

Investigation of the role of cullin 4A overexpression in human liver cancer

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Abstract. Cullin 4A (CUL4A) is the major component of cullin-RING-based E3 ubiquitin-protein ligase complexes, which regulate the ubiquitination of target proteins. The overexpression of CUL4A has been associated with the development and progression of various cancer types. However, a detailed understanding of the role of CUL4A in human liver cancer has not been determined by previous studies. In the present study, the association between human liver cancer and CUL4A expression was investigated. The expression of CUL4A in liver cancer tissues and paracancerous tissues of patients was investigated by reverse transcription-quantitative polymerase chain reaction, western blotting and immunohistochemical staining. Overexpression and knockdown of CUL4A were induced with an overexpression vector and small interfering RNA transfection, respectively, in human liver cancer cell lines, and the effects on cell proliferation were analyzed by a Cell Counting Kit-8 assay to investigate the role of CUL4A in human liver cancer. Cell migration, invasion, apoptosis and the cell cycle were also analyzed following transfection. The results of the present study revealed that the mRNA and protein expression of CUL4A was increased in the liver cancer tissues compared with the paracancerous tissues of 3 patients. Additionally, the results demonstrated that downregulation of CUL4A expression inhibited cell proliferation, migration and invasion, and increased the percentage of cell apoptosis, in HEPG2 and MHCC97-H cells, while CUL4A overexpression led to the opposite effects. Therefore, the results of the current study indicated that CUL4A may serve an important role in the development and progression of human liver cancer, and highlights the potential of CUL4A as a novel target in the diagnosis and treatment of human liver cancer and potentially other cancer types.

Introduction

Liver cancer is the third highest contributor to cancer mortality worldwide (1,2). A high rate of tumor metastasis and recurrence are major factors that contribute to the poor prognosis associated with liver cancer. Therefore, the efficacy of radiotherapy and chemotherapy is limited for the majority of patients with liver cancer following diagnosis, and surgical treatment is considered as the only available therapy method at the initial stage of liver cancer (3,4). Therefore, an improved understanding of the mechanism of liver cancer is required to identify novel prognostic molecular markers, as well as potential effective therapeutic targets, to improve the effect of therapy and patient survival rate.

It has previously been demonstrated that the accumulation of epigenetic and genetic alterations in hepatocytes, and uncontrolled cell proliferation and death, are essential for the progression and initiation of liver cancer (5). In liver cancer, chromosomal abnormalities are the most common form of genetic mutation, and numerous chromosomal regions that are frequently unstable have been identified in liver cancer (6,7). For example, a 13q34 amplification has been detected in several liver cancer cell lines, and this region comprises five genes, including transcription factor Dp-1, cullin 4A (CUL4A) and cell division cycle protein 16 (8). These genes may have potential as novel therapeutic targets for liver cancer; however, further investigation into the particular roles of these genes is required.

CUL4A is a single-copy gene and encodes an 87-kDa protein that belongs to the cullin family. High expression levels of CUL4A have been reported in the spleen and testis, with poor expression in the liver, lung and thymus (9). The CUL4A protein is able to bind to ring-box protein 1 and DNA damage-binding protein 1, forming the ubiquitin ligase E3 complex. This complex mediates the ubiquitination and degradation of particular substrates and has an important function in the maintenance of cellular physiology (10). The role of CUL4A in oncogenesis has received increased interest; amplification or overexpression of the CUL4A gene has been detected in various cancer types, including liver cancer (8), adrenocortical carcinoma (11) and pituitary adenomas (12). In 2015, Pan *et al* (13) reported an inverse correlation between the expression of the CUL4A gene and patient survival, while a positive correlation with lymphatic and venous invasion was

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identified. Additionally, the expression of CUL4A in liver cancer tissues was associated with hepatitis B virus (HBV) e-antigen (HBeAg) status in patients and may be upregulated by HBV in liver cancer cell lines *in vitro* (13). Furthermore, knockdown of CUL4A ameliorated the motility of liver cancer cell lines by regulating the expression of epithelial-mesenchymal transition (EMT)-associated genes (13). Recently, another group identified that a novel long noncoding (lnc)RNA, uc.134, repressed liver cancer progression by inhibiting the CUL4A-mediated ubiquitination of the large tumor suppressor kinase 1 (LATS1) protein, indicating that the application of uc.134 lncRNA may offer a promising treatment approach for liver cancer, and that CUL4A may serve an important role in liver cancer progression (14).

In the present study, the clinical relevance of CUL4A in liver cancer was primarily investigated. The results demonstrated that the expression levels of CUL4A in human liver cancer tissues were markedly increased compared with paracancerous tissues. CUL4A overexpression in liver cancer cell lines led to enhanced liver cancer cell proliferation, migration and invasion, while CUL4A knockdown suppressed the proliferation and motility of liver cancer cells, and significantly induced cell apoptosis, indicating that CUL4A may have the potential to serve as a novel therapeutic target for liver cancer.

Materials and methods

Ethical approval and consent. The present study was approved by the Committee on the Ethics of Animal Experiments and Human Subject Research of the First People's Hospital of Kunming (Kunming, China). All volunteers involved in the present study provided written informed consent.

Liver cancer samples. In the present study, liver cancer tissues and paracancerous tissues from 3 different patients (obtained from the First People's Hospital of Kunming, Kunming, China) were used to analyze the importance of CUL4A in liver cancer treatment. All samples originated from primary tumors and were collected from April-December 2016. The cancer tissue from patient 1 (age, 59; sex, male;) and patient 2 (age, 56; sex, female) were diagnosed as infiltrating liver cancer and the cancer tissue from patient 3 (age, 52; sex, male) was superficial liver cancer.

Cell culture. The liver cancer cell lines HEPG2 (hepatoblastoma cell line) (15) and MHCC97-H (hepatocellular carcinoma cell line) were employed in the present study and were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in high-glucose Dulbecco's modified Eagle's medium (HG-DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 0.1 g/ml streptomycin (Hyclone; GE Healthcare Life Sciences) in a humidified incubator at 37°C with 5% CO₂. Medium was replaced every other day and adherent cells were passaged by 1:4 dilution every 5-7 days.

