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Original Research

Knockdown of Angiopoietin-like protein 4 suppresses the development of colorectal cancer

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Abstract: Colorectal cancer, is the growth of cancer cells in the part of the colon. Angiopeptin is one of the growth factors in the human body that is particularly effective in the regulatory process. In this research, the regulatory role and its mechanism of Angiopoietin-like protein 4 (ANGPTL4) in colorectal cancer (CRC) metastasis, has been studied. Protein expression of ANGPLT4 was analyzed by immunohistochemistry in tumor samples and adjacent normal specimens of 40 patients with CRC cancer of various phases. A gene knockout test was conducted, two effective siRNA of ANGPTL4, named siRNA1 and siRNA2, were constructed and transfected into two CRC cell lines SW480 and HT-29 to block the expression of ANGPTL4. QRT-PCR and western blotting were used to validate the knockdown efficiency of the mRNA and proteins. Based on the results, the protein expression of ANGPTL4 was increased in human CRC tissues with the development of CRC. Knockdown of ANGPTL4 by siRNA in SW480 and HT-29 cells in vitro inhibited cell proliferation, promoted cell apoptosis, and suppressed the ability of cell migration and invasion. Besides, the sensitivity of CRC cells to Cisplatin was increased in the low ANGPTL4 expression group. ANGPTL4 might be a new potential therapeutic target for patients with CRC.

Key words: Angiopoietin-like 4; Colorectal cancer; Proliferation; Metastasis; Invasion; Cisplatin.

Introduction

Tumor cells are a population of host cells that lose their ability to reproduce and multiply indefinitely. Tumor tissue can absorb nutrients and oxygen through simple diffusion up to a range of 1 to 2 mm, and from this point onwards requires the formation of new feeding vessels (1, 2). In fact, in 1971, Folkman first hypothesized that tumor growth depended on angiogenesis. Subsequent research has shown that tumor growth and metastasis depend on the formation of new vessels and the need for tumor nutrition. For 30 years, it was thought that tumor regression was caused only by sprouting angiogenesis from existing vessels (3). Recently, other vascular mechanisms have been identified in tumors. One of these methods is vasculogenic mimicry (VM) vascular regeneration, a process in which tumor cells mimic the behavior of endothelial cells and form canal-like canal structures (4). This phenomenon was first identified in 1999 in skin cancer. In 2017, the role of high mobility protein A2 (HMGA2) proteins in the study of mimic vasculitis in gastric carcinoma were investigated. The effect of increasing the expression of HMGA2 protein and VM formation on clinical samples and cell categories as well as mice were studied. Based on the results, a higher rate of metastasis was observed in patients with VM-type vessels than in patients without it. It was also found that patients with HMGA2 protein expression have a shorter survival time than patients who do not have HMGA2 expression and therefore do not form VM (5, 6).

Colorectal cancer (CRC), also known as colon cancer or rectal cancer is the growth of cancer cells in the colon or rectum or part of the colon. This condition is caused by abnormal growth of cells that can invade or multiply in other tissues of the body (metastasis). Symptoms and signs of the disease can include blood in the stool (hematosis), changes in bowel movements, weight loss, and constant tiredness. CRC is one of the most common causes of cancer-related deaths worldwide, and its morbidity and mortality are increasing in developing countries (7-10).

Many CRCs are caused by lifestyle factors and aging, and a small number of cases are due to inherited genetic disorders. Risk factors such as diet, obesity, smoking and lack of physical activity are sufficient (11-13). Dietary factors that increase the risk of this disease include red meat and processed meats, as well as excessive alcohol consumption (14, 15). Another risk factor is inflammatory bowel disease, which includes Crohn's disease and ulcerative colitis. Some inherited conditions that cause CRC include inherited familial adenomatous polyps and nonspecific hereditary colon cancer, But these include less than five percent of cases. The disease usually begins with a benign tumor that eventually becomes cancerous (16, 17).

CRC can be diagnosed by taking a colon sample during a sigmoidoscopy or colonoscopy. Medical imaging is usually done after this to determine if the disease has spread. Screening is effective in reducing the risk of dying from CRC and is recommended from the age of 50 onwards until the age of 75. During a colonoscopy, the polyps are removed. Aspirin and other anti-inflammatory and non-steroidal anti-inflammatory drugs reduce the risk. Of course, their general use is not recommended due to side effects (18-20).

