Abstract. MicroRNAs (miRs) may have promotive or suppressive roles in various human cancers types, but the molecular mechanisms underlying the role of miR-124 in bladder cancer (BC) progression have remained largely elusive. In the present study, it was observed that miR-124 was significantly downregulated in BC tissues compared with that in adjacent non-neoplastic tissues. Furthermore, its expression was also reduced in several human BC cell lines (T24, HT-1376 and 5637) compared with that in the normal bladder epithelial SV-HUC-1 cell line. A low expression of miR-124 in BC patients was significantly associated with advanced malignancy and a poor prognosis. Caveolin 1 (CAV1) was identified as a novel target gene of miR-124, and the expression of CAV1 was negatively regulated by miR-124 in T24 cells. Furthermore, CAV1 was identified to be significantly upregulated in BC tissues and cell lines, and a negative correlation was observed between the expression of miR-124 and CAV1 in BC tissues. Furthermore, restoration of miR-124 expression significantly inhibited the proliferation, migration and invasion of T24 cells, and these effects were impaired following overexpression of CAV1. Taken together, the present results demonstrate that miR-124 has a suppressive role in the proliferation, migration and invasion of BC cells by targeting CAV1, which suggests that miR-124 is a potential therapeutic candidate for BC.

Introduction

Bladder cancer (BC) is a major tumor of the genitourinary tract and remains the cause of a considerable proportion of cancer-associated morbidity and mortality (1,2). Although great efforts have been made with respect to surgical resection combined with radiotherapy and chemotherapy, the overall survival time of patients with advanced BC remains unsatisfactory (3,4). In recent years, accumulating evidence has indicated that numerous oncogenes and tumor suppressors are dysregulated during the development and malignant progression of BC, some of which have been suggested to be potential therapeutic targets for BC (5-7).

MicroRNAs (miRs) are a class of non-coding RNAs of 18-25 nucleotides in length that act as key regulators of gene expression through binding to the 3' untranslated region (UTR) of their target mRNAs, eventually resulting in mRNA degradation or inhibition of protein translation (8-10). Various miRs have been implicated in a variety of physiological and pathological processes, including development and differentiation, cell proliferation and apoptosis, angiogenesis, cell motility and tumorigenesis (11-13). Furthermore, deregulated miRs have been implicated in various human cancer types, including miR-10 (14), miR-21 (15), miR-138 (16) and miR-576 (17), which have either promotive or suppressive roles in BC.

miR-124 has been demonstrated to act as a tumor suppressor in BC (18-20). It was reported to be frequently methylated in primary BC tissues in a tumor-specific manner (18). Xu et al (19) indicated that miR-124-3p inhibited the migration and invasiveness of BC cells by targeting Rho-associated protein kinase 1 (ROCK1). Wang et al (20) demonstrated that miR-124 exerted suppressive effects on cell proliferation, motility and angiogenesis of BC by targeting ubiquitin-like containing PHD and RING finger domains, 1 (UHRF1). In addition, Zhang et al (21) indicated that miR-124 inhibited BC growth by directly targeting cyclin D kinase (CDK4). However, the detailed regulatory mechanism of miR-124 in BC cells has remained to be fully elucidated.

Caveolin 1 (CAV1), a scaffolding protein, is the major component of the caveolae within plasma membranes that are present in various cell types (22). Increased expression of CAV1 was observed in high-grade BC, and CAV1 has been suggested to be a potential target for cancer prevention (22-24). However, the regulatory mechanism of CAV1 expression in BC remains elusive.

The aim of the present study was to investigate the underlying mechanism of the regulatory effects of miR-124 in BC progression.
Materials and methods

Tissue samples. The present study was approved by the Ethics Committee of the Third Xiangya Hospital, Central South University (Changsha, China). A total of 73 BC tissues and adjacent non-tumor tissues were collected at the Third Xiangya Hospital, Central South University (Changsha, China) between May 2010 and May 2011. Written informed consent was obtained from all patients. The clinicopathologic characteristics of the included BC patients are summarized in Table I. After surgical removal, tissues were immediately snap-frozen in liquid nitrogen.

Cell culture. The normal human bladder epithelial cell line SV-HUC-1 and the BC cell lines T24, HT-1376 and 5637 were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco’s modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% foetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂.

