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LncSNHG14 promotes the development and progression of bladder cancer by targeting miRNA-150-5p

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Abstract. – OBJECTIVE: To elucidate whether lncSNHG14 could influence the proliferative potential and cell cycle progression of bladder cancer cells *via* binding to microRNA-150-5p (miRNA-150-5p). We aim to investigate the potential mechanism of miRNA-150-5p in the occurrence and progression of bladder cancer (BCa).

PATIENTS AND METHODS: Expression levels of SNHG14 and miRNA-150-5p in BCa tissues and normal bladder tissues were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Their expressions in BCa cell lines were detected as well. Regulatory effects of NHG14 and miRNA-150-5p on proliferative potential and cell cycle progression were evaluated by cell counting kit-8 (CCK-8) and flow cytometry, respectively. Through the dual-luciferase reporter gene assay, binding conditions between SNHG14 and miRNA-150-5p, as well as between miRNA-150-5p and synaptic vesicle-associated membrane protein 2 (VAMP2), were verified. Finally, rescue experiments were performed to clarify whether SNHG14 regulated behaviors of BCa cells by absorbing miRNA-150-5p to degrade VAMP2.

RESULTS: SNHG14 was highly expressed in BCa tissues and cell lines. The overexpression of SNHG14 accelerated the proliferative potential and cell cycle progression of BCa cells. SNHG14 was confirmed to bind to miRNA-150-5p. MiRNA-150-5p remained a low expression in BCa tissues. Moreover, miRNA-150-5p overexpression suppressed proliferative potential and cell cycle progression of BCa cells, which could reverse the promotive role of SNHG14 on behaviors of BCa cells. Furthermore, VAMP2 was the target gene of miRNA-150-5p. VAMP2 overexpression reversed the biological function of miRNA-150-5p in inhibiting proliferative potential and cell cycle progression of T24 and UC9 cells.

CONCLUSIONS: LncSNHG14 overexpression accelerates proliferative potential and cell cycle progression of BCa cells through absorbing miRNA-150-5p to degrade VAMP2 expression.

Key Words:
Bladder cancer, LncSNHG14, MiRNA-150-5p, VAMP2.

Introduction

Transitional cell carcinoma (TCC) is the most common histopathological type of bladder cancer (BCa), accounting for more than 90% of BCa cases, followed by squamous cell carcinoma, adenocarcinoma, and undifferentiated carcinoma[1]. BCa is a malignancy in the urinary system. The incidence of male BCa is about three times higher than that of females[2]. Part of BCa patients die from complications[3]. Currently, the pathological mechanism of BCa remains unclear. The tumorigenesis of BCa is a complex process involving multiple environmental and genetic factors[4,5]. Clarification of the biological mechanism of BCa contributes to improve the prognosis of BCa patients.

Based on the length of the transcript, non-coding RNAs can be divided into small non-coding RNAs (transcripts <200 nt) and long non-coding RNAs (lncRNA, transcript 200 nt-10 Kb). LncRNAs account for the majority of non-coding RNAs, which were originally thought to be by-products of RNA polymerase II transcription without biological functions[6]. However, some studies revealed important regulatory functions of lncRNAs in epigenetic, transcriptional and post-transcriptional regulation. They participate in the X chromosome silencing, genomic imprinting, chromatin modification, transcriptional activation, transcriptional interference, intranuclear transport, etc.[7]. The significances of lncRNAs in tumors have also been identified. LncRNA CCAT1 is highly expressed in gastric cancer,

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which is closely related to *in vitro* proliferative and migratory potentials of tumor cells[8]. Over 23,000 lncRNAs in malignant hepatocellular carcinoma (HCC) are analyzed by the microarray, and about 3% of them are downregulated. Among them, MEG3 is downregulated by about 210-fold. Overexpression of MEG3 markedly suppresses the growth and induces apoptosis of HCC cells[9]. SNHG14 exerts a tumor-promoting role in NSCLC, and its high expression indicates a poor prognosis[10]. In gastric cancer, SNHG14 accelerates the occurrence and development of gastric cancer through enhancing the proliferative and invasive potentials but inhibiting cell cycle progression[11]. The specific function of SNHG14 in BCa, however, is rarely known.

In this study, SNHG14 was highly expressed in BCa. We aimed to explore its role in the development of BCa and its possible mechanism.