Treatments for CRC can include a combination of surgery, radiation therapy, chemotherapy, and targeted treatment. Surgery can be used to treat cancers limited to the colon wall, but cancer that has spread throughout the body is usually incurable, and management focuses on improving the quality of life and symptoms. Worldwide, CRC is the third most common type of cancer, accounting for 10 percent of all cancers. The disease is more common in developed countries and 65% of cases are found in these countries. The prevalence of this disease is lower in women than in men (21-23).

A complex multi-step process has been recognized in the process of CRC initiation, promotion and development, but its mechanism is still poorly understood. Therefore, elucidating the molecular mechanism of CRC is still a challenging issue (24).

Studies show that reactivating the p53 gene stops the growth of cancer cells in the gut and turns the cells into healthy tissue. 80 to 90% of colorectal tumors occur due to mutations in the same gene; Therefore, reversing the activity of this gene plays a significant role in stopping the growth of cancerous tumors. Animal studies show that reactivating this gene prevents tumor growth, and after six months, there is no longer any sign of the tumor in the body of laboratory mice (25, 26).

Angiogenesis is a physiological process in which new vessels grow from existing ones. During embryonic development, a primary vascular network is formed during a process called vasoconstriction. In this process, endothelial cells are re-formed as precursors of endothelial cells called angioblasts. In adults, Angiogenesis occurs only during physiological repair processes such as wound healing and ovarian cycle. In fact, in adults, endothelial cells are silent in adulthood but are able to be activated in response to appropriate factors. In other words, vasodilation is an essential process in the body's physiology that is regulated by the balance between the inductive and inhibitory factors of vasodilation, and if this balance is lost, the ground for diseases such as tumor growth and metastasis is provided. Stimulation for life, growth, and recovery play a key role, for example, in wound healing. However, the basis for the transformation of tumors from dormant to malignant is through this process (27, 28).

Tumor Angiogenesis is mediated by various molecules. The balance between progressive and inhibitory regulatory factors controls this process strongly. Because vasodilation is a good way to treat a tumor, researchers have tried to treat the tumor by designing therapeutic agents against the tumor. Recently, angiogenic inhibitors have been classified as endogenous (such as interleukin-8) and exogenous (such as Avastin). These factors interfere with the formation of arteries (28-32).

Angiopoietin is one of the growth factors in the human body that is particularly effective in the regulatory process. The gene for this protein is located on chromosome 8. Four types of angiopathy, such as ANGPT1, ANGPT2, and ANGPT4, have been discovered today. Angiogenesis plays a very important role in the physiological growth of tissues and tumors (29, 30, 33-35).

Angiogenesis is the process of forming new blood vessels from previous blood vessels. The formation of blood vessels or angiogenesis within tumors is essential for tumor growth and metastasis, which can lead to physiological growth or, in the case of diseases such as breast cancer, tumor growth. However, angiogenesis is regulated and balanced by specific molecular signaling pathways, and many stimulants and inhibitors, including exercise, may affect angiogenesis and eventually tumor growth and cancer (31, 36).

Evidence suggests that signal transducers and activators of transcription (STATs), especially STAT-3, play an important role in angiogenesis under physiological and pathological conditions in addition to cell survival in proliferation, differentiation, and cancer genes. STAT-3 has been reported to regulate many aspects of angiogenesis at the transcriptional level as an important multifunctional mediator (32, 37, 38).

