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Original Article

Effects of aquaporin-4 siRNA on the apoptosis of colon cancer cell line HT-29

Junlai Xue[1], Zhiwei Zhao[1], Xiangchun Xie[1], Shuhua Zhao[1], Liang Hu[2], Tongjun Liu[3]

[1]China-Japan Union Hospital of Jilin University, Changchun 130033, Jilin Province, PR China; [2]Changchun Children's Hospital, Changchun 130051, Jilin Province, PR China; [3]Jilin University, Changchun 130000, Jilin Province, PR China

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Abstract: Background: By inhibiting the expression of endogenous aquaporin-4 (AQP-4), we aimed to evaluate the effects of AQP-4 on the apoptosis of human colon cancer HT-29 cells and the expression of inhibitors of apoptosis proteins (IAPs). Methods: HT-29 cells were routinely cultured *in vitro*. The expression of endogenous AQP-4 was inhibited by siRNA technology, and the transfection efficiency of AQP-4-siRNA was detected by Western blot. The inhibition rate of cell proliferation was detected by MTT assay. The apoptotic rate was detected by flow cytometry. The expression levels of c-IAP1, c-IAP2, XIAP, NAIP, Survivin and Livin in the IAPs family in HT-29 cells transfected with AQP-4-siRNA were detected by real-time quantitative fluorescent PCR and Western blot. Results: Western blot showed that AQP-4 expression in HT-29 cells transfected with AQP-4-siRNA was down-regulated by 80%. MTT assay showed that HT-29 cells transfected with AQP-4-siRNA had significantly higher inhibition rate ((28.5±4.34)%) than that of the cells transfected with NS-siRNA ((1.24±0.14)%) (t=12.704, P<0.01). Flow cytometry showed that the apoptotic rate of the AQP-4-siRNA group ((11.73±1.25)%) significantly exceeded that of the NS-siRNA group ((0.87±0.14)%) (t=-11.837, P<0.01). Real-time quantitative fluorescent PCR and Western blot showed that compared with the NS-siRNA group, the AQP-4-siRNA had significantly lower levels of c-IAP1 (t=-3.145, P<0.01), c-IAP2 (t=-0.665, P<0.01), XIAP (t=-9.157, P<0.01) and Livin (t=-8.12, P<0.01) mRNA as well as c-IAP1 (t=-0.557, P<0.01), c-IAP2 (t=-4.778, P<0.01), XIAP (t=-6.576, P<0.01) and Livin (t=-6.243, P<0.01) protein expressions. XIAP expression decreased most obviously, while those of Survivin and NAIP hardly changed. Conclusion: AQP-4-siRNA promoted the apoptosis of HT-29 cells *in vitro*, probably by down-regulating c-IAP1, c-IAP1, XIAP and Livin mRNA and protein expressions.

Keywords: Colon cancer, aquaporin-4, apoptosis, inhibitor of apoptosis protein

Introduction

Colon cancer is a common malignant tumor worldwide, with the mortality rate ranking second [1]. Colon cancer usually has poor clinical outcomes due to the tendency to infiltration and metastasis requiring nutrients that are metabolized and exchanged in water. Meanwhile, water molecules are involved. Aquaporins (AQPs), as highly selective and specific water-transporting channels widely existing on cell membrane [2]. AQPs play important roles in tumor onset and progression, and participate in the apoptosis of tumor cells [3]. As crucial apoptotic factors, inhibitors of apoptosis proteins (IAPs) are involved in regulating the apoptosis of many types of cells. In this study, a member of the AQPs family, i.e. AQP-4, was specifically knocked down by RNA interference, the effects of which on the apoptosis of colon cancer cells and the expressions of members in the IAPs family were evaluated, aiming to provide experimental evidence for targeted treatment.

Materials and methods

Main reagents and cell line

MTT was purchased from Sigma (USA). DMEM/F12 culture medium and trypsin were bought from Gibco (USA). Real-time quantitative fluorescent RT-PCR kit was obtained from Promega (USA). AQP-4, c-IAP1, c-IAP2, XIAP, NAIP, Survivin, Livin and GAPDH antibodies were purchased from Santa Cruz (USA). PCR primers were synthesized by Sango Biotech (Shanghai)

Co., Ltd. FASCalibur flow cytometer was obtained from BD (USA). ABI 7500 PCR system was bought from ABI (USA). Differentiated human colon adenocarcinoma cells (HT-29 cells) were purchased from China Center for Type Culture Collection.

