Abstract. Colorectal cancer (CRC) is one of the most common types of tumor worldwide. The morbidity and mortality rates of CRC have increased significantly in adults <50 years of age. In the present study, the effects of homeobox A6 (HOXA6) on the proliferation, apoptosis, migration and invasion of CRC cells were investigated. The results of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis demonstrated that the expression of HOXA6 in CRC tumor tissue was higher than that in adjacent normal tissue. Appropriate cell lines and plasmids were selected by RT-PCR and western blot analyses, and recombinant plasmids were transfected into Caco2 or HT-29 cells. The results of RT-qPCR and western blot analyses demonstrated that the expression of HOXA6 was effectively enhanced, or inhibited, following transfection. The rate of cell proliferation was measured with cell counting kit-8, colony formation assay and 5-ethynyl-2'-deoxyuridine assay, apoptosis was detected using terminal deoxynucleotidyl transferase dUTP nick end labeling and flow cytometry assays, and migration and invasion were evaluated using Transwell and wound-healing assays. The results demonstrated that the upregulation of HOXA6 promoted cell proliferation, migration and invasion, but inhibited apoptosis, whereas the downregulated expression of HOXA6 produced the opposite effects. In addition, the expression levels of apoptosis- and epithelial-mesenchymal transition (EMT)-related proteins were examined. The results of the western blot analysis revealed that the upregulated expression of HOXA6 suppressed the expression of B-cell lymphoma-2 (Bcl-2)-associated X protein, caspase-3, poly(ADP-ribose) polymerase and E-cadherin, but promoted the expression of Bcl-2, N-cadherin and Vimentin, whereas the opposite effect was observed in cells with downregulated HOXA6. These results indicated that HOXA6 regulated apoptosis through the Bcl-2 signaling pathway, and regulated migration and invasion through the EMT process. In conclusion, the present study confirmed that HOXA6 was involved in the regulation of CRC, which may inform the development of strategies for the diagnosis and treatment of CRC.

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-associated mortality worldwide. Although the overall morbidity and mortality rates for CRC have been declining for decades, they have increased considerably in adults <50 years of age. In addition, the 5-year relative survival rate for patients with CRC diagnosed at an advanced stage is just 14%, as not all of the tumor can be surgically removed (1-3). In addition to old age and lifestyle factors, genetic changes are also a cause of CRC (4). In these cases of CRC, targeted therapy may be effective. Therefore, the identification of novel tumor markers may contribute to the diagnosis and treatment of CRC, such as the effect of the clinical application of carcinoembryonic antigen and carbohydrate antigen 19-9 (5).

The homebox (HOX) family includes a series of developmental genes. The encoded homeodomain proteins are important transcription factors that bind to specific DNA sequences as monomers or multimers to regulate cell differentiation, proliferation and apoptosis. At present, 39 human HOX genes have been identified, which are divided into four clusters: A, B, C and D. Previous studies have reported that HOX family genes are involved in the development of human malignancies and are associated with prognosis (6,7). It was previously observed that HOXA9, HOXA13 and HOXB7 were overexpressed in esophageal cancer, and promoted the proliferation of esophageal cancer cells (8-10). The expression levels of HOXA1, HOXA10, HOXA13, HOXB7 and HOXC6 were also found to be increased in gastric cancer tissue, and may promote cell proliferation and metastasis, in addition to being associated with prognosis (11-17). In CRC, HOXB7 was also reported to be expressed at a high level, and interacted with the mitogen-activated protein kinase and
phosphoinositide 3-kinase/AKT pathways to promote tumor cell proliferation (18).

HOXA6 is a member of the HOX family and encodes a DNA-binding transcription factor, which may regulate gene expression, morphogenesis and differentiation. Previous studies have reported that the HOXA6 gene is expressed at a high level in several types of malignant tumor, including cerebral glioma and acute myeloid leukemia, and that it is associated with cell invasion, proliferation, colony formation and chemosensitivity (19-20). HOX family members have been confirmed to be involved in tumor regulation, including in the occurrence and development of CRC. As HOXA6 is also involved in the regulation of certain types of malignant tumor, it is reasonable to suggest that HOXA6 may have a similar effect on tumor regulation in CRC. Based on this, the aim of the present study was to investigate the expression of HOXA6 in CRC, and the effect of the expression of HOXA6 on the proliferation, apoptosis, migration and invasion of CRC cells.

