miR-582-5p regulates cell stemness and recurrence in bladder cancer via targeting CD81

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Research Article

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Abstract

Background: Bladder cancer (BC) is a highly heterogeneous stem cell disease. Cancer stem cells (CSCs) are the drivers of tumor growth and recurrence, with the ability to self-renew, metastasize, and resist chemotherapy. However, the specific molecular mechanisms of CSCs driving BC recurrence and progression remains unclear.

Objective: To explore the underlying molecular mechanism of CSCs driving BC recurrence and progression.

Methods: Tumor xenograft model *in vivo* was established after 4-6-week-old male nude mice were subcutaneously injected with 5×10^6 of T24 and 5637 cells in 0.1 mL 50% Matrigel. Pearson correlation analysis analyzed the correlation between miR-582-5p and CD81, and which was furtherly verified by dual-luciferase reporter gene assay. Sphere formation assay, flow cytometry, immunohistochemistry (IHC), qRT-PCR and Western blot were carried to examine sphere formation, $ALDH^{high}$ populations, the level of genes and proteins. Multivariate analysis was carried to explore the factors associated with recurrence free survival of BC patients.

Results: MiR-582-5p was down-regulated in patients with BC, and miR-582-5p overexpression negatively correlated with BC stemness. Mechanically, miR-582-5p negatively targeted to CD81. Functionally, miR-582-5p overexpression inhibited BC stemness and recurrence via targeting CD81.

Conclusion: Our study illustrated that miR-582-5p inhibited cell stemness and recurrence via targeting CD81 in BC. Our findings illustrated the specific molecular mechanism of miR-582-5p inhibiting BC progression. MiR-582-5p may serve as the novel biomarker for BC clinical therapeutics and prognosis.

Highlights

- MiR-582-5p was down-regulated in patients with BC.
- MiR-582-5p negatively targeted to CD81.
- MiR-582-5p overexpression inhibited BC stemness via targeting CD81.
- MiR-582-5p inhibited recurrence via targeting CD81 in BC.

Introduction

Bladder cancer (BC) is considered to be the most common malignant tumor in the urinary system [1]. Cancer stem cells (CSCs) are the drivers of tumor growth and recurrence, with the ability to self-renew, metastasize, and resist chemotherapy [2]. CSCs are tumor-initiated clonal cells that can preserve cell heterogeneity and have the ability to self-renew and differentiate [3]. Several CSCs markers have been identified as the cause of BC progression, metastasis and recurrence, including CD44, ALDH1A1, KLF4, SOX2 and HMGA2 [4–6]. It is speculated that CSCs are partly responsible for the clinical features and

complex biological behavior of BC [7]. Therefore, understanding the role of CSCs in BC is crucial to gain insight into the mechanisms that lead to high recurrence and metastasis. Additionally, it will help identify new therapeutic avenues and promote better outcomes.

MicroRNA (miRNA) is a small, non-coding RNA approximately 20 bp in length that induce target mRNA degradation or translation inhibition by pairing with complementary bases in the 3'-untranslated region (3'-UTR) of the target mRNA [8]. MiRNAs have been widely proposed as potential therapeutic targets for BC. For instance, miR-150 was regulated by lncRNA CASC11 to promote cancer cell proliferation in BC [9]. Bi et al. reported that miR-31-5p promoted BC progression and recurrence by targeting RAB27A [10]. Moreover, Zhang et al. found that miR-34a/GOLPH3 axis reduced BC recurrence via reducing cancer stemness [11]. These findings indicated that miRNAs may be involved in BC progression by regulating cell stemness and recurrence. The regulatory mechanism of miR-582-5p in various cancers has been elucidated, including colorectal cancer, gastric adenocarcinoma, and blood cancer, etc. [12–14]. However, the specific molecular mechanism by which miR-582-5p regulates cell stemness and recurrence has not yet been elucidated in BC.

CD81 is a quadruple transmembrane protein molecule that is widely distributed in living organisms and participates in a variety of physiological responses [15]. CD81 is widely expressed in tumor cells, and its role in malignant cells and the host microenvironment has been preliminarily studied [16]. Studies have shown that CD81 affects the adhesion, differentiation, migration and invasion of tumor cells through cytokines[17]. To date, the regulatory mechanism of CD81 in BC has been initially elucidated. For example, CD81 increased matrix metalloproteinase expression by inhibiting ERK phosphate to promote BC cell invasion [18]. Li et al. proved that CD81 expression is associated with tumor biological aggressiveness and poor prognosis in primary BC, and is an important predictor of recurrence-free survival [19]. However, the specific role of CD81 in BC progression and recurrence remains unclear.

In this study, the regulatory effect of miR-582-5p on cell stemness and recurrence in BC and its underlying molecular mechanisms was investigated. We aimed to determine whether miR-582-5p regulates cell stemness and recurrence of BC via CD81. Our research provided a novel biomarker for BC clinical therapeutics and prognosis.

