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Original Article

Inhibitory effects of lentivirus mediated RNA interference targeting human AQP1 gene on the proliferation of human colon carcinoma SW480 cells and the expression of VEGF

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Abstract: Purpose: The objective of the present study was to construct the lentiviral expression vector for RNA interference (RNAi) of human AQPl gene in colon carcinoma SW480 cells, which may provide research foundation for investigating the mechanisms that AQP1 gene functions for the proliferation of human colon carcinoma SW480 cells and the expression of VEGF in the cells. Methods: Four effective sequences of RNAi targeting human AQPl gene were confirmed. Both sense and antisense Oligo DNA of the targeting sequences were designed, synthesized and cloned into the lentiviral vector pEGFP-N1-3FLAG. After transfected with lentiviral vector of T293 cells, and the titer of the lentivirus was tested. SW480 cells were infected with the lentivirus and the expression of AQPl mRNA and protein in SW480 cells was detected by real-time quantitative polymerase chain reaction (RT-PCR) and Western blotting. The propagation of SW480 cells was detected by MTT and expression of VEGF in SW480 cells was detected by RT-PCR. Results: A recombinant lentiviral vector expressing shRNA against AQP1 gene was obtained confirmed by DNA sequencing. The titre of virus was 2×10^9 TU/ml. AQP1 mRNA and protein expression in SW480 cells after infected with lentiviral vector was decreased remarkably. The mRNA expression was 60%, respectively, compared with the control group. And the lentiviral shRNA expression vector reduced cell pro1 if eration 5 d after stable AQP1 gene knock-down in SW480 cells. Meanwhile, the expression of VEGF mRNA was inhibited. Discussion: The lentiviral shRNA expression vector targeting human AQP1 gene capable of stable AQP1 gene knock-down SW480 cells has been successfully constructed, which provides a basis for further study of mechanisms that AQP1 gene acts for proliferation of colon carcinoma. And preliminary conclution was drawn that AQP1 gene may promote the growth of SW480 cells and the expression of VEGF.

Keywords: AQP1, colon carcinoma, RNA interference, lentiviral, VEGF

Introduction

Aquaporins are ubiquitous membrane channel proteins which present throughout nature. So far, there are 13 members (AQP0-AQP12) in the family of cloned mammal aquaporins. They have been identified and localized to various epithelial, endothelial and other tissues. Some researches have discovered that AQPs had an intimate connection with many diseases [1]. In recent years, researches have discovered that AQPs, especially AQP1 plays an important role in the growth, invasion and metastasis of tumor [1]. The tumor treatment is provided with a new clue in the way of suppressing the growth of tumor by aquaporin inhibitor [1]. RNA interference (RNAi) is a specific and powerful tool used to control the expression of specific genes in invertebrates, plants and mammalian cells, and has potential as a therapeutic strategy to reduce the expression of problem genes. Therefore, we hypothesized that AQP1 gene may have potential usefulness with the proliferation of human colon carcinoma. We constructed a lentiviral vector carrying shRNA aimed at AQP1 gene and transfected it into human colon carcinoma SW480 cells to investigate its efficacy for suppression of AQP1 in vitro and provide a new method for the further study on the relation between expression of AQP1 and VEGF.

Table 1. Four effective sequences of RNAi targeting AQP1

Vector	Effective sequences of RNAi
pSC-1	CACAACTTCAGCAACCACT
pSC-2	GCTGCCAGATCAGCATCTT
pSC-3	CCATTATGCTGGTGTATGT
pSC-4	GCAGGAACTTCTAGCTCAT

Materials and methods

Cultivation of SW480 colon carcinoma cells

SW480 human colon carcinoma cells obtained from the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences were cultured with DMEM/F12, supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 15% fetal bovine serum (FBS) at 37°C, 5% CO_2. Cells between passages 2~4 were used in the experiments.

