HOXB2 increases the proliferation and invasiveness of colon cancer cells through the upregulation of CCT6A

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Abstract. Colon cancer has a high mortality rate, thus there is an urgent need to develop novel therapeutic options for clinical management of the disease. Studies have revealed that chaperonin containing TCP1 subunit 6A (CCT6A) promoted the development of multiple types of cancer, and dataset analysis revealed that homeobox B2 (HOXB2) has the potential to modulate the expression of CCT6A. However, whether HOXB2 affects the proliferation, migration and invasion of colon cancer cells remains to be determined. A CCT6A knockdown colon cancer cell line was established and colony formation, wound healing and Transwell invasion assays were performed to assess proliferation, migration and invasion of the altered colon cancer cells. Subsequently, luciferase reporter gene assays and chromatin immunoprecipitation assays were performed to detect the relationship between HOXB2 and CCT6A. A HOXB2 overexpression colon cancer cell line was established and the proliferation, migration and invasion of these cells was determined using the same methods. Knockdown of CCT6A reduced the proliferation, migration and invasion of colon cancer cells. HOXB2 enhanced the expression of CCT6A in colon cancer cells by binding to the promoter of CCT6A. Overexpression of HOXB2 abolished the inhibitory effect of CCT6A knockdown on the proliferation, migration and invasion of colon cancer cells. HOXB2 increased the proliferation and invasiveness of colon cancer cells by increasing the expression of CCT6A.

Introduction

Colon cancer is a highly invasive and metastatic cancer. Colon cancer cells can spread to several organs in the body via the blood, where they colonize the new site and form a metastatic lesion. Metastasis underlies the death of 90% of patients with colon cancer (1). Epithelial-mesenchymal transition (EMT) is a key factor that endows a potent invasive ability on colon cancer cells (2). In this process, colon cancer cells gradually acquire the characteristics of mesenchymal cells and the increased expression of EMT-related proteins, such as vimentin and N-cadherin, promote the development of EMT in colon cancer cells (3). The occurrence of EMT is indicative of a poor prognosis in patients with colon cancer (4). Therefore, there is an urgent need to develop novel therapeutic options for the inhibition of the metastasis of colon cancer in the body and therefore alleviate the symptoms of colon cancer.

Chaperonin containing TCP1 subunit 6A (CCT6A) belongs to type II chaperone, which is a heteromorphic oligomeric protein widely existing in the cytoplasm and serves an important role in the assembly and folding of actin and tubulin (5). CCT6A is a protein that is associated with the development of several types of cancer (6). The expression of CCT6A is upregulated in the tissues of patients with Ewing sarcoma compared with that of the pericarcinomatous tissues (7). Overexpression of CCT6A can promote the transition from the G1 to S phase and therefore enhance the proliferation of hepatocellular carcinoma cells (8). Moreover, the expression of CCT6A has been revealed to be associated with a poor prognosis in patients with an adenocarcinoma of the colon (9). In addition, CCT6A has also been revealed to be associated with the depth of colorectal cancer invasion, tumor size and the occurrence of tumors (10). However, the effect of CCT6A on the development of colon cancer and the specific mechanism has not been studied thus far.

The homeobox genes encoded by HOX gene are basically some transcriptional regulatory factors, most of which play an important role in the physiological and pathological changes such as embryo development, cell differentiation and cell carcinogenesis (11). HOXB2 is a member of the HOX family, which is one of the critical genes involved in regulation of cell differentiation (12). However, the expression of HOXB2 is also related to the development of lung (13) and pancreatic cancer (14), and the upregulated expression of HOXB2 can induce the development of colorectal cancer (15). However, to the best of our knowledge, whether HOXB2 affects the proliferation and invasion of colon cancer

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cells by regulating the expression of CCT6A has not been determined.

Therefore, the relationship between HOXB2 and CCT6A was detected. Additionally, the effect of CCT6A on the development of colon cancer was assessed.

Materials and methods

Cell culture and treatment. Normal intestinal epithelial cells (HIEC cells) and colon cancer cell lines (Caco-2, LoVo, HCT116 and SW480 cells) were obtained from the American Type Culture Collection. All cell lines were cultured in RPMI-1640 medium (HyClone; Cytiva) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained in a humidified incubator at 37˚C with 5% CO₂. Cells were transfected with 20 nM CCT6A knockdown [short hairpin (sh)RNA-CCT6A#1 and shRNA-CCT6A#2] and interference control (shRNA-NC), HOXB2 overexpression (Ov-HOXB2) and overexpressed control group (Ov-NC), which were purchased from Shanghai GeneChem, Co., Ltd. Polybrene (Shanghai GeneChem, Co., Ltd.) was used as a transfection reagent. Transfections were performed using Lipofectamine® 2000 according to the manufacturer's protocol. After transfection for 48 h at 37˚C with 5% CO₂, transfection efficiencies were assessed via reverse transcription-quantitative PCR (RT-qPCR) and western blotting after 48 h.

