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Mechanism of LncRNA ROR promoting prostate cancer by regulating Akt

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Abstract. – OBJECTIVE: To investigate the expression of long-chain non-coding RNA ROR in prostate cancer (PCa), and to further study its possible underlying mechanisms in prostate cancer.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) was performed to detect the level of lncRNA ROR in 42 pairs of PCa tissues and adjacent normal tissues, and the correlation between ROR level and PCa pathological parameters was also evaluated. Besides, ROR expression in PCa cells was further verified by qRT-PCR, an knockdown model was constructed usi tivirus in PCa cell lines including PC-3 a cap. Cell counting kit-8 (CCK-8), transwell sion and cell scratch assay were used to anal the effect of ROR on the biologi function PCa cells and explore its unde chanism

RESULTS: QRT-PCR result mon ted that ROR levels in PCa tissue re not higher than that in normal ones the di statistically significant C ed with lowly-expresse OR, s with high ROR level had re ly more ced tumor stage, higher inc of lymph n distant metastasis. Si mpared wi egative control grou cell eration, invasion and metastasis bility of the knockdown group was sig ntly decrease addition, qRT-PCR re s indicated that the pression of Akt, the k otein e Akt signaling pathway, was sig ntly ced in si-ROR cell lines. Furthe e expe nt revealed that there was a egulat etween ROR and Akt.

NCL IS: cRNA ROR expression is ingly ed in PCa tissues or cells, and consider y associated with PCa stage, lym de and distant metastasis. Additional- R may promote PCa cell proliferon, invasion and migration by regulating Akt.

rds:
chain non-coding RNA, ROR, Akt, Prostate cancer.

duction

Pro cancer (PCa, most common malig- r in Western d loped countries, ranl second in Western countries such as Americ- men[1-3]. Prost ancer has a large difference in ncidence of erent races; for example, PCa h lower inci ce in China than in Western cou ver, after reform and opening up, with anges in dietary structure and the improvement of diagnostic techniques for prostate its incidence has been shown an increasing ch recent years[6,7]. Prostate cancer has a serious impact on the life expectancy of older men, both domestically and abroad. In particular, China has begun to enter an aging society, so the impact of prostate cancer on Chinese men's lifestyle has become more prominent[8]. However, early symptoms of PCa are concealed, which make many patients have been in a late stage at the time of diagnosis. But if diagnosed early, the harm of PCa to patients can be successfully alleviated, with a good chance of improving therapeutic effect and avoiding long-term complications[9]. With the rapid development of microarray technology and nucleic acid sequencing technology, a large number of non-coding RNA (ncRNA) transcripts have been discovered in eukaryotes, and these ncRNAs have become a crucial part of the intricate regulatory network in the body[10,11]. According to the length, ncRNA can be divided into long-chain non-coding RNA (LncRNA) and small RNA (siRNA, miRNA, piRNA). The former are RNAs with a transcript length of more than 200 nt, which are specifically expressed between various cells and do not encode proteins themselves, but can regulate the expression level of genes at various levels, initially thought to be the "noise" of transcription[12,13]. About 66% of the genome is transcribed into non-coding RNA. LncR-

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NA creates a complex network regulation system due to its abundance, location and diversity, which has led to the discovery that the differential expression of lncRNA is an important mechanism for the initiation, occurrence and development of cancer[14]. At present, gene chip technology is becoming more and more mature; in the mean time, more and more lncRNAs are being found, and the number of lncRNAs associated with prostate cancer is increasing. We have also found 13 lncRNAs with differential expression through gene chip technology[14]. Most of them have not been studied, among which, we selected lncRNA ROR as the research object. The biological function and related molecular mechanism of lncRNA ROR remain elusive; let alone its role in the progression of prostate cancer. We firstly identified the differential expression of lncRNA ROR in prostate cancer, and focused on its influence on biological behavior of prostate tumor as well as its mechanism of action. In this study, we analyzed the expression of lncRNA ROR in 42 pairs of PCa and adjacent tissues, and explored the effect of ROR on the biological function of PCa cells. Previous works have proved that ROR is able to accelerate the growth and metastasis of tum l ls, which may affect the development of tu this study, we aimed to investigate the exp on of lncRNA ROR in prostate cancer and to c its promoting effect on the malignant progress of prostate cancer by regulating A

Patients an ethod

Patients and Pros Tu mples

Prostate tumor es and t esponding adjacent normal ere collecte 42 patients who un en ical resectio and routine hemato yl and eo E) staining. All the samples w frozen and st a refrigerator at -80°C. subsequently used RNA extraction. Acc g to th h edition of the UICC/AJCC pr anc mor no metastasis (TNM) staging c patien re diagnosed as PCa by to oper thol al analysis, and no anti-tu- eatme radiotherapy or chemotherap s perfo before surgery. The study was ap by the Ethics Committee of our hospital a had signed informed consent.