Patients and Methods

Pathological Data of Subjects

Fresh BCa tissues and normal bladder tissues were surgically resected from 24 patients who were pathologically diagnosed with BCa. Their pathological data, including sex, age, tumor size, and tumor number were collected. None of the patients underwent preoperative treatments, and they denied family history. Patients volunteered to participate in the study and signed written informed consent. This study has been approved by the Ethics Committee of The Second Affiliated Hospital of Soochow University. Tissues were stored in liquid nitrogen.

Cell Culture

Normal bladder transitional epithelial cell line SV-HUC1 and BCa cell lines T24, UC9, PAL19, and UC19 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 μg/mL streptomycin (Gibco, Rockville, MD, USA) and placed in a 5% CO_2 incubator at 37°C.

Cell Transfection

An appropriate number of cells were inoculated into culture plates or flasks. Until the cell density reached 70%-80%, they were transfected with miRNA-150-5p mimics, pcDNA-SNHG14, pcDNA-VAMP2 (synaptic vesicle-associated membrane protein 2) or corresponding negative control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Complete medium was replaced at 5 h, and continually cultured for overnight.

RNA Extraction

50 mg of tissue were ground in liquid nitrogen with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA). About 5×10^6 cells were lysed in 1 mL of TRIzol. The homogenate was centrifuged in 0.2 mL of chloroform at 4°C, 12000 rpm for 10 minutes. Subsequently, the supernatant was mixed with isodose isopropanol and centrifuged at 4°C, 12000 rpm for 5 minutes. Finally, the precipitate was air dried, quantified, purified, and diluted in diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). Extracted RNA samples were preserved in a -80°C refrigerator.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The SYBR Green master mix, template, upstream/downstream primer, and DEPC water were formulated into a PCR reaction solution. PCR amplification reactions were: pre-denaturation at 95°C for 2 min, and then 40 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and finally extended at 72°C for 7 min. Primer sequences were as follows: MiRNA-150-5p, F: 5'-ACACTCCAGCTGGGTCTCCCAACCCTTGTA-3', R: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATGGTCAC-3'; U6, F: 5'-CTCGCTTCGGCAGCACATATA-3', R: 5'-AAATATGGAACGCTTCACGA-3'; SNHG14, F: 5'-GGGTGTTTACGTAGACCAGAACC-3', R: 5'-CTTCCAAAAGCCTTCTGCCTTAG-3';Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: 5'-GAAGAGAGAGACCCTCACGCTG-3', R: 5'-ACTGTGAGGAGGGGAGATTCAGT-3'; VAMP2, F: 5'-TGCACCTCCTCCAAATCTTA-3', R: 5'-CGATCATCCAGTTCCGATAG-3'.

Dual-Luciferase Reporter Gene Assay

The transcript 3'UTR sequence of VAMP2/SNHG14 was cloned into the pGL3 vector containing the luciferase reporter gene, which was the VAMP2/SNHG14 WT group. VAMP2/SNHG14 MUT group was constructed by mutating the core binding sequences using a site-directed mutagenesis kit. Cells were co-transfected with miRNA-150-5p mimics or negative control and VAMP2/SNHG14 WT or VAMP2/SNHG14

MUT, respectively. At 24 hours, cells were lysed and centrifuged at 10,000 g for 5 min. 100 µL of the supernatant was collected for determining the luciferase activity.

Cell Cycle Determination

Cells were collected at 72 h after transfection, digested, and resuspended in 70% alcohol. The suspension was fixed at 4°C for over 18 h. 1 mL suspension containing 1 $\times 10^6$ cells were incubated with 1 mL Propidium Iodide (PI) (50 µg/mL) and RNaseA (20 µg/mL) for 30 min at 37°C. Cell cycle progression was determined using flow cytometry.

Cell Counting Kit-8 (CCK-8)

Cells were inoculated at a 96-well plate with 1×10^4 cells per well. At 6 h, 24 h, 48 h, 72 h, and 96 h, 10 µL of CCK-8 reagent (Dojindo, Kumamoto, Japan) was supplied, respectively. After cell culture for 2 h, the absorbance value of each well at 450 nm wavelengths was measured by a microplate reader for plotting a growth curve.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., (Chicago, IL, USA) was utilized for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was used for analyzing the measurement data. $p<0.05$ was considered statistically significant.

