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Hsa circ ACTG1 Promotes Cell Growth of Bladder Cancer Through TGF-β1 Signalling Pathways by miR-744-5p

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ABSTRACT This study investigated the mechanism of circ ACTG1 in invasion and metastasis of patients with bladder cancer. As a result, the expression of circ ACTG1 expression was augmented in a model of bladder cancer. Circ ACTG1 promoted bladder cancer cell growth by miR-744-5p through the induction of TGF-β1/Jagged1/Notch. Si-miR-744-5p promoted cell growth of bladder cancer cells. Moreover, miR-744-5p attenuated the effects of circ ACTG1 on cell growth of bladder cancer cells. The inhibition of TGF-â1 also inhibited the effects of circ ACTG1 on cell growth of bladder cancer cells. Summary, the present findings reveal that circ ACTG1 promotes cell growth of patients with bladder cancer through TGF-â1/Jagged1/NOTCH signalling pathways by miR-744-5p.

INTRODUCTION

There are about 550,000 new bladder cancer (BC) patients worldwide every year, and 200,000 people die from it. Except for some special occupational exposure, smoking is the main cause of bladder cancer. With the increase of female smoking rate, the attributable risk of female bladder cancer has obviously increased. Transurethral resection of bladder tumour (TURBT) is widely used in clinics, and it is the standard operation for the treatment of NMIBC. The recurrence rate of superficial bladder tumours after operation is high. Proper choice of postoperative bladder infusion chemotherapy (BIC) drugs and regular and timely chemotherapy are not only important measures to prevent recurrence of bladder cancer (Ecke et al. 2022). Bladder cancer is a malignant tumor originating from urothelium, which is the most common cause of death caused by urinary system tumors in China. The treatment of bladder cancer includes surgical resection, radiotherapy and chemotherapy, and immunotherapy. Although these treatments have made significant progress in recent years, patients are still more likely to relapse or progress after treatment, and about 40 percent of patients have experienced multiple recurrences (Ecke et al. 2022). At present, the prognosis of bladder cancer patients is mainly predicted by histopathology diagnosis and tumor staging system, but the accuracy is not high (Ecke et al. 2022).

Worldwide, the number of new cases is as high as 430,000 every year, accounting for the 11th place of all malignant tumours (Ecke et al. 2022). It has the characteristics of high recurrence rate and poor prognosis. Accurate detection of tumour markers of bladder cancer have attracted the attention of scholars. The detection of tumour markers has the advantages of high sensitivity, portable operation, non-invasive and low cost (Pu et al. 2021; Hirano et al. 2022). Its results have become an important index for early diagnosis, classification, curative effect monitoring, recurrence and prognosis evaluation of malignant tumours. Tumour markers are substances, such as nucleic acids, protein, enzymes and sugars, which are produced by tumour cells (or cell membrane surfaces) or produced, secreted or shed into human body fluids or tissues by the immune response of the body to tumours. Bladder cancer is a common malignant tumour in the world, which seriously threatens human life and health. Overproliferation, apoptosis reduction and glycolysis are typical biological characteristics of tumour cells, and glycolysis provides energy for tumour cell activities (Pu et al. 2021; Hirano et al. 2022).

Transforming growth factor beta1 (TGF-β1) plays a dual role in tumorigenesis and tumour

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progression of BC (Wang et al. 2017). To be specific, tumour cells express a small amount of TGF-β1 in the early stage of tumorigenesis, where TGF-β1 plays a tumour-suppressing role to inhibit normal cell growth and tumorigenesis by suppressing cell cycle G1-S phase (Yang et al. 2012). However, both tumour cells and intra-tumoral stromal cells produce a large amount of TGF-β1 during tumour progression of BC (Wang et al. 2017). Due to the insensitivity of the inhibition of autocrine TGF-β1 by tumour cells, TGF-β1 tolerance exists within the tumour mass of BC (Yang et al. 2012). TGF-β1 acts on surrounding tissues (Liu et al. 2018b). The effects of TGF-β1 decrease the spatial restriction of tumour cell proliferation by inhibiting the proliferation of surrounding tissues, subsequently leading to accelerated tumour cell proliferation of BC (Liu et al. 2018b). TGF is a peptide cytokine secreted by multiple cells with multiple functional effects, which plays a role in cellular biological events such as differentiation, apoptosis, and proliferation. In addition, it also participates in regulating angiogenesis, damage repair, fibrosis, and tumor development by regulating the transcription and expression of downstream target genes (Liu et al. 2018b). Previous studies have shown that TGF-β broad involvement of pathways in mediating and regulating EMT processes (Liu et al. 2018b).

Jagged1 is one of the major ligands of Notch receptors on mammalian cell membranes (Hai et al. 2018). The expression of Jagged1 (a single-transmembrane glycoprotein) is widely distributed in various tissues (Qiu et al. 2015). Functionally, Jagged1 plays an important role in the growth, moreover maintaining normal hematopoietic progenitor cells and their proliferation. Moreover, the binding of Jagged1 to its receptor has recently been found to activate the Notch signalling pathway (Qiu et al. 2015).

Notch signalling exerts a wide and diverse effect on cell growth and development, either promoting or inhibiting cell proliferation, differentiation and apoptosis (Parajuli and Mittal 2018). Notch signalling pathway also actively interacts with other vital pathways involved in cancer progression, which is associated with the pathogenesis and progression of certain types of human tumours (Spino et al. 2018). Notch signalling might play a cancer-promoting role or tumour-suppressor effect in types of tumours (Kelliher and Roderick 2018). In addition, Notch signalling may have diverse effects even within the same tumour depending on different tumour stages (Kelliher and Roderick 2018). Studies have shown the abnormal expression of Notch signalling in certain brain tumours (sputum blastoma, neuroblastoma and meningioma), which is associated with the pathogenesis and progression of these brain tumours (Kelliher and Roderick 2018; Tchekneva et al. 2019). Thus, the Notch signalling pathway might be used as a novel target for tumours.

CircRNA is a non-coding RNA molecule that regulates gene expression by competitively binding to miRNA. The biogenesis of CircRNA mainly includes four models, that is, exon lasso splicing cyclisation, intron pairing cyclisation, inner lasso splicing cyclisation, RNA binding protein or trans-factor driven cyclisation (Yang et al. 2014). CircRNA is a single-stranded ring structure, which can form a double-stranded structure with some mRNA, thus regulating the expression of mRNA (Celik et al. 2013; Pu et al. 2020). Among them, CircRNA acts as a 'molecular sponge', which competitively combines with some miRNAs to promote the expression of target mRNA (Ma et al. 2022). Through the research on the mechanism of CircrRNA in bladder cancer, it is found that CircrRNA can indirectly regulate the downstream target mRNA, that is, the ceRNA mechanism pathway of CircRNA-mirna-mRNA type, by acting as a 'molecular sponge' of related miRNAs (Celik et al. 2013; Pu et al. 2020).

In recent years, it has been found that miRNA is abnormally expressed in many tumours (Zhang et al. 2018). Circulating miRNA mainly comes from the active secretion of cells, the cracking of circulating cells or the passive release due to apoptosis, and then it is coated in lipoprotein complexes such as microsomes or exosomes, which has good stability and can tolerate the action of RNase, acid and alkali environment and repeated freezing and thawing (Tchekneva et al. 2019; Xiao et al. 2019).

Objective of the Study

In this study, this experiment explored the mechanism underlying the effects of circ ACTG1 on bladder cancer cell invasion.

MATERIAL AND METHODS

Patients with Bladder Cancer

This study was approved by the Ethics Committee of the hospital. A total 24 of patients with bladder cancer and 24 of normal volunteers were obtained from between January 2017 and August 2017 (Table 1). All serum samples were collected and saved at -80 °C. Written informed consent was obtained from all participants enrolled in the current research.