Angiopoietin-like protein 4 (ANGPTL4) is a member of the angiopoietin family, which is highly expressed within adipose tissue, the liver, and the placenta (39-41). ANGPTL4 has been reported to exhibit different biological effects in normal and malignant cells, affecting lipid metabolism, glucose metabolism, vascular permeability, angiogenesis, wound healing, and tumorigenesis (42, 43). An increasing number of studies have been focused on the critical roles of ANGPTL4 in many aspects of tumorigenesis, including motility, metastasis, apoptosis, angiogenesis, regulation of tumor microenvironment, wound healing, vascular permeability and inflammation (44-49). Both proangiogenic effects and antiangiogenic effects of ANGPTL4 have been reported in different models. Studies have suggested that up-regulating ANGPTL4 could promote tumor occurrence and development in various cancers, including hepatocellular carcinoma (HCC) (50), CRC (51), breast cancer (2), prostate cancer (52), renal cell carcinoma (53) and Kaposi's sarcoma (54). However, studies have also pointed out that ANGPTL4 inhibits metastasis in melanoma cancer, prostate cancer, and hepatocellular carcinoma (48, 52). These inconsistent data suggest that additional research is needed to address the precise roles of ANGPTL4 in cancer progression. Up to now, the molecular mechanism of ANGPTLl4 in the progress of cancer remains largely unknown, particularly in CRC.

In this study, we attempted to determine the level of ANGPTL4 protein expression in human CRC tissues and the effects of down-regulation of ANGPTL4 by siRNA on tumor cell apoptosis, migration, and invasion. In addition, the role of ANGPTL4 in cisplatin-induced apoptosis in tumor cells is also one of the research priorities.

Materials and Methods

Sample preparation and cell culture

Tumor and corresponding non-tumor control tissues were collected from 40 CRC patients at the Sun Yat-sen

Memorial Hospital of the Sun Yat-sen University. There were 10 cases at stage I and IV respectively, 11 cases at stage II and 9 cases at stage III. This study was approved by the ethics committee of the Hospital, and written informed consent was obtained from all patients.

The human CRC cell lines SW480 and HT29 were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (Sigma Aldrich, St. Louis, MO, USA) under humidified conditions in 95% air, and 5% CO_2 at 37 ℃.

Knockdown of ANGPTL4 in CRC cell lines

For transfection, SW480 cells and HT29 cells were plated in 6-wells plates at a density of 1×10^6 cells per well without transfection were set to be the blank control group. A negative control siRNA (NC) and two siRNAs against ANGPTL4 (siRNA1, siRNA2; RiboBio Co. Ltd., Guangzhou, Guangdong) were designed and transfected into SW480 cells and HT29 cells using Lipofectamine 2000 reagents (Invitrogen, USA), according to the manufacturer's instructions. The sequence of siRNA1 was 5'-GGUGACUCUUGGCUCUGCC-3' (sense) and 5'-GGUGACUCUUGGCUCUGCC-3' (antisense). The sequence of siRNA2 was 5'-AGGGAAUCUUCUGGAAGAC-3' (sense) and 5'-GUCUUCCAGAAGAUUCCCU-3' (antisense).

Immunohistochemistry

Tumor and corresponding non-tumor control tissue specimens were fixed overnight in formalin solution, dehydrated in ethanol, embedded in paraffin, and cut into 5 μm sections. xylene and ethanol were used for treating the specimens in order to remove paraffin.

The slides were blocked with 5% normal goat serum and incubated with anti-ANGPTL4 (Abcam, ab196746Adipobioscience) at 4 ℃ overnight. After washing with PBS, slides were incubated for 1 h at room temperature with Goat anti-rabbit IgG H&L (HRP) secondary antibody (Abcam, ab205718Vector Laboratories; 1:3000). A DAB kit (Sigma-Aldrich, St. Louis, MO) was used to detect immunohistochemical reactions. Briefly, after IHC staining, if tissue was dyed brown, it would be recorded as positive immunostaining. Examination of slides was carried out under a phase-contrast microscope (Nikon, Tokyo, Japan), and the average integral optical density of each positively stained slide was measured using Image-Pro Plus 6.0 true-color image analysis system. Three areas were randomly selected from each section for measurement. To keep the results comparable, we made sure that the process of immunostained slides preparation was the same, including tissue fixation and staining.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA from SW480 cells and HT29 cells was purified by TRIzol (Invitrogen, USA) and reversely transcribed to cDNA using the First-Strand Synthesis System (Invitrogen, USA). Quantitative real-time PCR was done by the SYBR Green kit (Yeasen, China) in a 7500 Real-Time PCR system (Applied Biosystems), based on the manufacturer's guidelines. Relative transcript quantities were calculated by the $2^{-\Delta\Delta CT}$ way with GAPDH as the endogenous reference gene. The sequence of ANGPTL4 PCR primers was 5'-GG CGA GTT CTG GCT GGG TCT-3' (sense) and 5'-TGG CCG TTG AGG TTG GAA TG-3' (antisense). The sequence of GAPDH primers was: 5'-ATG TCG TGG AGT CTA CTG GC-3' (forward primer) and 5'-TGA CCT TGC CCA CAG CCT TG -3' (reverse primer).