Results

SNHG14 Expression Remained High in BCa

We examined SNHG14 expression in 24 cases of BCa tissues, and normal bladder tissues by qRT-PCR. SNHG14 expression remained higher in tumor tissues relative to controls (Fi-

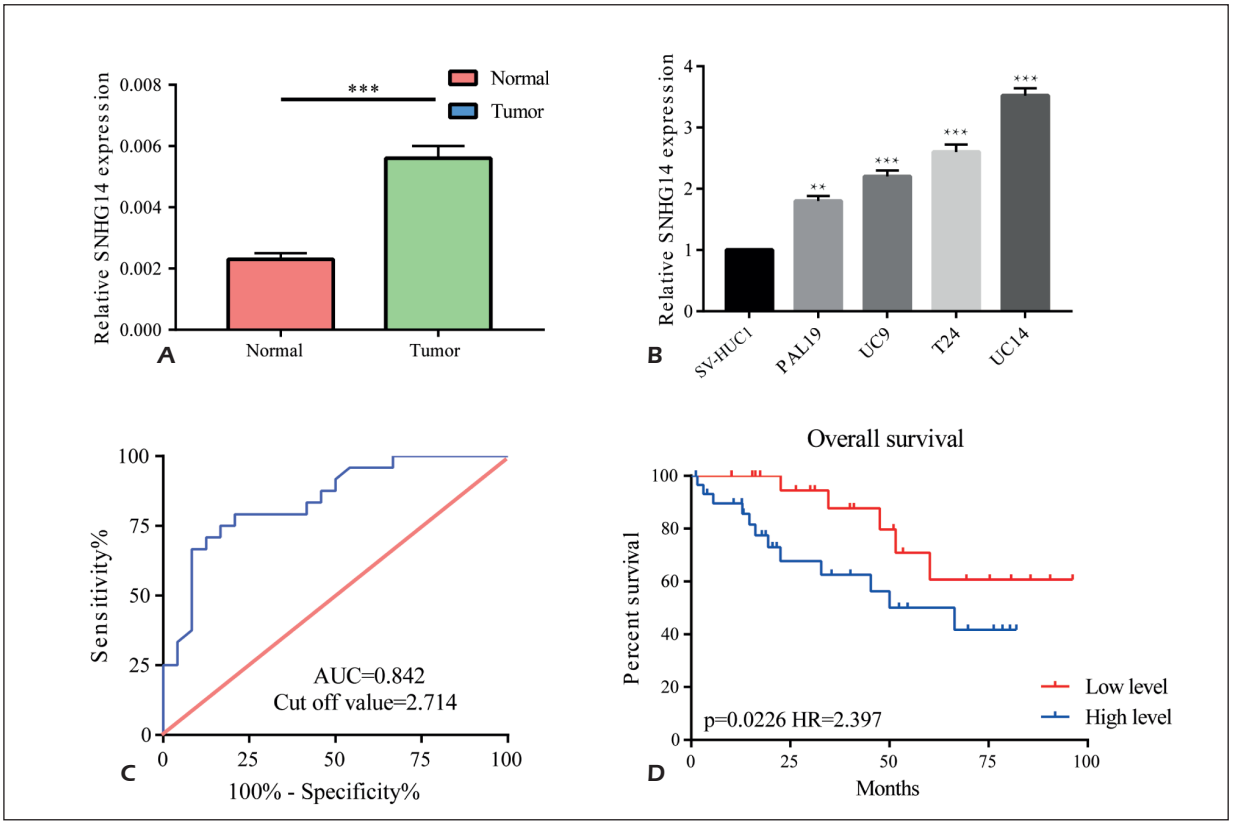

Figure 1. SNHG14 expression remained high in BCa. **A**, SNHG14 expression remained higher in BCa tissues relative to normal bladder tissues detected by qRT-PCR. **B**, SNHG14 was highly expressed in BCa cell lines than the normal bladder transitional epithelial cells. **C**, Survival curves suggested the potential of SNHG14 to be a biomarker for BCa (AUC=0.842, cut-off value=2.714). **D**, 5-year survival was higher in BCa patients with a low level of SNHG14 compared to those with a high level (p=0.0226, HR=2.397). **$p<0.01$, ***$p<0.001$.