Cell culture

HT-29 cells were cultured in DMEM/F12 culture medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 g/mL streptomycin, and incubated at 37°C in 5% CO_2 atmosphere with saturated humidity. The cells were digested with 0.25% trypsin solution (containing 0.02% EDTA) solution and passaged, and those in the logarithmic growth phase were selected.

AQP-4-siRNA transfection and experimental grouping

Full length of rat AQP-4 mRNA (entry: 012825) was searched in NCBI gene database, and a short RNA chain was designed based on the coding region by using a gene silencing software. Target gene DNA AGATCAGCATCGCCAAGTC is located at 326-345 bases of AQP-4 gene sequence. Sense strand for target dsRNA: 5'-AGAUCAGCAUCGCCAAGUCUU-3', anti-sense strand: 5'-UUUCUAGUCGUAGCGGUUCAG-3'. Non-specific control siRNA (NS-siRNA): 5'-GGUCUCACUCCCCAUAGAG-3'. HT-29 cells were inoculated onto 6-well plates at the density of 4×10^5/mL 24 h before transfection. Before transfection, the cells were washed by serum- and antibiotic-free DMEM/F12 culture medium. By using liposome-mediated method, AQP-4-siRNA mixture or NS-siRNA was diluted by DMEM/F12 culture medium. Meanwhile, Lipofectamine TM-2000 was diluted at corresponding proportions. Then the two diluents were mixed as a complex to transfect HT-29 cells. Transfection efficiency and expressions of other molecules were detected 24 h after transfection. The cells were divided into a control group (normally cultured), an NS-siRNA group (transfected with NS-siRNA) and an AQP-4-siRNA group (transfected with AQP-4-siRNA).

Cell proliferation rate detected by MTT assay

HT-29 cells in the logarithmic growth phase were digested with 0.25% trypsin solution (containing 0.02% EDTA) and inoculated onto 96-well plates at the density of 5×10^4/mL. Six replicate wells were set for each group. MTT (5 mg/mL, 20 μL) was added 4 h before the experiment ended, and the culture medium was discarded after another 4 h of culture. DMSO (150 μL) was added into each well, and the resulting crystals were completed dissolved by 15 min of shaking at room temperature. Absorbance (A) at 490 nm was measured with a microplate reader. The experiment was performed in triplicate.

Inhibition rate (%) = $(1\text{-}A_{\text{experimental group}}/A_{\text{control group}})\times100\%$

Cell apoptotic rate detected by flow cytometry

Cells were digested by 0.25% trypsin solution (containing 0.02% EDTA) and centrifuged at 4°C and 1672 r/min for 5 min (r=16 cm). Precooled 70% ethanol (1 mL) was added into the precipitate, and the mixture was gently blown, fixed overnight at 4°C, and centrifuged at 4°C and 1672 r/min for 10 min (r=16 cm). After the supernatant was discarded, the residue was washed twice by 1×PBS. Then the cells were resuspended by adding 1 ml of PI staining solution and stained in dark at 4°C for 30 min before flow cytometry. The experiment was performed in triplicate.

RNA extraction and real-time quantitative fluorescent RT-PCR

RNA was extracted from cells by the one-step method according to the instruction of Trizol reagent, with the purity and concentration determined. After RNA integrity was verified by 1% agarose gel electrophoresis, 1 μg RNA of each group was used for reverse transcription and real-time quantitative fluorescent PCR according to the kits' instructions. A 20 μL PCR reaction system was prepared, comprising 1 μL of reverse transcription product as the template for real-time quantitative fluorescent PCR, 10 μL of 2×UltraSYBR Mixture, 1 μL of 10 μmol/L upstream and downstream primers, and 8 μL of DNase-RNase water. Reaction parameters for PCR: Pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, 40 cycles in total. Fluorescent signals were collected at the extension stage of each cycle. After amplification, relative expression (RQ) of target gene was calculated by using GAPDH as