Overexpression and knockdown of CUL4A in liver cancer cell lines. To generate the CUL4A overexpression vector,

CUL4A-coding sequences were obtained by reverse transcription-polymerase chain reaction (RT-PCR) with the following primer sequences: CUL4A forward, 5'-CGG AATTCATGGCGGACGAGGCCCGCGGAA-3' and reverse, 5'-ACGGTACCTCAGGCCACGTAGTGGTAC TGAT-3'. The sequences were amplified using the following parameters: Initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 35 sec, 60°C for 35 sec and 72°C for 90 sec; followed by a final extension at 72°C for 5 min. Coding sequences were cloned into a pCI-based overexpression plasmid (Addgene, Inc., Cambridge, MA, USA). Human CUL4A small interfering (si)RNA (sequence, AAGAAG AUU AACACGUGCUGG) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA; cat. no. sc-44355). For the overexpression and knockdown of CUL4A, liver cancer cell lines (1x10⁶ cells/well) were cultured in a 6-well plate overnight at 37°C and were subsequently transfected with pCI-CUL4A vector (2 µg/ml) and CUL4A-siRNA (10 µM/ml), respectively, using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), while cells transfected with an empty pCI vector (2 µg/ml) or control-siRNA (10 µM/ml; sequence, AACAGUCGCGUUUGCGACUGG dTdT) served as the control groups. The detailed operation was performed according to the Lipofectamine[®] 2000 protocol. At 36 h post-transfection, cells were harvested for the subsequent experiments.

RT-quantitative PCR (RT-qPCR). RT-qPCR was performed as previously described (16,17). Briefly, the total RNA was extracted from HEPG2 cells and converted into cDNA. The high capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.) and PrimeScript[™] II High Fidelity One Step RT-qPCR Kit (Takara Biotechnology Co., Ltd., Dalian China) were used for reverse transcription and qPCR, respectively. Following an initial polymerase activation and denaturation step at 50°C for 2 min and 95°C for 5 min, respectively, the samples in each group underwent 40 amplification cycles of 95°C for 20 sec, 65°C for 10 sec and 72°C for 30 sec in the Light Cycler 480 instrument (Roche Diagnostics, Basel, Switzerland). Three independent experiments were performed. Results were quantified using the 2^{-ΔΔC_q} method (18). In the present study, 18s ribosomal (r)RNA was used for normalization and all measurements were performed in triplicate. The primer sequences (5'-3') were as follows: CUL4A, 5'-TCCTGT TCTTGGACCGCACCT-3' (forward) and 5'-ACCTGCAGG TCAGACAGCATGC-3' (reverse); and 18s rRNA, 5'-CCT GGATACCGCAGCTAGGA-3' (forward) and 5'-GCGGCG CAATACGAATGCCCC-3' (reverse).

Western blotting. Both tissue samples and cell samples were harvested with radioimmunoprecipitation assay lysis buffer (CST Biological Reagents Co., Ltd., Dalian, China) and the protein content of cell lysates in different groups was further detected with a bicinchoninic acid protein estimation kit (Pierce; Thermo Fisher Scientific, Inc.). Western blotting was performed as previously described (19). Briefly, Protein (15 µg/lane) was separated on 10% polyacrylamide gel, followed by transfer to a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin (BSA; Thermo Fisher Scientific, Inc.) for 2 h at room temperature

and subsequently incubated with the following primary antibodies overnight at 4°C: Anti-CUL4A (cat no. ab72548; 1:1,000; Abcam, Cambridge, UK), anti-E-cadherin (cat no. ab1416; 1:500; Abcam), anti-N-cadherin (cat no. ab18203; 1:500; Abcam), anti-claudin 3 (Cldn3; cat no. ab15102; 1:1,000; Abcam), anti-occludin (Ocldn; cat no. ab31721; 1:1,000; Abcam), anti-epithelial cell adhesion molecule (Epcam; cat no. ab71916; 1:800; Abcam), anti-Snail (cat no. ab53519; 1:500; Abcam), anti-Slug (cat no. ab27568; 1:500; Abcam), anti-vimentin (cat no. ab8978; 1:500; Abcam) and anti-GAPDH (cat no. ab8245; 1:10,000; Abcam). Subsequently, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse (cat no. sc-2005) or rabbit (cat no. sc-2357) immunoglobulin G secondary antibodies (1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Bands were visualized with an Amersham ECL kit (GE Healthcare, Chicago, IL, USA) and relative protein expression was quantified using Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunohistochemical staining. Prior to immunohistochemical staining of patient tissues, all tissue samples were fixed in 4% paraformaldehyde in PBS at room temperature for 36 h, and sectioned to 5 μ m thickness for staining, as previously described (20,21). For immunohistochemistry, sections were blocked with 5% BSA for 2 h at room temperature, and endogenous peroxidase activity was quenched with 3% H₂O₂ for 30 min at room temperature. A polyclonal primary antibody against CUL4A (cat no. ab72548; 1:200; Abcam) was employed. After 12 h of incubation at 4°C, the sections were washed three times with PBS and processed with a HRP-conjugated Streptavidin-Biotin complex kit (cat no. SA1040; Boster Biological Technology, Pleasanton, CA, USA) and 3',3'-diaminobenzidine solution, according to the manufacturer's protocol. Finally, the sections were observed using Axio Scope A1 (Carl Zeiss AG, Oberkochen, Germany) with AxioCAM MRc5 (Carl Zeiss AG) and the relative staining intensity of each group was processed with AxioVision software (version 4.7; Carl Zeiss AG).

Cell proliferation and cell cycle analysis. To evaluate cell proliferation ability, cells (1x10⁵ cells/well) were seeded into a 96-well plate and the proliferation index of each group was detected with the Cell Counting Kit-8 (CCK-8) method (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) as previously described (22,23). The following equation was used to measure cell proliferation ability: Proliferation index = absorbance of the experimental group-absorbance of blank group. The proliferation index of each group was measured at 0 h (Day 0), 24 h (Day 1), 48 h (Day 2) and 72 h (Day 3) after seeding. To analyze the cell cycle, 5x10⁶ cells were harvested and fixed with 70% ethanol for 30 min at 4°C. The cell samples were stained with 200 μ l propidium iodide (PI; Beyotime Institute of Biotechnology, Haimen, China) in the presence of RNase A (Beyotime Institute of Biotechnology) for 10 min at room temperature. Finally, the samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and Flowjo software (version 7.6.1; Flowjo, LLC, Ashland, OR, USA).