Table 1: Basic knowledge of patient with Glioma

Variables	*All patients (24)*	*All patients (24)*
Age (yr)		
≤55	12	12
>55	12	12
Gender		
Female	12	12
Male	12	12
Tumor Size (cm)		
≤3.0	10	
>3.0	14	
Edmondson Grade		
I -II	2	
III	9	
IV	13	

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Trizol reagent (Invitrogen, USA) was used to extract total RNA r. The PCR primers were as follows: circ ACTG1 F: 52 -ATGGAAGGAAACACGGCTC-32 and 52 -CACTCTGTTCTTCCGCCG-32 ; miR-744-5p F: 52 -AATGCGGGGCTAGGGCTA-32 and R: 52 -GTGCAGGGTCCGAGGT-32 ; and U6: F: 52 -GCTTCGGCAGCACATATACT-32 and R: 5'-GGAACGCTTCACGAATTTGC-3'. Relative gene expression was analysed by the 2-ΔΔCq method.

Cell Culture and Cell Transfection

HCV-29, 5637 and T24 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM,) with 10 percent foetal bovine serum (FBS) in a 37°C with 5 percent CO^2 condition. MiR-744-5p mimics, miR-744-5p inhibitor mimics and negative mimics were transfected into T24 cells using Lipofectamine 2000 (Invitrogen). Si-TGF-β1, si-Jagged1 or si-NOTCH1 and miR-744-5p inhibitor mimics were transfected into T24 cells using Lipofectamine 2000 (Invitrogen) for 48 hours.

Cell Counting Kit-8 (CCK8), Transwell Migration, Invasion Assays, LDH Activity and Caspase-3/9 Activity Levels

CK-8 assay (Dojindo, Kumamoto, Japan) added into each well and the culture plates were incubated for 1 hour. The absorbance was detected using a microplate reader (Bio-Rad Laboratories, Richmond, CA, USA) at 450 nm. The migratory and invasiveness were assessed using an 8-μm pore polycarbonate membrane Boyden chamber insert in a Corning Transwell apparatus (Corning, NY, USA). Cells were collected at 48 hours post-transfection, washed with PBS and mechanically dissociated into a single cell suspension. The cells were fixed with 100 percent methanol, stained with 0.05 percent crystal violet, washed with PBS, and invasion was observed under an inverted light microscope.

Cells were treated with lysis buffer (Beyotime) and protein concentration was determined with a BCA protein assay kit (Pierce, USA). 10 μg total protein was used to measure LDH activity and Caspase-3/9 activity levels using LDH activity and Caspase-3/9 activity levels kits (Beyotime).

Luciferase Assay

Cells were transfected with Circ ACTG1 and miR-744-5p plasmids or miR-744-5p and TGF-β1 3'-UTR-wild type and mutant plasmids. Renilla luciferase activity acted as the control after transfection for 48 hours. The activity was detected by using a luciferase assay kit (Beyotime Institute of Biotechnology, Shanghai, China).

Western Blot Analysis

Proteins were separated by SDS-PAGE and transferred into the polyvinylidene difluoride (PVDF) membranes (Invitrogen). Membranes were then incubated overnight with anti-TGF-β1 antibody (1:1000, Abcam, UK), Jagged1, NOTCH and actin antibody (Abcam, 1:1000) at 4°C. The membrane was incubated with goat anti-rabbit IgG-HRP antibodies for 1 hour and proteins were visualised by enhanced chemiluminescence.

Vivo Model

BALB/c nude mice were intraperitoneally injected with pentobarbital sodium (40 mg/kg) and inoculated subcutaneously from the right forelimb with 0.2 mL of cells (2 × 106 cells/mL). Every three days, tumour volume was measured using vernier calliper.

Immunofluorescence

Cell samples were incubated with antibodies (1:100, Abcam, UK), at 4 °C overnight. Cells were incubated with a secondary peroxidase-conjugated goat anti-rabbit-555 IgG (1:100, Santa Cruz Biotechnology) antibody at room temperature. Cells were stained with DAPI for 15 minutes in darkness. Samples were observed using fluorescence microscopes.

Statistical Analysis

The data are expressed using mean ± standard error in SPSS 20.0 software. Statistical significance is defined as p<0. 05. One-way analysis of variance (ANOVA) and Tukey's post test or Student's t-test were used to analyse comparison between groups.

RESULTS

The Expression of circ ACTG1 in Bladder Cancer Cell Lines and Clinical Samples

To analyse the mechanism and function of circular RNA affected bladder cancer, the researchers first found that the expression levels of circ ACTG1 was up-regulated in patients with bladder cancer (1.075 ± 0.339 vs 2.814 ±1.011, p < 0.01, Fig.1A, Table 2). Meanwhile, the expression level of circ ACTG1 was higher in bladder cancer cell lines than in that NHA cells (1±0.12 vs 2.45±0.07 vs 2.17±0.22, p < 0.01, Fig.1B, Table 2). Circ ACTG1 might be involved in the development of bladder cancer.

Table 2.: The expression of circ ACTG1 in Bladder cancer cell lines and clinical samples

	Normal	*Model*	
Circ ACTG1 expression	1.075 ± 0.339	2.814 ±1.011	
	HCV-29	*5637*	*T24*
Circ ACTG1 expression	1±0.12	2.45±0.07	2.17±0.22

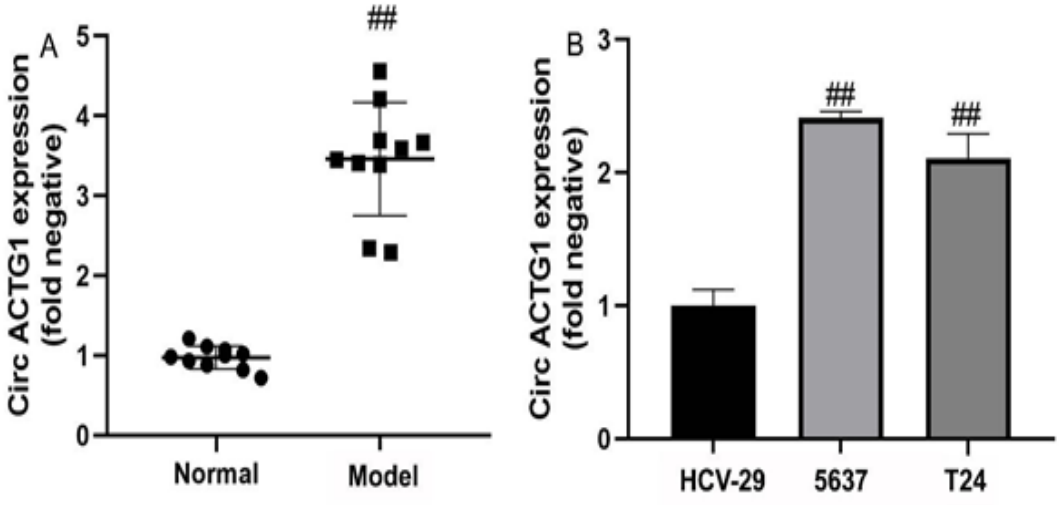

Fig. 1. Circ ACTG1 expression in patients with Bladder cancer or cell lines Circ ACTG1 expression levels was increased in patients with Bladder cancer using qRT-PCR method, circ ACTG1 expression (B) Bladder epithelial cells or bladder cancer cell lines. The transfection effects were confirmed by using qRT-PCR method. p<0.01 ## versus normal group or NHA group