Materials and methods

**Patient specimens.** A total of 16 CRC tumor and paired adjacent normal colorectal tissue samples were collected from the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) between September 28, 2017 and November 11, 2017 (Table I). Informed consent was signed by all patients, and ethics approval was obtained from the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

**Cell culture and transfection.** The Caco2, HCT116, SW480 and HT-29 human CRC cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (HyClone/GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (PAN-Biotech GmbH, Aidenbach, Germany) at 37˚C in 5% CO₂. Plasmids expressing HOXA6, short hairpin interfering RNA against HOXA6 (shHOXA6; sh1, 5'-CCTGAGTGTTTCTATTCTGATA-3'; sh2, 5'-CCTCGTGTTTCTATTCTGATA-3'; sh3, 5'-GGCGCGCAAATGAGTTCCTAT-3'; sh4, 5'-GACAAGACGTACACCTCACCT-3') (21) or a negative control (NC) sequence (non-targeting shRNA) were purchased from GeneCopeia, Inc. (Rockville, MD, USA). The CRC cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol (2X GoTaq qPCR Master mix (Promega Corporation) (nuclease-free water: 3.6 µl; upstream primer: 0.2 µl; downstream primer: 0.2 µl; 2X GoTaq qPCR Master Mix: 5 µl; cDNA: 1 µl). Thermocycling steps: 95˚C-10 min; 95˚C-15 sec; 60˚C-1 min, 40 cycles) and the data were analyzed using the 2^-ΔΔCq method (22). RT-PCR was performed with GoTaq polymerase (Promega Corporation) according to the manufacturer's protocol (2X GoTaq Green Master Mix: 5 µl; upstream primer: 0.6 µl; downstream primer: 0.6 µl; cDNA: 2 µl; nuclease-free water: 1.8 µl). Thermocycling steps: 95˚C-2 min; 95˚C-30 sec, 55˚C-30 sec, 72˚C-30 sec; 72˚C-3 min. HOXA6: 32 cycles, β-actin: 23 cycles.). The PCR products were analyzed with 2% agarose gel electrophoresis, and subjected to densitometric analysis with a gel imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Western blot analysis.** Proteins were extracted from the cells at 72 h post-transfection using RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). The protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology). Subsequent to 10% SDS-PAGE separation (40 µg), the proteins were transferred onto a PVDF membrane. The membrane was blocked in 5% skim milk at room temperature for 1-2 h, and then incubated at 4˚C overnight with the primary antibodies. Following incubation with appropriate horseradish peroxidase-conjugated anti-rabbit secondary antibodies (#7074, dilution: 1:3,000) (Cell Signaling Technology, Inc., Danvers, MA, USA) for 2 h at room temperature, the membranes were visualized with an ECL kit (Beyotime Institute of Biotechnology). The grayscale value of images was analyzed using Fusion software (Fusion FX, Vilber Lourmat). The primary antibodies for western blot analysis included rabbit anti-HOXA6 (YT2212, 1:800) and

<table>
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<th>Sample collection date</th>
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**Table I. Characteristics of the patients with colorectal cancer.**
antibodies for the detection of HOXA6 in CRC and normal colorectal tissue samples was performed by RT-qPCR analysis. The results showed that the mRNA levels of HOXA6 in the CRC tissues were significantly upregulated, compared with those in the paired normal colorectal tissue samples (P=0.0103) (Fig. 1A). Subsequently, the expression levels of HOXA6 in four CRC cell lines (Caco2, SW480, HCT116 and HT-29) were detected by RT-PCR analysis. The mRNA expression level of HOXA6 was highest in the HT-29 cells and lowest in Caco2 cells (Fig. 1B). Therefore, these two cell lines were selected for the subsequent experiments.

Verification of the knockdown efficiency of the shHOXA6 plasmid. To analyze the knockdown efficiency of four interference plasmids targeting HOXA6 (shRNA1/2/3/4-HOXA6), the expression of HOXA6 in transfected cells was examined by RT-PCR and western blot analyses. HOXA6 was expressed the least in the cells transfected with shRNA1-HOXA6, as confirmed by RT-PCR and western blot analyses (Fig. 1B). Based on these results, shRNA1-HOXA6 was selected as the most efficient interference plasmid for the remaining experiments.