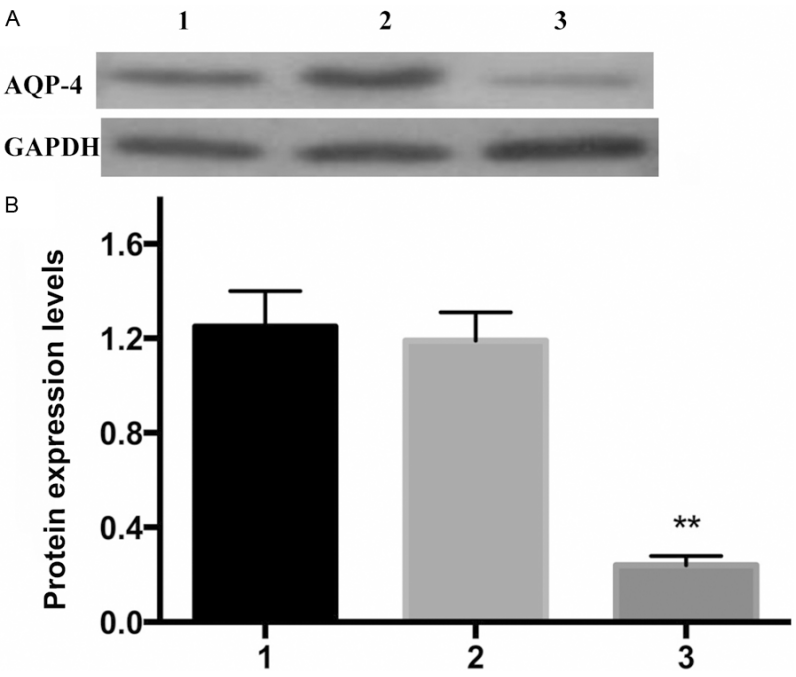

Figure 1. AQP-4 protein expressions in HT-29 cells. 1: Control group; 2: NS group; 3: AQP-4-siRNA group; A: AQP-4 expressions detected by Western blot; B: Integral scan for absorbance of bands in A, $A_{AQP-4\ protein}/A_{GAPDH}$; vs. NS group, **P<0.01.

Table 1. Inhibition rates of HT-29 cell proliferation 24 h after transfection (%, $\bar{x}\pm s$)

Group	Inhibition rate
Control	1.18±0.21
NS-siRNA	1.24±0.14
AQP-4-siRNA	28.5±4.34**

Vs. NS group, **P<0.01.

Table 2. Effects of transfection on apoptotic rates of HT-29 cells (%, $\bar{x}\pm s$)

Group	Apoptotic rate
Control	1.34±0.16
NS-siRNA	0.87±0.14
AQP-4-siRNA	11.73±1.25**

Vs. NS group, **P<0.01.

internal reference. Upstream primer for c-IAP1: 5'-GAAGACATCTCTTCATCGAGG-3', downstream primer: 5'-CCACAGGTGTATTCATCATGAC-3'; upstream primer for c-IAP2: 5'-TCCTAGCTGCAGATTCGTTC-3', downstream primer: 5'-GGTAACTGGCTTGAACTTGAC-3'; upstream primer for XIAP: 5'-GCACGAGCAGGGTTTCTTTATACTGGTG-3', downstream primer: 5'-CTTCTTCACAATACATGGCAGGGTTCCTC-3'; upstream primer for NAIP: 5'-CTGGGCCTAGATGCAGTTCAG-3', downstream primer: 5'-ACGGCTCATAAGTCACAAAAGTC-3'; upstream primer for Survivin: 5'-CTTTCTCAAGGACCACCGCA-3', downstream primer: 5'-GCCTCGGCCATCCGCT-3'; upstream primer for Livin: 5'-AGTTCCTGCTCCGGTCAAAA-3'; downstream primer: 5'-GCTGCGTCTTCCGGTTCTT-3'; upstream primer for GAPDH: 5'-TGAACGGGAAGCTCACTGG-3', downstream primer: 5'-GCTTCACCACCTTCTTGATGTC-3'.

