Original Article
Down-regulation of AIF1 inhibits gallbladder cancer cell proliferation, invasion, and EMT by regulating the TGF-β1/P38 pathway

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Abstract: Gallbladder carcinoma (GC) is a malignant tumor that occurs in the biliary system with a high rate of malignancy. Inflammatory factors play an important role in GC occurrence and progression. AIF-1 is an inflammatory factor secreted by peripheral blood mononuclear cells (PBMCs) and macrophages. It shows a correlation with inflammatory stimuli and inflammatory factors, and is involved in the occurrence of autoimmune diseases and tumors. Its expression is upregulated in several tumor types. However, the mechanism of AIF-1 in GC has not been fully elucidated. The gallbladder cancer NOZ cells were cultured *in vitro* and randomly divided into control, si-NC, and AIF1 siRNA groups. Real-time PCR was used to detect AIF1 expression. Tetrazolium salt colorimetry (MTT) was adopted to test cell proliferation. Capase-3 activity was selected to determine cell apoptosis. Transwell chamber assay was used to assess cell invasion. Western blot was adopted to detect E-cadherin, β-catenin, transforming growth factor-β1 (TGF-β1), and p38 expressions. Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels in the supernatant were analyzed by enzyme-linked immunosorbent assay (ELISA). Transfection of AIF1 siRNA significantly downregulated AIF1 expression in NOZ cells, inhibited tumor cell proliferation, enhanced Capase-3 activity, suppressed tumor invasion, increased E-cadherin expression, reduced β-catenin expression, downregulated TNF-α and IL-6 levels, decreased TGF-β1 expression, and suppressed phosphorylation of p38 compared with the control group (P < 0.05). Downregulation of AIF1 inhibited secretion of inflammatory factors, promoted cell apoptosis, and restrained cell proliferation, invasion, and epithelial-mesenchymal transition (EMT) of gallbladder cancer cells by regulating TGF-β1/P38 pathway.

Keywords: AIF1, gallbladder cancer, proliferation, EMT, TGF-β1/P38 pathway

Introduction

Gallbladder cancer (GC) is a malignant tumor that occurs in the biliary system with high malignancy. Its incidence ranks fifth in the digestive system malignancy worldwide with increasing trend in recent years [1, 2]. Early symptoms of GC contain right upper quadrant pain, jaundice, indigestion, and may be associated with radiation pain in the right shoulder, which are similar to calculous cholecystitis, occasionally accompanied by fever and right upper quadrant mass [3, 4]. GC is featured as concealed, rapid progress, and complicated factors. Most patients are in the late stage when diagnosed, which increases the difficulty of treatment [5, 6]. With the continuous advancements in the

treatment of gallbladder cancer, including surgery, radiotherapy, chemotherapy, and interventional treatment, individualized treatment trends have emerged. However, due to the severity of the condition and individual differences, the effect is still not significant [7, 8]. Gallbladder cancer has many inducing factors, which can be accompanied by gallbladder benign diseases including gallstones. Gallbladder cancer is closely related to chronic inflammatory stimulation. Inflammatory factors play an important role in the occurrence and progression [9]. Gallbladder cancer shows poor prognosis and high mortality, which is one of the clinical medical problems [10]. So far, the pathogenesis of gallbladder cancer has not been elucidated, so it is urgent to analyze the mecha-

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTACCTGCAGTTTGCTGG	TACCCGGATAGAATGTCTGGT
AIF1	TCCTAGACTCTCCCT	TGGTGTATAGATGGTGTT

nism of gallbladder carcinogenesis and development, which is conducive to the treatment of gallbladder cancer and may improve the prognosis.

AIF-1 is a newly discovered inflammatory mediator that is secreted by PBMCs and macrophages [11, 12]. AIF-1 has a role in regulating immune response, and is strongly correlated with inflammatory stimuli and inflammatory factors. It is involved in the occurrence of several diseases such as autoimmune diseases and tumors [13, 14]. AIF-1 is showed to be upregulated in various tumors such as liver cancer and gastric cancer [15, 16]. However, the mechanism of AIF-1 in gallbladder cancer has not been fully elucidated.

