Original Article

Long non-coding RNA LINC01559 serves as a competing endogenous RNA accelerating triple-negative breast cancer progression

Xue Yang, Yunqing Yang, Xueke Qian, Xiaodong Xu, Pengwei Lv [*]

Breast Surgery, The First Affiliated Hospital of Zhengzhou University, Henan, China

ARTICLE INFO

ABSTRACT

Background: Long non-coding RNA (lncRNA) is an endogenous RNA over 200 nt in length involved in gene regulation. LINC01559 is a novel lncRNA that has been identified as a fundamental player in human cancer. However, its role in triple-negative breast cancer (TNBC) remains unknown. Here, we explored the expression, function and clinical implication of LINC01559 in TNBC.

Methods: RNA expression was detected by qRT-PCR analysis. Cell Counting Kit-8 (CCK-8), 5-Ethynyl-2′-deoxyuridine (EdU), wound healing and Transwell assays were used to test cell viability, DNA synthesis rate, migration and invasion, respectively. The competing endogenous RNA (ceRNA) axis involved in LINC01559 was determined by RNA pull-down and luciferase reporter assays. The xenograft model was used to verify the function of LINC01559 *in vivo*.

Results: LINC01559 was significantly increased in TNBC tissues as compared to matched normal tissues, which was due to high levels of H3K4Me3 and H3K27Ac in the promoter region. Knockdown of LINC01559 inhibited TNBC cell proliferation, migration and invasion *in vitro*, and also retarded tumor growth and reduced lung metastasis *in vivo*. Mechanistically, LINC01559 served as a ceRNA that sponged miR-370-3p, miR-485-5p and miR-940, resulting in increasing the expression of a cohort of oncogenes, thus accelerating TNBC progression.

Conclusions: Our data provide a comprehensive analysis of LINC01559 in TNBC, we found that LINC01559 functioned as a carcinogenic ceRNA via sponging miRNAs. Targeting of LINC01559 may be a potential treatment for TNBC patients.

* *Corresponding author*. Breast Surgery, The First Affiliated Hospital of Zhengzhou University, 1, Jianshe E Rd, Erqi, Zhengzhou 45000, Henan, China.
    E-mail address: lvpwhn83@126.com (P. Lv).

At a glance of commentary

Scientific background on the subject

LINC01559 is a novel lncRNA that has been identified as a fundamental player in human cancer. However, its role in triple-negative breast cancer (TNBC) remains unknown. Here, we explored the expression, function and clinical implication of LINC01559 in TNBC.

What this study adds to the field

Our data suggest that LINC01559 functions as a novel driver of TNBC progression through serving as a carcinogenic ceRNA sponging multiple miRNAs. Targeting of LINC01559 may be a potential treatment for TNBC patients.

Breast cancer is one of the most common malignant tumors in women, according to statistics, the incidence of breast cancer accounts for 7—10% of all malignant tumors in the whole body [1]. Triple negative breast cancer (TNBC) is a special type of breast cancer that is negative for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2), accounting for about 15% of all breast cancer types [2]. TNBC is characterized by high histological grade, early onset age, large tumor volume, and high incidence of visceral and bone metastases [3]. The lack of ER, PR and HER2 expression means that endocrine therapy and HER2 targeted therapy are not effective for TNBC [4]. Although it is sensitive to chemotherapy, only about 20% of patients with TNBC have good chemotherapy effect through conventional adjuvant chemotherapy [5]. Therefore, the exploration for effective therapeutic targets is a hot spot in TNBC research, which will bring a new direction for TNBC diagnosis and treatment, so as to improve its poor prognosis.

Long non-coding RNA (lncRNA) is a kind of non coding RNA with a length of more than 200 nt and is the transcription product of RNA polymerase II [6]. Most of lncRNAs are distributed in the nucleus, and some are specifically distributed in the cytoplasm [7]. They are composed of most highly conserved promoter sequences, introns or exons containing the "K4—K36" domain, and also have secondary structures [8,9]. Although very few lncRNAs can translate peptides, they play the important roles in the process of gene coding, such as at transcriptional level, post-transcriptional level and epigenetic level, which can control gene expression [10]. New evidence suggests that abnormal lncRNA expression is one of the hallmarks of cancer [11]. LncRNAs function as oncogenes or tumor suppressors via affecting different targets and signaling pathways [12,13]. One of the universally acknowledged machine-processed of lncRNA action is as a "miRNA sponge", namely competing endogenous RNA (ceRNA) network, in which lncRNA sponges miRNA, isolating the repressive effect of miRNA on its targets, indirectly increasing mRNA expression [14]. The ceRNA network is frequently deregulated in various human cancers, for example, lncRNA KTN1-AS1 was upregulated in non-small cell lung cancer and promoted cell growth and inhibited apoptosis through sponging miR-130a-5p and elevating PDPK1, a key regulator of autophagy [15].