Western blot analysis

Cells were gathered and lysed with RIPA (Thermo, USA) comprising protease inhibitors (Invitrogen, USA). After centrifugation, the protein concentrations were determined by a BCA Protein Assay kit (Thermo Fisher Scientific., Rockford, IL, USA). An equal amount of total proteins was separated by 10% SDS PAGE and transferred onto polyvinyl difluoride (PVDF) membrane. Membranes were incubated with ANGPTL4 and β-actin (TDY051, TDY) primary antibody overnight at 4 ℃, and then incubated with secondary antibody for 1 h at room temperature. The proteins were visualized by an ECL detection kit (Thermo Scientific, USA).

Cell proliferation assay

Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide (MTT) assay following the manufacturer's menu. Briefly, a total of 2×10^4 treated cells were inoculated in each well of a 96-wells plate with complete medium for 24 h, then cells were washed with PBS and 20 μl of MTT solution was added. The absorbance was observed at 490 nm by a microplate reader (Sunrise™; Tecan Group Ltd., Switzerland). All experiments were performed in triplicate.

Flow-cytometric analysis of apoptosis

The ANGPTL4 siRNA transfected SW480 cells and HT-29 cells were added into 6-well plates and incubated overnight. The Apoptosis rate was measured by flow cytometry using Annexin-V/PI double staining (BD Biosciences, Franklin Lakes, NJ, USA). The flow cytometry results were analyzed by FlowJo software. Cells were segmented into four types, such as the viable cells, dead cells, the early-stage apoptotic cells, the late-stage apoptotic cells. The SW480 cells or HT29 cells apoptosis were analyzed in triplicates and repeated three times independently.

Cell migration and invasion assay

Cell migration and invasion assays were conducted by transwell chambers based on the manufacturer's instructions. Transfected SW480 cells and HT-29 cells (1×105 for the migration assay, 2×105 for the invasion assay) were inoculated in the upper compartment filled with serum-free media, and the lower compartment was filled with complete medium supplemented with 10% FBS. After incubation at 37 ℃ for 24 h, migratory and invasive cells on the bottom surface of the filters were fixed by 4% paraformaldehyde and stained with 5% Crystal Violet. Four random fields were counted for each group. The experiments were done in triplicate.

Cisplatin cytotoxicity assay

Si-RNA1, siRNA2, and negative control plasmids were transfected into SW480 and HT29 cells, respectively. Different concentrations of cisplatin (0, 2.5, 5.0, 10.0, 20.0, and 40.0) were used to treat the cells, and then the chemosensitivity of cisplatin and cell apoptosis were examined by MTT assay.

Statistical analysis

The data were analyzed by one-way ANOVA, the Student's t-test and variance test (ANOVA) using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). All data of this research were expressed as mean ± standard deviation (SD). The statistical significance was set at $P< 0.05$.

Results

Up-regulation of ANGPTL4 in CRC tissues

To investigate the roles of ANGPTL4 in CRC pathogenesis, Immunohistochemistry was used to analyze the expression level of ANGPTL4 in tumor and adjacent normal specimens from 40 patients with CRC (I phase, II phase, III phase, and IV phase). As shown in Figures 1A and 1B, the expression level of ANGPTL4 was increased in tissues of tumor/node/metastasis (TNM) stage I, II, III, and IV compared with those in normal tissues. Furthermore, we found that the higher TNM stage was associated with increased ANGPTL4 expression.

Expression of ANGPTL4 was decreased in transfected SW480 and HT29 cells

SW480 and HT29 cells were administrated with siRNA oligos targeting RNA1, RNA2, or negative control. ANGPTL4 mRNA (Figures 2A and 2B) and protein expression (Figures 2C and 2D) were significantly decreased in SW480 and HT-29 cells transfected with si-RNA1 and si-RNA2 than the blank or negative groups. These results demonstrated that the two siRNA was effective in down-regulating ANGPTL4 expression.

