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Original Article

Metadherin knockdown suppresses bladder cancer cell invasion and metastasis by inhibiting the epithelial to mesenchymal transition

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Abstract: Metadherin is over-expressed in several cancers and it is has been considered as an important oncogene. Recent studies have shown that elevated expression of metadherin is associated with poor prognosis in patients with transitional cell carcinoma, however, the potential role of metadherin in transitional cell carcinoma remains unknown. In this study, Paraffin sections of clinical bladder samples were evaluated by immunohistochemistry, Statistical analyses were applied to test for the relation between MTDH and E-cadherin, and the association of MTDH or E-cadherin with bladder cancer patients' clinicopathlogic features, respectively. After transfected with siRNAs, the expression of metadherin in T24 and 5637 cells were assessed by Real-time reverse transcription-PCR and Western blot. Moreover, the expression of Epithelial-to-mesenchymal transition (EMT)-related markers such as E-cadherin, N-cadherin, Vimentin were detected by Real-time reverse transcription-PCR and Western blot assay. Scratch wound assay and transwell matrix penetration assay were performed to determine migration and invasion of T24 and 5637 cells. We found that metadherin was over-expressed in clinical patients with transitional cell carcinoma, and the expression of metadherin and E-cadherin significantly correlated with Histopathological Grade (WHO, 2004), Clinical stage (UICC, 2002), and distant metastasis. There was a negative correlation between metadherin high expression and E-cadherin low expression in bladder cancer patients. In addition, we revealed that knock down metadherin in bladder cancer cells resulted in decreased regulation of N-cadherin, Vimentin, upregulation of E-cadherin. Furthermore, the siRNA-mediated down-regulation of metadherin resulted in decreased migration and invasion of T24 and 5637 cells. We speculate that MTDH knockdown might suppress migration and invasion in bladder cancer cells through the epithelial to mesenchymal transition.

Keywords: Metadherin, bladder cancer, invasion, metastasis, epithelial to mesenchymal transition

Introduction

Bladder cancer is the 6th most common cancer in the Western world and its incidence were still increased, Transitional cell carcinoma of bladder accounts for 90% of bladder cancer and is classified into superficial (75%) and muscle-invasive tumors (25%). Current clinical treatment strategies for transitional cell carcinoma of the bladder include radical transurethral resection (TUR) and intravesical perfusion for superficial cancers, radical cystectomy (RC) for muscle-invasive tumors, and dissection of the regional lymph nodes if the nodes show cancer positive, and followed by chemotherapy [1]. However, the recurrence rate and mortality of cancer patients is still high, even though the patients who undergoing satisfactory surgical resection and adequate chemotherapy. This cause of phenomenon is partly due to rapid metastasis after surgery. Therefore, metastasis is the most important reason for morbidity and mortality of bladder cancer. The disadvantage of current strategies for bladder cancer treatment suggests that the development of novel strategies against metastasis for this cancer therapy is urgently required.

Metadherin (MTDH, also known as AEG-1 or Lyric) was originally characterized as an HIV-1-inducible gene in primary human fetal astrocytes in 2002, or treated with tumor necrosis factor-α (TNF-α) [2]. MTDH is located in Chromosome 8q22 having 12 exons/11 introns, Genomic alterations are known to be happened in this hot spot in several cancer cells [3]. It has been found to be frequently over-expressed in primary cancers and its expression level is always associated with the progression and/or poor prognosis of various types of cancers, such as breast cancer, prostate cancer, non-small cell lung cancer, and hepatocellular carcinoma [4-7]. Several recent studies have revealed that MTDH plays a significant role in promoting proliferation, differentiation, apoptosis, invasion, migration, metastasis, and chemo-resistance [5, 8-11] via the regulation of several signaling pathways including Ha-ras, PI3K/Akt, NF-κB and Wnt/β-catenin [10, 12, 13].

In bladder cancer, previous studies have demonstrated that high expression of MTDH was found in 45% of bladder cancers and significantly associated with tumor grade and progression [14]. Over-expression of MTDH contributes to the neoplastic phenotype of bladder cancer cells by promoting survival, clonogenicity, and migration [14]. However, the expression of MTDH in bladder cancer cells and its precise role in invasion and migration is largely unknown, and there is currently no report regarding the role of MTDH in regulating EMT in bladder cancer cells.

In this study, we investigated the relation between MTDH and EMT related markers, the effect of knock down MTDH expression on regulation of N-cadherin, Vimentin and E-cadherin. The migration and invasion of T24 and 5637 cells after MTDH knockdown was investigated in follow-up experiments.

Materials and methods

Patients and tissue samples

This study was conducted on 136 paraffin-embedded primary transitional cell carcinoma of the bladder (TCC) patients who underwent transurethral resection (*n* = 99) and radical cystectomy (*n* = 37), the 136 tumor tissues and 37 adjacent normal tissues (ANT) (≥3 cm away from bladder cancer tissues) were collected from January 2000 to January 2005 at the Second Affiliated Hospital of Chongqing Medical University. Informed consent was obtained from all of the patients. The patients with bladder cancer included 90 males and 46 females, the patients' age ranged from 46 to 85 years (a median of 66.5 years). None of the patients received chemotherapy or radiation therapy before surgery. The pathological grade of patients was determined by two senior pathologists according to the criteria of the World Health Organization (WHO, 2004). The clinical stage was defined according to the Union for International Cancer Control classification (UICC, 2002). The clinic-pathologic features of these patients are shown in **Table 1**. The study was approved by the Ethics Committee of the Second affiliated Hospital of Chongqing Medical University.

Immunohistochemistry staining

The expression patterns of MTDH and E-cadherin in TCC and ANT were examined following standard immunohistochemistry protocol. Briefly, the sections were deparaffinized in xylene,rehydrated in serially graded ethanol (100, 95 and 75%) and rinsed in phosphate buffered saline (PBS). Antigen retrieval was performed by placed the sections in a boiling citric acid buffer (pH 6.0) once for 5 min, then the sections were incubated with 3% hydrogen peroxide for 20 min at room temperature to inhibit endogenous peroxidase activity, followed by incubation with 5% normal serum to block nonspecific binding. The sections were incubated with the primary antibodies: anti-MTDH (1:100; rabbit monoclonal antibody; Cell signaling Technology, USA) and E-cadherin (1:400; rabbit monoclonal antibody; Cell signaling Technology, USA) at 4°C overnight. After washing with a 0.01 mol/L concentration of PBS, the sections were treated with Biotinylated-HRP secondary antibody (1:200; Anti-rabbit IgG, Zhongshan Golden Bridge Biotechnology, China) for 30 min at room temperature, followed by further incubation with streptavidin-horseradish peroxidase complex and diaminobenzidine (DAB, Zhongshan Golden Bridge Biotechnology). Finally, the sections were counterstained with 10% Mayer's hematoxylin, dehydrated, and mounted. For negative controls, the primary antibody was replaced with PBS under the same conditions.