The Functions of circ ACTG1 In Vitro Model

Afterwards, this experiment explored the effects and function of circ ACTG1 on cell growth and proliferation in the in vitro model. The expression of of circ ACTG1 was increased in circ ACTG1 over-expression group, compared with negative group (1.02 ± 0.018 vs 3.56 ± 0.025, p < 0.01, Fig.2A, Table 3). Circ ACTG1 promoted cell proliferation and invasion of bladder cancer cell (1.78 ± 0.011 vs 3.12 ± 0.024; 1.05 ± 0.021 vs 1.89 ± 0.038; all p < 0.01, Fig.2B-2D, Table 3). In addition, over-expression of circ ACTG1 decreased LDH activity level and caspase-3/9 activity levels *in* bladder cancer cell (1.06 ± 0.014 vs 0.36 ± 0.22; 1.03 ± 0.041 vs 0.25 ± 0.061; 1.05 ± 0.088 vs 0.48 ± 0.091; all p <0.01, Fig. 2E-2G, Table 3). By contrast, the expression of circ ACTG1 was lower in circ ACTG1 down-regulation group than that of the negative group (1.04 ± 0.056 vs 0.45 ± 0.033; p < 0.01, Fig.2H, Table 3). Functionally, si-circ ACTG1 inhibited cell proliferation and invasion in bladder cancer cells (2.05 ± 0.042 vs 1.48 ± 0.033; 1.05 ± 0.015 vs 0.44 ± 0.065; all p < 0.01, Fig. 2I-2K, Table 3). Down-regulation of circ ACTG1 increased LDH activity level and caspase-3/9 activ-

Table 3: The function of circ ACTG1 in vitro model

Group	*Circ ACTG1 expression*	*Cell proli-feration*	*Cell invasion*	*LDH activity*	*Caspase-3 activity*	*Caspase-9 activity*
Negative	1.02 ± 0.018	1.78 ± 0.011	1.05 ± 0.021	1.06 ± 0.014	1.03 ± 0.041	1.05 ± 0.088
Circ ACTG1	3.56 ± 0.025	3.12 ± 0.024	1.89 ± 0.038	0.36 ± 0.22	0.25 ± 0.061	0.48 ± 0.091
Negative	1.04 ± 0.056	2.05 ± 0.042	1.05 ± 0.015	1.03 ± 0.011	1.07 ± 0.011	1.05 ± 0.017
Circ ACTG1 inhibitor	0.45 ± 0.033	1.48 ± 0.033	0.44 ± 0.065	5.23 ± 0.212	6.12 ± 0.17	4.32 ± 0.21

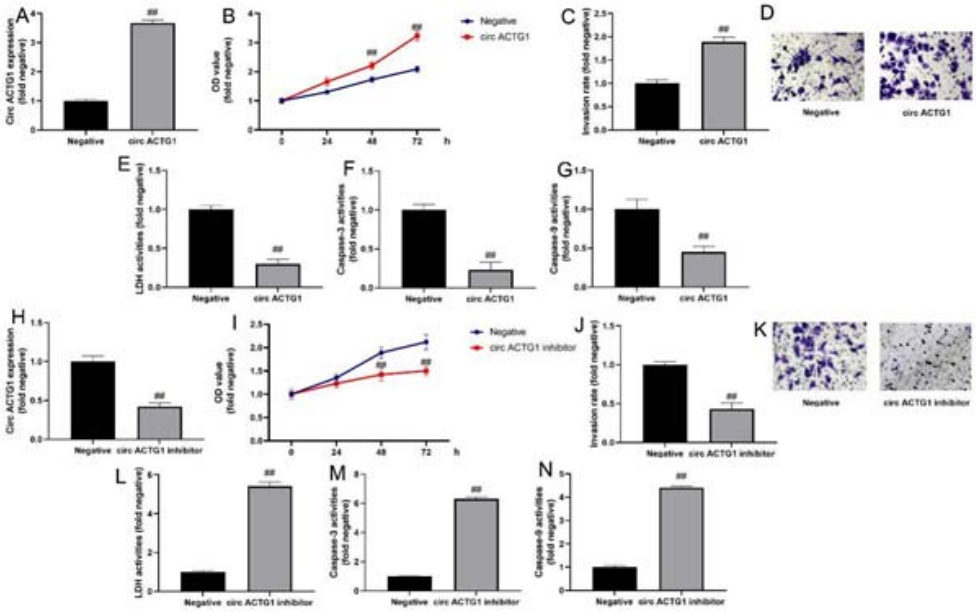

Fig. 2. The function of circ ACTG1 in vitro model
Circ ACTG1 increased the expression levels of circ ACTG1 (A), cell proliferation (B), invasion (C and D), LDH activity (E) and Caspase-3/9 activity levels (F and G) in T24 cells. Circ ACTG1 inhibitor reduced the expression levels of miR-744-5p (H), cell proliferation (I), invasion (J and K), LDH activity (L) and Caspase-3/9 activity levels (M and N) in T24 cells. Negative, negative mimics group; circ ACTG1 inhibitor, down-regulation of miR-744-5p expression group; circ ACTG1, over-expression of circ ACTG1 expression group. ##p<0.01 versus negative mimics group

ity levels in bladder cancer cells (1.03 ± 0.011 vs 5.23 ± 0.212; 1.07 ± 0.011 vs 6.12 ± 0.17; 1.05 ± 0.017 vs 4.32±0.21; all p < 0.01, Fig. 2L-2N, Table 3).

Circ ACTG1 Regulates Cell Growth of Bladder Cancer by miR-744-5p

The expression levels of miR-744-5p were measured in bladder cancer cell lines with qRT-qPCR. The results of qRT-qPCR uncovered that miR-744-5p expression levels was reduced in patients with bladder cancer (1.05 ± 0.321 vs 0.325 ± 0.142, p < 0.01, Fig.3A, Table 4). Meanwhile, miR-744-5p expression levels were lower in bladder cancer cell lines (5637 and T24) than those in NHA cells (1.00 ± 0.15 vs 0.42 ± 0.11 vs 0.38 ± 0.17, all p < 0.01, Fig.3B, Table 4). The expression of circ ACTG1 was negatively correlated with miR-744-5p expression (Fig.3C, Table 4). The putative binding sites of miR-744-5p on the sequence of wild-type (WT) circ ACTG1 and luciferase activity levels was decreased in miR-744-5p + circ ACTG1-WT group (1.00 ± 0.05 vs 0.31 ± 0.08; all p <0.01, Fig.3D-3E, Table 4). Circ ACTG1 decreased the expression of miR-744-5p while si-circ ACTG1 increased miR-744-5p expression in bladder cancer cells (1.00 ± 0.07 vs 0.42 ± 0.09; 1.01 ± 0.09 vs 3.15 ± 0.17; all p < 0.01, Fig.3F-3G, Table 4) MiR-744-5p might be involved in the regulatory roles of circ ACTG1 on bladder cancer.

Table 4: MiR-744-5p could regulate cell proliferation and invasion in vitro model of Bladder cancer

Group	*Normal*	*Model*	
MiR-744-5p expression	1.05 ± 0.321	0.325 ± 0.142	
Group	*HCV-29*	*5637*	*T24*
MiR-744-5p expression	1.00 ± 0.15	0.42 ± 0.11	0.38 ± 0.17
Group	*Negative*	*MiR-744-5p-Circ ACTG1-wt*	
Luciferase reporter	1.00 ± 0.05	0.31 ± 0.08	
Group	*Negative*	*Circ ACTG1*	
MiR-744-5p expression	1.00 ± 0.07	0.42 ± 0.09	
Group	*Negative*	*Circ ACTG1 inhibitor*	
MiR-744-5p expression	1.01 ± 0.09	3.15 ± 0.17	