Lines and Reagents

human PCa cells (PC-3, DU-145, 22RV1, Lncap) and one human normal prostate matrix immortalized cell (WPMY-1) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). F-12k and 1640 medium (HyClone, South Logan, UT, USA) and bovine serum (FBS, Gibco, Rockvill were purchased from Life Tech gies (Gai thersburg, MD, USA). Cells we tured in an incubator with 5% CO_2 at 37°C, culture medium was F-12k or 1640 taini fetal bovine serum (FBS).

Transfection

The lentivirus c ing egative ntrol sequence or the R k wn se ce (si-ROR) was pu ed from ha na Company (Shan China). Cel seeded in 6-well pla n to a cell nsity of 40%, and then ansfecti s performed according to the ufacturer's t tions. Subsequently, ce harvested 48 s later for quantitat Real-time polymerase chain reaction (qRT-R) analysis a unction experiments.

y Form n Assay

e collected after 48 h of transfection, 200 cells were seeded in each well f a 6-well plate and cultured in complete medium ks. The medium should not be replaced as possible in the previous week to avoid cell adhesion and then changed twice a week. After 2 weeks, when cell colony formed, the cells were washed twice with phosphate-buffered saline (PBS) and fixed in 2 ml of methanol for 20 minutes. After removing methanol, cells were washed with PBS and stained with 0.1% crystal violet staining solution for 20 minutes. Next, the cells were photographed and counted under a light-selective environment after washed 3 times with PBS.

Transwell Cell Migration and Invasion Assay

After transfection for 48 hours, cells were digested, centrifuged and resuspended in medium without fetal bovine serum (FBS) to adjust the density to 5 x 10^5 cells/mL. A cell suspension of 200 uL (1 x 105 cells) was added to the upper chamber, and 700 uL of a medium containing 20% fetal bovine serum (FBS) were added to the lower chamber. According to the different migration abilities of each cell line, they were cultured in the incubator for a specific time. Transwell chamber was taken out, washed 3 times with 1 x PBS, and placed in methanol for 15 min of cell fixation. After the chamber was stained in 0.2% crystal violet for 20 min, the cells on the upper surface of the chamber

were carefully wiped off with water and a cotton swab. The cells were placed under a microscope to observe and photograph, and 10 fields of view were randomly selected for counting and statistical analysis was performed.

Wound Healing Assay

The cells underwent 48 hours of transfection were digested, centrifuged and resuspended in medium without FBS to adjust the density to 5 x 10^5 cells/mL. The density of the plated cells was determined according to the size of the cells (the majority of the number of cells plated was set to 50,000 cells/well), and the confluency of the cells reached 90% or more the next day. After scratching in the middle of the culture plate, PBS was used to gently rinse cells for 2-3 times and low-concentration serum medium (such as 1% FBS) was added. Lastly, observation was performed after 24 hours. The difference in cell healing ability was judged according to the migration area.

Quantitative Real-Time Phosphate Buffered Saline (qRT-PCR)

Total RNA was extracted from PCa cell[illegible]s and tissues using TRIzol reagent (Invitroge[illegible]sbad, CA, USA), and RNA was reverse trans[illegible]ed into cDNA using Primescript RT Reagent (T[illegible]Ra, Otsu, Shiga, Japan). QRT-PCR reactions w[illegible] performed using SYBR® Premix [illegible]TaqTM (T[illegible]KaRa, Otsu, Shiga, Japan) an[illegible]Plus Re-al-time PCR System (Appli[illegible]iosyst[illegible], Foster City, CA, USA). The follo[illegible]e prime[illegible]used for qRT-PCR reaction[illegible]R[illegible]ery[illegible] ACGAGAGGACCG[illegible]-3', [illegible]se: 5'-GC-CAAGTTCTAGA[illegible]GC-3'; [illegible] forward: 5'-TGCCACTGC[illegible]AGAAGAA[illegible]everse: 5'-GCTCACA[illegible]GC[illegible]TACCCA-3', β-actin: forward: 5'-C[illegible]TGGCA[illegible]AGCACAAT-3', reverse: 5'-[illegible]CTGATCCAC[illegible]TGCTGGAA-3'. Data a[illegible]is was performed u[illegible]g ABI Step One softw[illegible]and re[illegible]e expression levels of mRNA we[illegible]ulat[illegible]sing th[illegible] $2^{-\Delta\Delta Ct}$ method.