Cell migration and invasion assay. In the present study, the migration and invasion of liver cancer cells were measured with Transwell plates (8 μ m pore filter, Costar; Corning Incorporated, Corning, NY, USA) as previously described (16,17). Briefly, the liver cancer cells were seeded onto the upper insert at a concentration of 1x10⁵ cells per insert in serum-free medium (HG-DMEM). The insert covered with Matrigel was used for the invasion assay, while a normal insert was used for the migration assay. Lower chambers were filled with HG-DMEM containing 10% FBS as a chemoattractant; cells were incubated for 48 h at 37°C. Non-invading cancer cells were removed by swabbing the top layer and cancer cells that had migrated through the gel and attached to the lower surface of the membrane were stained with 0.5% crystal violet for 20 min at 37°C. The number of cancer cells in four randomly selected microscopy fields under a light microscope (magnification, x100) was counted for each group.

Cell apoptosis assay. In the present study, a cell apoptosis assay was performed using a fluorescein isothiocyanate (FITC)-Annexin V/PI cell apoptosis assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, 5x10⁶ cancer cells in each group were dissociated into single cells with trypsin and washed with PBS, followed by incubation with 200 μ l FITC-Annexin V and PI solution. Cancer cells incubated without the addition of any reagents were used as the negative control group. Finally, all cell samples were analyzed using a FACSCalibur cytometer (BD Biosciences) and Flowjo software (version 7.6.1; Flowjo, LLC).

Statistical analysis. In the present study, the results are presented as the mean \pm standard error of the mean and statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Unpaired Student's t-tests were used to compare the means of two groups. One-way analysis of variance with Bonferroni's correction was used to compare the means of three or more groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of CUL4A in liver cancer tissue and paracancerous tissue. In the present study, liver cancer tissues and paracancerous tissues from 3 different patients were harvested to analyze the association between CUL4A and liver cancer. The results of RT-qPCR and western blotting demonstrated that the expression levels of CUL4A were significantly higher in liver cancer tissues compared with in paracancerous tissues (P<0.05), with similar patterns observed in the 3 different patients (Fig. 1A and B). The phenomenon was further confirmed by immunohistochemical staining as the results indicated strong positive staining of CUL4A in the liver cancer tissues, while staining of CUL4A in the paracancerous tissues was weak, and quantification of staining demonstrated significantly higher CUL4A expression in liver cancer tissues compared with paracancerous tissue (Fig. 1C). Therefore, these results indicated that human liver cancer tissues exhibited higher expression of CUL4A compared with normal tissue.

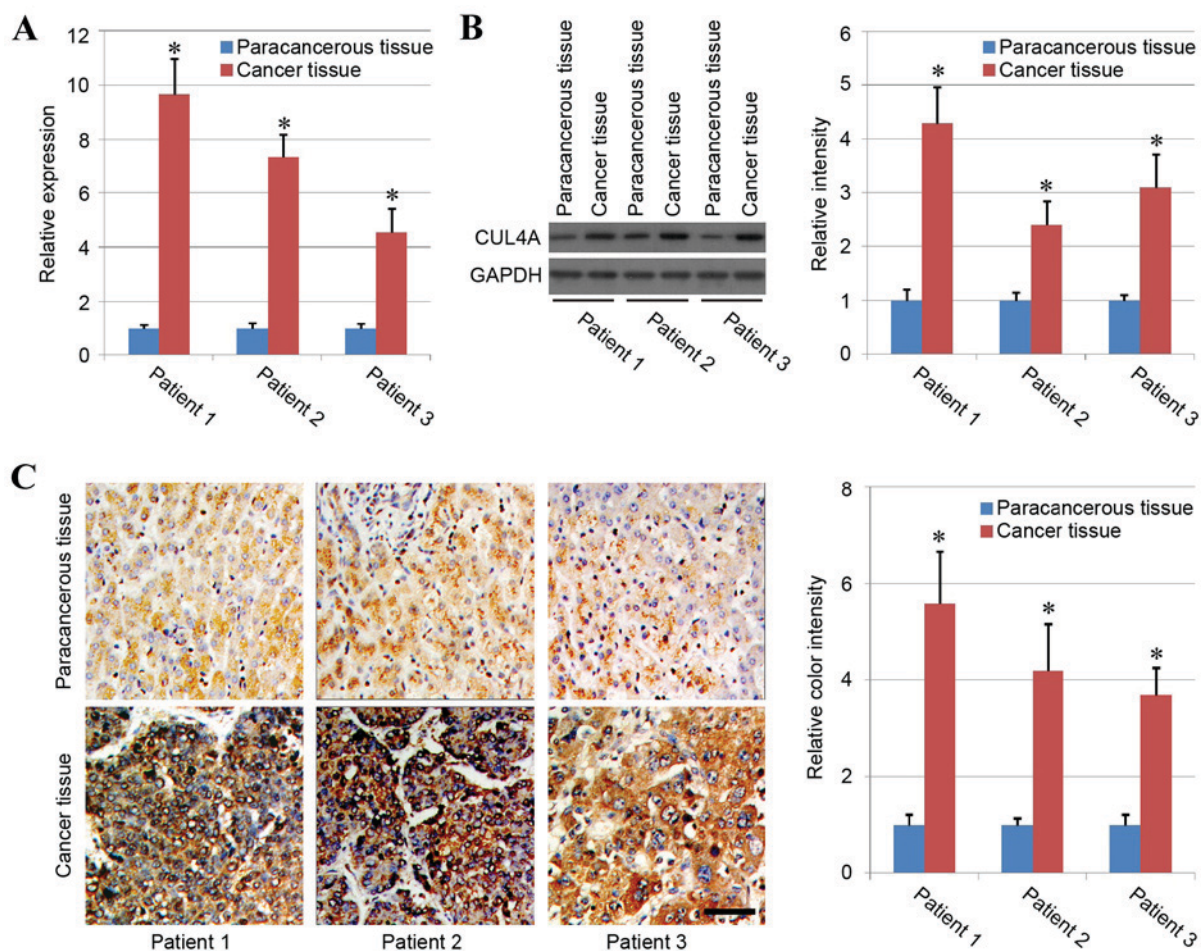


Figure 1. Expression of CUL4A in human liver cancer tissues and paracancerous tissues. The expression of CUL4A in human liver cancer tissues and paracancerous tissues was evaluated by (A) reverse transcription-quantitative polymerase chain reaction, (B) western blotting and (C) immunohistochemical staining. Scale bar, 50 μ m. Similar results were obtained in three independent experiments. Results are presented as the mean \pm standard error of the mean. * $P < 0.05$ vs. paracancerous tissue group. CUL4A, cullin 4A.

