MiR-4644 is upregulated in plasma exosomes of bladder cancer patients and promotes bladder cancer progression by targeting UBIAD1

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Abstract: Exosome-encapsulated microRNAs (miRNAs) have been identified as potential cancer biomarkers and pro-tumorigenic mediators for several cancers. However, the miRNA profiling in BCa-Exo (exosomes from plasma of patients with bladder cancer) has not yet been explored. Hence, the aim of this study was to analyze the miRNA profiling in BCa-Exo and to explore the function and mechanism of the selected miR-4644 in BCa progression. Of the 8 differentially expressed miRNAs in BCa-Exo relative to NC-Exo (exosomes from plasma of normal control subjects), hsa-miR-4644 was the only upregulated (fold change >2.0, \( P < 0.05 \)) miRNA, which was further confirmed to be up-regulated in plasma of BCa patients and BCa cell lines. Further in vitro assays demonstrated that miR-4644 mimic promoted, whereas miR-4644 inhibitor suppressed BCa cell proliferation and invasion. miR-4644 negatively regulated expression of UBIAD1 (UbiA prenyltransferase domain-containing protein 1) by directly binding to its 3'-UTR region. UBIAD1 overexpression effectively abrogated the promoting effects of miR-4644 mimic on BCa proliferation, migration, and invasion. Additionally, intratumoral injection of miR-4644 antagonir downregulated miR-4644 expression in tumors and suppressed tumorigenesis in mouse xenografts. Collectively, miR-4644 promotes BCa progression by targeting UBIAD1. miR-4644 may be an important therapeutic target for BCa treatment.

Keywords: Exosome, miR-4644, UBIAD1, bladder cancer

Introduction

Bladder cancer (BCa) is one of the most prevalent malignancies of the urinary system, accounting for approximately 3% of total cancer-related death [1]. Despite the improvement in the treatment, the 5-year survival rate is still unsatisfactory. Hence, it is indispensable for us to find novel diagnostic indicators and therapeutic targets to improve the clinical efficacy of treatment for BCa.

Exosomes are nanoscale membrane vesicles that contain bioactive molecules such as lipids, proteins, mRNAs, and microRNAs (miRNAs) and have been regarded as a crucial means of intercellular communication [2]. Exosome-encapsulated miRNAs have been identified as potential cancer biomarkers and pro-tumorigenic mediators during carcinogenesis [3-5]. To date, many studies have highlighted the important role of miRNAs in BCa diagnosis and progression [6-8]. However, the miRNA profiling in BCa-Exo (exosomes from plasma of patients with BCa) has not yet been explored. In the present study, we performed a miRNA microarray to analyze the miRNA profile of BCa-Exo and NC-Exo (exosomes from plasma of normal controls) and found that hsa-miR-4644 was the only upregulated miRNA in BCa-Exo. A previous study suggested that miR-4644 in salivary exosome could be used as a potential biomarker for pancreatobiliary tract cancer [9]. However, the functional role and the underlying mechanism of miR-4644 in BCa have not been explored.

Our bioinformatics analysis (Targetscan) revealed that UbiA prenyltransferase domain-containing protein 1 (UBIAD1, also referred to as TERE1) was a putative target of miR-4644 by harboring two putative miR-4644 binding sites in the 3'-UTR of its mRNA. UBIAD1 has been shown to inhibit the proliferation of BCa cells
Thus, in the present study, we examined the functional role of miR-4644 in regulating BCa cell behaviors and elucidated whether UBIAD1 was involved in this process. Our study provides new information regarding miR-4644 as a novel therapeutic target for BCa treatment.

Materials and methods

Patients and sample collection

Peripheral blood was withdrawn from patients with pathologically confirmed BCa and age-matched healthy volunteers as normal control (NC). The patients who underwent chemotherapy or radiotherapy before blood sampling were excluded. The clinicopathological characteristics of bladder patients, including age, gender, and cancer stage is shown in Table 1. There were 57 patients, with 12 in Ta, 13 in T1, 15 in T2, and 10 in T3, and 7 in T4. Peripheral blood was collected into tubes and centrifugated at 1,000× g for 10 min to obtain plasma. The obtained plasma was stored at -80°C prior to exosome isolation. This study was approved by the Research Ethics Committee of the First Affiliated Hospital of Zhengzhou University and all tested individuals signed an informed consent form.

