

Original Article

The knockdown of MTDH expression inhibits human bladder cancer proliferation and invasion through the JAK1/STAT3 signaling pathway in T24 cells

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Abstract: MTDH is overexpressed in many malignant tumors and is closely related to the occurrence and development of tumors. The purpose of this study is to explore the effects of the knockdown of the MTDH gene on the proliferation and metastasis of human bladder cancer in T24 cells. shRNA plasmids targeting MTDH were constructed and transfected into T24 cells. The effects of gene silencing were confirmed by qPCR (Quantitative real-time PCR) and Western blotting. An MTT assay was used to determine the effects of MTDH on the proliferation of the T24 cells. The cell apoptosis rate was determined using Hoechst 33342. We additionally determined the expressions of caspase-3, JAK (Janus Activated Kinase) 1, P-JAK1, STAT (Signal transducers and activators of transcription) 3, P-STAT3, and MTDH using Western blotting, and the secretions of the tumor invasion-related proteins (MMP2 and MMP9) were determined using ELISA. The results showed that MTDH RNAi was constructed and transfected into the T24 cells successfully. Compared to the control groups, the MTDH, P-JAK1, and P-STAT3 proteins were reduced significantly, but the level of caspase-3 was clearly increased in the MTDH RNAi groups. Cell apoptosis was significantly increased in the MTDH RNAi groups. The secretions of MMP2 and MMP9 were decreased, and the cells' ability to proliferate and invade decreased significantly after MTDH RNAi was transfected into the T24 cells. In conclusion, we constructed an shRNA plasmid targeting MTDH, and it was successfully transfected into T24 cells. The knockdown of MTDH expression may inhibit proliferation and invasion via the JAK1/STAT3 pathway in T24 cells. Therefore, MTDH may be a new target for the genetic treatment of human bladder cancer.

Keywords: MTDH, bladder cancer, signaling pathway, JAK1, STAT3

Introduction

The incidence of bladder cancer ranks sixth in terms of malignancy, and its occurrence and development are related to smoking, environmental pollution, and other factors [1, 2]. It is estimated that there are approximately 429,000 new cases and 165,000 deaths because of bladder cancer worldwide every year [3, 4]. It is generally divided into non muscle invasive and myometrial invasive urothelial carcinoma [5]. The current treatments mainly include surgical treatment, the intravesical instillation of chemotherapy drugs, and systemic

chemotherapy [6, 7]. However, postoperative recurrence, an increase in the degree of malignancy, and ineffective chemotherapy have been difficult problems in the clinical treatment. Therefore, it is necessary for cancer researchers to do further research on the pathogenesis of bladder cancer to determine prognostic and predictive biomarkers to better help the doctors with early diagnosis, postoperative recurrence monitoring, and the treatment of bladder cancer [8, 9].

Metadherin (MTDH), also known as astrocyte elevated gene-1 (AEG-1), is a single transmem-

brane protein [10-12]. It is increased significantly in a variety of human malignant tumors, such as colorectal carcinoma, lung cancer, pancreatic cancer, glioma, papillary thyroid cancer, and oral squamous cell carcinoma [12-18]. In addition, patients with MTDH overexpression have poor prognoses [19-21]. The overexpression of MTDH promotes the proliferation and invasion of tumor cells, but with the down-regulation of MTDH expression, the proliferation of tumor cells is inhibited [19, 22], which suggests that MTDH expression is closely related to the occurrence and development of tumor cells. The knockdown of MTDH expression can inhibit the growth of tumor cells.

MTDH can be an important target for gene therapy. However, studies on MTDH in bladder cancer are rare. We knocked down its expression using RNA interference (RNAi) technology, and we explored its inhibitory effects on proliferation and invasion and the related mechanisms in T24 cells, laying a foundation for subsequent drug and gene therapy research on bladder cancer.