Evaluation of immunohistochemistrystaining

The sections were observed and scored separately by two independent pathologists, who were blinded to the clinicopathologic characteristics and patients' profile. MTDH and E-cadherin expression was determined by com-

Table 1. Correlation between clinicopathlogic feature and expression of MTDH, E-cadherin in bladder urothelial carcinoma

Characteristics		MTDH		E-cadherin	
		Positive	Negative	Abnormal	Normal
Total number	136	87	49	116	20
Age(y)		χ^2=0.577, P=0.448		χ^2=1.463, P=0.226	
< 50	58	35 (60.34)	23 (39.66)	47 (81.03)	11 (18.97)
≥ 50	78	52 (66.67)	26 (33.33)	69 (88.46)	9 (11.54)
Gender		χ^2=0.804, P=0.37		χ^2=0.001, P=0.977	
Male	82	50 (60.98)	32 (39.02)	70 (85.37)	12 (14.63)
Female	54	37 (68.52)	17 (31.48)	46 (85.19)	8 (14.81)
Tumor size (cm)		χ^2=1.433, P=0.23		χ^2=0.295, P=0.587	
≤3	74	44 (59.46)	30 (40.54)	62 (83.78)	12 (16.22)
>3	62	43 (69.35)	19 (30.65)	54 (80.10)	8 (12.90)
Tumor quantity		χ^2=0.505, P=0.477		χ^2=0.223, P=0.637	
Unifocal	75	46 (61.33)	29 (38.67)	63 (84)	12 (16)
Multifocal	61	41 (67.21)	20 (32.79)	53 (86.89)	8 (13.11)
Distant metastasis		χ^2=9.699, P=0.002		χ^2=5.025, P=0.025	
Negative	112	65 (58.04)	47 (41.96)	92 (82.14)	20 (17.86)
Positive	24	22 (91.67)	2 (8.33)	24 (100)	0 (0)
TNM (UICC) Clinical stage		χ^2=11.091, P=0.001		χ^2=9.409, P=0.002	
Ta-T1	80	42 (52.5)	38 (47.5)	62 (77.5)	(22.5)
T2-T4	56	45 (80.36)	11 (19.64)	54 (96.43)	2 (3.57)
Histopathological Grade (WHO 2004)		χ^2=14.530, P=0.002		χ^2=12.44, P=0.006	
UP	33	16 (48.48)	17 (51.52)	23 (69.70)	10 (30.30)
PUNLMP	48	32 (66.67)	16 (33.33)	40 (83.33)	8 (16.67)
LG	29	15 (51.72)	14 (48.28)	27 (93.10)	2 (6.90)
HG	26	24 (92.31)	2 (7.69)	26 (100)	0 (0)

UP urothelial papilloma, PUNLMP papillary urothelial neoplasm of low malignant potential, LG low-grade papillary urothelial carcinoma, HG highgrade papillary urothelial carcinoma.

bining the proportion of positively stained tumor cells and the intensity of staining [15]. The cell proportion was scored as follows: 0, no positive cells; 1, <10% positive cells; 2, 10-50% positive cells; and 3, >50% positive cells. The intensity of staining was graded according to the following criteria: 0 (no staining); 1 (weak staining = light yellow); 2 (moderate staining = yellow brown); and 3 (strong staining = brown). The staining index (SI) was calculated as the product of the proportion of positive cells and the staining intensity score. Using this method of assessment, we evaluated MTDH and E-cadherin expression in the sections by calculating SI scores of 0, 1, 2, 3, 4, 6 or 9. An optimal cutoff value was identified: an SI score of ≥4 was used to define tumors as high expression and an SI score of <3 were used to define low expression.

Cell lines and culture

Human bladder cancer cell lines (muscle-invasive cell lines T24 and 5637) were purchased from Shanghai Cell Bank of Chinese Academy of Sciences. Both cell lines were cultured in RPMI-1640 (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), cultured in a humidified incubator at 37°C with 5% CO_2.

Small interfering RNA (siRNA) experiments

The small interfering RNA (siRNA) for MTDH silence was synthesized by Genepharma Technology Co., Ltd. (Shanghai, China). The sequences of siRNA are as follows: siRNA1 (MTDH-744) forward: 5'-GCUGUUCGAACACCUCAAATT-3', reverse: 5'-UUUGAGGUGUUCGAACAGCTT-3'; and siRNA2 (MTDH-1432) forward: 5'-GCCGUA-

AUCAACCCUAUAUTT-3', reverse: 5'-AUAUAGGG-UUGAUUACGGCTT-3'. The experiments included two experiment groups and one negative control group and one control group, two experiment groups were transfected with MTDH-744, MTDH-1432 respectively, conventional cultured cell treated with lipofectamine RNAiMax reagent served as control group, Non-targeting siRNA was used for negative control group, its sequence is: forward: 5'-UUCUUCGAACGU-GUCACGUTT-3', reverse: 5'-ACGUGACACGUUC-GGAGAATT-3'. Four groups of cells were transfected with lipofectamine RNAiMax reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). As described previously, before transfection, 1.5×10^5 cells were plated per 6-well plate in media containing 10% FBS to reach 50% confluency, siRNA were incubated with Opti-MEM (Invitrogen) and lipofectamine RNAiMax reagent (Invitrogen) following the manufacture's protocols, and transient transfection of the siRNA was performed to result in a final siRNA concentration of 100 nM for T24 and 5637 cells. The cells were harvested for Real-Time PCR Analysis after 48 hours of transfection and for Western blot analysis after 72 hours of transfection.

