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Original Article

Impact of AQP-5 on the growth of colorectal cancer cells and the underlying mechanism

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Abstract: Aquaporin 5 (AQP-5) is highly expressed in colorectal cancer tissue and associated with colorectal cancer development and prognosis. Here, we explored the effects of AQP-5 on colorectal cancer cell proliferation and apoptosis and the underlying mechanism by inhibiting endogenous AQP-5 expression in the human colorectal cancer cell lines COLO 205 and SW480. These cells were transfected with an AQP-5-siRNA, and transfection efficiency and its effects on AQP-5 expression were assessed by immunofluorescence and PCR, respectively. Then, cell proliferation was assessed via the MTT assay, apoptosis was assessed by Annexin V-FITC/PI and TUNEL assays, and expression changes in Bax and Bcl-2 were assessed by RT-PCR and western blotting. Transfection with AQP-5-siRNA reduced AQP-5 expression by up to 62%. The MTT assay showed that cell proliferation was significantly inhibited by AQP-5-siRNA transfection compared to that in NS-siRNA-transfected cells ($P < 0.05$). Flow cytometry analysis revealed that the percentage of apoptotic AQP-5-siRNA-transfected cells was significantly higher than that of NS-siRNA-transfected cells ($P < 0.05$). Real-time quantitative RT-PCR and western blotting showed that AQP-5-siRNA significantly increased the Bax/Bcl-2 mRNA and protein ratios compared with those following NS-siRNA transfection. Thus, AQP5-siRNA promotes apoptosis of colorectal cancer cells, which may be associated with Bax/Bcl expression.

Keywords: Colorectal cancer, aquaporin-5, apoptosis, Bax/Bcl

Introduction

Colorectal cancer is the third most common cancer and the fourth leading cause of cancer death worldwide [1], and its incidence is increasing each year. In China, the incidence and mortality rates of colon cancer are also increasing [2]. The first-line treatment for colon cancer involves comprehensive treatment with surgery and adjuvant chemotherapy; however, recurrence and metastasis are major causes of treatment failure [3]. Although some recent progress has been made in both diagnosis and treatment, colorectal cancer continues to have a huge impact on human lives and health [3]. Therefore, new targeted therapeutic interventions are greatly needed.

In recent years, an increasing number of studies has shown that the growth, development, invasion, and metastasis of tumors is dependent on the tumor microenvironment and metabolism [4]. Water channels play a significant role in tumor development, and this finding has been very important for the development of antitumor therapies [5]. Aquaporins (AQPs) are membrane water transport channels that play a role in secretion and absorption in epithelial cells. Some aquaporin subtypes also transport other molecules, such as glycerol and urea. Currently, there are 13 known types of AQPs in mammals, which are mainly divided into 3 categories [6, 7]: 1. classical AQPs, which are primarily water-selective channels, including AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8; 2. aquaglyceroporins, including AQP3, AQP7, AQP9, and AQP10, which can also transport glycerol and other small solutes; and 3. non-classical AQPs, including AQP11 and AQP12, which have been localized within cells;

however, their selectivity has not been elucidated. In recent years, the roles of AQPs in tumor development have gained increasing attention. Previous studies have shown that AQPs are strongly expressed in tumor cells of different origins and play key roles in tumor biology, including tumor-associated edema, tumor cell migration, tumor proliferation, and tumor angiogenesis [8, 9]. The expression of AQPs in various tumor types differs because of their tissue-specific localization. Recently, the expression of AQP-5 was found to be upregulated in colon cancer tissues. AQP5 expression in colon cancer tissues is related to tumor prognosis, suggesting that AQP5 overexpression is involved in the development of colorectal tumors [10]. AQP5 was reportedly involved in colorectal carcinogenesis [11, 12] and affected the phosphorylation of extracellular signal-regulated kinase-1/2 [13] and p38 MAPK signaling [14] in colorectal cancer cells. In this study, we determined the impact of an AQP-5-targeting siRNA on the proliferation and apoptosis of colorectal cancer cells in order to explore the roles of AQP-5 in the treatment of colon cancer and its regulatory mechanism.

Materials and methods

Materials

The human colorectal cancer cell lines COLO 205 and SW480 were purchased from ATCC (Manassas, VA, USA). DMEM culture medium was purchased from GIBCO (Grand Island, NY, USA). Fetal bovine serum was purchased from Lanzhou MinHai Biological Engineering Co., Ltd. (Lanzhou, China). The anti-AQP-5, -Bcl-2, -Bax, and -β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Annexin V-FITC and propidium iodide (PI) were purchased from Yisheng Biotechnology (Shanghai, China).

Cell culture and treatment

COLO 205 and SW480 cells were cultured in DMEM high-glucose culture medium supplemented with 10% inactivated fetal bovine serum at 37°C in a 5% CO_2 incubator and treated prior to reaching 80% confluence. The cells in the adherent cell culture were passaged once every 2-3 days. Cells in the logarithmic growth phase were used for experiments.

