Effects of cullin 4B on the proliferation and invasion of human gastric cancer cells

FENG HE1, XIU-MEI CHENG2 and WEN-LONG GU1

1Department of Medical Oncology, The Affiliated Jianhu Hospital of Nantong University, Jianhu People's Hospital, Yancheng, Jiangsu 224700; 2Department of Basic Medicine, Jiangsu Vocational College of Medicine, Yancheng, Jiangsu 224000, P.R. China

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Abstract. The major aim of the present study was to explore the effects of cullin 4B (CUL4B) on the proliferation and invasion of human gastric cancer cells. Gastric tumor tissues and paired adjacent non-tumor tissues were obtained from 21 gastric cancer patients, and gastric cancer cell lines (AGS, MGC-803, KATO-III, MKN-45, SGC-7901, BGC-823 and MKN-74) were cultured. BGC-823 cells were transfected with CUL4B small interfering (si)RNA or control siRNA. Reverse transcription-quantitative polymerase chain reaction analysis was performed to detect the mRNA expression of CUL4B. Western blot analysis was performed to measure the protein levels of Wnt, β-catenin, glutathione synthase kinase (GSK)-3β, caspase-3 and cyclin E. MTT and Transwell assays were performed to examine cell proliferation and invasion following CUL4B knockdown. In addition, the effect of CUL4B knockdown on the cell cycle and apoptosis of BGC-823 cells was evaluated by flow cytometric analysis. The results indicated that compared with the adjacent non-tumor tissues and a normal gastric epithelial cell line, gastric cancer tissues and cell lines exhibited significantly higher expression of CUL4B. Knockdown of CUL4B in gastric cancer cells suppressed cell proliferation, caused G1 arrest and inhibited cell invasion. Silencing of CUL4B also resulted in decreased Wnt and β-catenin expression, but increased expression of GSK-3β, caspase-3 and cyclin E. These results indirectly demonstrate that CUL4B enhances the proliferation and invasion abilities of gastric cancer cells by upregulating the constituent factors Wnt and β-catenin, as well as by negatively regulating the mRNA and protein expression of GSK-3β, caspase-3 and cyclin E. The potential mechanism of CUL4B highlighted in the present study may be helpful for the treatment of patients with gastric cancer.

Introduction

Gastric cancer (GC) is one of the most common cancer types and the second leading cause of cancer-associated death worldwide (1). More than 70% of GC cases occur in developing countries, Eastern Asia has half the world's total cases of GC, with 42% of all GC cases occurring in China (2,3). Thus, GC represents a considerable burden particularly among Chinese populations, and there is an urgent requirement to identify an effective method of treating GC.

Cullin 4B (CUL4B) is the largest in the class of Cullin-RING ubiquitin ligases (CRLs), and is regarded as the scaffolding protein in modular CRLs. It has been reported to be upregulated in most human cancers, including cervical, colorectal and hepatocellular carcinomas (4). CUL4B participates in the regulation of a broad spectrum of biological processes (5). Previous studies have indicated that CUL4B has a significant role in numerous aspects of cellular activity (6). For instance, CUL4B was demonstrated to regulate the intracellular dioxin-dependent receptor signaling pathway (7). CUL4B mutant carrier-derived cells are induced upon degradation and ubiquitination of camptothecin by the presence of topoisomerase I (8). In addition, CUL4B accumulates in the nucleus during cell differentiation, and its knockdown increases β-catenin levels in various rodent cell types (9,10). CUL4B has also been identified to downregulate cyclin E (11,12). Jiang et al (4) demonstrated that CUL4B may serve as a novel prognostic marker, it was significantly associated with processes involved in colon cancer pathogenesis and progression, and to be correlated with the depth of invasion, lymph node metastasis, histological differentiation, distant metastasis, vascular invasion and advanced tumor stage. CUL4B was also reported to promote the proliferation and inhibit apoptosis of osteosarcoma cells (13). In addition, downregulation of CUL4B reduced the proliferation, colony formation and invasiveness of hepatocellular carcinoma cells (14). In line with this, another study demonstrated that CUL4B promoted tumorigenesis as well as the proliferation and invasion of various human cancer types (15). However, the biological functions of CUL4B in GC have largely remained
elusive. In the present study, the functions of CUL4B in GC were investigated.

The Wnt family contains 19 types of secreted glycoprotein, which primarily transmit signals through 7-pass trans-membrane receptors of the frizzled family (16). β-catenin forms part of the canonical Wnt signaling pathway (Wnt/β-catenin pathway), and together they regulate various cellular activities including the cell cycle, communication, differentiation and apoptosis (17). Recent studies have indicated that the β-catenin signaling pathway in glioma cells is involved in tumor cell development (18-20).

