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Circ_0061140 promotes metastasis of bladder cancer through adsorbing microRNA-1236

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Abstract. – OBJECTIVE: The purpose of this study was to investigate the expression characteristics of circular RNA circ_0061140 in bladder cancer (BCa), and to further explore its effects on invasiveness and migration capacity of BCa cells, as well as its possible potential mechanism.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine the expression level of circ_0061140 in tumor tissu[illegible]ples and paracancerous ones collected [illegible]patients with BCa, and the interplay be[illegible]en circ_0061140 level and the clinical indica[illegible]as well as the prognosis of BCa patients w[illegible]analyzed. Meanwhile, qRT-PCR was also us[illegible]to verify circ_0061140 expres[illegible]BCa ce[illegible]lines. In addition, a circ_0[illegible]40 [illegible]ckdown model was constructed us[illegible]Lentiv[illegible]in BCa cell lines, including T24[illegible]4 253j, [illegible]of-fect of circ_0061140 on [illegible]ell f[illegible]its underlying mecha[illegible]ms [illegible]ored using Cell Counting Kit-8[illegible]K-8), t[illegible]ll, and cell wound healing as[illegible].

RESULTS: qP[illegible]lts showed [illegible]the expression leve[illegible]ci[illegible]1140 in tumor tissues of BCa patie[illegible]s was [illegible]kably higher than that in a[illegible]ent tissues, [illegible]the difference was stat[illegible]ally significant. [illegible]pared with patients [illegible]low e[illegible]pression of circ_0061140, patient[illegible]h high[illegible]pression of circ_0061140 had wo[illegible]ogn[illegible]and hi[illegible]er incidence of lymph node[illegible]nt me[illegible]sis. Compared with those in[illegible]egati[illegible]ontrol group, the prolif-[illegible]n an[illegible]as well as the metastasis[illegible]ity of [illegible]ells in the sh-circ_0061140 grou[illegible]were rem[illegible]kably attenuated. In addition, bio[illegible]and Luciferase reporter gene a[illegible]rated that circ_0061140 can spe-[illegible]cally bind to microRNA-1236. At the same [illegible], the results of qPCR revealed that the ex-p[illegible]on levels of circ_0061140 and microR-NA[illegible] were negatively correlated in the tumor tissues of BCa patients. Finally, cell recovery expe[illegible]ent in[illegible]d that silencing microRNA-1236 reversed th[illegible]ct of the knockdown of [illegible]1140 on the [illegible]y of BCa cells to p[illegible]rate and invade, suggesting that the two [illegible]regulate each other.

CONCLUSIONS: Circ_0061140 level was found [illegible]arkably eleva[illegible]in BCa tissues, as well as in c[illegible]es, which [illegible]s closely relevant to the incid[illegible]f lym[illegible]node or distant metastasis of BCa p[illegible]addition, circ_0061140 may enhance the proliferation rate and invasion ability [illegible]BCa cells through the modulation of microR-[illegible]

Key Words:
Circular RNA, Circ_0061140, MicroRNA-1236, BCa, Invasion.

Introduction

Bladder cancer is the most common malignant tumor of the urinary system, and its incidence rate ranks ninth in the world[1,2]. It is the eighth major cause leading to cancer-related death in the United States in 2015, while 40,000 patients die of BCa each year in Europe[2,3]. BCa metastasis is the main cause resulting in death, and the 5-year survival rate of patients with metastasis is only 8.1%[4,5]. To worsen the patients' prognosis, muscular-invasive BCa in about 1/3 of patients has already metastasized during treatment[6,7]. Although surgical techniques and chemotherapies for BCa have made significant advancement in the past decade, the mortality of patients with metastatic BCa has not been remarkably improved[8,9]. Invasion and metastasis of BCa may be affected by a complex molecular regulatory network, but this regulatory network has not been defined yet[10,11].

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Therefore, further research on the molecular regulatory mechanism of BCa in the process of invasion and metastasis and exploration of new therapeutic targets can better contribute to the promotion of BCa treatment and the improvement of the prognosis of patients with BCa[10,12,13].

Circular RNA (circRNA) is a kind of RNA molecule that binds end-to-end covalently and cyclically, which was discovered by electron microscopy 40 years ago in the cytoplasm of eukaryotic cells[14,15]. Although there have been some reports about circRNA since then, circRNA has been considered as a by-product of faulty gene splicing in the past due to the limited detection methods of circRNA[16,17]. With the rapid progress of high-throughput sequencing and bioinformatics technology, large amounts of circRNA were found in human, rats, nematodes, and other species, as well as in cells, through large-scale and more in-depth analysis of transcriptome data[18,19]. Unlike linear mRNAs normally formed by downstream and upstream exons splicing forwards, circRNA is formed by end-to-end connections of exons or introns *via* back-splicing[20,21]. Therefore, this study proved that circRNA production might be an active regulatory process during gene transcription, rather than just the "noise" in the splicing process, and also suggested that circRNA might have a potential function to regulate gene expression[22-24].

MicroRNA (miRNA) is a non-coding RNA with a length of 22-25 nucleotides, which can regulate the expression level of target gene mRNA before and after transcription, it has very important biological functions[25,26]. The abnormal expression of miRNA in BCa can promote or inhibit the development, invasion, metastasis, and other aspects of BCa[3]. Recently, some studies have found that circRNA can act as a "miRNA sponge", binding miRNAs without degradation and negative regulation on miRNA expression[27,28]. At present, the specific mechanism of circRNA expression in BCa and its role as "miRNA sponge" have not been clearly clarified. In this study, high-throughput sequencing and bioinformatics analysis of circRNA were conducted in human BCa tissues and normal bladder tissues. It was found that circ_0061140 was remarkably expressed in BCa tissues, and its remarkable existence and relatively low expression were verified in both tissues and cells, suggesting that circ_0061140 may be an oncogene in BCa. Therefore, the possible role of circ_0061140 in bladder cancer was investigated, and the possible effects of the knockdown on circ_0061140 on the biological behavior of bladder cancer cells were evaluated, so as to explore the relationship between circ_0061140 and miRNA as "miRNA molecular sponge" as the entry point, as well as the regulatory mechanism of circ_0061140, thus providing a new target for targeted therapy of bladder cancer.

