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[Aaron Huang](#) , Wayne C. Waltzer , Michael Hung , [Frank Darras](#) , Adam M. Kressel , [Victor Romanov](#) *

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Article

Identification of circCIAO1(5) and circMALAT1 as Novel Biomarkers for Bladder Cancer Monitoring Based on the Binding to miR-101-3p

Aaron Huang, Wayne C. Waltzer, Michael Hung, Frank Darras, Adam M. Kressel and Victor Romanov *

Department of Urology, Renaissance School of Medicine, SUNY at Stony Brook, Stony Brook, NY 11794, USA

* Correspondence: victor.romanov@stonybrookmedicine.edu

Simple Summary

Bladder cancer is a significant malignancy characterized by high rates of recurrence and progression, highlighting the need for reliable non-invasive biomarkers. Urine represents an optimal source of biomarkers for bladder cancer surveillance. Circular RNAs are a class of non-coding RNAs with a stable, covalently closed structure generated by back-splicing are widely distributed in biological fluids. However, the identification and validation of clinically relevant circular RNAs remain labor- and time-intensive. This study aims to simplify circular RNAs selection and identify functionally relevant candidates. Circular RNAs can regulate gene expression by acting as microRNA sponges. Using database-based screening, we identified two circular RNAs with high binding affinity for miR-101-3p, a known tumor suppressor in bladder cancer. Sequestration of miR-101-3p by these circRNAs enhanced cancer-related activity by limiting its interaction with key oncogenes. Importantly, both circRNAs exhibited distinct expression patterns in urine from bladder cancer patients compared with controls, supporting their potential utility as non-invasive biomarkers for disease monitoring.

Abstract

Background and Objectives: Bladder cancer (BCa) is characterized by high rates of recurrence and progression, underscoring the need for reliable non-invasive biomarkers. Circular RNAs (circRNAs) are covalently closed non-coding RNAs generated by back-splicing and are stable in biological fluids, including urine. Increasing evidence implies circRNAs in BCa pathogenesis; however, identification of clinically relevant circRNAs remains labor-intensive. This study aimed to streamline circRNA selection and identify functionally relevant urinary circRNAs in BCa. **Methods:** Using a database-screening approach, we identified circRNAs with high predicted affinity to miR-101-3p, a tumor-suppressive microRNA in BCa. Candidate circRNAs were prioritized based on: (i) strong miR-101-3p binding potential; (ii) derivation from genes involved in BCa tumorigenesis; and (iii) origination from exonic or long non-coding RNA sequences. The potential contribution of Argonaute-2 (Ago2) binding sites to circRNA–miRNA complex stability was also evaluated. Expression levels were assessed in urine samples and BCa cell lines, and functional relevance was examined using molecular and cellular assays. **Results:** circCIAO1(5) and circMALAT1 fulfilled all prioritization criteria and exhibited distinct Ago2-binding site profiles. Both circRNAs were upregulated in urine from BCa patients and in aggressive BCa cell lines and showed differential expression between remission and recurrent disease. CircCIAO1(5) demonstrated higher-affinity binding to miR-101-3p, while RNA immunoprecipitation confirmed interactions of both circRNAs with miR-101-3p and Ago2. Functional assays revealed enhanced proliferation, motility, and invasion upon circRNA expression, consistent with miR-101-3p sequestration and derepression of miR-101-3p target oncogene-EZH2. **Conclusions:** circCIAO1(5) and circMALAT1 represent promising urinary biomarkers for BCa, illustrating the value of bioinformatics-guided circRNA discovery and significance of circRNA-mediated regulatory mechanisms in BCa biology.

Keywords: bladder cancer; CIAO1; MALAT1; circular RNA; liquid biopsy

1. Introduction

Bladder cancer (BCa) is a common and aggressive malignancy of the urinary tract, and current diagnostic tools such as cystoscopy and urine cytology remain either invasive or insufficiently sensitive, particularly for low-grade tumors. Therefore, developing reliable non-invasive biomarkers is a major clinical need. Circulating nucleic acids are promising candidates, because of relative simplicity and reliability of analysis [1–3]. In recent years, circular RNAs (circRNAs), a class of covalently closed non-coding RNAs generated through back-splicing of precursor mRNAs, have emerged as promising biomodulators in cancer research. Their high stability, tissue-specific expression, and presence in body fluids such as urine make them attractive candidates for biomarker development [4–10]. Functionally, many circRNAs act as microRNA (miRNA) sponges, modulating the activity of miRNAs and thereby regulating downstream gene expression [11–15]. Dysregulated circRNAs have been implicated in various aspects of cancer biology, including proliferation, invasion, metastasis, and therapy resistance [4,6,7]. Among numerous miRNAs involved in tumorigenesis, miR-101-3p has been recognized as a tumor suppressor in several cancers, including BCa. It regulates the expression of oncogenes such including EZH2, thereby influencing cellular proliferation, motility, and epithelial–mesenchymal transition[16–18]. Downregulation of miR-101-3p has been associated with more aggressive tumor behavior and a poorer prognosis in BCa. CircRNAs could bind and sequester miR-101-3p, therefore playing a significant role in BCa progression [18–20]. We employed a bioinformatic strategy to identify circRNAs originated from BCa-related parental genes and with strong binding affinity for miR-101-3p.

Selected candidates were differed in their genomic origins: circCIAO1(5) is generated from exon 5 of the *CIAO1* protein-coding gene, whereas circMALAT1 originates from the *MALAT1* long non-coding RNA (LncRNA), whose biological activity is mediated through RNA-based interactions.[21–23]. CircRNAs derived from exon and lnc-RNAs more likely function as miRNA sponge. The presence of Ago binding sites, short RNA sequences on circRNAs that are complementary to the “seed region” of specific miRNAs, suggests that Ago2 may play a role in circRNA action. Although, circRNA-Ago interaction is primarily mediated via the miRNA, not direct Ago–RNA binding, and number of Ago sites itself will not increase direct interaction with microRNA, but it can increase functionality of circRNA -microRNA interactions not directly. Ago2 acts as a bridge and is also capable of recruiting other Ago proteins that may play a role in circRNA action [24–26]. Therefore, 2 selected circRNA candidates in addition to other differences possessed distinct numbers of Ago binding sites. Both were examined for their differential expression in urine samples of BCa patients and healthy individuals, as well as in BCa cell lines with varying tumorigenic potential. Additionally, we assessed their ability to bind miR-101-3p in vitro, to regulate oncogenic pathways, and to influence malignant cellular phenotypes.

The present study aimed to assess the potential of circCIAO1(5) and circMALAT1 as non-invasive urinary biomarkers for BCa and to elucidate their mechanistic roles in BCa progression through miR-101-3p sponging. Furthermore, we examined how the binding affinity to the target miRNA and the number of Ago2 binding sites influence the functional properties of these circRNAs. A deeper understanding of the circRNA-miRNA interplay may not only facilitate the development of reliable diagnostic tools but also provide novel insights into the molecular mechanisms driving BCa pathogenesis.

2. Materials and Methods

2.1. Selection of Potential Candidates from Databases

Potential circRNA targets of miR-101-3p were identified using the *starBase* database (miRNA–circRNA interaction module) [27]. CircRNAs with a Target-Directed miRNA Degradation (TDMD) score greater than 1.1 were considered high-affinity binders. Among these, circRNAs whose parental genes exhibited differential expression between bladder cancer (BCa) tissue and normal urothelium (based on the *starBase* Pan-Cancer dataset, The Cancer Genome Atlas (TCGA) and published data) and originated from exons or lnc-RNA were selected as candidates.

To further investigate the potential influence of number of Ago2 binding sites on circRNA function, two circRNAs meeting these criteria were chosen for detailed analysis: circCIAO1(5) (containing 3 Ago2 binding sites) and circMALAT1 (containing 88 Ago2 binding sites).

2.2. Cell Culture

Human BCa cells (T24 and 5637) and human uroepithelial immortalized UROtsa cells were obtained from ATCC (Manassas, VA, USA). T24 and 5637 cells were cultivated in RPMI 1640 medium (Gibco, USA) containing 10% fetal bovine serum, and UROtsa cells were grown in DMEM medium (Gibco, USA) containing 10% FBS[28]. All cells were cultured at 37°C in a 5% CO₂ incubator.