Transfection of AQP-5-siRNA

An AQP-5-specific small interfering RNA (AQP-5-siRNA) was designed and synthesized as the following oligonucleotides: siRNA #1 sense strand: 5'-AAAACTCTGCGAACACGGCCCCTGTCTC-3' and antisense strand: 5'-AAGGCCGTGTTCGCAGAGTTCCTGTCTC-3'; siRNA #2 sense strand: 5'-AAGAGCAGCCAGTGAAGTAGACCTGTCTC-3' and antisense strand: 5'-AATCTACTTCACTGGCTGCTCCCTGTCTC-3'. The sequence of the non-specific control siRNA (NS-siRNA) was 5'-GGUCUCACUCCCCAUAGAGTT-3'. The cells were inoculated into 6-well plates at a density of 4×10^5 cells/mL 24 h prior to transfection. Before transfection, the cells were washed with serum-free, antibiotic-free DMEM/F12 medium. Next, the cells were transfected using the liposome-mediated method. According to the reagent instruction manual, the AQP-5-siRNA complex or control NS-siRNA and the Lipofectamine 2000 Transfection Reagent were diluted with serum-free, antibiotic-free DMEM/F12 medium. The two dilutions were mixed to form a complex that was used to transfect the cells. The transfection efficiency and expression of other molecules were determined 24 h after transfection. The experimental groups included normal cultures of COLO 205 and SW480 cells (control group), NS-siRNA-transfected COLO 205 and SW480 cells (NS-siRNA group), and AQP-5-siRNA-transfected COLO 205 and SW480 cells (AQP-5-siRNA group).

Cell proliferation assay

Cell proliferation was assessed by the MTT assay. A 96-well plate was inoculated with 5×10^3 cells, 200 μL of culture medium was added to each well, and the plate was incubated overnight. The cells were cultured for 24 h after transfection, and then MTT reagent (5 mg/mL) was added. The supernatant was discarded after 4 h of incubation. Then 150 μL of dimethyl sulfoxide was added to each well, and the absorbance was measured. The assay was repeated three times.

Flow cytometry assay for apoptosis

Apoptosis was detected by using the Annexin V-FITC/PI Apoptosis Detection Kit according to the manufacturer's instructions. Each well of the 6-well plate was inoculated with 10^5 cells. The medium was discarded after 24 h of incu-