Cell transfection. T24 cells were transfected with miR-124 mimics (HmiR0126-MR04), scrambled miR mimics (miR-NC; CmiR0001-MR04), a miR-124 inhibitor (HmiR-AN0074-SN-10) or a negative control (NC) inhibitor (CmiR-AN0001-SN; all Guangzhou FulenGen Co. Ltd., Guangzhou, China). In another experiment, cells were co-transfected with miR-124 mimics and blank pcDNA3.1 vector (Yearchbio, Changsha, China) or with miR-124 mimics and the pcDNA3.1-CAV1 open reading frame plasmid (Yearchbio). All transfections were performed using Lipofectamine™ 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. Cells were then cultured for 48 h prior to use in the subsequent assays.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues and cell lines using TRIzol reagent (Thermo Fisher Scientific, Inc.). For the conversion of RNA into complementary (c)DNA, a RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania) was used according to the manufacturer’s protocol. To determine the expression of miR and mRNA, real-time PCR was performed using a PrimeScript® miRNA RT-PCR kit (Takara), respectively, and the Green RT-PCR kit (Takara), respectively, and the 2-ΔΔCT method (25). The primer sequences were as follows: CAV1 forward, 5'-GGC ACCCTAAAACACCTCAAC-3' and reverse, 5'-ATGCGGG TCAAATCTCTGTGTGC-3'; GAPDH forward, 5'-GGAGCG AGATCCCCTCCCAAT-3' and reverse, 5'-GGCTGTGTTGT CATACTTCTCATGG-3'; miR-124-3p forward, 5'-CGG GTAGCAGGTCTGCTAGT-3' and reverse, 5'-AAACCC CTCCTCTCGGAGTCT-3'; U6 forward, 5'-CTGCCTTCGG GCAGCACA-3' and reverse, 5'-AAGCCTTACAGGAAY YGGCT-3'.

Western blot analysis. Tissues and cells were lysed with cold radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.). The protein concentration was determined using a BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. The proteins (50 µg) were separated by 12% SDS-PAGE and were then transferred onto a polyvinylidene difluoride membrane (Thermo Fisher Scientific, Inc.). After incubation with PBS containing 5% non-fat milk at 4°C overnight, the membrane was incubated with rabbit antibodies against CAV1 (1:100; cat. no. ab2910) and GAPDH (1:50; cat. no. ab9485; both Abcam, Cambridge, MA, USA) at 4°C overnight. After washing in PBS containing Tween-20 (PBST) for 10 min, the membrane was incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000; cat. no. ab6721; Abcam) at room temperature for 40 min. After washing in PBST for 10 min, chemiluminescent detection was performed using a Novex™ ECL Chemiluminescent Substrate Reagent kit (Thermo Fisher Scientific, Inc.). The protein expression was analyzed using Image-Pro Plus software 6.0 (Media Cybernetics, Rockville, MD, USA) and is presented as the density ratio versus GAPDH.

MTT assay. T24 cells (10⁴ cells/well) were seeded in 96-well plates and cultured at 37°C for 0, 24, 48 or 72 h. Subsequently, T24 cells were incubated with MTT (0.5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C for 4 h. The cell supernatants were discarded and 150 mM dimethylsulfoxide (Sigma-Aldrich; Merck KGaA) was added to dissolve the formazan. The optical density was determined using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 570 nm.

Wound healing assay. T24 cells (10⁵ cells/well) were seeded in 6-well plates and cultured at 37°C to 100% confluence. Cells were scraped with a pipette tip to generate wounds. After washing in PBS, the cells were cultured in DMEM at 37°C for 48 h. The wound was observed and images were captured under a microscope (Olympus, Tokyo, Japan).

Transwell assay. For the cell invasion assay, 24-well plates with 8-µm pores and Matrigel-coated Transwell inserts (8-µm; BD Biosciences, San Jose, CA, USA) were used. T24 cells (150 µl, 10⁵ cells/ml) in serum-free DMEM were seeded in the upper chambers, and 750 µl DMEM containing 10% FBS was added to the lower chamber. After incubation for 48 h, the cells on the upper surface of the Transwell chamber were scraped off with cotton swabs. Invading cells were fixed with methanol at room temperature for 30 min, followed by staining with 1% crystal violet at room temperature for 30 min. Finally, images of the cells were captured under a microscope.

Bioinformatics prediction and dual-luciferase reporter assay. The putative target genes of miR-124 were predicted by TargetScan (www.targetscan.org), PicTar (pictar.mdc-berlin.de) and miRanda (www.microrna.org). The mutant type (MT) of the CAV1 3’UTR was constructed with a Quick-C hange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Subsequently, the wild-type (WT) or MT of the CAV1 3’UTR was constructed by PCR and inserted into the multiple cloning site in the psiCHECK vector (Promega Corp., Madison, WI, USA).
Madison, WI, USA). Next, T24 cells were co-transfected with miR-124 mimics or miR-NC and with the WT-CA V1-3'UTR or MT-CA V1-3'UTR plasmid using Lipofectamine™ 2000. The luciferase activity was examined using the Dual-luciferase Reporter Assay System (Promega Corp.) after transfection for 48 h according to the manufacturer's protocol. Renilla luciferase activity was normalized to firefly luciferase activity.