CUL4A overexpression and knockdown in liver cancer cell lines. To further investigate the role of CUL4A in the biological function of human liver cancer cells, HEPG2 and MHCC97-H cancer cell lines were employed in the present study. Overexpression of CUL4A was induced with pCI vector transfection (overexpression group) and knockdown of CUL4A in liver cancer cells was performed with using siRNA (siRNA group). Following transfection, the mRNA and protein expression of CUL4A was determined with RT-qPCR and western blotting, respectively, to evaluate the overexpression and siRNA efficiency, and the results confirmed significant upregulation of CUL4A in the overexpression group and downregulation of CUL4A expression in the siRNA group compared with the control group ($P < 0.05$; Fig. 2A and B), indicating the enhancing effect of the pCI-CUL4A vector and inhibiting function of the siRNA on CUL4A expression in liver cancer cell lines.

Effect of CUL4A on liver cancer cell proliferation. The present study analyzed the differences in cell proliferation ability among the control, overexpression and siRNA groups. The CCK-8 detection assay indicated that the proliferation index of the overexpression group was higher compared with the control group, while the index of the siRNA group

was significantly lower compared with the control group, indicating that the expression of CUL4A may be essential for liver cancer cell proliferation and downregulation of CUL4A expression may inhibit the proliferation ability of liver cancer cells (Fig. 3A).

In addition, the effects of CUL4A overexpression and CUL4A-siRNA on the cell cycle of HEPG2 and MHCC97-H cancer cells were analyzed by fluorescence-activated cell sorting. The results demonstrated that compared with the control group, CUL4A overexpression reduced the percentage of G_0/G_1 phase and increased the percentage of S phase MHCC97-H cells ($P < 0.05$; Fig. 3B and C), but exhibited no notable effects in HEPG2 cells (Fig. 3B and C). Conversely, treatment with CUL4A-siRNA increased the percentage of G_0/G_1 phase cells and decreased the percentage of S phase and G2/M phase cells compared with the control group ($P < 0.05$; Fig. 3B and C), therefore inhibiting cell proliferation ability in both liver cancer cell lines.

Effect of CUL4A on liver cancer cell apoptosis. The degree of cell apoptosis in different groups was further analyzed with Annexin V-FITC/PI double staining in the present study. In the HEPG2 and MHCC97-H cell lines, the downregulation of CUL4A expression led to

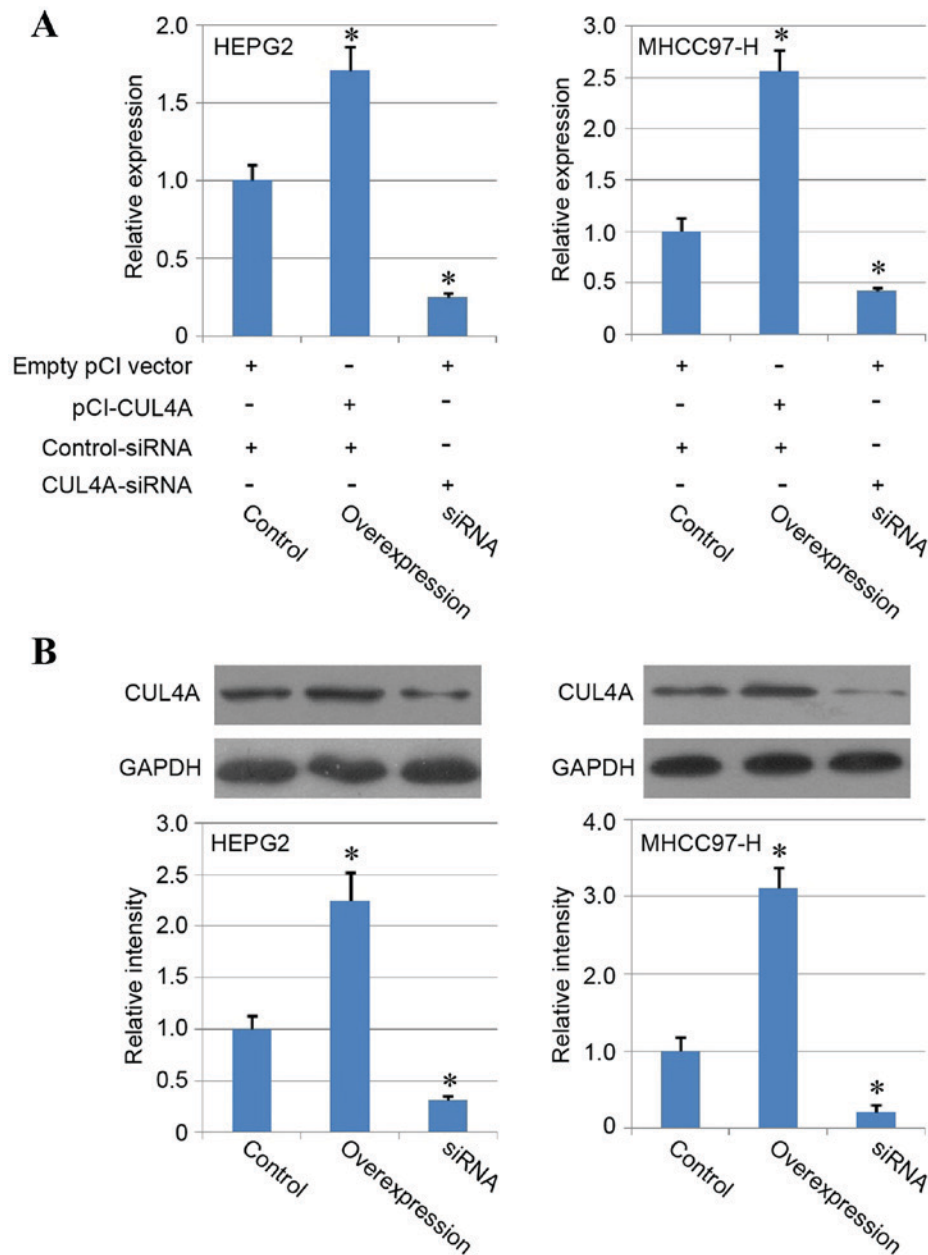


Figure 2. Evaluation of CUL4A overexpression and CUL4A knockdown in human liver cancer cell lines. The expression of CUL4A in control, overexpression and siRNA groups was evaluated by (A) reverse transcription-quantitative polymerase chain reaction and (B) western blotting in HEPG2 and MHCC97-H liver cancer cell lines. Similar results were obtained in three independent experiments. Results are presented as the mean \pm standard error of the mean. * $P < 0.05$ vs. control group. CUL4A, cullin 4A; siRNA, small interfering RNA.

an increased percentage of early-stage apoptotic cells (Annexin V-FITC-positive and PI-negative cells; $P < 0.05$) and late-stage apoptotic cells (Annexin V-FITC-positive and PI-positive cells; $P < 0.05$) compared with the control group (Fig. 4A and B). Additionally, CUL4A overexpression only decreased the percentage of late-stage apoptotic cells in MHCC97-H cells weakly ($P < 0.05$), with no notable alterations induced by CUL4A overexpression in HEPG2 cells (Fig. 4A and B).