Materials and methods

Main reagents and instruments

The gallbladder cancer cell line NOZ was purchased from the Health Science Research Resources Bank (HSRRB). DMEM medium, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Hyclone. Dimethyl sulfoxide (DMSO) and MTT powder were purchased from Gibco. Trypsin-EDTA was purchased from Sigma. The Capase-3 active kit was purchased from Boster. The Transwell chamber was purchased from Corning. PVDF membrane was purchased from Pall Life Sciences. EDTA was purchased from Hyclone. Western blot related chemical reagents were purchased from Beyotime. ECL reagents were purchased from Amersham Biosciences. Rabbit anti-human E-cadherin, β-catenin, TGF-β1, and p38 monoclonal antibodies, and mouse anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody were purchased from Cell Signaling. The RNA extraction kit and the reverse transcription kit were purchased from Axygen. Lipo2000 reagent was purchased from Invitrogen. TNF-α and IL-6 ELISA kits were purchased from R&D. The Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad Corporation. The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI. The SW-CJ ultra-clean workbench was purchased

from Suzhou Sutai Purification Equipment Engineering Co., Ltd.

Methods

NOZ cell culture and grouping

Gallbladder cancer cell line NOZ preserved in liquid nitrogen was resuscitated and cultured. The NOZ cells in 2nd-8th generation were used for the experiment. The cells were randomly divided into 3 groups, including a control group, a si-NC group that was transfected with AIF1 siRNA negative control, and a AIF1 siRNA group that was transfected with AIF1 siRNA.

Liposome transfection of AIF1 siRNA

AIF1 siRNA negative control and AIF1 siRNA were transfected into NOZ cells, respectively. The AIF1 siRNA negative control and the AIF1 siRNA sequences were synthesized by Genepharma. The AIF1 siRNA sequence was 5'-GUAUCUGCGAUUAUCCAAT-3'. The AIF1 siRNA negative control sequence was 5'-ACCUCGCGAGUGUGUAAUG-3'. AIF1 siRNA negative control and AIF1 siRNA liposome were separately added to 200 μl of serum-free DMEM medium and incubated at room temperature for 15 minutes. The mixed lipo2000 was separately mixed and incubated at room temperature for 30 minutes. The mixture was added to the cells in 6-well plate together with 1.6 ml of serum-free DMEM medium and cultured in a 5% CO_2 incubator at 37°C for 6 hours. The medium was replaced and the cells were further cultured for 48 hours.

Real-time PCR

Total RNA was extracted from the cells by Trizol and reverse transcribed to cDNA. The primers were designed by PrimerPremier software and synthetized by Invitrogen (**Table 1**). Real-time PCR was performed at 55°C for 1 minute, followed by 35 cycles of 90°C for 25 seconds, 56°C for 40 seconds, and 72°C for 20 seconds. GAPDH was selected as internal reference. The relative expression of mRNA was calculated by $2^{-\Delta Ct}$ method.

MTT assay

The NOZ cells in logarithmic phase were seeded in 96-well plate at 3000 cells/well and added with 20 μl MTT for 4 hours. Then, the plate was added with 150 μl DMSO for 10 min-
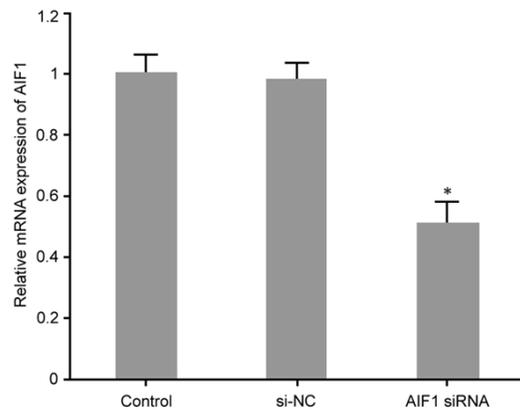
Figure 1. Impact of regulating AIF1 on AIF1 expression in NOZ cells. *P < 0.05, compared with control.

utes and tested at 570 nm to obtain the absorbance value (A).

Caspase 3 activity detection

Caspase 3 activity in each group of cells was examined by the kit according to the kit instructions. The cells were trypsinized and centrifuged at 4°C and 600 g for 5 minutes. Next, the cells were added with lysate on ice for 15 minutes and centrifuged at 4°C and 20000 g for 5 minutes. Then the cells were treated with 2 mM Ac-DEVD-pNA and the optical density (OD) value was detected at 405 nm wavelength.

Transwell assay

According to the instruction, the serum-free DMEM medium was replaced. After 24 hours, the Transwell chamber was coated with the 50 mg/L Matrigel diluted at 1:5 and air-dried at 4°C. 100 μl of tumor cell suspension prepared by serum-free medium and 10% FBS DMEM medium were added to the inner and outer and inner of chamber with 3 replicate wells in each group. Then the chamber was placed in a 24-well plate. After 48 hours of culture, the Transwell chamber was washed with PBS, and the cells above the membrane were removed. Next, the membrane was fixed in ice ethanol. After staining with crystal violet, the cells in the lower layer of the microporous membrane were counted. The experiment was repeated three times.

