MicroRNA-497 inhibits the proliferation, migration and invasion of human bladder transitional cell carcinoma cells by targeting E2F3

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Abstract. Accumulating evidence indicates that microRNAs (miRNAs) play critical roles in regulating cellular processes, such as cell growth and apoptosis, as well as cancer progression and metastasis. Low expression of miR-497 has been observed in breast, colorectal and cervical cancers. Human bladder transitional cell carcinoma (BTCC) progression typically follows a complex cascade from primary malignancy to distant metastasis, but whether the aberrant expression of miR-497 in BTCC is associated with malignancy, metastasis or prognosis remains unknown. In the present study, we found that miR-497 was markedly downregulated in BTCC tissue samples when compared with that noted in adjacent normal tissues, and low expression of miR-497 was correlated with poor prognosis in BTCC patients. We also found that overexpression of miR-497 inhibited the proliferation, migration and invasion of bladder cancer cells by downregulating E2F3 (an miR-497 target gene) mRNA and protein and that siRNA against E2F3 inhibited cell proliferation, migration and invasion, which was similar to the effect of miR-497 overexpression in the BTCC cells. Our experimental data indicated that miR-497 mediates the in vitro proliferation, migration and invasion of BTCC cells. Together, these results suggest that miR-497 may represent a novel prognostic indicator, a biomarker for the early detection of metastasis and a target for gene therapy of BTCC.

Introduction

Human bladder transitional cell carcinoma (BTCC) is one of the most frequent malignant tumors and its incidence is increasing worldwide (1). Despite many advances in cancer diagnosis and treatment in recent decades, the outcome of patients with advanced BTCC remains poor. There is, therefore, a tremendous and urgent need for elucidating the underlying molecular mechanisms of BTCC tumorigenesis and for developing novel therapeutic strategies, including treatments aimed at specific molecular targets.

MicroRNAs (miRNAs), which are short, highly conserved non-coding RNAs, regulate the expression of messenger RNAs (mRNAs) by binding to their 3'-untranslated region (3'-UTR) (2,3). miRNAs play a critical role in processes related to cancer by acting as oncogenes or tumor suppressors in the regulation of carcinogenesis, metastatic capacity and drug resistance. Low expression of miR-497 has been observed in breast, colorectal and cervical cancers (4). Recent studies have shown that miR-497 suppresses angiogenesis and invasion of ovarian cancer by targeting vascular endothelial growth factor A (5). Li et al reported that miRNA-497 modulates gastric cancer cell proliferation and invasion by repressing eIF4E (6). Notably, a recent study by Itesako et al found that miR-497 significantly inhibited cancer cell proliferation, migration and invasion in two bladder cell lines by targeting BIRC5 and WNT7A (7). However, the relationship between miR-497 and the prognosis of BTCC patients, and whether miR-497 targets other target genes to regulate BTCC cell growth and invasion are undefined.

Transcription factor E2F3, a key regulator of G1/S phase transition, plays major roles in regulating cell cycle progression (8,9). Previous studies have shown that E2F3 overexpression is critical for the growth and survival of bladder cancer cells, and is generally correlated with poor outcomes (3,10). Furthermore, BTCC patients with higher levels of E2F3 also show rates of higher mortality compared with patients with lower levels of E2F3 (3,11), suggesting that E2F3 is important for BTCC development. To date, several miRNAs, such as miR-34a, miR-20a, miR-125b and miR-217, have been confirmed to suppress cell growth and survival by targeting E2F3 in tumor cells (3,12-16).

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Abbreviations: BTCC, bladder transitional cell carcinoma; miRNAs, microRNAs; miR-497, microRNA-497

Key words: human bladder transitional cell carcinoma, miR-497, cancer progression, E2F3
However, the relationship between miR-497 and E2F3 in BTCC is poorly understood.

In the present study, we found that miR-497 was significantly downregulated in BTCC cell lines and tissues. Furthermore, we identified E2F3 as a target gene of miR-497 and showed that upregulation of miR-497 inhibited cell proliferation and invasion by directly downregulating E2F3 expression in T24 and UM-UC-3 cells in vitro. Our data indicated that miR-497 functions as a tumor suppressor in BTCC development and serves as a prognostic marker for BTCC.