Materials and methods

Cell lines, cell cultures and reagents

SV-HUC-1, EJ, T24, and BIU87 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The EJ, T24, and BIU87 cells are human urothelial cell carcinoma cells. The SV-HUC-1 cells are normal human uroepithelium cells. Trypsin and lipofectamine 2000 were obtained from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Caspase-3, JAK1, P-JAK1, STAT3, P-STAT3, β -actin, and their secondary antibodies were obtained from Boster Biological Technology, Ltd. (Wuhan, China).

SiRNA for MTDH

4 SiRNA for MTDH RNAi was synthesized by the Wuhan Cell Marker Biotechnology Co., Ltd. The sequence is as follows, p1 GTTAGCCGTAATCAACCCT, p2 GATGATGAATGGTCTGGGT, p3 CCAAGTCAAATACCAAGCA, p4 CGTGATAAGGTGCTGACTG. Negative interference plasmid sequence: p5 AATTCTAATACGACTCACTATAG.

Cell culture and transfection

The cells were cultured in DMEM supplemented with 10% FBS. They were cultured continuously

in a cell incubator at 37°C and with 5% carbon dioxide. About 2000 cells were inoculated in a six-well plate the day before the transfection, and the cells were transfected according to the instructions of Lipofectamine-2000. The cells were divided into three groups: the blank control group (the cells were transfected with empty plasmids), the negative control group (the cells were transfected with plasmids containing the negative interference sequence p5), and the MTDH RNAi group (the cells were transfected with plasmids containing four siRNA p1-p4).

Quantitative real-time PCR (qPCR)

After the T24 cells were transfected for 48 hours, the total RNA was extracted from the T24 cells using Trizol Reagent (Invitrogen, California, USA). The first strand cDNA was synthesized from 1 μ g total RNA according to the Prime Script RT kit's instructions (CoWin Biosciences, Jiangsu Province). The qPCR used SYBR Green dye (Takara, Dalian, China), and the PCR cycling parameters (40 cycles) were: denaturation (95°C, 15 s), annealing (60°C, 30 s) and extension (72°C, 30 s). The primers used in the qPCR were as follows: MTDH, 5'-CGAGGAACAGAAGAAGAAGAACC-3' (sense) and 5'-CCTGAAGAAACCTGAAGTGTAGAAT-3' (antisense). All the results were calculated using the standard $2^{-\Delta\Delta CT}$ method. The experiment was divided into three groups: the blank control group (the cells were transfected with empty plasmids), the negative control group (the cells were transfected with plasmids containing the negative interference sequence p5), and the MTDH RNAi group (the cells were transfected with plasmids containing four siRNA p1-p4).

Western blot analysis

The T24 cells were collected and divided into 3 groups: the blank control group, the negative control group, and the MTDH RNAi group. After treatment with cell lysate, we added an appropriate amount of SDS-PAGE loading buffer, heated it in boiling water for 5 min, performed electrophoresis on the gel, and transferred it to the polyvinylidene fluoride (PVDF) membrane (Emd Millipore, Bedford MA, USA) using the wet transfer method; blocked it overnight with 5% skim milk at 4°C, washed it with Tris-Buffered Saline Tween-20 (TBST), and incubated it with antibodies against MTDH (Santa Cruz Bio-

technology, Inc., Santa Cruz, CA, USA), caspase-3, JAK1, P-JAK1, STAT3, P-STAT3, and β -actin at room temperature for 1 hour. The membranes were incubated for 1 h with a secondary antibody (1:8000). After being washed with TBST, the proteins were quantified using an enhanced chemiluminescence kit.

MTT assay

The experimental groups were the same as before. An MTT assay was used to evaluate the proliferation of the different cell groups. T24 cells in the logarithmic growth phase were collected, and the cell density was adjusted after digestion and inoculation into four 96-well culture plates, which were cultured overnight. The second day, the cells were transfected. Then the plates were taken out after being transfected for 24 h, 48 h, 72 h, 96 h, and we added 20 μ L MTT (5 mg/mL, Sigma) to each well, then we incubated the wells for 4 h. Then we added 150 μ L dimethyl sulfoxide (DMSO) to each well. The absorbance of the sample was determined using a microplate spectrophotometer (Thermo) at 490 nm.