Recently, almost at the same time, a novel lncRNA, LINC01559, has been identified as a critical driver in different cancers, including gastric cancer [16], hepatocellular carcinoma [17] and pancreatic cancer [18,19]. Nevertheless, its role in TNBC remains unexplored. In the present study, we found that LINC01559 was also increased in TNBC, it promoted TNBC cell proliferation and invasion both *in vitro* and *in vivo*. Importantly, we further investigated the underlying mechanisms of its dysregulation and pro-oncogenic effect.

Materials and methods

TNBC tissue samples

We collected a total of 96 pairs of TNBC and corresponding non-tumor normal tissues from patients pathologically diagnosed with TNBC between January 2013 to September 2018 at The First Affiliated Hospital of Zhengzhou University. Patients with other serious diseases or who had received any anti-tumor therapy before surgical resection, such as radiotherapy, chemotherapy, neoadjuvant chemoradiotherapy, immunotherapy, etc., were excluded. The clinicopathological characteristics of above patients were shown in Table 1. We obtained written informed consent from each patient, and followed them up every six months. This study was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University.

qRT-PCR analysis

Total RNA was isolated from surgically resected fresh tissues and cultured cells by using TRIzol (Invitrogen, CA, USA)

Table 1 Association of LINC01559 expression with clinical features in 96 triple-negative breast cancer patients.

Parameters	Total (n = 96)	LINC01559 expression		p value
		Low (n = 48)	High (n = 48)	
Age (years)				
≤ 40	28	12	16	0.369
> 40	68	36	32	
Menopause				
Yes	40	22	18	0.408
No	56	26	30	
Tumor size (cm)				
≤ 2	44	28	16	**0.014**
> 2	52	20	32	
Lymph node metastasis				
Negative	36	23	13	**0.035**
Positive	60	25	35	
TNM stage				
I	25	20	5	**<0.001**
II	48	24	24	
III	23	4	19	

The statistical method was chi-square test.
The median value of LINC01559 expression was used to divide the high and low LINC01559 groups.
p < 0.05 was indicative of statistical significance (bold font).

according to the manufacturer's instructions. Cell nucleus/cytoplasm fraction isolation was performed using the Nuclear and Cytoplasmic Extraction Kit (Thermo, MA, USA) according to the supplier's recommendation. The first-strand cDNA was generated by using MMLV transcriptase (Promega, WI, USA) with random primers. Real-time qRT-PCR was performed on a CFX96 real-time PCR detection system (Bio-Rad, CA, USA). The fold change of each gene was calculated by $2^{-\Delta\Delta Ct}$ method. U6 and GAPDH were used as control references for nuclear and cytoplasmic fragments, respectively. The detailed primer sequences are presented in Supplementary Table 1.

Cell culture and transfection

Two TNBC cell lines MDA-MB-231 and BT-20 and one normal MCF-10 A cells were all commercially purchased from American Type Culture Collection (ATCC), which were regularly authenticated by STR profiling and tested for mycoplasma contamination every two months. Both of them were cultured in RPMI 1640 medium with 10% FBS at 37 °C with 5% CO_2. Cell transfection was conducted using Lipofectamine 3000 (Invitrogen) as per supplier's instructions. Three miRNA inhibitors (miR-370-3p, miR-485-5p and miR-940) were commercially purchased from RiboBio (Guangzhou, China).