Materials and methods

Cell lines and culture. Human BTCC cell lines (T24, 5637, BIU-87 and UM-UC-3) and a human bladder urothelium cell line (SV-HUC-1) were purchased from the Chinese Science Institute (Shanghai, China). All cell lines were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Gibco, Gaithersburg, MD, USA). Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Tissue specimens. Eighty paired tissue samples from BTCCs and the corresponding adjacent normal tissues were collected from the Department of Pathology of Shengjing Hospital. All tissue specimens were confirmed by pathology. The collection and use of tissues followed procedures that were in accordance with the ethical standards formulated in the Declaration of Helsinki. Patients provided informed consent prior to tissue collection. The present study was approved by the Institutional Research Ethics Committee of China Medical University.

Transfection of miRNA mimics and small interfering RNAs. An miR-497 mimic (referred to as miR-497) and negative control (NC) duplex were used for further gain-of-function experiments. A small interfering RNA (siRNA) duplex (siE2F3) targeting E2F3 mRNA was used for RNA interference experiments. All RNA duplexes and oligos were obtained from RiboBio (Guangzhou, China).

Cells (5x10⁵) were seeded into 6-well plates (Nest Biotechnology, Hong Kong, China). At 70% confluence, the cells were subjected to transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions (17). The sequences of the RNA duplexes and oligos were as follows: miR-497 mimics, 5'-CAGCAGCAGCACACTG TGG-3' (sense), and 5'-GTGCAAGAGTGCCAGGT-3' (antisense); U6, 5'-GTCCTATCCAGTCCAGGGTGCCAGG TGCACTGGATACGACAAAAATATGGAAC-3' (antisense); and 5'-TGGCGGTTCGCTGCAGTGCAGG TGCACTGGATACGACAAAAATATGGAAC-3' (antisense). U6 was used as an internal control. All results are representative of three independent assays, and the expression levels of miR-497 were calculated by the 2^ΔΔCt method.

Cell Counting Kit-8 assay. Cell proliferation was measured using a Cell Counting Kit-8 assay (CCK-8) (Solarbio, Beijing, China). Cells transfected with miR-497 mimics, NC, siRNAs and relative controls were seeded in 96-well plates (Corning, Corning, NY, USA) at 2x10³ cells/well. At 0, 24, 48 and 72 h, 10 µl CCK-8 reagent was added to each well. After 2 h of incubation, the absorbance was measured at 450 nm as previously described (3). All results are representative of three independent assays.

Colonies formation assay. After transfection, 300 cells were seeded into 6-well plates containing 2 ml DMEM supplemented with 10% FBS/well and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 7-10 days. After staining with a 0.1% crystal violet solution for 10 min, the numbers of colonies were counted. All results are representative of three independent assays.

Cell migration and invasion assays. At 90% confluency, a sterile pipette tip was used to make a scratch through each well. Cells were photographed under a microscope at 0 and 24 h for comparison. We used Transwell invasion assays to evaluate the invasive ability of cells as previously reported (3). An inverted microscope was used to observed cell invasion. The invasion assay was terminated when the cells crossed into the lower well. After the Matrigel was scraped off, the number of cells in the bottom well was counted. Images of cells stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were obtained. Each treatment was performed in triplicate.

Dual-luciferase reporter assays. Dual-luciferase reporter assays were performed as previously reported (18). Briefly, T24 cells were seeded in 96-well plates and co-transfected with a pMIR-Report luciferase vector, pRL-TK Renilla luciferase vector and miR-497 mimics, as previously reported (17). After 48 h, the luciferase activities were determined with the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA). Renilla luciferase activity was used as an internal control. After normalization to Renilla luciferase activity, the firefly luciferase activity was analyzed. All results are representative of three independent assays.