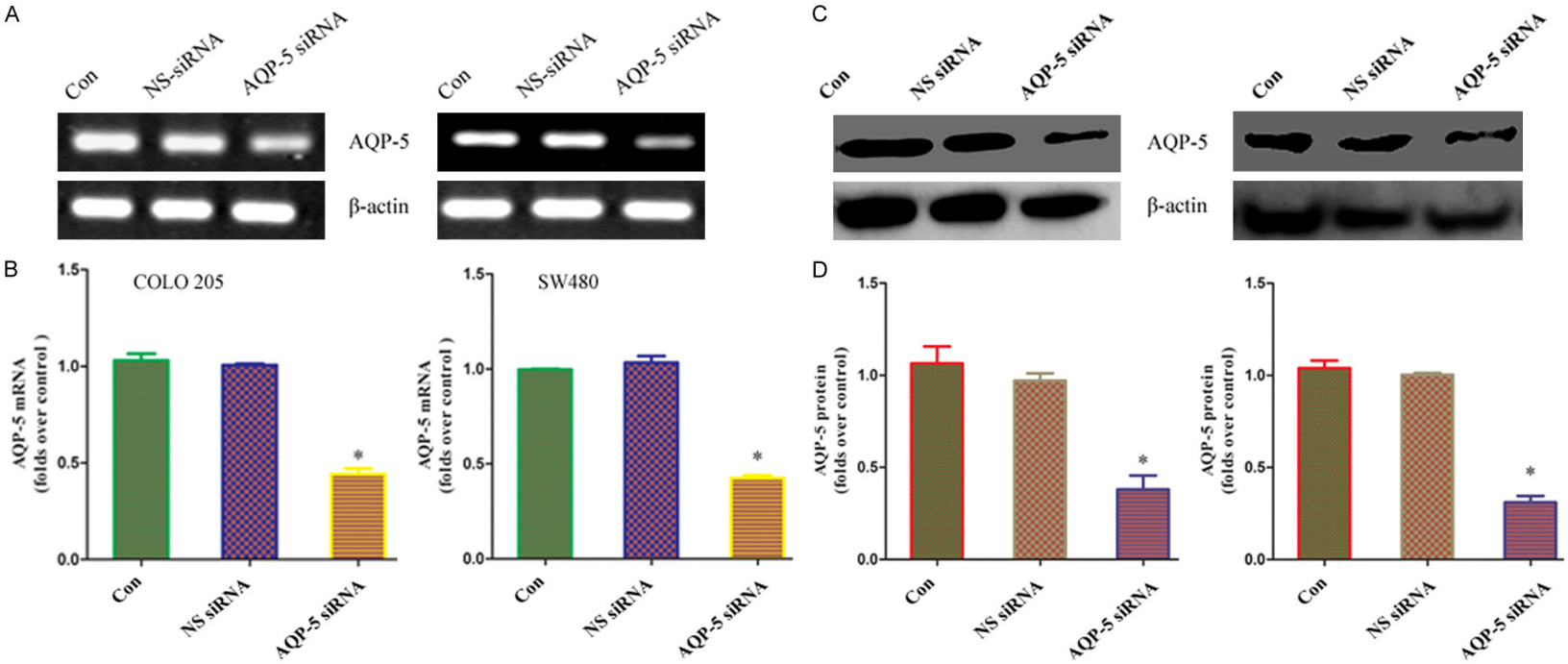

Figure 1. Transfection efficiency of AQP-5-siRNA. Transfection efficiency was assessed 24 h after AQP-5-siRNA and non-specific control siRNA (NS-siRNA) were transfected into COLO 205 and SW480 cells. A, C: Determination of AQP-5 expression in control, AQP-5-siRNA-transfected, and NS-siRNA-transfected cells by using western blotting and RT-PCR. β-actin was used as housekeeping gene for standardisation in this analysis. B, D: Results of statistical analysis. The results relative to control cells are presented as a standard ratio. *indicates $P < 0.05$.

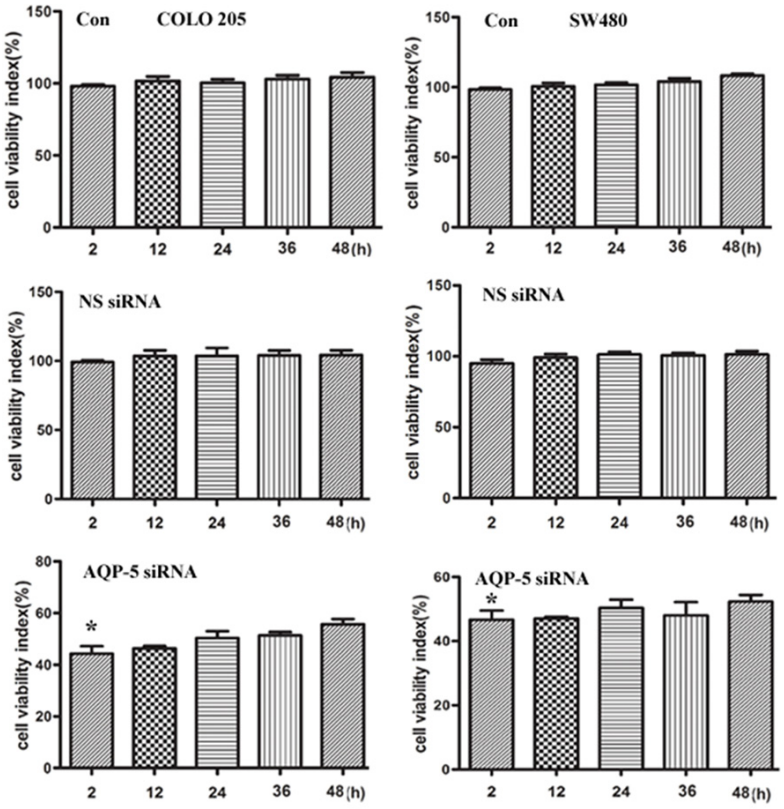

Figure 2. Reduction of cell proliferation by AQP-5-siRNA. The cell proliferation of control, AQP-5-siRNA-transfected, and NS-siRNA-transfected cells was assessed using the MTT assay at 2, 12, 24, 36, and 48 h after AQP-5-siRNA and non-specific control siRNA (NS-siRNA) were transfected into COLO 205 and SW480 cells. *indicates $P < 0.05$.

bation, and the wells were washed once with PBS, followed by transfection and a second 24-h incubation. The cells were trypsinized and centrifuged, and then stained with Annexin V and propidium iodide in the dark for 15 min. The percentage of apoptotic cells in each sample was determined by flow cytometry.

Transmission electron microscopy (TEM)

After washing, treated cells were incubated in 2.5% pentanediol at 4°C for 2 h. Next, the cells were treated with 1% osmium tetroxide and dehydrated with a graded series of ethanol. The cells were then embedded in epoxy, and 70-nm ultrathin sections were prepared using a microtome (Leica, Wetzlar, Germany). After counterstaining with uranyl acetate and lead citrate, the cells were observed under a HITACHI H-7650 transmission electron microscope (TEM; Hitachi, Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV.