Elisa

ELISA was used to test TNF-α and IL-6 contents in the serum. A total of 50 μl diluted standard substance were added to each well to establish standard curve. Next, the plate was added with 50 μl sample and washed for five times. Then the plate was incubated in 50 μl conjugate reagent at 37°C for 30 minutes. At last, the plate was tested at 450 nm to obtain the OD value. The OD value of standard substance was used to prepare the linear regression equation, which was adopted to calculate the concentration of samples.

Western blot

The cells were lysed on ice and quantified by BCA method. The isolated proteins were electrophoresed using 10% SDS-PAGE. The gel was transferred to PVDF membrane by semi-dry transfer method at 100 mA for 1.5 hours. After blocked by 5% skim milk for 2 hours, the membrane was incubated in p-p38, p38, E-cadherin, β-catenin, and TGFβ1 (1:1000, 1:1500, 1:1000, 1:2000, and 1:1000) primary antibodies (Cell signaling Technology, Danvers, MA, USA) at 4°C overnight. After incubated in secondary antibody (1:2000) avoid of light for 30 minutes, the membrane was imaged using chemiluminescence reagent for 1 minute and analyzed by image processing system software and Quantity one software. The experiment was repeated for four times (n=4).

Statistical analysis

All data analyses were performed on SPSS 16.0 software. The measurement data were presented as mean ± standard deviation and compared by Dunnett's method or one-way ANOVA. P < 0.05 was considered as statistical difference.

Results

The impact of regulating AIF1 on AIF1 expression in NOZ cells

The expression of AIF1 affected by transfection of AIF1 siRNA into NOZ cells was tested by Real-time PCR. AIF1 siRNA significantly inhibited AIF1 mRNA expression in gallbladder cancer cells (P < 0.05). In contrast, transfection of AIF1 siRNA negative control did not affect AIF1 mRNA expression (P > 0.05) (**Figure 1**).
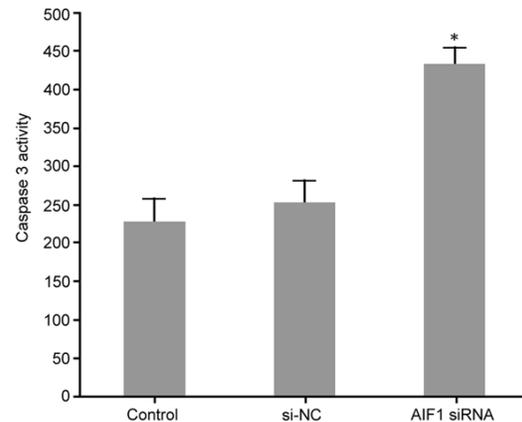
The influence of regulating AIF1 on NOZ cell proliferation

The effect of AIF1 siRNA on NOZ cell proliferation was detected by MTT assay. Downregulation of AIF1 expression suppressed NOZ cell prolif-
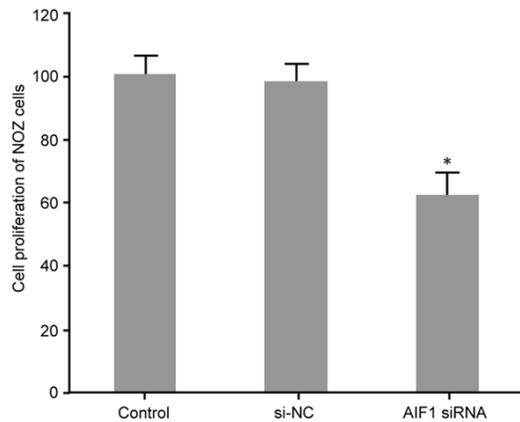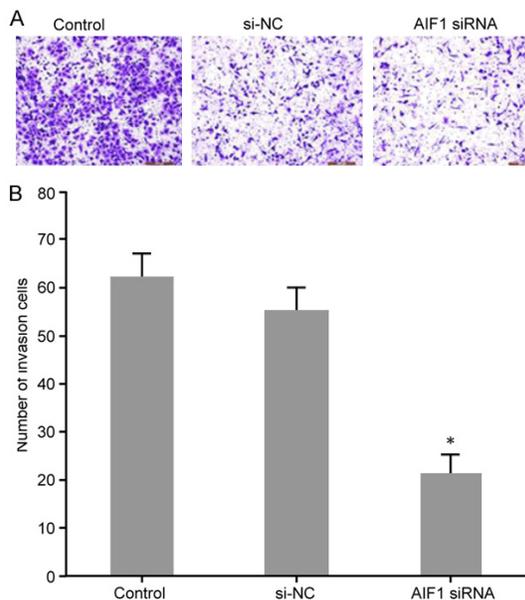
Figure 2. Influence of regulating AIF1 on NOZ cell proliferation. *P < 0.05, compared with control.