Statistical analysis. Values are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS 20 (IBM Corp., Armonk, NY, USA). Differences between two groups were analyzed using Student's t-test. The differences among >2 groups were analyzed using analysis of variance followed by Tukey's post-hoc multiple comparisons test. The Chi-square test was used to examine the association between gene expression and the clinical characteristics. The Wilcoxon signed-rank test was performed to assess the survival of patients with low and high miR-124 expression, and a log-rank test was also used. Pearson correlation analysis was performed to assess the correlation between miR-214 and CA V1 expression levels. P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of miR-124 in BC. In the present study, it was observed that the expression levels of miR-124 were markedly reduced in BC tissues compared with those in adjacent non-tumor tissues (Fig. 1A). Consistent with these results, miR-124 was also significantly downregulated in the T24, HT-1376 and 5637 BC cell lines compared with that in SV-HUC-1 normal human bladder epithelial cells (Fig. 1B). These results indicate that miR-124 is downregulated in BC.

The clinical significance of miR-124 expression in BC was then studied. Based on the mean expression value of miR-124, these BC patients were divided into the high expression group and the low expression group. Further investigation revealed that low expression of miR-124 was significantly associated with lymph node metastasis and advanced clinical stage in BC (Table I). Furthermore, the BC patients with low expression of miR-124 exhibited a shorter overall survival time compared with those with a high expression of miR-124 (Fig. 1C). These results suggest that the downregulation of miR-124 in BC patients may predict a poor prognosis.

CAV1 is a novel target gene of miR-124 in BC cells. The potential target genes of miR-124 were then studied. As presented in Fig. 2A, CAV1 is a putative target gene of miR-124. To confirm the targeting association between miR-124 and CAV1, a luciferase reporter plasmid containing WT-CAV1-3'UTR or MT-CAV1-3'UTR was generated (Fig. 2B). The results of the luciferase reporter gene assay indicated that the luciferase activity was significantly reduced in T24 cells that were co-transfected with the miR-124 mimics and the WT-CAV1-3'UTR luciferase reporter plasmid, which was eliminated by transfection of cells with the MT-CAV1-3'UTR
luciferase reporter plasmid (Fig. 2C). Accordingly, it was demonstrated that CAV1 is a target gene of miR-124 in T24 cells.

The effects of miR-124 on the expression of CAV1 in T24 cells were then assessed. T24 cells were transfected with miR-124 mimics or miR-NC. After transfection, the miR-124 levels were significantly increased in the miR-124 group compared with those in the miR-NC group (Fig. 3A). RT-qPCR and western blot analysis indicated that the mRNA and protein levels of CAV1 were significantly reduced in the miR-124 group compared with those in the miR-NC group (Fig. 3B and C). It was therefore indicated that miR-124 exerts a suppressive effect on CAV1 expression in T24 cells. To further confirm these results, T24 cells were transfected with a miR-124 inhibitor or an NC inhibitor. After transfection, the miR-124 levels were markedly reduced in the miR-124 inhibitor group compared with those in the NC inhibitor group (Fig. 3D). RT-qPCR and western blot results indicated that the
knockdown of miR-124 significantly increased the mRNA and protein levels of CAV1 in T24 cells compared with those in the NC inhibitor group (Fig. 3E and F). Therefore, miR-124 negatively regulates CAV1 expression in T24 cells by binding to the 3'UTR of CAV1 mRNA.

Upregulation of CAV1 is inversely correlated with miR-124 expression in BC. The expression of CAV1 in BC tissues was then assessed. RT-qPCR and western blot analysis demonstrated that the mRNA and protein levels of CAV1 were markedly increased in BC tissues compared with those
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in adjacent non-tumor tissues (Fig. 4A and B). Furthermore, CAV1 was also significantly downregulated in the T24, HT-1376 and 5637 BC cell lines compared with that in the SV-HUC-1 normal human bladder epithelial cells (Fig. 4C and D). Of note, an inverse correlation was observed between the CAV1 and miR-124 expression levels in BC tissues (Fig. 4E). Based on these results, the decreased expression of miR-124 may contribute to the upregulation of CAV1 in BC.

Restoration of miR-124 inhibits the malignant phenotypes of BC cells, which is attenuated by CAV1 overexpression. The present study further assessed the regulatory role of miR-124 regarding the malignant phenotypes of T24 cells. An MTT assay, wound healing assay and a Transwell assay demonstrated that overexpression of miR-124 significantly decreased the proliferation, migration and invasiveness of T24 cells (Fig. 5A-C), which suggests that miR-124 may have suppressive effects on the growth and metastasis of BC.