Verification of the expression of HOXA6 in transfected cells. The Caco2 cells were transfected with plasmids expressing HOXA6 or an NC and HT-29 cells were transfected with shHOXA6 or NC plasmids. The expression of HOXA6 was detected by RT-qPCR and western blot analyses. The results showed that the mRNA and protein expression levels were significantly upregulated in the Caco2-HOXA6 cells (P=0.0008 and 0.0012) and suppressed in the HT-29-shHOXA6 cells (P=0.0225 and 0.0026) (Fig. 1C and D).

HOXA6 enhances the proliferation rate of CRC cells. To examine the effect of HOXA6 on CRC cell proliferation, CCK-8, colony formation and EdU assays were performed. The CCK-8 assay demonstrated that the Caco2-HOXA6 cells grew significantly faster than the Caco2-NC cells (P<0.0001), whereas the growth rate of the HT-29-shHOXA6 cells was significantly lower, compared with that of the HT-29-NC cells (P<0.0001) (Fig. 2A). In addition, the colony formation

Cell proliferation assay. Cell proliferation was assessed using a cell counting kit-8 (CCK-8) assay. The stably transfected cells, including the Caco2-HOXA6, Caco2-NC, HT-29-shHOXA6 and HT-29-NC cells, were seeded in a 96-well plate at a density of 4x10³ cells/well, and cultured overnight. CCK-8 reagent was then added into the wells and the absorbance at 450 nm was detected following culture for 24, 48 and 72 h.

For the colony formation assay, the stably transfected cells were seeded in a 6-well plate at a density of 500 cells/well. Following culture for 2 weeks, the clones were fixed with paraformaldehyde and stained with gentian violet. The numbers of clones containing >50 cells were counted under an inverted phase contrast microscope (Leica, Wetzlar, Germany).

A 5-ethynyl-2'-deoxyuridine (EdU) assay was also performed to measure cell proliferation ability. An EdU kit (RiboBio Co., Ltd., Guangzhou, China) was used according to the manufacturer's protocol. Images were captured with a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Cell migration and invasion. Cell migration and invasion were measured using Transwell assays. To determine the rate of cell migration, 200 µl of RPMI-1640 medium containing 5x10⁴ cells was added into the upper chamber, and 700 µl RPMI-1640 containing 10% FBS was added into the lower chamber. Following incubation for 48 h, the migrated cells were fixed and stained with crystal violet for 15 min. Subsequently, the cells were viewed and counted under an inverted phase contrast microscope in five fields of view. The invasion ability was measured with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The Matrigel was diluted with RPMI-1640 and added to the upper chamber (100 µl/well). Following solidification of the Matrigel, 100 µl of RPMI-1640 containing 1x10⁵ cells was added into the upper chamber; the invasion rate of cell migration, 200 µl of RPMI-1640 medium and 100 µl RPMI-1640 containing 10% FBS were added to the lower chamber. Following incubation for 24 h, the migrated cells were viewed and counted using an inverted microscope.

Cell apoptosis. Flow cytometry was used to detect the rate of apoptosis of Caco2 and HT-29 cells. At 48 h post-transfection, the cells were harvested with EDTA-free trypsin and suspended in PBS buffer. The cells were stained with an Annexin V-FITC/PI kit (BD Biosciences) according to the manufacturer's protocol, and immediately analyzed by flow cytometry.

Apoptosis was also measured using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer's protocol. Following TUNEL staining, the cells were stained with DAPI for 5 min and washed with PBS. A fluorescence microscope (Leica) was used to capture images.