Databases. The GEPIA (http://gepia.cancer-pku.cn/) database predicted the relationship between HOXB2 and CCT6A. The CCLE database (portals.broadinstitute.org/ccle) detected the expression of CCT6A in colon cancer cells. JASPAR (jaspar.genereg.net/) was used to predict the relationship between HOXB2 and CCT6A.

Cell Counting Kit-8 (CCK-8) assays. Cell suspensions were plated into 96 well plates (8x10³ cells/well). After cells had adhered, 10 µL CCK-8 solution (Dojindo Molecular Technologies, Inc.) was diluted using culture medium and added to the 96-well plates and cells were incubated for 1 h at 37˚C. Finally, the absorbance at a wavelength of 450 nm was detected using a spectrophotometer (Thermo Fisher Scientific, Inc.).

Colony formation assays. Cell suspensions were plated in 60-mm culture dishes (300 cells per dish) and cultured for 2 weeks at 37˚C. Subsequently, cells were fixed using 70% ethanol solution at room temperature for 15 min, after which, cells were stained using 0.005% crystal violet at room temperature for 30 min (Thermo Fisher Scientific, Inc.). The number of colonies with >50 cells was counted by an inverted phase contrast light microscope (magnification, x200; Olympus Corporation).

Wound healing assays. Cells were plated (1x10⁶ cells/well) in 6-well plates and cultured with serum-free medium for 12 h. Once cells reached 80% confluence, a tweezer was used to create a scratch in the monolayer of cells and the images of the scratch were captured using a light microscope (magnification, x200; Olympus Corporation). After a 24 h incubation in serum-free medium at 37˚C, images of the scratch were captured and the width was measured using ImageJ software (version 146; National Institutes of Health).

Transwell assays. Cells were cultured in serum-free medium for 12 h, then suspended in culture medium. Matrigel (BD Biosciences) was diluted with serum-free RPMI-1640 medium at 37˚C for 30 min and added to the upper chamber of a Boyden insert (8-µm pores; Corning, Inc.). Subsequently, 600 µl RPMI-1640 medium supplemented with 10% FBS was added to the lower chamber. The suspended cells (5x10⁵ cells/well) were added to the upper chamber and incubated for 24 h at 37˚C. The cells which had invaded through the Matrigel and migrated across the membrane were fixed with 4% paraformaldehyde for 15 min at room temperature and stained using 0.1% crystal violet at room temperature for 45 min and observed using a light microscope (magnification, x200; Olympus Corporation).

Chromatin immunoprecipitation (ChIP). Total RNA was extracted using TRIzol® buffer (Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (cat. no. R022B; Takara Bio, Inc.), according to the manufacturer's protocol (cat. no. R022B; Takara Bio, Inc.). DNA (8-40 µg) was diluted with DNase-free water (cat. no. R0021; Beyotime Institute of Biotechnology) and incubated with the primary antibody (1-5 µg) at 4˚C overnight. The primary antibody was an anti-HOXB2 antibody (1:200, cat. no. ab220390; Abcam). Next, the DNA that had bound to HOXB2 was collected using DNA extraction buffer in DNase-free water and amplified using qPCR to detect CCT6A.

 Luciferase reporter assay. Cell were plated in 6-well plates and after the cells had adhered, 0.5 µg vectors containing the 3'-untranslated region (UTR) of wild-type (WT) CCT6A or mutant (MUT) 3'-UTR CCT6A, with control vector or HOXB2 overexpression vector and pMIR-Renilla vector (Shanghai GeneChem Co., Ltd.) were co-transfected with the aforementioned kit (Polybrene Shanghai GeneChem, Co., Ltd.) into the cells (1x10⁶ cells/well) and cells were incubated for 48 h at 37˚C. Finally, the luciferase activity was detected using a Renilla-Glo® Luciferase Assay System (cat. no. E2710; Promega Corporation) at room temperature and a spectrophotometer at 490 nm (Thermo Fisher Scientific, Inc.). Renilla luciferase activity was used to normalize the firefly luciferase activity.