Patients and Methods

Patients and Tissue Samples

The tumor tissue samples, and corresponding adjacent tissues were collected from 42 BCa patients undergoing surgical resection and then, analyzed *via* routine H&E staining. All specimens were frozen and stored in refrigerator at -80°C for preparation of RNA extraction. According to the 8th edition of UICC/AJCC BCa TNM staging criteria, all patients were diagnosed as BCa by postoperative pathological analysis, and no adjuvant treatment, such as radiotherapy or chemotherapy was performed before surgery. The study was approved by the Ethics Committee of the Hospital and all patients signed informed consent. This study was conducted in accordance with the Declaration of Helsinki.

Cell Lines and Reagents

Six human BCa cells (T24, 253j, 5637, J82, RT4, UMUC3) and one human bladder epithelial immortalized cell (SV-HUC-1) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), and Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) and fetal bovine serum (FBS; Gibco, Rockville, MD, USA) were purchased from Life Technologies (Gaithersburg, MD, USA). BCa cell lines were cultured in DMEM medium high glucose medium containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). All cells were cultured in a 37°C, 5% CO_2 incubator, and passaged with 1% trypsin + ethylene diamine tetraacetic acid (EDTA; Yangze Pharm. Co. Ltd., Taizhou, China) for digestion when grown to 80%-90% confluence.

Transfection

The control group (sh-NC) and the lentivirus containing the circ_0061140 knockdown sequence (sh-circ_0061140) were purchased from Shanghai Jima Company (Shanghai, China). The cells were seeded in 6-well plates and grown

to a cell density of 40%, and then transfection was performed according to the manufacturer's instructions. After 48 h, the cells were collected for qPCR analysis and cell function experiments.

Cell Counting Kit-8 (CCK-8) Assay

The transfected cells after 48 h were collected and plated into 96-well plates at 2000 cells per well. The cells were cultured for 24 h, 48 h, 72 h, and 96 h respectively, and then added with CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent. After incubation for 2 h, the optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

Transwell Assay

After 48 h of transfection, the cells were trypsinized and resuspended in the serum-free medium. Following cell counting, the diluted cell density was adjusted to 2.0×10^5/mL, and the transwell chamber containing Matrigel and no Matrigel was placed in a 24-well plate. 200 μL of the cell suspension was added in the upper chamber, while 500 μL of a medium containing 10% FBS was added to the lower chamber. After incu[illegible] in a 37°C incubator for 48 hours, the c[illegible] was removed, fixed with 4% paraformal[illegible]de for 30 minutes, and stained with crystal viol[illegible] 15 minutes. Subsequently, the cells were was[illegible] with phosphate-buffered saline (PBS), and t[illegible] inner surface of the basemen[illegible]e of the chamber was carefully cl[illegible]d to [illegible]ove the inner layer cells. The per[illegible]ted cell[illegible]ined in the outer layer of the bas[illegible]me chamber were obser[illegible]un[illegible]microscope, and 5 fields of view[illegible]e rando[illegible]lected.

Cell Wound [illegible] Assay

The cells [illegible] trans[illegible]n for 48 h were digested, ce[illegible]fuged, and re[illegible]nded in medium without [illegible] to adjust the de[illegible] to 5-10^5 cells/mL. T[illegible] densit[illegible]f the plated cells was determin[illegible]ccordi[illegible] their size (the majority of the num[illegible]f [illegible] plated[illegible]s set to 50000 cells/well), a[illegible] confl[illegible]y of the cells reached [illegible]r mo[illegible]n[illegible]ay. After the stroke, cells we[illegible]sed ge[illegible]th PBS for 2-3 times and observ[illegible]gain after[illegible]4 of incubation with low-conce[illegible] medium (such as 1% FBS).

[illegible]antitative Real-Time Polymerase [illegible]n Reaction (qRT-PCR)

[illegible]otal RNA was extracted from BCa cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and then reversely transcribed into cDNA using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Ja[illegible] was performed using SYBR® Pre[illegible]Ex Taq (TaKaRa, Otsu, Shiga, Japan), a[illegible]tepOne Plus Real Time-PCR System (App[illegible]iosystems, Foster City, CA, USA). The fol[illegible] prim-ers were used for qPCR [illegible]ion: circ[illegible]140 forward: 5'-CGAAC[illegible]AGGACCGA[illegible]; reverse: 5'-GCC[illegible]TTCT[illegible]TAAGC[illegible]; microRNA-1236: [illegible]ard: [illegible]CCAA[illegible]CAG-CCTCTTCCCCT[illegible]3', [illegible]e: [illegible]ATG-GTTGTTCAC[illegible]TCC[illegible]-3'; [illegible]dherin: forward: 5'[illegible]CTTTCT[illegible]GGTC-3', reverse: [illegible]AGCCTGG[illegible]GCTAGG-GT-3'; N[illegible]dher[illegible]orward: 5'-AGATGGCG-ACAAGGATCGTC[illegible]verse: 5'-CGCGATC-CAG[illegible]TTTAGT-3'; [illegible]imentin: forward: 5'[illegible]ACGAGAAAG[illegible]AGTGG-3', reverse: [illegible]TCTCGGCTTCATCTTTGAGC-3'; MMP9: [illegible]ward: 5'-TT[illegible]AACCTTTGAGGGCGA-3', [illegible]rse: 5'-CA[illegible]GGCGTCGTCAATCACC-3'; β[illegible]: forw[illegible]5'-CCTGGCACCCAGCA-CA[illegible]se: 5'-GCTGATCCACATCT-GCTGG[illegible], U6: forward: 5'-ATTGGAAC-[illegible]ATACAGAGAAGATT-3', reverse: 5'-GGAAC-[illegible]ACGAATTTG-3'. Data analysis was per-[illegible]using ABI Step One software (Applied Biosystems, Foster City, CA, USA) and the relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method.