Real-time PCR analysis

Total RNA from T24 and 5637 cells after transfection was extracted using the Trizol reagent (Takara Biotechnology, Dalian, China), and 1 μg of RNA from each sample was used for cDNA synthesis (PrimeScript™ RT reagent Kit with gDNA Eraser), followed by PCR amplification; Real-time Quantitative PCR was done according to the manufacturer's protocol from Takara Biotechnology (SYBR® Premix Ex Taq™ (Tli RNaseH Plus), ROX plus). The primer sequences used for Real-Time PCR were as followed (Sangon Biotech, Shanghai, China)): MTDH: forward, 5'-GGGGAAGGAGTTGGAGTGAC-3'; reverse, 5'-GTAGACTGAGAAACTGGCTCAGCAG-3'. E-cadherin: forward, 5'-TCGCTTACACCATCCT-CAGC-3'; reverse, 5'-AGGGAAACTCTCTCGGTC-CA-3'. N-cadherin: forward, 5'-TCGGGTAATCCT-CCCAAATC-3'; reverse, 5'-CCACAAACATCAGCAC-AAGG-3'. Vimentin: forward, 5'-AGAGAACTTTG-CCGTTGAAGC-3'; reverse, 5'-ACGAAGGTGAC-GAGCCATT-3'. The geometric mean of housekeeping gene GAPDH was used to normalize the variability at expression levels.

Western blot analysis

After 72 hours of transfection, cells of each group were harvested, Total proteins were extracted using the total protein extraction kit (Beyotime, Shanghai, China): the cells were washed three times with iced-cold PBS, and lysed with RIPA lysis buffer (Beyotime, Shanghai, China), then the concentration of proteins were measured with the BCA Protein Assay kit (Enhanced BCA Protein Assay Kit, Beyotime, Shanghai, China). 30 μg of protein was loaded and separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime, Shanghai, China), and transferred to PVDF membranes (Millipore, Bedford, USA). The primary antibodies included MTDH (1:10000, Abcam, USA), Epithelial-Mesenchymal Transition (EMT) Antibody Sampler Kit (Cell signaling Technology , USA), (N-Cadherin, 1:1000, E-Cadherin, 1:1000, Vimentin, 1:1000, β-actin (1:2000), HRP-linked secondary antibody, 1:2000) were used to detect the target of protein. β-actin (Cell signaling Technology, USA) was used as loading control. This program was followed by an additional 1 h of incubation with HRP-linked secondary antibodies (Cell signaling Technology, USA) in blocking buffer (1:2000), Finally, the protein bands were detected using an ECL system, (Millipore, Bedford, USA) and quantified by densitometry using Quantity One software (Bio-Rad) according to the manufacturer's instructions. The results were expressed as protein/β-actin absorbance ratio.

Scratch wound assay

T24 and 5637 cells were placed in a 6-well plate (1×10^6 cells/well), respectively. Cell lines were transiently transfected with MTDH siRNA, Scrambled siRNA was considered as negative control group, Mock transfection was considered as control group, and all the experiments were performed in triplicates. When cells were grown to 100% confluence, the wounding line of approximately 200 μm in diameter was created with a 10 μL plastic filter tip. To eliminate dislodged cells, culture medium was removed and wells were washed with PBS three times. The width of the wound was measured under a microscope at 0, 24 hours after the scratch to assess the migration ability of the cells.

Table 2. Expression of MTDH, E-cadherin in bladder urothelial carcinoma and adjacent normaltissues (%)

Group		MTDH		E-cadherin	
		r=-0.722, P=0.001			
		Positive	Negative	Abnorma	Normal
		χ^2=13.121	P < 0.001	χ^2=95.784	P < 0.001
TCC	136	87 (64.00)	49 (36.00)	116 (85.29)	20 (14.71)
ANT	37	2 (5.41)	35 (94.59)	0 (0.00)	37 (100.00)

Transwell matrix penetration assay

Invasion of T24 and 5637 cells were assayed using the Matrigel Invasion Chamber (Corning Costar, Cambridge, MA, USA). 2×10^4 cells were seed into the upper chamber coated with 50μl Matrigel (BD Matrigel TM) in a 24-well plate with serum-free medium. Medium containing 10% FBS were used as the chemo-attractant in the lower chamber. Transwell filter were incubated at 37°C for 24 h, followed by removal of noninvasive cells inside the upper chamber with a cotton swab. Invasive cells on the lower membrane surface were fixed in 70% ethanol, stained withGiemsa's azure eosin methylene blue, and counted under a light microscope at least in ten random visual fields per well.

Statistical analysis

Results were presented as means ± standard deviation (SD); all statistical analyses were carried out using SPSS 18.0 software (SPSS, Inc., Chicago, IL). Chi-square test were used to evaluate the relationship between MTDH and E-cadherin protein expression and clinicopathological parameters, Bivariate correlations between MTDH and E-cadherin expression was analyzed by Spearman's correlation coefficients. The statistical differences between treatment groups were determined using a Student t test when comparing 2 treatment groups, or a one-way ANOVA followed by Tukey's method when comparing more than 2 treatment groups. P < 0.05 was considered statistically significant and the experiments were repeated at least three times.

Results

Expression of MTDH and E-cadherin in bladder cancer tissues and adjacent normal tissues

MTDH protein was positive expressed in 64.0% (87/136) of the bladder cancer samples (**Table 2**) and mainly localized in the cytoplasm of primary cancer cells (**Figure 1A** and **1B**). With only a minority of primary cancer cells also stained in the nucleus, metastatic tumors showed a high percentage of MTDH staining in the nucleus (**Figure 1C**). Whereas it was weakly detected in other cancer samples and adjacent normal tissues (49/136, 36.0 % and 2/37, 5.41%) (**Figure 1D** and **1E**). The expression of MTDH in bladder cancer tissues and adjacent normal tissues statistically significant (χ^2=13.121, P < 0.001, **Table 2**).

In contrast, E-cadherin protein was highly expressed in adjacent normal tissues (37/37,100%, **Figure 1F**) than bladder cancer samples (**Figure 1G** and **1H**) (20/136, 14.71%, χ^2= 95.784, P < 0.001, **Table 2**). In normal urothelium, E-cadherin is expressed homogeneously with a typical membranous staining at cell-cell borders, the results of E-cadherin expression in Ta-T1 and T2-T4 bladder lesions are summarized in **Table 1**. Of 80 Ta-T1 tumors, 18 cases (22.5%) had a similar staining pattern referred as normal staining (similar to normal urothelium, homogeneously observed at cell-cell borders), whereas 62 specimens (77.5%) showed an abnormal E-cadherin expression (heterogeneous or negative). Of these 62 specimens, only 4 cases (5%) were completely negative and 58 cases (72.5%) were heterogeneous, with both positive and negative areas in the same tumor. Among the 56 T2-T4 tumors, only 2 case (3.57%) showed normal expression of E-cadherin, and the prevailing abnormal pattern was heterogeneous (54 cases, 96.43%). Completely negative tumors are infrequent (1/56, 1.79%).