Materials And Methods

Specimen

From March 2016 to May 2018, after receiving the written informed consent, Nanjing Drum Tower Hospital affiliated to Nanjing University provided BCa tumors and normal bladder tissues. Tumor tissue and adjacent normal tissue from 84 patients were collected according to institutional protocols. After analysis, it was found that miR-582-5p expressionwas lower in BC tissues compared with normal tissues (Fig. 1A). Moreover, of the 84 patients, 39 had no recurrence at 3 years, and 45 had relapsed, and miR-582-3p was lower in the relapsed tissues than in the non-relapsed tissues (Fig. 1B). The Ethics Committee

of Nanjing Drum Tower Hospital approved to carry out this study and obtained the written and informed consent of all patients. All experiments were conducted in accordance with approved guidelines.

Cell lines and culture

American Type Culture Collection (ATCC, Manassas, VA, USA) provided human BC cell lines, T24 and 5637. Cells were cultivated within RPMI1640 medium contained 10% FBS and 1% P/S (Gibico, NY, USA), and incubated within the humid incubator under 37°C and 5% CO_2 conditions.

Cell transfection

General Biosystems (Anhui, China) synthesized miR-582-5p mimics and NC mimics. Sequences for CD81 were generated by PCR and inserted into pCS2-CMV vector (GenePharma, Shanghai, China) for CD81 overexpression. According to the manufacturer's instructions, these segments were transfected into T24 and 5637 cells cultured to 80% confluence with Lipofectamine™ 3000 Transfection Reagent (Invitrogen, California, USA).

qRT-PCR

miRNA was extracted from BC tissues and cell lines by using mirVana microRNA Isolation kits (Invitrogen, California, USA). The Taqman microRNA assay kit (Invitrogen) was performed to detected miR-582-5p levels. U6 RNA was used as an endogenous control. The crease change was calculated by $2^{-\Delta\Delta Ct}$ method. The whole process was repeated three times. The primerswere shown in Table 1.

Western blot

The total T24 and 5637cell lytic was prepared with RIPA cleavage buffer (Beyotime, Nanjing, China). 10% SDS-PAGE gel separated protein and then was transferred to the PVDF membrane. TBST and 5% skimmed milk powder were used to seal the PVDF membrane. Then, the PVDF membrane was incubated overnight with specific antibodies (Abcam, Cambridge, UK), including anti-CD44, anti-KLF4, anti-ALDH1A1, anti-SOX2, anti-HMGA2 and anti-CD81 at 4°C overnight. β-actin was used as the endogenous control. The second day, after incubation with secondary antibody, the bands were detected by GEL imaging system (Bio-Rad), and the quantification of proteins was analyzed by the software Image J.

Bioinformatics and dual-luciferase reporter gene assay

Firstly, the potential binding sites of miR-582-5p downstream was predicted by using starbase bioinformatics software. Clone the wild (WT) or mutant (MUT) sequence of CD81 3'-UTR into a pGL3-M vector (Promega, WI, USA) to build CD81-3'-UTR-WT or CD81-3'-UTR-MUTvectors. Lipofectamine™ 3000 Transfection Reagent (Invitrogen, California, USA) was used to co-transfect these vectors and miR-582-3p mimics or NC mimics into T24 and 5637 cells. Following 48 h, luciferase activity was assessed.

Sphere formation assay

T24 and 5637 cells were digested by trypsin and washed 3 times in PBS. $1 \subseteq 10^4$ cells were inoculated in 6-well cell culture plates containing DMEM/F12 medium (Gibico, NY, USA). After 9–12 days of culture, the

spheres were photographed and counted with diameters greater than 50 μm.

Flow cytometry

ALDEFLUOER kit (Stem Cell Technologies, Vancouver, Canada) was performed to identify cells with high ALDH enzyme activity. In detail, 1 mL single cell suspension was added into 5 μL of activated ALDEFLUOER reagent for 40 min at 37℃. Moreover, Diethylaminobenzaldehyde (DEAB) served as a negative control. Finally, FACS Calibur system was used for flow cytometry, Cellquest graphics software was used for data acquisition and analysis.

Immunohistochemistry (IHC)

The tumor tissues were dewaxed and rehydrated, and then sealed with 0.3% hydrogen peroxide. After antigen repair, the sections were treated with sealing solution. Subsequently, the slices were incubated with the first antibody against CD81 overnight at 4 ℃, and then the second antibody coupled with peroxidase (1:100, Bioss, Beijing, China) was placed at 25℃ for 1 h. Finally, the sections were developed with DAB and observed under microscope.

Statistical analysis

The mean ± standard deviation (SD) represents data from three independent experiments. Student t-test for two groups and Tukey's multiple comparison test for multi-group comparison. When $P < 0.05$, the difference is statistically significant.