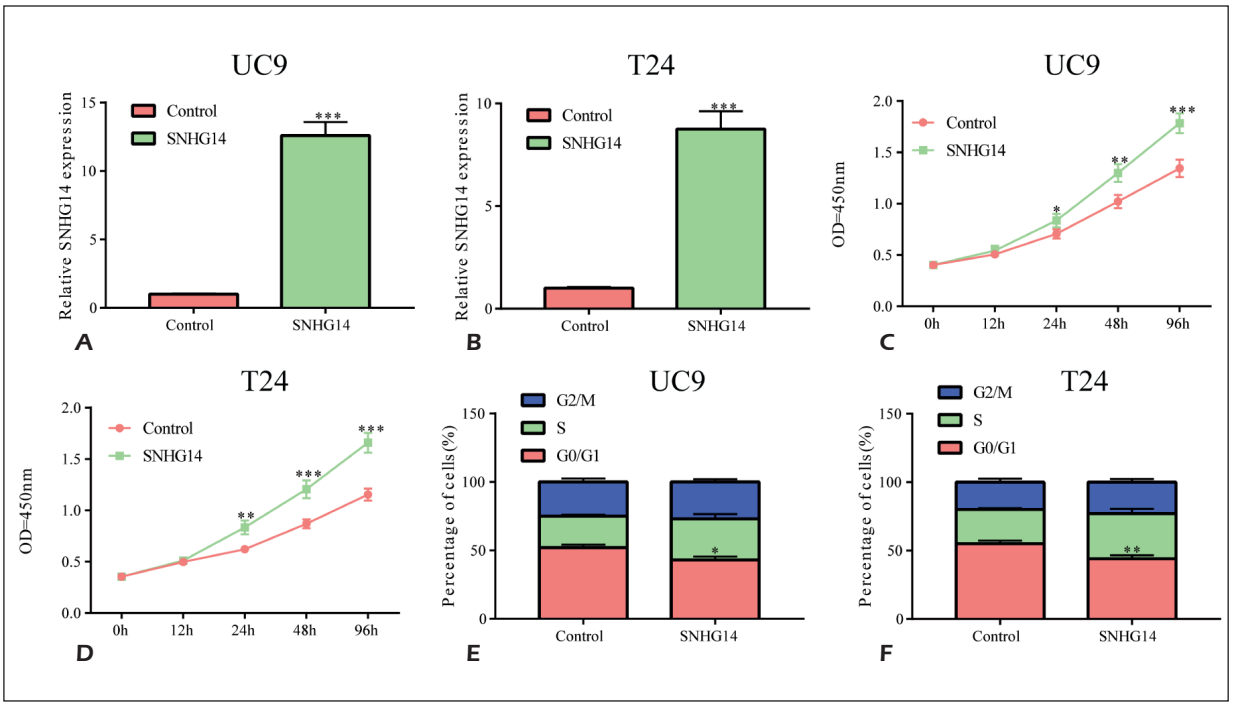

Figure 2. SNHG14 accelerated proliferative and cell cycle progression of BCa cells. **A-B**, Transfection efficacy of pcDNA-SNHG14 in UC9 and T24 cells was determined by qRT-PCR. **C-D**, CCK-8 assay revealed an increased viability in UC9 and T24 cells overexpressing SNHG14. **E-F**, FCM data revealed that SNHG14 overexpression remarkably accelerated cell cycle progression of UC9 and T24 cells. **p*<0.05, ***p*<0.01, ****p*<0.001.

gure 1A). Identically, SNHG14 was also highly expressed in BCa cells than the normal bladder transitional epithelial cells (Figure 1B). By collecting the clinical data of enrolled BCa patients, the plotted survival curves indicated the diagnostic potential of SNHG14 for BCa (AUC=0.842, cut-off value=2.714, Figure 1C). Moreover, 5-year survival tended to be higher in BCa patients with a low level of SNHG14 compared to those with a high level (p=0.0226, HR=2.397, Figure 1D). The above data suggested that SNHG14 was closely related to the occurrence and development of BCa.

SNHG14 Accelerated Proliferative and Cell Cycle Progression of BCa Cells

Since SNHG14 was highly expressed in BCa, we next elucidated the potential function in regulating cellular behaviors of BCa cells. First of all, the transfection efficacy of pcDNA-SNHG14 was determined by qRT-PCR. Transfection of pcDNA-SNHG14 effectively upregulated SNHG14 expression in UC9 and T24 cells (Figure 2A, B). The CCK-8 assay revealed an increased viability in UC9 and T24 cells overexpressing SNHG14 (Figure 2C, 2D). Meanwhile, SNHG14 overexpression remarkably accelerated cell cycle progression of BCa cells (Figure 2E, 2F).