2.3. Patient Specimens

Urine samples and 8 matched tumors were collected from 145 patients diagnosed with BCa from March 2022 to September 2025. In addition, urine specimens were obtained from 25 healthy donors from March 2022 to August 2025. Ninety-three of these patients were male. The age range of patients and healthy volunteers was 28-95 years. The pathological types were confirmed by professional pathologists. Written informed consent was acquired from all patients, and this study was approved by the IRB committee of Stony Brook School of Medicine.

2.4. RNA Extraction, Reverse Transcription, and RT-qPCR

Total RNA from tissues, cells and, in ten cases, from pelleted circulated cells in liquid samples was extracted with TRIzol Reagent (Invitrogen, USA) following the manufacturer’s instructions. RNA from urine was extracted with Urine Cell-free circulating RNA kit (Norgen Biotech, Canada) or by using the protocol for nucleic acid preparation (CleanNA, The Netherlands) adjusted in our laboratory for the isolating of free nucleic acids from urine. Briefly: (i) urine was centrifuged at 3000Xg for 10 min and supernatant was used for sample preparation; (ii) 20 µl of magnetic beads (CleanNGS) and 3 ml of Buffer K (Norgen Biotech, Canada) were added to 15 ml of urine and incubated on rocking platform for 30 min at RT; (iii) beads with bound nucleic acid were washed 5 times with 1 ml of 75% EtOH; (iv) nucleic acids were eluted with 30 ul of water or (v) RNA was then purified using miRNeasy Mini Kit (QIAGEN, USA) and eluted in 15 µl of water. After that, RNA was reverse transcribed into cDNA with Evo cDNA kit (BioVision, USA) using random primers, dT primers for control or stem-loop primer: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCAGT for miR-103-3p. All primers were synthesized by IDT (USA). One divergent primer for circRNA detection was designed by spanning back-splicing junction (BSJ). This is especially critical for detection circRNA derived from single exon. Reverse divergent primer was designed as described before [29]. Sequences of primers for RT-qPCR are provided (Table 1).

Table 1. Primers for RT-qPCR.

Template		Primer 5'-3'	
circCIAO1(5) BSJ has_circ_0055631	div	Forward	AATGAACAAGCTCTTAGCTTCTGC
		Reverse	TTCATTGCCTGGTAGATACTGAC
CIAO1	conv	Forward	AGGTCCTCCCTTCCAGTTT
		Reverse	ATCCCCAGTTGCATCACAG
circMALAT1 a BSJ has_circ_0002082	div	Forward	GCTGGTGTATTTTATAGAACTTTGTC
		Reverse	CCTTTTACTCTGATCATAATCTCCC
circMALAT1 b has_circ_0002082	div	Forward	CAGCTGAGTGATAAAGGCTGAG
		Reverse	AATTTGTCTTTCCTGCCTTAAAGT
MALAT1	conv	Forward	ACCTCTTAGACAGGTGGGAGA
		Reverse	TTAAAACCCACAGGCACCC
EZH2	conv	Forward	TGTTTCTGTGTTCTTCCGCTT
		Reverse	CACCTCTTTCATACGCTTTTCTG
β -ACT	conv	Forward	CACCATTGGCAATGAGCGGTTT
		Reverse	AGGTCTTTGCGGATGTCCACGT
U6	conv	Forward	GCTTCGGCAGCACATATACTAAAT
		Reverse	CGCTTCACGAATTTGCGTGTCTAT
miR-101-3p	conv	Forward	TAGAGTACTGTGATAACTGAA
		Univ Reverse	CCAGTGCAGGGTCCGAGGTA

The circular nature of circCIAO1(5) and circMALAT1 was confirmed by Sanger sequencing across the back splicing junction site. RT-qPCR was conducted using SYBR Green Master Mix (Biotum, USA) on ABI 4300 system (Applied Biosystems, USA). U6 and β -Actin served as internal references for RT-qPCR.

2.5. RNase R Treatment

Total RNA was incubated for 30 minutes at 37°C with or without 3 U/ μ g RNase R (Epicentre Technologies, USA) followed by RNase R inactivation at 65°C for 20 min. K⁺ was replaced with Li⁺ in the reaction buffer to improve the efficiency of linear RNA degradation. As shown previously, replacing was sufficient to enable RNase R to proceed through these sequences and fully degrade the linear RNAs [30]. RNA was then purified using miRNeasy Mini Kit (Qiagene, USA) and eluted in 15 μ l of water.

2.6. Molecular Cloning and Cell Transfection

CircCIAO1(5) siRNA was synthesized by IDT based on the sequence of mature circCIAO1(5)-CD.Ri.497024.13.1 SEQ1 and CD.Ri.497024 Ri.13.1 SEQ2CircMALAT1 was synthesized by IDT based on the sequence of the mature circMALAT1-CD.Ri.493950.13.1 SEQ1 and CD.Ri.493950.13.1 SEQ2. All 4 siRNAs encompassed junction area. Scrambled negative control DsiRNA (IDT, USA) served as non-related siRNA (siRNA nr). MiR-103-3p mimics were ordered from Qiagene (USA)- gene globe ID MSY0000099. MiR-101-3p Inhibitor was synthesized by IDT(USA)

CircRNA mini (mc2) expression plasmid (Addgene 206218) was used for cloning circCIAO1(5) and circMALAT1 between a pair of BsmBI restriction sites [31]. CircCIAO1(5) was amplified by PCR with:

Forward primer: 5'-GCGTCTCA TCAG CTCTTAGCTTCTGCCAGCTA-3'
Reverse primer: 5'-TCGTCTCA TTAC TGTTCATTGCCTGGTAGATA-3'
circMALAT1 with:

Forward primer: 5'-GCGTCTCA TCAG AAACCTTTGTCTGCGAACACT-3'
Reverse primer: 5'-TCGTCTCA TTAC CTAAAAATACACCAGCAAAA-3.

Amplification from cDNA performed with a high-fidelity DNA polymerase NEB Q5 DNA polymerase. The PCR amplicon is then digested with BsmBI, recovered using a DNA purification kit, and ligated with BsmBI-digested mc2 using T4 DNA ligase.

Plasmid DNA was isolated from overnight cultures (37°C, 230 rpm, 20 hours) of chemically competent *E. coli* (TOP10- or Stbl3) using MiniPrep Qiagene kit (USA)

A total of 2×10^5 5637 or T24 cells were seeded into six-well plates. The next day small RNAs were transfected into BCa cells with Lipofectamine RNAiMAX reagent (Invitrogen, USA). Cloned circCIAO1(5) and circMALAT1 overexpressing construct and the corresponding control (original MC2 MCS vector) were transfected into BCa cells with Viafect (Promega, USA). After 24 hours, RT-qPCR was performed to evaluate the transfection efficiency.

2.7. Wound-Healing Assay

Wound-healing assays were performed as previously described [32]. Transfected or non-treated cells were cultured to 85–95% confluency in 24-well plates. The scratch was made using a 200 μ L tip. Next, the cells were washed three times with PBS, and 2 ml serum-free medium was added. Photographs of the cell scratches were obtained using a microscope camera at 0 h and 24 h to calculate the cell scratch healing rate [32]. The experiments were performed in triplicate.

2.8. Transwell Invasion Assay

For the Transwell invasion assay, 100 μ L serum-free culture medium containing 2×10^4 cells were plated into the upper chamber of a 24-well plate, which was precoated with Matrigel (BD Biosciences, USA). 600 μ L medium containing 10% FBS was then added to the lower chamber. After incubation for 24 h, the cells that migrated to the membrane of the upper chamber were fixed with 4% paraformaldehyde and stained with 1% crystal violet. The invasive cells were treated with 10% acetic acid and spectrometric absorbance at 525 nm was determined with plate reader [29,33].

2.9. Dual-Luciferase Reporter Assay

The wild-type fragment of circCIAO1(5) and circMALAT1 wt., capable of binding to miR-101-3p as predicted with CircInteractome software [34], and the EZH2 sequence that can potentially bind miR-101-3p as determined by miRDB software [35], along with their mutated versions were inserted into the pmiRGLO plasmid (Addgene 78131)[24,33]. To construct dual-luciferase reporter plasmids we used single nucleotide oligo bridge technology and NEbuilder HiFi system (NEB, USA). Fragments (Figure 5A) were cloned into pmiRGLO vector using NheI and XbaI restriction sites. Plasmids were then transfected into BCa cells with or without miR-101-3p mimic using Viafect transfection reagent (Promega, USA). Twenty-four hours later, firefly luciferase and Renilla luciferase were detected by using a Dual-Lumi™ Luciferase Assay Kit (Promega, USA), and the assays were independently repeated three times.