CUL4A promotes liver cancer cell migration and invasion. The present study also analyzed the variations in cell migration and invasion ability among the different treatment groups. The same tendencies were observed for the results of both

evaluations. CUL4A overexpression increased cell migration and invasion ability in the two liver cancer cell lines and the CUL4A-siRNA group exhibited a significant reduction in cell migration and invasion ability, compared with the control group ($P < 0.05$; Fig. 5A and B). These results indicated a key role of CUL4A in liver cancer migration and invasion.

As EMT has been regarded as the key process in cancer cell migration and invasion (24), the expression of certain key genes associated with EMT was analyzed by western blotting to confirm the effect of CUL4A in HEPG2 cells. The present study demonstrated that the protein expression of epithelial genes (E-cadherin, Cldn3, Oc1n and Epcam) was upregulated in the CUL4A-siRNA group and the expression of mesenchymal genes (N-cadherin, Snail, Slug and vimentin)

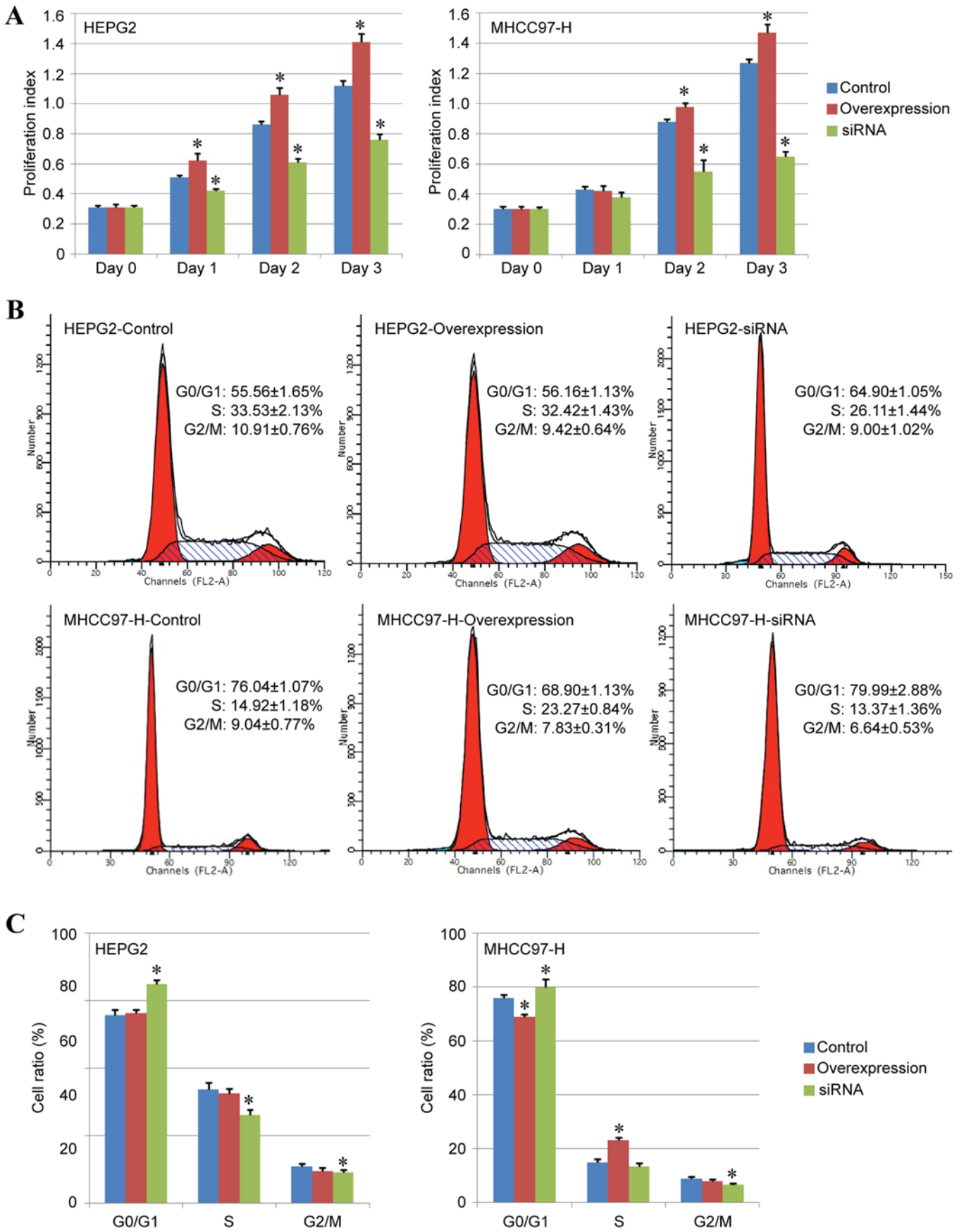


Figure 3. Effect of CUL4A on cell proliferation ability. (A) Evaluation of the effects of CUL4A overexpression and CUL4A knockdown on the cell proliferation of HEPG2 and MHCC97-H liver cancer cells using a Cell Counting Kit-8 assay. (B) Propidium iodide staining followed by flow cytometry was performed to analyze the cell cycle distribution in control, CUL4A overexpression and CUL4A-siRNA HEPG2 and MHCC97-H cells. (C) Percentage of HEPG2 or MHCC97-H cells in each phase. Similar results were obtained in three independent experiments. Results are presented as the mean ± standard error of the mean. *P<0.05 vs. control group. CUL4A, cullin 4A; siRNA, small interfering RNA.

