miR-92b targets DAB2IP to promote EMT in bladder cancer migration and invasion

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Abstract. Muscle-invasive or metastatic bladder cancer (BCa) has a very poor prognosis; however, its mechanisms remain largely unknown. Previous studies have discovered multiple microRNAs (miRs) that are involved in BCa progression and regarded as potential biomarkers or therapeutic targets. In this study, we demonstrated that miR-92b could uniquely promote cell migration and invasion of BCa cells, but had no effect on cell proliferation. Mechanistically, our data provided evidence to verify that miR-92b was able to directly target DAB2IP, a well-known tumor suppressor, and inhibit epithelial-mesenchymal transition of BCa cells. Moreover, the increased expression levels of miR-92b were negatively correlated with DAB2IP, and predicted poor prognosis of patients with BCa. Overall, this study reveals a new promising biomarker and its mechanisms contributing to BCa invasion or metastasis.

Introduction

Bladder cancer (BCa) is one of the most common malignant diseases around the world. In the United States, the estimated new cases in 2015 were 74,000, and the deaths caused by BCa about 16,000. Statistical data from 2004 to 2010 in the United States showed that, the five-year survival rate for localized BCa was 69%, for regional BCa 34%, but for distant BCa only 6%, which means distant metastasis is the main cause of death in BCa (1). Thus, further understanding of the mechanism leading to BCa invasion and metastasis is still needed.

MicroRNAs (miRs) are single-strand small non-coding RNAs which take part in different processes of biological evaluation. During cancer progression, miRs not only affect cancer cell behavior such as proliferation, migration and invasion, but are also used to predict the prognosis of patients. A growing number of studies have discovered the dysregulated expression of miRs and the underlying mechanisms in BCa invasion or metastasis (2). For example, our previous study demonstrated that tumor-suppressive miR-145 could inhibit the invasion of BCa cells by targeting P21-activated kinase 1 (PAK1) (3). On the other hand, another miR, miR-92b, acts as an oncogene in certain malignancies including glioma, non-small cell lung cancer, and oral squamous cell carcinoma (4-8); however, its role in BCa remains poorly understood.

DAB2IP, a member of the RAS-GTPase-activating protein (RAS-GAP) family (9), is downregulated in multiple cancer types, including BCa. Our and other previous studies have shown that DAB2IP loss in BCa tissues is not only associated with the intravesical pirarubicin chemoresistance, but also facilitates tumor invasion and metastasis (10,11). More importantly, DAB2IP may be a promising biomarker to predict the prognosis of patients with BCa (12). However, the mechanism of DAB2IP downregulation in BCa is not yet clarified.

In this study, we demonstrated that miR-92b could significantly promote cell migration and invasion of the BCa cell line 5637, but had no effect on cell proliferation. Furthermore, our data provided evidence to verify that miR-92b could inhibit epithelial-mesenchymal transition (EMT) of BCa cells. Mechanistically, we showed that the tumor suppressor, DAB2IP, is a direct target of miR-92b. Indeed, restoration of DAB2IP expression could reverse all these biological effects of miR-92b in BCa cells. Moreover, we analyzed The Cancer Genome Atlas (TCGA) database and found that the expression level of miR-92b in BCa tissues was higher than normal, and there was a negative correlation between miR-92b level and DAB2IP expression. Importantly, miR-92b upregulation alone or combination with DAB2IP downregulation could predict a lower recurrence-free survival (RFS) of patients with BCa. Taken together, these results suggest that miR-92b acts as a critical onco-miR in BCa progression.

Materials and methods

Cell culture and transfection. The BCa cell line 5637 and human embryonic kidneys (HEK) cell line 293T were main-
The 5637 cells were cultured in RPMI-1640 and 293T were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂.

miR-92b mimic, miR-negative control (miR-NC) mimic, miR-92b inhibitor and miR-NC inhibitor were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). DAB2IP cDNA and its vector, DAB2IP shRNA and its scrambled shRNA have been described in our previous studies (11). Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

**Real-time quantitative PCR (RT-qPCR).** Total RNA was isolated from cells using TRIzol reagent (Life Technologies, Rockville, MD, USA). For miRNA RT-qPCR, miScript II RT kit (Qiagen, Valencia, CA, USA) was used for reverse transcription, and U6 was used as an internal control. The primer sequence for miR-92b was 5'-TAT TGC ACT CGT CCC GGC CTC C-3' [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China]. Reverse transcription for other genes was performed in our previous studies (11). Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

**Wound healing assay.** Wounds were scratched using a 200-µl pipette tips when transfected cells in six-well plate were grown to 100%, and the culture medium was changed to serum-free medium. The wound closure was monitored by inverted microscope after 24 h.