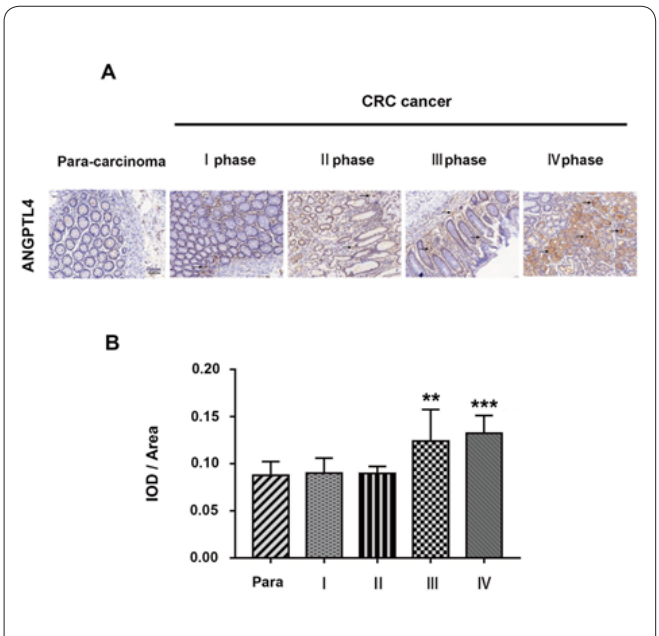

Figure 1. ANGPTL4 was up-regulated in CRC tissues. Representative immunohistochemistry staining **(A)** of ANGPTL4 in tumor tissues and para-carcinoma tissues from 40 patients with CRC (I phase, II phase, III phase, and IV phase) and the statistic results of IOD/Area **(B)**. ANGPTL4 positive signals were stained brown (arrow). **p< 0.01 ****p< 0.0001 compared with Para-carcinoma groups.

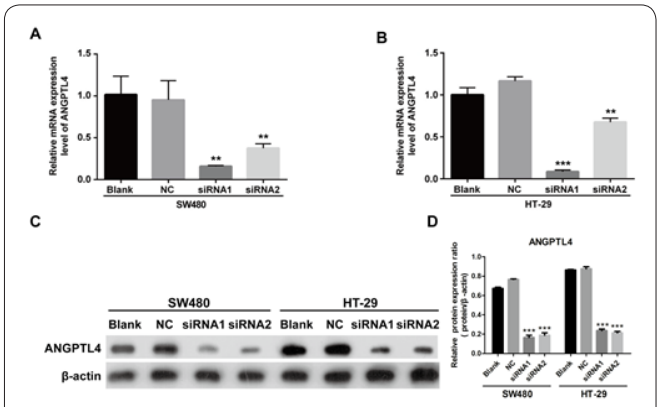

Figure 2. The ANGPTL4 expression levels in the siRNA transfected CRC cells. Relative mRNA levels **(A, B)** and protein levels **(C, D)** of ANGPTL4 in SW480 and HT-29 cells transfected with negative control, si-RNA1 or si-RNA2. *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001 compared with negative control (NC) groups.