Dual-Luciferase Reporter Assay

HEK293T cells were seeded in 24-well plates and co-transfected with microRNA-1236 mimic/NC and pMIR Luciferase reporter plasmids. Before this, the plasmid was paired with the circ_0061140 mutation binding site 3'UTR by insertion of other wild type circ_0061140, and the mutation binding site was constructed into pMIR. The plasmid was then introduced into the cells using Lipofectamine 2000 according to the manufacturer's protocol. After 48 h of transfection, the reporter Luciferase activity was normalized to control firefly Luciferase activity using a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

The continuous variables were analyzed using the *t*-test, and the categorical variables were analyzed using the χ^2 test or Fisher's exact probability method. Kaplan-Meier method was used to evaluate the prognosis survival time of

patients, and the difference between different curves was compared by Log-rank test. The program was processed using the Statistical Product and Service Solutions (SPSS) 22.0 (SPSS IBM Corp., Armonk, NY, USA), and the data were expressed as mean ± standard deviation. $p<0.05$ was considered to be statistically significant.

Results

Circ_0061140 Was Highly Expressed in BCa Tissues and Cell Lines

Circ_0061140 expression in 42 pairs of BCa tumor tissue specimens and their adjacent paracancerous ones was evaluated by qPCR. The results indicated that circ_0061140 level was remarkably elevated in tumor samples compared with the adjacent ones, and the difference was statistically significant (Figure 1A). Similarly, circ_0061140 was also found remarkably higher in the BCa cell line than in the normal human bladder epithelial immortalized cells (SV-HUC-1) (Figure 1B).

Circ_0061140 Expression Was Correlated with the Incidence of Lymph Node or Distance Metastasis in BCa Patients

According to the qPCR resu[lts] 0061140 expression, the above-me[ntioned] t[he] sam-ples were divided into [high] expr[ession] and low expression group. [The] num[ber] group was counted and the interplay between circ_0061140 level and the age, sex, pat[holog-]ical stage, lymph node or distant m[etastasis] patients with BCa were analyzed [as] shown [in] Table I, high expression of ci[rc_]0061140 was positively correlated with the [incidence] of BCa lymph node or distant metastasis. [At the] same time, relevant follow-up [data] were co[llected] t[o] estimate the associatio[n be]tween circ_0[061140] and the prognosis of [pat]ients [with] BCa. A[s a] result, Kaplan-Mei[er] survival curves [re]vealed that high expres[sion] of 0061140 [was] remarkably ass[ociated] with poor p[rog]nosis of BCa patients ($p<0.05$, Figu[re 1C]).

Knockdown of Circ_0061140 Inhibited Cell Proliferation and Invasion

In order to clarify the influence of circ_0061140 on cell proliferation, invasion, and migration ability of BCa cells, circ_0061140 knockdown cell model was successfully constructed, and qPCR was used to verify the interference efficiency (Figure 2A). Later, CCK-8 test confirmed that the proliferation rate of BCa cells remarkably decreased after knocking down circ_0061140 compared with the sh-NC group (Figure 2B). Moreover, transwell and cell wound healing assays demonstrated that after the knockdown of circ_0061140, the cell crawling ability and the number of BCa cells transferred in the transwell chamber was remarkably reduced, suggesting that the cell invasive and migratory abilities were both inhibited (Figure 2C, 2D). The results of qPCR showed that the EMT assay could change

Table I. Association [of] c_0061140 exp[ression] with clinicopathologic characteristics of bladder cancer.

Param[eters]	[Num]ber of cases	circ_0061140 expression		*p*-value
		Low (%)	High (%)	
Age (y				0.780
<	15	9	6	
	27	15	12	
Ge[nder]				0.721
Mal[e]	20	12	8	
[F]emale	22	12	10	
[T]				0.463
[T]2	26	16	10	
[T]4	16	8	8	
[Ly]mphatic metastasis				**0.047**
[N]o	28	19	9	
Yes	14	5	9	
[Di]stance metastasis				**0.002**
[M]0	31	22	9	
[M]1	11	2	9	