**Transwell migration and invasion assay.** The 5637 cells were digested 24 h later after transfection. For migration assay, 5x10⁴ cells in 200 µl serum-free medium were seeded in the upper chamber of the Transwell without Matrigel. For invasion assay, 1.2x10⁵ cells in 200 µl serum-free medium were seeded in the upper chamber of the Transwell with Matrigel. The lower chamber was filled with 600 µl medium with 20% FBS. After 24-h incubation at 37°C with 5% CO₂, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of migrated or invaded cells was counted using a microscope in three random fields.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.** The 5637 cells were digested 24 h after transfection, and 2x10³ cells in 200 µl complete medium were seeded into 96-well plates/well, then the cells were incubated for 24, 48, 72 and 96 h before 20 µl MTT was added to each well and incubated for another 4 h in 37°C. Then the medium was discarded and 150 µl DMSO was added, after 10 min of shaking, the absorbance of 490 nm was measured using the microplate autoreader (Bio-Tek Instruments, Winooski, VT, USA).

Western blot analysis. Cells were washed with cold PBS and then lysed in RIPA buffer containing protease inhibitor. Then total protein was loaded into 10% SDS-PAGE gel and blotted onto nitrocellulose membranes. The membrane was blocked with 5% skim milk for 1 h, then incubated with primary antibodies against DAB2IP (Abcam, Cambridge, UK), E-cadherin, N-cadherin, vimentin, matrix metalloproteinase (MMP) 2 and MMP9 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) overnight at 4°C. After washing with TBST three times, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Then the bands were visualized by ECL system (Bio-Rad Laboratories, Inc.). Loading differences were normalized using a monoclonal GAPDH antibody (KangChen Bio-tech, Inc., Shanghai, China).

Dual-luciferase reporter assay. Wild-type (WT) and mutation (MUT) of DAB2IP 3'-UTR reporter plasmids were designed and obtained from GenePharma (Shanghai, China). To perform the dual-luciferase reporter assay, WT or MUT DAB2IP 3'-UTR plasmids were transfected into 293T cells with miR-92b mimic or inhibitor together using Lipofectamine 2000. After 24-h incubation, luciferase assay was carried out using the dual-luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

### Table I. Primers and sequences used in this study.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primers (5'-3')</th>
<th>Reverse primers (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>ATGGGGAAGGTGAAAGTCGG</td>
<td>GACGGTGCCATGGGAATTTGC</td>
</tr>
<tr>
<td>CDH1</td>
<td>TGCCCAAGAAATGAAAAAGG</td>
<td>GTGTAATGGCAGATCGGTTC</td>
</tr>
<tr>
<td>CDH2</td>
<td>ACAGTGGCCACCTAACAAGG</td>
<td>CCGAGATTGGGTGATAATG</td>
</tr>
<tr>
<td>VIM</td>
<td>GAGAACTTGGCCTGGAAGC</td>
<td>GTCCTCCTGAATGGTCCCAATC</td>
</tr>
<tr>
<td>MMP2</td>
<td>GATACCCCTTTTGAGGTAGGA</td>
<td>CCTTCTCCAAAGGTCCCATGC</td>
</tr>
<tr>
<td>MMP9</td>
<td>CATTTCGACGATGACGGTTGT</td>
<td>CCGGTTAGAGTCTCTGCC</td>
</tr>
<tr>
<td>SNAI1</td>
<td>GACACTATGGCCCGCTTCTT</td>
<td>TCCTGTGATTTGAGGTCTCCAGT</td>
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<tr>
<td>SNAI2</td>
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<td>CTCAGATTTGACCTGTCTGCAAATG</td>
</tr>
<tr>
<td>ZEB1</td>
<td>GAAAGTGATCCACGCAATGGAA</td>
<td>TTGGCCGCGGTGATAACAGAG</td>
</tr>
<tr>
<td>ZEB2</td>
<td>AATGCAAGAAGGTGAGAACAGG</td>
<td>CTGCTGATGTCGAACTTGAAGA</td>
</tr>
<tr>
<td>TWIST1</td>
<td>GTCGGCAGTCTTACGAGGAG</td>
<td>GCTTGAGGGTCTGATCTTGTCT</td>
</tr>
</tbody>
</table>

Note: The table includes the forward and reverse primers used in the study, with GAPDH used as both an internal control and a reference gene.
Bioinformatics. miRNA expression and gene expression data in BCa tissues were downloaded from TCGA database (https://genome-cancer.ucsc.edu/proj/site/hgHeatmap/) at February 24, 2015, the data files were used to analyze the expression levels of miR-92b, DAB2IP, and their correlation with the overall survival (OS) and RFS of patients.