SNHG14 Exerted its Biological Function Through Sponging MiRNA-150-5p

A potential binding site for SNHG14 and miRNA-150-5p was found through online prediction (Figure 3A). Furthermore, we verified their binding condition by dual-luciferase reporter gene assay (Figure 3B, 3C). To clarify whether SNHG14 exerted its functions by targeting miRNA-150-5p, BCa cells were co-overexpressed with miRNA-150-5p and SNHG14. As the experimental data revealed, SNHG14 overexpression promoted proliferative rate and cell cycle progression of UC9 cells, which was partially reversed by miRNA-150-5p overexpression (Figure 3D-3F). Similar results were yielded in T24 cells as well (Figure 3G-3I). We indicated that SNHG14 regulated behaviors of BCa cells through sponging miRNA-150-5p.

MiRNA-150-5p Exerted its Biological Function Through Degrading VAMP2

Since miRNA functions are achieved by degrading their target genes, we searched for the

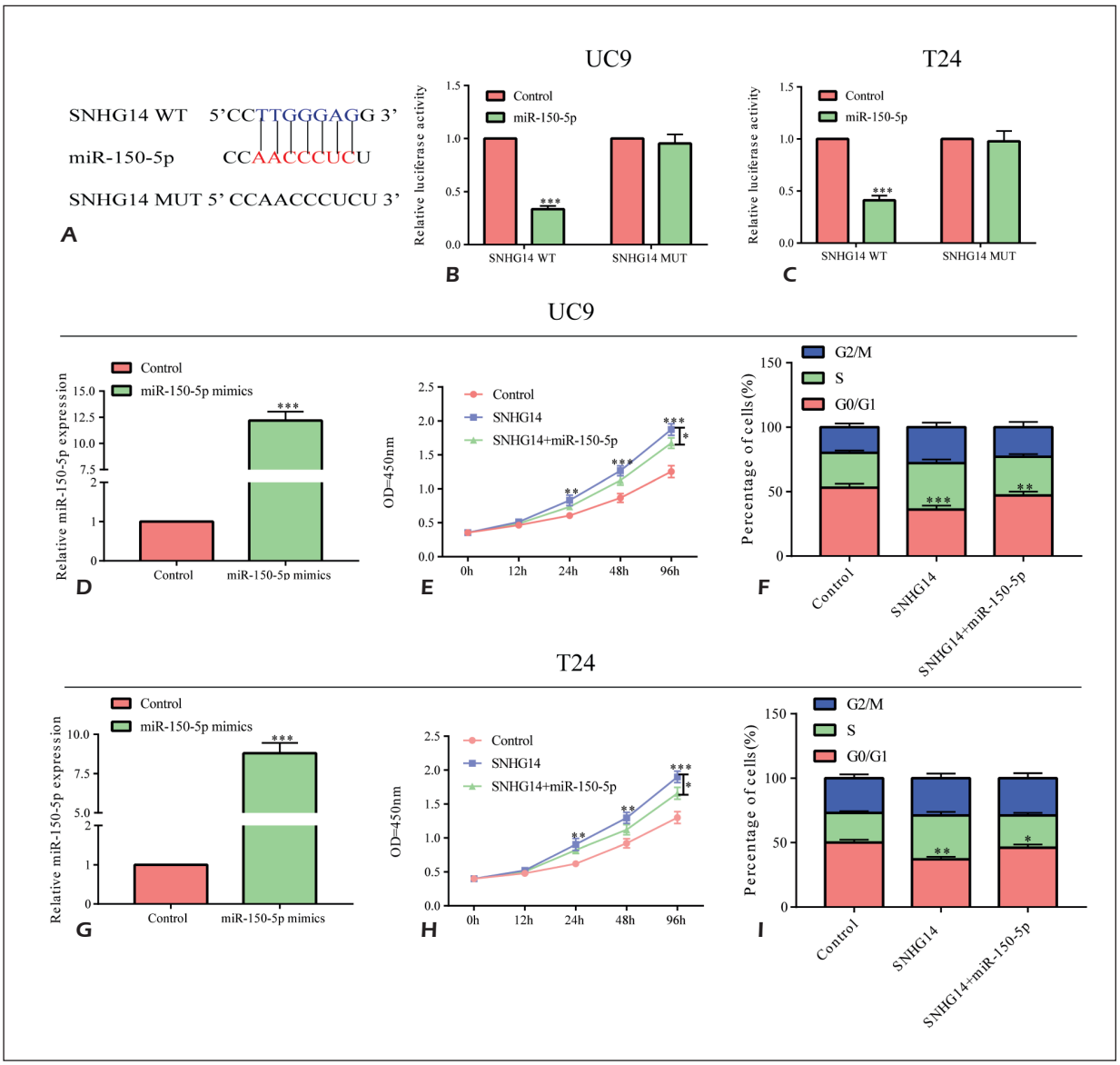

Figure 3. SNHG14 exerted its biological function through sponging miR-150-5p. **A**, A potential binding site for SNHG14 and miR-150-5p was found through online prediction. **B-C**, Dual-luciferase reporter gene assay verified the binding condition between SNHG14 and miR-150-5p. **D**, Transfection of miR-150-5p mimics upregulated miR-150-5p expression in UC9 cells. **E-F**, SNHG14 overexpression promoted proliferative potential and cell cycle progression of UC9 cells, which was then reversed by miR-150-5p overexpression. **G**, Transfection of miR-150-5p mimics upregulated miR-150-5p expression in T24 cells. **H-I**, SNHG14 overexpression promoted the proliferative potential and cell cycle progression of T24 cells, which was then reversed by miR-150-5p overexpression. *p<0.05, **p<0.01, ***p<0.001..

potential target gene for miRNA-150-5p through bioinformatics. VAMP2 was finally selected for subsequent verification (Figure 4A). Our experiments further showed their binding condition (Figure 4B, 4C). Subsequently, rescue experiments were conducted to elucidate whether miRNA-150-5p could influence BCa cells by degrading VAMP2. Overexpression of miRNA-150-5p in UC9 cells markedly suppressed proliferative potential and cell cycle progression, but was reversed by VAMP2 overexpression (Figure 4D-4F). Identically, we also observed the same trends in T24 cells (Figure 4G-4I). It is suggested that SNHG14/miRNA-150-5p/VAMP2 axis regulated BCa cell behaviors, thus participating in the development of BCa.

