miR-24-3p regulates bladder cancer cell proliferation, migration, invasion and autophagy by targeting DEDD

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Abstract. microRNAs (miRNAs), a class of small non-coding RNA molecules, can regulate gene expression by interacting with the 3'-untranslated regions (3'UTR) of target genes and influence various biological processes. We investigated the potential role of miR-24-3p in the development of bladder cancer by regulating DEDD, a member of the death effector domain-containing protein family. First, we found that miR-24-3p was highly expressed and that DEDD was expressed at a low level in bladder cancer tissues compared with that in adjacent bladder tissues by qRT-PCR (P<0.0001). Second, we found that miR-24-3p promoted the proliferation ability of bladder cancer cells using the MTT assay and colony forming assay; and showed that miR-24-3p accelerated the migration and invasion of bladder cancer cells using migration and invasion assays (P<0.05). Moreover, miR-24-3p inhibited apoptosis of bladder cancer cells, as shown by flow cytometry (P<0.05). Western blot results demonstrated that miR-24-3p participated in autophagy of bladder cancer cells by DEDD. In addition, the tumor formation assay showed that miR-24-3p promoted the growth of bladder tumor in vivo. Furthermore, the luciferase reporter gene assay indicated that miR-24-3p suppressed DEDD gene transcription. Therefore, our study indicated that miR-24-3p promoted bladder cancer progression by inhibiting DEDD.

Introduction

Human bladder cancer is prevalent throughout the world. According to statistics, human bladder cancer is the fourth leading cause of malignancy in men and currently the tenth most malignant tumor in women (1). According to its pathological features, bladder cancer can be divided into two major groups: urothelial carcinoma (UC) and invasive bladder cancer. UC is the most widespread bladder cancer, and it frequently recurs, but rarely progresses (2,3); invasive bladder cancer easily progresses to distant metastases (4). However, because the pathophysiological mechanisms underlying bladder cancer are not clear, the appropriate treatment to increase bladder cancer survival rates is limited. Consequently, molecular mechanism studies concerning the development mechanisms of bladder cancer are urgent.

microRNAs (miRNAs) are a subgroup of endogenous non-coding small RNAs that are 19-23 nucleotides in length and are involved in post-transcriptional regulation by targeting the 3'UTR of mRNA to affect biological processes (5-8). An increasing number of studies have shown that miRNAs act as a group of regulatory genes and participate in the development and progression of various diseases, such as the progression of tumorigenesis (6,9-11). Many studies have revealed that various miRNAs, such as miR-126, miR-143, miR-145, miR-23b, and miR-144-5p, are related to the development of bladder cancer (12-16). The mechanism and function of miR-24-3p have also been studied in glioma (17), lung cancer (18), hepatocellular carcinoma (19), colorectal cancer (20), and breast cancer (21). However, the functional relevance of miR-24-3p in bladder cancer is not fully understood.

DEDD, a member of the death effector domain-containing protein family, participates in biological processes, such as cell apoptosis, cell cycle, and cell mitosis (22,23). Increasing evidence has shown that the epithelial-mesenchymal transition (EMT), a crucial development process, promotes cancer invasion and metastasis (24,25). Studies have found that DEDD serves as a novel tumor repressor and can suppress EMT and metastasis in breast and colon cancers (26,27). Therefore, DEDD may act as a prognostic marker and potential therapeutic target for the treatment of cancer metastasis. However, the mechanism and function of DEDD have not been reported in bladder cancer. To further study the anti-metastatic roles of DEDD in bladder cancer, our study investigate the expression level of DEDD in bladder cancer tissues and the interaction relationship between DEDD and miR-24-3p in bladder cancer.

Here, we confirmed that miR-24-3p was highly expressed and that DEDD was expressed at a low level in bladder cancer tissues. miR-24-3p promoted the abilities of proliferation, migration and invasion; inhibited apoptosis; participated in autophagy of bladder cancer cells by LC3, DEDD, and p62.
**in vitro**; and promoted the growth of bladder tumor in vivo. Furthermore, miR-24-3p suppressed DEDD gene transcription and accelerated proliferation and invasion through DEDD in bladder cancer cells. Therefore, miR-24-3p could be a pivotal potential therapeutic target for the treatment of bladder cancer.