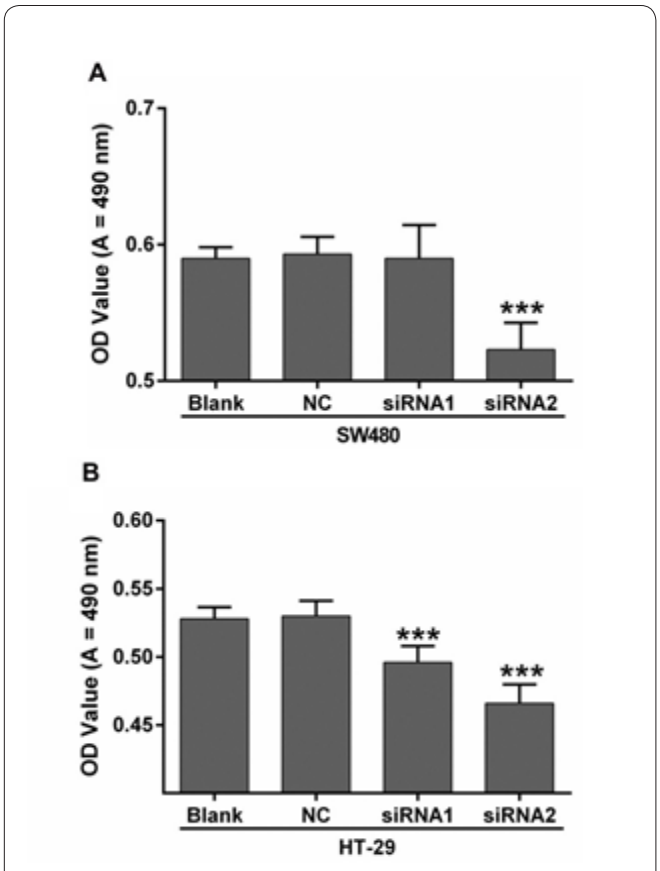

Figure 3. Suppression of cell proliferation after ANGPTL4 knockdown. The transfected SW480 cell **(A)** and HT-29 cell **(B)** proliferation for each group (negative control, si-RNA1 group or si-RNA2 group) measured by MTT. ***p< 0.001 compared with NC groups.

Knockdown of ANGPTL4 suppressed the proliferation ability of CRC cells

Cell proliferation was measured using the MTT assay. The results indicated that knockdown of ANGPTL4 significantly inhibited cell proliferation in both SW480 cells (Figure 3A) and HT-29 cells (Figure 3B) compared to the blank or negative groups.

Cell apoptosis is induced by ANGPTL4 knockdown

Flow cytometry assay was used to explore whether cell apoptosis could be affected by ANGPTL4 knockdown. We found that the apoptotic rate of SW480 (Figure 4A) cells and HT-29 (Figure 4B) cells transfected with siRNA1 or siRNA2 were increased compared to the control group and the blank group. It suggested that knockdown of ANGPTL4 promoted cell apoptosis in CRC cell lines.

Suppression of cell migration and invasion after ANGPTL4 knockdown

Transwell assays were performed to detect the effects of ANGPTL4 knockdown on the migration and invasion ability of SW480 and HT-29 cells. As the results indicated, the ability of cell migration and invasion was significantly decreased in SW480 and HT-29 cells which were transfected with siRNA1 or siRNA2 compared to the blank or negative groups (Figures 5A and 5B).

Knockdown of ANGPTL4 sensitized CRC cells to cisplatin

To study the effect of ANGPTL4 on the sensitivity of anti-tumor drugs in CRC cells, the MTT assay was employed. The results revealed that knockdown of ANGPTL4 significantly decreases the viability of SW480 cells (Figure 6A) and HT-29 cells (Figure 6B) in response to cisplatin in a dose-dependent manner. With higher concentrations of cisplatin, the cell growth inhibition rate increases. Those results indicated that knockdown of ANGPTL4 enhances the sensitivity of human CRC cells to cisplatin.