Results

MiR-582-5p was down-regulated in patients with BC

Firstly, we analyzed miR-582-5p level in BC patients. Results showed that compared with paired control, miR-582-5p was lower expression in BC tissues (Fig. 1A). qRT-PCR analysis furtherly indicated that miR-582-5p was down-regulated in BC patients which were recrudescent within 3 years (Fig. 1B). Taken together, these findings suggest that miR-582-5p was down-regulated in patients with BC, and miR-582-5p level was negatively related to BC recurrence.

MiR-582-5p overexpression negatively correlated with cell stemness in BC

Subsequently, miR-582-5p mimics were respectively transfected into T24 and 5637 cells for miR-582-5p overexpression (Fig. 2A). Analysis showed that miR-582-5p overexpression decreased the levels of CSCs markers including CD44, KLF4, ALDH1A1, SOX2 and HMGA2 in T24 and 5637 (Fig. 2B). Consistently, flow cytometry analysis indicated that $ALDH^{high}$ populations in both T24 and 5637 cells were decreased by miR-582-5p overexpression (Fig. 2C and 2D). Moreover, findings showed that miR-582-5p overexpression decreased spheres' number (Fig. 2E). To sum up, miR-582-5p overexpression decreased the CSC subpopulation in BC.

MiR-582-5p negatively targeted to CD81

MiR-582-5p regulates gene transcription by binding to the 3'-UTR of the target mRNA [12]. To find target of miR-582-5p, starbase software was applied to predict the downstream targets of miR-582-5p. Aa binding site between miR-582-5p and CD81 was found (Fig. 3A). Subsequently, Analysis indicated that miR-582-5p overexpression inhibited CD81 protein levels in T24 and 5637 cells (Fig. 3B). Analysis revealed that CD81-WT reported gene' luciferase activity was inhibited by co-transfection of miR-582-5p mimics, but CD81-MUT reported gene' luciferase activity was not changed by co-transfection of miR-582-5p mimics in T24 and 5637 cells (Fig. 3C). In total, miR-582-5p negatively targeted to CD81 in T24 and 5637 cells.

MiR-582-5p overexpression inhibited cell stemness via targeting CD81 in BC

We next examined whether miR-582-5p affected BC stemness via CD81, pcDNA-CD81 was transfected into T24 and 5637 cells for CD81 overexpression (Fig. 4A). As showing in Fig. 4B, the levels of CSC markers were down-regulated by miR-582-5p overexpression, while CD81 overexpression reversed the downward trend (Fig. 4B). Subsequently, flow cytometry analysis indicated that miR-582-5p overexpression decreased $ALDH^{high}$ populations in T24 and 5637 cells, whereas CD81 overexpression reversed the downward trend (Fig. 4C and 4D). Similarly, spheres number from T24 and 5637 cells was decreased by miR-582-5p overexpression, but which was reversed by CD81 overexpression (Fig. 4E). In conclusion, miR-582-5p overexpression inhibited BC stemness via targeting CD81.

MiR-582-5p inhibited recurrence via targeting CD81 in BC

For further explore the effect of miR-582-5p/CD81 axis on BC recurrence, CD81 level in BC recurrence tissues was measured. Results showed that compared with control, CD81 was highly expressed in recurrence tissues (Fig. 5A). As shown in Fig. 5B, miR-582-5p was negatively correlated with CD81 expression in recurrence tissues. What's more, miR-582-5p level was negatively correlated with recurrence free survival of BC patients (Fig. 5C). Inversely, CD81 level was positively correlated with recurrence free survival of BC patients (Fig. 5D). Therefore, we concluded that miR-582-5p inhibited recurrence in BC via targeting CD81.

Discussion

Tumor recurrence is common in cancer. Increasing evidence shows that a large number of CSCs within heterogeneous tumors may be positive factors for tumor recurrence [20]. In this paper, our findings indicated the underlying molecular mechanism of miR-582-5p inhibited cell stemness and recurrence in BC. Our research provided a novel biomarker for the treatment of BC and prognosis.

There is increasing evidence that abnormal expression of miRNAs is closely related to tumor progression, the underlying mechanism may be through regulation of tumor cell stemness. Lin et al. reported that miR-

5188 reduces nuclear translocation of β-catenin by targeting FOXO1, which in turn promotes Wnt signaling and downstream tumor stemness [21]. Tang et al. found that miR-204 promotes stem cell function by interacting with ANXA2 to activate WNT/β-catenin signaling in breast cancer [22]. Our findings revealed that miR-582-5p was lowly expressed in BC tissues, indicating miR-582-5p may play a negative role in BC progression. Functional studies showed that miR-582-5p overexpression inhibited BC cell stemness. Moreover, miRNAs are also closely associated with the recurrence of BC, including miR-31-5p, miR-1178-3p and miR-130a-3p etc. [10, 23, 24]. Our findings indicated that miR-582-5p was down-regulated in BC patients which were recrudescent within 3 years, and miR-582-5p level was negatively correlated with recurrence free survival of BC patients. These results illustrated that miR-582-5p inhibited cell stemness and recurrence in BC. Mechanically, miRNA regulates gene levels horizontally after transcription and transcription by binding to 3'-UTR of the target mRNA [25]. Our results indicated that miR-582-5p negatively targeted to CD81.