Preparation of shRNAs and transfection

Four chemically synthesized AQP1 gene-specific SiRNA duplexes were designed. Each target site was searched with NCBI Blast to confirm specificity only to AQP1. The follows siRNA sequence used are listed in **Table 1**. The pEGFP-N1-3FLAG vector enhanced green fluorescence protein (GFP) was purchased from Shanghai Genechem Co., Ltd. (China). For transfection, the cells were seeded into 6-well plates at 1.2×10^7 cells/20 ml with fresh medium without antibiotics and were incubated at 37°C with 5% CO_2. The medium was changed the following 24 h until 70-80% confluence was achieved. The cells were transfected with 4.0 μg lentiviral DNA (AQP1-pEGFP-N1-3FLAG group [KD]), non-specific siRNA mock lentiviral DNA (negative control group [NC]) and blank group [CON], by 10 μl lipofectamine 2000 in 2 ml serum-free OPTI-MEMI medium (Invitrogen, USA) according to the manufacturer's instructions. At 6 hours after transfection, the medium was replaced by normal medium containing 15% FBS and the transfection efficiency was determined and then the cells were collected for functional assay.

AQP1 protein expression in SW-480 cells by western blot

The transfected and non-transfected cells were harvested at 48 hours after transfection, then were washed twice with cold PBS and lysed with cold lysis buffer plus protease inhibitors. The protein was separated by 12% SDS-PAGE gel and transferred onto polyvinylidene fluoride (PVDF). The membranes were blocked with 5% skim milk in Tris-Cl buffered saline (TBS-T, 0.1% Tween-20). The blots were incubated with an affinity-purified anti-rabbit polyclonal AQP1 (1:400) at 4°C overnight. Immunoblots were washed and then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:10000) at room temperature for 1 h. Signals were detected using a chemiluminescence detection system. β-actin staining served as the internal standard for all membranes.

AQP1 mRNA expression in SW-480 cells by RT-PCR

The total RNA from the transfected and non-transfected cells was extracted at 48 hours after transfection using a Trizol Reagent (Invitrogen USA) according to the manufacturer's protocol. RT-PCR was performed according to the protocol. The primers were designed and synthesized. The AQP1 primer sequences: 5'-TTAACCCTGCTCGGTCCTTTG-3' (sense), 5'-AGTCGTAGATGAGTACAGCCA-3' (antisense), amplified fragment was 114 bp; β-actin primer sequences: 5'-GGCGGCACCACCATGTACCCT-3' (sense), 5'-AGGGGCCGGACTCGTCATACT-3' (antisense), amplified fragment was 202 bp. All PCR products sample was analyzed by electrophoresis in 8% polyacrylamide gel and the results was processed by gel image analysis system.

MTT assay

Cell proliferation was measured by the MTT assay. The cells were seeded into wells of 96-well flat-bottom plates in triplicate and allowed to adhere overnight. MTT in PBS was added to each well. The plates were then incubated for 1 d at 37°C. Following 2, 3, 4 and 5 d of culture, proliferative activity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5 bromide assay using a microplate reader. All experiments were done in quadruplicate.

VEGF mRNA expression in SW-480 cells by RT-PCR

TThe VEGF primer sequences: 5'-CAACATCACCATGCAGATTATGC-3' (sense), 5'-CCCACAGGGATTTTCTTGTCTT-3' (antisense), amplified fragment was 133 bp; β-actin primer sequences: 5'-GGCGGCACCACCATGTACCCT-3' (sense), 5'-AGGGGCCGGACTCGTCATACT-3' (antisense),