Hoechst 33342 staining assay

The experimental groups were the same as before. First, 4×10^4 /ml cells were inoculated into six-well plates and incubated overnight in an incubator. On the second day, the cells were transfected, and the supernatant was removed after they were transfected for 48 h. They were fixed with 4% paraformaldehyde for 15 mins. 1 ml Hoechst 33342 (3 μ g/ml Boster Biological Technology, Ltd, Wuhan, China) was added to the hole, avoiding light. After being shaken for 10 minutes, the apoptotic cells were observed in a fluorescence inverted microscope, and the apoptosis rate was calculated.

Enzyme linked immunosorbent assay (ELISA)

The bladder cancer cells at the logarithmic phase were inoculated into a 6-well plate. Three groups were set up in the experiment. They were the blank control, the negative control, and the MTDH RNAi group. After 48 hours of culturing, the cell supernatant was collected for quantification (according to the instructions).

Statistical analysis

SPSS software version 18.0 was used for the statistical analysis. The study data were expressed as the means \pm standard deviations. Student's *t*-tests and one-way ANOVA tests were used for the statistical analysis. When the *P* value < 0.05, the differences were considered statistically significant.

Results

Knockdown of MTDH expressions by RNAi

The levels of the MTDH mRNA were 0.110 ± 0.031 , 0.812 ± 0.09 , 0.916 ± 0.056 , and 0.836 ± 0.035 respectively in the SV-HUC-1, EJ, T24, and BIU87 cells. The mRNA of MTDH in the SV-HUC-1 cells was significantly lower than it was in the EJ, T24, and BIU87 cells ($P=0.033$, $P=0.033$, $P=0.003$, and $P=0.006$, respectively), but there were no significant statistical differences in EJ, T24, or BIU87 ($P=0.323$). Before the transfection, the highest level of MTDH mRNA was found in the T24 cell line (**Figure 1A**), and then the T24 cells were selected for the RNAi experiments. After being transfected for 48 h, compared to the control cells, the levels of MTDH mRNA were significantly down-regulated in the RNAi group (**Figure 1B**).

The effect of MACC1 knockdown on the expressions of caspase-3, P-JAK1, JAK1, P-STAT3, STAT3, and MTDH

In the RNAi group, the level of caspase-3 was markedly enhanced, but the P-JAK1, P-STAT3, and MTDH levels were markedly declined compared to the blank control and the negative control groups. However, the JAK1 and STAT3 levels did not change significantly in the RNAi group compared to the blank control group and the negative control group, (**Table 1** and **Figure 2**).

The knockdown of MTDH inhibited proliferation and induced apoptosis

After the T24 cells were transfected for 48 h, an MTT assay showed the proliferation rate of T24 in the MTDH RNAi group was significantly lower than it was in the blank control and negative control groups ($P=0.010$ and $P=0.002$, respectively), (**Figure 3**). We evaluated the apoptosis intensity using Hoechst 33342. The