### Materials and methods

#### Cell lines and transfection.
Human ureter epithelium (HCV29) cells, the human bladder cancer cell line T24, human bladder transitional cell carcinoma (UM-UC-3) cells, human bladder cancer (HBC) cells, and malignant bladder carcinoma (BLU87) cells were purchased from College of Life Science, Hunan Normal University, China. All of the cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), penicillin (100 U/ml; Invitrogen), and streptomycin (100 µg/ml; Invitrogen). All of the cultures were maintained at 37°C with 5% CO₂. For treatment, according to the manufacturer's protocol, T24 and HBC cells (2x10⁵ cells/well) were seeded in 6-well plates and were transfected with 200 µl of mature miR-24-3p mock, mimic, scramble, or inhibitor (GenePharma Co., Ltd., Shanghai, China) using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) for 72 h. Similarly, T24 and HBC cells were transfected with scramble and vector, miR-24-3p and pcMV6, and miR-24-3p and DEDD with Lipofectamine 3000, respectively.

#### Clinical specimens.
Our study obtained ethics committee approval from the First Affiliated Hospital of Henan University of Science and Technology. We collected the bladder cancer tissues and adjacent non-cancerous tissues samples from the First Affiliated Hospital of Henan University of Science and Technology between 2014 and 2016. All of the tissue samples were snap-frozen at -80°C. Each patient provided written informed consent.

#### Quantitative real-time reverse transcription PCR (qRT-PCR).
According to the manufacturer's instructions, total RNA was extracted from bladder cancer tissues, matched adjacent non-cancerous tissues and treated T24 and HBC cells using the RNaseasy Plus Mini kit. cDNAs were synthesized using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher). The samples were normalized to U6 according to their miRNA and GAPDH for mRNA. As previously described (28), the mRNA expression levels were analyzed using the SYBR-Green PCR Master Mix kit (Takara). For the detection of mRNA, the primer sequences for GAPDH were: 5'-TGG TCG TCA TGG GTG TGA AC-3' (the forward primer) and 5'-ATG GCA TGG ACT GTG GTC AT-3' (the reverse primer); the primer sequences for DEDD were: 5'-TGG TCG TCA TGG GTG TGA AC-3' (the forward primer) and 5'-CCG CAG TGT GAT GTC ACA TG-3' (the reverse primer); the primer sequences for GAPDH were: 5'-TTG GGA ATG GAT GGC TCT GT-3' (the forward primer) and 5'-ATG TCA ACC TTT GGC CCA TA-3' (the reverse primer). For the detection of miRNA, the primer sequence for miR-24-3p was 5'-CCT CCA GCC CTC TAA AAC AG-3' (the forward primer) and 5'-CCG CAG TGT GAT GTC ACA TG-3' (the reverse primer); the primer sequences for PKA1 were: 5'-TCC CCA GCC CTC TAA AAC AG-3' (the forward primer) and 5'-CCG CAG TGT GAT GTC ACA TG-3' (the reverse primer); the primer sequences for PKA1 were: 5'-TTG GGA ATG GAT GGC TCT GT-3' (the forward primer) and 5'-ATG TCA ACC TTT GGC CCA TA-3' (the reverse primer). For the detection of miRNA, the primer sequence for miR-24-3p was 5'-CCT CCA GCC CTC TAA AAC AG-3' and that for U6 was 5'-ACG CAA ATT CGT GAA GCG TT-3'.

#### Western blot analysis.
Treated T24 and HBC cells were lysed using RIPA buffer containing a protease inhibitor cocktail (P8340; Sigma). Equivalent protein samples were separated on 8% SDS-PAGE gels and were then transferred to PVDF membranes (Millipore, Billerica, MA, USA). The PVDF membranes were incubated in 5% skim milk (BD Biosciences) for 2 h with a primary antibody at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The results were obtained using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscatway, NJ, USA). In this study, the used primary antibodies were anti-LC3 (1:200; MBL International Co., Woburn, MA, USA), anti-DEDD (1:200; ab56480, Abcam), and anti-P62 (1:5000, Progen, GP62-C); the anti-GAPDH antibody (1:4000, Beverly, MA, USA) was used as an internal control.

#### Luciferase reporter assays.
Treated T24 and HBC cells (5x10⁴ cells/well) were cultured in 24-well plates and were co-transfected with miR-24-3p and the wild-type pGL3-promoter-DEDD reporter vector or mutant pGL3-promoter-DEDD reporter vector as well as a renilla plasmid (RL-SV40) using Lipofectamine 3000 (Invitrogen). The renilla plasmid was used as an internal control. According to the manufacturer's instructions, the luciferase activities were measured by the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

#### Colony forming unit (CFU) assay.
For the colony forming unit assay, T24 and HBC cells (5x10⁴ cells/well) were co-transfected with miR-24-3p mock, mimics, scramble, or inhibitors and cultured into a 10-cm culture dish with complete DMEM medium for 12 days. Next, the colonies were fixed using methanol and dyed using Giemsa dye solution. The colony forming units were observed and photographed.