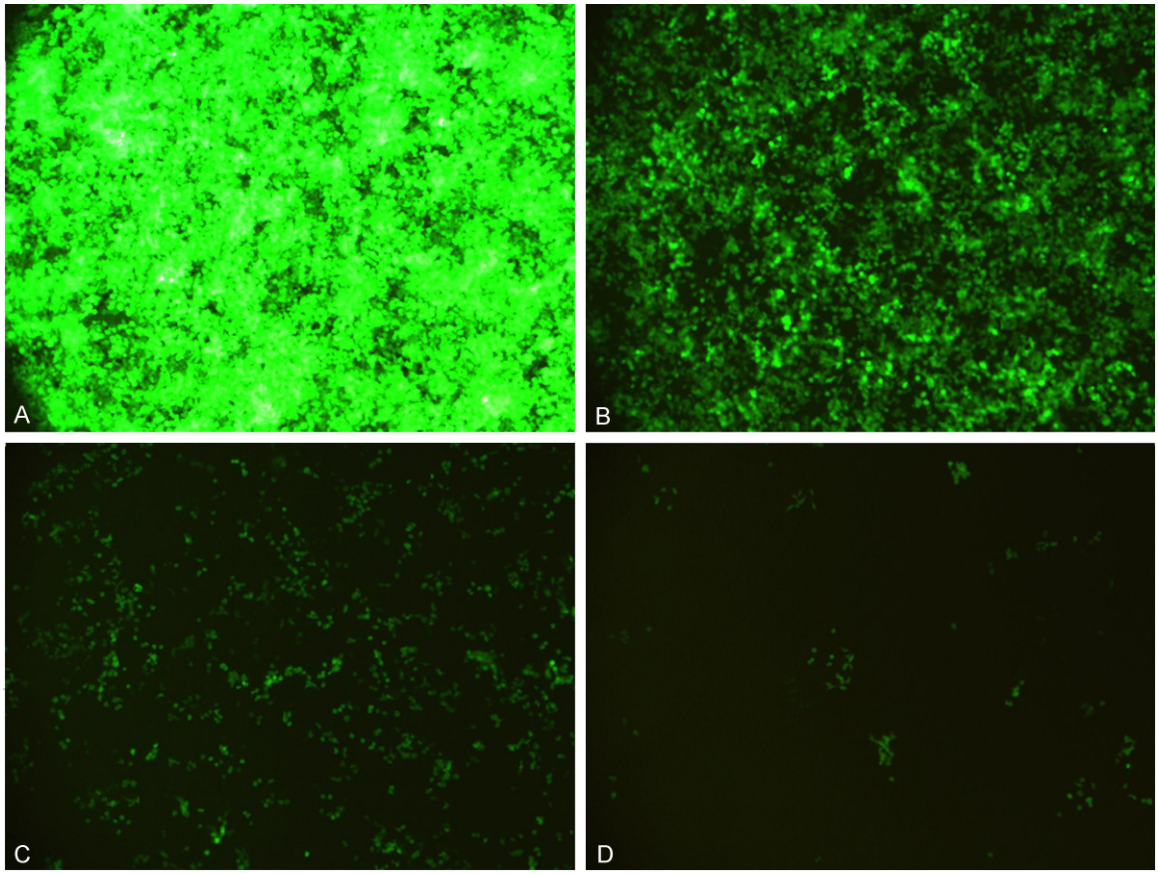

Figure 1. The titer of lentiviral AQP1-RNAi-LV. A. 1E-0 uL 100×g; B. 1E-1 uL 100×g; C. 1E-2 uL 100×g; D. 1E-3 uL 100×g.

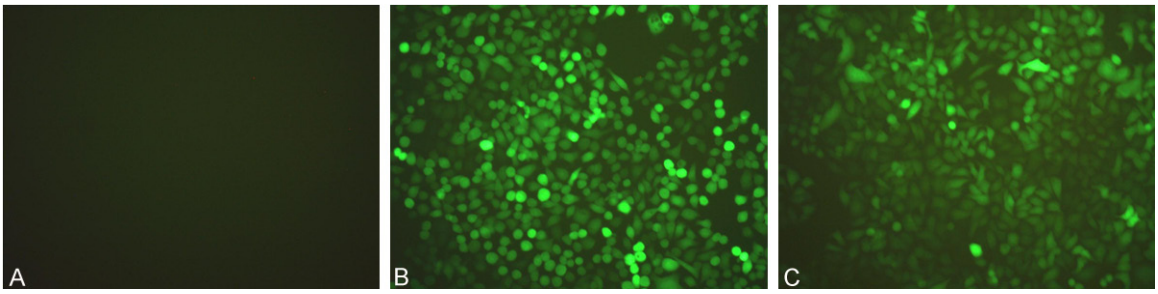

Figure 2. Fluorescence of SW480 cells transfected with lentiviral AQP1-RNAi-LV. A. con 200×g; B. kd 200×g; C. nc 200×g.

amplified fragment was 202 bp. All PCR products sample was analyzed by electrophoresis in 8% polyacrylamide gel and the results was processed by gel image analysis system.