CD81 is a cell surface protein that is involved in a variety of cellular functions.. As for the pathogenesis of cancer, CD81 has been proven to play a vital role in the recurrence of multiple cancer [26]. Hong et al. showed that CD81 overexpression accelerated melanoma cell migration and invasion and increased the recurrence rate of patients [27]. CD81 is up-regulated and is associated with poor recurrence free survival [28]. Our results indicated that CD81 was up-regulated in recurrence tissues, and miR-582-5p level was negatively correlated with recurrence free survival of BC patients. Furthermore, we showed for the first time that CD81 is involved in cell stemness in BC, specifically as overexpression of CD81 rescues the suppression of cell stemness caused by overexpression of miR-852. These findings suggest that miR-582-5p inhibited cell stemness and recurrence via targeting CD81 in BC.

Summary, our research illustrated the role of miR-582-5p in cell stemness and recurrence in BC and the potential molecular mechanism. This study indicated that miR-582-59/CD81 axis participate in the regulation of cell stemness and recurrence in BC. These findings may provide a new strategy for BC treatment.

Declarations

Ethics approval and consent to participate

The Ethics Committee of Nanjing Drum Tower Hospital approved to carry out this study and obtained the written and informed consent of all patients. All experiments were conducted in accordance with approved guidelines.

Data availability statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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Conflict

There are no conflicts of interest to declare.

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Table S1

Table S1. Oligonucleotide Sequences for this Study

	Direction	Sequences
miRNA primers		
miR-582-5p	Reverse transcription (5’-3’)	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGTAACTG
	qPCR (5’-3’)	ACACTCCAGCTGGGTTACAGTTGTTCAACCA
RNU6	Reverse transcription (5’-3’)	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAACGCTTC
	qPCR (5’-3’)	ACACTCCAGCTGGGACGCAAATTCGTGAAG
PCR primers		
pCDH-CD81	Forward (5’-3’)	CTAGCTAGCATGGGAGTGGAGGGCTGCAC
	Reverse (5’-3’)	ATTTGCGGCCGCTTAATGTATTAAAGGGTTGG
psiCheck2-CD81-3’UTR-wt	Forward (5’-3’)	AATTCTAGGCGATCGCTCGAGGGCCCCGCAGCTCTGGCC
	Reverse (5’-3’)	ATTTTATTGCGGCCAGCGGCCGCTAGCATGCCTGATGTTCCTTC
psiCheck2-CD81-3’UTR-Mut	Overlap Forward (5’-3’)	GTCCTTTCGCCAACGTCGCCTTACCAGTTAATCACAACATC
	Overlap Reverse (5’-3’)	GATGTTGTGATTAACTGGTAAGGCGACGTTGGCGAAAGGAC