Glycogen synthase kinase-3β (GSK-3β) is one of a wide range of cellular serine/threonine kinases that are involved in regulating Wnt/β-catenin signaling (21,22). It was revealed that the inhibition of GSK-3β activates Wnt and β-catenin signaling via dephosphorylation, and that GSK-3β acts as an inhibitor in the Wnt signaling pathway (23). Another study suggested that GSK-3β has a key role in this interaction, since it is a negative regulator of β-catenin and a positive regulator of nuclear factor (NF)-κB by targeting the proteasome degradation of the primary inhibitor of NF-κB, IκB (24).

Caspase-3 is a key enzyme that induces apoptosis during ontogenesis and homeostasis of multicellular organisms. It is an important potential drug target in the treatment of apoptosis disturbance (25). Activated caspase-9 cleaves pro-caspase-3 into its active form, which induces cell apoptosis (26). The activities of caspase-3 and caspase-9 were demonstrated to be increased in the GC cell lines SGC7901/ADR (27). Upregulation of cleaved (c)-caspase-3 and c-caspase-9 and suppression of glutathione synthase kinase (GSK)-3β have also been observed in ganetespib-treated cells (28).

Cyclin E alterations are associated with the pathogenesis of numerous cancer types (29-31). Several studies have demonstrated that knockdown of CUL4B induces a significant accumulation of cyclin E (12) and a prolonged the S phase, leading to inhibition of cell proliferation. Together, these studies suggest that CUL4B may target cyclin E degradation to inhibit cell proliferation (11).

Therefore, the present study investigated the potential role of CUL4B in the proliferation and invasion of GC cells through regulating the expression of Wnt/β-catenin, GSK-3β, caspase-3 and cyclin E with the aim of providing an understanding of the mechanisms of CUL4B in GC progression. The results may contribute to the improvement of therapeutic strategies for GC.

Materials and methods

Ethics statement. All experimental protocols were in accordance with the principles of the Helsinki Declaration and approved by the Clinical Research Ethics Committee of the Affiliated Jianhu Hospital of Nantong University (Nantong, China). Prior to enrollment, written informed consent was obtained from all patients.

Paired gastric tumor tissues and adjacent non-tumor tissues were surgically extracted from 21 patients at the Affiliated Jianhu Hospital of Nantong University (Nantong, China) from March 2014 to February 2017. All tissues were derived from patients without adjuvant therapy to avoid treatment-induced effects on gene expression. The adjacent non-tumor tissues were extracted at least 3 cm away from the apparent edge of the tumor tissues. After extraction, all clinical samples were immediately snap-frozen in liquid nitrogen and maintained at -80°C until RNA extraction.

GC cell lines, culture and clinical samples. Three GC cell lines, AGS, MGC-803 and KATO-III, were purchased from the American Type Culture Collection (Manassas, VA, USA). A further four GC cell lines, MKN-45, SGC-7901, BGC-823 and MKN-74, and the normal gastric epithelial cell line GES-1, were purchased from the Chinese Academy of Sciences Type Culture Collection (Shanghai, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) in a tissue culture incubator with 5% CO₂ at 37°C.

Lentiviral transfection of CUL4B small interfering (si)RNA. In the present study, 7 different GC cell lines were selected to examine the tumor-suppressant effect of siRNA against CUL4B in GC. The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) results indicated that the expression of CUL4B was highest in BGC-823 cells, so that this cell line was used for the subsequent knockdown experiments.

Lentiviruses expressing CUL4B siRNA or control siRNA were purchased from Sunbio Medical Biotechnology Co., Ltd. (Shanghai, China). BGC-823 cells, 100 pmol lentivirus and 8 µg/ml polybrene were added into the culture medium at a multiplicity of infection (MOI) of 10-15. Following incubation overnight, the medium was replenished with normal medium. Cell cultures were maintained for another 5-7 days to stabilize the lentiviral transduction. Subsequently, the transduction efficiency was verified by RT-qPCR.

RNA extraction and RT-qPCR. Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse-transcribed into complementary (c)DNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Real-time qPCR was performed using the TaqMan Gene Expression Assay (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, to detect relative mRNA expression levels of CUL4B, Wnt, β-catenin, GSK-3β, caspase-3 and cyclin E. The reaction conditions were set as follows: Initial denaturation at 95°C for 10 min, followed by 32 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 45 sec. The 2^(-ΔΔCq) method (32) was used to calculate relative mRNA expression. GAPDH was used as the internal control. All primer sequences are presented in Table I.