Statistical analysis

The relative gene expression analysis was calculated by using aΔΔCt method for experiments with both biological and technical replicates. First Ct values of technical replicates were averaged (step 1). Then, ΔCt was calculated by normalizing to Ct (target gene) to (Reference gene). Next average ΔCt values were calculated for the treatment control (step 3). ΔΔCt was then calculated by normalizing ΔCt from step 2 to average ΔCt in step 3. Relative gene expression was calculated (step 5) as 2-ΔΔCt (Livak and Schmittgen, 2001) before averaging and calculating the standard deviation (step 6).

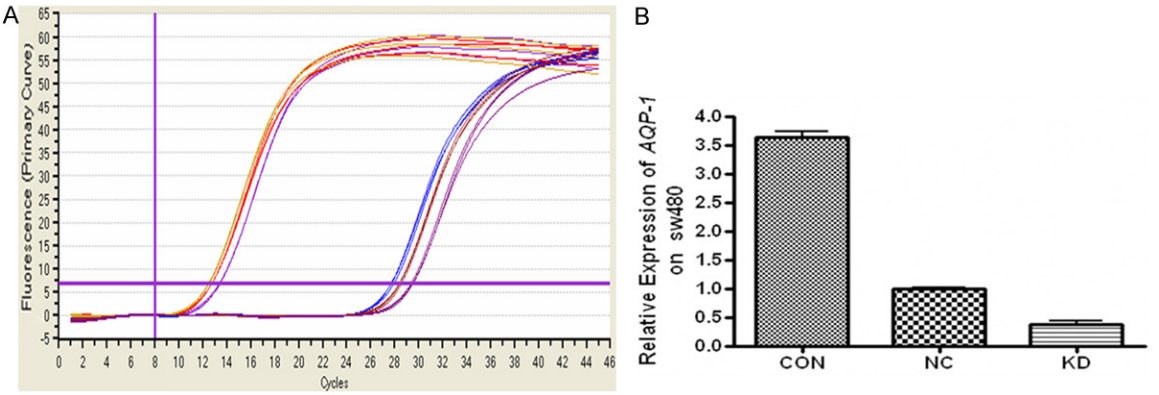

Figure 3. Expression of AQP1 mRNA by RT-PCR. A. Fluorescence (primary curve); B. Relative expression of AQP-1.

Table 2. Effects of AQP1 siRNA on tumor proliferation by the MTT assay (OD490 value)

OD490 value	OD490	CON	NC	KD
Average value	day 1	0.211±0.021	0.196±0.022	0.214±0.042
	day 2	0.448±0.011	0.279±0.025	0.233±0.021
	day 3	0.987±0.023	0.339±0.031	0.249±0.024
	day 4	1.581±0.087	0.387±0.026	0.152±0.002
	day 5	2.496±0.104	0.624±0.059	0.338±0.009

Table 3. Quantitative results, expression of AQP1 mRNA by RT-PCR

	Quantitative results				
	Actin Ct	AQP1 Ct	ΔCt	-ΔΔCt	2-ΔΔCt
CON	13.55	27.46	13.91	1.61	3.053
CON	13.51	27.55	14.04	1.48	2.789
CON	13.42	27.78	14.36	1.16	2.235
NC	12.65	28.21	15.56	-0.04	0.973
NC	12.66	28.22	15.56	-0.04	0.973
NC	12.71	28.15	15.44	0.08	1.057
KD	12.43	29.32	16.89	-1.37	0.387
KD	12.42	29.26	16.84	-1.32	0.401
KD	12.42	29.28	16.86	-1.34	0.395

All the values were expressed as mean ± SD (standard deviation). Statistical analysis was performed using SPSS12.0, *P* values less than 0.05 were considered statistically significant.