TUNEL assay

Twenty-four hours after transfection, the cells were fixed in 4% paraformaldehyde for 30 min, and endogenous peroxidase activity was inactivated by treatment with 3% H_2O_2. Terminal deoxynucleotidyltransferase was added to the slides and incubated at 37°C in a humid chamber for 1 h for in situ hybridization of DNA fragments. After diaminobenzidine (DAB) staining and counterstaining with hematoxylin, the slides were observed and photographed under a microscope.

RT-PCR

Total RNA was extracted from the cells using TRIzol reagent. In total, 2 μg of RNA was reverse transcribed into first-strand cDNA using the RevertAid First Strand cDNA Synthesis Kit. The primer sequences were as follows: Bcl-2 (376 bp) forward primer: 5'-CCGGGAGATCGTGATGAAGT-3' and reverse primer: 5'-ATCCCAGCCTCCGTTATCCT-3'; Bax (284 bp) forward primer: 5'-CCAAGAAGCTGAGCGAGTGTC-3' and reverse primer: 5'-TGAGGACTCCAGCCACAAAGA-3'; β-actin (179 bp) forward primer: 5'-ATCGTGCGTGACATTAAGGAGAAG-3' and reverse primer: 5'-AGGAAGGAAGGCTGGAAGAGTG-3'. The PCR conditions were as follows: an initial incubation at 42°C for 1 h to synthesize cDNA, followed by denaturation at 94°C for 5 min and 22 cycles of 94°C for 30 s, 61°C (Bcl-2) or 62°C (Bax, β-actin) for 30 s, and 72°C for 30 s. After the last cycle, the reaction was incubated at 72°C for 10 min. The housekeeping gene β-actin was used as an internal reference.

Western blotting

In total, 5×10^5 cells in logarithmic growth phase were added to 0.5 mL of pre-chilled cell lysis

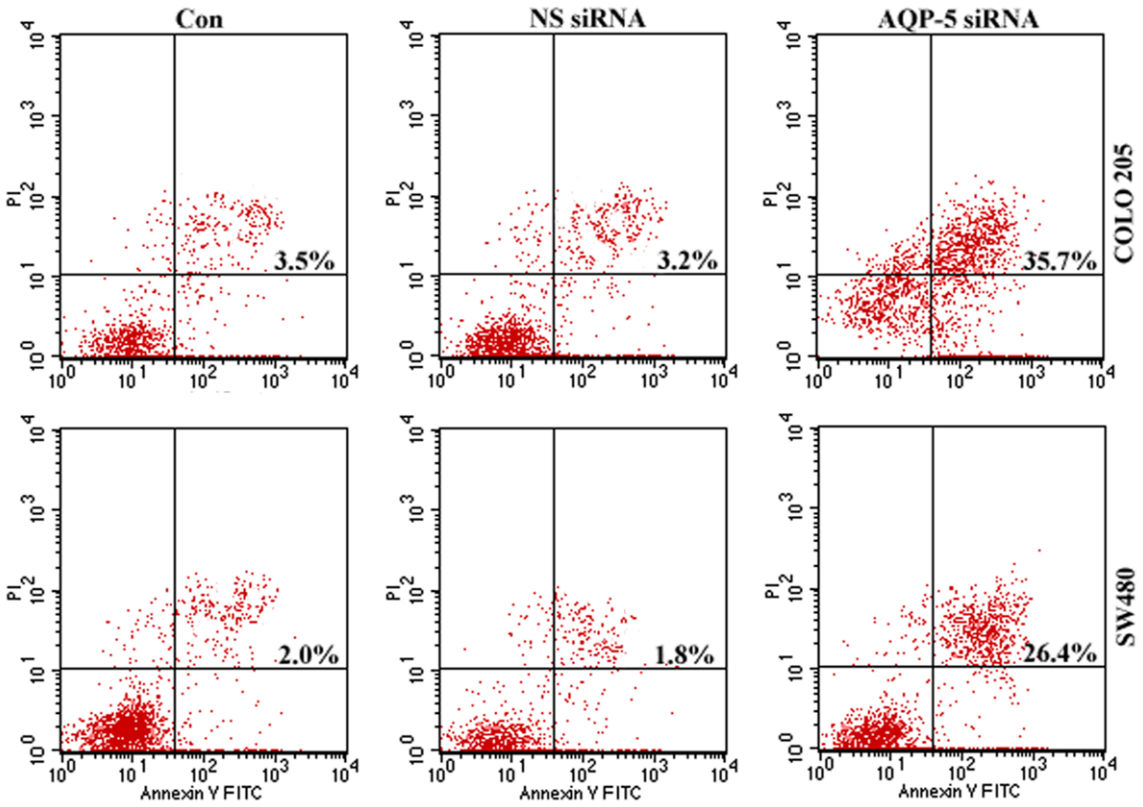

Figure 3. Promotion of cell apoptosis of AQP-5-siRNA transfection by flow cytometry. The apoptotic rate of samples measured by flow cytometry 24 h after AQP-5-siRNA and non-specific control siRNA (NS-siRNA) were transfected into COLO 205 and SW480 cells. The results showed the following: control COLO 205 cells, 3.5%; NS-siRNA transfected COLO 205 cells, 3.2%; AQP-5-siRNA transfected COLO 205 cells, 35.7%; control SW480 cells, 2.0%; NS-siRNA transfected SW480 cells, 1.8%; AQP-5-siRNA transfected SW480 cells, 24.6%. The transfection of AQP-5-siRNA significantly increased the percentage of apoptotic cells ($P < 0.05$).