Stable LINC01559 knockdown cell lines

Two shRNA oligonucleotides targeting LINC01559 were designed and cloned into the pLV2-U6-Puro lentiviral vector. Then, the lentiviral vector was infected into MDA-MB-231 and BT-20 cells in the presence of infection enhancer polybrene. 48 h later, the fluorescence signals were observed under the fluorescence microscope. And stable cell lines were screened by adding puromycin to the culture medium, the knockdown efficiency was determined by qRT-PCR analysis.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assays were carried out by using the SimpleChIP® Plus Kit according to the manufacturer's instruction (Cell Signaling Technology). In brief, protein was cross-linked to DNA with 1% formaldehyde for 10min at room temperature, followed by inactivation using glycine for 5min. The micrococcal nuclease was added to digest DNA into a length of 200−1000 bp. Then, the antibodies against Histone H3 (#ab1791, Abcam), H3K27Ac (#ab4729, Abcam) and H3K4Me3 (#ab8580, Abcam) were added and incubated overnight. Lastly, the immunoprecipitated DNA was eluted and enriched. Quantification was calculated as a percentage relative to the input DNA from equation 1

$$[\text{Input Ct}-\text{Target Ct}] \times 100 \ (\%) \tag{1}$$

CCK-8 and EdU assays

MDA-MB-231 and BT-20 cells were plated onto 96-well plates and then cultured 3 days for CCK-8 assay. During this time,

10 μL CCK-8 solution was added into each well, followed by incubation at 37 °C for 1 h. Then, the cell culture plate was taken out and the absorbance value of each well was measured with a microplate reader. For detecting DNA synthesis rate, EdU staining assay was conducted by using Cell-Light™ EdU kit purchased from RiboBio as per manufacturer's instructions.

Wound healing and Transwell assays

For wound healing assay, a sterile pipette tip was used to generate three vertical scratches in each 6-well plate. Then, cells were cultured for 24 h and the migratory distance of each well was recorded. For Transwell assay, coated basement membrane: Matrigel matrix adhesive was diluted 1:8 and coated with the upper surface of Transwell chamber bottom membrane. 200 μL cell suspension was added to upper chamber and 600 μL culture medium containing 10% FBS was added to lower chamber. After 20 h of incubation, cells in the upper layer of the basement membrane were carefully wiped with a cotton swab, cells in the lower layer were fixed with 4% paraformaldehyde for 20 min, and stained with crystal violet solution for 15 min. Photographs were taken under an inverted microscope. Each sample was randomly counted in 10 fields, and the mean value was taken for statistical analysis.

RNA pull-down and immunoprecipitation (RIP) assays

LINC01559 was *in vitro* transcribed using T7 High Yield RNA Synthesis Kit (Ambion, TX, USA) according to the manufacturer's instructions. The transcribed RNA was labeled using RNA 3′-End Biotinylation Kit (Themo, MA, USA). Antisense-LINC01559 was biotinylated and served as a control. TNBC cell lysates were collected, added with above probes and incubated overnight with agitation. Then, the streptavidin magnetic beads were added and incubated at room temperature for 30 min with agitation. The miRNAs bound by LINC01559 probe were eluted and extracted using TRIzol reagent for qRT-PCR analysis. Besides, RIP assay was carried out by using Magna RIP™ RNA-Binding Protein Immunoprecipitation kit (Millipore, MA, USA) with 5 μg anti-AGO2 antibody (#186733, Abcam) as the manufacturer's instructions.

Luciferase reporter assay

The wild-type and mutant sequences between LINC01559 and miR-370-3p, miR-485-5p or miR-940 were cloned into pmirGLO luciferase vector (Promega, WI, USA). After that, miR-370-3p, miR-485-5p or miR-940 mimics were co-transfected with above vectors into MDA-MB-231 and BT-20 cells using Lipofectamine 3000. After 48 h, the dual-luciferase reporter assay was conducted using a commercial kit (Promega) as per standard protocols.

Tumor xenografts in vivo

All animal procedures were approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Zhengzhou University. Five BALB/c nude mice per group were used to establish experimental models of

subcutaneous neoplasia and lung metastasis. For *in vivo* tumorigenesis, 1×10^7 MDA-MB-231 cells with or without LINC01559 knockdown in 0.2 mL PBS were subcutaneously injected into nude mice. After five weeks of growth, all mice were sacrificed and tumor tissues were photographed and weighed. For tail vein injection, 1×10^7 MDA-MB-231 cells with or without LINC01559 knockdown in 0.2 mL PBS were injected into the lateral tail vein of nude mice. After 6 weeks of injection, the mice were killed by cervical dislocation and the lungs were collected to count surface metastases under a dissecting microscope. Extracted lungs were embedded in paraffin using the routine method for hematoxylin&eosin (H&E) staining.