#### MTT assay.
T24 and HBC cells (3x10⁵ cells/well) transfected with miR-24-3p mock, mimics, scramble, or inhibitors were seeded in 96-well plates for 0, 12, 24, 48, and 72 h. Next, 20 µl of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) solution was added into each well. After 4 h, formazan was dissolved in a dimethyl sulfoxide (DMSO) solution. A microplate reader was used to measure the absorbance at 570 nm.

#### Flow cytometric analysis of the cell apoptosis.
The treated T24 and HBC cells were resuspended in PBS and were stained with FITC-Annexin V and propidium iodide (PI). The flow cytometry results were analyzed using FlowJo software. The cells were segmented into four types, viable cells, dead cells, early-stage apoptotic cells, and late-stage apoptotic cells.

#### Migration and invasion assays.
According to the manufacturer’s instructions, the migration abilities of the treated T24 and HBC cells were carried out in 24-well Transwell chambers with 8-µm pore cell culture inserts (Costar). The treated cells (5x10⁵ cells/well) in serum-free media were seeded into the upper chamber, and DMEM medium with 10% FBS was seeded into the lower chambers for 24 h in an incubator at 37°C with 5% CO₂. Thereafter, the migratory cells were segmented into four types, viable cells, dead cells, early-stage apoptotic cells, and late-stage apoptotic cells.
were fixed, stained and counted under a microscope. For the invasion assay, 10 µl of 1:5 diluted matrigel (BD Biosciences) was pre-paved into Transwell inserts before the experiment at 37°C for 2 h.

**Tumor formation in nude mice.** The Institutional Committee for Animal Research approved the animal experiments of this study, and we performed tumor formation in nude mice according to the Institutional Animal Care and Use Committee. The flanks of 5-week-old BALB/c athymic nude mice were subcutaneously implanted with T24 cells (1x10⁷ cells in 100 µl) transfected with miR-24-3p mimics, mock, inhibitor or scramble for 0, 8, 16, 24, 32, 40, and 48 days. The tumor volume and tumor weight were measured.

**Statistical analysis.** The data were analyzed by Student’s t-test and analysis of variance (ANOVA) using Prism6 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS 15.0 software (SPSS, Chicago, IL, USA). All of the results are presented as the means ± SD. A P-value <0.05 was considered to indicate a statistically significant difference.

**Results**

**miR-24-3p is overexpressed, and DEDD expression is low in bladder cancer.** We collected bladder cancer tissues and paired non-carcinoma tissues from 26 patients. The mRNA expression levels of miR-24-3p and DEDD were measured by qRT-PCR. The results indicated that the expression level of miR-24-3p was increased in bladder cancer tissues (n=26) compared with that in para-carcinoma tissues (n=26) (P<0.0001) (Fig. 1A); the mRNA expression level of DEDD was decreased in bladder cancer tissues (n=26) compared with that in para-carcinoma tissues (n=26) (P<0.0001) (Fig. 1B). We also found that the mRNA expression level of miR-24-3p was increased in human ureter epithelium (HCV29) cells compared with that in bladder cancer cells (T24, UM-UC-3, HBC, and BLU87) (P<0.0001). The highest level of DEDD was found in T24 cells (P<0.0001) (Fig. 1C). The mRNA expression level of DEDD was decreased in human ureter epithelium (HCV29) cells compared with that in bladder cancer cells (T24, UM-UC-3, HBC, and BLU87) (P<0.0001). The lowest level of DEDD was found in T24 cells (P<0.0001) (Fig. 1D).