buffer and incubated on ice for 30 min. After centrifugation, the supernatant was collected and the protein content was measured. The proteins were separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane by semi-dry transfer. Next, the membrane was blocked by immersion in Tris-buffered saline with Tween 20 (TBST) containing 5% skim milk and overnight incubation with the primary antibody at 4°C. On the following day, the membrane was incubated with the secondary antibody conjugated to horseradish peroxidase (1:2000; Santa Cruz) at 25°C for 2 h, and then an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Amersham, UK) was used for staining. The membrane was photographed, and the results were analyzed.

Statistical analysis

Each experiment was repeated at least 3 times. Data were expressed as means ± SD and analyzed using ANOVA and Student's t-test. *P* values less than 0.05 were considered statistically significant.

Results

Transfection efficiency of AQP-5-siRNA and its effects on AQP-5 expression

A synthetic AQP-5 small interfering RNA (AQP-5-siRNA) was transfected into COLO 205 and SW480 cells. Non-specific control siRNA (NS-siRNA)-transfected cells were included as negative controls. The immunofluorescence results indicated a transfection efficiency of 62%. Western blotting and RT-PCR showed that AQP-5 was highly expressed in NS-siRNA-transfected cells, whereas the expression of AQP-5 in AQP-5-siRNA-transfected COLO 205 and SW-480 cells was significantly down regulated ($P < 0.05$). These results indicate that the AQP-5-siRNA used in this study efficiently inhibited the expression of AQP-5. The difference in the AQP-5 expression levels between the NS-siRNA-

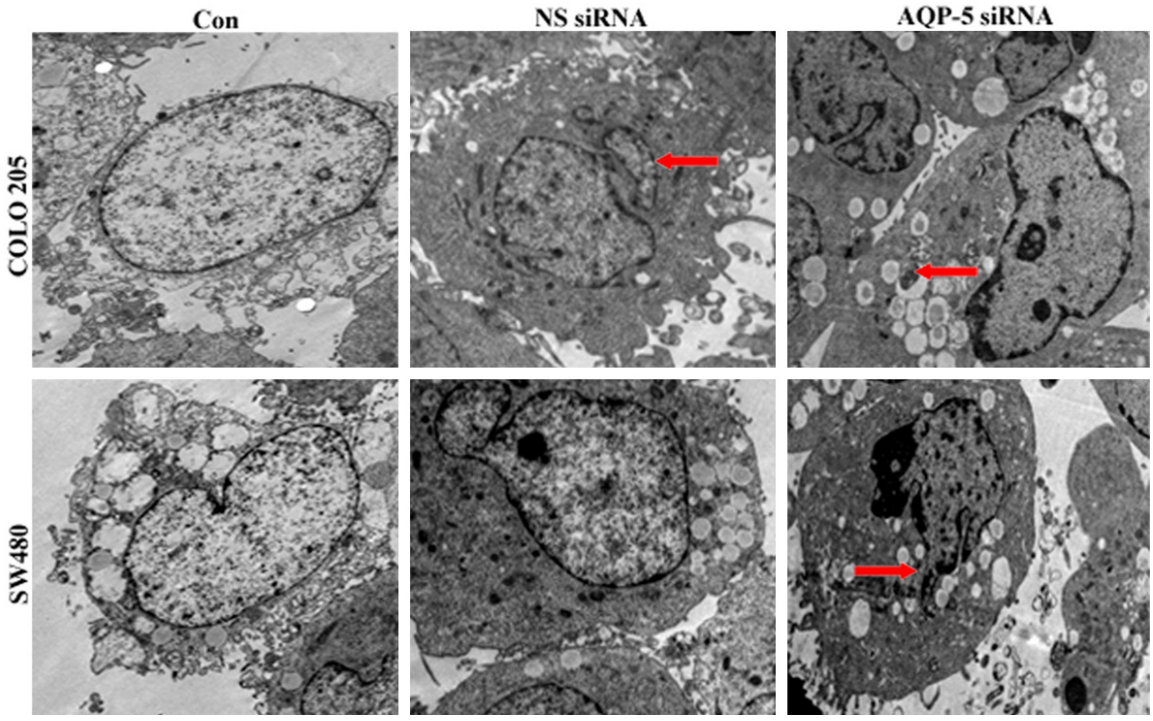

Figure 4. Induction of cell apoptosis of AQP-5-siRNA transfectionby transmission electron microscopy. The subcellular structures were observed by transmission electron microscopy (TEM) 24 h after AQP-5-siRNA and non-specific control siRNA (NS-siRNA) were transfected into COLO 205 and SW480 cells. Apoptotic bodies were indicated by red arrows. The subcellular structures of control cells and NS-siRNA transfected cells were normal. AQP-5-siRNA-transfected cells appeared to have a high degree of chromatin condensation and marginalization, resulting in apoptotic bodies.

transfected cells and untransfected control cells was not statistically significant ($P > 0.05$, **Figure 1**).