Results

Lentiviral vector construction and transfection

The lentiviral vector pEGFP-N1-3FLAG enhanced green fluorescence protein (GFP) was purchased from Shanghai GeneCham Co., Ltd (China). Four target sites within AQP1 gene were chosen from the human AQP1 mRNA sequence (GenBank, accession NM198098.1). Each target site was searched with NCBI Blast to confirm specificity only to AQP1. The four different shRNAs designated was constructed by Shanghai Gene Cham Co., Ltd (China). The sequences were listed in **Table 1**. After transfected with lentiviral vector of T293 cells, the titer of lentivirus was 2×10^9 TU/ml. The transfected efficiency was above 80% (**Figures 1** and **2**).

Inhibition effect of AQP1 gene expression by siRNA

RT-PCR and Western blot were performed to assess the knockdown efficiency after transfection. Transfection with the AQP1 specific siRNA caused AQP1 protein and mRNA expression to decrease of the levels in the groups of SW-480 cells respectively (**Figure 3**; **Tables 3**, **4**).

The MTT assay was used to quantify the effects of siRNA targeting AQP1 on SW-480 cell growth. As shown in **Table 2**, siRNA targeting AQP1 caused a decrease in cell viability of SW-480 cells. The growth curves of three groups were drawn as **Figure 4**. In contrast, AQP1 knockdown showed distinctly decrease on the viability of SW-480 cells compared with the control (**Figure 6**; **Tables 5**, **6**).

Inhibition effect of VEGF mRNA expression by siRNA

Meanwhile, the expression of VEGF mRNA was inhibited after siRNA targeting AQP1. The result was shown in **Figure 5**.

Table 4. Paired sample test, expression of AQP1 mRNA by RT-PCR

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Pair1 NC-KD	0.60067	0.04843	0.02796	0.48636	0.72697	21.697	2	0.002
Pair2 CON-KD	2.29800	0.42029	0.24265	1.25394	3.34333	9.470	2	0.011
Pair3 CON-NC	1.69133	0.46374	0.26774	0.53933	2.84333	6.317	2	0.024

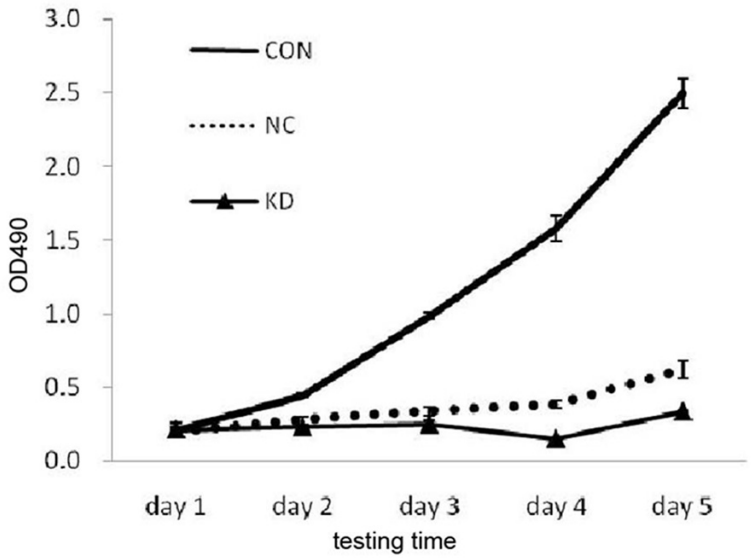

Figure 4. Effects of AQP1 siRNA on tumor proliferation by the MTT assay.

Discussion

When Agre and his colleagues were identifying human RH blood type antigen in 1988, they accidentally discovered a new type of 28 kD transmembrane protein [1]. During 1991-1992, Agre and his colleagues cloned and identified this protein molecule, and demonstrated that this protein had water transporting function. Later, the similar channels which transfer water only were discovered in animal, plant and microorganism, and the channels were named water channel protein. Cloned water channel family in mammals had 13 members (AQP0-AQP12). More and more research discovered that AQPs had intimate connection with many diseases [2, 3].