Association between MTDH and E-cadherin expression and the clinicopathologic characteristics of the bladder cancer patients

As shown in **Table 1**, the association between MTDH protein expression and clinicopathological characteristics of bladder cancer was performed. Over-expression MTDH significantly correlated with increased Pathological grade(WHO 2004) and Clinical stage (UICC 2002) (χ^2= 14.530, P=0.002, and χ^2= 11.091, P=0.001, respectively). Furthermore, MTDH protein expression was also associated with distant metastasis (χ^2=9.699, P=0.002). No correlation was found between the expression

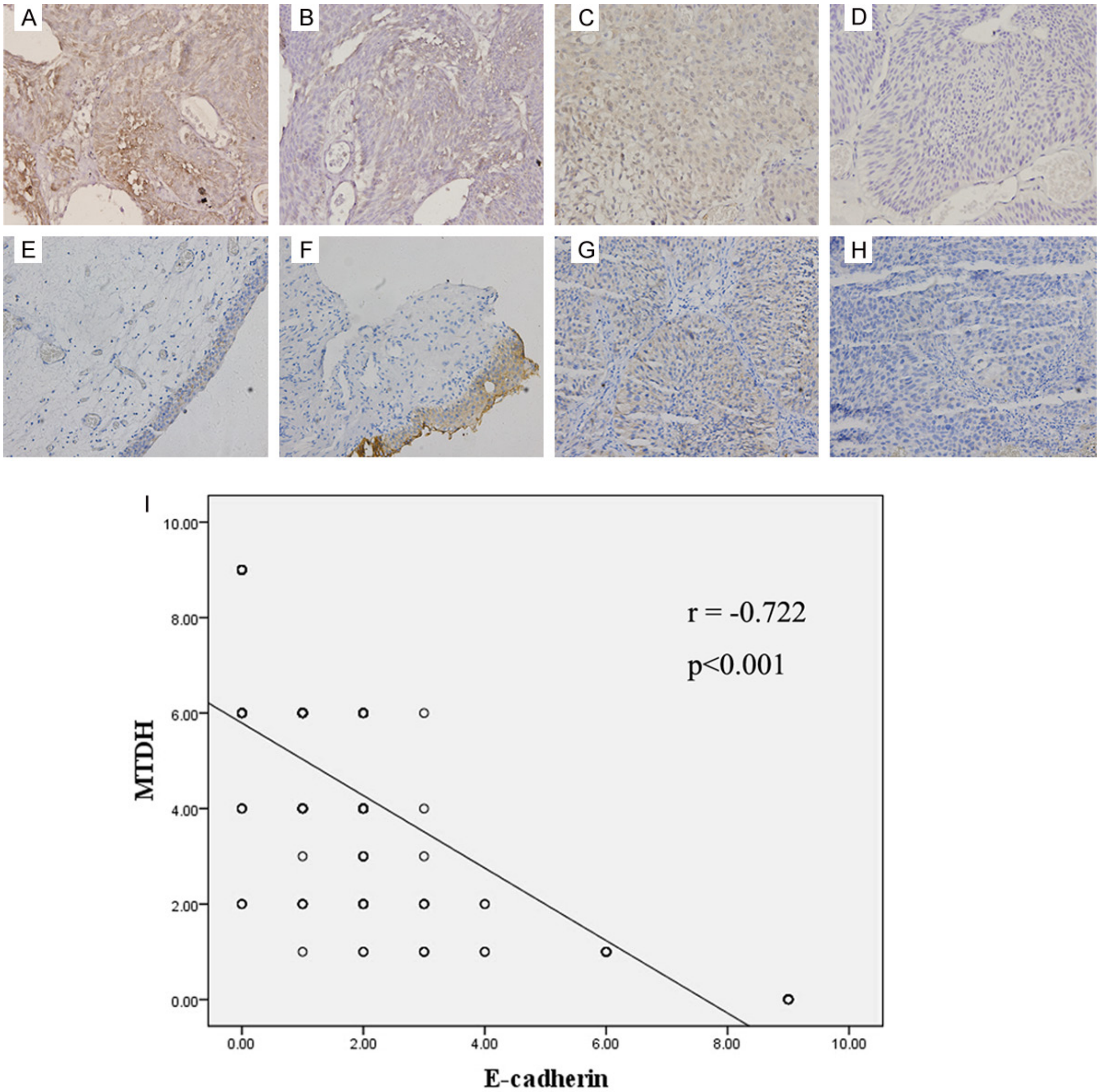

Figure 1. A-E. Representative immunostaining of MTDH protein (magnification×200). A. In high-grade urothelial carcinoma, it is Strong positive stained in the cytoplasm of primary cancer cells. B. In low-grade urothelial carcinoma, it is weakly positive stained in the cytoplasm of primary cancer cells.C. In metastatic tumors, it is mainly localized in the nucleus of primary cancer cells. D. It is weakly expressed in cancer samples. E. It is weakly expressed in benign tissues. F-H. Representative immunostaining of E-cadherin protein (magnification×200). F. In normal urothelium, only localized in cell-cell borders. G. In low-grade urothelial carcinoma, weakly expressed in the cytoplasm of primary cancer cells. H. In high-grade urothelial carcinoma, negative expressed in primary cancer cells. I. Representative a significant correlation between MTDH high expression and E-cadherin abnormal expression in bladder cancer patients, which was confirmed by Spearman correlation analysis.

level of MTDH protein and patient age (χ^2=0.577, P=0.448,) or Gender (χ^2=0.804, P=0.37), Tumor size (χ^2=1.433, P=0.231), Tumor quantity (χ^2=0.505, P=0.477).

As shown in **Table 1**, E-cadherin protein expression was also associated with clinicopathological features such as Pathological grade(WHO 2004) and Clinical stage (UICC 2002) (χ^2= 12.44, P=0.006, and χ^2=9.409, P=0.002, respectively). Furthermore, E-cadherin protein expression was also associated with distant metastasis (χ^2=5.025, P=0.025). However, E-cadherin protein expression was also not associated with other clinicopathological features such as age (χ^2=1.463, P=0.226), gender