Statistical analysis. Each experiment was independently repeated three times. Statistical analysis was performed with SPSS version 22 (IBM SPSS, Armonk, NY, USA). All data are presented as the mean ± standard deviation and were analyzed using Student's t-test (two groups) or two-way analysis of variance [for the results of CCK-8 assay (Fig. 2A) and wound-healing assay (Fig. 4B)] followed by Bonferroni's test. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of HOXA6 in human CRC tissues and cell lines. The detection of the mRNA expression of HOXA6 in CRC and normal colorectal tissue samples was performed by RT-qPCR analysis. The results showed that the mRNA levels of HOXA6 in the CRC tissues were significantly upregulated, compared with those in the paired normal colorectal tissue samples (P=0.0103) (Fig. 1A). Subsequently, the expression levels of HOXA6 in four CRC cell lines (Caco2, SW480, HCT116 and HT-29) were detected by RT-PCR analysis. The mRNA expression level of HOXA6 was highest in the HT-29 cells and lowest in Caco2 cells (Fig. 1B). Therefore, these two cell lines were selected for the subsequent experiments.

Verification of the expression of HOXA6 in transfected cells. The Caco2 cells were transfected with plasmids expressing HOXA6 or an NC and HT-29 cells were transfected with shHOXA6 or NC plasmids. The expression of HOXA6 was detected by RT-qPCR and western blot analyses. The results showed that the mRNA and protein expression levels were significantly upregulated in the Caco2-HOXA6 cells (P=0.0008 and 0.0012) and suppressed in the HT-29-shHOXA6 cells (P=0.0225 and 0.0026) (Fig. 1C and D).

HOXA6 enhances the proliferation rate of CRC cells. To examine the effect of HOXA6 on CRC cell proliferation, CCK-8, colony formation and EdU assays were performed. The CCK-8 assay demonstrated that the Caco2-HOXA6 cells grew significantly faster than the Caco2-NC cells (P<0.0001), whereas the growth rate of the HT-29-shHOXA6 cells was significantly lower, compared with that of the HT-29-NC cells (P<0.0001) (Fig. 2A). In addition, the colony formation assay showed that the Caco2-HOXA6 cells grew significantly faster than the Caco2-NC cells (P<0.0001), whereas the growth rate of the HT-29-shHOXA6 cells was significantly lower, compared with that of the HT-29-NC cells (P<0.0001) (Fig. 2A).
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The EdU assay demonstrated that the Caco2-HOXA6 cells formed more colonies than the Caco2-NC cells (P<0.0001), whereas the HT-29-shRNA cells formed fewer colonies than the HT-29-NC cells (P=0.0004) (Fig. 2B). The EdU assay demonstrated that the upregulated expression of HOXA6 promoted cell proliferation (P=0.0301), whereas the downregulation of HOXA6 suppressed cell proliferation (P=0.0310) (Fig. 2C). These results indicated that the expression of HOXA6 enhanced the proliferative capacity of the CRC cells.

HOXA6 inhibits apoptosis in CRC cells. The effect of HOXA6 on CRC cell apoptosis was evaluated with flow cytometry and a TUNEL assay. The results of the two assays revealed that the rate of apoptosis of the Caco2-HOXA6 cells was inhibited, compared with that of the Caco2-NC cells (P<0.05), whereas the rate of apoptosis of the HT-29-shRNA cells was significantly higher, compared with that of the HT-29-NC cells (P<0.01) (Fig. 3A and B). Western blot analysis was performed to detect the protein expression levels of PARP, Bcl-2, Bax and caspase-3. The protein expression levels of cleaved PARP, Bax and cleaved caspase-3 were inhibited in the Caco2-HOXA6 cells and increased in the HT-29-shRNA cells, whereas the expression of Bcl-2 was increased in the Caco2-HOXA6 cells and suppressed in the HT-29-shHOXA6 cells, compared with levels in the NC cells (Fig. 3C). These results demonstrated that HOXA6 inhibited the apoptosis of the CRC cells.

Expression of HOXA6 enhances CRC cell migration and invasion. Transwell and wound-healing assays were performed to analyze the effect of HOXA6 on CRC cell migration and invasion. The results of the Transwell assay showed that the numbers of migrated and invaded Caco2-HOXA6 cells were significantly increased, whereas the numbers of migrated and invaded HT-29-shHOXA6 cells were suppressed, compared with those in the NC (Fig. 4A). The wound-healing assay demonstrated that the Caco2-HOXA6 cells migrated at a faster rate than the Caco2-NC cells (P=0.0186), whereas the HT-29-shHOXA6 cells migrated at a slower rate than the HT-29-NC cells (P=0.0003) (Fig. 4B). The results of the western blot analysis showed that the expression levels of N-cadherin and Vimentin increased, whereas the expression of E-cadherin decreased in the Caco2-HOXA6 cells, with the opposite pattern observed in the HT-29-shHOXA6 cells (Fig. 4C). These results confirmed that HOXA6 enhanced the migration and invasion of the CRC cells.
Discussion