Immunocytochemistry. Cells (1x10⁶ cells/well) were plated in 6-well plates and after they had adhered, 4% paraformaldehyde was used to fix the cells at 4˚C for 24 h. Subsequently, 0.5% Triton X-100 (Beyotime Institute of Biotechnology) was used to permeabilize the cells and 5% BSA (Beyotime Institute of Biotechnology) was used to block non-specific binding at room temperature for 1 h. A primary antibody was diluted with 5% BSA and incubated with the cells at 4˚C overnight. The primary antibody used was an anti-Ki-67 (1:200; product code ab16667; Abcam). Next, the cells were incubated at 37˚C for 1.5 h with the goat polyclonal secondary antibody to Rabbit IgG (heavy chain and light chain) Alexa Fluor® 488 (1:3,000; cat. no. ab150077; Abcam) in a dark room for 1.5 h.
Finally, the nucleus of these cells was stained with 1 mg DAPI for 10 min at room temperature [Invitrogen; Thermo Fisher Scientific, Inc.]. The fluorescence was observed using a laser scanning confocal microscope (magnification, x200; Olympus Corporation).

**RT-qPCR.** Total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.). Subsequently, RNA was reverse transcribed into cDNA using a commercial kit, according to the manufacturer's protocol (cat. no. R022B; Takara Bio, Inc.). cDNA was amplified using an ABI 7500 system (Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: The thermocycling conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 10 sec, 55°C for 10 sec, and 72°C for 30 sec. qPCR was performed using a SYBR-Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). And the results were analyzed with the 2−ΔΔct method (16). The sequences of the primers used were: CCT6A forward, 5′-TGACGACCTAAGTCCTGAAGT-3′ and reverse, 5′-ACAGAACGGAGGTTGTTACATT-3′; HOXB2 forward, 5′-CGAGTGCAACAGGGTGAGCC-3′ and reverse, 5′-GTTGACCCTTCTGTTAGG-3′; GAPDH forward, 5′-CCAGATCTCATGTTGTCCG-3′ and reverse, 5′-AGCCTTCTCCATGGTGTTGAA-3′.

**Western blotting.** Protein samples were collected using RIPA lysis buffer (Beyotime Institute of Biotechnology). Next, the concentration of these samples was determined using the BCA (Beyotime Institute of Biotechnology) method. Proteins (30 µg/lane) were resolved using 10% SDS-PAGE (Beyotime Institute of Biotechnology) and subsequently transferred to a PVDF membrane (MilliporeSigma). Next, these membranes were blocked with 5% BSA at 37°C for 1.5 h and incubated with primary antibodies at 4°C overnight. The primary antibodies (1:1,000) used in the present study were CCT6A (cat. no. ab110905), HOXB2, E-cadherin (cat. no. ab40772), N-cadherin (cat. no. ab18203), vimentin (cat. no. ab9789) and GAPDH (cat. no. ab8245) (all purchased from Abcam). The following day, the membranes were incubated at 37°C for 1.5 h with the secondary antibody HRP-conjugated anti-mouse antibody (goat IgG; 1:5000; cat. no. ab150113; Abcam). Finally, the bands were developed using ECL substrate (MilliporeSigma). Protein expression levels were semi-quantified using ImageJ software (version 1.46; National Institutes of Health).

**Statistical analysis.** Analysis was performed using GraphPad Prism version 7.0 (GraphPad Software, Inc.). Data are presented as the mean ± standard deviation of three repeats. Differences between groups were compared using an unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**CCT6A expression is upregulated in colon cancer tissues.** The expression of CCT6A in colon cancer tissues was detected. Data from GEPIA revealed that the expression of CCT6A in colorectal cancer tissues was higher than that in the para-carcinoma tissues (Fig. 1A). Furthermore, the data from GEPIA also revealed that higher levels of CCT6A were associated with lower survival rates in these patients (Fig. 1B). Data from the CCLE database (portals.broadinstitute.org/ccle) showed that the expression of CCT6A was also higher in colorectal cancer cells compared with normal cells (Fig. 1C). Next, the expression of CCT6A in colon cancer cells was detected using RT-qPCR (Fig. 1D) and western blotting (Fig. 1E). The results revealed that the expression level of CCT6A in colon cancer cell lines (Caco2, LoVo, HCT116 and SW480 cells) was higher compared with the HIEC cells.