**miR-24-3p promotes cell proliferation and invasion in bladder cancer cells.** The impact of miR-24-3p on the proliferation and invasion abilities of bladder cancer cells was detected in T24 and HBC cells transfected with miR-24-3p mimics, mock, inhibitor or scramble. The mRNA expression levels of miR-24-3p and DEDD were measured by qRT-PCR. Our results indicated that the transfection effects of miR-24-3p mimics and inhibitor were high (P<0.05) (Fig. 2A).
miR-24-3p promotes cell migration, inhibits apoptosis, and participates in autophagy. We further studied the cell migration and apoptosis that were affected by miR-24-3p in bladder cancer cells. First, the migration ability of T24 and HBC cells transfected with miR-24-3p mimics, mock, inhibitor or scramble were measured by the migration assay. The results indicated that the migration of T24 and HBC cells transfected with miR-24-3p mimics were significantly increased compared with mock (P<0.05); the cell migration capacity of T24 and HBC cells transfected with the miR-24-3p inhibitor was significantly decreased compared with scramble (P<0.05) (Fig. 3A and B). Second, cell apoptosis was measured by Annexin V-FITC/PI staining. The results showed that apoptosis T24 and HBC cells transfected with miR-24-3p mimics was significantly decreased compared with that of the mock (P<0.05); apoptosis of T24 and HBC transfected with miR-24-3p inhibitor was significantly increased compared with that of scramble (P<0.05) (Fig. 3C and D). Furthermore, the western blot results showed that miR-24-3p upregulated the protein expression levels of LC3 and p62 and downregulated the protein expression level of DEDD in T24 and HBC cells (Fig. 3E). These results indicated that miR-24-3p participated in autophagy by repressing...
DEDD and then increasing LC3 and p62 in bladder cancer cells.

miR-24-3p suppresses DEDD gene transcription in bladder cancer. To further explore the mechanism of miR-24-3p in bladder cancer, we studied the relationship between miR-24-3p and DEDD. According to TargetScan, the miR-24-3p target sites in the sequence of DEDD were found (Fig. 4A). As shown in Fig. 4B, the target sites between miR-24-3p and the DEDD 3'UTR among human and other species were also analyzed. Therefore, we hypothesized that miR-24-3p may directly bind to DEDD. The luciferase reporter gene assay was used to measure the luciferase activity of the DEDD 3'UTR. The results showed a significant decrease in fluorescence activity after cotransfection of miR-24-3p and the wild-type DEDD 3'UTR vector (P<0.01), but a not significant change in the mutant DEDD 3'UTR vector (Fig. 4C). The qRT-PCR results revealed that miR-24-3p inhibited the mRNA expression level of DEDD T24 and HBC cells (P<0.05) (Fig. 4D).

miR-24-3p promotes the growth of bladder tumor in vivo. To assess the effect of miR-24-3p on tumorigenesis in vivo, T24 cells transfected with miR-24-3p mimics, mock, inhibitor or scramble were implanted subcutaneously into nude mice. The tumor volume was calculated, and the tumor weight was measured at 0, 8, 16, 24, 32, 40, and 48 days. The results showed that mice injected with cells transfected with miR-24-3p mimics were larger than control mice (P<0.05)
Figure 4. miR-24-3p suppresses DEDD gene transcription in bladder cancer. (A) According to the TargetScan, the miR-24-3p target site in the sequence of DEDD was predicted. (B) The target site between miR-24-3p and the DEDD 3'UTR among a variety of species was predicted. (C) The fluorescence activity of the DEDD 3'UTR was measured by the luciferase reporter gene assay in T24 cells that were cotransfected with wild-type DEDD 3'UTR and miR-24-3p or mutational type DEDD 3'UTR and miR-24-3p, respectively (**P<0.01). (D) qRT-PCR was used to determine the mRNA expression level of PAK1 in T24 and HBC cells transfected with miR-24-3p mimics, or scramble, respectively (*P<0.05).

Figure 5. miR-24-3p promotes the growth of bladder tumors in vivo. (A) The tumor volume was counted in nude mice with T24 cells transfected with miR-24-3p mimics, mock, inhibitor or scramble at 0, 8, 16, 24, 32, 40, or 48 days (*P<0.05). (B) The tumor weight was measured in nude mice with T24 cells transfected with miR-24-3p mimics, mock, inhibitor or scramble (*P<0.05). (C) miR-24-3p increased the tumor size in nude mice. Athymic mice were treated, and the tumors were removed and photographed. (D) The treated tumors in nude mice were removed, and RNA was extracted; the mRNA expression level of miR-24-3p was detected by qRT-PCR (*P<0.05).
miR-24-3p accelerates the proliferation and invasion through DEDD in bladder cancer cells. We further investigated whether miR-24-3p expression influenced the bladder cancer cell proliferation and invasion through DEDD. T24 and HBC cells were transfected with scramble and vector, miR-24-3p and pcMV6, and miR-24-3p and DEDD, respectively. The qRT-PCR results indicated that the mRNA expression level of DEDD was decreased significantly in T24 and HBC cells transfected with miR-24-3p and pcMV6 compared with scramble and vector (P<0.05); the mRNA expression level of DEDD was increased significantly in T24 and HBC cells transfected with miR-24-3p and DEDD compared with those transfected with miR-24-3p and pcMV6 (P<0.05) (Fig. 6A). The western blot results also indicated that miR-24-3p inhibited the protein expression level of DEDD (Fig. 6B).