E2F3 rescue experiments. The E2F3-coding sequence excluding the 3’UTR was inserted into a pReceiver vector (GeneCopoeia) to construct the pReceiver-E2F3 vector. The BTCC cells were co-transfected with miR-497 or NC and pReceiverE2F3 or an empty pReceiver vector. BTCC cells were harvested at 48 h after transfection. Cell proliferation and invasion were determined and E2F3 protein expression was analyzed by western blotting as described above.
Western blot analysis. Proteins were isolated from the cells or tissues by mechanical disruption and a Mammalian Cell Lysis kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The proteins were separated on 1 mm NuPage Novex 10% Bis-Tris gels using a NuPage MOPS SDS Buffer kit (Life Technologies, Carlsbad, CA, USA) followed by electrotransfer to 0.2-mm nitrocellulose membranes (Pall, Port Washington, WI, USA). Non-specific binding sites were blocked with 5% bovine serum albumin in phosphate-buffered saline (PBS) for 1 h at room temperature. The membranes were then incubated with a diluted primary antibody (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight. After three washes with PBS containing 0.5% Tween-20, the membranes were incubated with a diluted secondary antibody (GE Healthcare, Buckinghamshire, UK) at room temperature for 2 h. Signals were visualized with enhanced chemiluminescent reagent (Amersham Biosciences, Piscataway, NJ, USA). As a protein loading control, the blots were stripped and stained for GAPDH using an anti-GAPDH antibody (1:2,000; Abcam, Cambridge, MA, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). All statistical analyses were performed by one-way analysis of variance (ANOVA) (SPSS 18.0). miRNA target prediction and the associated mRNA pathway analysis.
were performed using ingenuity pathway analysis and TargetScan. Differences between treatments were assessed using Fisher's least significant difference test. Significant differences were considered at $P<0.05$.

### Results

**miR-497 is downregulated in BTCC cell lines and tissues.**

We detected the expression of miR-497 in four human BTCC cell lines (T24, 5637, BIU-87 and UM-UC-3) by RT-PCR and found that miR-497 was downregulated in BTCC cell lines T24, 5637, BIU-87 and UM-UC-3 compared with that noted in a normal bladder epithelial cell line, SV-HUC-1 (Fig. 1A). The levels of miR-497 were also detected in 80 human BTCC specimens and adjacent normal tissues by RT-PCR. The results showed that miR-497 was downregulated in the human BTCC specimens compared with that noted in their adjacent normal tissues (Fig. 1B). Moreover, a low level of miR-497 expression was correlated with tumor size (Fig. 1C), node metastasis (Fig. 1D), invasion (Fig. 1E), differentiation (Fig. 1F) and poor prognosis (Fig. 1G).

**miR-497 suppresses BTCC progression in vitro.**

To examine the role of miR-497 in cell proliferation, migration and invasion, T24 and UM-UC-3 cell lines were transfected with miR-497 mimics or NC. Increased expression of miR-497 upon transfection was confirmed by RT-PCR (Fig. 2A). As shown in Fig. 2B, ectopic miR-497 expression suppressed proliferation of the BTCC cells as analyzed by the CCK-8 assay. Consistent with the effects on cell proliferation, the capacity for colony formation by both cell lines was robustly compromised by miR-497 transfection compared with the
corresponding control cells (Fig. 2C). As shown in Fig. 2D, the migratory ability of the miR-497 mimic groups was lower than that of the NC groups. Similar to the effect on cell migration, the capacity for invasion of both cell lines was significantly decreased by miR-497 transfection compared with that noted in the corresponding control cells (Fig. 2E). Taken together, our results revealed that miR-497 inhibited BTCC cell proliferation, migration and invasion in vitro.

miR-497 directly targets E2F3 in BTCC cells. Next, we identified the miR-497 target gene to gain further insight into the molecular mechanisms of miR-497 in BTCC proliferation, migration and invasion. The public database-TargetScan (http://www.targetscan.org) was used to predict the potential target of miR-497. Due to a critically conserved binding site, E2F3 was selected for further examination (Fig. 3A).

To investigate the relationship between miR-497 and E2F3, the protein expression of E2F3 was measured in BTCC cell lines with differential expression of miR-497. E2F3 protein levels in T24 and UM-UC-3 cells were significantly decreased after overexpression of miR-497 mimics (Fig. 3B). To confirm whether E2F3 is a direct target of miR-497, we constructed a dual-luciferase reporter vector with the putative E2F3 3'UTR target site for miR-497, which was downstream of the luciferase gene (pMir-E2F3-Wt), and a deletion mutant of 7 bp in the seed region (pMir-E2F3-Mut). As shown in Fig. 3C and D, luciferase activity assays showed that miR-497 significantly suppressed the activity of Wt, but not Nut reporters in the T24 and UM-UC-3 cells. In addition, the relative expression levels of E2F3 were inversely correlated with the relative expression levels of miR-497 in the BTCC tissues (Fig. 3E). These results strongly demonstrated the specificity of miR-497 to target E2F3.