MTDH in T24 cells

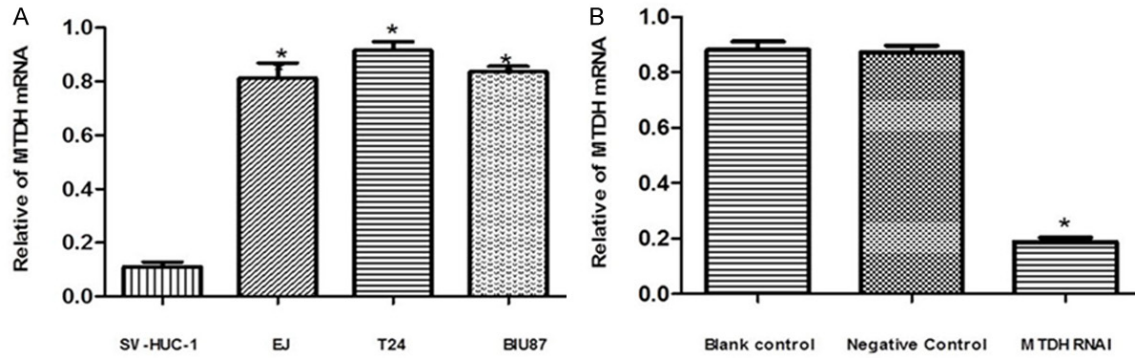


Figure 1. The down-regulation of MTDH by MTDH RNAi in T24 cells. The highest level of MTDH mRNA was found in the T24 cell line (A), the best inhibitory effects of MTDH were identified in the MTDH RNAi group cells using qPCR (B), experiments which were both performed three times independently. *P < 0.05 vs. the control groups.

Table 1. p value of caspase-3, P-JAK1, JAK1, P-STAT3, STAT3 and MTDH in the blank control, negative control, and the MTDH RNAi groups

Variable	P value		
	Blank control vs. Negative control	Blank control vs. MTDH RNAi	Negative control vs. MTDH RNAi
Caspase-3	0.555	0.004	0.011
P-JAK1	0.970	0.022	0.003
JAK1	0.760	0.161	0.436
P-STAT3	0.305	0.006	0.001
STAT3	0.152	0.604	0.097
MTDH	0.821	0.003	0.003

apoptotic cells showed cytoplasm reduction, nuclear pyknosis, and a bright blue fluorescence reaction in the blank control groups (Figure 4A), the negative control groups (Figure 4B), and the MTDH RNAi groups (Figure 4C). In the study, cell apoptosis was significantly increased in the MTDH RNAi groups, compared to the blank control and negative control groups (P=0.003 and P=0.006, respectively), (Figure 4D).

The knockdown of MTDH inhibited the secretions of MMP2 and MMP9

After the T24 cells were transfected for 48 h, ELISA showed that the secretion capacity of MMP2 in MTDH RNAi group was significantly reduced, compared to the blank control and negative control groups (P=0.032 and P=0.006, respectively), (Figure 5A). The MMP9 level in the MTDH RNAi group was significantly reduced compared to the blank control and negative control groups (P=0.029 and P=0.014, respectively), (Figure 5B).

Discussion

An earlier study found that MTDH was overexpressed in many types of human cancers and is an important oncogene [19-22]. Studies have found that MTDH may induce apoptosis and metastasis by activating different signaling pathways. In oral squamous cell carcinoma, MTDH induced the activation of the p38 and NF- κ B signaling pathways, thereby inhibiting tumor invasion and metastasis

[17]. Wang found that MTDH knockdown inhibited the activity of the AKT signaling pathway, and down-regulated MMP-2 expression, which could result in ovarian cancer cell migration and invasion [23]. Other scholars found that the knockdown of MTDH could inhibit the proliferation and induce the apoptosis of tumor cells through the MAPK pathways [24, 25]. Some researchers found that MTDH induced the epithelial- and mesenchymal transitions, leading to the development of multiple lymph node metastases in lung cancer [26].

In this study, we designed the interference sequence of MTDH, and it was successfully transfected into T24 cells. We found that their apoptosis rate was increased and their proliferation rate was decreased. The apoptosis protein (caspase-3) increased significantly after the MTDH RNAi. Therefore, it was speculated that MTDH was a tumorigenic gene, and it promotes the proliferation of bladder cancer cells.