During carcinogenesis and its progression, metabolism is notably active. Hence, water channel plays an important role in the survival of tumor cells and tissue. Tumor angiogenesis has distinct relationship with tumor metastasis and relapse and it is an important indicator to assess whether the patient was recovered. At present, many researches have demonstrated there is abundant AQP1 expression in many tumor tissues, cell lines and micro-vascular endothelial cells [4]. Hence, we can imply that AQP1 has much to do with the increase of vascular permeability, it can promote the tumor angiogenesis, strengthen the invasion and metastasis ability of tumor [5-8]. In this thesis, we researched the effects of aquaporin inhibitor acetazolamide on xenograft tumor growth of colon cancer in nude mice and its mechanisms through animal experiments, aimed to supply theoretical foundation for clinic application of aquaporin inhibitor.

In Moon's research of the expression of AQP1 in mucosal epithelium, colorectal adenoma, colon cancer tissue and colon cancer cell lines, he discovered that normal colon epithelium had low-level or had no AQP1 expression, and AQP1 expression in adenocarcinoma tissue was higher than that in adenoma, and this phenomenon had no connection with pathological staging. Therefore, they stated that high expression of AQP1 is related to colorectal carcinoma development and subtype change of water channel protein [9].

Domenico Ribatti and his partners used chick chorioallantoic membrane to simulate angiogenesis. From 6-14 d during chick embryo incubation, AQP1 expression was detected by using immunohistochemistry (IHC) and Western-blot analysis. Results demonstrated that AQP1 had intimate connection with angiogenesis. Further research demonstrated that AQP1 plays an

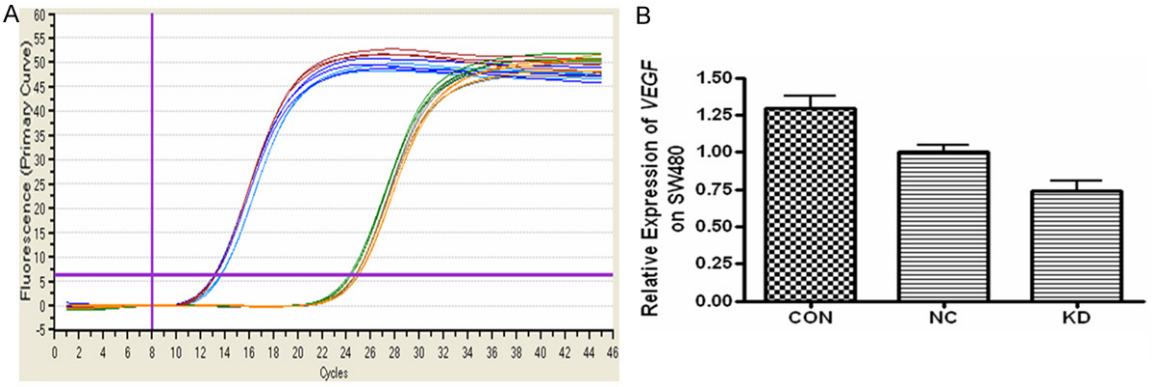

Figure 5. Expression of VEGF mRNA by RT-PCR. A. Fluorescence (primary curve); B. Relative expression of VEGF.

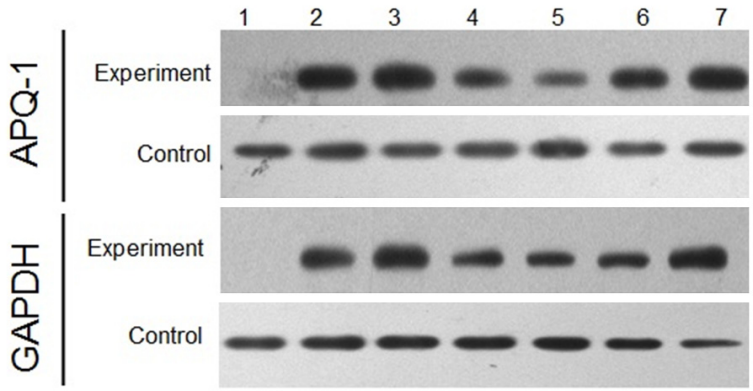

Figure 6. Western blot of AQP1 gene 1: CON; 2-3: NC; 4-7: KD.

