cells (Fig. 4A and B), while the expression levels of N-cadherin and vimentin were significantly decreased (Fig. 4). These results suggested that CDC23 may affect LC metastasis by regulating the EMT process in LC cells.

Knockdown of CDC23 inhibits tumour growth in vivo. A tumour xenograft model was established to assess the role of CDC23 *in vivo*. *In vivo* experiments were further conducted to evaluate the effect of CDC23 knockdown on LM3 cells in mice (Fig. 5A). The results indicated that the tumour volumes in the sh-CDC23 group were significantly lower compared with those in the sh-NC group (Fig. 5B). In addition, the tumour weights were higher in the sh-NC group compared with the sh-CDC23 group (Fig. 5C). Western blot assays also revealed that the protein expression level of E-cadherin was increased in the sh-CDC23 group compared with sh-NC, while those of N-cadherin and vimentin were decreased instead (Fig. 5D). These results were consistent with those from the *in vitro* experiments.

Discussion

The role of CDC23 in cancer has attracted the attention of the scientific community (21,23). Interestingly, a previous study assessed whether CDC23 is a tumour suppressor gene by searching for CDC23 mutations in 5q-abnormal myeloid leukaemia cells (30). However, experimental results suggested that CDC23 may not be involved in the progression of myeloid leukaemia characterized by chromosome 5 abnormalities (30).