As CAV1 was indicated to be a target gene of miR-124 and its expression was negatively regulated by miR-124 in T24 cells, it was speculated that CAV1 may be involved in the miR-124-mediated malignant phenotypes of T24 cells. To test this hypothesis, T24 cells were co-transfected with miR-124 mimics and the pcDNA3.1-CAV1 expression plasmid. Cells that were co-transfected with a miR-124 inhibitor and the blank pcDNA3.1 vector served as the control group. As indicated in Fig. 6A and B, the mRNA and protein expression levels of CAV1 were significantly reduced in the miR-124+CAV1 group compared with those in the miR-124+blank group. Further investigation revealed that the proliferation, invasiveness and migration of T24 cells were significantly increased in the miR-124+CAV1
group compared with those in the miR-124+blank group. Therefore, the overexpression of CAV1 impaired the suppressive effects of miR-124 on the malignant phenotypes of T24 BC cells.

Discussion

The underlying molecular mechanisms of the role of miR-124 in BC progression have remained to be fully clarified. In the present study, it was observed that miR-124 was significantly downregulated in BC tissues compared with that in adjacent non-tumor tissues. Furthermore, its expression levels were also reduced in several human BC cell lines (T24, HT-1376 and 5637) compared with those in SV-HUC-1 normal bladder epithelial cells. A low expression of miR-124 in BC patients was significantly associated with advanced malignancy and a poor prognosis. CAV1, which is upregulated in BC, was identified as a novel target gene of miR-124 in T24 cells. Restoration of miR-124 expression significantly inhibited T24 cell proliferation, migration and invasion, while the overexpression of CAV1 impaired these suppressive effects of miR-124 on T24 cells.

Previous studies have demonstrated that miR-124 generally acts as a tumor suppressor in certain common human cancer types (26-28). For instance, miR-124 inhibits the proliferation of glioblastoma cells and induces differentiation of brain tumor stem cells (29). An et al (30) demonstrated that miR-124 inhibits glioma cell migration and invasion via the inhibition of ROCK1. Zhang et al (31) reported that miR-124 inhibits the proliferation, invasion, migration and epithelial-mesenchymal transition (EMT) of cervical carcinoma cells by targeting astrocyte-elevated gene-1. Of note, Huang et al (32) demonstrated that knockdown of miR-124 promoted neuroblastoma cell differentiation, cell cycle arrest and apoptosis, which suggests that it may have an oncogenic role in neuroblastoma. These dual roles of miR-124 are probably due to its different target genes in different cancer types. Furthermore, miR-124 was reported to be a tumor suppressor in BC, and several target genes, including UHRF1, CDK4 and ROCK1, have been identified in BC cells (19-21). The present study indicated that miR-124 was significantly downregulated in BC tissues and cell lines, which may be due to high levels of methylation (18). However, the clinical significance of miR-124 expression in BC has remained to be elucidated. In the present study, it was observed that the reduced expression of miR-124 in patients with BC was significantly associated with lymph node metastasis, an advanced clinical stage and a shorter survival time.
CAV1 was then identified as a target gene of miR-124 in BC T24 cells. CAV1, a scaffolding protein, is the major component of caveolae within plasma membranes in most cell types (33,34). CAV1 may link integrin subunits to the tyrosine kinase FYN, which is the initiating step in the coupling of integrins to the Ras-extracellular signal-regulated kinase pathway and the promotion of cell cycle progression (35,36). It has been reported that CAV1 was significantly upregulated in high-grade BC and that CAV1 expression is correlated with tumor grade and squamous cell differentiation of BC (23,24).

Kunze and Schlott (37) indicated a lack of hypermethylation of CAV1 in primary adenocarcinomas and signet ring cell carcinomas of the urinary bladder. In this study, it was also observed that the expression levels of CAV1 were markedly increased in BC tissues and cell lines compared with those in adjacent non-tumor tissues and SV-HUC-1 normal human bladder epithelial cells, respectively. Furthermore, the expression of CAV1 was inversely correlated with miR-124 expression in BC tissues, which suggests that the increased expression of CAV1 may be due to the reduced expression of miR-124 in BC. In
A lentiviral sponge for miRNA -21 diminishes migration, at least partly by directly targeting CA V1, which has an inhibitory role in BC cell proliferation, invasion and migration. These results confirm the present hypothesis that expression of CA V1 impaired the inhibitory effects of miR-124 upregulation on the proliferation, invasiveness and migration of T24 cells. These results confirm the present hypothesis that CA V1 acts as a downstream effector of miR-124 in BC cells and highlight the significance of the miR-124/CAV1 axis in BC.

In conclusion, the present study demonstrated that miR-124 has an inhibitory role in BC cell proliferation, invasion and migration, at least partly by directly targeting CAV1, which suggests that the miR-124/CAV1 axis may be a potential therapeutic target in BC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WZ and LH collected clinical samples and wrote the manuscript. YD, YZ and JW conducted the experiments and statistical analyses. BL designed the current study and revised this manuscript.

Ethical approval and consent to participate

The present study was approved by the Ethics Committee of Third Xiangya Hospital, Central South University (Changsha, China). Written informed consent was obtained from all patients.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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