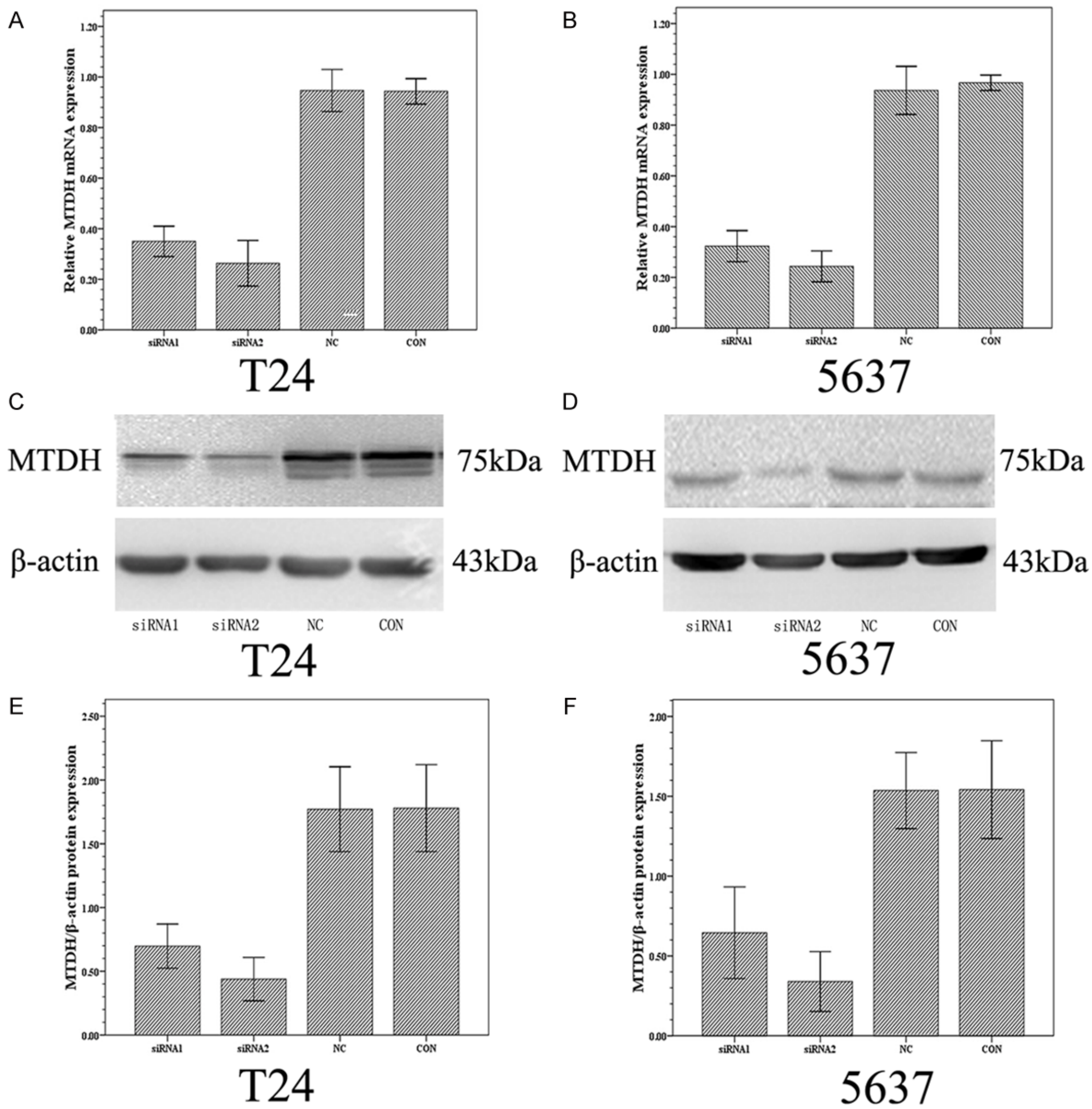

Figure 2. A, B. Representative the relative MTDH mRNA expression in siRNA1, siRNA2, NC, CON groups in T24 and 5637 cells, C, E. Representativethe relative MTDH protein expression in siRNA1, siRNA2, NC, CON groups in T24, D, F. Representativethe relative E-cadherin protein expression in siRNA1, siRNA2, NC, CON groups in 5637 cells.

(χ^2=0.001, *P*=0.977), tumor size (χ^2=0.295, *P*=0.587), tumor number (χ^2=0.223, *P*=0.637).

Association between MTDH and E-cadherin expression in bladder cancer patients

As shown in **Figure 1**, There was a negative correlation between MTDH high expression and E-cadherin abnormal expression in bladder cancer patients, which was confirmed by Spearman correlation analysis (r=-0.722, P<0.001 **Table 2**).

Decreased expression of MTDH mRNA and protein in bladder cancer cells

Based on the above findings that MTDH is involved in clinicopathological features such as metastasis of TCC and negatively correlated with E-cadherin (a biomarker of EMT) expression, we further investigated the functional role of MTDH *in vitro*. In order to determine whether MTDH plays an important role in bladder tumor cells, we transfect the siRNA1 and siRNA2 to T24 and 5637 to generate the MTDH decreased