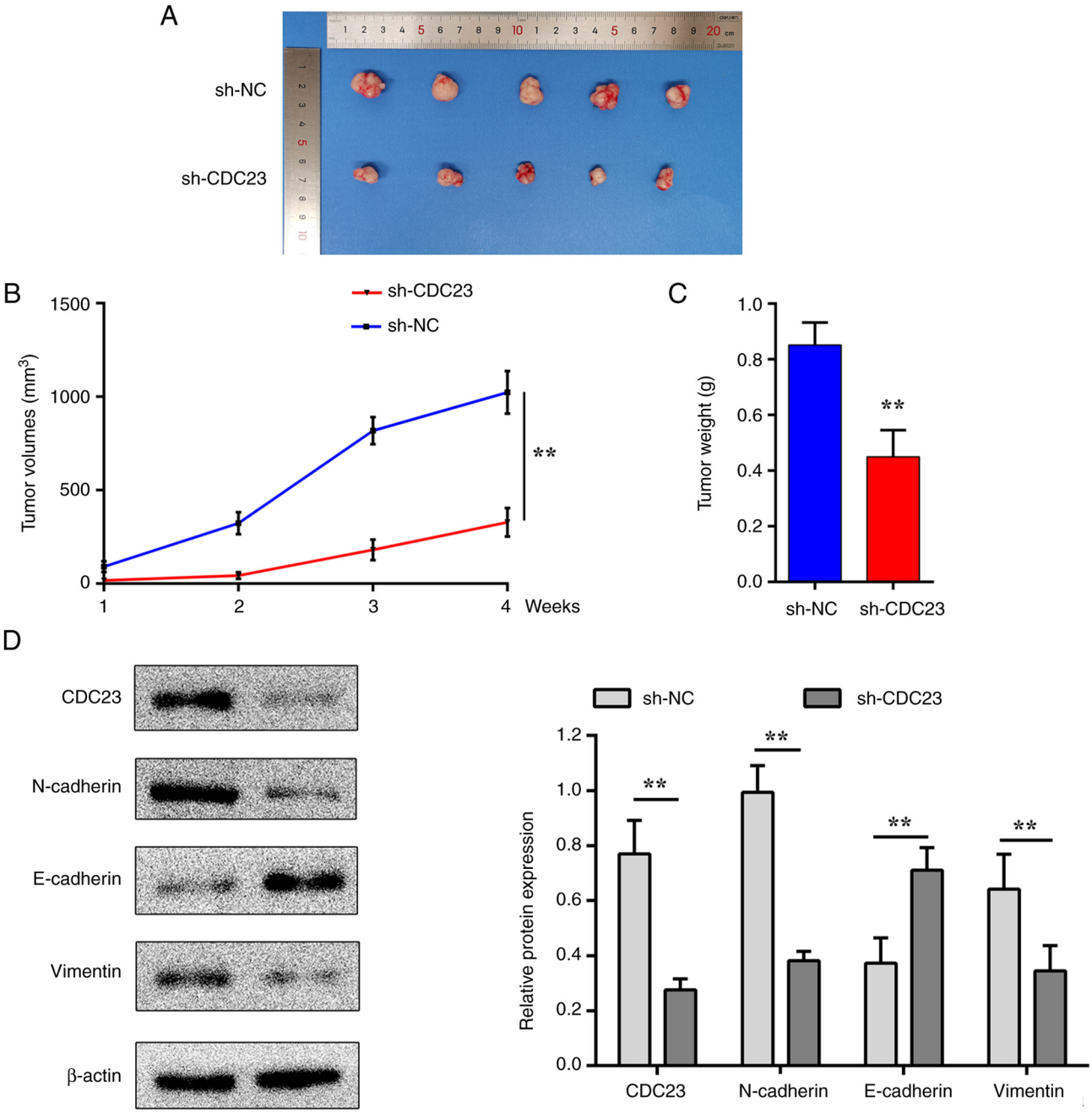

Figure 5. *In vivo* effect of CDC23 knockdown in LM3 cells. (A) Xenograft tumours in nude mice. (B) Tumour growth curve and (C) tumour weights in the sh-NC and sh-CDC23 groups. (D) Expression of CDC23, N-cadherin, vimentin and E-cadherin in tumour tissue. **P<0.01. CDC23, cell division cycle 23; NC, negative control; sh, short hairpin.

Thus, in the present study, it was experimentally evaluated whether CDC23 suppresses or promotes LC.

To determine whether CDC23 is specifically expressed in LC, the protein expression level of CDC23 was examined and found to be significantly higher in LC cell lines compared with a normal liver cell line. This finding may suggest that CDC23 may impact the LC cell phenotype and functions. Migration and proliferation are closely associated with the high mortality rate of cancer, and these processes are also main obstacles for successfully curing cancer (31-33). Therefore, whether CDC23 affects the LC cell phenotype was investigated in the present study. The experimental results indicated that the proliferation and migration of the HepG2 and LM3 cell lines were significantly inhibited after CDC23 gene knockdown. The results suggested that high CDC23 expression was closely associated with the proliferation and migration of LC cells.

The metastatic capacity of tumour cells is closely related to the high morbidity and mortality of cancer, with 50% of 1,438 patients with breast cancer developing metastasis within 10 years, and it is also a main hurdle in preventing cancer regression (31). EMT is a key cellular process required for embryonic development and its role in initiating and promoting tumour cell metastasis and invasion, as well as the underlying mechanism, have received increasing attention (34,35). The metastasis of tumour cells involves EMT, directed invasion of the surrounding tissue of tumour cells, invasion of tumour cells

into the blood circulation, lymphatic circulation and vascular exudation (36). EMT is regulated by numerous calcium-dependent cell adhesion molecules that regulate epithelial properties, including E-cadherin, N-cadherin and vimentin (29,37). These EMT markers have been confirmed to be associated with the progression of various tumours, including breast cancer (38), laryngeal squamous cell carcinoma (39) and pancreatic carcinoma (40). However, the functions of these three proteins (E-cadherin, N-cadherin and vimentin) on tumour metastasis is not the same. For example, overexpression of E-cadherin is involved in inhibiting tumour metastasis, whilst overexpression of N-cadherin and vimentin is involved in promoting tumour metastasis (38-40). Moreover, multiple studies have confirmed that the functions of these three proteins in liver cancer are similar to those aforementioned (41-43).

The experimental results of the present study indicated that inhibition of CDC23 expression in LC cell lines significantly reduced cancer cell invasion. Notably, it was hypothesized that the mechanism by which CDC23 regulates tumourigenesis involves tumour EMT and metastasis. In the present study, the expression of EMT markers was investigated. The results indicated that the expression of E-cadherin was significantly increased while the expression levels of N-cadherin and vimentin were significantly decreased after knockdown of CDC23 in HepG2 and LM3 cells. The aforementioned results revealed that CDC23 may regulate LC progression through EMT *in vitro*. However, how CDC23 regulates EMT is not clearly established and the lack of CDC23 overexpression experiments is a limitation of the present study.

However, the biological environment is also relevant, and whether these *in vitro* results can be verified *in vivo* still requires further investigation. *In vivo* experiments were performed to confirm the effect of the CDC23 knockdown that was observed in the *in vitro* experiments. Nude mice were injected with cells that had been transduced with sh-NC or sh-CDC23. After analysis, significant differences in the tumour dimensions and weight between the two groups were observed. In addition, western blot assays revealed that the protein expression of E-cadherin was increased, while the expression levels of CDC23, N-cadherin and vimentin were decreased in the sh-CDC23 group compared with sh-NC. These results indicated that CDC23 knockdown in LC cells inhibited tumour growth *in vivo*. Thus, the *in vitro* results were consistent with the *in vivo* results.

In conclusion, the present study demonstrated that CDC23 is highly expressed in LC cell lines. In addition, CDC23 emerged as a regulator of the malignant biological behaviour of LC cell lines through modification of the expression of EMT markers, which may reveal a novel target for further studying LC growth and metastasis.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YL and JX designed the study. YZ, LL, CF and WH analysed the data. YZ and LL contributed to performing the experiments and wrote the manuscript. YL and JX confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal study was approved by the Ethics Committee of The First Affiliated Hospital of Nanchang University (Nanchang, China; approval no. 202112020).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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