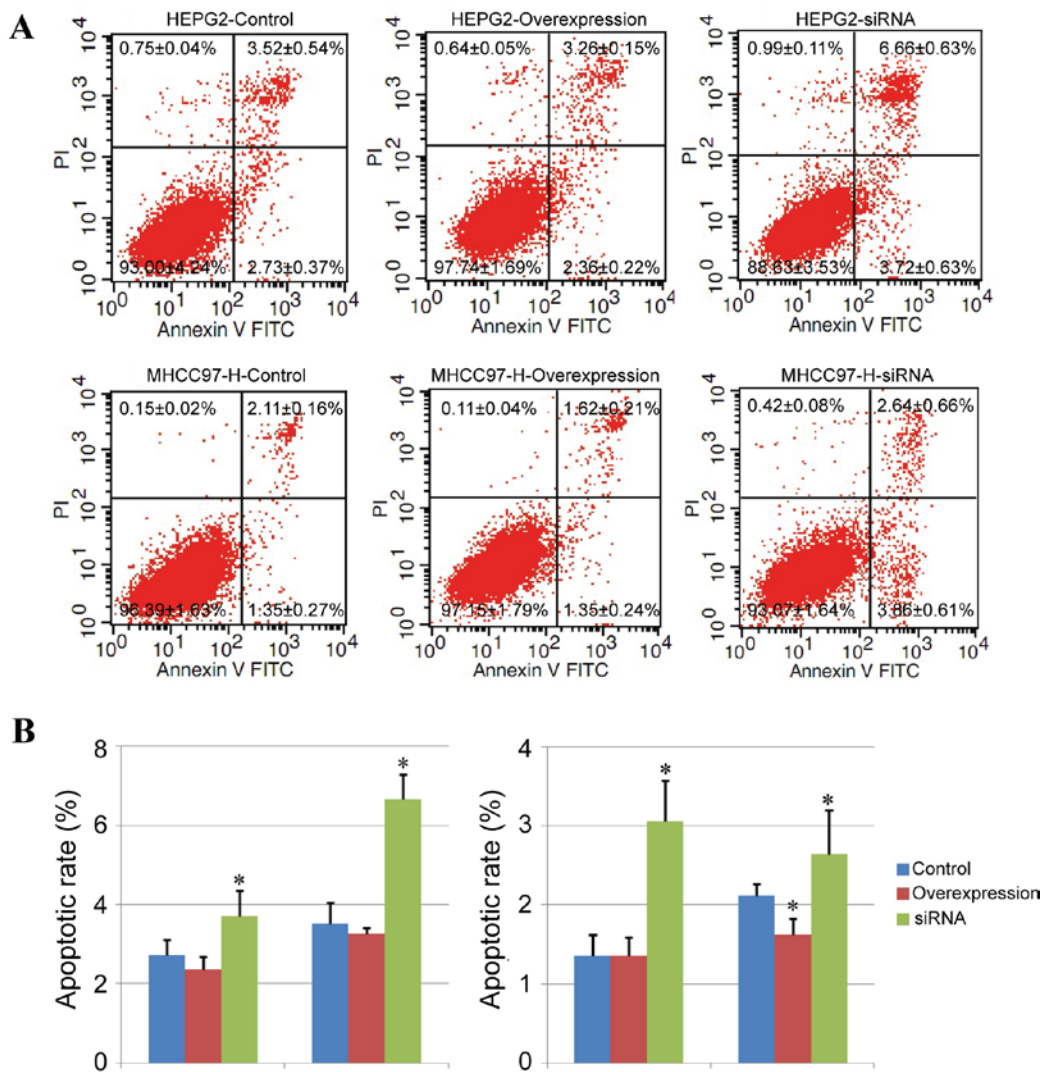


Figure 4. Cell apoptosis analysis in HEPG2 and MHCC97-H liver cancer cells using Annexin V-FITC/PI staining and flow cytometry. (A) Cell apoptosis analysis by flow cytometry. Early-stage apoptotic cells are presented in the lower right quadrants (Annexin V-FITC positive and PI negative) and late-stage apoptotic cells are presented in the upper right quadrants (Annexin V-FITC and PI positive) (B) Calculated apoptotic rate (%) of early and late stage apoptotic cells. Similar results were obtained in three independent experiments. Results are presented as the mean \pm standard error of the mean. FITC, fluorescein isothiocyanate; PI, propidium iodide; siRNA, small interfering RNA.

was reduced in the CUL4A siRNA group ($P < 0.05$; Fig. 5C), compared with the control group, indicating that CUL4A may affect liver cancer cell migration and invasion by regulating EMT. However, the overexpression of CUL4A exhibited few effects on the expression of most mesenchymal or epithelial genes.

Discussion

Previous studies have indicated that CUL4A serves an important function in the progression of various cancer types (10,12). However, to the best of our knowledge, previous studies have not extensively investigated the role of CUL4A in human liver cancer. Recently, an inverse correlation was reported between the expression of CUL4A and patient survival, and a positive correlation with lymphatic and venous invasion (13). In addition, the expression of CUL4A in liver cancer tissues was associated with patient HBeAg status, and knockdown of CUL4A ameliorated the motility of liver cancer cell lines by

regulating the expression of EMT-associated molecules (13). Recently, another group identified that a novel lncRNA, uc.134, may repress liver cancer progression by inhibiting the CUL4A-mediated ubiquitination of the LATS1 protein, indicating that the application of uc.134 lncRNA may offer a promising treatment approach for liver cancer and that CUL4A may have an important role in liver cancer progression (14). In the present study, CUL4A was observed to exhibit increased expression in human liver cancer tissues compared with in paracancerous tissues. In addition, the inhibition of CUL4A using siRNA led to the reduction of cell proliferation, cell migration and invasion, and enhanced the percentage of cell apoptosis, indicating the key function of CUL4A in liver cancer progression.

However, a detailed understanding of the molecule mechanism of CUL4A function in human liver cancer remains unclear. For example, further investigation is required to determine why the expression level of CUL4A may be upregulated in liver cancer. Numerous complex genetic and