Table 5. Quantitative results, expression of VEGF mRNA by RT-PCR

	Quantitative results				
	Actin Ct	VEGF Ct	ΔCt	-ΔΔCt	2-ΔΔCt
CON	13.57	24.84	11.27	0.163	1.120
CON	13.8	24.8	11	0.433	1.350
CON	13.8	24.74	10.94	0.493	1.407
NC	12.99	24.4	11.41	0.023	1.016
NC	13.02	24.58	11.56	-0.127	0.916
NC	13.28	24.61	11.33	0.103	1.074
KD	12.95	25.08	12.13	-0.697	0.617
KD	13.09	24.96	11.87	-0.437	0.739
KD	13.21	24.86	11.65	-0.217	0.860

important role in angiogenesis and cell migration and AQP1 was abundant in tumor tissues, cell lines and micro-vascular endothelial cells [10]. Other researches had demonstrated that tumor angiogenesis and metastasis could be reduced through inhibiting AQP1 (AQP1 protein inhibitor, RNA interference, gene knockout). Hence, we can imply that AQP1 plays an important role in the increase of tumor vascular permeability, and AQP1 can promote tumor angiogenesis, strengthen invasion and metastasis of tumor [5-8].

In a recent research, Saadoun and her partners established water channel protein (aquaporin-1, AQP1) gene knockout transgenic mice model and seeded tumor in these mice. Results were that mice tumor angiogenesis in model was distinctly lower than that in normal mice; the volume of tumor in model was smaller than that in normal mice, the progression situation and speed were also slower than control group. The results demonstrated that AQP1 may promote carcinogenesis and progression through promoting tumor angiogenesis [6]. This hypothesis had already been verified in many researches, and our experiment verified that aquaporin inhibitor acetazolamide could significantly suppress xenograft tumor growth of colon cancer in nude mice. Hence, the author perceived that AQP1 was involved in the progression of colon cancer and its function mechanisms were related to its promotion of tumor angiogenesis.

As a gene-silencing tool, RNAi represents an exciting new technology and has been used for functional analysis of genes in mammalian cells. In this study, we first established recombinant lentiviral expression vectors encoding AQP1-specific siRNA and successfully transfected them into SW480 human colon cancer

Table 6. Quantitative results, expression of VEGF mRNA by RT-PCR

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Pair1 NC-KD	0.26333	0.11894	0.06867	-0.03213	0.55879	3.835	2	0.062
Pair2 CON-KD	0.55367	0.05431	0.03135	0.41876	0.68857	17.658	2	0.003
Pair3 CON-NC	0.29033	0.16909	0.09762	-0.12970	0.71037	2.974	2	0.097

cells. Our results showed that AQP1 expression was attenuated by transfecting with AQP1 specific siRNA vectors. We successfully used vector-based RNAi to suppress AQP1 gene expression. The transfection efficiency was 80%. Our results indicated that the expression of AQP1 at mRNA and protein levels effectively, specifically, and rapidly down-regulated in SW480 cells after transfected with AQP1 specific siRNA vectors. The levels of AQP1 inhibited by pEGFP-N1-3FLAG vector were 60%. The difference between control group and experimental group was considered to be statistically significant ($P<0.05$). Compared with control group, AQP1 protein and mRNA expression were reduced significantly. Our results suggested that AQP1 gene could be silenced by RNAi post-transfection. These data also suggested that siRNA was highly specific and efficient in AQP1 gene silencing in SW480 human colon cancer cells.

We also examined the effect of AQP1 siRNA on the expression of VEGF gene at mRNA level by RT-PCR, and found that the expression of VEGF mRNA was inhibited after siRNA targeting AQP1. But we have no explanation as to why the difference of the consequence between NC and KD, CON and NC are not statistically significant. It still needs to be further studied.

In conclusion, the lentiviral vectors of shRNA for the AQP1 specifically inhibited both mRNA and protein expression in human colon carcinoma SW480 cells, which result in suppressing the growth of colon carcinoma cells. Therefore, we believe more and more drug research will choose AQP1 as target point, aimed to further enhance clinical curative effect of cancer [11, 12].

Disclosure of conflict of interest

None.

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