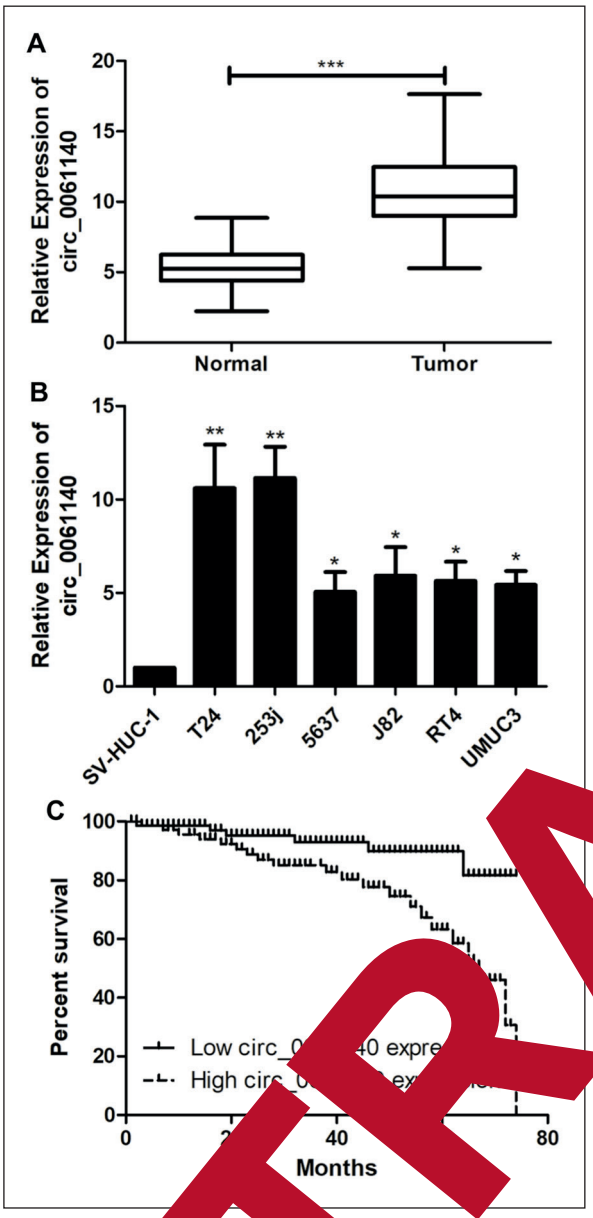

Figure 1. Circ_0061140 is [illegible] expressed in bladder cancer tissu[illegible]d cell lines. **A,** [illegible]R is used to detect the differ[illegible]in expression of ci[illegible]061140 in bladder cancer [illegible]s and [illegible]jacent non-tumor tissues. **B,** qRT-PCR [illegible]d to de[illegible]e expression level of circ_0061140 in l[illegible]cancer [illegible] lines. **C,** The Kaplan Meier survival curve [illegible]cancer p[illegible]ts based on circ_0061140 expressio[illegible] that th[illegible]gnosis of patients with high [illegible]ion is [illegible]an[illegible]orse than that of low expression g[illegible]ata are [illegible], *p<0.05, **p<0.01, ***p<0.001.

[illegible] knock[illegible]wn of circ_0061140, which demonstrated circ_0061140 knockdown increased the [illegible]sion of E-cadherin; but decreased the expression of N-cadherin, Vimentin, and MMP9 (Figure 2E).

Circ_0061140 Was Bound to MicroRNA-1236

To analyze the underlying mechan[illegible] croRNA-1236 and circ_0061140 i[illegible]ncing t[illegible] malignant progression of BCa[illegible]nformatics and Luciferase reporting assay [illegible]erformed. The result confirmed that circ_0[illegible] could specifically bind to microR[illegible]1236 an[illegible]eg[illegible] ulate its expression (Fig[illegible]3A, 3B). In ac[illegible] it was found from qP[illegible] that th[illegible]ockdown[illegible]f circ_0061140 rema[illegible]y enh[illegible]d the [illegible]RNA expression level [illegible]f i[illegible]1236, i[illegible]ating that there may [illegible]t a cl[illegible]eract[illegible]between circ_0061140 [illegible] microRN[illegible]gure 3C). Subsequen[illegible] tissue verifi[illegible]n indicated that mic[illegible]A-[illegible]vel was remarkably lower in BCa tumor tissu[illegible]n that in normal ones, and [illegible]fference wa[illegible]istically significant (F[illegible]3D). Furthermor[illegible] circ_0061140 was [illegible]d remarkably less expressed in the BCa cell [illegible] than in bl[illegible]r normal cells (SV-HUC-1) [illegible]re 3E). Ad[illegible]onally, in order to further fi[illegible]ut the a[illegible]iation between the expression of mi[illegible]6 and the prognosis of patients with BCa, relevant follow-up data were collected and the Kaplan-Meier survival curves revealed [illegible]xpression of microRNA-1236 was sig[illegible]ca[illegible]y associated with poor prognosis of BCa patients (p<0.05, Figure 3F). At the same time, based on the expression levels of circ_0061140 and microRNA-1236 in BCa tissues, it was concluded that there may exist a significant negative correlation between them (Figure 3G).

MicroRNA-1236 Modulated Circ_0061140 Expression in Human Prostate Cancer Cells

In order to further understand the interaction between circ_0061140 and microRNA-1236, circ_0061140 and microRNA-1236 were simultaneously knocked down in the BCa cell lines. And then, qPCR was conducted to examine circ_0061140 level in each group. As a result, the downregulation of microRNA-1236 was found to partially restore circ_0061140 expression in BCa cells (Figure 4A). Subsequently, CCK-8 experiment verified that silencing of microRNA-1236 counteracted the effect of knockdown of circ_0061140 on BCa cell proliferation (Figure 4B). At the same time, transwell invasion assay demonstrated that silencing microRNA-1236 was able to elevate the decreased number of BBC cells transferred in transwell chamber induced by knockdown of