siE2F3 inhibits the proliferation, migration and invasion of BTCC cells. As shown in Fig. 4A and B, compared with NC, siE2F3 decreased the expression of E2F3 at both the mRNA and protein levels. To reveal the biological function of E2F3, siE2F3 was stably transfigured into the T24 and UM-UC-3 cells. As a result, we found that siE2F3 could reproduce an effect on cell proliferation, migration and invasion similar to that of miR-497 (Fig. 4C-F).

Discussion
Recent studies have demonstrated the potential of miRNAs as promising prognostic biomarkers (2,3). Targeting miRNAs is an effective approach for the treatment of advanced cancers (2-4). Moreover, aberrant expression of miRNAs has been identified in bladder cancer, which plays crucial roles in tumorigenesis and cancer progression. miR-497 is a recently discovered miRNA that is downregulated in bladder, gastric and breast cancers (19-24). In the present study, we found that miR-497 was markedly downregulated in the BTCC cell lines. Our results indicate that low expression of miR-497 is significantly associated with BTCC progression.

We also analyzed the relative expression of miR-497 in BTCC tissues. miR-497 expression was decreased in cancer tissues, and the low expression of miR-497 was significantly associated with tumor size, node metastasis, invasion, differentiation, as well as a poor prognosis. These results imply that miR-497 expression may function as an independent prognosis factor that is significantly associated with the overall survival rates of BTCC patients and BTCC progression. Our results
were consistent with a previous study by Zhao et al who showed that downregulation of miR-497 is associated with a poor prognosis in renal cancer (25). Further studies demonstrated that upregulation of miR-497 significantly decreased cell proliferation, the colony formation rate, wound healing and migration rates, indicating that miR-497 inhibits the proliferation, migration and invasion of BTCC cells.

Next, we explored the molecular mechanism by which miR-497 acts as a suppressor in BTCC progression. Luciferase reporter assays and western blotting were employed to confirm that E2F3 is direct target of miR-497 in BTCC cells. E2F3, which is known as a transcription factor, has a central role in linking cell cycle proteins, such as cyclins, cyclin-dependent kinases and pRB, to the expression of genes involved in cell growth and survival (26-29).

E2F3 gene amplification and protein overexpression have been extensively studied in bladder cancer (10). High levels of E2F3 expression have been observed in approximately one-third of BTCC with E2F3 overexpression increasing with tumor grade and stage (10). Hence, the mechanism of E2F3 upregulation, except for gene RNA inference, recent reports showed several miRNAs (miR-141, miR-199a-5p, miR-125 and miR-200c) may be involved (30-34). In the present study, western blot analysis and RT-PCR demonstrated downregulation of E2F3 by miR-497, which inhibited the cell proliferation, migration and invasion of BTCC cells. To test whether miR-497 inhibits proliferation, migration and invasion of BTCC cells through targeting E2F3, the expression of E2F3 was knocked down using specific siRNAs. siE2F3 could emulate the effects of miR-497 overexpression on cell proliferation, migration and invasion.
growth, migration and invasion. All these results suggest that miR-497 may act as a suppressor of BTCC cells by inhibiting the expression of E2F3. Therefore, we proposed that downregulation of miR-497 may enhance the proliferation and invasion of BTCC cells and subsequently facilitate the development of BTCC through upregulating the expression of E2F3. The rescue effect of E2F3 expression partly reversed the inhibition of cell proliferation, migration and invasion induced by miR-497. In the present study, we demonstrated the direct role of E2F3 in the proliferation, migration and invasion of BTCC cells, which were regulated by miR-497. The specific mechanism will be revealed in a future study.

In conclusion, our data demonstrated altered expression of miR-497 in human BTCC cell lines and BTCC tissues, and
that low levels of miR-497 correlate with poor prognoses in patients. Our data also showed that miR-497 suppresses the proliferation and invasion of BTCC cells, possibly through modulating the target gene E2F3. These findings suggest that miR-497 may be a valuable biomarker for BTCC progression, and the miR-497-E2F3 axis may be a novel therapeutic target for BTCC.

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References