Analysis of the downstream miRNAs of LINC01559

Three online tools including miRanda (http://www.microrna. org/), PITA (https://genie.weizmann.ac.il/pubs/mir07/mir07_ data.html) and RNAhybrid (http://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid) were used to predict the miRNAs bound by LINC01559. miRNAs concurrently predicted by these three databases were used for subsequent experimental validation.

Statistic analysis

Each experiment was repeated at least three times, and the statistics were analyzed using the SPSS 21.0 software and figures were generated using Graphpad Prism v7.0 software. The difference between groups were analyzed by Student's t-test (2 groups) and one-way analysis of variance (ANOVA) (>2 groups).

The survival curve was generated by Kaplan—Meier method and analyzed by Log-rank test. In addition, the survival curve based on LINC01559 level from Kaplan—Meier database (http://kmplot. com/analysis/index.php?p=service&start=1) was generated using auto select best cutoff value, the False Discovery Rate was 10%. Pearson correlation coefficient was used to test the correlation between LINC01559 expression and H3K4Me3 or H3K27Ac enrichment on LINC01559 promoter. $p < 0.05$ was indicative of statistical significance.

Results

LINC01559 is a upregulated lncRNA in TNBC

We first performed qRT-PCR analysis in 96 pairs of TNBC and precancerous normal tissues, as shown in Fig. 1A, LINC01559 was significantly increased in TNBC tissues as compared to adjacent normal tissues (ANT). Then, we analyzed the subcellular localization of LINC01559, the results showed that LINC01559 was dominantly located in the cytoplasm in both MDA-MB-231 and BT-20 TNBC cell lines [Fig. 1B, C]. Clinically, high LINC01559 was positively linked to larger tumor size, advanced TNM stage and lymph node metastasis [Table 1]. Importantly, patients with high LINC01559 had a shorter survival time than patients with low LINC01559 [Fig. 1D], which was confirmed by the data from Kaplan—Meier database (http://kmplot.com/analysis/index.php?p=background) [Fig. 1E]. These data suggest that LINC01559 is frequently
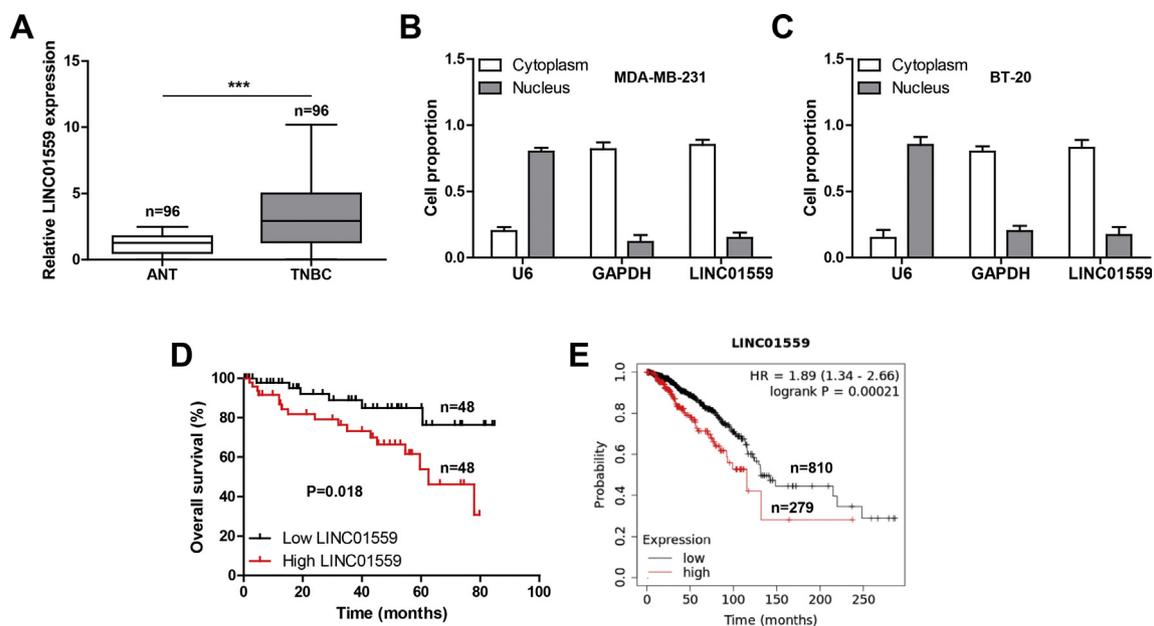
Fig. 1 LINC01559 is frequently increased in TNBC. (A) qRT-PCR analysis of LINC01559 expression in 96 paired TNBC and adjacent normal tissues (ANT). (B, C) qRT-PCR analysis detecting the location of LINC01559 in MDA-MB-231 (B) and BT-20 (C) cell lines. (D) The survival curve of TNBC patients based on the median expression of LINC01559 in our cohort. (E) Kaplan—Meier database (http://kmplot.com/analysis/index.php?p=background) analyzing the correlation between LINC01559 expression and the overall survival, which was plotted using the best performing threshold automatically analyzed by the database, the False Discovery Rate (FDR) was 10%. Data was the mean ± standard deviation (SD) of at least three independent experiments carried out in triplicate. ***$p < 0.001$.