Statistical analysis. GraphPad Prism version 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze the difference between experimental groups. Quantitative data are presented as mean ± SD, and the differences between two groups were compared by the two-tailed Student's t-test. A P<0.05 was considered to be statistically significant.

Results

miR-92b promotes BCa cell migration and invasion in vitro. To explore the role of miR-92b in BCa, we transfected the BCa cell line 5637 with miR-92b mimic or inhibitor and examined its biological function in BCa. As showed in Fig. 1A, RT-qPCR assay detected the change of miR-92b expression in the 5637 cells 48 h after transfection. Subsequently, miR-92b upregulation could significantly enhance the wound healing
rate; whereas, miR-92b inhibitor decreased this rate (Fig. 1B). Moreover, the Transwell migration and invasion assay further proved that miR-92b promoted the ability of migration and invasion in 5637 cells, but downregulation of miR-92b abolished cell migration and invasion (Fig. 1C; P<0.001). However, similar treatment with miR-92b mimic or inhibitor had no effect on cell proliferation by MTT assays (Fig. 1D), indicating a unique role of miR-92b in BCa invasion and metastasis, but not tumor growth.

**miR-92b promotes EMT in BCa cells.** EMT is well-known to play an important role in tumor invasion and metastasis by endowing cells with a more motile and invasive phenotype (13,14). Both transcriptional and post-transcriptional regulatory mechanisms contribute to this complex network during the EMT process, and substantial evidence demonstrates that miRs are crucial regulators of this network (15). Herein, we further demonstrated that miR-92b mimic or inhibitor had no effect on cell proliferation by MTT assays (Fig. 1D), indicating a unique role of miR-92b in BCa invasion and metastasis, but not tumor growth.

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**DAB2IP is a direct target of miR-92b.** Mechanically, miR works mainly by binding to the 3′-UTR (un-translated region) of its target mRNA, by which miR degrades the mRNA or suppresses its translation. To elucidate the target gene of miR-92b in BCa, we searched TargetScan and PicTar, and both tools predicted DAB2IP as a potential target of miR-92b. RT-qPCR and western blot assays were performed, and demonstrated that miR-92b downregulated both DAB2IP mRNA and protein levels (Fig. 3A and B). Also, by co-transfection the
reporter plasmid carrying WT DAB2IP 3'-UTR with miR-92b mimic or inhibitor in 293T cells, we found that miR-92b significantly reduced its luciferase activity. In contrast, this suppressive effect was abolished when the miRNA binding sequence in DAB2IP 3'-UTR was mutated (Fig. 3C). These results suggest that DAB2IP is a direct target of miR-92b.

DAB2IP mediates the biological effects of miR-92b in BCa cells. To determine whether DAB2IP is critical for cell migration and invasion of BCa cells by miR-92b, we restored or knocked down DAB2IP expression in miR-92b mimic- or inhibitor-transfected 5637 cells. Indeed, as shown in Fig. 4A, DAB2IP overexpression could abolish the increased wound healing rate induced by miR-92b in the 5637 cells. In contrast, knockdown of DAB2IP by shRNA restored the ability of wound healing in the 5637 cells transfected with miR-92b inhibitor. Moreover, Transwell migration and invasion assays showed similar results after manipulation of DAB2IP expression levels (Fig. 4B and C; P<0.001, P<0.01 and P<0.05). Thus, these data demonstrate that DAB2IP mediates the biological functions of miR-92b in BCa as an important downstream target.

DAB2IP reverses the phenotype of EMT induced by miR-92b. Our recent studies have discovered the tumor-suppressive roles of DAB2IP in the regulation of cell apoptosis, survival (16), autophagy (17), DNA repair (18), EMT (19) and cancer stem cells (CSCs) (20), all of which may contribute to the tumor initiation and progression. Indeed, as shown in Fig. 5, restoration of DAB2IP in miR-92b mimic-transfected 5637 cells dramatically increased E-cadherin expression, but decreased vimentin and MMP9 expression, however, knockdown of DAB2IP in miR-92b inhibitor-transfected 5637 cells resulted in a decrease of E-cadherin expression but an increase of vimentin and MMP9 expression. These data support that miR-92b could target DAB2IP and induce EMT for BCa migration and invasion.