C-IAP1, c-IAP2, XIAP and livin expressions detected by western blot

Cells were washed three times with pre-cooled 1× PBS, resuspended in lysates on ice, vortexed for 30 min, and centrifuged at 4°C and 8000 r/min for 10 min (r=16 cm), from which the supernatant was collected into a new EP tube. Proteins were then quantified by the modified Lowry method. Same quantities of total proteins from each group were subjected to SDS-polyacrylamide gel electrophoresis, electronically transferred to a PVDF membrane, blocked in 5% skimmed milk at room temperature, incubated with diluted primary antibodies for target proteins at 4°C overnight, rinsed three times by shaking with TTBS at room temperature, incubated with corresponding secondary antibodies for 2 h, and color-developed by the chemiluminescence method. Integral scan for the absorbance of each band was finally carried out. GAPDH was used as internal reference. The absorbance of each protein was thereafter compared with that of the internal reference.

Statistical analysis

All data were analyzed by SPSS11.5. The experimental data conforming to the normal distribution with variance homogeneity were expressed as $\bar{x}\pm s$. Means of different groups were compared by one way ANOVA. Inter-group comparisons were performed by the Dunnett's test,

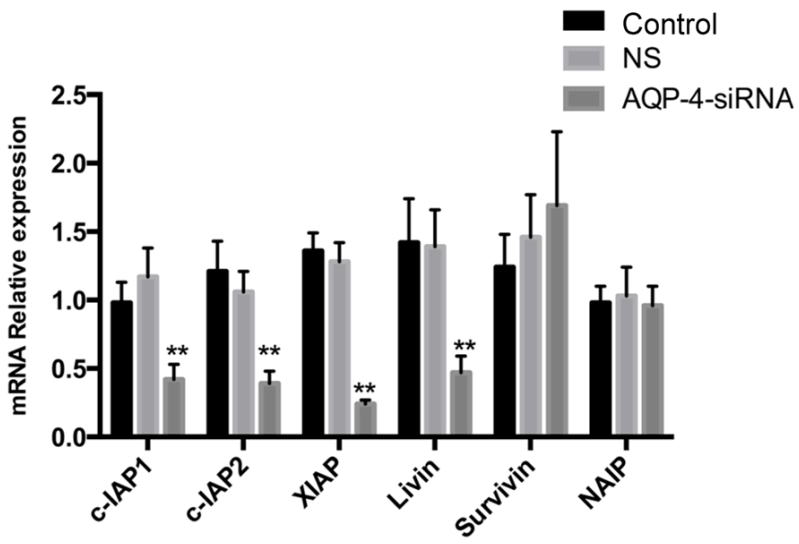

Figure 2. Effects of AQP-4-siRNA on mRNA expressions of c-IAP1, c-IAP2, XIAP, Livin, Survivin and NAIP in the IAPs family vs. NS group, **P<0.01.

and the Dunnett's T3 method was employed in the case of variance heterogeneity. Comparison of two independent samples was conducted by t test. α=0.05 was considered statistically significant.

Results

Effects of AQP-4-siRNA on AQP-4 expression in HT-29 cells

HT-29 cells were transfected with AQP-4-siRNA and NS-siRNA as the negative control. **Figure 1** shows that AQP-4 expressions are significantly different in the three groups (F=45.054, P<0.01). AQP-4 was highly expressed in the NS-siRNA group, whereas that in the AQP-4-siRNA group was significantly lower (decreased by 80%, t=9.416, P<0.01), suggesting that AQP-4-siRNA effectively inhibited AQP-4 expression in HT-29 cells. Such expressions in the NS-siRNA and control groups were similar (t=-1.157, P=0.302).

Effects of AQP-4-siRNA on HT-29 cell proliferation

Twenty-four hours after transfection, MTT assay (**Table 1**) showed that the proliferation inhibition rate of the AQP-4-siRNA group ((28.5±4.34)%) significantly exceeded that of the NS-siRNA group ((1.24±0.14)%) (t=12.704, P<0.01), indicating that inhibition of AQP-4 expression was able to suppress the proliferation of HT-29 cells. The NS-siRNA and control ((1.18±0.21)%) groups had similar inhibition rates (t=-0.720, P=0.475).