2.10. RNA Immunoprecipitation (RIP) Assay

As a competitive endogenous RNA (ceRNA), circRNA can compete with miRNA. miRNA is usually combined with AGO2 protein to form RNA-induced silencing complex (RISC), thus regulating the expression of target genes. Because AGO2 can combine with circRNAs and miRNAs, the RNA-protein complex can be precipitated under the action of AGO2 protein antibody by RNA immunoprecipitation (RIP) experiment. RIP assay was conducted with a RIP kit (Millipore, USA) according to the manufacturer's instructions. Coprecipitated RNAs were subsequently identified through RT-qPCR utilizing specific primers [24].

2.11. Statistical Analysis

Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method, with normalization to the internal reference gene and calibration to the control group. Statistical analyses were performed on ΔC_t values. Differences in ΔC_t values between normal and tumor tissues were assessed using an unpaired two-tailed Student's t-test (or Mann–Whitney U test, as appropriate). Data are presented as individual values with mean $\pm$ SEM (or median with interquartile range). Data among the three

independent patient groups were analyzed using one-way ANOVA with Kruskal–Wallis’s test and Dunn’s correction for non-normally distributed data. Experimental data were analyzed by GraphPad Prism 5.0.1 software. P value <0.05 was considered statistically significant.

3. Results

3.1. Bioinformatic Selection of Potential circRNA Biomarker Candidates

As shown on Figure 1 (upper box) the group of circRNA were selected based on the high binding affinity to miR-101-3p (TDMD score > 1.1) [27]. In addition, for this study we considered circRNA derived from exons and lnc-RNAs. Inside this group circRNA derived from genes related to BCa progression were determined and used for further analysis (Figure 1 second box).

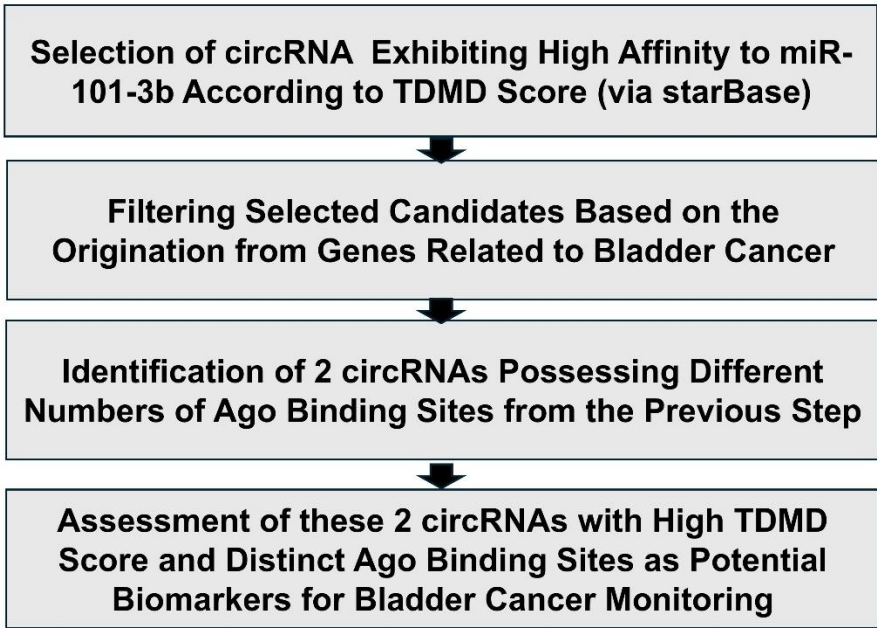


Figure 1. Workflow illustrates the bioinformatic pipeline for identification circRNA candidates with potential utility in BCa monitoring.

This assessment was based on the differential expression of genes in tumor and normal urothelium (TCGA database reports amplification of CIAO1 in 3% tested cases, while MALAT1 in 2%). The starBase Pan Cancer option reported difference of CIAO1 expression 4.25 for cancer versus 3.7 (Log2) for normal urothelium. For MALAT1 expression difference was reported as insignificant in starBase. Nevertheless, amplification of this gene in BCa was reported in the literature [36,37]. CircCIAO1(5) originated from exon 5, whereas circMALAT1 from lnc-RNA.

Since we also aimed to examine the role of Ago binding to successful biomarker candidate selection, 2 candidates with different number of Ago binding sites from the group of circRNA that met described requirements were chosen. Therefore, circCIAO1(5) and circMALAT1 qualified for the current study and were selected for validation as biomarkers for BCa monitoring and for functional study (Figure 1 box 4).

3.2. Characterization of CircCIAO1(5) and circMALAT1 in BCa and Urothelial Carcinoma Cell Lines

CircRNA hsa_circ_0055631, designated as circCIAO1(5) by new classification [38], was formed through back-splicing of exon 5 of the CIAO1 gene on human chromosome 2, whereas hsa_circ_0002082 (circMALAT1), was formed through back-splicing of long non-coding RNA (lncRNA) of the MALAT1 gene on human chromosome 11. Since there are no clear guides about

naming circRNA derived from non-coding RNAs [38], we select this label for hsa_circ_0002082 for this study.

Sanger sequencing validated the back-splicing site of circCIAO1(5), and circMALAT1 (Figure 2A)

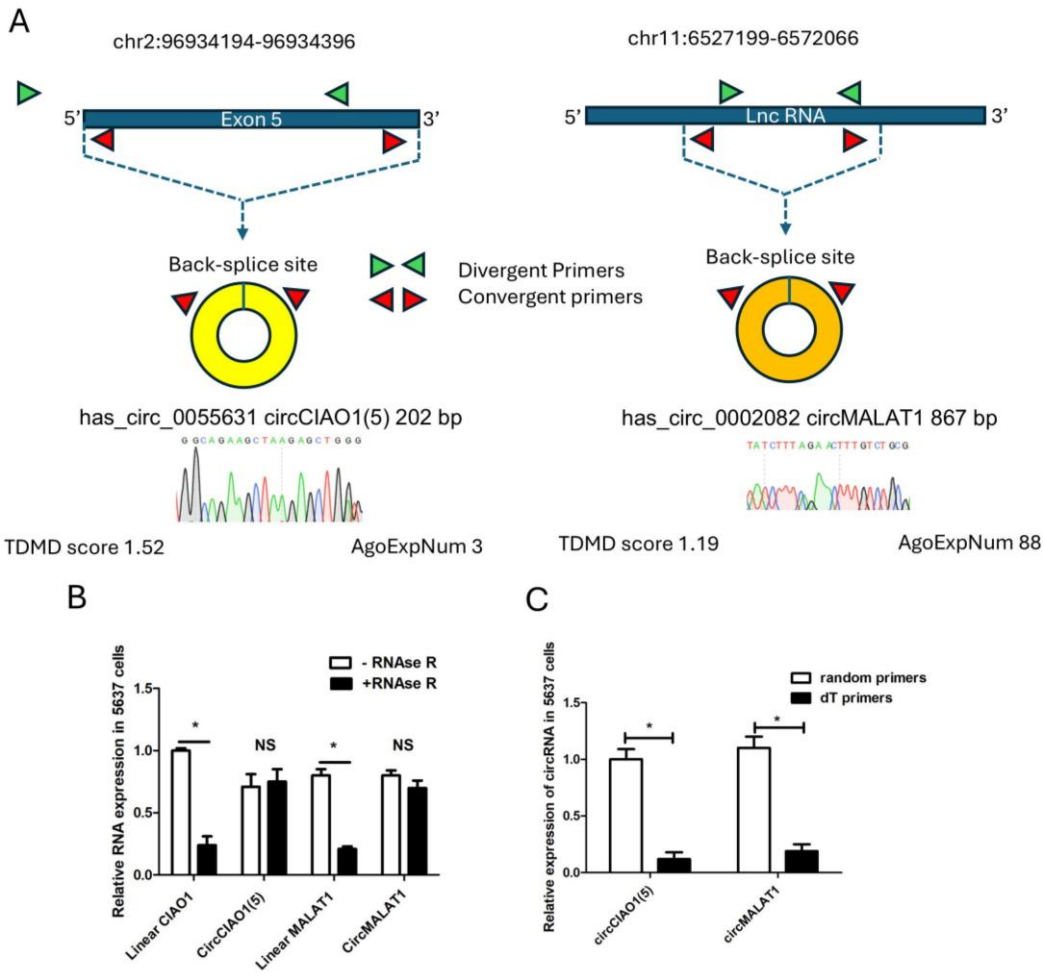


Figure 2. Identification and characterization of circCIAO1(5) and circMALAT1. **(A)** Schematic diagram of circCIAO1(5) and circMALAT1 and Sanger sequencing of back-spliced site, **(B)** RNase R treatment, **(C)** reverse transcription with dT and random primers. * P<0.05.