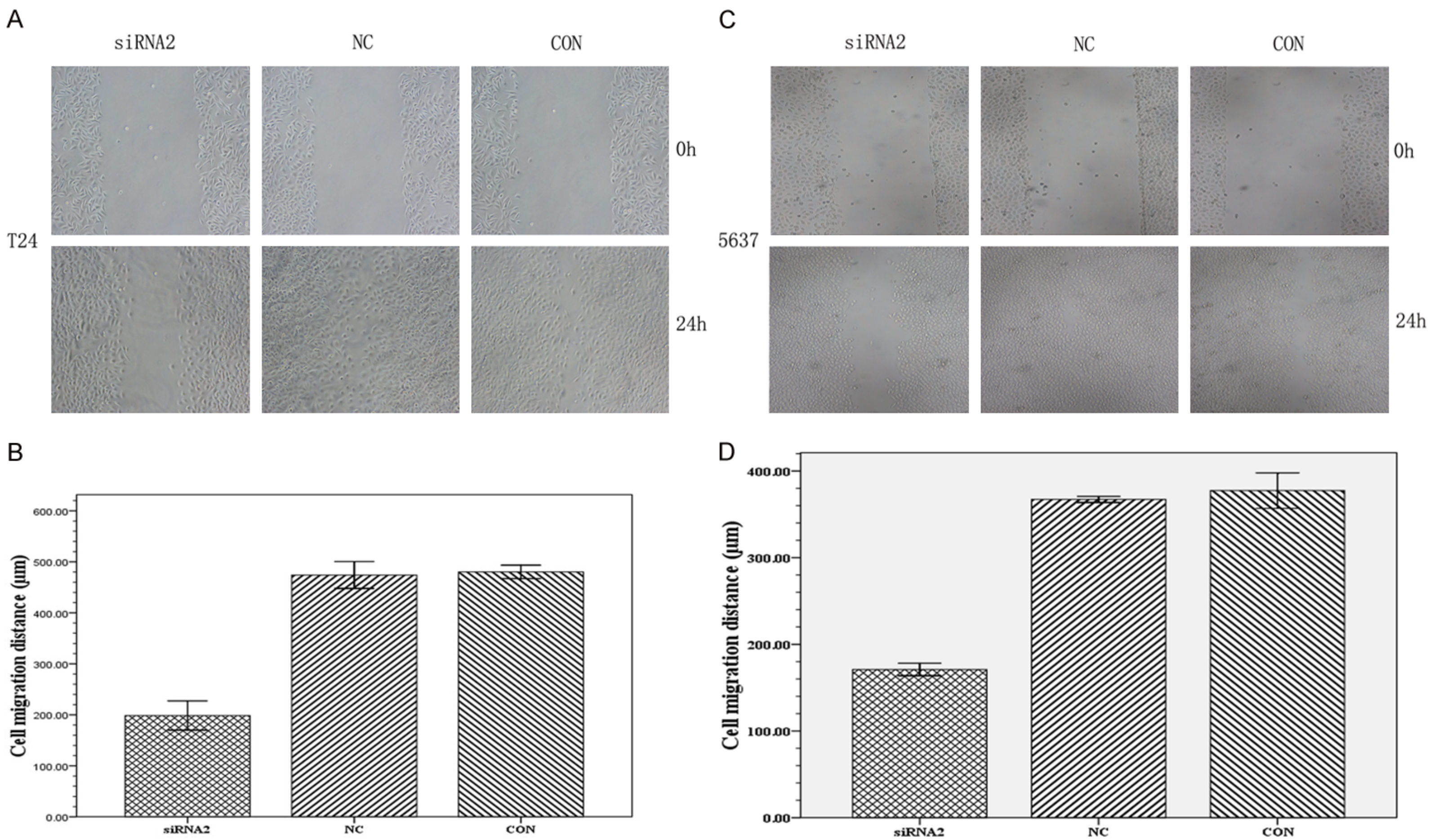

Figure 3. A, B. Representative the migration distance of T24 cells when treated with siRNAs (siRNA2, NC and CON), Compared with control group, siRNA2 treatment group had significant inhibition on the migration of cells at 24 h time point. C, D. Representative the migration distance of 5637 cells treated with siRNAs (siRNA2, NC and CON), Compared with control group, siRNA2 treatment group had dramaticallyinhibition on the migration of cells at 24 h time point.

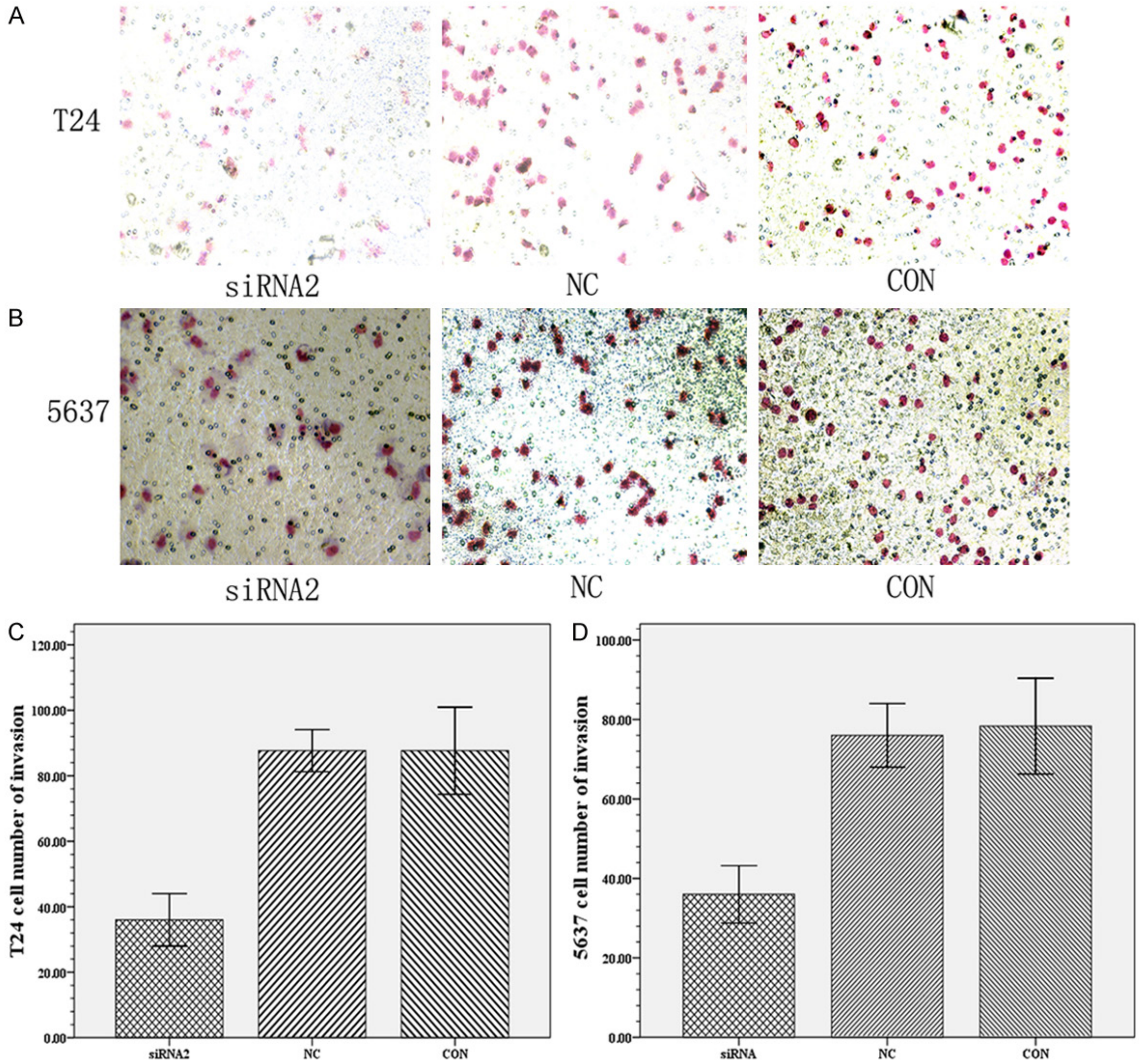

Figure 4. A, C. Representative the mean number of invasive T24 cells when treated with siRNAs (siRNA2, NC and CON), it was 43.7 per field of view in siRNA2 group compared with control groups (87.5 per field of view). B, D. Representative the mean number of invasive 5637 cells when treated with siRNAs (siRNA2, NC and CON), it was 37.0 per field of view in siRNA2 group compared with control groups (76.5 per field of view).

expression. The decreased expression of MTDH in transfected T24 was confirmed by RT-PCR and western blot analysis.