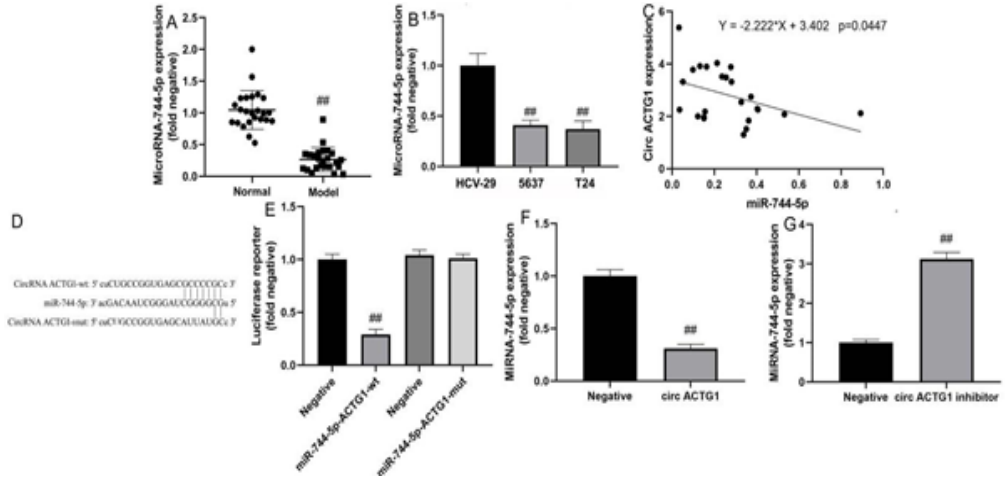

Fig. 3. Circ ACTG1 regulates cell growth of Bladder cancer by miR-744-5p
MiR-744-5p expression levels was reduced in patients with Bladder cancer (A), miR-744-5p expression (B) , Correlation between the expression of miR-744-5p and Circ ACTG1 was evaluated by Pearson's correlation test (C), The putative binding sites of miR-744-5p on the Circ ACTG1 wild-type (WT) or mutated sequence are shown (D), luciferase reporter assay (E), miR-744-5p expression levels by over-expression of circ ACTG1 (F) or down-regulation of circ ACTG1 (G). Negative, negative mimics group; circ ACTG1 inhibitor, down-regulation of miR-744-5p expression group; circ ACTG1, over-expression of circ ACTG1 expression group. ##p<0.01 versus negative mimics group

MiR-744-5p Regulates Cell Proliferation of Bladder Cancer in vitro

The researchers further explored the effects of miR-744-5p on cell proliferation and invasion in the *in vitro* model of bladder cancer. After transfection of miR-744-5p mimics into T24 cells, the expression levels of miR-744-5p were increased in comparison with normal group (1.00 ± 0.04 vs 4.35 ± 0.25, p < 0.01, Fig.4A, Table 5). MiR-744-5p reduced cell proliferation and invasion, and increased LDH activity and Caspase-3/9 activity levels in T24 cells, compared with the negative group (2.05 ± 0.15 vs 1.37 ± 0.07; 1.00 ± 0.11 vs 0.41 ± 0.18; 1.01 ± 0.03 vs 4.21 ± 0.11; 1.04 ± 0.12 vs 3.32 ± 0.32; 1.02 ± 0.15 vs 4.47 ± 0.23; all p < 0.01, Fig. 4B-4G, Table 5). However, after transfection of miR-744-5p inhibitor into T24 cells, and the expression levels of miR-744-5p were decreased, compared with normal group (1.02 ± 0.11 vs 0.39 ±0.09, p < 0.01, Fig. 4H, Table 5). Si-miR-744-5p further promoted cell proliferation and invasion, and reduced LDH activity and Caspase-3/9 activity levels in T24 cells (1.98 ± 0.05 vs 3.15 ± 0.14; 1.04 ± 0.07 vs 0.23 ± 0.08; 1.00 ± 0.07 vs 0.42 ± 0.05; 1.01 ± 0.09 vs 0.48 ± 0.07; all p < 0.01, Fig.4I-4N, Table 5).

Table 5. Circ ACTG1 regulates cell growth of Bladder cancer by miR-744-5p

Group	*Circ ACTG1 expression*	*Cell proli-feration*	*Cell invasion*	*LDH activity*	*Caspase-3 activity*	*Caspase-9 activity*
Negative	1.00 ± 0.04	2.05 ± 0.15	1.00 ± 0.11	1.01 ± 0.03	1.04 ± 0.12	1.02 ± 0.15
miR-744-5p	4.35 ± 0.25	1.37 ± 0.07	0.41 ± 0.18	4.21 ± 0.11	3.32 ± 0.32	4.47 ± 0.23
Negative		1.02 ± 0.11	1.98 ± 0.05	1.04 ± 0.07	1.00 ± 0.07	1.01 ± 0.09
miR-744-5p inhibitor	0.39 ±0.09	3.15 ± 0.14	0.23 ± 0.08	0.42 ± 0.05	0.48 ± 0.07	

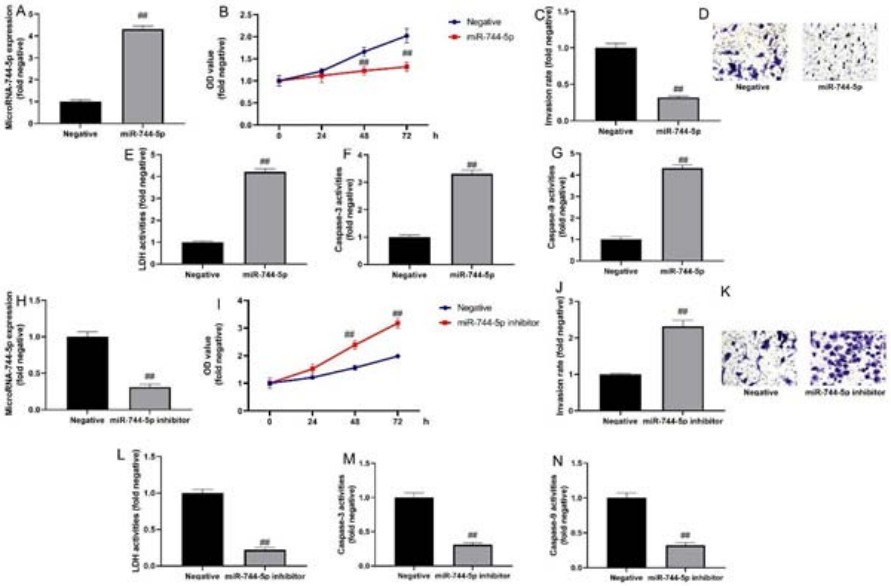

Fig. 4. MiR-744-5p could regulate cell proliferation and invasion in vitro model of Bladder cancer
MiR-744-5p mimics increased the expression levels of miR-744-5p (A), cell proliferation (B), invasion (C and D), LDH activity (E) and Caspase-3/9 activity levels (F and G) in T24 cells. MiR-744-5p inhibitor the expression levels of miR-744-5p (H), cell proliferation (I), invasion (J and K), LDH activity (L) and Caspase-3/9 activity levels (M and N) in T24 cells. Negative, negative mimics group; MiR-744-5p inhibitor, down-regulation of miR-744-5p expression group; miR-744-5p, over-expression of miR-744-5p expression group. ##p<0.01 versus negative mimics group

Circ ACTG1 Regulates Cell Growth of Bladder Cancer via TGF-β1/Jagged1/NOTCH Signalling Pathways by miR-744-5p

To probe the mechanism of miR-744-5p on cell growth of bladder cancer, 3'-UTR of TGF-β1 was complementary to the seed sequence of miR-744-5p and luciferase activity levels were decreased in T24 cells following miR-744-5p+ TGF-β1-WT, in comparison with the negative group (1.01 ± 0.03 vs 0.41 ± 0.07, $p < 0.01$, Fig. 5A-5B, Table 6). Over-expression of miR-744-5p suppressed the protein expression of TGF-β1, Jagged1 and NOTCH1 in T24 cells (1.05 ± 0.07 vs 0.41 ± 0.12; 1.01 ± 0.05 vs 0.54 ± 0.14; 1.01 ± 0.11 vs 0.33 ± 0.15; all $p < 0.01$, Fig. 5C-5F, Table 6). Moreover, circ ACTG1 induced the protein expression of TGF-β1, Jagged1 and NOTCH1 in T24 cells in comparison with the negative group (1.01 ± 0.03 vs 3.52 ± 0.14; 1.03 ± 0.01 vs 2.24 ± 0.21; 1.02 ± 0.01 vs 3.11 ± 0.11; all $p < 0.01$, Fig. 5G-5J, Table 6).