**Knockdown of CCT6A suppresses the proliferation of colon cancer cells.** The expression of CCT6A in SW480 cells was higher than that in the other colon cancer cells. Therefore, SW480 cells were selected for subsequent experiments. CCT6A-knockdown SW480 cells were established. As revealed in Fig. 2A and B, the expression of CCT6A in SW480 cells in the knockdown group was lower compared with the control cells. The inhibitory effect of shRNA-CCT6A#2 was greater than that of shRNA-CCT6A#1. Therefore, shRNA-CCT6A#2 was selected for subsequent experiments. Next, the viability and proliferation of these cells was detected using CCK-8 and colony formation assays, respectively. The results revealed that knockdown of CCT6A induced a decrease in viability and restricted the formation of colonies of SW480 cells compared with the control cells (Fig. 2C and D). Next, the expression of Ki-67 in these cells was also determined using immunofluorescence analysis. The results revealed that knockdown of CCT6A suppressed the expression of Ki-67 in SW480 cells compared with the control cells (Fig. 2E).

**Knockdown of CCT6A inhibits the migration and invasion of colon cancer cells.** Changes in the migratory and invasive abilities of SW480 cells after the knockdown of CCT6A were next determined. The wound healing and Transwell invasion assays revealed that the migration and invasion of SW480 cells were reduced following knockdown of CCT6A compared with the control cells (Fig. 3A-C). Furthermore, the expression of N-cadherin and vimentin was also suppressed, whereas that of E-cadherin was increased in the CCT6A-knockdown SW480 cells compared with the control cells (Fig. 3D).

**HOXB2 activates the expression of CCT6A in colon cancer cells.** Using JASPAR [jaspar.genereg.net/], it was predicted that HOXB2 could bind to the promoter of CCT6A and affect the expression of CCT6A (Fig. 4A and B). According to the results of RT-qPCR and western blotting, the expression of HOXB2 was higher in colon cancer cells (SW480 cells) compared with the HIEC cells (Fig. 4C and D). Next, HOXB2 was overexpressed in SW480 cells, and the results revealed that the expression of HOXB2 in SW480 cells in the overexpression group was higher than that in cells of the negative control group (Fig. 4E and F). Moreover, luciferase reporter assays revealed that the luciferase activity was highest in the CCT6A WT and HOXB2 overexpression system (Fig. 4G). ChIP analysis demonstrated that HOXB2 bound to the promoter region of CCT6A (Fig. 4H), and western blotting and RT-qPCR analysis also revealed that overexpression of HOXB2 rescued the expression of CCT6A in the CCT6A-knockdown SW480
cells compared with the shRNA-CCT6A + OV-NC group (Fig. 4I and J).

Overexpression of HOXB2 abolishes the effects of CCT6A knockdown on the proliferation, migration and invasion of colon cancer cells. The effect of HOXB2 on the proliferation, migration and invasion of SW480 cells was next determined. The viability of CCT6A-knockdown SW480 cells and number of colonies formed were rescued following overexpression of HOXB2 compared with the shRNA-CCT6A + OV-NC group (Fig. 5A and B). Moreover, the expression of Ki-67 in CCT6A-knockdown SW480 cells was rescued following overexpression of HOXB2 compared with the shRNA-CCT6A + OV-NC group (Fig. 5C). Furthermore, the wound healing and Transwell assays revealed that overexpression of HOXB2 abolished the inhibitory effect of CCT6A knockdown on the migration and invasion of SW480 cells compared with the shRNA-CCT6A + OV-NC group (Fig. 6A and B). The expression levels of N-cadherin and vimentin were rescued, whereas the levels of E-cadherin in SW480 cells were decreased following overexpression of HOXB2 compared with the shRNA-CCT6A + OV-NC group (Fig. 6C).
Discussion

Colon cancer is a common malignant tumor of the digestive tract and the incidence and mortality rates of colon cancer have increased in recent years (17). With changes in lifestyle and eating habits, the incidence of colon cancer in China is also increasing and there is a trend of a decreasing age of onset (18). Thus far, the molecular mechanisms of colon cancer have not been fully elucidated. It has been suggested that the onset of colon cancer is the result of a combination of genetic and environmental factors (19). Moreover, environmental factors, intestinal homeostasis, dietary choices, tobacco, alcohol and physical exercise are crucial factors influencing the onset of colon cancer (20). At present, the clinical treatment of colon cancer is still based on surgery, while chemotherapy and radiotherapy are used as adjuvant treatments (21). However, the effect of this treatment strategy is extremely limited for patients with advanced colon cancer.
The postoperative metastasis of colon cancer cells is the critical cause of poor prognosis (22). Blood, peritoneal and distant lymph node metastases are the primary means of postoperative colon cancer metastasis (23). Through invasion of cancer cells, new tumors may be formed and this will lead to the deterioration of the condition. Therefore, there is an urgent need to identify novel targets to suppress the metastasis of colon cancer cells.