Figure 4. Impact of regulating AIF1 on Caspase 3 activity of NOZ cells. *P < 0.05, compared with control.

Figure 3. Effect of regulating AIF1 on NOZ cell invasion. A. Transwell assay detection of AIF1 on NOZ cell invasion. B. Cell invasion analysis. *P < 0.05, compared with control.

eration compared with the control group (P < 0.05), while transfection of AIF1 siRNA negative control failed to affect cell proliferation (P > 0.05) (**Figure 2**).

The effect of regulating AIF1 on NOZ cell invasion

Transwell chamber was adopted to detect the effect of AIF1 siRNA on NOZ cell invasion. Transfection of AIF1 siRNA markedly attenuated NOZ cell invasion (P < 0.05) (**Figure 3**).

The impact of regulating AIF1 on the Caspase 3 activity of NOZ cells

The effect of AIF1 siRNA on Caspase 3 activity in gallbladder cancer cell line NOZ was analyzed. Downregulation of AIF1 expression increased Caspase 3 activity in NOZ cells (P < 0.05) (**Figure 4**).

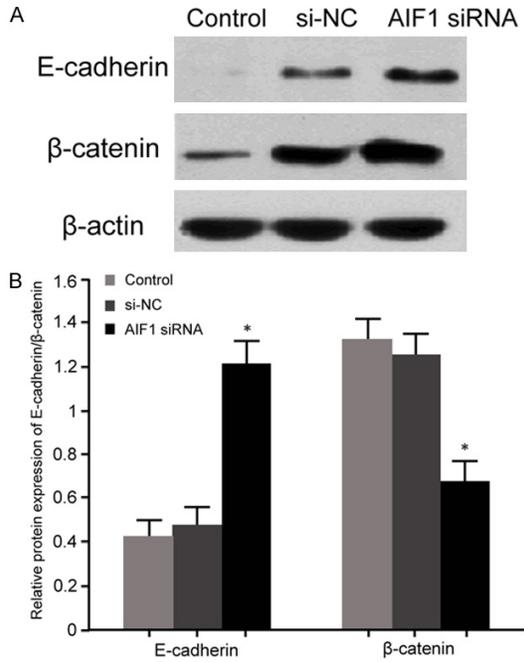*The influence of regulating AIF1 on E-cadherin/β-catenin*

The effect of AIF1 on E-cadherin/β-catenin in NOZ cells was assessed by Western blot. AIF1 siRNA transfection upregulated E-cadherin and decreased β-catenin levels compared with the control group (P < 0.05) (**Figure 5**).

The effect of regulating AIF1 on TNF-α and IL-6 in the supernatant of NOZ cells
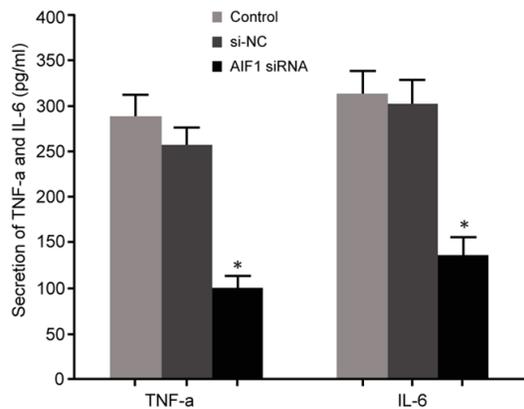
The effect of AIF1 on the expressions of TNF-α and IL-6 in the supernatant of NOZ cells was analyzed by ELISA. AIF1 siRNA transfection significantly inhibited expression of TNF-α and IL-6 in NOZ cells (P < 0.05) (**Figure 6**).