Table 6: Circ ACTG1 regulates cell growth of Bladder cancer via TGF-β1/Jagged1/NOTCH signaling pathways by miR-744-5p

Group	*Negative*	*miR-744-5p TGF-β1WT*
Luciferase activity	1.01 ± 0.03	0.41 ± 0.07
Protein expression	Negative	miR-744-5p
TGF-β1	1.05 ± 0.07	0.41 ± 0.12
Jagged1	1.01 ± 0.05	0.54 ± 0.14
NOTCH1	1.01 ± 0.11	0.33 ± 0.15
Protein expression	Negative	Circ ACTG1
TGF-β1	1.01 ± 0.03	3.52 ± 0.14
Jagged1	1.03 ± 0.01	2.24 ± 0.21
NOTCH1	1.02 ± 0.01	3.11 ± 0.11

Circ ACTG1 Promoted Cancer Cell Growth of Vivo Model

Moreover, the in vivo model was used to analyse the functions of circ ACTG1 on cell growth in bladder cancer. The expression of circ ACTG1 was

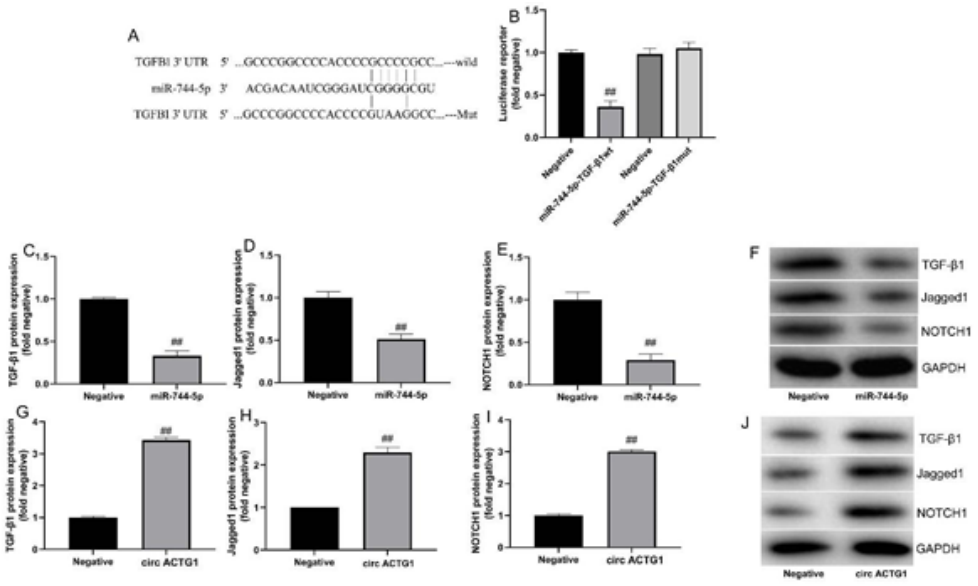

Fig. 5. Circ ACTG1 regulates cell growth of Bladder cancer via TGF-?1/Jagged1/NOTCH signaling pathways by miR-744-5p

3'-UTR of TGF-?1 is complementary to the miR-744-5p seed sequence (A), luciferase activity levels was decreased in T24 cells (B), over-expression of miR-744-5p suppressed TGF-?1, Jagged1 and NOTCH1 protein expression by statistical analysis (C, D and E) and western blotting analysis (F); Circ ACTG1 induced TGF-?1, Jagged1 and NOTCH1 protein expression by statistical analysis (G, H and I) and western blotting analysis (J). Negative, negative mimics group; miR-744-5p, over-expression of miR-744-5p expression group; circ ACTG1, over-expression of circ ACTG1 expression group. ##p<0.01 versus negative mimics group

increased after circ ACTG1 (1.02 ± 0.04 vs 3.34 ± 0.15, p < 0.01, Fig.6A, Table 7). As shown in Figure 6B-6F, over-expression of circ ACTG1 promoted cancer cell growth, increased tumour weight, induced the expression of ki67 expression and reduced that of miR-744-5p *in vivo*, compared with those in the negative group (1.01 ± 0.11 vs 0.46 ± 0.12; 1.00 ± 0.04 vs 0.41 ± 0.14; 415 ± 18 vs 825 ± 47; all p < 0.01, Fig. 6B-6F, Table 7). Over-expression of circ ACTG1 induced the protein expression of TGF-β1, Jagged1 and NOTCH1, and reduced caspase-3/9 activity levels *in vivo* (1.01 ± 0.02 vs 3.46 ± 0.05; 1.00 ± 0.01 vs 3.15 ± 0.09; 1.02 ± 0.03 vs 2.88 ± 0.05; 1.00 ± 0.04 vs 0.58 ± 0.15; 1.00 ± 0.08 vs 0.47 ± 0.07; all p < 0.01, Fig. 6G-6L, Table 7).

Table 7: Circ ACTG1 promoted cancer cell growth of vivo model

Group	*Negative*	*Circ ACTG1*
Circ ACTG1 expression	1.02 ± 0.04	3.34 ± 0.15
Tumor volume	1.01 ± 0.11	0.46 ± 0.12
miR-744-5p expression	1.00 ± 0.04	0.41 ± 0.14
Tumor weight	415 ± 0.18	825 ± 0.47
Protein expression		
TGF-â1	1.01 ± 0.02	3.46 ± 0.05
Jagged1	1.00 ± 0.01	3.15 ± 0.09
NOTCH1	1.02 ± 0.03	2.88 ± 0.05
Caspase-3 activity	1.00 ± 0.04	0.58 ± 0. 15
Caspase-9 activity	1.00 ± 0.08	0.47 ± 0.07

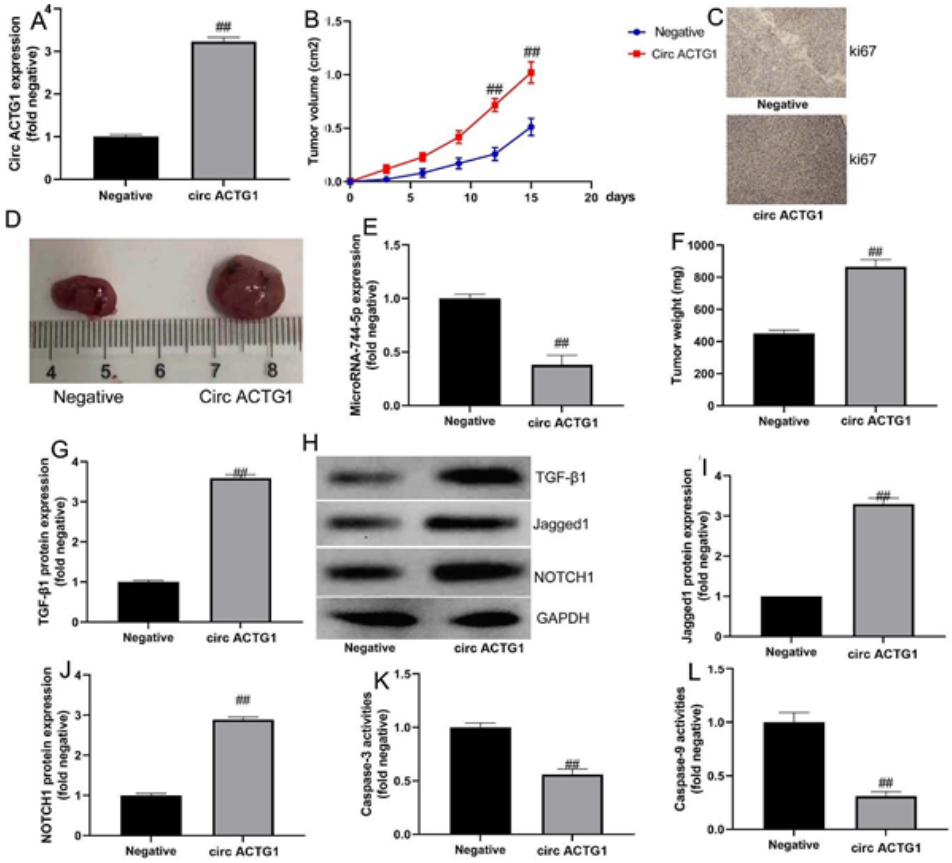

Fig. 6. Circ ACTG1 promoted cancer cell growth of vivo model
The expression levels of Circ ACTG1 (A), Tumor volume (B), ki67 expression (C), Tumor vivo model (D), miR-744-5p expression levels (E), Tumor weight (F), TGF-?1, Jagged1 and NOTCH1 protein expression by statistical analysis (G, H and I) and western blotting analysis (J), Caspase-3/9 activity levels (K and L) in vivo model. circ ACTG1, over-expression of circ ACTG1 expression group. ##p<0.01 versus negative mimics group

miR-744-5p Attenuates the Effects of circ ACTG1 on Bladder Cancer Cell Proliferation in Bladder Cancer