CCT6A is a protein that enhances the development of multiple types of cancer (24). Higher levels of CCT6A can promote the proliferation of hepatocellular carcinoma cells by inducing the transition from the G$_1$ phase to the S phase (8). In addition, the expression of CCT6A has been revealed to be upregulated in breast cancer tissues and is associated with a poor prognosis of patients with breast cancer (24). Additionally, the expression of CCT6A has also been revealed to increase drug resistance of melanoma cells and promote the proliferation of these cells (25). Furthermore, CCT6A was revealed to be an inhibitor of SMAD2; and it promoted the proliferation and invasion of non-small cell lung cancer cells by inhibiting the expression of SMAD2 (26).

In the present study, it was demonstrated that knockdown of CCT6A expression reduced the proliferation, migration and invasion of colon cancer cells by promoting the expression of EMT-related proteins (N-cadherin and vimentin). These results also indicated that CCT6A is a promoting factor of colon cancer.

HOXB2 is a transcription factor that has been revealed to enhance the occurrence and development of ovarian cancer (27). Aberrant expression of HOXB2 has been observed in multiple types of cancer, such as leukemia, breast and liver cancer and gastric carcinoma (28). Additionally, another study indicated that upregulated expression of HOXB2 can induce the occurrence and development of bladder cancer (11). At present, there has been no literature report on the specific relationship between HOXB2 and CCT6A, which is also the innovation of our study. The transcription factor HOXB2 binding to the CCT6A promoter was revealed in the JASPAR database. By querying UniProt and using a dual luciferase assay, it was revealed that HOXB2 binds to the promoter region of CCT6A.

Figure 3. Inhibition of CCT6A restricts the migration and invasion of colon cancer cells. (A) Wound healing and (B) Transwell assays were performed to detect the migration and invasion of colon cancer cells. (C) Statistical analysis of cell invasion and migration. (D) Western blotting was performed to determine the expression of epithelial-mesenchymal transition-related proteins in colon cancer cells. *P<0.01 and **P<0.001. CCT6A, chaperonin containing TCP1 subunit 6A; shRNA, short hairpin RNA; NC, negative control.
and upregulates the expression of CCT6A in colon cancer cells. Furthermore, overexpression of HOXB2 abolished the inhibitory effect of CCT6A knockdown on the proliferation, migration and invasion of colon cancer cells. These results indicated that HOXB2 could promote the proliferation and invasiveness of colon cancer cells by promoting the expression of CCT6A.

The present study also has certain limitations. In our experiments, SW480 cells were only selected and selecting another cell line for verification will render the results more rigorous. Another cell line will be selected for verification in a future study. In addition, the results should be further verified in animal experiments in the future. Finally, the downstream regulation mechanism of HOXB2 and CCT6A should be further explored.

In conclusion, the effect of CCT6A on the proliferation, migration and invasion of colon cancer cells was assessed. The results revealed that HOXB2 promoted the proliferation and invasion of colon cancer cells by directly targeting and activating the expression of CCT6A in colon cancer cells. These results highlighted CC6TA and HOXB2 as novel potentially druggable targets.
Figure 5. Overexpression of HOXB2 rescues the proliferation of colon cancer cells. (A) Cell Counting Kit-8 was performed to detect the viability of colon cancer cells. (B) The proliferation of colon cancer cells was determined with the colony formation assay. (C) Immunofluorescence was performed to detect the expression of Ki-67 in colon cancer cells. *P<0.05, **P<0.01 and ***P<0.001. ##P<0.01 vs. shRNA-ccT6a + Ov-NC. ccT6a, chaperonin containing TCP1 subunit 6A; shRNA, short hairpin RNA; Ov, overexpressing; NC, negative control.

Figure 6. Overexpression of HOXB2 rescues the invasion of colon cancer cells. (A) Wound healing and (B) Transwell assays were performed to determine the migration and invasion of colon cancer cells. (C) Western blotting was used for the detection of the expression of epithelial-mesenchymal transition-related proteins in colon cancer cells. *P<0.01 and ***P<0.001. CCT6A, chaperonin containing TCP1 subunit 6A; shRNA, short hairpin RNA; Ov, overexpressing; NC, negative control.
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Availability of data and materials

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

Authors’ contributions

XY and LC wrote the manuscript and analyzed the data. YT and WY carried out the experiments, supervised the present study, searched the literature and revised the manuscript. All authors read and approved the final manuscript. XY and LC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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