Effects of AQP-4-siRNA on HT-29 cell apoptosis

Flow cytometry (**Table 2**) showed that the three groups had significantly different cell apoptotic rates (F=147.312, P<0.01). The apoptotic rate of the AQP-4-siRNA group ((11.73±1.25)%) was significantly higher than that of the NS-siRNA group ((0.87±0.14)%) (t=-11.837, P<0.01), suggesting that inhibition of AQP-4 expression promoted HT-29 cell apoptosis. The apoptotic rates of the AQP-4-siRNA and control ((1.34±0.16)%) groups were also significantly different (t=-12.532, P<0.01), but the NS-siRNA and control groups had similar outcomes (t=-1.073, P=0.183).

Effects of AQP-4-siRNA on the expressions of members in the IAPs family

The results of real-time quantitative fluorescent RT-PCR and Western blot are exhibited in **Figures 2** and **3** respectively. Compared with the NS-siRNA group, the mRNA expression levels of members in the IAPs family, i.e. c-IAP1, c-IAP2, XIAP and Livin, significantly decreased in the AQP-4-siRNA group (t=-3.145, -0.665, -9.157 and -8.12, P<0.01). The protein expression levels also significantly dropped (t=-0.557, -4.778, -6.576 and -6.243, P<0.01). The expressions of Survivin and NAIP barely changed. Such members in the NS-siRNA and control groups had similar expression levels.

Discussion

IAPs, which are endogenous, lead to tumors by inducing insufficient cell apoptosis when overexpressed in human body [4]. It is feasible to treat these diseases by altering IAPs expressions that affect cell apoptosis.

As membrane transport proteins with the molecular weights of approximately 30 kD, AQPs can specifically transport water mole-