To further confirm the role of miR-744-5p on invasion by circ ACTG1 in bladder cancer, miR-744-5p and circ ACTG1 plasmids were co-transfected into T24 cells. As shown in Figure 7A, miR-744-5p plasmid increased the expression of miR-744-5p in T24 cells following circ ACTG1 (1.00 ± 0.05 vs 0.23 ± 0.07 vs 0.71 ± 0.08, all $p < 0.01$, Table 8). MiR-744-5p suppressed the protein expression of TGF-β1, Jagged1 and NOTCH1 in T24 cells following circ ACTG1 compared with circ ACTG1 group (1.00 ± 0.01 vs 3.11 ± 0.08 vs 1.56 ± 0.05, 1.01± 0.02 vs 3.87 ± 0.11 vs 1.46 ± 0.07, 1.01 ± 0.01 vs 2.89 ± 0.05 vs 1.87 ± 0.03, all $p < 0.01$, Fig. 7B-7E, Table 8). MiR-744-5p attenuated the effects of circ ACTG1 on promotion of cell proliferation and invasion, and inhibition of LDH activity and Caspase-3/9 activity levels in bladder cancer *cell*, compared with si-miR-744-5p group (1.00

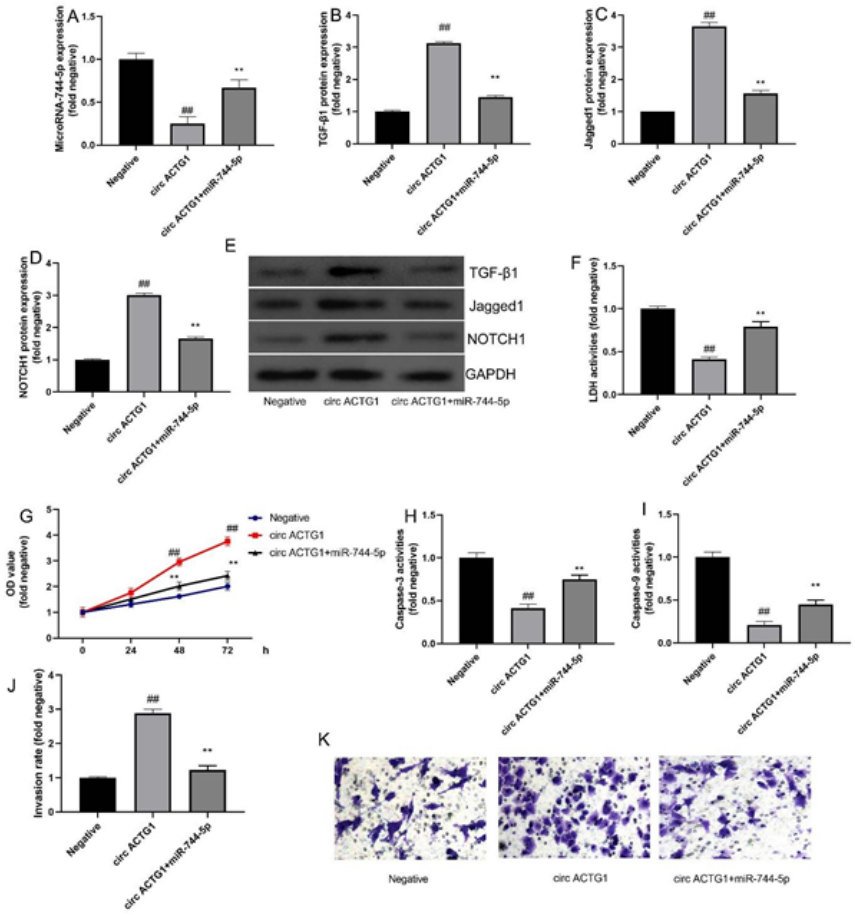

Fig. 7. MiR-744-5p reduced the effects of circ ACTG1 on cell proliferation and invasion in vitro model of Bladder cancer MiR-744-5p expression (A), TGF-β1, Jagged1 and NOTCH1 protein expression by statistical analysis (B, C and D) and western blotting analysis (E), cell proliferation (F), invasion (G and H), LDH activity (I) and Caspase-3/9 activity levels (J and K) in T24 cells. Negative, negative mimics group; circ ACTG1, over-expression of circ ACTG1 expression group; circ ACTG1+miR-744-5p, over-expression of circ ACTG1 expression and miR-744-5p group. ##p<0.01 versus negative mimics group, **p<0.01 versus over-expression of circ ACTG1 expression group

± 0.03 vs 0.42 ± 0.05 vs 0.86 ± 0.06, 1.88 ± 0.04 vs 3.92 ± 0.12 vs 2.19 ± 0.08, 1.00 ± 0.07 vs 0.48 ± 0.07 vs 0.78 ± 0.06, 1.00 ± 0.06 vs 0.26 ± 0.05 vs 0.52 ± 0.05, 1.00 ± 0.04 vs 2.87 ± 0.17 vs 1.34 ± 0.18, all $p < 0.01$, Fig.7F-7K, Table 8).

Table 8: MiR-744-5p reduced the effects of circ ACTG1 on cell proliferation and invasion in vitro model of Bladder cancer

Group	*negative*	*Circ ACTG1*	*Circ ACTG1 +miR-744-5p*
MiR-744-5p expression	1.00 ± 0.05	0.23 ± 0.07	0.71 ± 0.08
Protein expression			
TGF-β1	1.00 ± 0.01	3.11 ± 0.08	1.56 ± 0.05
Jagged1	1.01± 0.02	3.87 ± 0.11	1.46 ± 0.07
NOTCH1	1.01 ± 0.01	2.89 ± 0.05	1.87 ± 0.03
LDH activity	1.00 ± 0.03	0.42 ± 0.05	0.86 ± 0.06
Cell proliferation	1.88 ± 0.04	3.92 ± 0.12	2.19 ± 0.08
Caspase-3 activity	1.00 ± 0.07	0.48 ± 0.07	0.78 ± 0.06
Caspase-9 activity	1.00 ± 0.06	0.26 ± 0.05	0.52 ± 0.05
Cell invasion	1.00 ± 0.04	2.87 ± 0.17	1.34 ± 0.18

The Inhibition of TGF-β1 Attenuated the Effects of Anti-miR-744-5p on Invasion in Bladder Cancer In Vitro

To confirm the roles of TGF-β1 on invasion in bladder cancer *in vitro*, si-TGF-β1 and miR-744-5p inhibitor mimics were co-transfected into T24 cells. As shown in Figure 8A-8D, si-TGF-β1 suppressed the protein expression of TGF-β1, Jagged1 and NOTCH1 in T24 cells following down-regulation of miR-744-5p (1.00 ± 0.02 vs 3.02 ± 0.03 vs 1.55 ± 0.03, 1.00 ± 0.03 vs 3.61 ± 0.04 vs 2.17 ± 0.05, 1.00 ± 0.05 vs 3.33 ± 0.05 vs 1.68 ± 0.04, all $p < 0.01$, Table 9), compared with down-regulation of miR-744-5p group. Additionally, the inhibition of TGF-β1 attenuated the effects of anti-miR-744-5p on the promotion of cell proliferation and invasion, and inhibition of LDH activity and Caspase-3/9 activity levels in bladder cancer *in vitro*, in comparison with those in down-regulation of miR-744-5p group (1.92 ± 0.04 vs 3.45 ± 0.02 vs 2.51 ± 0.03, 1.00 ± 0.02 vs 3.18 ± 0.05 vs 1.26 ± 0.04, 1.00 ± 0.03 vs 0.27 ± 0.07 vs 0.78 ± 0.07, 1.00 ± 0.03 vs 0.48 ± 0.04 vs 0.82 ± 0.04, 1.00 ± 0.02 vs 0.47 ± 0.04 vs 0.68 ± 0.03, all $p < 0.01$, Fig.8E-8J, Table 9).