RNase R treatment assays demonstrated the enhanced stability of circCIAO1(5) and circMALAT1 compared to the corresponding mRNAs (Figure 2B).

In addition, comparison of RT-qPCR results obtained using either an oligo(dT) primer or a random primer for reverse transcription revealed that circCIAO1(5) and circMALAT1 markedly higher expressed when random primers were used (Figure 2C). This primer-dependent difference supports the circular nature of analyzed RNAs.

3.3. CircCIAO1(5) and circMALAT1 are Expressed at Higher Levels in Urine from Patients with Recurrent and Progressive Tumors

Expression of circCIAO1(5) in BCa patient urine was significantly higher compared with urine from control median 1.2 vs 4.8, $P < 0.0001$ for circCIAO1(5) and 1.8 vs 7.3 $P < 0.000$ for circMALAT1 (N=25). For several samples, RT-qPCR results were unreliable, likely due to low RNA quality or quantity. (Figure 3A) To evaluate the diagnostic performance of the biomarkers receiver operating characteristic (ROC) curves were generated. Areas under the ROC curves (AUCs) were compared using DeLong's test for two ROC curves using easyROC software. CircCIAO1(5) showed an AUC of 0.8 (95% CI 0.73–0.94), while circMALAT1 achieved an AUC of 0.86 (95% CI 0.85–0.996). DeLong's test

revealed no statistically significant difference between the two ROC curves ($P > 0.05$) (Figure 3B). Minor deviation of the ROC curve below the diagonal at extreme threshold values reflects instability at the distribution tails and does not affect the overall diagnostic performance. A limitation of this study is the modest sample size, which may restrict statistical power; nevertheless, the observed AUC values support the diagnostic potential of circCIAO1(5) and circMALAT1 warrant validation in larger, independent cohorts.

To determine whether the urinary circRNAs originated from the bladder tumor, we quantified circCIAO1(5) and circMALAT1 in eight paired bladder tumor tissues and corresponding urine samples. CircCIAO1(5) was detected in eight pairs, in one case, the urinary circCIAO1(5) level was below the detection threshold, whereas circMALAT1 was detected in all nine. (Figure 3C).

To assess whether circCIAO1(5) and circMALAT1 expression correlated with clinical status, we analyzed their levels in urine from patients with distinct clinical courses. Each BCa patient was classified into one of three groups based on their clinical follow-ups: remission, recurrence, or progression. Remission was defined as the absence of detectable BCa during surveillance following surgery. Recurrence was defined as the reappearance of BCa of the same or lower grade and stage during surveillance. Progression was defined as the development of a tumor with higher grade or stage compared with the initial diagnosis. Twelve age-matched samples (55–70 years) were selected for each group.

CircCIAO1(5) levels were ~3.5-fold higher in urine from patients with recurrent tumors and ~3.1-fold higher in those with progressive disease compared with patients in remission. CircMALAT1 levels were increased ~2.5-fold in the recurrence and progression groups (Figure 3C). We next examined circCIAO1(5) and circMALAT1 expression in urothelial cell lines. Both circRNAs showed elevated expression in the aggressive BCa cell lines 5637 and T24 compared with the non-transformed urothelial line UROtsa. A non-related circRNA was used as a negative control for comparison (Figure 3E).

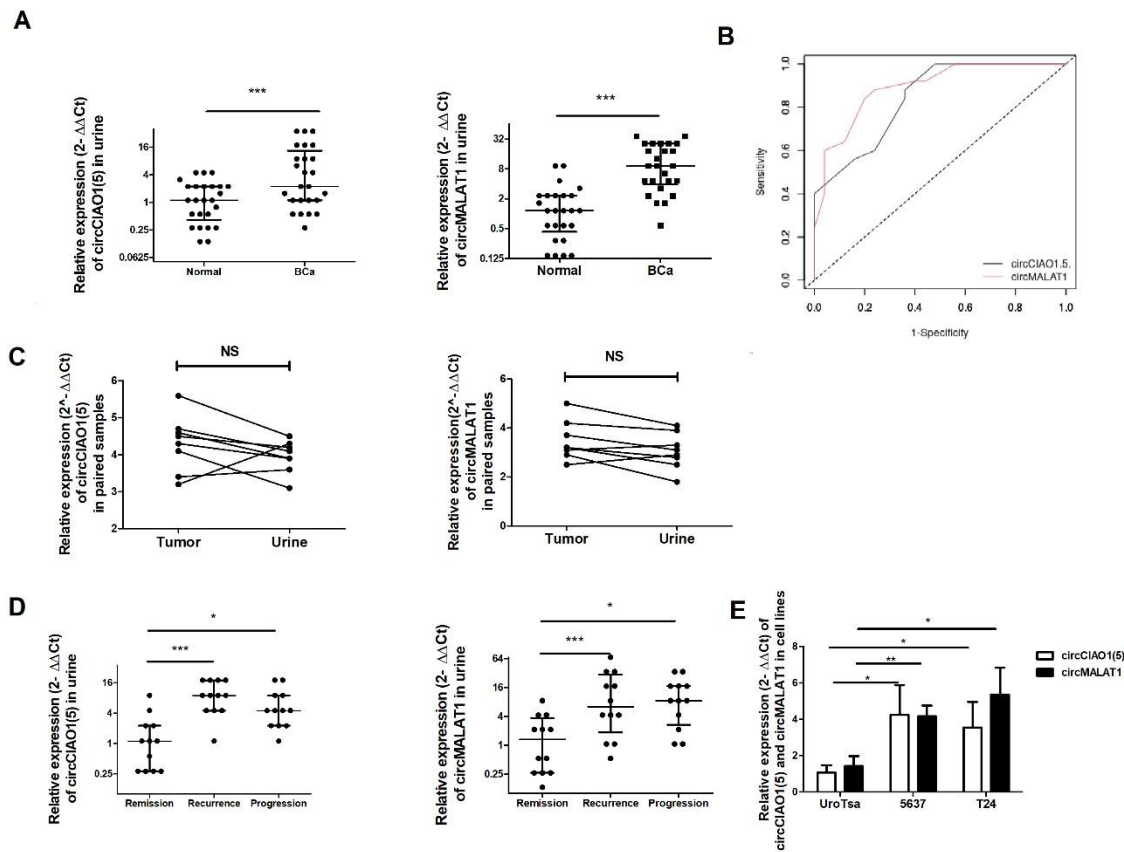


Figure 3. CircCIAO1(5) and circMALAT1 in BCa. (A) Relative CircCIAO1(5) and circMALAT1 expression in the urine of BCa patients and control group (25 samples in each group). (B) ROC curve for circCIAO1(5) and

circMALAT1 for BCa samples and control (25 samples per group) (C) circCIAO1(5) and circMALAT1 expressions in pairs of urine-tissue samples, (D) circCIAO1(5) and circMALAT1 are expressed at higher levels in urine samples related to recurrent and progressive tumor. (E) circCIAO1(5) and circMALAT1 expression in BCa cell lines, NS—non-significant difference, data are shown as the means SD of three independent experiments, * $p < 0.05$. *** $p < 0.0001$. qPCR data were analyzed using the $\Delta\Delta Ct$ method. Statistical analyses were performed on $\Delta\Delta Ct$ values. For graphical representation expression was expressed as fold change ($2^{-\Delta\Delta Ct}$) for interpretation.