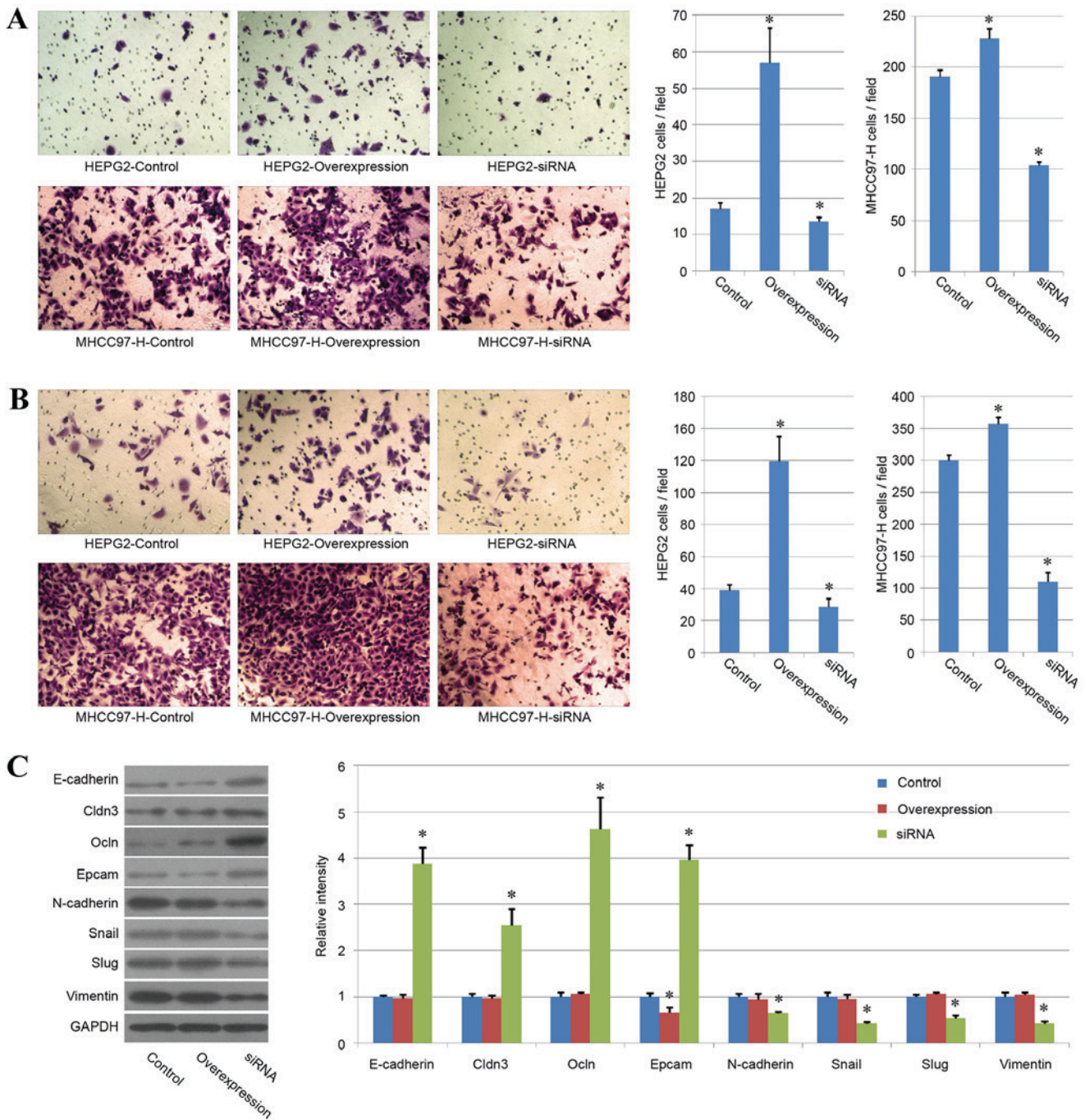


Figure 5. Cell migration and invasion assay. The effects of CUL4A overexpression and CUL4A knockdown on (A) cell migration and (B) invasion ability in HEPG2 and MHCC97-H liver cancer cells. (C) Western blot analysis of key proteins expressed during epithelial-mesenchymal transition in HEPG2 liver cancer cells. Similar results were obtained in three independent experiments under a light microscope (magnification, x100). Results are presented as the mean ± standard error of the mean. *P<0.05 vs. control group. CUL4A, cullin 4A; siRNA, small interfering RNA; Cldn3, claudin 3; Ocln, occludin; Epcam, epithelial cell adhesion molecule.

epigenetic alterations were previously reported in hepatocytes during liver cancer progression, and those alterations led to transformation of normal hepatocytes and resulted in hepatocarcinogenesis (6). Thus, other factors may contribute to the upregulation of CUL4A in liver cancer progression. HBV infection has been considered as the most important cause of liver cancer worldwide. Recently, one study indicated that the majority of liver cancer cases were HBsAg-positive and that HBV infection directly upregulated the expression of CUL4A in liver cancer cells, indicating the regulatory role of HBV on

the expression of CUL4A (13). However, the exact mechanisms underlying the function of HBV in CUL4A regulation require *in vitro* and *in vivo* investigation.

In present study, the results demonstrated that CUL4A overexpression increased the proliferation of human liver cancer cell lines, while the downregulation of CUL4A suppressed cell proliferation and enhanced cell apoptosis. However, different cell lines revealed different results in the present study. For example, CUL4A overexpression increased the percentage of S phase and reduced the percentage of G₀/G₁

cells, and decreased the percentage of late-stage apoptotic cells in MHCC97-H cells, but demonstrated no notable effects in HEPG2 cells. These varying effects require further investigation. Thus, the basal expression levels of CUL4A in various cell lines may be different and cancer cells with high expression levels of CUL4A may exhibit a certain degree of tolerance to CUL4A overexpression. However, investigation of additional cell lines is required to confirm this hypothesis, as well as the mechanism underlying this phenomenon.

CUL4A overexpression did not result in significant alteration of mesenchymal or epithelial gene expression. However, CUL4A overexpression significantly increased cell migration and invasion ability in the two liver cancer cell lines. This may be due to the high expression of mesenchymal genes and low expression of epithelial genes that is typically observed in liver cancer cell lines (25-27). Therefore, CUL4A overexpression may have been unable to increase mesenchymal or decrease epithelial gene expression further. However, CUL4A overexpression still increased cell viability by promoting cell proliferation and inhibiting cell apoptosis. Cell migration and invasion ability was also promoted, likely through other signaling pathways. This hypothesis requires further investigation to elucidate the mechanisms of CUL4A overexpression on migration, invasion and viability.

The present study confirmed the association between the expression of CUL4A and human liver cancer, and indicated that CUL4A may represent a novel target in the treatment of human liver cancer. Further analysis for each pathway associated with CUL4A expression and an enhanced understanding of the regulatory mechanism of those genes in various cancer cells may contribute to the development of novel drugs or gene therapy methods for the treatment of patients with liver cancer and potentially other types of cancer.