Isolation and characterization of plasma-derived exosomes

Exosomes were isolated from plasma of NC subjects or BCa patients using differential centrifugation, size-exclusion chromatography on Sephadex G50 columns, and ultracentrifugation, as previously described [11, 12]. Approximately 10 mL of plasma was collected for exosome isolation. The exosome pellet was resuspended in PBS and protein concentration was measured using Bradford protein assay. Isolated exosomes were immediately used for subsequent experiments or stored at 4°C.

For identification, the morphologic characteristics of exosomes were observed by transmission electron microscopy (TEM) as previously described [13]. The particle size distribution of exosomes was determined by Nanosight. The protein levels of exosomal surface markers CD9, CD63, and Tsg101 were measured by western blot.

miRNA microarray analysis

miRNA microarray analysis was performed by Shanghai Biotechnology Corporation (Shanghai, China). miRNA expression profiles in the collected BCa-Exo and NC-Exo were analyzed using the Human miRNA microarray kit (Agilent Technologies, Spain), and the differentially expressed miRNAs were screened out based on the filtration criteria of fold change >1.0 or <1.0 and P<0.05.

Cell lines and culture

The immortalized human bladder epithelial cell line SV-HUC-1 (ATCC, Manassas, VA, USA) and human BCa cell lines (T24, J82, 5637, and UMUC3; ATCC) were cultured in complete DMEM (Gibco) containing 10% FBS (Gibco), 100 U/mL penicillin and 0.1 mg/mL streptomycin in a humidified 95% air and 5% CO₂ atmosphere at 37°C.

Cell transfection

The UBIAD1 overexpression vector (pcDNA3.1-UBIAD1), an empty pcDNA3.1 vector, miR-4644 mimic, mimic NC, miR-4644 inhibitor, inhibitor NC, UBIAD1 siRNA (si-UBIAD1) and scramble control siRNA (si-Ctrl) were purchased from GenePharma (Shanghai, China). BCa cells at 80-90% confluence were transfected with the indicated constructs, miRNAs, and siRNAs using Lipofectamine™ 2000 (Invitrogen).

The sequences were as follows: miR-4644 mimic (forward, 5'-UGGAGAGAGAAAAGAGAGAGAAG-3'; reverse, 5'-CUUCUGUCUCUUUUCUCUCUCCA-3'), miR-4644 inhibitor (5'-CUUCUGUCUCUUUUCUCUCUCCA-3'), negative control mimic (mimic NC; forward, 5'-UCACAAACCUCUCAGAGAGUAGA-3', reverse, 5'-UCUCACGUAGAGAGUAGA3'.

Table 1. Clinicopathological characteristics of bladder patients (n=57)

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<th>Value</th>
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<td>T3</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 1. Clinicopathological characteristics of bladder patients (n=57)
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CUUUCUAGGGUUGUGA-3'), inhibitor NC (5'-UCUACUCUUUCUAGGGUUGUGA-3'), si-UBIAD1 (5'-GUAAUUUGGUCACUATT-3'), si-NC (5'-UUCUCGAACGUGUCACGUTT-3').

Quantitative real-time PCR

Total RNA was extracted from plasma or BCa cells using Trizol reagent (Invitrogen). TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to reverse-transcribe RNA to cDNA. qRT-PCR was performed using the Taqman Universal PCR Master Mix (Thermo Fisher Scientific). For internal control, cel-miR-39 was applied for the detection of miRNAs in plasma samples, while U6 was used as the internal control of miRNAs in BCa cells. The UBIAD1 mRNA level was normalized to that of GAPDH.

Western blot

Proteins were extracted from isolated exosome with M-PER buffer (Pierce Biotechnology) or from BCa cells using RIPA lysis buffer (Beyotime). Proteins were separated by electrophoresis through 12% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. Finally, an ECL kit (Perkin Elmer, Waltham, MA, USA) was used to visualize protein bands. The primary antibodies and secondary antibodies were purchased from Abcam (Cambridge, MA).

RNA pull-down assay

The biotin-labeled miR-4644 and random pull-down probe sequences as NC were designed and synthesized. T24 cells were washed twice with pre-cooled PBS solution, and the cells were lysed in 1 mL 0.1% NP40 lysis containing protease inhibitor and centrifuged at 12000× g for 5 min to extract supernatant. Biotinylated RNAs were then incubated with streptavidin agarose-treated magnetic beads (Life Technologies, Thermo Fisher Scientific) and the cell lysates at room temperature for 1 h. The precipitated UBIAD1 mRNA level was detected by qRT-PCR.