Impact of an AQP-5-targeting siRNA on COLO 205 and SW480 cell proliferation

The difference in the proliferation of COLO 205 and SW480 cells transfected with AQP-5-siRNA or NS-siRNA was assessed by using the 3-(4,5)-dimethylthiazol (2 yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Proliferation of AQP-5-siRNA-transfected COLO 205 and SW480 cells was significantly lower than that of NS-siRNA-transfected cells and control cells ($P < 0.05$). These results suggest that inhibition of AQP-5 expression inhibits the proliferation of COLO 205 and SW480 cells. The difference in the proliferation of NS-siRNA-transfected and control cells was not statistically significant ($P > 0.05$, **Figure 2**).

Apoptotic effects of AQP5-siRNA transfection

To investigate whether inhibition of cell proliferation by AQP-5-siRNA was caused by apoptosis, we first detected the apoptotic changes in COLO 205 and SW480 cells using flow cytometry. The results showed that transfection with AQP-5-siRNA significantly increased the percentage of apoptotic cells compared with that in untransfected control cells ($P < 0.05$, **Figure 3**).

This finding was confirmed by transmission electron microscopy (TEM) and a TUNEL assay. AQP-5-siRNA-transfected cells exhibited a high degree of chromatin condensation and marginalization, resulting in apoptotic bodies. These findings were consistent with the flow cytometry results; AQP-5-siRNA transfection significantly enhanced apoptosis (**Figures 4**, **5** and **Table 1**).

Expression of Bax/Bcl-2

Bax and Bcl-2 are pro-apoptotic and anti-apoptotic proteins, respectively. To investigate the impact of AQP-5-siRNA transfection on the ratio of Bax to Bcl-2, we used reverse transcription (RT)-PCR and western blotting to determine the ratio of Bax to Bcl-2 at the mRNA and protein

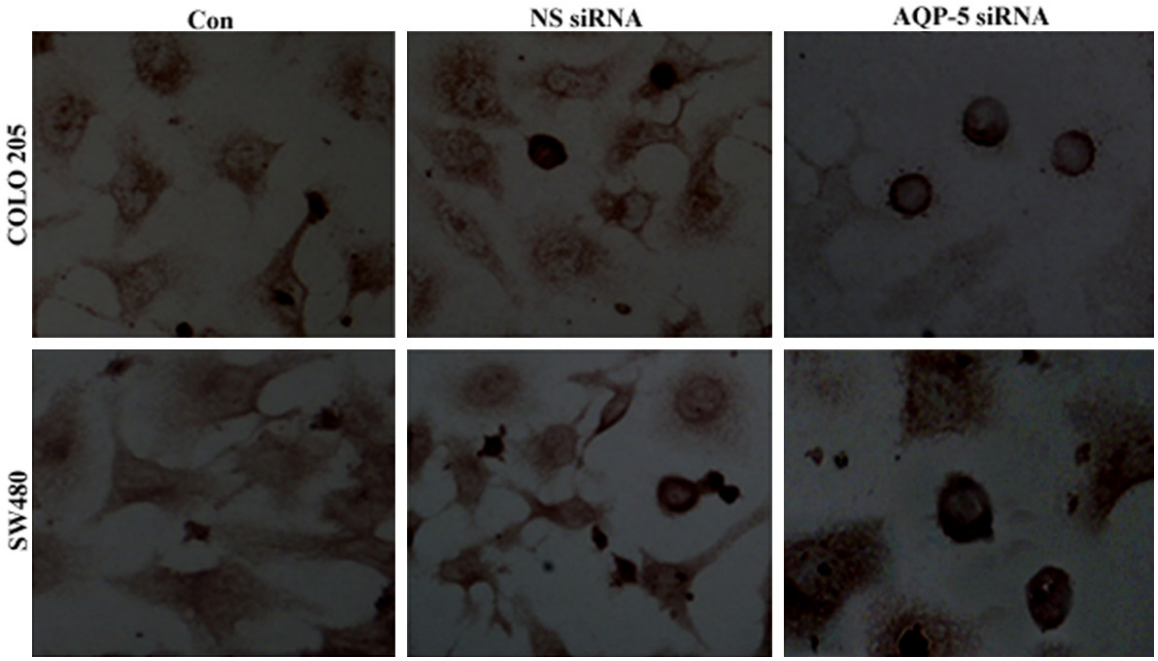

Figure 5. Induction of cell apoptosis of AQP-5-siRNA transfection by TUNEL assay. Cell apoptosis was assessed using the TUNEL assay 24 h after AQP-5-siRNA and non-specific control siRNA (NS-siRNA) were transfected into COLO 205 and SW480 cells. The number of apoptotic AQP-5-siRNA transfected cells was significantly higher than the control cells and NS-siRNA-transfected cells.