As shown in **Figure 2A**, MTDH mRNA levels were inhibited when cells were infected the MTDH siRNAs in T24, the relative MTDH mRNA expression in siRNA1, siRNA2, NC, CON groups in T24 were 0.3500±0.03, 0.2633±0.04509, 0.9467±0.04163 and 0.9433±0.02517, respectively. (T24, F=310.363, P<0.001), As shown in **Figure 2B**, MTDH mRNA levels were inhibited when cells were infected the MTDH siRNAs in 5637, the relative MTDH mRNA expression in siRNA1, siRNA2, NC, CON groups in 5637 was 0.3233±0.03055, 0.2433±0.03055, 0.9367±0.04726 and 0.9667±0.01528, respectively. (5637, F=415.679, P<0.001) Furthermore, the observed reduction in mRNA level was accompanied by a similar diminution of the protein level. As shown in **Figure 2C**, **2E**, MTDH protein levels were inhibited when cells were transfected the MTDH siRNAs in T24, the relative MTDH protein expression in siRNA1, siRNA2, NC, CON groups was 0.6971±0.0866, 0.4377±0.08513, 1.7712±0.16614 and 1.7-

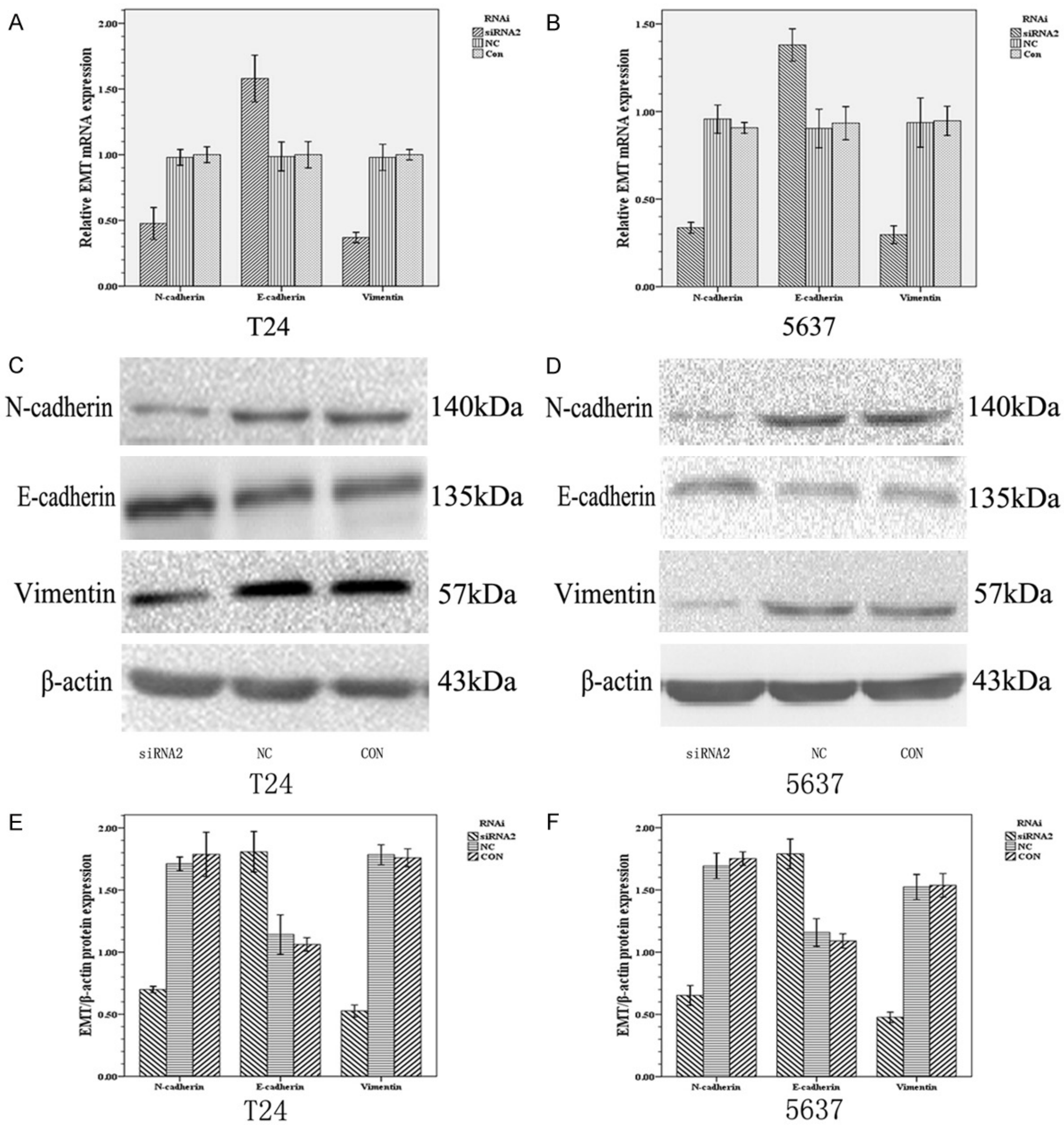

Figure 5. A, B. Representative the mRNA expression of EMT markers, N-cadherin, vimentin were downregulated and E-cadherin was upregulated in siRNA2 group, C, E. Representative the protein expression of EMT markers, N-cadherin, vimentin were decreased and E-cadherin was increased in T24. D, F. Representative the protein expression of EMT markers, N-cadherin, vimentin was decreased and E-cadherin was increased in 5637 cells.

792±0.17045, respectively (T24, F=83.601, *P*<0.001). As shown in **Figure 2D**, **2F**, MTDH protein levels were inhibited when cells infected the MTDH siRNAs in 5637, the relative MTDH protein expression in siRNA1, siRNA2, NC,CONgroupswas0.6455±0.11544,0.3403±0.07543, 1.5359±0.09593 and 1.5416±0.12316, respectively. (5637, F=105.141, *P*<0.001), According to above findings that MTDH mRNA and protein levels were inhibited more than 70% when transfect with siRNA2, so we selected siRNA2 for following study.