upregulated in TNBC, which may be linked to TNBC progression.

High LINC01559 expression in TNBC is due to gain of H3K4me3 and H3K27ac

Through analyzing the UCSC Genome Browser (http://genome.ucsc.edu/) [20], we found high enrichment of H3K4me3 and H3K27ac (transcriptional activation markers) on the LINC01559 promoter region [Fig. 2A], hinting that these histone modifications may be linked to high LINC01559 expression. To verify above observation, we performed ChIP assay, the results showed that H3K4me3 and H3K27ac were indeed abundantly occupied on LINC01559 promoter in both MDA-MB-231 and BT-20 cells in comparison to normal MCF-10 A cells [Fig. 2B, C]. Consistently, high H3K4me3 and H3K27ac on LINC01559 promoter were also observed in TNBC tissues as compared to matched normal tissues, and LINC01559 expression was strongly positively correlated with H3K4me3 (r = 0.759, p = 0.001) or H3K27ac (r = 0.64, p = 0.01) enrichment [Fig. 2D−G]. These results indicate that LINC01559 expression is regulated by histone modification.

Knockdown of LINC01559 inhibits TNBC cell proliferation, migration and invasion in vitro

To study the biological function of LINC01559 in TNBC, we constructed the stable LINC01559 knockdown TNBC cell lines using lentiviral vector, the results of qRT-PCR assay verified the knockdown efficiency in both MDA-MB-231 and BT-20 cells [Fig. 3A]. Then, we performed a series of functional assays. As shown in Fig. 3B, C, cell viability was significantly weakened after depletion of LINC01559. Similarly, the DNA synthesis rate of LINC01559-silenced TNBC cells was remarkably slowed down as compared to control TNBC cells [Fig. 3D, E]. Moreover, knockdown of LINC01559 resulted in a significant reduction in the migration distance of TNBC cells, as illustrated by wound healing assay [Fig. 3F, G]. And the invasion ability was also dramatically attenuated after LINC01559 silencing [Fig. 3H, I]. These data suggest that LINC01559 is a driver of TNBC malignant phenotype.

LINC01559 is a ceRNA that sponges miR-370-3p, miR-485-5p and miR-940

Given that LINC01559 is a cytoplasmic lncRNA, we thus inferred that LINC01559 may function as a ceRNA. To test this hypothesis, we performed RIP assay using anti-AGO2 antibody. The results showed that a large amount of LINC01559 was enriched by AGO2 in both MDA-MB-231 and BT-20 cells [Fig. 4A]. Through intersecting the prediction results of miRanda (http://www.microrna.org/) (113 miRNAs predicted bound by LINC01559) [21], PITA (https://genie.weizmann.ac.il/pubs/mir07/mir07_data.html) (68 miRNAs predicted bound by LINC01559) [22] and RNAhybrid (http://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid) (47 miRNAs predicted bound by LINC01559) [23] software, we found five miRNAs that may be sequentially complementary to LINC01559, which were used for experimental validation [Fig. 4B]. Then, RNA pull-down assay was carried out by using LINC01559 probe, the results showed that miR-370-3p, miR-485-5p and miR-940 were synchronously enriched by LINC01559 in both MDA-MB-231 and BT-20 cells [Fig. 4C, D]. The binding sites between LINC01559 and above three miRNAs were shown in Fig. 4E−G, we mutated them one by one to perform luciferase reporter assay. The results showed that overexpression of miR-370-3p, miR-485-5p or miR-940 could significantly reduce the luciferase activity of wild-type LINC01559 vector, but did not affect that of the mutant one [Fig. 4H, I]. Besides, LINC01559 knockdown led to a distinct upregulation in miR-370-3p, miR-485-5p and miR-940 [Fig. 4J, K]. Importantly, the levels of the validated target oncogenes of above three miRNAs were significantly reduced after LINC01559 knockdown, and these effects were partially eliminated by silencing these miRNAs, respectively [Fig. 4L−N]. Functionally, knockdown of miR-370-3p, miR-485-5p or miR-940 effectively rescued the attenuated cell viability [Fig. 4O] and invasion [Fig. 4P] caused by LINC01559 depletion. These results demonstrate that LINC01559 sponges miR-370-3p, miR-485-5p and miR-940 in TNBC cells.