The occurrence and development of tumors are associated with several factors, including behavioral and environmental factors. The occurrence of tumors is the result of the accumulation of mutations in a number of genes over an extended period of time. The identification of affected genes may provide a basis for the early diagnosis and treatment of cancer. HOXA6, a member of the HOX family, has been reported to be involved in the regulation of certain types of malignancy. The present study investigated the effect of the expression of HOXA6 on CRC cell proliferation, apoptosis, migration and invasion, and examined the expression of associated proteins. Initially, the RT-qPCR results demonstrated that HOXA6 was upregulated in CRC samples compared with normal tissues. Subsequently, by upregulating and downregulating the expression of HOXA6 with plasmid transfection, it was found that the upregulated expression of HOXA6 promoted proliferation, migration and invasion, and inhibited apoptosis, whereas the downregulated expression of HOXA6 had the opposite effects. Finally, the expression levels of associated proteins were detected. The upregulated expression of HOXA6 increased the expression levels of cleaved PARP, cleaved caspase-3, Bax, N-cadherin and Vimentin, but decreased the expression levels of Bcl-2 and E-cadherin. The downregulated expression of HOXA6 induced the opposite results. These results suggested that HOXA6 promoted proliferation and metastasis, and inhibited apoptosis in CRC.

Apoptosis is the process of programmed cell death, which eliminates unnecessary or unhealthy cells from the body. Cancer cells avoid apoptosis, and gain an advantage in survival and proliferation by promoting anti-apoptotic mechanisms and downregulating pro-apoptotic programs (23). Apoptosis is regulated by the Bcl-2 and caspase families, particularly caspase-3. The activation of caspase-3 is necessary for efficient apoptosis (24). The balance between pro-apoptotic and anti-apoptotic Bcl-2 family proteins is also significant in apoptosis. Previous studies have identified that the Bcl-2/Bax ratio was...
increased in tumor tissues, causing a reduced rate of apoptosis (25,26). PARP is cleaved by caspase-3 during apoptosis, which means that PARP cleavage is an important indicator of apoptosis and caspase-3 activation (27,28). This is consistent with the experimental results of the present study described above.

Tumor metastasis is the main cause of the poor prognosis of CRC. Following the determination of changes in migration and invasion, the expression of epithelial-mesenchymal transition (EMT)-related proteins was detected. It has been demonstrated that EMT initiates the isolation of cancer cells from primary carcinomas, which subsequently migrate and spread further (29,30). Its core features are a decreased expression of E-cadherin and increased expression of Vimentin. The experimental results in the present study confirmed that an increase in the expression of HOXA6 promoted EMT.
The findings of the present study suggested that HOXA6 was associated with the proliferation, apoptosis, migration and invasion of CRC cells, and further elucidated the potential mechanisms by which HOXA6 inhibited apoptosis and promoted migration and invasion. However, there were number of shortcomings in the present study and the potential mechanism underlying the regulation of cell proliferation requires further investigation. The number of clinical samples was insufficient, and additional samples are required to verify the expression of HOXA6 in colorectal tumors. There was also a lack of relevant clinical information regarding the study participants. In addition, HOXA6, as a transcription factor, mediates its biological effect by regulating the expression of downstream genes, which may provide a direction for further investigations.

In conclusion, the expression of HOXA6 was demonstrated to be associated with the proliferation, apoptosis, migration and invasion of CRC cells through experiments involving HOXA6 knockdown or transfection. These results suggest that HOXA6 is involved in the regulation of CRC, which may inform the development of strategies for the diagnosis and treatment of CRC.

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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

SW and FW made substantial contributions to the study conception, design, acquisition of data, analysis and interpretation of the data and were involved in the drafting of the
manuscript. ZJ was involved in revising the manuscript critically for important intellectual content. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent was signed by all patients, and ethics approval was obtained from the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References