3.4. Knockdown of circCIAO1(5) and circMALAT1 Inhibits BCa Cell Proliferation, Motility and Invasion while Their Overexpression Promoted These Functions

To investigate the biological roles of circCIAO1(5) and circMALAT1 in BCa, we designed two siRNAs targeting the back-splice junctions of circCIAO1(5) and circMALAT1 (see Section 2) and transfected them into 5637 or T24 cells. Relative to a non-targeting scrambled siRNA, both siRNAs efficiently reduced circCIAO1(5) and circMALAT1 expression by several folds in 5637 cells (Figure 4A, B).

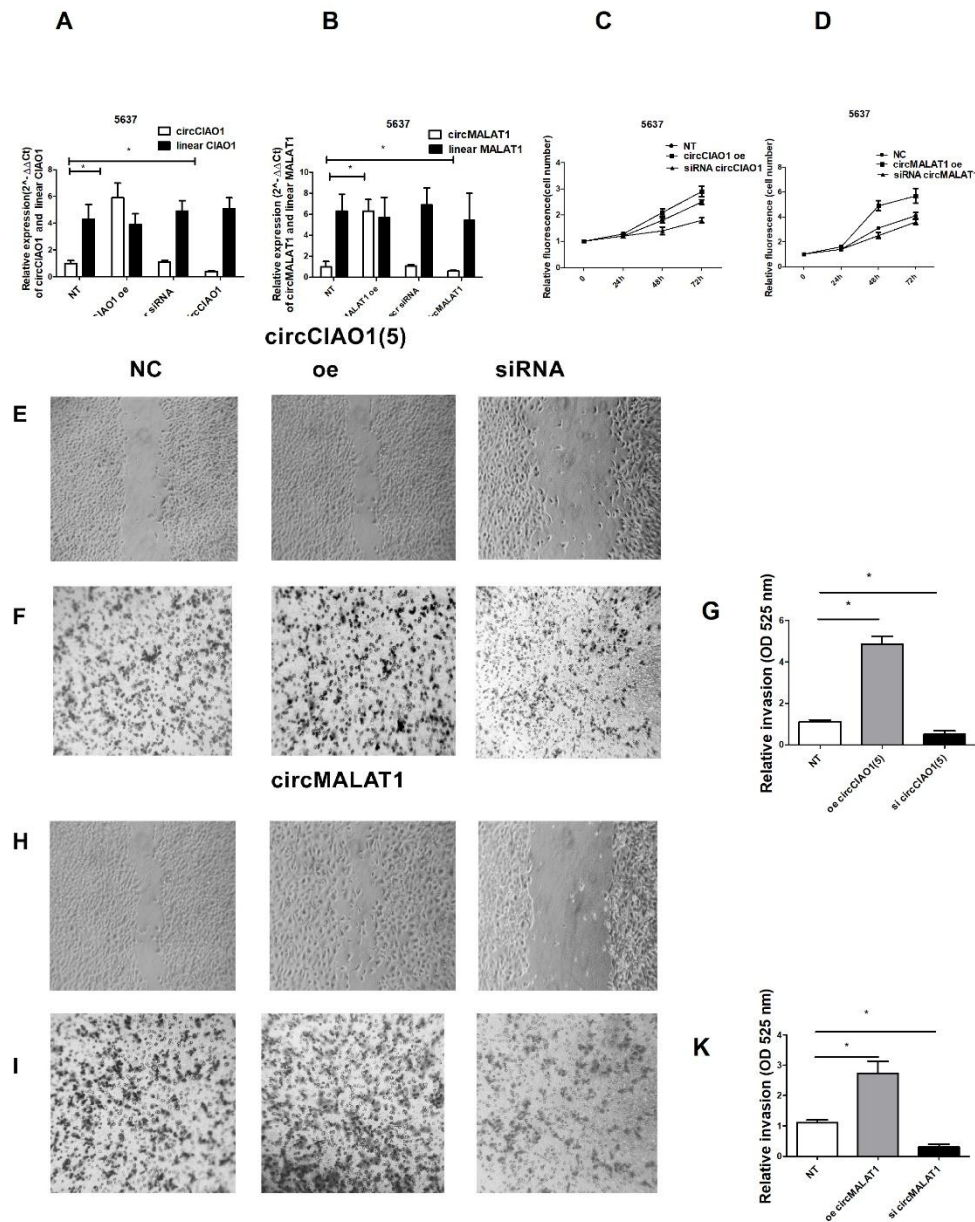


Figure 4. Effect of CircCIAO1 and circMALAT1 on proliferation, motility and invasion of BCa cells. (A) CircCIAO1(5) in 5637 cells following overexpression or siRNA-mediated silencing; linear *CIAO1* RNA served as

a control. **(B)** CircMALAT1 in 5637 cells following overexpression or inhibition; linear *MALAT1* RNA served as a control. **(C, D)** Proliferation of 5637 cells upon modulation of circCIAO1(5) **(C)** or circMALAT1 **(D)**. **(E)** Representative images of cell motility upon modulation of circCIAO1(5) **(F, G)**. **(H)** Representative images and quantification of cell invasion showing dependence on circCIAO1(5). **(I, K)** Representative images of cell motility demonstrating circCIAO1(5)- and circMALAT1-dependent regulation. circMALAT1-dependent regulation. Representative images and quantification of cell invasion showing dependence on circCIAO1(5). Magnification, 100×. qPCR data were analyzed using the $\Delta\Delta C_t$ method. Statistical analyses were performed on $\Delta\Delta C_t$ values. For graphical representation expression was expressed as fold change ($2^{-\Delta\Delta C_t}$) for interpretation. Data is presented as means $\pm$ SD from three independent experiments. $p < 0.05$. *** $p < 0.0001$.

We also constructed overexpression plasmids by inserting the circRNA-producing exons between intronic sequences previously shown to enhance back-splicing. RT-qPCR confirmed that transfection of these plasmids increased circCIAO1(5) and circMALAT1 levels by approximately eightfold in both cell lines, while the empty vector control had no effect (Figure 4A, B). Importantly, expression of the corresponding linear mRNAs remained largely unchanged following either siRNA-mediated knockdown or plasmid-driven overexpression (Figure 4A, B).

We next assessed the impact of circCIAO1(5) and circMALAT1 expression on BCa cell behavior—including proliferation, motility, and matrix invasion. In 5637 and cells, siRNA-mediated silencing of circCIAO1(5) and circMALAT1 significantly reduced cell proliferation, whereas overexpression of either circRNAs enhanced proliferation (Figure 4C, D).

In addition, In 5637 cells, overexpression of circCIAO1(5) and circMALAT1 enhanced cellular motility, whereas knockdown of either circRNA exerted a more pronounced effect, leading to a significant suppression of this activity (Figure 4E, G). Matrix invasion increased following circRNA overexpression and was reduced upon transfection with the corresponding siRNAs (Figure 4E, F, G and I, K)).

3.4. *CircCIAO1(5) and circMALAT1 May Act as a Sponge for miR-101-3p in BCa Cells*

Bioinformatic analysis identified putative miR-101-3p binding sites within circCIAO1(5) and circMALAT1 (Figure 5A). To validate these interactions, luciferase reporter assays were performed using constructions containing the predicted binding regions or their corresponding mutant sequences. In 5637 and T24 cell lines, co-transfection with miR-101-3p mimics significantly decreased luciferase activity of the wild-type reporters, whereas no reduction was observed for the mutant constructions. Luciferase activity of the circCIAO1(5) reporter decreased by approximately 60%, compared with a ~30% reduction for the circMALAT1 reporter (Figure 5B), supporting differential binding affinity of the two selected circRNAs for miR-101-3p.

Further evidence supporting circRNA–miRNA interaction was obtained using AGO2 RNA immunoprecipitation (RIP) in 5637 cells. qRT-PCR analysis demonstrated a marked enrichment of circCIAO1(5) and circMALAT1 in AGO2 immunoprecipitated relative to IgG controls. Notably, circMALAT1 showed higher enrichment than circCIAO1(5), which is consistent with its greater number of predicted AGO2-binding sites (88 versus 3). miR-101-3p was also robustly enriched in AGO2 pulldown, reaching levels exceeding those of circMALAT1, likely to reflect the generally higher abundance of miRNAs relative to circRNAs in cells (Figure 5C).