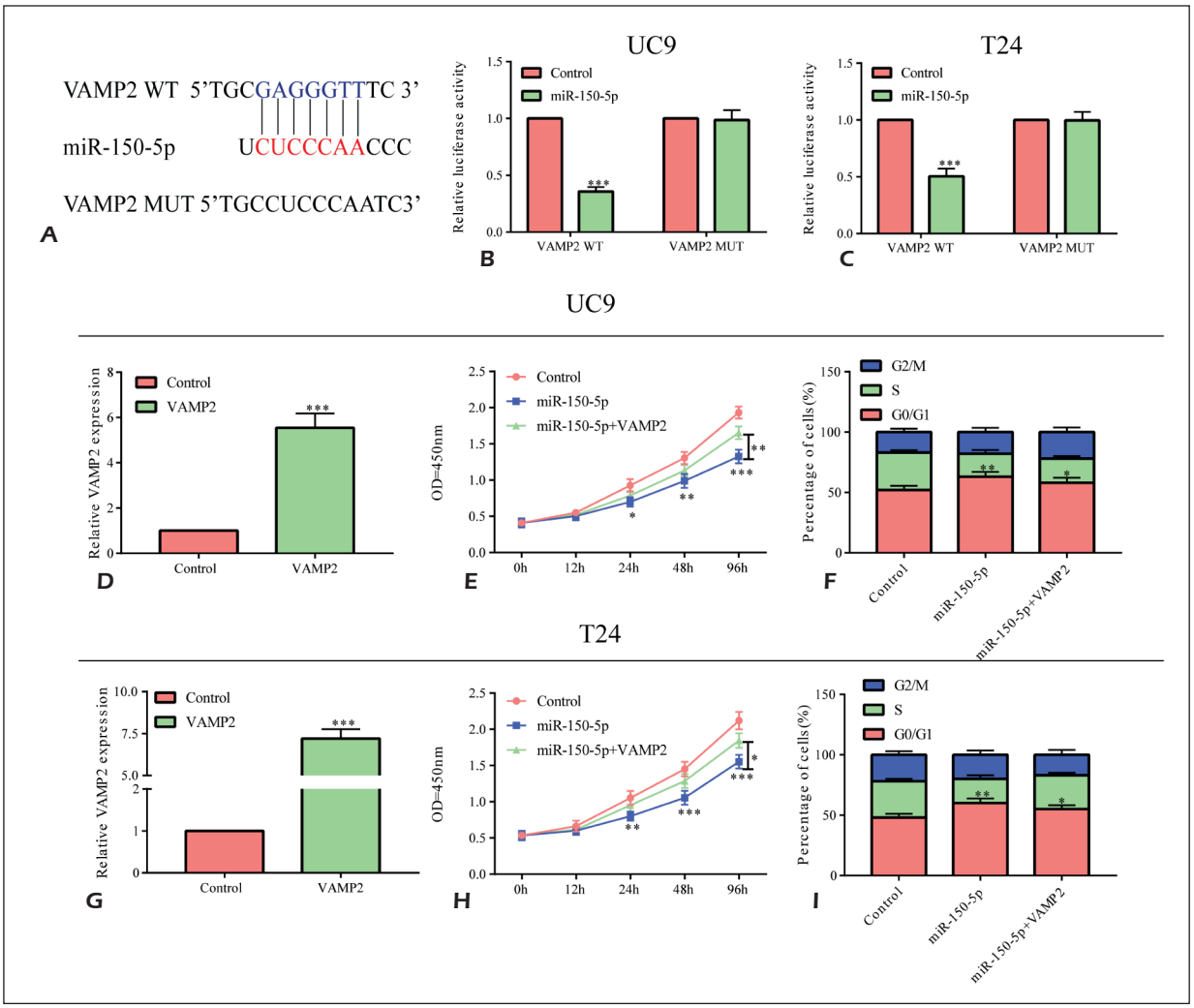

Figure 4. MiR-150-5p exerted its biological function through degrading VAMP2. **A**, A potential binding site for VAMP2 and miR-150-5p was found through online prediction. **B-C**, Dual-luciferase reporter gene assay verified the binding condition between VAMP2 and miR-150-5p. **D**, Transfection of pcDNA-VAMP2 upregulated VAMP2 expression in UC9 cells. **E-F**, Overexpression of miR-150-5p in UC9 cells markedly suppressed proliferative potential and cell cycle progression, but was further reversed by VAMP2 overexpression. **G**, Transfection of pcDNA-VAMP2 upregulated VAMP2 expression in T24 cells. **H-I**, Overexpression of miR-150-5p in T24 cells markedly suppressed proliferative potential and cell cycle progression, but was further reversed by VAMP2 overexpression. *p<0.05, **p<0.01, ***p<0.001.

Discussion

In addition to the traditional miRNA-RNA regulatory mechanisms, the reverse RNA-miRNA mechanism has emerged in recent years, which is known as the competitive endogenous RNA (ceRNA) hypothesis[12]. A part of the transcripts (ceRNAs) is capable of endogenously binding to miRNAs, thereafter altering expressions of target genes. Nearly 20,000 protein-coding genes in human genomes have been confirmed to possess abundant microRNA response elements (MREs)[13]. MicroRNA mainly inhibits mRNA expression and function. The regulatory effect of ceRNA is achieved by competitively binding to the target mRNAs with common MREs, so as to attenuate the inhibitory function on the target mRNAs. Otherwise, inhibition of ceRNA will lead to the downregulation of target genes[14].