Cell proliferation assay

Following transfection with the designated plasmids or siRNAs, cells were plated in 96-well plates for the indicated time. Then 20 μL of MTT (Sigma-Aldrich, 5 mg/mL) was added into each well. After 4 h of incubation, the medium was replaced with 150 μL dimethyl sulfoxide. After 10 min, the optical density (OD) at 490 nm was measured by a spectrophotometer.

Transwell migration and invasion assay

Cell invasion was assessed by 24-well Transwell plates (8-μm pore size, Corning, NY, USA) pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA) as previously described [14]. The migration assay was conducted the same way as the invasion assay described above, except that the upper chambers were coated without Matrigel.

Dual-luciferase reporter assay

The putative miR-4644 binding site at the 3'-UTR of UBIAD1 was cloned into a pMIR-REPORT vector (Ambion; Thermo Fisher Scientific). For luciferase reporter assay, 293T cells were co-transfected with hsa-miR-4644 or mimic NC, wild-type or mutant luciferase constructs, and pRL-TK (internal control; Promega, Madison, WI, USA) using Lipofectamine 2000 (Invitrogen). The luciferase activity 48 h post-transfection was analyzed using the dual-luciferase reporter assay system (Promega).

In vivo xenograft experiments

BALB/c nude mice (six-week-old; male) were randomly divided into the following 3 groups: control, antagonmir-NC, and miR-4644 antagonmir, with 6 mice in each group. T24 cells (3×10^6 cell/mouse) were injected subcutaneously into the right flank of BALB/c nude mice in their backs. After 1 week, 25 nM of miR-4644 antagonmir (RiboBio Co., Guangzhou, China; diluted in 100 μL PBS) and 25 nM of antagonmir-NC (diluted in 100 μL PBS) were injected intratumorally three times per week for 2 weeks. Mice in the control group were treated with PBS instead. Tumor diameters were measured every week. The tumor volume was calculated. After 6 weeks, the mice were sacrificed, and tumors were excised; the weight of the tumor was measured. All experimental procedures were performed in strict accordance with the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health.
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Statistical analysis

All statistical analyses were performed using SPSS version 20.0. The differences between two groups were analyzed using Student's t-test. The differences between three or more groups were analyzed by one-way ANOVA. P-value <0.05 was considered statistically significant.

Results

Characterization of NC-Exo and BCa-Exo

TEM revealed that the vesicles from the plasma of NC subjects or BCa patients exhibited a “round-shaped” appearance (Figure 1A). Furthermore, data from NanoSight measurements showed that these vesicles were uniform in size (30-150 nm in diameter) (Figure 1B). Moreover, western blot analysis showed that exosomes in both groups were positive for the exosomal surface markers CD9, CD63, and Tsg101 (Figure 1C).

Expression profiles of miRNAs in BCa-Exo and expression of selected miRNAs in BCa plasma

The miRNA profile of NC-Exo and BCa-Exo was analyzed using a miRNA microarray. Volcano plot (Figure 2A) and cluster analysis (Figure 2B) revealed 8 miRNAs that were differentially
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expressed in BCa-Exo compared with NC-Exo, of which 1 was upregulated (hsa-miR-4644; fold change >2.0, P<0.05) and 7 miRNAs were downregulated (hsa-miR-4298, hsa-miR-4499, hsa-miR-4669, hsa-miR-6756-5p, hsa-miR-6809-5p, hsa-miR-6812-5p, and hsa-miR-7107-5p; fold change <-1.0, P<0.05). The fold change values of 8 selected miRNAs and their p-values are shown in Table 2.

To verify the results from miRNA array data, the top 3 miRNAs (hsa-miR-4499, hsa-miR-4669,
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Table 2. The most differentially expressed microRNAs in BCa-Exo

<table>
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<th>P value</th>
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and hsa-miR-4298; fold change >2.0 or <2.0, P<0.05) that were significantly upregulated and downregulated were selected and analyzed in plasma from 57 BCa patients and 25 normal controls by qRT-PCR analysis. Consistent with miRNA array data, qRT-PCR further confirmed that expression of hsa-miR-4298 and hsa-miR-4669 was significantly downregulated, whereas expression of hsa-miR-4644 was notably upregulated in most plasma samples from BCa patients when compared with the Normal group (Figure 2C). Furthermore, miR-4644 expression was upregulated along with the disease progressed to the advanced stage (Figure 2D). Conversely, UBIAD1 mRNA level was significantly downregulated in most plasma samples from BCa patients (Figure 2E). Moreover, a negative correlation between miR-4644 level and UBIAD1 mRNA level in BCa plasma samples was observed (Figure 2F).