Figures

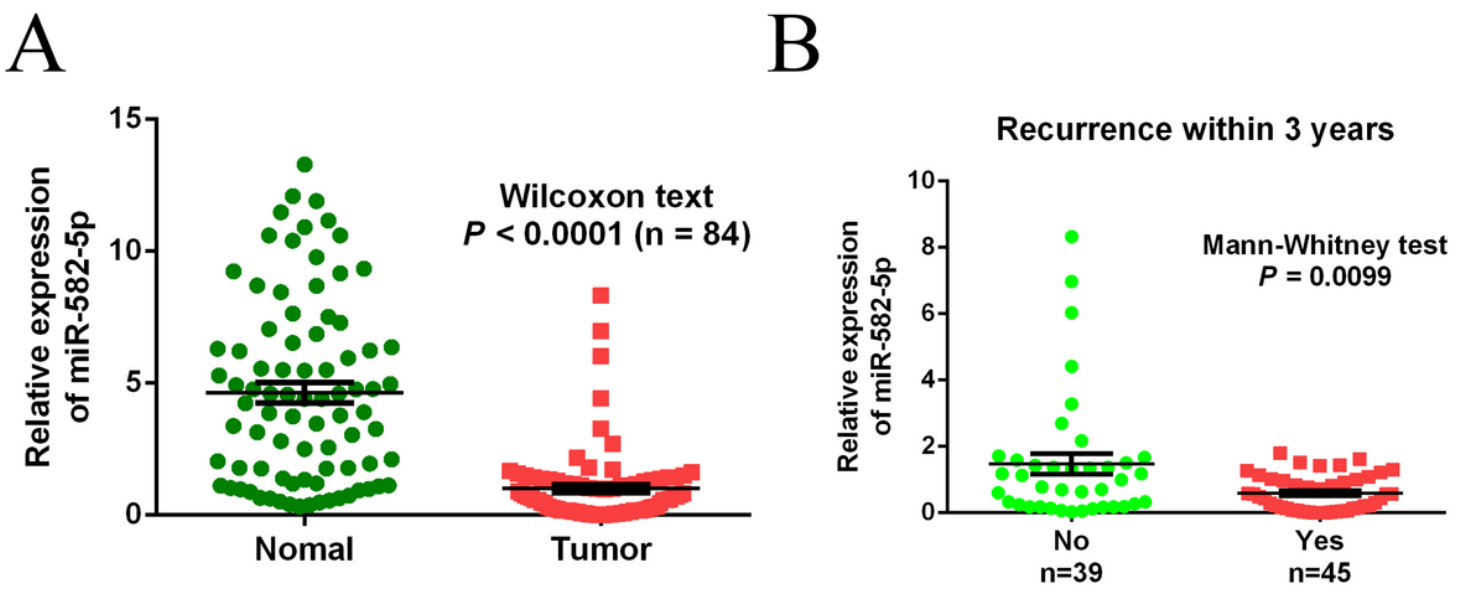

Figure 1

miR-582-5p was down-regulated in patients with BC. A, miR-582 expression level in paired BC tissues and their adjacent normal tissues (n=48). B, miR-582-5p level was detected in BC patients which were recrudescent (n=45) or not (n=39) within 3 years.