Western Blot Assay

[illegible] trans[illegible]ells were lysed using cell lysi[illegible]ffer, si[illegible]d on ice for 30 minutes, and cen[illegible]ed at 14,000 x g for 15 minutes at 4°C. T[illegible]oncentration was calculated by bi-choninic acid (BCA) Protein Assay Kit (Pier-[illegible]Rockford, IL, USA). The extracted proteins w[illegible]eparated using a 10% sodium dodecyl sulp[illegible]ate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Western blot analysis was performed according to[illegible]d procedures. The primary antibodie[illegible] and glyceraldehyde 3-phosphate d[illegible]drogenase (GAPDH), and the secondary[illegible]odies were anti-mouse and anti-rabbit, whic[illegible]all purchased from Cell Signalin[illegible]Techno[illegible]Danvers, MA, USA).

Statistical Analysis

The continuous va[illegible]y analyz[illegible]sing the *t*-test, while[illegible]ca[illegible]l vari[illegible]s were using the χ^2[illegible]or Fishe[illegible]ac[illegible]obability method. Ka[illegible]eier method[illegible]rformed to analyze th[illegible]of patients, and the difference bet[illegible]en diffe[illegible]urves was compared by Log-ra[illegible]test. The pro[illegible]was processed using th[illegible]ical Product [illegible] Service Solutions ([illegible]S) 22.0 (IBM, Armonk, NY, USA) program [illegible] the data wa[illegible]xpressed as mean ± standard [illegible]ation ($\bar{x}$±s). [illegible]05 was considered to be sta-ti[illegible]ly signifi[illegible].

Results

ROR was Highly Expressed in PCa Tissues and Cell Lines

We examined the level of ROR in 42 pairs of PCa tissues as well as their adjacent tumor-free tissues and PCa cell lines by qRT-PCR. The results showed that ROR expression levels were appreciably increased in PCa tissues compared with adjacent tumor-free tissues. The difference was statistically significant (Figure 1A). Compared with human normal prostate matrix immortalized cells (WPMY-1), ROR was also found notably higher in PCa cells (Figure 1B).

ROR Expression was Correlated with Clinical Stage, Lymph Node and Distance Metastasis in PCa Patients

According to the results of qRT-PCR of 42 pairs of ROR expression in PCa tissues and paracancerous ones, the samples were divided into two groups, which were high expression one and low expression one. The number of each group was counted and the relationship between ROR level and age, sex, clinical stage of PCa patients, condition of lymph node or distant metastasis were analyzed. Table I indicated that highly expressed ROR was positively correlated with the above clinical parameters.

Table I. Association of lncRNA ROR expression with clinicopathologic characteristics of prostate cancer.

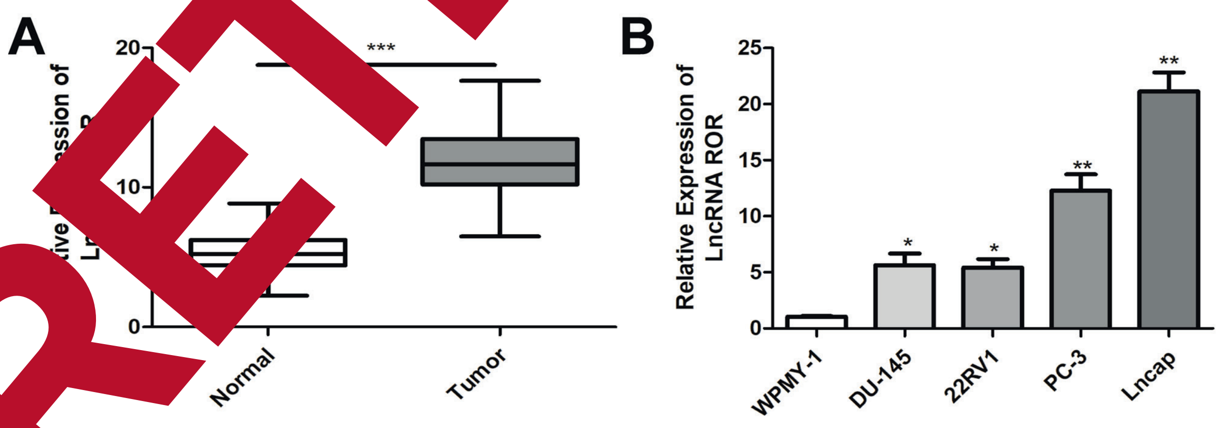
Parameters	Number of cases	ROR expression		[illegible]
		Low (%)	High (%)	
Age (years)				0.780
< 60	15	9	6	
≥ 60	27	15	12	
Gender				[illegible]
Male	20	12	8	
Female	22	12	[illegible]	
T stage				0.044
T1-T2	26	18	[illegible]	
T3-T4	16	6	[illegible]	
Lymph node metastasis				[illegible]
No	28	19	9	
Yes	14	5	9	
Distance metastasis				0.002
No	31	22	9	
Yes	11	2	[illegible]	