LncRNA HULC attenuates the inhibitory effect on PRKACB by binding to miR-372, thereafter promoting the phosphorylation of CREB in liver cancer. The phosphorylated CREB subsequently upregulates HULC expression, forming a regulatory loop of HULC/miR-372/PRKACB/p-CREB axis[15]. PTC-SC3 downregulates the tumorigenic miR-574-5p in

thyroid tumors, leading to cell growth inhibition, cell cycle arrest, and apoptosis induction in thyroid tumor cells[16]. As a ceRNA, PTCSC3 participates in the development of thyroid tumor *via* absorbing miR-574-5p. SNHG14, as an oncogene, regulates the expression of miR-203 in renal clear cell carcinoma, thereby affecting N-WASP (Neural Wiskott-Aldrich Syndrome Protein) expression[17].

In this study, SNHG14 was highly expressed in BCa, which promoted the proliferative potential and cell cycle progression of BCa cells. A potential binding of SHHG14 to miRNA-150-5p was predicted by bioinformatics analysis. Thus, we speculated that SNHG14 may be a ceRNA absorbing miRNA-150-5p to exert its biological function. Previous studies[18,19] have pointed out the tumor-suppressor role of miRNA-150-5p. Here, we found miRNA-150-5p expression remained low in BCa. SNHG14 was capable of binding to miRNA-150-5p in BCa cells. Notably, miRNA-150-5p overexpression partially reserved the promotive effect of SNHG14 on proliferative potential and cell cycle progression of BCa cells.