The impact of regulating AIF1 on TGF-β1/p38 signaling pathway

Western blot was used to detect the effect of AIF1 on TGF-β1/P38 signaling pathway in NOZ cells. AIF1 siRNA transfection restrained TGF-β1 expression and suppressed P38 phosphorylation compared with the control group (P < 0.05) (**Figure 7**).
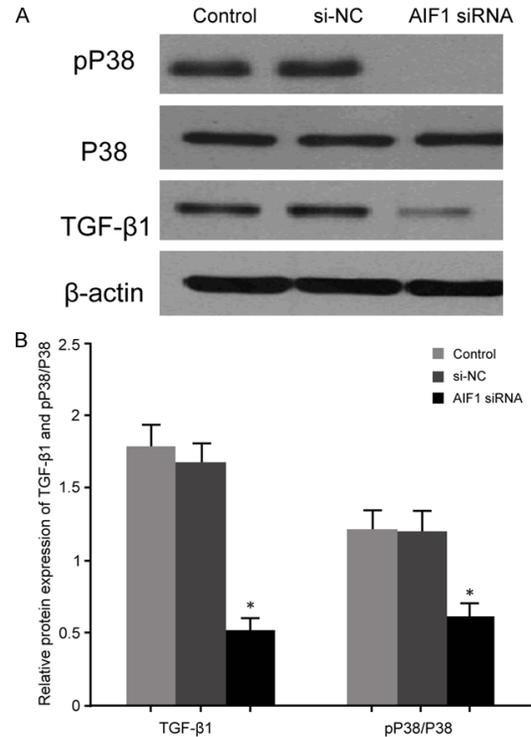
A

B

Figure 5. Influence of regulating AIF1 on E-cadherin/β-catenin. A. Western blot detection of E-cadherin/β-catenin expression; B. E-cadherin/β-catenin expression analysis. *P < 0.05, compared with control.

A

B

Figure 7. Impact of regulating AIF1 on TGF-β1/p38 signaling pathway. A. Western blot detection of TGF-β1/p38 signaling pathway expression; B. TGF-β1/p38 signaling pathway expression analysis. *P < 0.05, compared with control.

Figure 6. Effect of regulating AIF1 on TNF-α and IL-6 in the supernatant of NOZ cells. *P < 0.05, compared with control.

Discussion

Inflammation plays an important role in the occurrence and development of tumor. The inflammatory factors secreted by immune cells are involved in the formation of tumor microenvironment, and are widely associated with tumor progression, metastasis and immune escape [17]. Increased expression of AIF1 in gliomas and mammary ductal epithelial cell carci-

noma promotes tumor cell proliferation and migration [14-16]. Multi-factor and multi-gene regulatory factors are involved in the occurrence and development of gallbladder carcinoma. The metastasis of gallbladder cancer is the focus of refractory tumors. Therefore, finding the relevant pathogenesis and molecular targets of gallbladder cancer can provide effective treatment [18]. This study demonstrated that downregulation of AIF1 expression in gallbladder cancer cells inhibited cell proliferation, increased Caspase 3 activity, enhanced cell apoptosis, and restrained cell invasion. TNF-α and IL-6 are important inflammatory factors involved in tumorigenesis [19]. Downregulation of AIF1 in gallbladder cancer cells inhibited the expressions of TNF-α and IL-6, suggesting that AIF1 participates in the regulation of inflammation and thus inhibits tumor progression. EMT refers to the transformation of epithelial cells into a mesenchymal phenotype through a specific procedure, which plays a crucial role in tumor invasion and metastasis. EMT is accompanied by reduced expression of cell adhesion

molecules (such as E-cadherin) [20]. The adhesion factor β-catenin is involved in the regulation of tumor cell proliferation, differentiation, and apoptosis [21]. Down-regulation of AIF1 promoted expression of E-cadherin and decreased expression of β-catenin in gallbladder cancer cells, suggesting that AIF1 may be involved in regulation of EMT in gallbladder cancer.

TGF-β1/P38 signaling pathway plays a role in inflammation, tissue repair, and embryonic development. It has been found to have important regulatory effects on cell growth, differentiation, and immune function. By activating the p38 signaling pathway, it promotes p38 phosphorylation and facilitates cell proliferation and differentiation [22, 23]. Downregulation of AIF1 can inhibit the expression of TGF-β1 and restrain the phosphorylation level of p38 signaling pathway, thereby regulating the occurrence and progression of gallbladder carcinoma. This result suggested that AIF1 can regulate inflammation through the TGF-β1/P38 signaling pathway to exert its inhibitory effect on the development of gallbladder carcinoma. The goal of this study was to explore the expression of AIF1 in clinical patients with gallbladder carcinoma in further in-depth study, thus to provide a basis for elucidating the mechanism of AIF1 in gallbladder carcinoma.

Conclusion

Downregulation of AIF1 inhibits secretion of inflammatory factors, promotes cell apoptosis, and restrains cell proliferation, invasion, and epithelial-mesenchymal transition (EMT) of gallbladder cancer cells by regulating TGF-β1/P38 pathway.

Disclosure of conflict of interest

None.

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