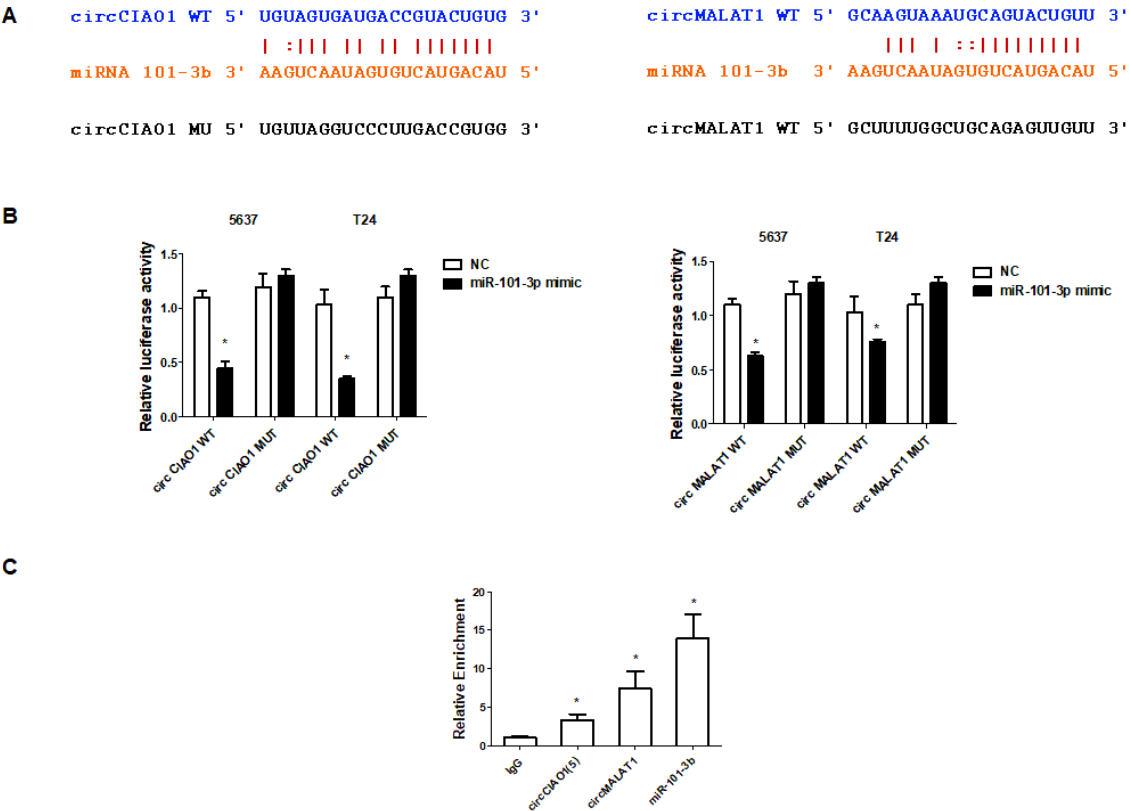


Figure 5. Interaction of circCIAO1(5) and circMALAT1 with miR-101-3p. **(A)** Predicted miR-101-3p binding motifs within circCIAO1(5) and circMALAT1. **(B)** Dual-luciferase reporter assays demonstrating the interaction between circCIAO1(5) (left) or circMALAT1 (right) and miR-101-3p. **(C)** AGO2 RNA immunoprecipitation (RIP) showing enrichment of circRNAs and miR-101-3p in AGO2 complexes. Data is presented as means ± SD from three independent experiments. *P* < 0.05.

3.5. miR-101-3p Expression Is Correlated with BCa Progression and Suppresses Proliferation, Migration, and Invasion of BCa Cells

Using RT-qPCR, we found suppressed expression of miR-101-3p in the urine of BCa patients and in more aggressive BCa cell lines (Figure 6A, B). Proliferation of 5637 cells was modulated by miR-101-3p expression (Figure 6C). Mobility of 5637 was increased when miR-101-3p inhibitor was expressed while transfection with miR-101-3p mimic impaired it (Figure 6D). For 5637 cells, a trend of increasing invasion after transfection with miR-101-3p inhibitor and decreasing with miR-101-3p mimic was observed (Figure 6E, F).

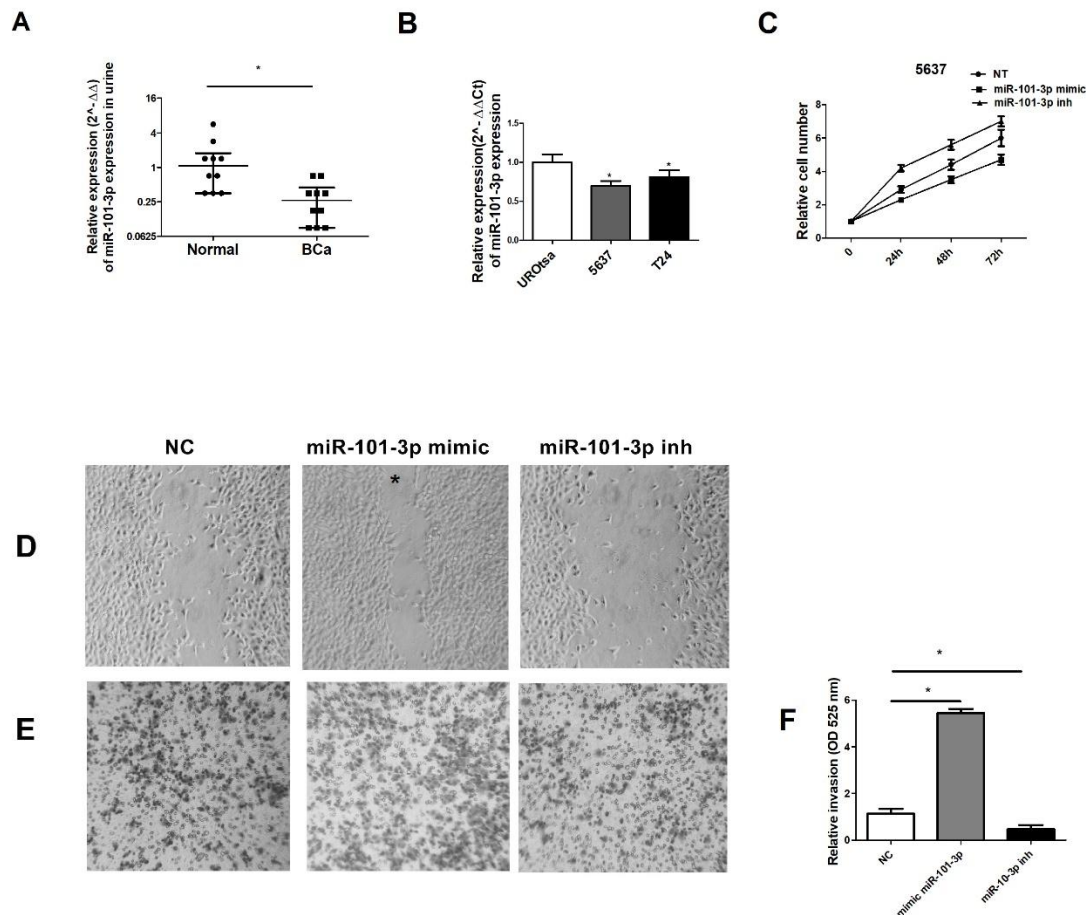


Figure 6. miR-101-3p expression is elevated in BCa and regulates proliferation, motility, and invasion of BCa cells. **(A)** miR-101-3p expression levels in urine samples from BCa patients and controls. **(B)** miR-101-3p expression in urothelial and BCa cell lines. **(C)** Effects of miR-101-3p modulation on proliferation of 5637 cells. **(D)** Regulation of 5637 cell motility by miR-101-3p **(E, F)** Regulation of 5637 cell invasion by miR-101-3p. Magnification, 100×. Data is presented as means ± SD from two independent experiments. *P* < 0.05.