Knockdown of LINC01559 inhibits TNBC growth and metastasis in vivo

Lastly, we explored the *in vivo* function of LINC01559 through subcutaneous and tail vein injection of control and LINC01559-silenced MDA-MB-231 cells into nude mice. The results showed that the tumor volume and size in LINC01559-silenced group were significantly smaller than those in control group [Fig. 5A−C]. Likewise, the average number of lung metastases in LINC01559-silenced group was evidently less than that in control group [Fig. 5D, E]. These data indicate that depletion of LINC01559 represses TNBC progression, which is consistent with *in vitro* data.

Discussion

TNBC is a highly heterogeneous and malignant disease with poor prognosis. At present, the pathogenesis of TNBC is still poorly understood. In the current study, we found a TNBC-related lncRNA, LINC01559, which was upregulated in TNBC tissues by high H3K4me3 and H3K27ac enrichment on its promoter. LINC01559 promoted TNBC cell malignant behaviors both *in vitro* and *in vivo*. In terms of mechanism, LINC01559 was identified as a ceRNA that sponging miR-370-3p, miR-485-5p or miR-940, resulting in upregulation of a cohort of proto-oncogenes, thereby facilitating TNBC progression. Our data demonstrate that LINC01559 is a novel driver of TNBC, dysregulation of LINC01559/miR-370-3p/miR-485-5p/miR-940 axis may be responsible for TNBC tumorigenesis and dissemination.