We further predicted and analyzed the potential target gene of miRNA-150-5p through an online website, and VAMP2 (vesicle-associated membrane protein 2) was selected for further verification. Studies have found that VAMP2 knockdown could suppress the proliferative ability of liver cancer cells, showing a tumor-promoting role of VAMP2[20]. Since ceRNA functions are achieved by miRNA-mediated regulation of downstream target genes, we subsequently detected VAMP2 expression in BCa tissues. VAMP2 expression was upregulated in BCa, and further verified to bind to miRNA-150-5p. Rescue experiments revealed that VAMP2 overexpression partially reversed the inhibitory effects of miRNA-150-5p on the proliferative potential and cell cycle progression of BCa cells.

Conclusions

We showed that the lncSNHG14 overexpression accelerates proliferative potential and cell cycle progression of BCa cells through absorbing miRNA-150-5p to degrade VAMP2 expression.

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Conflict of Interests

The authors declare that they have no conflict of interest.

References

1) Grayson M. Bladder cancer. Nature 2017; 551: S33.
2) Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115-132.
3) Smith RA, Andrews K, Brooks D, DeSantis CE, Fedewa SA, Lortet-Tieulent J, Manassaram-Baptiste D, Brawley OW, Wender RC. Cancer screening in the United States, 2016: A review of current American Cancer Society guidelines and current issues in cancer screening. CA Cancer J Clin 2016; 66: 96-114.
4) Knowles MA, Hurst CD. Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. Nat Rev Cancer 2015; 15: 25-41.
5) Wu J, Wang J. HMGN5 expression in bladder cancer tissue and its role on prognosis. Eur Rev Med Pharmacol Sci 2018; 22: 970-975.
6) Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. Cell 2009; 136: 629-641.
7) Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet 2009; 10: 155-159.
8) Yang F, Xue X, Bi J, Zheng L, Zhi K, Gu Y, Fang G. Long noncoding RNA CCAT1, which could be activated by c-Myc, promotes the progression of gastric carcinoma. J Cancer Res Clin Oncol 2013; 139: 437-445.
9) Braconi C, Kogure T, Valeri N, Huang N, Nuovo G, Costinean S, Negrini M, Miotto E, Croce CM, Patel T. microRNA-29 can regulate expression of the long non-coding RNA gene MEG3 in hepatocellular cancer. Oncogene 2011; 30: 4750-4756.
10) Zhang Z, Wang Y, Zhang W, Li J, Liu W, Lu W. Long non-coding RNA SNHG14 exerts oncogenic functions in non-small cell lung cancer through acting as a miR-340 sponge. Biosci Rep 2018.
11) Liu Z, Yan Y, Cao S, Chen Y. Long non-coding RNA SNHG14 contributes to gastric cancer development through targeting miR-145/SOX9 axis. J Cell Biochem 2018; 119: 6905-6913.
12) Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell 2011; 146: 353-358.
13) Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009; 19: 92-105.
14) Jens M, Rajewsky N. Competition between target sites of regulators shapes post-transcriptional gene regulation. Nat Rev Genet 2015; 16: 113-126.
15) Milligan MJ, Lipovich L. Pseudogene-derived lncRNAs: emerging regulators of gene expression. Front Genet 2014; 5: 476.

16) Liu Y, Yue P, Zhou T, Zhang F, Wang H, Chen X. LncRNA MEG3 enhances (131)I sensitivity in thyroid carcinoma via sponging miR-182. Biomed Pharmacother 2018; 105: 1232-1239.

17) Liu G, Ye Z, Zhao X, Ji Z. SP1-induced up-regulation of lncRNA SNHG14 as a ceRNA promotes migration and invasion of clear cell renal cell carcinoma by regulating N-WASP. Am J Cancer Res 2017; 7: 2515-2525.

18) Sakr M, Takino T, Sabit H, Nakada M, Li Z, Sato H. miR-150-5p and miR-133a suppress glioma cell proliferation and migration through targeting membrane-type-1 matrix metalloproteinase. Gene 2016; 587: 155-162.

19) Yu J, Feng Y, Wang Y, An R. Aryl hydrocarbon receptor enhances the expression of miR-150-5p to suppress in prostate cancer progression by regulating MAP3K12. Arch Biochem Biophys 2018; 654: 47-54.

20) Wang G, Fang X, Han M, Wang X, Huang Q. MicroRNA-493-5p promotes apoptosis and suppresses proliferation and invasion in liver cancer cells by targeting VAMP2. Int J Mol Med 2018; 41: 1740-1748.