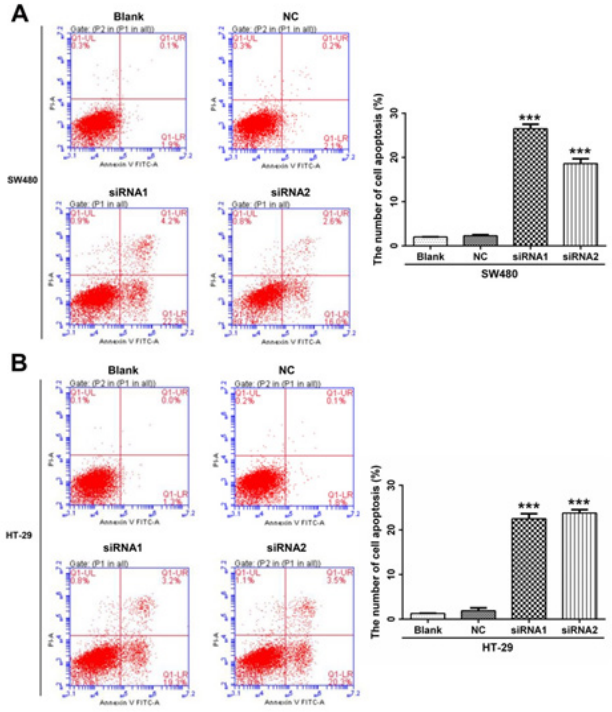

Figure 4. Effects of ANGPTL4 knockdown on CRC cell apoptosis. Flow cytometry was performed to measure the apoptosis ratio of SW480 cells **(A)** and HT-29 cells **(B)** transfected with negative control, si-RNA1 or si-RNA2. ***p< 0.001 compared with NC groups.

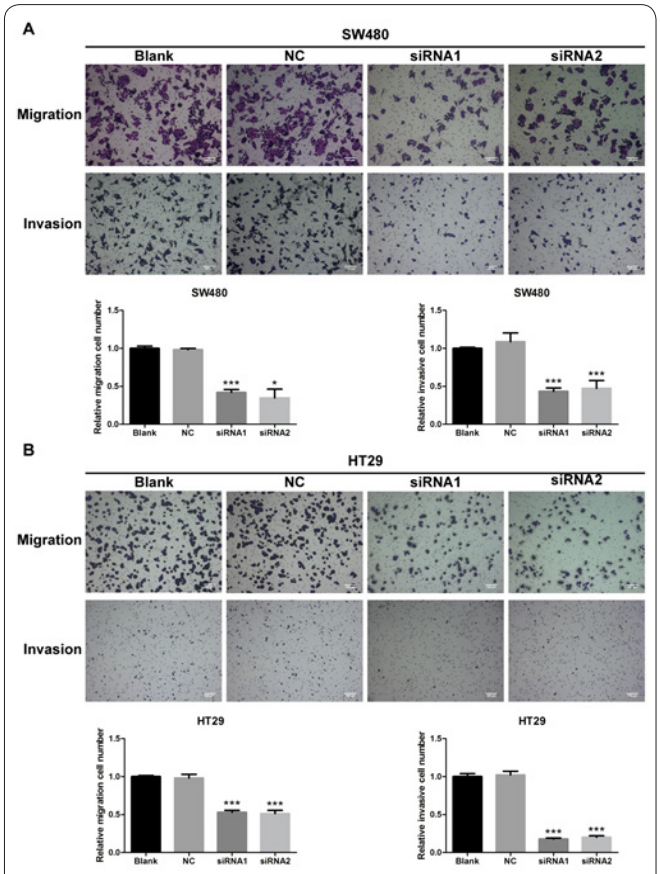

Figure 5. The migration and invasion abilities in the different cell groups. Transwell assays were performed to detect the effects of ANGPTL4 knockdown on the migration and invasion ability of SW480 cells **(A)** and HT-29 cells **(B)** transfected with negative control, si-RNA1 or si-RNA2. **p< 0.01 or ***p< 0.001 compared with NC groups.

Discussion

As a secretory protein expressed in the perinecrotic regions of different human tumors, ANGPTL4 plays a key role in the existence and development of different cancer. There is evidence that ANGPTL4 plays a significant role in tumorigenesis and contributes to a variety of biological functions in various tumors. Previous studies on the biological role of ANGPTL4 in oncology have revealed that it plays an essential role in tumorigenesis of Oral Kaposi's Sarcoma (54, 55), which could promote tumor angiogenesis in malignant gliomas (56), and inhibits the growth of liver cancer cells (57). Galaup et al confirmed that ANGPTL4 prevents the metastatic process by inhibiting vascular activity as well as tumor cell motility and invasiveness (55). Yet, the role of ANGPTL4 in CRC remains unclear. Several studies about the molecular events of ANGPTL4 in the CRC metastatic process have been reported. It suggested that PGE2 enhanced ANGPTL4 expression via EP1 receptor signaling, thus promotes CRC cell proliferation (58),