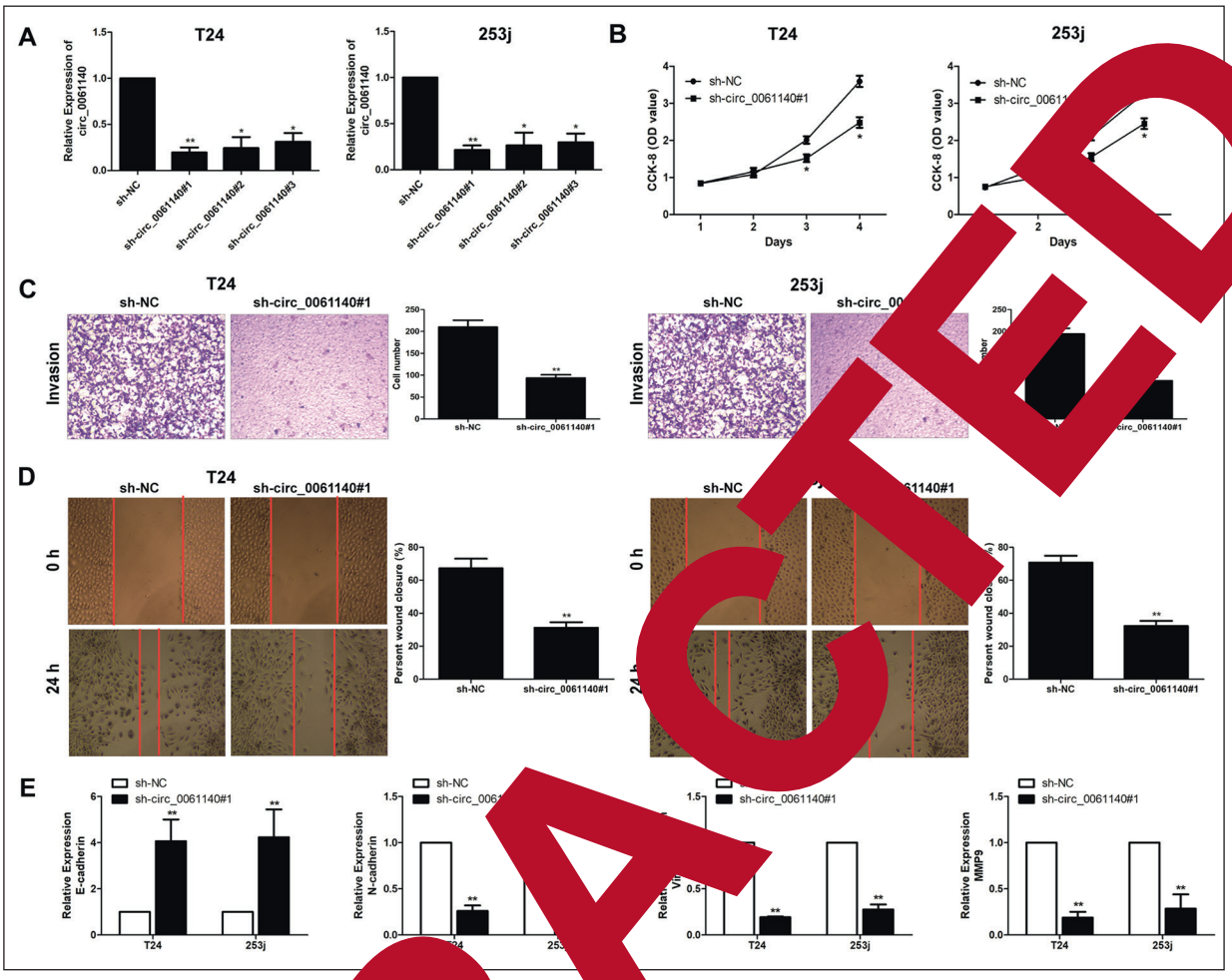

Figure 2. Silencing circ_006114 bits t oliferation invasion of bladder cancer cells. **A,** qRT-PCR verifies the interference efficiency of circ_ 40 after fection of c c_0061140 knockdown vector in T24 and 253j cell lines. **B,** Effect on transfection of blad cer cel on of the circ_0061140 knockdown vector in T24 and 253j cell lines detected *via* CCK-8 a ay. bladder cancer cells after knockdown of circ_0061140 in T24 and 253j cell lines (×40) dete *ia* tra vasion assay. **D,** Effect of knockdown of circ_0061140 on the crawling ability of bladder cancer cells in nd 253j c ×40) examined *via* cell wound healing assay. **E**, Expression levels of the EMT assay after knockdo irc_0061140 *vi* Data are mean ± SD, **p*<0.05, ***p*<0.01.

circ_0061 (Figure 4C), larly, cell wound healing y also indicated the cell healing a was tored by downregulation of mi NA-12 igure 4D).

Discussion

C RNAs hav been discovered by scientists in a special endogenous non-cod- RNA, g dually becoming a hotspot in the A region[14-16]. Unlike other linear RNAs with a c 5 'cap and 3' poly A tail, circRNAs form a c nt ring structure with no 5' and 3' ends, or adenosine tails[16-18]. CircRNAs were first discovered in RNA viruses in 1970, but only a fraction of circRNAs were accidentally discovered in the following 30 years[14,18]. Due to their low expression levels, these circRNAs were considered as by-products of abnormal RNA splicing[18,19]. Fortunately, with the development of novel RNA sequencing technology and bioinformatics, many characteristics of circRNAs, such as endogenesis, sequence conservative, and stability, have been revealed[19,21]. In this study, through circRNA sequencing on BCa and normal bladder tissues, hundreds of circRNA with differential expression between BCa tissues and normal bladder tissues were discovered. Differential expression of circRNA has also been found in other cancers, such