Knockdown of ROR Inhibited Cell Proliferation, Migration and Invasion

To explore the effects of ROR on PCa cell proliferation, migratory and invasive capacity, we first successfully constructed a ROR knock[illegible] model and verified it using qRT-PCR [illegible] 2A). Subsequently, we used cell-cloning [illegible]riments to investigate the effect of ROR o[illegible] proliferation of PCa cells, and found that the [illegible] proliferation rate of the si-ROR [illegible] was sign[illegible]ficantly lower than that of th[illegible] (Figure 2B). In addition, using th[illegible]answel[illegible]gration assay and wound heali[illegible]st, the [illegible] of transmembrane PCa c[illegible]lls [illegible]tr[illegible] of the si-R[illegible] group was extensively less [illegible] the NC gr[illegible] suggesting that the migrat[illegible]bility of [illegible]s with down-regulated ROR was [illegible]gure 2D). Meanwhile, the cells in the si[illegible] group also showed a poor healing [illegible]bility (Figure 2C).

[illegible]own of ROR Changed Akt Expression

To analyze the potential mechanisms of Akt and ROR in influencing the malignant progression of PCa, we examined Akt expression in 42 pairs of PCa tissues and their corresponding normal ones using qRT-PCR. The results indicated that



[illegible] **1.** LncRNA ROR was highly expressed in prostate cancer tissues and cell lines. ***A,*** QRT-PCR was used to detect the di[illegible]al expression of LncRNA ROR in prostate cancer tissues and adjacent non-tumor tissues; ***B,*** QRT-PCR was used to det[illegible]ct the expression level of LncRNA ROR in prostate cancer cell lines. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

compared to adjacent tissue, the expression level of Akt in PCa tissue was considerably elevated, with the difference statistically significant (Figure 3A). Furthermore, ROR level was also notably high in PCa cells compared to WPMY-1 (Figure 3B). Therefore, we analyzed the expression of ROR and Akt both on gene and protein levels in tissues and found a clear correlation between the two (Figure 3C). Next, using qRT-PCR method, we demonstrated that the level of Akt was down-regulated after ROR knockdown (Figure 3D).

Akt Modulated ROR Expression in Human Prostate Cancer Cells

Subsequently, to figure out the regula between ROR and Akt, we transfect rexpression-plasmid into ROR kno wned-PCa cells. The expression of Akt was ed by qRT-PCR and Western Blot, respectiv lts reve-aled that overexpression of A restore le of knockdown of ROR in PC lls (Figure Figure 4B). Subsequen throu cell cl experiments, we foun t over ession of Akt