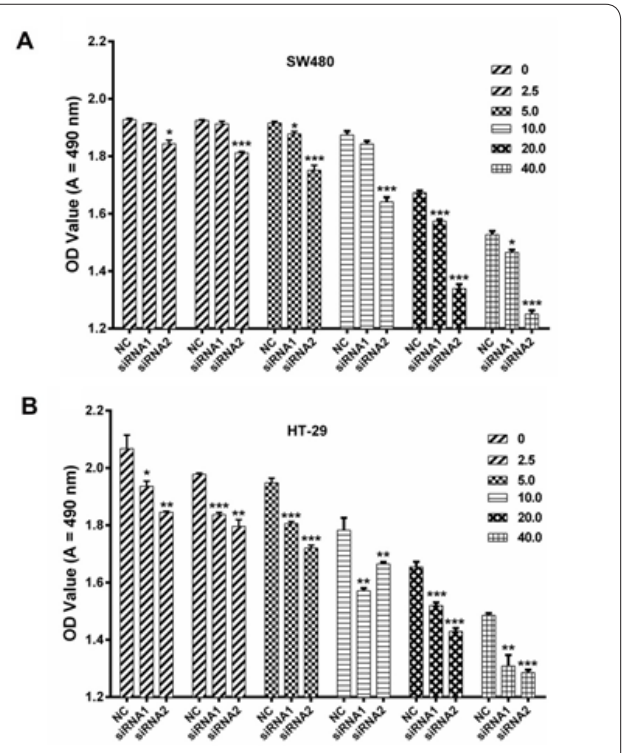

Figure 6. ANGPTL4 knockdown promoted the chemosensitivity of CRC cells to cisplatin in vitro. The chemosensitivity of CRC cell lines SW480 **(A)** and HT-29 **(B)** to cisplatin was measured by MTT assay in the different cell groups following treatment with different concentrations of cisplatin, respectively. ***p< 0.001 compared with NC groups.

and Li et al. (2015) revealed that higher expression of ANGPTL4 accelerated CRC progression by up-regulation of BMP7 (59).

In the present study, we detected the protein expression of ANGPTL4 in CRC tumors and normal colorectal tissues by immunohistochemistry. Our results demonstrated that ANGPTL4 expression in CRC tissue was significantly up-regulated compared with normal colorectal tissues. The function of ANGPTL4 in colon cancer cell lines was then investigated by knockdown of its expression in vitro.

In vitro study, we constructed two effective siRNA of ANGPTL4, named siRNA1 and siRNA2. Then the SW480 and HT-29 cell lines were transfected with siRNA1 or siRNA2 to block the expression of ANGPTL4. We validated the knockdown efficiency of mRNA and protein by qRT-PCR and western blotting, respectively. The results showed that ANGPTL4 mRNA and protein expression were significantly reduced in SW480 and HT-29 cells transfected with siRNA1 and siRNA2 compared to the blank or negative groups.

To further determine the biological functions of ANGPTL4 in CRC cancer, we applied MTT, flow cytometry, and transwell assay in the ANGPTL4 knockdown CRC cell lines. Results from these assays indicated that knockdown of ANGPTL4 by siRNA inhibits cell proliferation, promotes cell apoptosis, and suppresses cell migration and invasion in both SW480 and HT-29 cells. Chemotherapy is an effective treatment for patients with CRC cancer and cisplatin is widely used for CRC patients around the world. Studies have demonstrated that ANGPTL4 increases melanoma cell survival in cisplatin-induced apoptosis (55-65). But whether ANGPTL4 affects the efficacy of cisplatin in human CRC remains unknown. In this study, we treated SW480 and HT-29 cells with various concentrations of cisplatin after the knockdown of ANGPTL4 by siRNA1 or siRNA2. The results showed that the block of ANGPTL4 obviously enhanced the sensitivity to cisplatin in both SW480 and HT-29 cells dose-dependently (66). New technologies about gene expression such as genome editing (67-71) can also be used in this regard (72-74).

In conclusion, the data in this study revealed that knockdown of ANGPTL4 inhibits the development of CRC by inhibiting CRC cell proliferation, promoting apoptosis, suppressing migration and invasion. In addition, with the knockdown of ANGPTL4, CRC cells showed higher chemosensitivity to cisplatin, which was positively correlated with high apoptosis induction.

Acknowledgments

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