Knock down of MTDH suppressmigration of bladder cancer cells in vitro

We conducted the wound healing assay to assess the migration of bladder cancer cells, as shown in **Figure 3A**, **3B**. The migration distance of T24 cells treated with siRNAs (siRNA2, NC and CON), was 198.5967± 14.33271 μm, 474.1167±13.15342 μm, and 480.29±6.52666μm, respectively. Compared with control group, siRNA2 treatment group had significant inhibition on the migration of cells at 24 h time point (F=553.279, P<0.001). As shown in **Figure 3C**, **3D**. The migration distance of 5637 cells treated with siRNAs (siRNA2, NC and CON), was 171±3.6 μm, 367.2±1.8 μm, and 377.4±10.23523 μm, respectively. Compared with control group, siRNA2 treatment group had dramaticallyinhibition on the migration of cells at 24 h time point (F=1006.937, P<0.001). The results suggest knock down MTDH suppressed migration of transitional cell carcinoma cells.

Knockdown of MTDH suppressinvasion of bladder cancer cells in vitro

To evaluate the invasion ability of bladder cancer cells *in vitro*, the transwell invasion assay were conducted, we counted the number of invasive cells in different groups (siRNA2, NC and CON). As shown in **Figure 4A**, **4C**, the mean number of invasive T24 cells in group of siRNA2 was 43.7 per field of view compared with control groups (87.5 per field of view) (P<0.01). As shown in **Figure 4B**, **4D**, The mean number of invasive 5637 cells in group of siRNA2 was 37 per field of view compared with control groups (76.5 per field of view) (P<0.01). The results suggest knock down MTDH suppress invasion of TCC cells.

Knockdown of MTDH led to acquisition of epithelial markers and reduction of mesenchymal markers.

To further confirm the relationship between MTDH and EMT process, we examined the changes of EMT markers between MTDH-siRNA and parental cells using RT-PCR and Western blot. RT-PCR showed that epithelial marker E-cadherin was upregulated while the mesenchymal N-cadherin and vimentin were downregulated in MTDH-siRNA cells of T24 (**Figure 5A**, F=40.551, P<0.001) and 5637 (**Figure 5B**, F=22.485, P<0.001). Western blot analysis further showed that the expression of N-cadherin and vimentin were decreased in MTDH-siRNA cells, which was accompanied by increased E-cadherin expression in T24 (F=689.971, P<0.001) and 5637 (F=105.141, P<0.001), Both of these analyses revealed that TCC cells with inhibited MTDH expression displayed down-regulated N-cadherin and vimentin and up-regulated E-cadherin.

Discussion

Although the new therapeutic strategies of bladder cancer have made great progress, the unfavorable biological behaviors, especially prone to invasion and metastasis, are still puzzles clinical treatment, resulted in unsuccessful therapy and unsatisfactory prognosis. So it is crucial to understand the molecular mechanisms leading to invasion and metastasis of bladder cancer cells.

Epithelial to mesenchymal transition (EMT) was known as a crucial morphogenetic process in which cells undergo an important transition from a polarized epithelial phenotype to a mesenchymal phenotype. It is characterized by loss of polarity and epithelial markers including tight junctional and cell-cell adhesion molecules, and gain of polarity and mesenchymal markers (such as vimentin and N-cadherin) [8]. E-cadherin is a characteristic biological marker of epithelial phenotype and the deletion of E-cadherin is an important symbol of EMT [16]. Therefore, the research of E-cadherin has an important significance in the study of EMT, the epithelial marker E-cadherin may indicates the occurrence of EMT, Cadherins are a family of calcium-dependent transmembrane glycoproteins that mediate cellular adhesion [17]. Loss or reduced expression of E-cadherin expression and aberrant expression of N-cadherin ('cadherin-switch') is a characteristic feature of epithelial-mesenchymal transition (EMT), a process associated with cancer progression. Jager et al [17] first time demonstrated that N-cadherin gene expression strongly correlates with tumor stage and grade in bladder cancer samples. Furthermore, gain of N-cadherin expression proved to be a risk factor in superficial bladder carcinoma. In our cytological study, E-cadherin was negatively correlated with mRNA and protein expression of N-cadherin, which also verified the occurrence of cadherin-switch.

Immunohistochemical staining revealed that the expression of MTDH and E-cadherin expression were both significantly associated with clinical stage, pathological grade and distant metastasis. Furthermore, the expression level of MTDH increased gradually from normal bladder epithelium to high grades of histopathogical bladder cancers. The results partly accorded with the studies of Yang et al [14] and Zhou et al [18], they reported that expression of MTDH correlated with cancer pathologic stage and World Health Organization classification of bladder cancer. Previous study has revealed that decreased E-cadherin correlates with poor survival in patients with bladder cancers [19], but the relationship between MTDH and E-cadherin has not been studied. In the present study, we found that MTDH was negatively correlated with E-cadherin in bladder tumor tissues; therefore, we suggest that MTDH is closely related to EMT and caused the occurrence of EMT. In addition, knock down MTDH promote the occurrence of Mesenchymal to epithelial transition (MET) in bladder cancer cells. So we suspect that co-expression of MTDH/E-cadherin may be a novel distinctive marker to predict the prognosis of bladder cancer.

The invasion and metastasis of malignant tumor is a complex, multi-step, multi factor and sequential process, which includes departing from the primary site of tumor, entering into the surrounding matrix, entering the circulation or lymph system, and adhering to the vascular or lymphatic vessel or lymphatic endothelial cell wall [22]. EMT is an important mechanism for tumor cells to obtain the ability to infiltrate, it can decrease tumor cell adhesion ability, break through the basement membrane, enter the blood circulation, and then circulating tumor cells in the implantation site to form a metastatic tumor [23]. Therefore, we suggest that MTDH can promote the invasion and migration of cells through enhanced EMT, Knock down of MTDH suppressesmigration and invasion of bladder cancer cells in vitro.

To investigate the mechanisms by which MTDH influenced biological behaviors in cancer cells, the activation of possible signal pathways were evaluated in previous studies, such as Ha-ras, PI3K/Akt, NF-κB and Wnt/β-catenin signaling pathway, Behrens J et al [20] have reported β-catenin is a key downstream effector of the Wnt signaling pathway, Xu et al [21] have reported inhibition of AEG-1 reduced phosphorylation of AKT and glycogen synthase kinase (GSK)-3 beta and decreased the level of β-catenin, lymphoid enhancer binding factor 1, and cyclin D1 in gastric cancer. We speculate that E-cadherin is regulated by MTDH via complex signal pathway in bladder cancer, but the specific signal pathway needs further study.

In a word, to the best of our knowledge, we are the first to provide evidence that MTDH could suppress migration and invasion by inhibiting the epithelial to mesenchymal transition in bladder cancer cells. Thus, MTDH may represent a promising target for developing a novel treatment strategy for bladder cancer.

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Disclosure of conflict of interest

None.

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