Table 9. The inhibition of TGF-â1 reduced the effects of anti-miR-744-5p on invasion in vitro model of Bladder cancer

Group	*Negative*	*MiR-744-5p*	*MiR-744-5 p + si-TGF-â1*
Protein expression			
TGFβ1	1.00 ± 0.02	3.02 ± 0.03	1.55 ± 0.03
Jagged1	1.00 ± 0.03	3.61 ± 0.04	2.17 ± 0.05
NOTCH1	1.00 ± 0.05	3.33 ± 0.05	1.68 ± 0.04
Cell proliferation	1.92 ± 0.04	3.45 ± 0.02	2.51 ± 0.03
Cell invasion	1.00 ± 0.02	3.18 ± 0.05	1.26 ± 0.04
LDH activity	1.00 ± 0.03	0.27 ± 0.07	0.78 ± 0.07
Caspase-3 activity	1.00 ± 0.03	0.48 ± 0.04	0.82 ± 0.04
Caspase-9 activity	1.00 ± 0.02	0.47 ± 0.04	0.68 ± 0.03

DISCUSSION

According to the latest data from GLOBOCAN, bladder cancer accounts for 3 percent of global cancer diagnoses and 4.4 percent of male cancer diagnoses (Lee et al. 2022a). In China, the mortality rate of male bladder cancer is 4 times that of female, which is one of the main malignant tumours threatening the health of Chinese residents (Lee et al. 2022b). At present, the treatment of bladder cancer is mainly surgery (Kotolloshi et al. 2022). After surgery, bladder cancer needs infusion chemotherapy or immunotherapy. Repeated endoscopic and radiological examinations during the follow-up are essential to confirm whether the tumour recurs, metastasises or worsens (Madej et al. 2022). Its incidence rate is increasing year by year, and the incidence population tends to be younger. Therefore, to find suitable biological indicators for judging the condition of bladder cancer patients can provide reference for clinical treatment and play an important role in improving the prognosis of patients (Madej et al. 2022). At present, it is often treated by surgery, radiotherapy and chemotherapy, but because of its strong metastatic capacity, the prognosis of patients is often poor. In this study, circ ACTG1 expression was augmented in a model of bladder cancer. Meanwhile, miR-744-5p was reduced in the model of bladder cancer. Liu et al. (2018a) have suggested that miR-744 advanced gastric cancer cell apoptosis. These findings suggest that Circ ACTG1/miR-744 promotes bladder cancer cell proliferation.

CircRNA is a highly conserved circular non-coding RNA. Circ-LRBA is a newly discovered circRNA, which is encoded by the LRBA gene located on chromosome 4q31. 3. At present, there is little research on circ-LRBA. It is known that circ-LRBA is specifically up-regulated in ependymoma, which may be involved in regulating the progression of

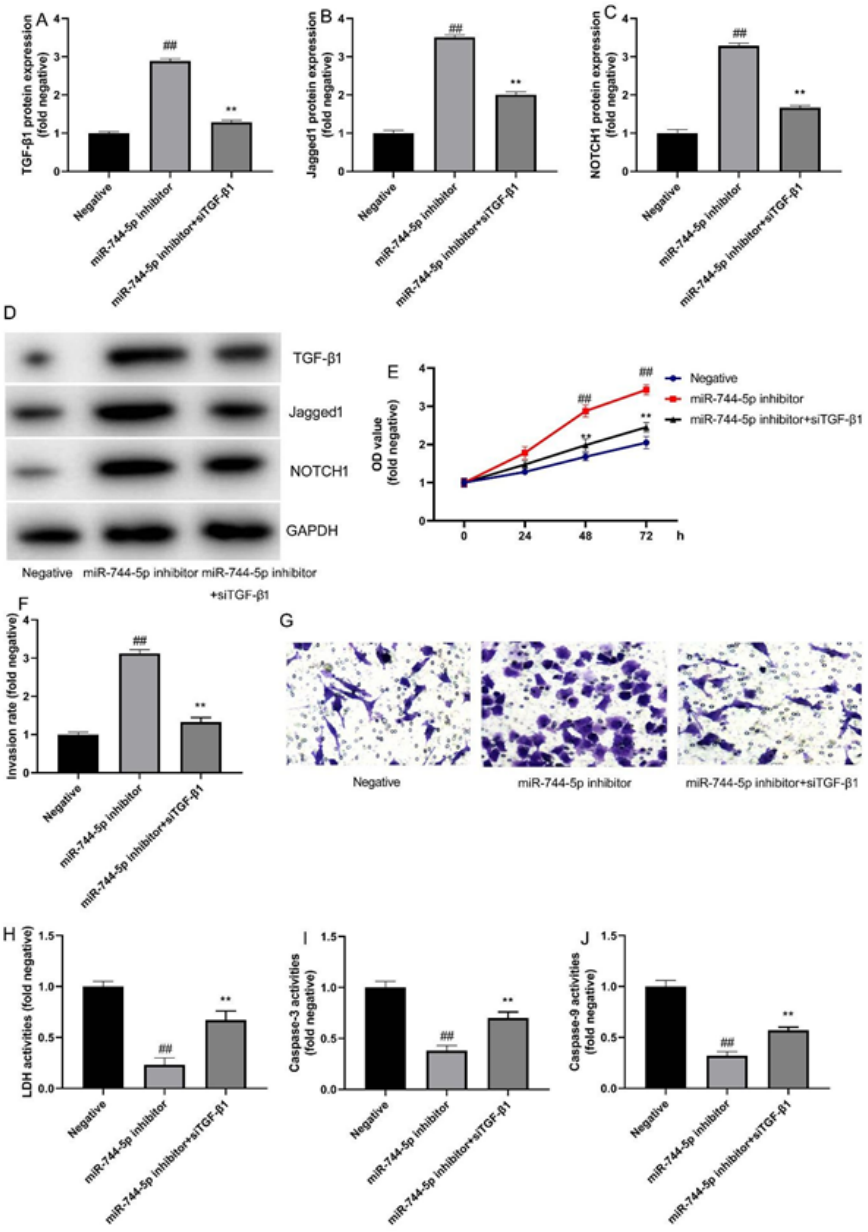

Fig. 8. The inhibition of TGF-β1 reduced the effects of anti-miR-744-5p on invasion in vitro model of Bladder cancer TGF-β1, Jagged1 and NOTCH1 protein expression by statistical analysis (A, B and C) and western blotting analysis (D), cell proliferation (E), invasion (F and D), LDH activity (H) and Caspase-3/9 activity levels (I and J) in T24 cells. Negative, negative mimics group; MiR-744-5p inhibitor, down-regulation of miR-744-5p expression group; Si- TGF-?1, Si- TGF-β1 and down-regulation of miR-744-5p expression group. ##p<0.01 versus negative mimics group, **p<0.01 versus down-regulation of miR-744-5p expression group