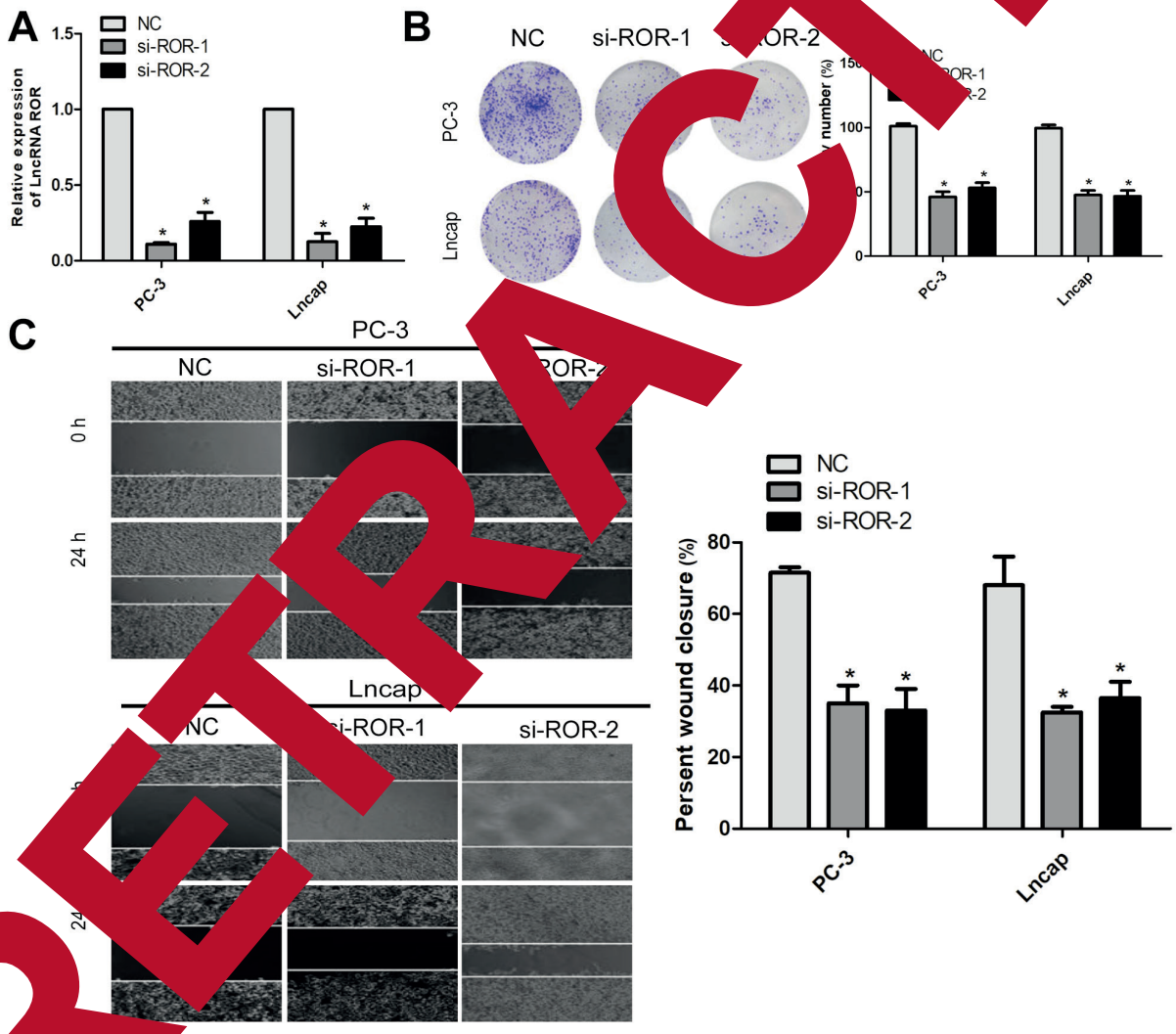


Figure 2. LncRNA ROR affected prostate cancer cell proliferation and invasion and migration. ***A,*** QRT-PCR validated the interference efficiency of LncRNA ROR after transfection of si-ROR in PC-3 and Lncap cell lines; ***B,*** Results of cell colony formation assay showed the effect of knockdown of ROR on prostate cancer cell proliferation in PC-3 and Lncap cell lines; ***C,*** Cell scratch assay was used to detect the effect of knock-downed ROR on the healing ability of prostate cancer cells in PC-3 and Lncap cell lines.