Flow cytometric analysis. Cells (2x10⁵/2 ml) were seeded in 6-well plates. Cells were centrifuged after 48 h of transfection and fixed with 70% ice-cold ethanol at 4°C for 24 h. Next, cells were harvested and washed with cold PBS twice [containing 100 mg/ml RNase A (EN0531; Thermo Fisher Scientific, Inc.) and 50 mg/ml propidium iodide (PI)] in the dark for 30 min at 4°C. The cell cycle distribution was analyzed using a FACScan laser flow cytometer (BD Biosciences,
Table I. Primers used for quantitative polymerase chain reaction.

| Gene         | Forward primer (5'-3') | Reverse primer (5'-3') |
|--------------|------------------------|------------------------|
| CUL4B        | CAAACGGCTAGCCAAATCTT   | CAGTTTTTGCCAGGTTCATCTG |
| GSK-3β       | AGACGCCCTCCTGTATTTATGT | CGTGGCAAGATTTCCAAGG    |
| Caspase-3    | AGAGGGATCGTTGTAGAAGTC  | ACAGTCCAGTCTGTACCACG   |
| Wnt          | AGTTTCATCGAATCCTGAC    | CATCTCGGAATACGCTG      |
| S-catenin    | ATTTAAGCTGAGGAGGCCAC   | TCCGAGCAGATCCACAGA     |
| Cyclin E     | CAATGACCCGGACAGATTTC   | CATGGAGGGGAGTTGGA      |
| GAPDH        | ATCATCCTGGCTTCTACTGG   | GTCAGTCCACCACCTGAC     |

GSK, glutathione synthase kinase; CUL4B, cullin 4B.

Franklin Lakes, NJ, USA) equipped with CellQuest software version 3.1 (BD Biosciences). All experiments were performed in triplicate. Similarly, cells were transfected for 48 and 96 h, then harvested and suspended in medium at a concentration of 2x10^5 cells/ml. Annexin V-fluorescein isothiocyanate (5 µl) and PI (5 µl) were added to the cells, followed by incubation in the dark at 4˚C for 15 min. Subsequently, cell apoptosis was measured using flow cytometry.

Cell proliferation assay. MTT dye is reduced to a blue formazan product by respiratory enzymes that are only active in viable cells. The amount of blue formazan formed is indicative of cell proliferation. Thus, cell proliferation was analyzed using MTT as follows: Following transfection in the 6-well plates for 24, 48 or 72 h, 2,000 BGC-823 cells at the logarithmic growth phase were seeded into 96-well plates in 200 µl DMEM and 20 µl MTT was added to each well, followed by incubation at 37˚C for a further 4 h. Subsequently, the medium was removed, 150 µl dimethyl sulfoxide added and the plates were agitated in the dark for 10 min. The absorbance value of each well was measured at a wavelength of 570 nm.

Transwell migration assay. Transwell inserts pre-coated with Matrigel (pore size, 8 µm; BD Biosciences) were used for the cell invasion assay. At 48 h after transfection, cells were collected for serum starvation and suspended in FBS-free RPMI-1640 cell culture medium (Thermo Fisher Scientific, Inc.). A total of 300 µl of a cell suspension (10^6 cells/ml) was added into each of the upper wells of the Transwell invasion chamber. RPMI-1640 culture medium with 10% FBS (500 µl) was added to the lower chambers. Cells were then incubated for 24 h at 37˚C with 5% CO₂. Subsequently, cells on the lower membrane of the inserts were fixed in 4% paraformaldehyde at 4˚C and stained with 0.05% crystal violet for 10 min at room temperature. Images of at least four randomly selected fields per membrane were captured under a microscope and the numbers of invaded cells were quantified using ImageJ software version 1.51j8 (National Institutes of Health, Bethesda, MD, USA).