MTDH in T24 cells

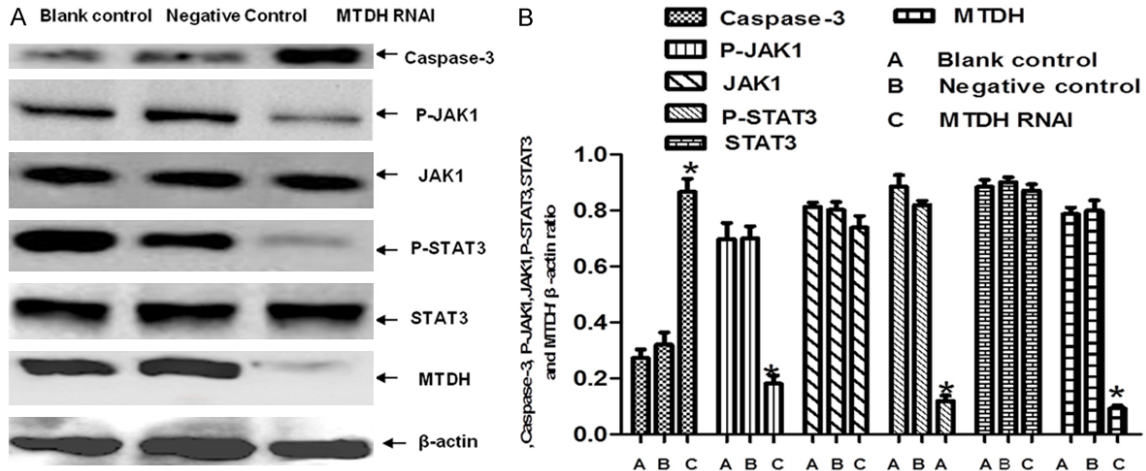


Figure 2. The expressions of the cell proteins in the different groups. The results showed that the level of caspase-3 was markedly enhanced, but the P-JAK1, P-STAT3, and MTDH levels were markedly declined in the MTDH RNAI group, compared to the other groups. Compared to other groups, JAK1 and STAT3 did not change significantly in the MTDH RNAI group. *($P < 0.05$ vs. control groups).

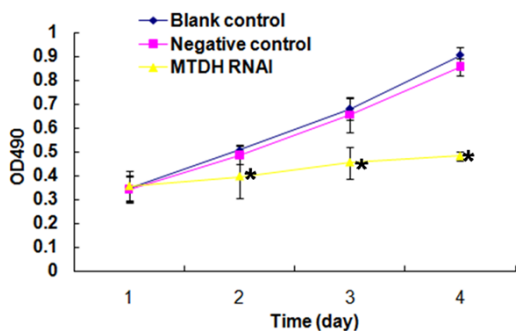


Figure 3. The effects of MTDH RNAI on proliferation in the T24 cells using the MTT. The MTDH RNAI group showed a significant decline in the cell growth rate compared to the negative control group. *($P < 0.05$).

Matrix metalloproteinases (MMPs) are tumor invasion-related proteins, and they can promote the invasion and metastasis of tumor cells by degrading the extracellular matrix [27, 28]. MMP2 and MMP9 are important members of the MMPs family. They are overexpressed in a variety of tumors, and their invasion and metastasis abilities have been improved significantly [27, 28]. In our study, MMP-2 and MMP-9 were downregulated by MTDH RNAi, indicating that the knockdown of MTDH could inhibit metastasis in T24 cells.

STAT3 is in an inactive form in the cytoplasm, and it can be activated by the phosphorylation of Janus kinase (JAK) [29, 30]. Phosphorylated

STAT3 protein can be translocated into the nucleus, bind to DNA, and regulate cell survival, angiogenesis, proliferation, and invasion [31, 32]. Previous studies found that the JAK1/STAT3 signaling pathway is involved in the proliferation and invasion of various tumor cells [33, 34]. It indicated that MTDH knockdown could inhibit proliferation through the JAK1/STAT3 pathway in T24 cells.

Our study showed that the phosphorylation levels of JAK1 and STAT3 were significantly inhibited after MTDH RNAI. However, JAK1 and STAT3 did not change significantly. Previous studies found that the knockdown of MTDH inhibits Stat3 activation in tumor cells [35]. Zhang found that STAT3 could upregulate MMP-2/9 expression and increase invasion ability in cancer cells [36].

Combined with the previous studies, this indicated that the reduced phosphorylated JAK1 and STAT3 proteins might reduce invasion and downregulate MMP-2 and MMP-9 in T24 cells. Thus, our study suggests that the knocking down of the MTDH expression inhibits human bladder cancer invasion through the JAK1/STAT3 signaling pathway in T24 cells.