Gene expression is strictly controlled at the transcriptional level, in which abnormal histone modification plays an important role [24]. Histone can maintain DNA structure, protect genetic information, its N-terminal domain extends from the nucleosome and interacts with other regulatory proteins and DNA [25]. Emerging evidence suggests that histone modification imbalance can lead to tumorigenesis [26], and loss or
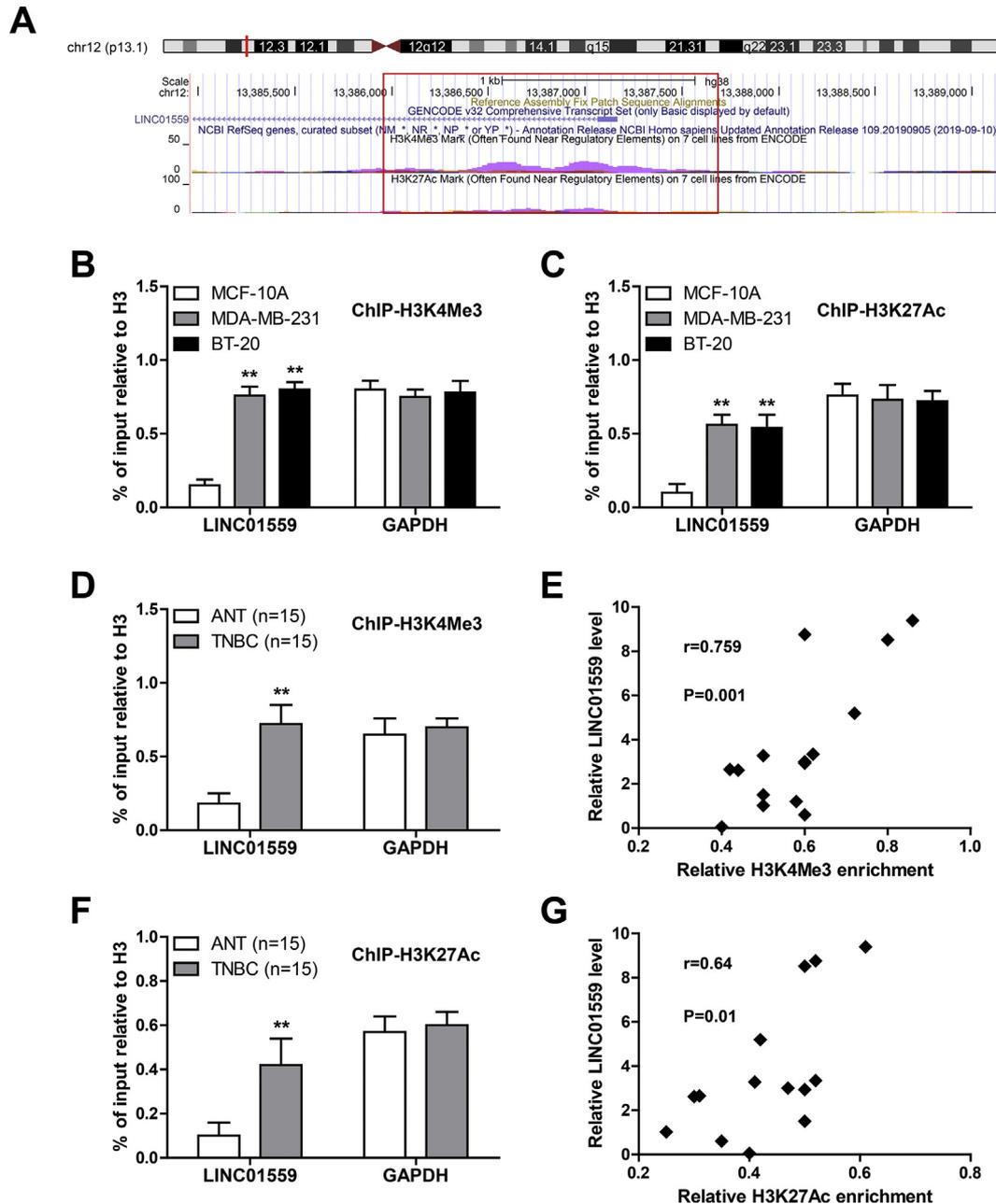
Fig. 2 LINC01559 is regulated by H3K4me3 and H3K27ac. (A) UCSC Genome Browser showing the high enrichment of H3K4me3 and H3K27ac on LINC01559 promoter. The red box indicates the peaks of H3K4me3 and H3K27ac. (B, C) ChIP assay using anti-H3K4me3 (B) or -H3K27ac (C) antibody in normal and TNBC cells, followed by qPCR analysis of LINC01559 promoter enrichment, GAPDH promoter was used as a reference control. (D) ChIP assay using anti-H3K4me3 antibody in 15 paired TNBC and normal tissues. (E) The correlation between LINC01559 expression and H3K4me3 enrichment. (F) ChIP assay using anti-H3K27ac antibody in 15 paired TNBC and normal tissues. (G) The correlation between LINC01559 expression and H3K27ac enrichment. ANT = adjacent normal tissue, Data was the mean ± standard deviation (SD) of at least three independent experiments carried out in triplicate. **$p < 0.01$.