miR-4644 promotes BCa cell proliferation and invasion

We next evaluated the biological roles of the most upregulated miRNA, miR-4644, in BCa cell proliferation and invasion. qRT-PCR results showed that miR-4644 expression was significantly higher in BCa cells (T24, J82, 5637, and UMUC3) than in SV-HUC-1 cells (Figure 3A). MTT assay demonstrated that the growth of both T24 and J82 cells was significantly facilitated following transfection with miR-4644 mimic (Figure 3B). Transwell invasion assays indicated that the number of invaded cells in T24 and J82 cells transfected with miR-4644 mimic was notably more than those transfected with mimic NC (Figure 3C). In contrast, miR-4644 inhibitor transfection significantly inhibited cell proliferation and invasion of T24 and J82 cells (Figure 3B, 3C).

miR-4644 directly targets and downregulates UBIAD1 expression

UBIAD1 has been shown to inhibit the proliferation of BCa cells [10]. Our bioinformatics analysis (Targetscan) revealed that UBIAD1 was a putative target of miR-4644. Thus, we examined whether miR-4644 regulated BCa cell behaviors by targeting UBIAD1. In turn, RNA pull-down and a luciferase reporter assay were performed to analyze the interaction between miR-4644 and UBIAD1. Data from RNA pull-down assay confirmed the direct interaction between miR-4644 and UBIAD1 (Figure 4A). Furthermore, Data from luciferase reporter assay revealed that miR-4644 mimic notably reduced WT luciferase activity to less than 40% of levels in mimic NC-transfected cells, suggesting that UBIAD1 3'-UTR was indeed directly targeted by miR-4644. Furthermore, the activities of the Mut1 construct and the Mut2 construct were over 50% of that of the control. Moreover, Mut1+ Mut2 construct disrupting both binding sites completely blocked the downregulation of luciferase activity (Figure 4B). These results indicated that miR-4644 targeting to UBIAD1 mRNA was through binding to both sites on the 3'-UTR.

Furthermore, results from qRT-PCR (Figure 4C) and western blot (Figure 4D) demonstrated that the mRNA and protein levels of UBIAD1 in T24 cells were significantly decreased following transfection with miR-4644 mimic, whereas markedly increased upon transfection with miR-4644 inhibitor. Consistent results were observed in J82 cells (Figure 4E, 4F).
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These data suggested that miR-4644 directly targeted and downregulated UBIAD1 expression.

UBIAD1 inhibits BCa cell proliferation and invasion

To gain further insight into the biological role of UBIAD1 in BCa, we assessed the effect of UBIAD1 overexpression and knockdown on BCa cell proliferation and invasion. MTT assay demonstrated that UBIAD1 overexpression by pcDNA3.1-UBIAD1 transfection significantly suppressed the growth of both T24 and J82 cells (Figure 5A). Transwell invasion assays indicated that the number of invaded cells in T24 and J82 cells transfected with pcDNA3.1-UBIAD1 was notably less than those transfected with vector control (Figure 5B). In contrast, UBIAD1 knockdown significantly facilitated cell proliferation and invasion of T24 and J82 cells (Figure 5A, 5B).

Figure 3. miR-4644 promotes BCa cell proliferation and invasion. (A) qRT-PCR analysis of miR-4644 in human bladder epithelial cell line SV-HUC-1 and human BCa cell lines (T24, J82, 5637, and UMUC3). Cell proliferation (B) and invasion (C) in T24 and J82 cells transfected with miR-4644 mimic, miR-4644 inhibitor, and corresponding controls were evaluated by MTT assay and Transwell invasion assay, respectively. *P<0.05, **P<0.01 vs. SV-HUC-1 or mimic NC, #P<0.01 vs. inhibitor NC. Data are expressed as the mean ± standard deviation (n=3).
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miR-4644 facilitates BCa cell proliferation, migration, and invasion by targeting UBIAD1

Finally, we tested whether UBIAD1 was involved in the miR-4644 mimic-induced BCa cell proliferation, migration, and invasion. MTT assay (Figure 6A) and Transwell assay (Figure 6B, 6C) revealed that UBIAD1 overexpression effectively attenuated the miR-4644 mimic-mediated promotion of cell proliferation, migra-
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Antagomir-4644 suppressed tumorigenesis in mouse xenografts