Our research provided a theoretical basis for targeting MTDH in the treatment of bladder cancer. However, there are still some limits to this study. This study was only carried out with

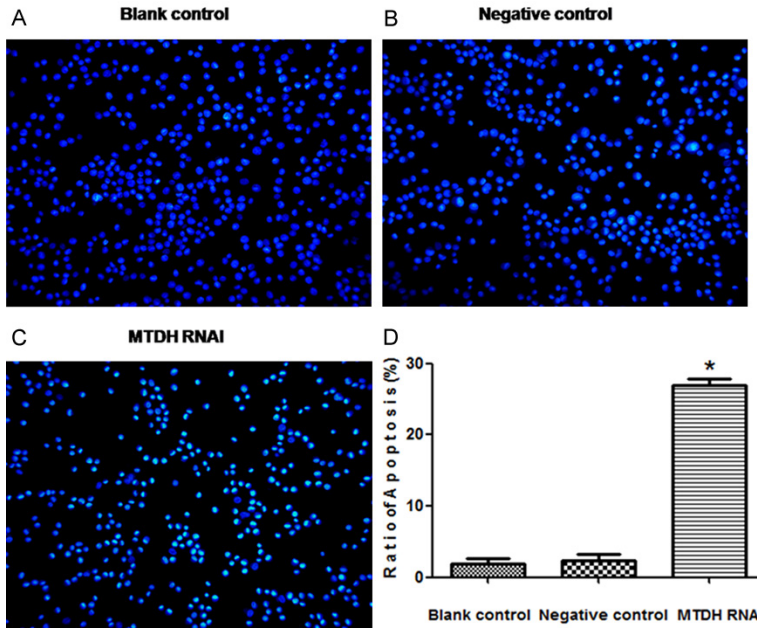


Figure 4. The effects of MTDH RNAi on apoptosis in the T24 cells. Cell apoptosis was determined using Hoechst 33342. The apoptotic cells showed cytoplasm reduction, nuclear pyknosis, and a bright blue fluorescence reaction in the blank control groups (A) (100×), negative control groups (B) (100×), and MTDH RNAi groups (C) (100×). Compared to the blank control and negative control groups, cell apoptosis was significantly increased in the MTDH RNAi group (D). (*P < 0.05).

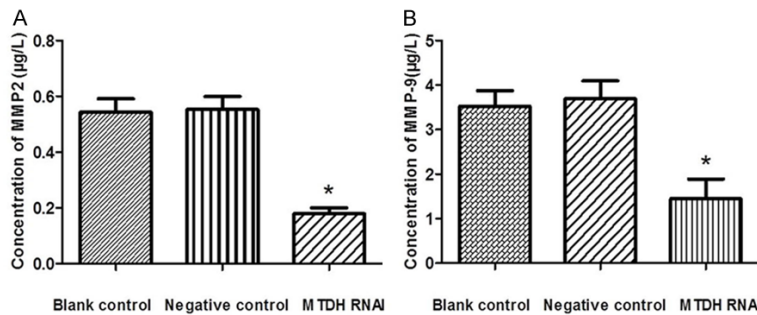


Figure 5. The effects of RNAi on the invasion protein of T24 cells. MTDH RNAi inhibited the secretion of MMP2 in the T24 cells (A). MTDH RNAi decreased the secretion of MMP9 in T24 cells (B). *(P < 0.05 vs. the control groups).

cell experiments in vitro, but not in vivo. Our findings will be further verified in vivo to determine their safety and effectiveness.

Conclusion

We constructed an shRNA plasmid targeting MTDH, and it was successfully transfected into T24 cells. The present study demonstrated that knocked down MTDH expression inhibits the proliferation and invasion of T24 cells via the JAK1/STAT3 pathway. Therefore, MTDH

may be a new target for the genetic treatment of bladder cancer.

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Disclosure of conflict of interest

None.

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