Table 1. Detection of apoptotic changes by using TUNEL assay after AQP-5-siRNA transfection

	TUNEL-positive cells (apoptotic index) (%)	
	COLO 205	SW480
Control	2.02 ± 0.67	1.62 ± 0.30
NS-siRNA	3.82 ± 0.92	2.70 ± 0.15
AQP-5-siRNA	12.72 ± 0.47*	14.00 ± 0.24*

All values are expressed as mean ± S.D. *P < 0.05 Control or NS-siRNA VS AQP-5-siRNA group.

levels. The results showed that the ratio of Bax to Bcl-2 at both the mRNA and protein level in AQP-5-siRNA-transfected COLO 205 and SW480 cells was significantly higher than that in the control cells (**Figures 6** and **7**).

Discussion

AQPs are specific channels for highly efficient and selective transport of water molecules and are widely present in the cell membranes of prokaryotes and eukaryotes [15]. Tumor development involves a loss of the normal regulation of cell growth at the genetic level due to various tumorigenic factors. Tumors rely on various metabolic processes involving water molecules, and AQPs can rapidly and specifically transport water molecules [16]. AQP1-12 is widely expressed in the brain, skin, fat, liver, kidney, pancreas, muscle, ovary, testis, spleen, lung, and tissues where body fluid absorption and secretion occurs [17]. AQPs are also involved in the reabsorption of water in the kidneys, gland secretion, intestinal lipid absorption, and function at the blood-air barrier, blood-brain barrier, and in cerebrospinal fluid formation [17]. Additionally, AQPs are involved in the stress response, tissue injury, infection, and tumor formation [16]. Different AQPs are expressed in different types of tumors because of their tissue-specific localization, for example, AQP1 expression is often associated with brain tumors [18]. In a study of AQP3-null mice, deletion of the *AQP3* gene induced resistance to carcinogen-induced skin tumors [19]. In addition, AQP3-facilitated glycerol transport contributes to ATP production during cell proliferation and tumorigenesis [19]. High expression levels of AQP5 in breast [20], stomach [21], liver [22], lung [23], and cervical [24] cancers are associated with poor prognosis. The results of our previous study [10] showed that AQP5

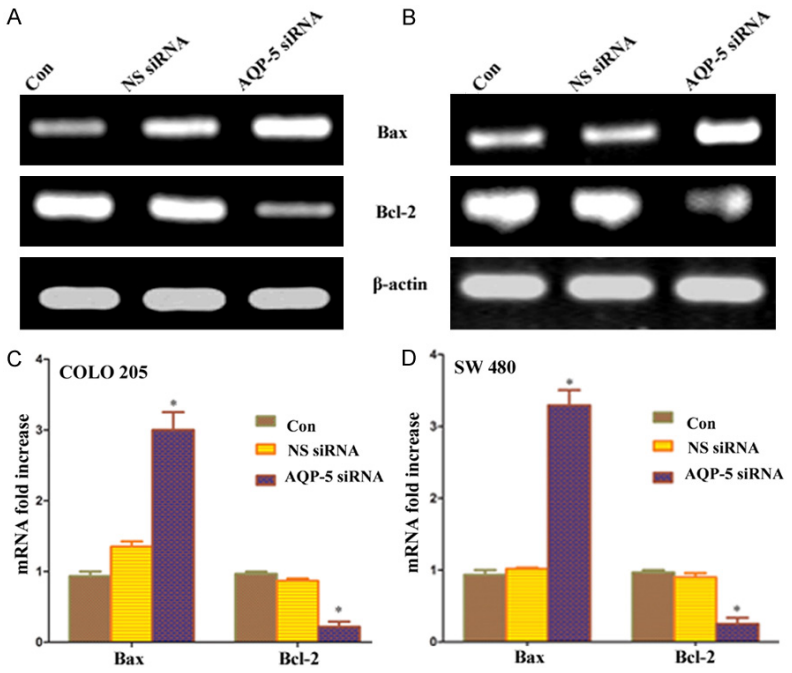

Figure 6. AQP-5-siRNA transfection significantly increased the mRNA expression ratio of Bax/Bcl-2. The mRNA expression increased 24 h after AQP-5-siRNA and non-specific control siRNA (NS-siRNA) were transfected into COLO 205 and SW480 cells. A and B: The mRNA expression ratio of Bax/Bcl-2 in control COLO 205 and SW480 cells, AQP-5-siRNA-transfected COLO 205 cells, and NS-siRNA-transfected COLO 205 cells were determined using RT-PCR. C and D: Results of statistical analysis. The results relative to control cells are presented as a standard ratio. *indicates $P < 0.05$.