To ascertain the functional role of miR-4644 in vivo, subcutaneous tumors formed by T24 cells in BALB/c nude mice were administered miR-4644 antagonir once every 3 days for 2 weeks. Importantly, in vivo xenograft tumor growth in nude mice was effectively inhibited by intratumor injection of miR-4644 antagonir, as evidenced by a significant decrease in tumor weight (Figure 7B) and tumor volume (Figure 7A and 7C) in the miR-4644 antagonir group relative to the antagonir-NC group. Expectedly, intratumor injection of miR-4644 antagonir significantly decreased miR-4644 expression in tumors (Figure 7D). However, the mRNA and protein levels of UBIAD1 in tumors were notably increased following intratumor injection of miR-4644 antagonir (Figure 7E and 7F).
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A

B

C

mimic NC+Vector
mimic NC+UBIAD1
mimic NC+Vector
mimic NC+UBIAD1

Vector+miR-4644 mimic
miR-4644 mimic+UBIAD1
Vector+miR-4644 mimic
miR-4644 mimic+UBIAD1

Vector+miR-4644 mimic
miR-4644 mimic+UBIAD1
Vector+miR-4644 mimic
miR-4644 mimic+UBIAD1

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Discussion

To the best of our knowledge, this was the first report demonstrating that miR-4644 was upregulated in plasma from BCa patients and that miR-4644 promoted BCa progression via targeting UBIAD1.

Exosomes could serve as a source of diagnostic and prognostic biomarkers for multiple cancers [15], one of which the most exciting marker for cancer diagnosis is miRNA [4, 16]. In this study, we performed miRNA microarray in exosome samples from the plasma of BCa patients to analyze differentially expressed miRNAs. Our microarray results showed that the only upregulated miRNA in BCa-Exo was hsa-miR-4644, which was further confirmed by qRT-PCR to be upregulated in plasma of BCa patients. However, more research is warranted to elucidate the potential of exosomal miR-4644 as a biomarker for predicting the clinical behavior of BCa.

Dysregulation of miRNAs is associated with the progression of BCa [1, 6, 7, 17]. For example, Zhang et al. [7] observed that miR-154 expression was notably decreased in BCa tissues and cell lines, and acted as a tumor suppressor in BCa by directly targeting autophagy-related gene 7 (ATG7). MiR-4644 was a recently identified miRNA, whose functions have not been characterized. A previous study suggested that miR-4644 in salivary exosome could be used as a potential biomarker for pancreatobiliary tract cancer [9]. However, until recently, there were few studies concerning miR-4644. In the present study, we selected miR-4644 to examine its biological function in BCa. Our in vitro gain- and loss- of function assays suggested that miR-4644 promoted BCa cell proliferation, migration, and invasion. Furthermore, antagomir-4644 suppressed tumorigenesis in mouse xenografts. These findings demonstrated the pro-tumoral role of miR-4644 in BCa, which merits further investigation.

UBIAD1 protein has been identified as a tumor suppressor in BCa [18]. Recently, Xu et al. [10] suggested that UBIAD1 suppressed the BCa cell proliferation by regulating H-Ras intracellular trafficking via interaction with the C-terminal domain of H-Ras. Consistent with this, our findings revealed that UBIAD1 overexpression significantly inhibited, whereas UBIAD1 knockdown facilitated BCa cell proliferation and invasion. Using luciferase reporter assay, we confirmed that UBIAD1 was a target of miR-4644, which can negatively regulate UBIAD1 mRNA and protein levels. More significantly, the restoration of UBIAD1 in miR-4644-overexpressed BCa cells rescued the promoting effects of miR-4644, indicating that miR-4644 promoted BCa cell proliferation and invasion via targeted silencing of UBIAD1.

In conclusion, we demonstrated that miR-4644 was upregulated in plasma of BCa patients and its derived exosomes. Furthermore, miR-4644 promoted BCa progression by targeting UBIAD1. Our findings contribute to a
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Figure 7. Antagomir-4644 suppresses tumorigenesis in mouse xenografts. The BALB/c nude mice were injected subcutaneously with T24 cells and then administered PBS (control), antagomir-NC or miR-4644 antagomir via intratumoral injection. After 6 weeks, the tumors were exercised. (A) Images of representative tumors excised from mice. (B) Tumor weight. (C) Tumor volume (mm$^3$) was calculated every week. qRT-PCR analysis of miR-4644 (D), qRT-PCR analysis of UBIAD1 mRNA level (E), and western blot analysis of UBIAD1 protein level (F) in tumor tissues excised from mice in each group. **P<0.01 vs. antagomir-NC. N=6 in each group.
better understanding of the molecular mechanisms occurring in bladder tumorigenesis and progression.

Acknowledgements

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Disclosure of conflict of interest

None.

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