3.6. miR-101-3p Suppressed BCa Cell Proliferation, Migration, and Invasion by Targeting EZH2

MiR-101-3p was identified as a tumor suppressor gene in BCa [17]. Candidate target genes of miR-101-3p were predicted using TargetScan, mirMAP and ENCORI Top binders were selected by combined search (Figure 7A). Six genes: EZH2 LCOR GPAM CADM1 ZC3H11A TGFBR3 were present in all 3 databases. Further filtration of the list was expressed in analyzing expression alteration and role in survival in BCa (TGGA). Only ZC3H11 showed low amplification in BCa and was excluded. Survival analysis showed that only EZH2 and TGFBR alteration impaired survival in BCa. However, EZH2 emerged as the most biologically and clinically relevant candidate in BCa, supported by strong experimental validation and its established oncogenic role and was therefore considered as a candidate for miR-101-3p binding and regulation [16,39–41].

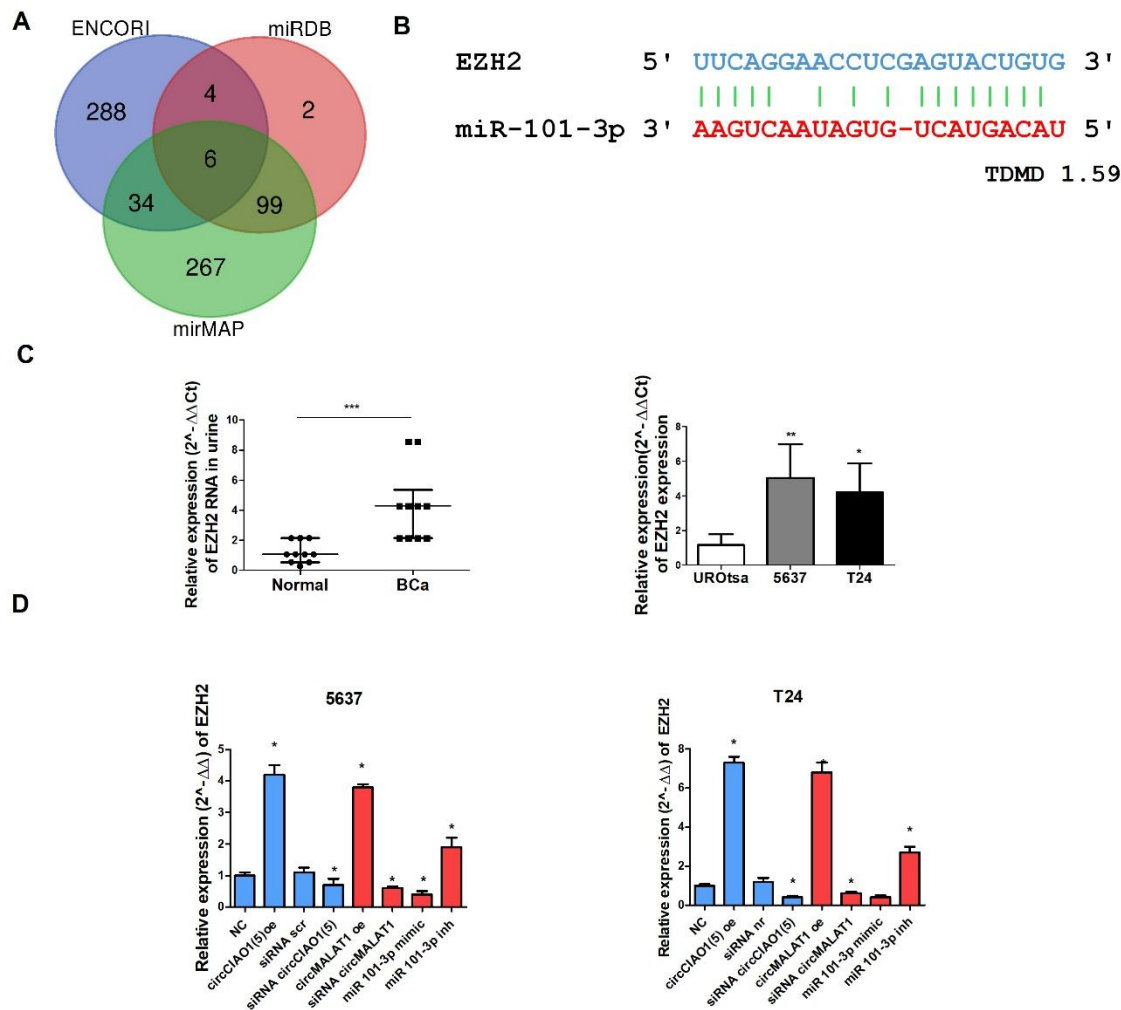


Figure 7. EZH2 is regulated by circCIAO1(5) and circMALAT1 through miR-101-3p. **(A)** Venn diagram for bioinformatic search of miR-101-3p binders, **(B)** Predicted miR-101-3p binding motifs within the 3'UTR of EZH2, indicating its potential regulation by miR-101-3p. **(C)** Relative EZH2 mRNA expression in urine samples from BCa patients, controls and in urothelial and BCa cell lines, **(D)** Regulation of EZH2 mRNA expression by circCIAO1(5), circMALAT1, and miR-101-3p in 5637 and T24 cells. For **(C left)** data presented as median with interquartile range, for **C right** and **(D)**, data are presented as means $\pm$ SD from three independent experiments. *= $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, for **(D)** * -compared with NC.

3.7. CircCIAO1(5) and circMALAT1 Regulate EZH2 Expression in BCa Cells

We found that EZH2 was overexpressed in tumor urine samples as compared with control (Figure 7B). EZH2 RNA levels were also elevated in BCa cell lines with higher malignant potency (Figure 7C). CircCIAO1(5) and circMALAT1 overexpression in 5637 and T24 cells resulted in increase in EZH2 mRNA levels. Conversely, siRNA-mediated circCIAO1(5) and circMALAT1 silencing led to a reduction in EZH2 expression (Figure 7D). Consistent with the known interaction between miR-101-3p and EZH2, miR-101-3p overexpression significantly decreased EZH2 expression, whereas inhibition of miR-101-3p activity induced an increase in EZH2 mRNA levels (Figure 7D).

4. Discussion

A common strategy for identifying circRNA biomarker candidates involves comparing circRNA expression profiles between normal and pathological samples and selecting transcripts that are differentially expressed between these groups. Although circRNA microarray profiling is a powerful

discovery tool, it remains relatively costly, labor-intensive, and time-consuming. Moreover, commercial arrays include only predefined circRNA catalogs, limiting the identification of novel or less-characterized candidates [42–45].

One mechanism through which circRNAs exert regulatory functions is the sequestration (“sponging”) of functional miRNAs [18,46]. Although this mechanism is well established for a small number of circRNAs—most notably CDR1as, which contains more than 60 miR-7 binding sites [47,48]—transcriptome-wide studies suggest that most circRNAs rely on weaker and often noncanonical forms of miRNA engagement.

In this study, we pursued a streamlined bioinformatics-based selection strategy counting the possible interactions of circRNA with miRNA of interest (miR-101-3p)[12,13]. Candidate circRNAs were identified directly from public databases based on three criteria: (i) predicted binding affinity to miR-101-3p; (ii) documented involvement of their parental genes in BCa development and progression and (iii) exon or lnc-RNA origin of selected circRNA. Exonic circRNAs, which arise from protein-coding exons, are predominantly localized in the cytoplasm, where can sequester miRNAs. Similarly, circRNAs derived from lncRNAs—such as those produced from MALAT1 or H19—may in some cases also exert sponge activity, provided they accumulate in the cytoplasm and harbor functional miRNA-binding motifs. These circRNA classes contribute to post-transcriptional regulation through miRNA sequestration, with their functional impact determined by circRNA abundance, subcellular localization, and the number and accessibility of embedded miRNA response elements [49,50]. Since many circRNA present in much less copy number than microRNA-sponging phenomenon still need detailed confirmations.

To prove interactions of circCIAO1(5) and circMALAT1 with miR-101-3b in this study we showed direct binding in vitro as well as coexistence with AGO2 protein.