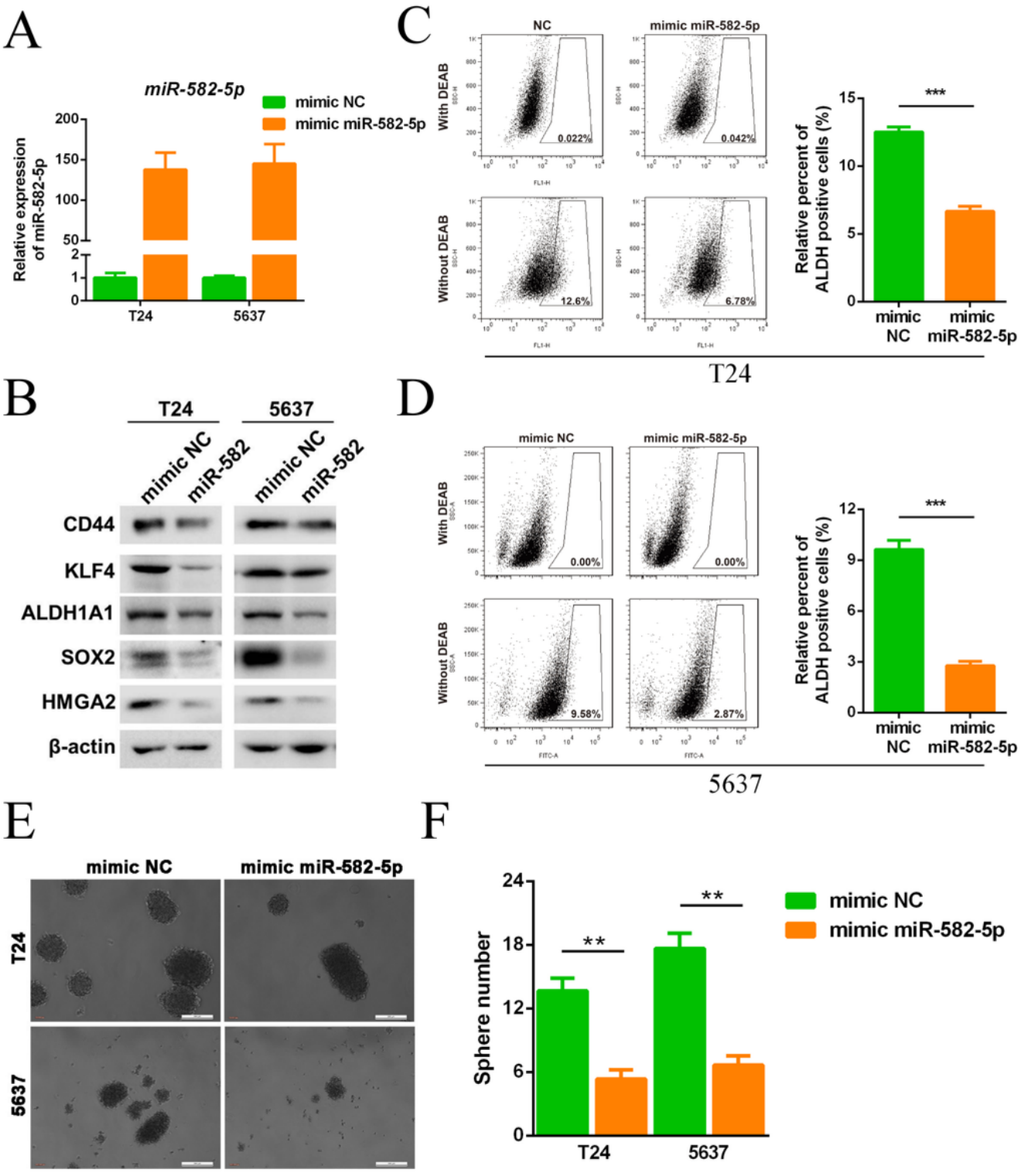

Figure 2

miR-582-5p overexpression negatively correlated with cell stemness in BC. A, miR-582-5p level was detected by qRT-PCR. B, the levels of CSC markers including CD44, KLF4, ALDH1A1, SOX2 and HMGA2 in T24 and 5637 xenografts were measured by Western blot. C-D, the percent of ALDH positive cells in T24 and 5637 xenografts was evaluated by flow cytometer. E-F, Sphere formation assay was performed to

evaluate sphere formation of T24 and 5637 cells. *$P < 0.05$ indicates statistical significance. Data are presented as mean ± SD of three replicate experiments (n=3).

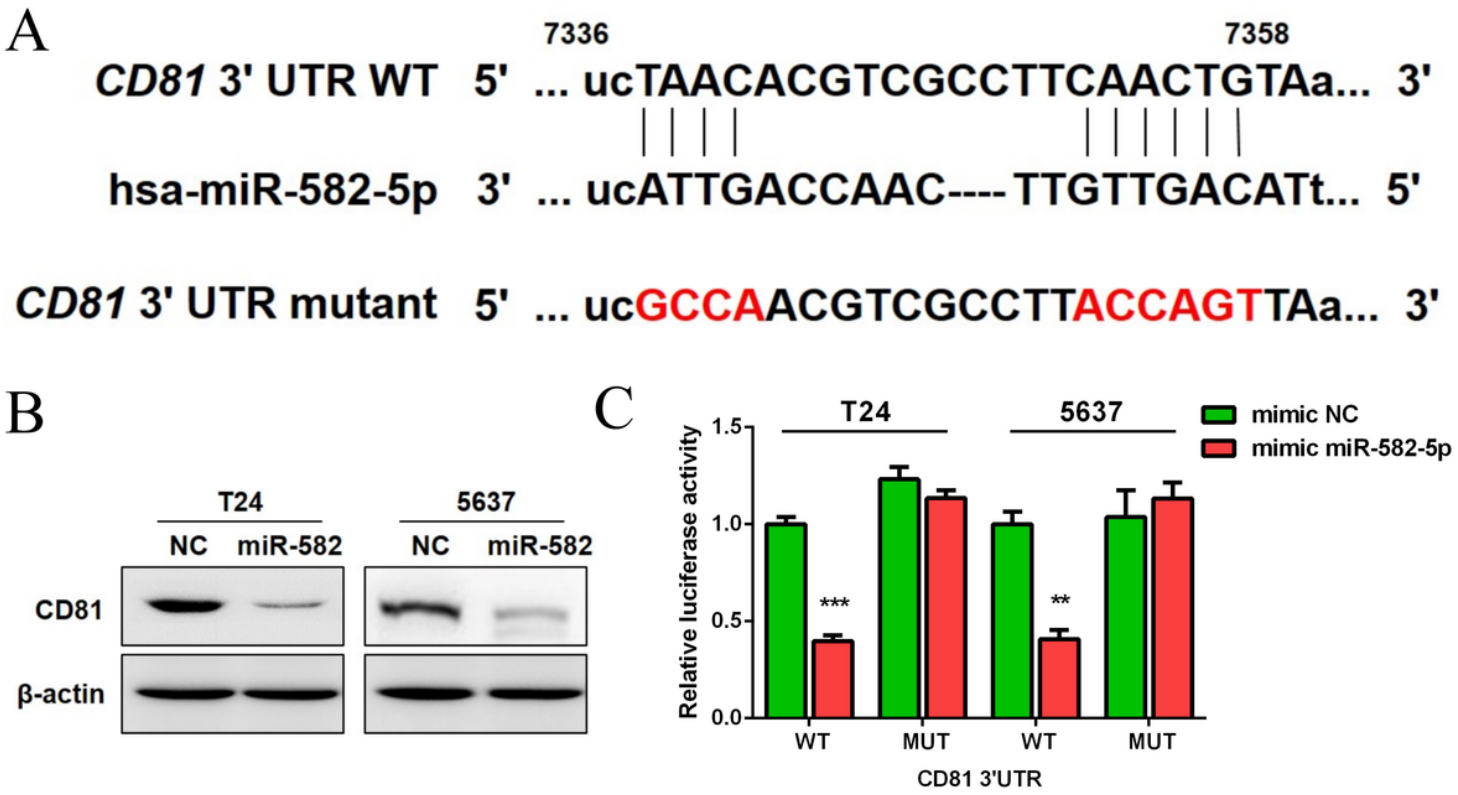

Figure 3

miR-582-5p negatively targeted to CD81. A, the downstream targets of miR-582-5p was predicted by starbase software. B, CD81 level in T24 and 5637 cells transfected with miR-582-5p mimics was measured by Western blot. C, Dual-luciferase reporter was carried to confirm the binding relationship between miR-582-5p and CD81. *$P < 0.05$ indicates statistical significance. Data are presented as mean ± SD of three replicate experiments (n=3).