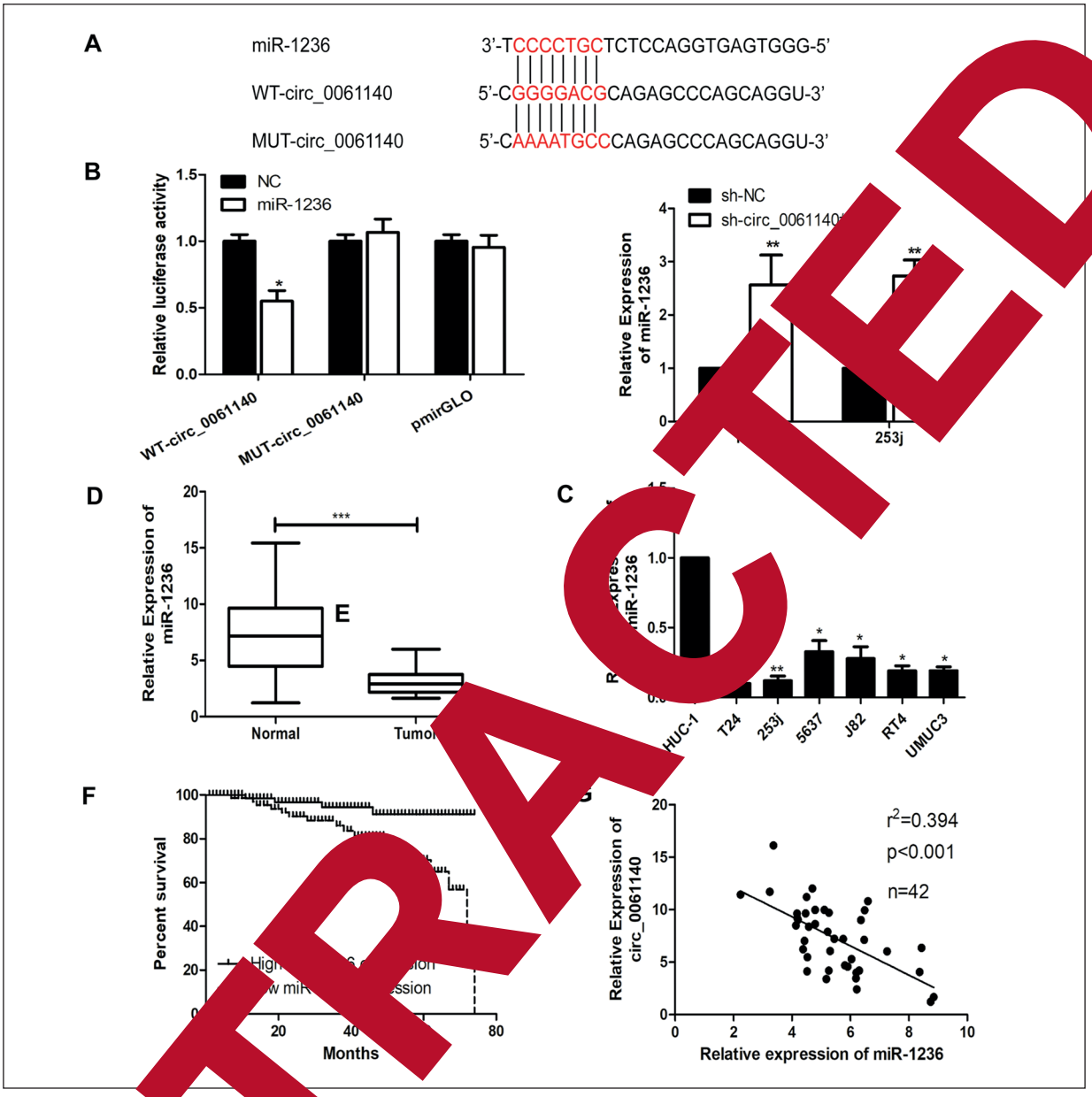

Figure 3. C 0061140 speci inds to miR-1236. **A,** Schematic representation of the specific binding sequence of circ_0061 miR-1236. **B,** Luci eporter gene assay reveals that circ_0061140 can specifically bind to miR-1236. **C,** qRT-PC ifies the ransfection effic ncy of miR-1236 after transfection of circ_0061140 knockdown vector in T24 and 253j cell li , qRT-P s used to detect the expression of miR-1236 in bladder cancer tissues and adjacent tissues. **E,** qRT-PCR is u detect t pressio evel of miR-1236 in bladder cancer cell lines. **F,** Kaplan Meier survival curve of bladder cancer patie R-1236 ssion shows that patients with low expression group have significantly worse prognosis than high exp oup. **G** e is a significant negative correlation between the expression levels of circ_0061140 and miR- bla er s. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

as liver cancer, esophageal squa- s cell c inoma, basal cell carcinoma, and ngeal carcinoma[15,16,20,22,27]. Since circRNA ex- n has complex tissue and cell specificity, ev e same circRNA expression is not the same in different cells or at different developmental stages[22-24]. Nevertheless, it is recognized that the dysregulated circRNA has the potential to regulate gene expression[23,24].

Finding and analyzing the abnormal expression of circRNA in BCa will help improve the diagnosis and treatment of BCa and thus ad-