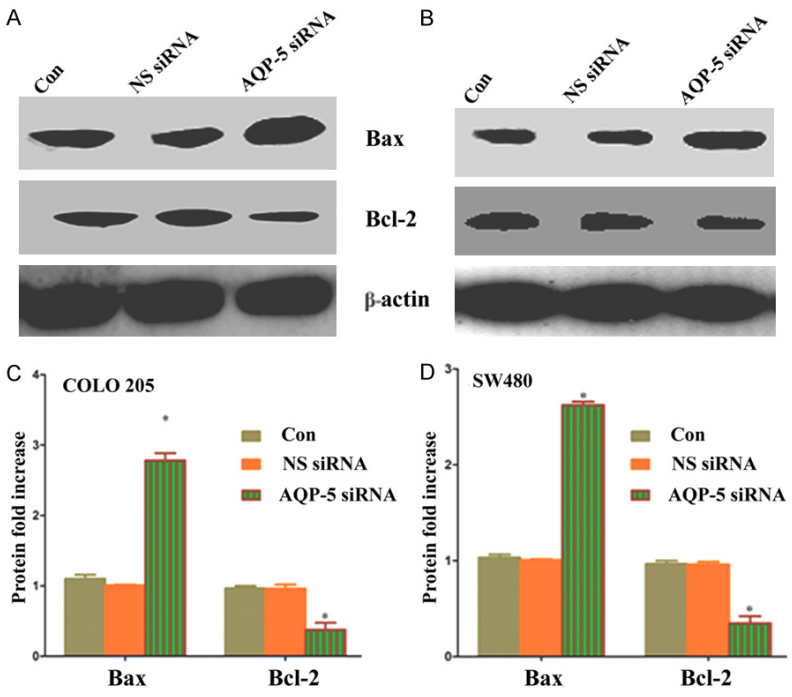

Figure 7. AQP-5-siRNA transfection significantly increased the protein expression ratio of Bax/Bcl-2. The protein expression ratio increased 24 h after AQP-5-siRNA and non-specific control siRNA (NS-siRNA) were transfected into COLO 205 and SW480 cells. A and B: The expression ratio of Bax/Bcl-2 in control COLO 205 and SW480 cells, AQP-5-siRNA-transfected COLO 205 and SW480 cells, and NS-siRNA-transfected COLO 205 and SW480 cells were determined by western blotting. C and D: Results of statistical analysis. The results relative to the control cells are presented as a standard ratio. *indicates $P < 0.05$ in t-test.

expression was upregulated in colorectal cancer cells. Furthermore, in this study, we found silencing AQP5 expression enhanced cell apoptosis. These findings increase our understanding of the role of AQP5 as a therapeutic target.

AQPs play a key role in maintaining water balance and regulating various physiological and pathological processes [25]. AQP5 is a 21-24-kDa protein that was initially thought to be the major structural protein of caveolae in cell membranes and was shown to be a key molecule in oncogenic transformation and malignant progression [26, 27]. However, the specific mechanism underlying the promotion of tumor development by AQP5 remains unclear. Previous studies have shown that high APQ5 expression can promote cell proliferation, inhibit cell apoptosis, reset the cell cycle, and promote epithelial-mesenchymal transition and cell migration [28]. The alterations in the biological behaviors of tumors induced by AQP5 may be regulated by several signaling pathways leading to cell transformation, including the Src family of tyrosine

kinases, epidermal growth factor receptor, Wnt signaling pathway, and Erk1/2-mediated signal transduction [28-30].

The clinical significance of AQP5 in colorectal cancer has not yet been determined. In a previous study [10], we found that the AQP5 expression was significantly higher in colorectal tissues than in paracancerous and normal mucosal tissues and that AQP5 expression was associated with a higher TNM stage, lymph node metastasis, and distant metastasis. In addition, the expression level of AQP5 is related to the number of circulating tumor cells in the peripheral blood of patients [10]. Our current findings indicate that *in vitro* inhibition of AQP5 expression can promote apoptosis of colorectal cancer cells and that the mechanism is associated with Bax/Bcl expression. Our preliminarily study confirmed the possible roles of AQP5 in colorectal cancer via interference of AQP5 expression. However, the specific mechanism underlying the effect of AQP5 on the development of colorectal cancer requires further analyses, including molecular biology, cell biology, and animal experiments, which are the current research focus for our group.

In conclusion, our results suggest that silencing of AQP5 expression through transfection of AQP5-siRNA promotes apoptosis of colorectal cancer cells *in vitro* and the underlying mechanism is associated with Bax/Bcl expression. Therefore, AQP5 is a potential therapeutic target for colorectal cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

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