Figure continued

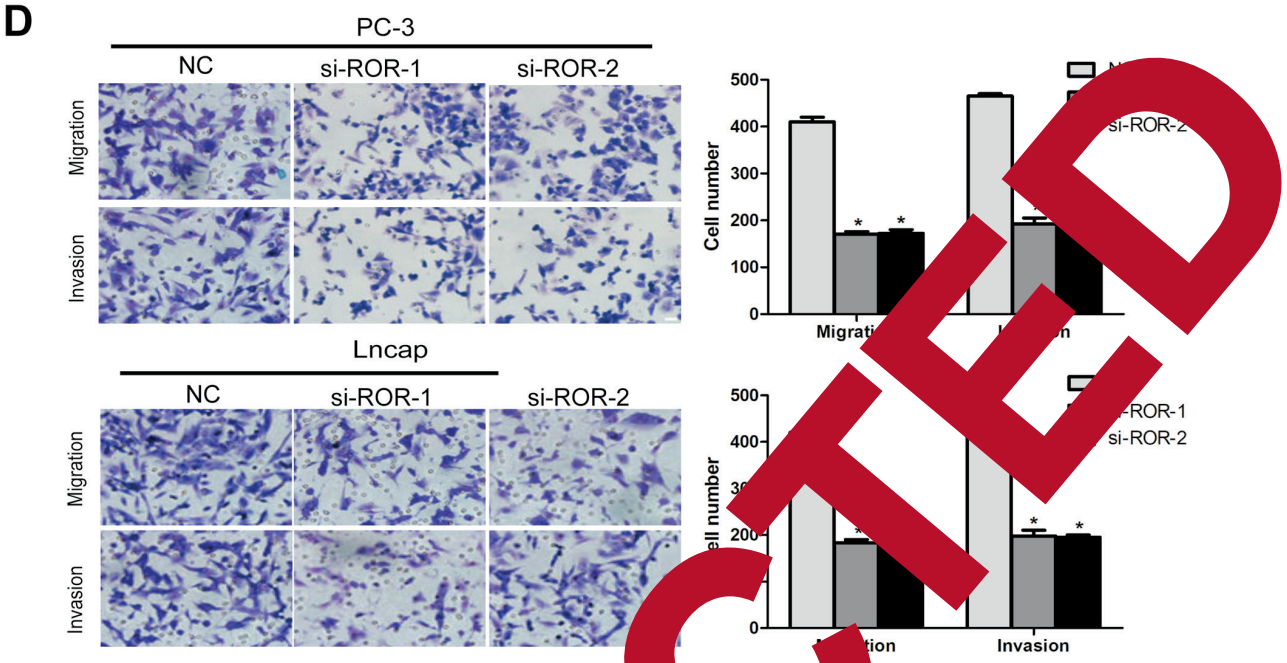


Figure 2. *Continued.* ***D,*** Transwell assay was used to detect the e f knock-dow OR on the ability of cancer cells to invade and migrate in PC-3 and Lncap cell lines. Data were expres ean ± SD 0.05.

could counteract the effect of knockdown o R on PCa cell proliferation rate (Figures 4C).

Discussio

As a common malig tumor, rly diagnosis, metastasis, rec ce treatment of advan Ca urgery have become the focus current re h[1-3]. Scholars[4,7,9] have fou lncRNA pla tal role in various di s, ding tumors. Multiple abnormally ex essed-ln As in PCa may play a crucial in the diagnos atment and prognosis Ca. Therefore, find these lncRNAs in P d ana g its correlation with clinical pr is wil p to im rove the diagnosis and treatm Ca, and rove the prognosis of ients.

RNA -scale ncRNAs that can regul variety iological processes. They are wi istributed and generally more than 200 h . However, most of them do not get ability of encoding proteins due to the lack valid open reading frame (ORF)[9,10]. The role o NA in tumors is still in infancy and has grad ally become a hot spot. It has been found that some LncRNAs exert a crucial influence on could counteract the effect of knockdown of ROR expression of tumors. They are abnormally expressed in various malignant tumors such as liver cancer, lung cancer, and prostate cancer, and have played a considerable role in the occurrence and development of malignant tumors, even some lncRNAs can be used as a marker for tumor diagnosis and prognosis[11-14]. Therefore, finding abnormal expression of lncRNA in prostate cancer and analyzing its function will help to elevate diagnosis and treat level of prostate cancer and improve the prognosis of patients[15,16]. Currently, lncRNA related to prostate cancer is being gradually discovered, including PCA3, PCEGM1, etc. These lncRNAs are gradually being applied to clinical diagnosis and prognosis evaluation[17-19]. In this study, RNA was extracted from pathologically diagnosed tumor specimens and adjacent normal tissues of patients with prostate cancer, and 13 lncRNAs were found to be associated with prostate cancer by gene chip hybridization screening. Further literature review provided that LncRNA ROR gene is closely related to the development of colorectal and esophageal cancer, but its association with prostate cancer remains elusive[15,16]. Our research focused on the lncRNA ROR expression in PCa tissues and its role in the development of this tumor. We first verified the level of ROR in 42 pairs of PCa tissues and adja-