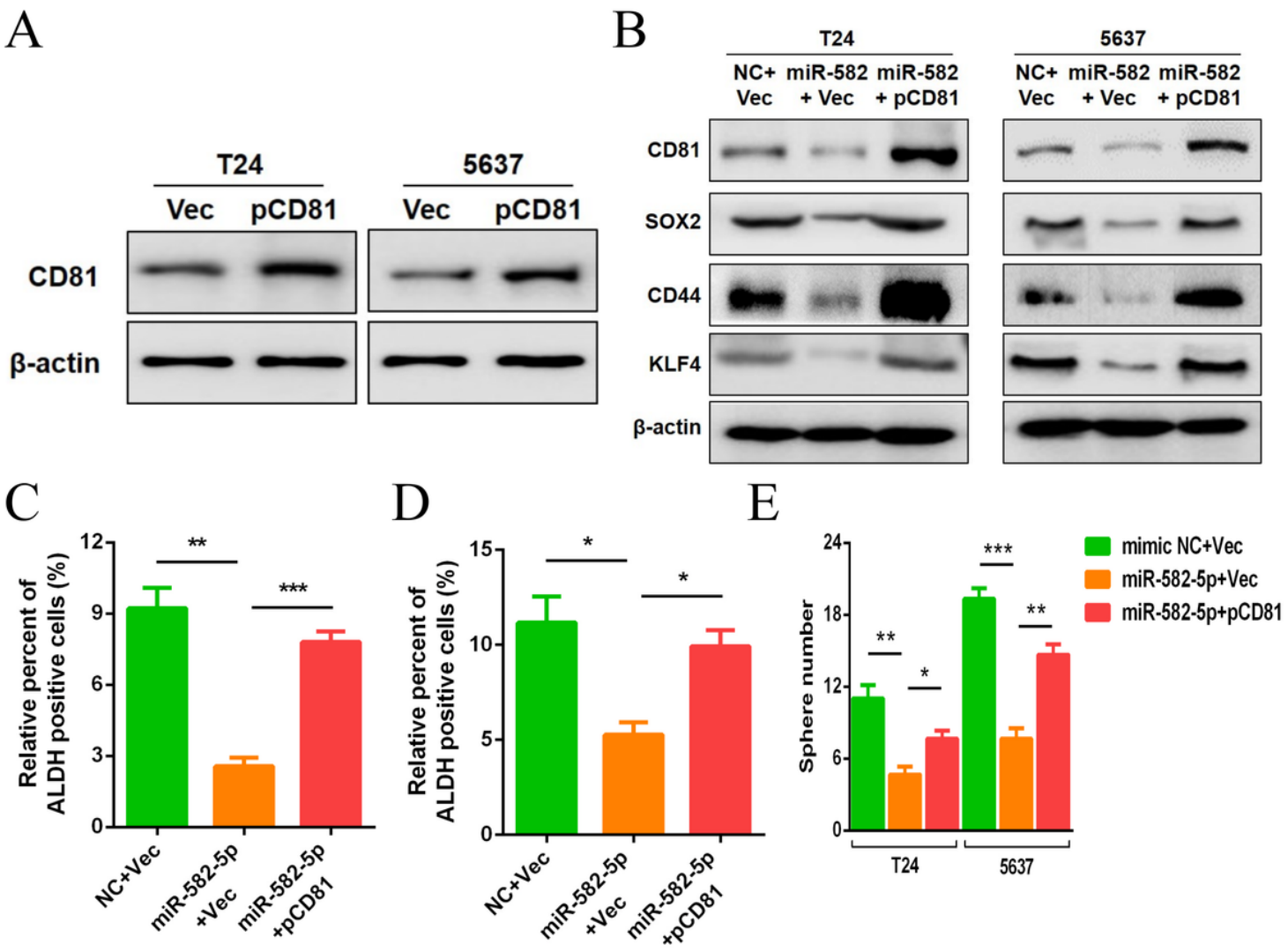

Figure 4

miR-582-5p overexpression inhibited cell stemness via targeting CD81 in BC. A, CD18 protein level in T24 and 5637 cells transfected with pcDNA-CD18. B, the levels of CSC markers including CD44, KLF4, ALDH1A1, SOX2 and HMGA2 in T24 and 5637 cells transfected with miR-582-5p mimics and pcDNA-CD18. C-D, the percent of ALDH positive cells in T24 and 5637 cells transfected with miR-582-5p mimics and pcDNA-CD18 was evaluated by flow cytometer. E, Sphere formation assay was performed to evaluate sphere formation of T24 and 5637 cells transfected with miR-582-5p mimics and pcDNA-CD18. *$P < 0.05$ indicates statistical significance. Data are presented as mean ± SD of three replicate experiments (n=3).