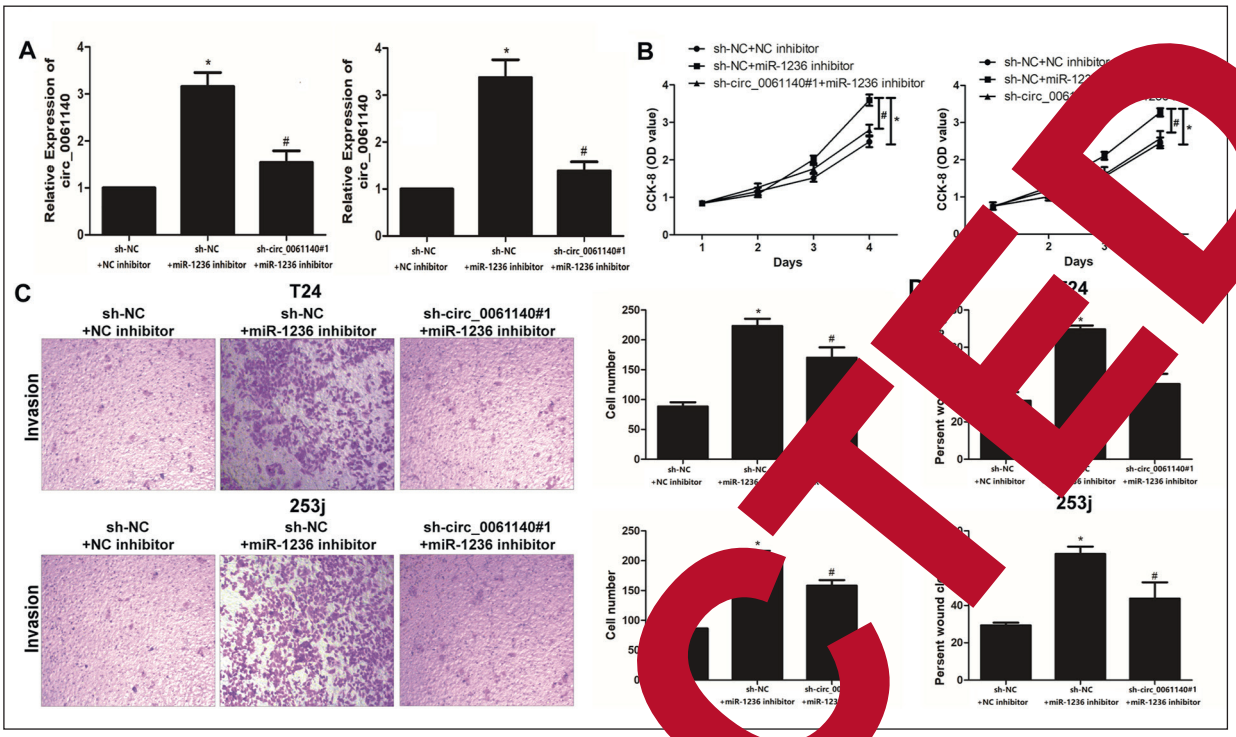

Figure 4. Circ_0061140 regulates the expression profile of miR-1236 in [illegible]cer cell lines. **A,** The expression level of circ_0061140 after co-transfection of circ_0061140 [illegible]1236 in bladder cancer cell lines is detected by qRT-PCR. **B,** The role of co-transfection of circ_0061140 and miR-1[illegible]n of bladder cancer cell proliferation detected *via* CCK-8 assay. **C,** The role of co-transfection of circ_0061[illegible]d mi[illegible]egulation of bladder cancer cell invasion ability (40×) detected *via* transwell invasion assay. **D,** Cell w[illegible]heali[illegible]ed to detect the crawling ability of bladder cancer cells after co-transfection of circ_0061140 and miR-12[illegible] mean ± SD, *#$p < 0.05$.

vance the prognosis. At p[illegible]nt, th[illegible]rcRNA associated with bladde[illegible]ncer is[illegible]dually discovered and applied [illegible]lini[illegible] and prognosis predi[illegible]n. [illegible]ay hybridization screening [illegible]ysis and [illegible] literature review have su[illegible]d that the [illegible]sion of circ_0061140 [illegible]ly related t[illegible] the occurrence and [illegible]evelop[illegible] of ovarian cancer, but the re[illegible]nship betw[illegible]irc_0061140 and BCa wa[illegible]t clear. In our [illegible], we detected the e[illegible]ssion [illegible]el of circ_0061140 in BCa tiss[illegible]and c[illegible]nes and explored its role in the [illegible]n[illegible]t of B[illegible]s well. As a result, circ_00[illegible] expres[illegible] was found remarkably [illegible]ulate[illegible] p[illegible]tively correlated with the [illegible]ce of l[illegible]ode or distant metastasis of BCa[illegible]ggesting [illegible]at circ_0061140 may act as a ca[illegible]g gene in BCa. In order to fur[illegible] investig[illegible]e the effect of circ_0061140 on the [illegible]logical functions of BCa cells, lentivirus was [illegible]o construct a circ_0061140 knockdown mo[illegible]The results of CCK-8, transwell invasion, and cell wound healing assays demonstrated that circ_0061140 could promote the invasion and metastasis of BCa and perform important functions in BCa, but the specific molecular mechanism remains elusive.

Further, it was found that reverse complementary sequences, including reverse repeat sequences and exon jumps, play a significant role in the formation of circRNAs[23,24]. Subsequently, some research reports revealed that circRNA can act as the sponge of miRNA to regulate its selective splicing and modulate the expression of target genes, and can be inhibited at its binding site of miRNA, which is the mechanism of sponge action[24]. The condition of being a miRNA molecule sponge is that the transcript contains the binding site of miRNA or the miRNA response elements. Similar to mRNA and lncRNA, circRNA also contains miRNA binding sites, but different from them, circRNA contains more miRNA binding sites and is not easily degraded, which makes circRNA a more ideal sponge for miRNA molecules[28,29]. Therefore, through bioinformatics analysis and Luciferase reporter gene

assay, it was found that circ_0061140 could specifically bind to microRNA-1236. In this study, in order to prove whether circ_0061140 promotes the malignant progression of BCa by regulating microRNA-1236, the expression changes of microRNA-1236 after knockdown of circ_0061140 were detected by qPCR. The results indicated that the expression level of microRNA-1236 was remarkably increased after downregulation of circ_0061140, which suggested a mutual regulatory relationship between circ_0061140 and microRNA-1236. In addition, it was found through the recovery experiment that silencing microRNA-1236 could offset the effect of knocking down circ_0061140 on BCa cell functions. Consequently, with the continuous deepening of research, further understanding of the cell biological functions of circ_0061140 and microRNA-1236 and their roles in the development of BCa will be more helpful for the diagnosis, treatment and prognosis evaluation of this tumor.

Conclusions

In summary, circ_0061140 is remarkab[illegible]ly expressed in BCa tissues and cell lines, [illegible]ch is significantly correlated with the inciden[illegible]f lymph node or distant metastasis of BCa patie[illegible] In addition, circ_0061140 may [illegible]ote malig[illegible]nant progression of BCa by [illegible] microRNA-1236 expression.

Conflict of Intere[illegible]

The Authors declare [illegible]ey have no co[illegible] interests.