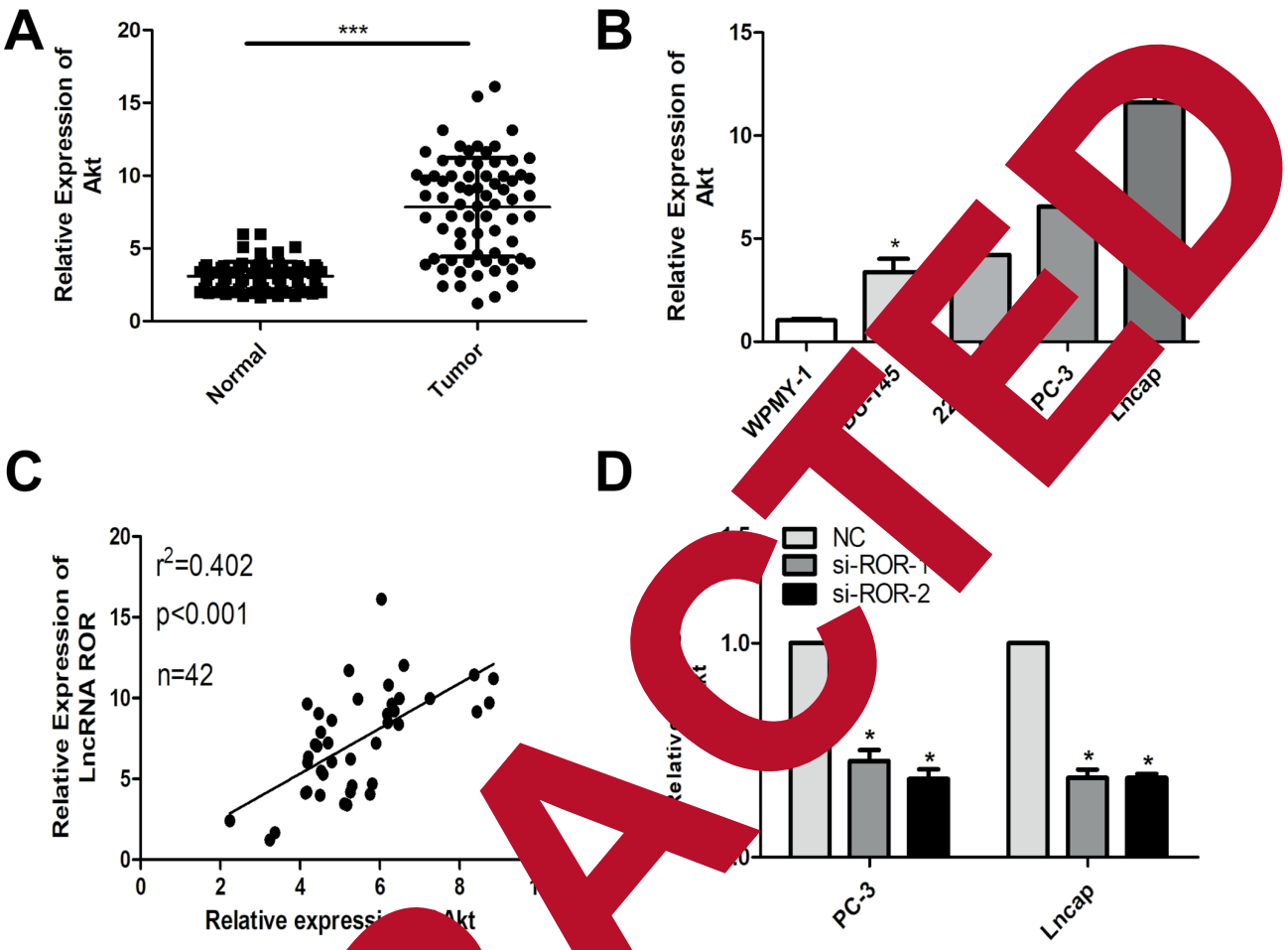


Figure 3. Akt was highly expres… prosta… ncer tissu… d cell lines. ***A,*** QRT-PCR was used to detect Akt expression in prostate cancer tissues and a… nt non-tu… tissues; ***B,*** QRT-PCR was used to detect Akt expression in prostate cancer cell lines; ***C,*** LncRNA ROR i… e can… nificantly positively correlated with the expression level of Akt; ***D,*** QRT-PCR verified the i… fe… y of RO… ter transfection of si-ROR in PC-3 and Lncap cell lines. Data were expressed as mean ± SD… 0.05, *… ***$p<0.001$.

cent tissu… nd the result… cated that ROR level w… considerably up-re… ated and positivel… rrelate… ith PCa TNM stage, lymph no… dista… etastas… From this we believe that R… play a… in promoting the pro-…sion …a. T… ther explore the effects …R on … gical function of PCa, we con… cted a R… knockdown model using lentivi… Cell colony formation experiments, invas… …ion assay and wound healing test …owed that ROR exerted an important influence …e occurrence and development of PCa, but its sp… molecular mechanism remains unclear. Akt … a serine kinase, also known as protein kinase B (PKB) or A and C related protein kinases (RAC.PK) because its amino acid composition in kinase activity region is very similar to PKA and PKC[20,21]. There are three subtypes of Akt, including Aktl, Akt2 and Akt3. Among them, Aktl is widely present in various tissues. Akt2 is mainly found in insulin-like tissues, while Akt3 is more strictly distributed in the brain and testes[21,22]. The Akt protein consists of three parts: pleckstrin homology domain (PH domain), the intermediate kinase domain and the carboxy-terminal regulatory region. Among them, the PH domain of Akt is highly similar to that of some other signaling molecules which are capable of binding 3-phosphatidylinositol[22,23]. In this experiment, in order to clarify whether ROR promotes the progres-