Expression and clinical significance of miR-92b in patients with BCa. To evaluate the role of miR-92b in clinic, we searched the TCGA database to analyze its expression and clinical significance. First, we found 19 paired BCa and adjacent normal tissues with available miR-92b and DAB2IP expression data. As shown in Fig. 6A, the expression of miR-92b was higher in BCa tissues than normal, while the expression of DAB2IP was lower in BCa tissues. Also, there was a negative correlation between miR-92b and DAB2IP expression. Importantly, we further observed a significant association between miR-92b level and RFS of patients with BCa. Although there was no statistical correlation between miR-92b expression levels and the OS of patients with BCa (Fig. 6B and C, left panel), miR-92b upregulation alone or combination with DAB2IP downregulation could specifically predict a lower RFS of patients (Fig. 6B and C, right panel). These clinical data suggest miR-92b and DAB2IP as good prognostic markers for patients with BCa.

Discussion

BCa is a major cause of morbidity and mortality worldwide, in which muscle-invasive BCa (MIBC) with a high frequency of
metastasis has very poor prognosis for patients. Understanding and clarifying these mechanisms of cancer metastasis will help to develop new therapeutic strategies and improve patient survival. Recently, several whole-genome studies provided a comprehensive landscape of molecular alterations in BCa, especially intrinsic basal and luminal MIBC subtypes associated with different chemotherapy sensitivity or prognosis were well characterized (21,22). In our previous studies, we developed unique isogenic T24-t sublines and discovered a complex regulatory network contributing to BCa metastasis.
For example, HIF-1α-regulated MMP-1 expression, Slug-mediated cadherin switch, and PI3K/Akt/GSK3β/β-catenin signaling cascade-initiated ZEB1 transcription all endowed BCa cells with the mesenchymal and CSC-like properties (23-25). In the present study, we further explore the roles of miRs, another crucial regulator in this network, and first provide the evidence to show that miR-92b induces EMT in BCa.

miRs have been reported to play critical roles in different cancer progression, including BCa. For example, miR-100 targeted the fibroblast growth factor receptor 3 (FGFR3) to maintain BCa cell viability under conditions of hypoxia stress (26). Our previous study also demonstrated that the tumor suppressive miR-145 inhibited invasion of BCa cells via targeting PAK1 (3). Herein, we reported the function of another important miR, miR-92b, in BCa migration and invasion via targeting DAB2IP.

DAB2IP, also named ASK1-interacting protein 1 (AIP1), acts as a crucial tumor suppressor in different human malignancies, such as prostate cancer (27), breast cancer (28), lung cancer (29), gastrointestinal tumor (30), liver cancer (31), pancreatic cancer (32), medulloblastoma (33) and esophageal carcinoma (34). It plays vital roles in different aspects of biological activities, including cell apoptosis or survival (16), autophagy (17), DNA repair (18), EMT (19) and CSC (20), all of which may contribute to the tumor initiation, growth, metastasis and therapeutic resistance. Until now, we have known that DAB2IP gene transcription is usually regulated by epigenetic mechanisms, such as DNA methylation and histone modification (35). Also, DAB2IP expression could be modulated by Skp2-mediated proteasome degradation (36). Recently, several studies demonstrated that different miRs could target DAB2IP 3’-UTR and suppress its expression. For example, Xu et al reported that miR-889 promoted proliferation of esophageal squamous cell carcinomas through DAB2IP (34), while another study showed that miR-32 induced radioresistance by targeting DAB2IP and regulating autophagy in prostate cancer (37). In this study, we further revealed that DAB2IP could also be targeted by miR-92b in BCa and facilitated EMT for tumor cell migration or invasion, indicating a complex upstream regulation for DAB2IP in different cancer types.

The importance of miR-92b in cancer development has been established by other studies. In neuroblastoma, miR-92b could be regulated by MYCN, which then inhibits secretion of the tumor suppressor DKK3 (38). By directly targeting Nemo-like kinase and DKK3, miR-92b controls glioma cell proliferation, invasion and apoptosis (6). In non-small cell lung cancer, miR-92b targets PTEN and RECK to regulate cell growth and cisplatin chemosensitivity (39). Also,

![Figure 6](image_url)
miR-92b functions as a potential oncogene by targeting Sma3 in glioblastomas (5). Moreover, in hepatic progenitors, aberrant expression of miR-92b is associated with proliferation increase and differentiation arrest (40). We are the first to show the role of miR-92b in BCA cells based on both gain-of-function and loss-of-function strategies. In addition, our analysis of clinical data from TCGA database also showed the increased miR-92b levels along with decreased DAB2IP expression in BCA specimens predicted a poor RFS rate of patients, which indicates that miR-92b may be another good prognostic molecular marker for patients with BCA in future.

Taken together, our studies revealed the critical role of miR-92b in modulating migration and invasion of BCA cells in vitro, in which its direct downstream target gene DAB2IP regulates the EMT phenotype. Importantly, our findings provide a molecular and clinical basis highlighting miR-92b as a potential biomarker in predicting the outcome of BCA.

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References