Refere[illegible]

1) An[illegible]s, Ferla[illegible] J, Soerjomataran[illegible] I, Znaor A, Jemal [illegible]ay F. Bl[illegible]er cancer incidence and mortali[illegible] globa[illegible]rview a[illegible]d recent trends. Eur Urol [illegible]71[illegible]108.
2) Bell[illegible] Bladd[illegible]ncer. Hematol Oncol Clin [illegible]orth A[illegible]5: [illegible]xiii-xiv.
3) [illegible]ruch J, [illegible]mand S, Fisch M, Lotan Y, Noon [illegible] Resnick MJ, [illegible]hariat SF, Zlotta AR, Boorjian SA. [illegible] bladder cancer: a collaborative re[illegible]ogy, biology, and outcomes. Eur Urol 2016; 69: 300-310.
4) [illegible]oegemann M, Aydin AM, Bagrodia A, Krabbe LM. [illegible]spects and progress of immunotherapy for [illegible]dder cancer. Expert Opin Biol Ther 2017; 17: 1417-1431.
5) Funt SA, Rosenberg JE. Systemic, perioperative management of muscle-invasive bladder cancer and future horizons. Nat Rev Clin On[illegible] 221-234.
6) Guijarro A, Hernandez V, Llore[illegible]. The anatomical limits and oncological [illegible]fit of lymphadenectomy in muscle invasiv[illegible]er cancer. Actas Urol Esp 2017; 41: 2[illegible]4-291.
7) Del BG, Sternberg CN. [illegible]emic che[illegible] in muscle invasive a[illegible]etastatic blad[illegible]cer: present and fu[illegible] Urolo[illegible]017; 84: [illegible]141.
8) Cumberbatch K, He[illegible] Z, G[illegible] BA. Emerging dr[illegible]for [illegible] (blad[illegible] cancer. Expert Opi[illegible]erg Drug[illegible] 22[illegible]-164.
9) Choe J, [illegible]i-Amirfarzan [illegible]ni SH, Shinagare[illegible] Ramaiya NH, [illegible]jewski KM. Update[illegible] the [illegible]gist in non-muscle-invasive, musc[illegible]-invasive, [illegible]etastatic bladder cancer. Ab[illegible] Radiol (NY) [illegible]2: 2710-2724.
10) [illegible]s-Uzunova ES, Bo[illegible]her R, Croce CM, Jen[illegible]ster G, Visakorpi T, Calin GA. Long noncoding RNA in prostate, b[illegible]der, and kidney cancer. Eur Urol 2014; 65: 114[illegible]51.
11) [illegible]iefker-Radtke [illegible]rachal adenocarcinoma: a clini[illegible]'s guide[illegible]reatment. Semin Oncol 2012; 39: [illegible]
12) Buttigliero C, Tucci M, Vignani F, Scagliotti GV, Di Maio M. Molecular biomarkers to predict re[illegible]nse to neoadjuvant chemotherapy for bladder [illegible]er. Cancer Treat Rev 2017; 54: 1-9.
13) Enokida H, Yoshino H, Matsushita R, Nakagawa M. The role of microRNAs in bladder cancer. Investig Clin Urol 2016; 57 Suppl 1: S60-S76.
14) Wang D, Yang S, Wang H, Wang J, Zhang Q, Zhou S, He Y, Zhang H, Deng F, Xu H, Zhong S, Fu L, Tang J. The progress of circular RNAs in various tumors. Am J Transl Res 2018; 10: 1571-1582.
15) Hua X, Sun Y, Chen J, Wu Y, Sha J, Han S, Zhu X. Circular RNAs in drug resistant tumors. Biomed Pharmacother 2019; 118: 109233.
16) Xia L, Song M, Sun M, Wang F, Yang C. Circular RNAs as biomarkers for cancer. Adv Exp Med Biol 2018; 1087: 171-187.
17) Zhang Z, Yang T, Xiao J. Circular RNAs: promising biomarkers for human diseases. EBioMedicine 2018; 34: 267-274.
18) Hsiao KY, Sun HS, Tsai SJ. Circular RNA - new member of noncoding RNA with novel functions. Exp Biol Med (Maywood) 2017; 242: 1136-1141.
19) Bonizzato A, Gaffo E, Te KG, Bortoluzzi S. CircRNAs in hematopoiesis and hematological malignancies. Blood Cancer J 2016; 6: e483.
20) Xu XY, Zhou LL, Yu C, Shen B, Feng JF, Yu SR. Advances of circular RNAs in carcinoma. Biomed Pharmacother 2018; 107: 59-71.
21) Gao Y, Zhao F. Computational strategies for exploring circular RNAs. Trends Genet 2018; 34: 389-400.

22) Kristensen LS, Hansen TB, Veno MT, Kjems J. Circular RNAs in cancer: opportunities and challenges in the field. Oncogene 2018; 37: 555-565.

23) Greene J, Baird AM, Brady L, Lim M, Gray SG, McDermott R, Finn SP. Circular RNAs: biogenesis, function and role in human diseases. Front Mol Biosci 2017; 4: 38.

24) Rong D, Sun H, Li Z, Liu S, Dong C, Fu K, Tang W, Cao H. An emerging function of circRNA-miRNAs-mRNA axis in human diseases. Oncotarget 2017; 8: 73271-73281.

25) Acunzo M, Romano G, Wernicke D, Croce CM. MicroRNA and cancer--a brief overview. Adv Biol Regul 2015; 57: 1-9.

26) Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov 2017; 16: 203-222.

27) Zhang Y, Liu H, Li W, Yu J, Li J, Shen Z, Li G. CircRNA_100269 is downregulated in gastric cancer and suppresses tumor cell growth by targeting miR-630. Aging (Albany NY) 2017; 9: 1585-1594.

28) Jin X, Feng CY, Xiang Z, Chen YP, Li CircRNA expression pattern and circRNA-miRNA-mRNA network in the pathogenesis of nonalcoholic steatohepatitis. Oncotarget 2016; 7: 66455-66467.

29) Kulcheski FR, Christoff AP, Margis R. Circular RNAs are miRNA sponges and can be used as a new class biomarker. J Biotechnol 2016; 238: 42-51.