In conclusion, the present study indicated that the mRNA and protein expression levels of CUL4A were markedly higher in human liver cancer tissues compared with human paracancerous tissues. The overexpression of CUL4A in human liver cancer cells increased the cell proliferation, cell migration and invasion, and reduced the percentage of cell apoptosis, while CUL4A knockdown exhibited opposing effects. The results of the present study indicated the key function of CUL4A expression in liver cancer, as well as the potential of CUL4A in the diagnosis and treatment of human liver cancer.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Author's contributions

LL conceived, designed and supervised the experiments. GC, XZ, ZT, DW, DL, PZ and JC performed the experiments. The data were analyzed by GC, JC, FW and QL. GC and LL contributed the reagents, materials and analysis tools. GC and LL wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by the Committee on the Ethics of Animal Experiments and Human Subject Research of the First People's Hospital of Kunming. All volunteers involved in the present study provided written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 136: E359-E386, 2015.
2. Altekruse SF, Henley SJ, Cucinelli JE and McGlynn KA: Changing hepatocellular carcinoma incidence and liver cancer mortality rates in the United States. *Am J Gastroenterol* 109: 542-553, 2014.
3. Tang A, Hallouch O, Chernyak V, Kamaya A and Sirlin CB: Epidemiology of hepatocellular carcinoma: Target population for surveillance and diagnosis. *Abdom Radiol (NY)* 43: 13-25, 2018.
4. Xu IM, Lai RK, Lin SH, Tse AP, Chiu DK, Koh HY, Law CT, Wong CM, Cai Z, Wong CC and Ng IO: Transketolase counteracts oxidative stress to drive cancer development. *Proc Natl Acad Sci USA* 113: E725-E734, 2016.
5. Nishida N and Goel A: Genetic and epigenetic signatures in human hepatocellular carcinoma: A systematic review. *Curr Genomics* 12: 130-137, 2011.
6. Liu M, Jiang L and Guan XY: The genetic and epigenetic alterations in human hepatocellular carcinoma: A recent update. *Protein Cell* 5: 673-691, 2014.
7. Nishida N and Kudo M: Clinical significance of epigenetic alterations in human hepatocellular carcinoma and its association with genetic mutations. *Dig Dis* 34: 708-713, 2016.
8. Yasui K, Arii S, Zhao C, Imoto I, Ueda M, Nagai H, Emi M and Inazawa J: TFDPI, CUL4A, and CDC16 identified as targets for amplification at 13q34 in hepatocellular carcinomas. *Hepatology* 35: 1476-1484, 2002.
9. Hori T, Osaka F, Chiba T, Miyamoto C, Okabayashi K, Shimbara N, Kato S and Tanaka K: Covalent modification of all members of human cullin family proteins by NEDD8. *Oncogene* 18: 6829-6834, 1999.
10. Sharma P and Nag A: CUL4A ubiquitin ligase: A promising drug target for cancer and other human diseases. *Open Biol* 4: 130217, 2014.
11. Dohna M, Reincke M, Mincheva A, Allolio B, Solinas-Toldo S and Lichter P: Adrenocortical carcinoma is characterized by a high frequency of chromosomal gains and high-level amplifications. *Genes Chromosomes Cancer* 28: 145-152, 2000.
12. Xu Y, Wang Y, Ma G, Wang Q and Wei G: CUL4A is overexpressed in human pituitary adenomas and regulates pituitary tumor cell proliferation. *J Neurooncol* 116: 625-632, 2014.
13. Pan Y, Wang B, Yang X, Bai F, Xu Q, Li X, Gao L, Ma C and Liang X: CUL4A facilitates hepatocarcinogenesis by promoting cell cycle progression and epithelial-mesenchymal transition. *Sci Rep* 5: 17006, 2015.

14. Ni W, Zhang Y, Zhan Z, Ye F, Liang Y, Huang J, Chen K, Chen L and Ding Y: A novel lncRNA uc.134 represses hepatocellular carcinoma progression by inhibiting CUL4A-mediated ubiquitination of LATS1. *J Hematol Oncol* 10: 91, 2017.
15. López-Terrada D, Cheung SW, Finegold MJ and Knowles BB: Hep G2 is a hepatoblastoma-derived cell line. *Hum Pathol* 40: 1512-1515, 2009.
16. Liu P, Feng Y, Dong D, Liu X, Chen Y, Wang Y and Zhou Y: Enhanced renoprotective effect of IGF-1 modified human umbilical cord-derived mesenchymal stem cells on gentamicin-induced acute kidney injury. *Sci Rep* 6: 20287, 2016.
17. Liu P, Cai J, Dong D, Chen Y, Liu X, Wang Y and Zhou Y: Effects of SOX2 on proliferation, migration and adhesion of human dental pulp stem cells. *PloS One* 10: e0141346, 2015.
18. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
19. Tao S, Liu P, Luo G, Rojo de la Vega M, Chen H, Wu T, Tillotson J, Chapman E and Zhang DD: p97 Negatively regulates NRF2 by extracting ubiquitylated NRF2 from the KEAP1-CUL3 E3 complex. *Mol Cell Biol* 37: e00660-e00616, 2017.
20. Cai J, Zhang Y, Liu P, Chen S, Wu X, Sun Y, Li A, Huang K, Luo R, Wang L, *et al*: Generation of tooth-like structures from integration-free human urine induced pluripotent stem cells. *Cell Reg (Lond)* 2: 6, 2013.
21. Liu P, Feng Y, Dong C, Yang D, Li B, Chen X, Zhang Z, Wang Y, Zhou Y and Zhao L: Administration of BMSCs with muscone in rats with gentamicin-induced AKI improves their therapeutic efficacy. *PloS One* 9: e97123, 2014.
22. Liu P, Feng Y, Dong C, Liu D, Wu X, Wu H, Lv P and Zhou Y: Study on therapeutic action of bone marrow derived mesenchymal stem cell combined with vitamin E against acute kidney injury in rats. *Life Sci* 92: 829-837, 2013.
23. Zhao L, Feng Y, Chen X, Yuan J, Liu X, Chen Y, Zhao Y, Liu P and Li Y: Effects of IGF-1 on neural differentiation of human umbilical cord derived mesenchymal stem cells. *Life Sci* 151: 93-101, 2016.
24. Heerboth S, Housman G, Leary M, Longacre M, Byler S, Lapinska K, Willbanks A and Sarkar S: EMT and tumor metastasis. *Clin Transl Med* 4: 6, 2015.
25. Jayachandran A, Dhungel B and Steel JC: Epithelial-to-mesenchymal plasticity of cancer stem cells: Therapeutic targets in hepatocellular carcinoma. *J Hematol Oncol* 9: 74, 2016.
26. Panebianco C, Saracino C and Pazienza V: Epithelial-mesenchymal transition: Molecular pathways of hepatitis viruses-induced hepatocellular carcinoma progression. *Tumour Biol* 35: 7307-7315, 2014.
27. Ogunwobi OO and Liu C: Therapeutic and prognostic importance of epithelial-mesenchymal transition in liver cancers: Insights from experimental models. *Crit Rev Oncol Hematol* 83: 319-328, 2012.



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