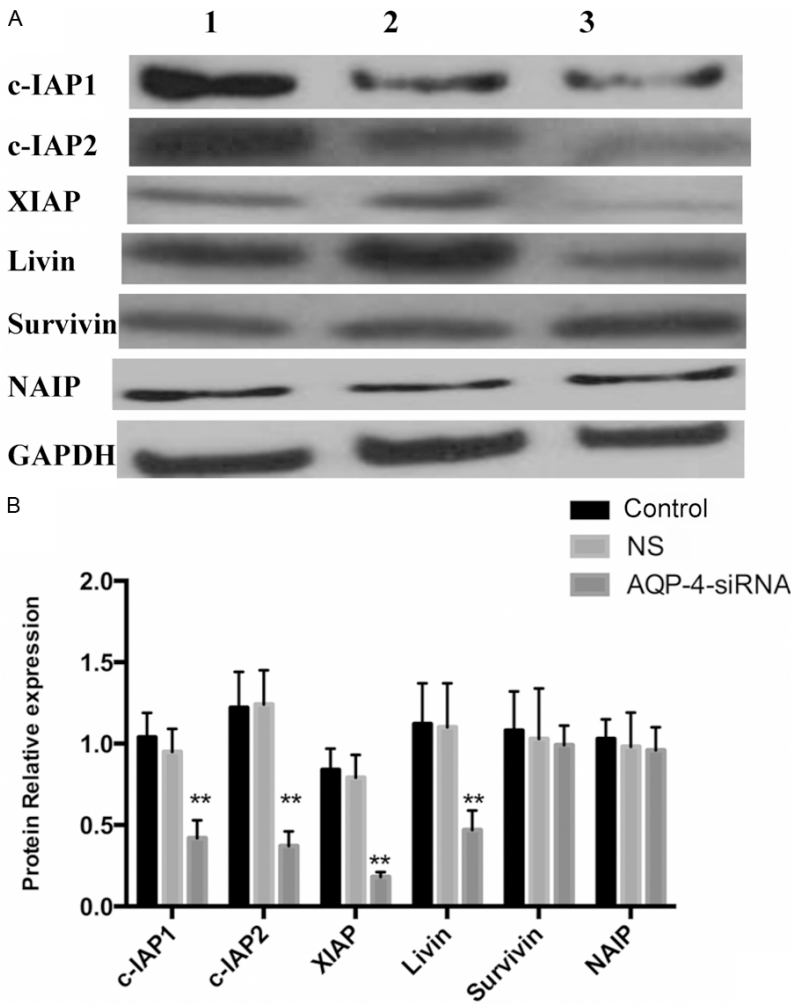

Figure 3. Effects of AQP-4-siRNA on protein expressions of c-IAP1, c-IAP2, XIAP, Livin, Survivin and NAIP in the IAPs family. A: Protein expressions, 1: Control group, 2: NS group, 3: AQP-4-siRNA group; B: Histogram of protein expressions, vs. NS group, **P<0.01.

cules [5]. Excessive proliferation of tumor cells is bound to enhance metabolism to which water is indispensable. Given abnormally increased expressions in many tumors, AQPs have attracted particular attention regarding the influence on tumor cells and the underlying mechanisms [6]. Moon et al. [7] detected the expressions of AQP-1, AQP-3 and AQP-5 in epithelia from normal colorectal mucosa, colorectal adenomas, as well as colorectal tissues and cell lines. These proteins were lowly or not expressed in normal colorectal epithelia, and the expressions in cancer tissues surpassed those in adenomas, being related with pathological staging. They also reported that overexpressions of AQPS participated in the onset and progression of colorectal cancer, probably playing key roles in tumor invasion. Therefore, water channels were indeed involved in colorectal cancer. AQP-4 expression is evidently increased in tumor tissues, especially in those with lymphatic metastasis [8]. AQP-4 has recently been found [9] to be overexpressed in glioma, being closely associated with tumor metastasis and angiogenesis. In this study, the effects of AQP-4 expression on the apoptosis of colon cancer cells and members in the IAPs family were preliminarily evaluated.

Being efficient, lowly toxic and highly specific, RNA interference technology cleaves double-stranded RNA into 19~25 nt siRNA fragments through nuclease, forming silencing complexes that recognize and degrade mRNA sharing homologous sequences. As a result, gene expressions are specifically silenced [10]. This technology has been widely applied in genotherapy [11, 12]. We herein blocked endogenous AQP-4 expression by RNA interference to observe the influence on the apoptosis of differentiated human colon adenocarcinoma cells (HT-29 cells). AQP-4-siRNA managed to inhibit as high as 80% of AQP-4 expression.

Thus, AQP-4-siRNA also suppressed the proliferation of HT-29 cells and promoted their apoptosis. Apoptosis is co-regulated by pro- and anti-apoptotic proteins that are balanced under normal conditions. If the expressions of pro-apoptotic proteins increase and (or) those of anti-apoptotic proteins decrease, cells undergo apoptosis that is however inhibited in the opposite case. Besides, we herein explored the effects of inhibiting endogenous AQP-4 expression on those of members in the IAPs family. C-IAP1 and c-IAP2 can directly inhibit the activi-

ties of Caspase-3, Caspase-7 and Caspase-9 [13]. XIAP, which is stable and efficient, is the most effective Caspase inhibitor hitherto by blocking apoptosis induced by many factors. NAIP in highly expressed in breast cancer patients and related with their poor prognosis [14]. As a new member of this family, Livin is highly expressed in many tumor tissues, inhibiting cell apoptosis by suppressing Caspase and by activating the TAK1/JNK1 pathway [15]. Functioning as a potent apoptosis-inhibiting factor in the IAPs family, Survivin plays essential roles in tumor onset and progression. In this study, inhibiting endogenous AQP-4 expression down-regulated the expressions of c-IAP1, c-IAP1, XIAP and Livin. Nevertheless, the expressions of Survivin and NAIP did not change obviously. Hence, we postulated that in HT-29 cells, there may be other molecules regulating the expressions of these IAPs.

In conclusion, inhibiting endogenous AQP-4 expression may induce the apoptosis of HT-29 cells by down-regulating c-IAP1, c-IAP1, XIAP and Livin mRNA and protein expressions.

Disclosure of conflict of interest

None.

Address correspondence to: Tonjun Liu, Jilin University, Changchun 130000, Jilin Province, PR China. E-mail: liutongjunju@163.com

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