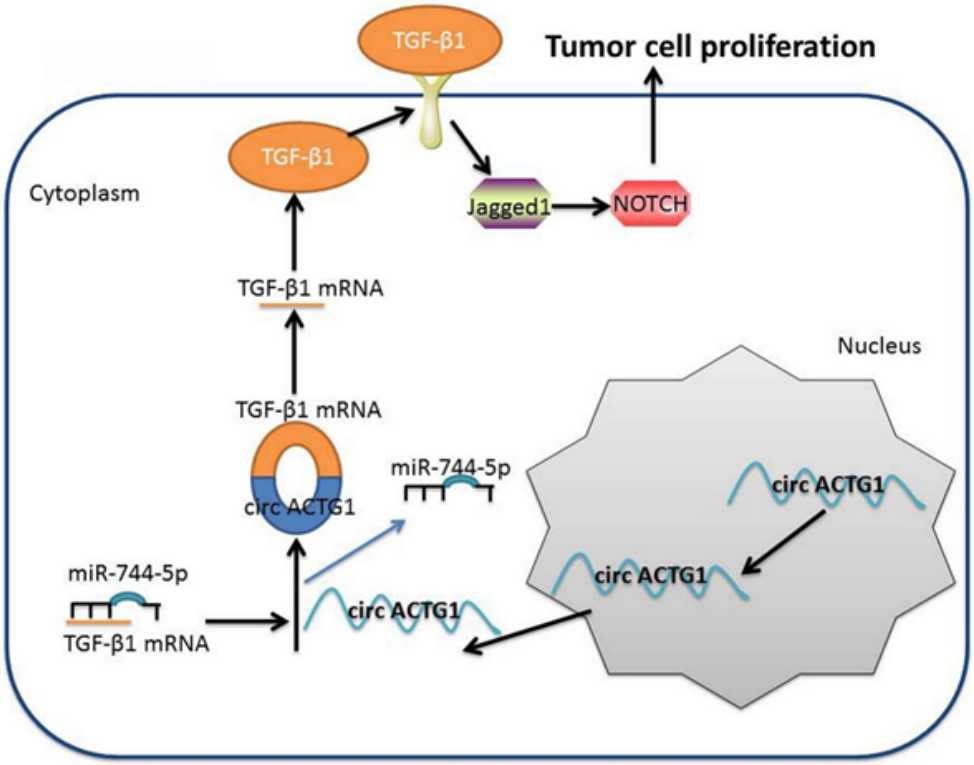

Fig. 9. Circular RNA has circ ACTG1 promotes cell growth and invasion of patients with Bladder cancer through TGF-β1/ Jagged1/NOTCH signaling pathways by miR-744-5p

ependymoma. CircRNA can combine with MicroRNA (miRNA) to influence downstream signal transduction and gene expression, and then regulate biological behaviours such as proliferation and apoptosis of tumour cells. CircRNA can affect the expression of downstream factors through targeted regulation of miRNA, thus affecting many pathological or physiological processes (Celik et al. 2013). It is found that miRNA exists stably in the body fluid of tumour patients, and it is insensitive to RNase, acid and alkali environment, long-term storage at room temperature (>24 hours) and repeated freezing and thawing, and has the potential of tumour marker (Zhang et al. 2018; Guo et al. 2019). With the development of high-throughput sequencing and bioinformatics technology, it has been found that circRNA exists widely in eukaryotes and can play a role as a sponge molecule of miRNA. It can also bind with RNA binding proteins to participate in the process of tumorigenesis and development. CircRNA can relieve the inhibition of miRNA on target genes by adsorbing miRNA.Circ ACTG1 promoted bladder cancer cell growth. In addition, si-miR-744-5p also promoted cell growth and Caspase-3/9 activity levels in T24 cells. Circ ACTG1 regulated bladder cancer cell growth by miR-744-5p. Kleemann et al. (2018) have shown that miR-744-5p induces ovarian cancer cells death. Similarly, circ ACTG1/ miR-744 axis has also been found to be involved in bladder cancer cell growth in the current study.

Subsequent studies have revealed that Notch is expressed in many species of invertebrates and vertebrates, with highly-conserved structure of its family members (Wang et al. 2014; Min et al. 2015). Notch receptor family also plays a critical role in determining cell fate (Min et al. 2015). To be specific, Notch signalling can promote or inhibit cell proliferation, differentiation and apoptosis in different cell types (Wang et al. 2014). Notch signalling also is associated with pathogenesis and progression of

some brain tumours. In the present study, the researchers showed quieting TGF-β1 diminished the effects of circ ACTG1 on invasion in vitro model of bladder cancer. As a result, circ ACTG1 was identified as the binding protein of TGF-β1 in gastric cancer. Nevertheless, further experiments are needed to elucidate the exact mechanism.

TGF-β1 can further promote bladder cancer progression by regulating cell proliferation and angiogenesis, suppressing immune surveillance, recruiting inflammatory cells, and maintaining stemness of bladder cancer stem cells (Yang et al. 2012; Wang et al. 2017). After binding with type II receptor of TGF-β1, activate and recruit type I receptor and bind to form a complex. During the formation of this complex, type I receptor is activated, further activate downstream transcription factor Smads and bind to it, leading to the phosphorylation of Smad protein, accelerating the transfer of activated protein into the cell nucleus and the synergistic effect of transcription factors, effectively playing the role of regulating the cell junction complex. In addition, by blocking TGF-β1/Smad signaling pathway can effectively mediate the regulation process of EMT, and inhibiting tumor cell migration and invasion has gradually become a strategy with great potential to prevent and treat tumor metastasis (Yang et al. 2012; Wang et al. 2017). Therefore, targeting the TGF-β1 signalling pathway has become a therapeutic strategy of bladder cancer. In this study, circ ACTG1 suppressed the protein expression ofTGF-β1, Jagged1 and NOTCH1 protein expression in T24 cells. and The inhibition of TGF-β1 attenuated the function of anti-miR-744-5p on invasion in bladder cancer *in vitro*. Martin et al. (2011) identified miR-744 could direct the post-transcriptional regulation of TGF-β1. Circ ACTG1 regulates the expression of TGF-β1/ Jagged1/ NOTCH in bladder cancer.

Jagged1 also plays an important role in mammalian development, which has recently been found to have a major impact on tumorigenesis (Hai et al. 2018). To be specific, in hematopoietic malignancies and solid tumours, Jagged1 can control cell proliferation, apoptosis (Kim et al. 2015). However, Jagged1 plays a dual role, either promoting or inhibiting carcinogenesis, which may depend on the tumour tissue type and the role of different signalling pathways (Kim et al. 2015). Here, the researchers revealed that the quieting of Jagged1 attenuated the effects of circ ACTG1 on bladder cancer cell proliferation. These results illustrated that circ ACTG1 could function as an oncogene and regulate cell survival via TGF-β1/ Jagged1/NOTCH signalling pathways by miR-744-5p in bladder cancer.

CONCLUSION

To the researchers' knowledge, they investigated the relationship of circ ACTG1 in bladder cancer, and circ ACTG1 of patients with bladder cancer through TGF-β1/ Jagged1/NOTCH signalling pathways by miR-744-5p (Fig. 9). Therefore, further investigations on miR-744-5p will provide a broader perspective and novel perspectives for therapy of bladder cancer.

RECOMMENDATIONS

The present findings reveal that circ ACTG1 promotes cell growth of patients with bladder cancer through TGF-β1/Jagged1/NOTCH signalling pathways by miR-744-5p. miR-744-5p might provide broader prospects and novel perspectives for bladder cancer.

ABBREVIATIONS

miRNA: microRNA
TGF-β1: Transforming growth factor beta1
CircRNAs: Circular RNAs
CCK8: Cell Counting Kit-8
PBS: phosphate-buffered saline
DMEM: dulbecco's modified eagle medium
FBS: foetal bovine serum
qRT-PCR: Quantitative Reverse Transcription-Polymerase Chain Reaction
PVDF: polyvinylidene difluoride
LDH: lactate dehydrogenase
BSA: bovine serum albumin

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