The qRT-PCR results showed that the mRNA expression level of miR-24-3p was increased significantly in T24 and HBC cells transfected with miR-24-3p and pcMV6 compared with that in scramble and vector (P<0.05); the mRNA expression level of miR-24-3p was decreased significantly in T24 and HBC cells transfected with miR-24-3p and DEDD compared with miR-24-3p and pcMV6 (P<0.05) (Fig. 6C). The bladder cell proliferation ability was further detected by the colony forming unit assay. We found that miR-24-3p inhibited the proliferation ability of T24 and HBC cells and that DEDD promoted the proliferation ability of T24 and HBC cells mediated by miR-24-3p (P<0.05) (Fig. 6D). We also found that miR-24-3p promoted the invasion ability of T24 and HBC cells and that DEDD inhibited the invasion ability of T24 and HBC cells mediated by miR-24-3p (P<0.05) (Fig. 6E). Therefore, as shown in Fig. 6F, miR-24-3p accelerated cell proliferation, migration, invasion and autophagy and inhibited apoptosis by inhibiting DEDD and activating p62 in bladder cancer.

Discussion

miRNAs serve as a class of small non-coding RNAs that regulate mRNAs according to the 3'UTR. Several studies have demonstrated that miRNAs are involved in multiple biological functions, including cell proliferation, migration, metastasis, and inflammation, as well as tumor angiogenesis, by targeting mRNAs in cancer (29,30). Various studies have indicated that miRNAs participate in the occurrence and development of diversified diseases (6,9,11). A handful of
studies has found that various miRNAs are involved in the development of bladder cancer (31). For example, miR-126 and miR-182 are related to urinary bladder cancer (12); miR-125b inhibits the development of bladder cancer through E2F3 (32); miR-143 serves as a tumor inhibitor of bladder cancer and reverses the resistance to epidermal growth factor (EGF) receptor (33).

miR-24-3p has been studied in many cancers. For example, miR-24-3p promotes cell proliferation in glioma cells via MXI1 (17); the diagnosis and prognosis of miR-24-3p have been investigated in HBV-related hepatocellular carcinoma (19); miR-24-3p inhibits VP16-DDP resistance in small cell lung cancer via ATG4A(18). However, the functional relevance of miR-24-3p in bladder cancer is still not known. In our study, we indicated that the expression level of miR-24-3p was increased in bladder cancer tissues compared with para-carcinoma tissues. We also found that miR-24-3p promotes cell proliferation, migration and invasion and inhibited apoptosis in bladder cancer cells. In addition, we found that miR-24-3p promotes the growth of bladder tumors in vivo.

DEDD, an important effector molecule for cell death signaling receptors, plays critical roles in cell apoptosis, the cell cycle, and cell mitosis (22,23). A recent study also reported that DEDD can weaken the EMT and inhibits tumor growth and metastasis (26). Studies have found that DEDD inhibits transforming growth factor-β1 signaling (34). DEDD has also been correlated with apoptosis (35,36) and reverses EMT by activating selective autophagy (26,27). In our study, we found that miR-24-3p suppressed DEDD gene transcription. miR-24-3p accelerated proliferation and invasion through DEDD in bladder cancer cells.

Autophagy, also called type 2 non-apoptotic cell death, is a prominent mechanism of self-destruction, distinguished by a catabolic process beginning with the formation of autophagosomes and degradation of cytoplasmic material in the autophagy-lysosome by lysosomal enzymes to affect health and disease (37-40). Various studies have suggested that LC3 can serve as an autophagosome marker, and p62 has been shown to be related to autophagy (41-43). Our study showed that miR-24-3p participated in autophagy through DEDD, LC3 and p62 in bladder cancer cells.

In summary, we found that miR-24-3p was highly expressed and that DEDD was expressed at a low level in bladder cancer tissues. miR-24-3p promoted proliferation, migration and invasion and inhibited apoptosis in bladder cancer cells. miR-24-3p also participated in the autophagy of bladder cancer cells through LC3, DEDD, and p62. We found that miR-24-3p promoted the growth of bladder tumors in vivo. Furthermore, miR-24-3p suppressed DEDD gene transcription, miR-24-3p accelerated proliferation and invasion through DEDD in bladder cancer cells. Therefore, our study indicated that miR-24-3p promoted bladder cancer progression by inhibiting DEDD. Thus, miR-24-3p could be a pivotal potential therapeutic target for the treatment of bladder cancer.

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References