Western blot assay. Radioimmunoprecipitation assay lysis buffer (Gibco; Thermo Fisher Scientific, Inc.) was used to extract the total protein of cells and tissues. The protein concentration in samples was measured using a bicinchoninic acid assay kit (Keygentec, Nanjing, China) according to the manufacturer's protocol. Proteins (30 µg per lane) were separated by 10% SDS-PAGE and immediately transferred onto polyvinylidene difluoride membranes (FPFP33; Beyotime Institute of Biotechnology, Haimen, China), which were then incubated for 1 h with Tris-buffered saline containing TWEEN-20 and 5% skimmed milk. The membranes were incubated with primary antibodies against CUL4B (cat. no. ab67035; 1:1,000 dilution), Wnt (cat. no. ab72583; 1:4,000 dilution), β-catenin (cat. no. ab6302; 1:4,000 dilution), GSK-3β (cat. no. ab32391; 1:5,000 dilution), caspase-3 (cat. no. ab13847; 1:500 dilution), cyclin E (cat. no. ab33911; 1:1,500 dilution) and anti-GAPDH (cat. no. ab9485; 1:5,000 dilution) (all from Abcam, Cambridge, MA, USA) at 4˚C overnight. GAPDH was used as the internal control. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (cat. no. ab13168; 1:1,000 dilution) and anti-rabbit secondary antibody (cat. no. ab191866; 1:1,000 dilution) at room temperature for 1 h. Then the membranes were developed with ECL under the tablet cassette (FFC83; Beyotime Institute of Biotechnology). The X-ray films (FF081; Beyotime Institute of Biotechnology) were scanned with HP Scanjet G3110 and the ImageJ analysis software version 1.51j8 (National Institutes of Health) was used to quantify protein expression. Relative expression was calculated by comparison with the internal reference protein. Each experiment was performed three times.

Statistical analysis. Data analyses were performed using SPSS software version 21.0 (IBM Corp., Armonk, NY, USA). Values are expressed as the mean ± standard deviation and were determined to follow a normal distribution. Therefore, the differences between two groups were analyzed using Student’s t-test, while differences between multiple groups were assessed by one-way analysis of variance followed by the Dunnett’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

CUL4B expression in tissue samples from GC patients and adjacent control samples, 7 GC cell lines and a normal cell
First, the CUL4B mRNA levels in tissue samples from GC patients and adjacent control tissues (n=21) were quantified. It was identified that CUL4B levels were significantly elevated in the GC tissues compared with those in the controls (P<0.01; Fig. 1A). Based on these results, the following experiments focused on the expression of CUL4B in cell lines in vitro to explore its role and the underlying mechanisms. CUL4B mRNA expression was therefore detected in different cell lines. The RT-qPCR results indicated an apparent increase in CUL4B expression in the GC cell lines (AGS, MGC-803, KATO-III, MKN-45, SGC-7901, BGC-823 and MKN-74) compared with that in the normal gastric epithelial cell line GES-1 (P<0.05 or P<0.01; Fig. 1B). The BGC-823 cell line exhibited the most marked overexpression of CUL4B, and thus, BGC-823 cells were selected for subsequent experiments.

Effect of CUL4B siRNA on multiple malignant phenotypes of BGC-823 cells. The results of RT-qPCR indicated a marked reduction in CUL4B mRNA levels in the CUL4B siRNA group when compared with those in the controls (P<0.01; Fig. 2A). The MTT assay revealed that CUL4B siRNA-transfected BGC-823 cells exhibited a significantly decreased growth rate compared with that of the control siRNA-transfected cells and NC cells (P<0.05 at 24 h, P<0.01 at 48-72 h; Fig. 2B). Therefore, knockdown of CUL4B was indicated to markedly reduce the proliferation/survival rate of BGC-823 cells.

To explore possible mechanisms via which CUL4B knockdown reduces BGC-823 cell proliferation, the effect of CUL4B siRNA on the apoptotic rate of BGC-823 cells was determined. The results indicated that the early apoptotic rate was significantly higher when CUL4B was inhibited in BGC-823 cells (P<0.01, CUL4B siRNA vs. NC and siRNA control; Fig. 3). Subsequently, the distribution of cells within cell cycle stages (G1, S and G2 phases) was determined by flow cytometry. Cells treated with CUL4B siRNA exhibited a significant increase in the percentage of cells in the G1 phase and a decrease in the percentage of cells in the S phase (P<0.01; Fig. 4), but the G2 phase population was not significantly different among the three groups. These results suggested that knockdown of CUL4B promoted cell proliferation by inhibiting the cell cycle and inducing apoptosis.

The invasive ability of BGC-823 cells transfected with CUL4B siRNA decreased when compared with that of the cells in the NC and control siRNA groups (P<0.01; Fig. 5). These results indicated that CUL4B has a key role in the progression of GC.