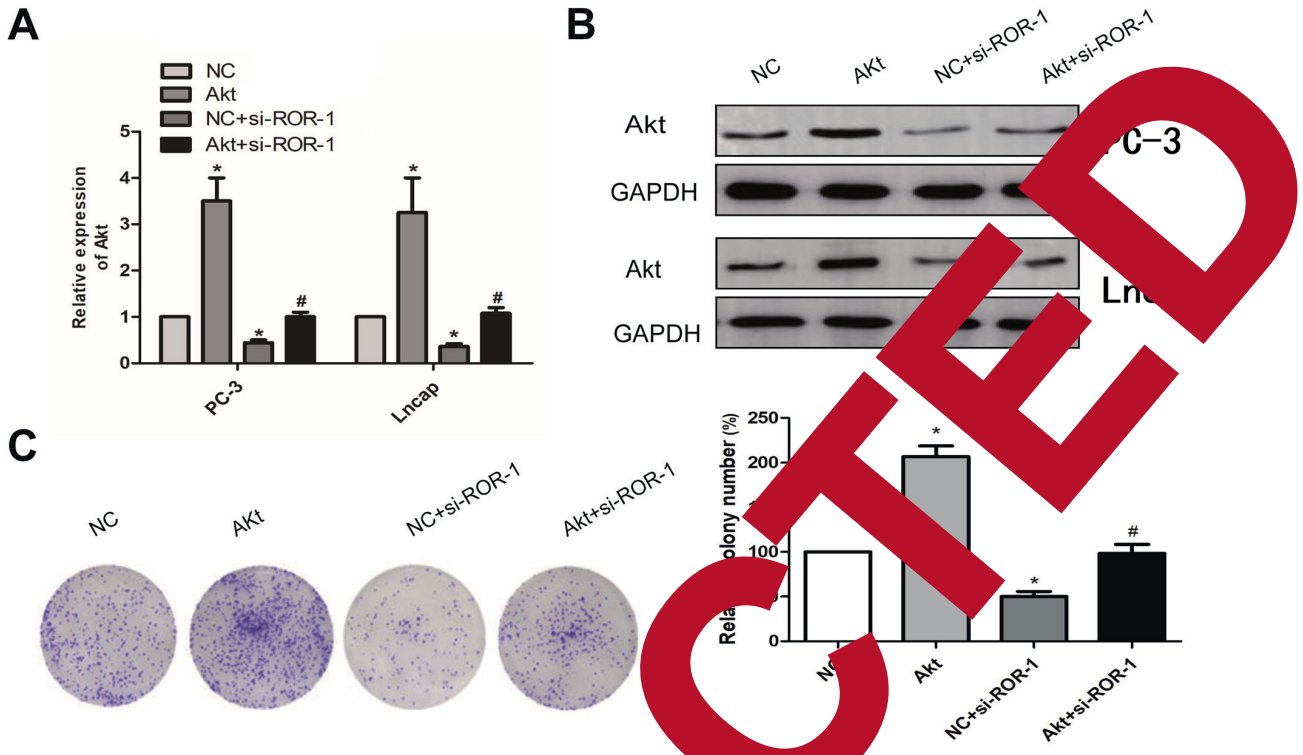


Figure 4. LncRNA ROR could regulate the expressi… Akt in prostate ca… ssues and cell lines. ***A,*** Akt gene levels were detected by qRT-PCR in LncRNA ROR and Akt c… ll lines; ***B,*** Akt protein levels were detected by western blot test in LncRNA ROR and Akt co-transfected cell l… ***C,*** C… rmation experiments examined the role of co-transfection of LncRNA ROR and Akt in regulating prost… ncer c… on. Data were expressed as mean ± SD, *$p<0.05$.

sion of PCa by regulating Ak… ined the expression of Akt after kno… wn of …R using Western Blot assay. Re… indicat… the expression level of the ab… ote… drastically after RO… lock… uggesting a relationship betwe… OR and … addition, we also found t… cell rescue … iments that overexpr… n … counteracted the role of knockdo… f ROR … cells. As the research cont… s to deepen, … understanding of the b… gical function of A… gene and its role in th… velopm… of tumors will be more conduc… the … gnosis, …eatment and prognosis of tu… s undo… dly brings good news …any … pa… s and their families, and … new … dawn to human conquest of c… er.

Conclusions

…etected the expression level of LncRNA ROR… was remarkably upregulated in PCa tissues and cells, and notably correlated with PCa TNM stage, lymph node or distant metastasis. In addition, LncRNA ROR may have the ability to promote the malignant progression of PCa by regulating Akt.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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