We further examined the relevance of AGO-binding site abundance as a determinant of circRNA functionality and potential biomarker utility. AGO proteins play a central role in miRNA-mediated gene silencing by stabilizing miRNA–target interactions and facilitating base pairing with target transcripts, including circRNAs. The number of AGO-binding sites contributes to the avidity and stability of the circRNA-miRNA complex and may determine the extent to which a circRNA can sequester a given miRNA. Importantly, Ago2 enrichment on a circRNA does not necessarily indicate strong sponging capacity. Rather, such interactions may represent transient binding events, seedless low-affinity contacts, or protein-mediated recruitment of [51]. Consequently, circRNAs with limited predicted seed sites—but detectable AGO2 occupancy—may still modulate miRNA activity. Because molecular sponging of tumor-suppressive miRNAs represents one of the key mechanisms through which circRNAs may influence cancer, AGO-binding site composition is an important parameter for prioritizing circRNAs for functional characterization [14,26].

Using this approach, we identified and validated circCIAO1(5) and circMALAT1 with different binding affinity, number of Ago2 sites and gene origine in BCa cell lines, tumor tissues, and, critically, in urine samples from patients. To confirm the circular nature of these transcripts, we verified their back-splice junctions and demonstrated resistance to RNase R digestion, supporting their bona fide circular structure.

As no previous data existed regarding the expression of these circRNAs in normal urothelium, we quantified their levels in clinical urine specimens and observed higher expression of both circCIAO1(5) and circMALAT1 in samples from patients with recurrent or progressive disease. In most cases these circRNAs were detectable in urine samples (15 ml) by conventional RT-qPCR. Two circRNAs examined here differ substantially in several properties: (i) predicted binding strength to miR-101-3p (TDMD scores: 1.52 for circCIAO1 and 1.19 for circMALAT1); (ii) number of Ago2-binding sites (3 for circCIAO1 and 88 for circMALAT1); (iii) transcript size (202 bp vs. 887 bp, respectively); and (iv) the nature of their parental genes (a protein-coding exon for circCIAO1 vs. lncRNA for circMALAT1). Both circRNAs showed potential use as biomarkers for predicting tumor progression. However, at this stage of the study it is difficult to conclude which circRNA characteristics are more important for the performance of the selected candidate as biomarker or

miRNA sponge. Integrating all database-annotated features of the candidate circRNAs will markedly improve the robustness of our selection strategy. Nonetheless, because in this study these features overlap in function and predictive value and only 2 candidates were considered, it was difficult to disentangle the relative contribution of each individual feature. Following study is based on more restrictive candidate selection and on larger sample sets that will investigate the role of some characteristics separately.

Manipulating circCIAO1(5) and circMALAT1 expression in BCa cells demonstrated that several tumor-associated phenotypes depend, at least in part, on their expression levels. Functional assays showed that both circRNAs contribute to the regulation of cell proliferation, motility, and invasion, consistent with their involvement in BCa progression. The modest impact of circRNA overexpression, assuming a miRNA-sponging mechanism, may be attributed to a limited number of miRNA molecules available for sequestration.

These circRNAs were selected for experimental validation based on their predicted high-affinity interactions with the tumor-suppressive miRNA miR-101-3p. In line with this, miR-101-3p overexpression in BCa cells reduced expression of the oncogenic target EZH2, highlighting a mechanistic axis in which circCIAO1(5) and circMALAT1 may modulate EZH2 levels through miR-101-3p sequestration. Notably, increased MALAT1 expression was detected in BCa and regulation of cisplatin resistant was linked to MALAT1 RNA binding (sponging) to miR-101-3p [19].

In this study, we also demonstrated that the expression of EZH2 is influenced by circCIAO1(5) and circMALAT1 levels (Figure 7). Together, circCIAO1(5), circMALAT1, miR-101-3p, and EZH2 form a regulatory network that may contribute to BCa cell proliferation, motility, and invasion (Figure 8).

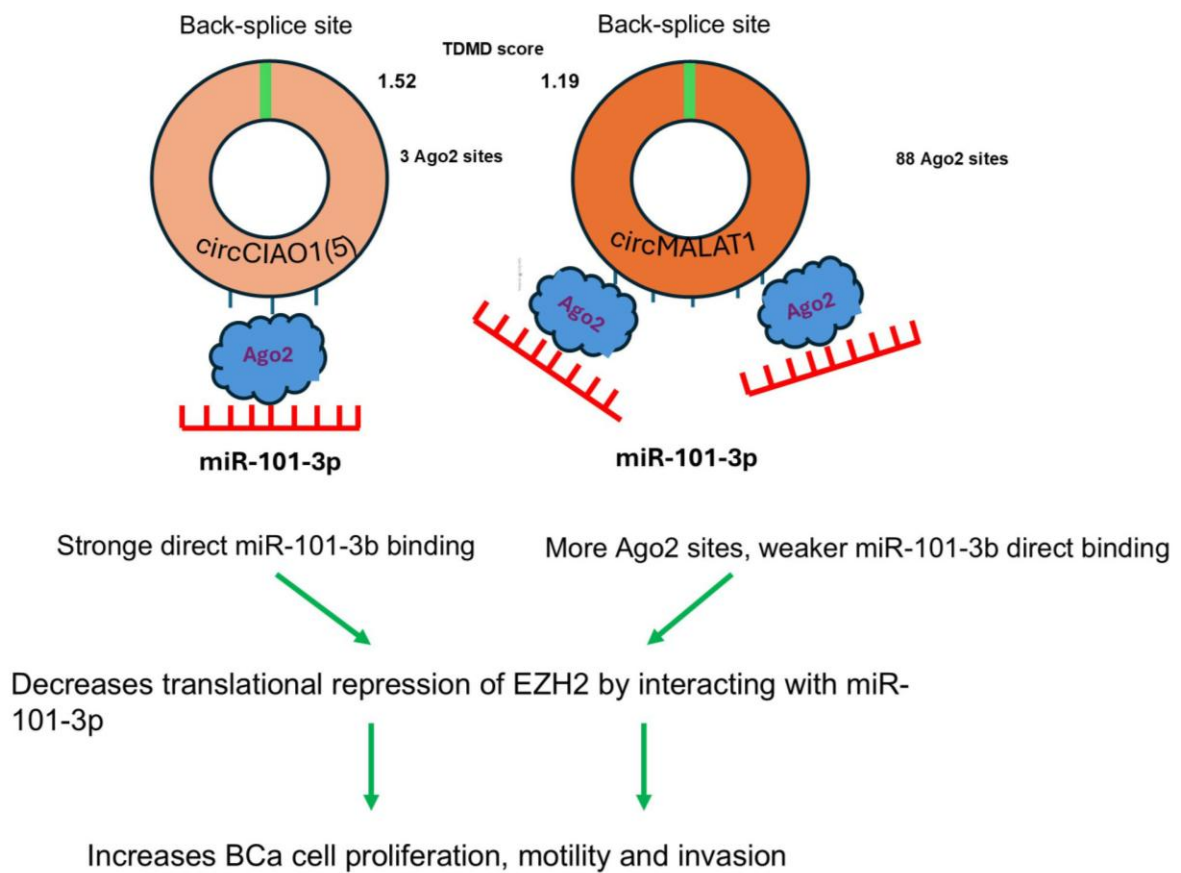


Figure 8. Schematic diagram for possible mechanism connecting circCIAO1(5) and circMALAT1 with miR-101-3p and EZH2 activity in BCa.

Although our findings are preliminary, circCIAO1(5) and circMALAT1 show potential as non-invasive biomarkers for monitoring clinical status in BCa. Future studies incorporating longitudinal follow-up will be required to determine whether these circRNAs can reliably predict recurrence and progression.

5. Conclusions

This study revealed the tumor-promoting effects and potential mechanisms of action of circCIAO1(5) and circMALAT1. Elevated levels of these circRNAs in BCa specimens support their suitability as candidate diagnostic or prognostic biomarkers. Collectively, these findings support the hypothesis that circCIAO1(5) and circMALAT1 may influence BCa cell behavior by modulating miR-101-3p activity. A reduction in the pool of free, functional miR-101-3p within cells may lead to depression of EZH2, promoting a more malignant phenotype. Currently, no circRNA-based biomarker tests have received commercial approval, and the full extent of circRNA activity in gene regulation remains incompletely understood. Nevertheless, the inherent stability of circRNAs and their reliable detectability in biologically active fluids underscore their promise as non-invasive biomarker candidates.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.”.

Data Availability Statement: The datasets generated and/or analyzed during this current study are not publicly available due to the need for further research but are available from the corresponding author upon reasonable request.

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