Expression of Wnt/β-catenin, GSK-3β, caspase-3 and cyclin E in BGC-823 cells. The effect of CUL4B on the expression of Wnt/β-catenin, GSK-3β, caspase-3 and cyclin E was then assessed in order to investigate the mechanism by which CUL4B is involved in the growth and migration of GC cells. As presented in Figs. 6 and 7, western blot analysis and RT-qPCR revealed that the expression of Wnt and β-catenin was significantly decreased at the mRNA and protein level following
Figure 3. Downregulation of the CUL4B gene promotes apoptosis of BGC-823 cells. After staining with Annexin V-FITC and PI, apoptotic cells were analyzed using a flow cytometer. The percentages of apoptotic cells (in Q2 and Q4) were displayed in the bar graph. Annexin V assay was performed three times and values are expressed as the mean ± standard deviation of three independent experiments. "P<0.01, CUL4B siRNA vs. NC and control siRNA. CUL, cullin; NC, negative control; siRNA, small interfering RNA; FITC, fluorescein isothiocyanate; PI, propidium iodide; Q, quadrant.

Figure 4. (A) Changes in the cell cycle distribution of BGC-823 cells in the CUL4B knockdown group in the G1, S and G2/M phase. (B) Compared with the NC group, the BGC-823 cell cycle distribution exhibited an increase in the G1 phase and a decrease in the S phase population. "P<0.01, CUL4B siRNA vs. NC and control siRNA. CUL, cullin; NC, negative control; siRNA, small interfering RNA; PI, propidium iodide.
CUL4B knockdown. By contrast, significant increases in the protein and mRNA levels of GSK-3β, caspase-3 and cyclin E were observed following knockdown of CUL4B. These results indicated that CUL4B exerts its effects in BGC-823
cells at least in part by regulating the Wnt/β-catenin signaling pathway. CUL4B was indicated to regulate downstream proteins, including GSK-3β, caspase-3 and cyclin E.

Discussion

GC is a heterogeneous, multifactorial disease. The incidence and mortality rate of GC in East Asia (Japan, China and Korea) displays geographic variations (33). GC is the second most frequently diagnosed cancer type and the third leading cause of cancer-associated death in China (34). There is an urgent requirement to reduce the mortality rate of GC patients by developing effective treatment strategies.

The present study identified higher levels of CUL4B in GC tissues and cell lines compared with those in adjacent normal tissues and normal cells. CUL4B overexpression has been reported in several types of solid tumor, including osteosarcoma, as well as esophageal, lung, gastric, colon, pancreatic and cervical carcinomas (15). The present study assessed the effect of CUL4B knockdown on GC cells via CUL4B siRNA transfection of BGC-823 cells. It was identified that CUL4B knockdown led to an increased expression of GSK-3β, caspase-3 and cyclin E, but decreased expression of Wnt and β-catenin. The results also demonstrated that knockdown of CUL4B inhibited BGC-823 cell proliferation and invasion and significantly induced apoptosis. These results suggest that silencing of the CUL4B gene may be of valuable in the treatment of human GC.

Evidence of the carcinogenicity of CUL4B is accumulating. CUL4B may contribute to GC by activating Wnt/β-catenin signaling and induce apoptosis (13), and to result in the ubiquitination of β-catenin leading to its rapid degradation (37).

Previous studies have indicated that astragalin induces apoptosis in A375P and SK-MEL-2 melanoma cells via activation of caspase-9/3 and inhibition of Sry-related HMG-Box gene 10 signaling (38). The present study indicated that knockdown of CUL4B results in the upregulation of caspase-3. It is well known that caspase-3 (and its cleavage) has a role in apoptosis, and it was demonstrated that apoptosis was induced by knockdown of CUL4B, so there is probably a link between the two observations. The results of the present study demonstrated that caspase-3 has an important role in the apoptosis and progression of GC cells.

Cell proliferation is mainly regulated by the cell cycle, which has been formally divided into distinct sequential phases (G0/G1, S, G2 and M) (39). It has been demonstrated that GSK-3β degrades cyclin D1 (40,41). The results of the present study indicated that CUL4B siRNA reduced the proliferation of BGC-823 cells by inducing cell cycle arrest in G1 phase. In addition, the apoptotic rate was significantly increased following CUL4B knockdown, and the invasive capacity of the cells was reduced. The molecular mechanism of the effects CUL4B silencing was indicated to involve upregulation of the expression of GSK-3β, caspase-3 and cyclin E, as well as downregulation of Wnt and β-catenin expression levels. The present results suggest that CUL4B siRNA has potential applications in the treatment of GC.

In conclusion, the present study investigated the biological function of the CUL4B gene in GC and proposed a possible mechanism based on previous studies. It enhanced the current knowledge regarding the role of CUL4B in tumorigenesis. However, further study is required to define the mechanisms by which cullin proteins are involved in gastric tumorigenesis and oncogenesis. The present results may contribute
to the development of novel diagnostic and therapeutic strategies for GC.

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