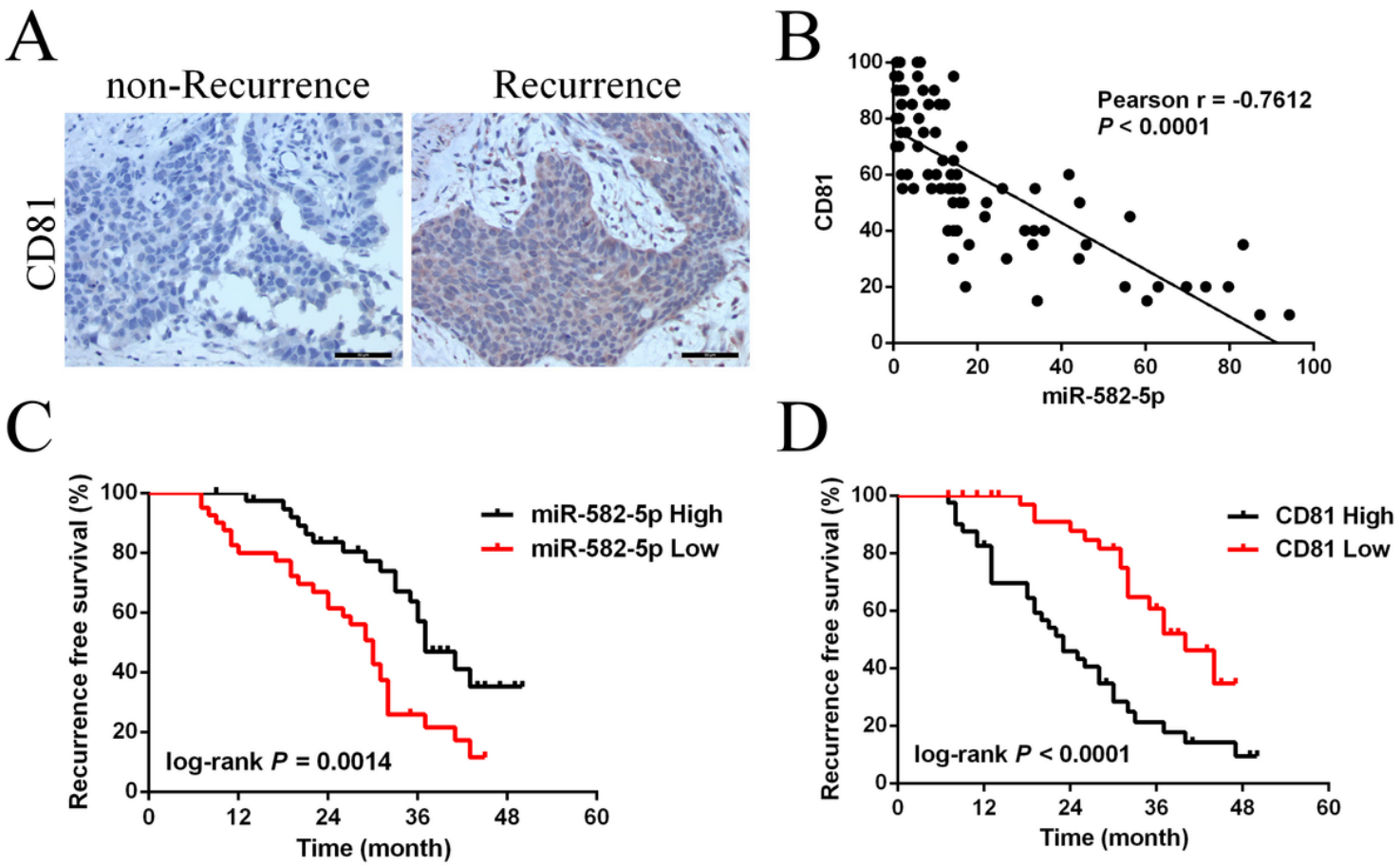

Figure 5

miR-582-5p inhibited recurrence via targeting CD81 in BC. A, CD81 level in recurrence tissues was assessed by IHC. B, the correlation between miR-582-5p or CD81 level. (C-D) Recurrence free survival for all 80 BC patients who underwent radical cystectomy with expression profile of miR-582 high (n = 40) versus miR-582 low (n = 40), CD81 high (n = 40) versus CD81 low (n = 40).