gain of methylation and acetylation of histone H3 residues has been proved to be involved in gene silencing or activation [27]. For example, methylation at H3K4 and H3K36 and mono-methylation of H3K27 can activate gene transcription, while methylation at H3K9 and H3K79 and dimethylation and tri-methylation at H3K27 can inhibit the transcription of target genes [28,29]. On the other hand, histone H3 acetylation adds acetyl group to lysine and makes lysine positively charged,
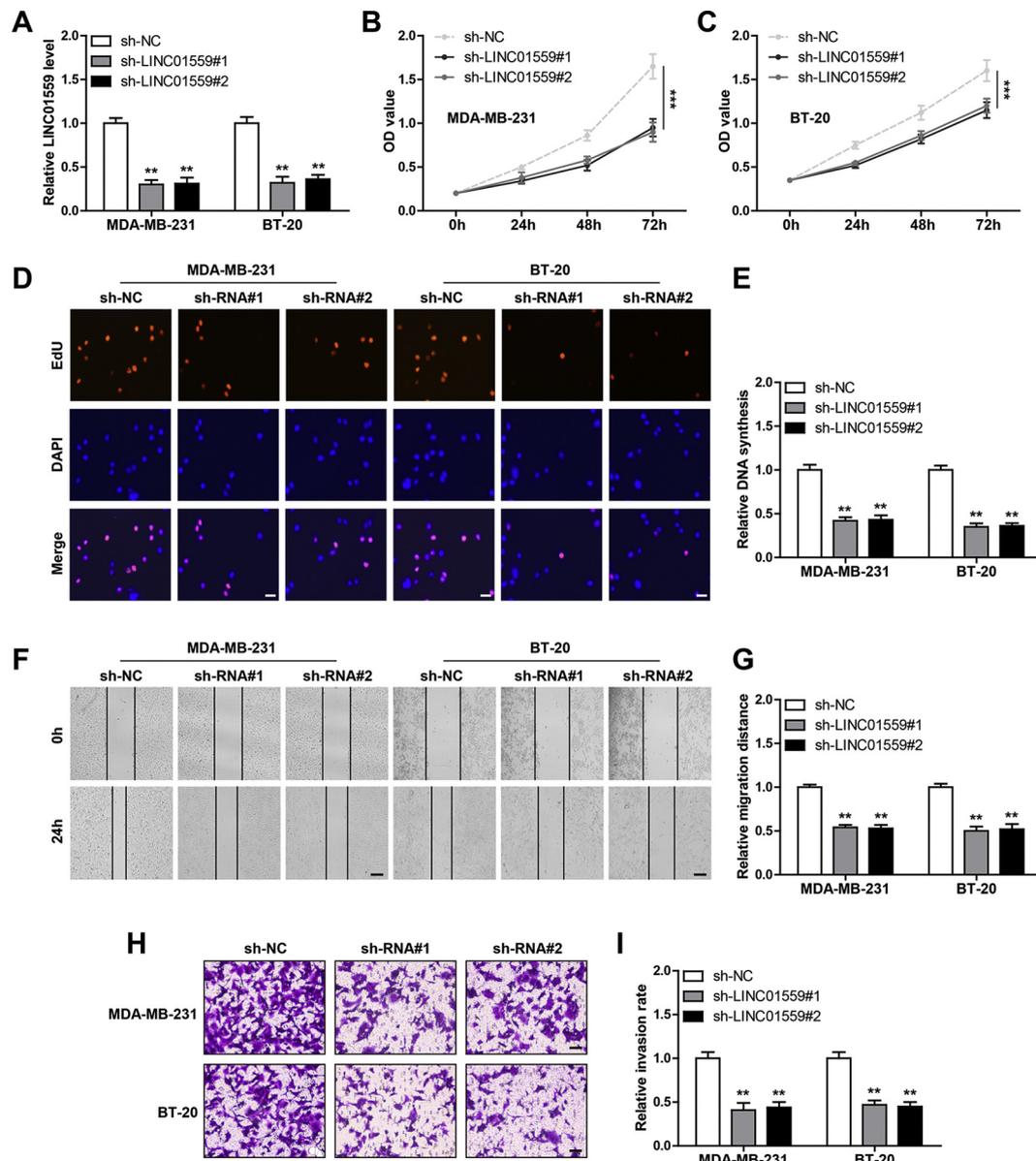
Fig. 3 LINC01559 knockdown represses TNBC cell malignant phenotype. (A) qRT-PCR analysis detecting the knockdown efficiency of LINC01559 in MDA-MB-231 and BT-20 cells using lentiviral vector. (B, C) CCK-8 assay testing cell viability after knockdown of LINC01559. (D, E) EdU assay testing DNA synthesis rate of TNBC cells after knockdown of LINC01559. (F-I) Wound healing and Transwell assays detecting cell migration and invasion after knockdown of LINC01559. NC = negative control, Data was the mean ± standard deviation (SD) of at least three independent experiments carried out in triplicate. The relative level was compared between the sh-LINC01559 group and the NC group. Scale bar = 50 μm, **$p < 0.01$, ***$p < 0.001$.

leading to chromatin structure opening and promoting gene transcription, thus high H3 acetylation is a marker of gene active transcription [30]. Up to now, many genes including lncRNAs have been found to be regulated by histone modification, such as HOXC-AS3 [31], SATB2-AS1 [32], SAMMSON [33] and UCA1 [34]. Herein, by *in silico* analysis, we found high enrichment of H3K4me3 and H3K27ac on LINC01559 promoter. ChIP assay confirmed this finding via using specific anti-H3K4me3 and -H3K27ac antibodies, in which more H3K4me3 and H3K27ac occupations were observed on LINC01559

promoter in both TNBC tissues and cells in comparison to respective normal controls. Moreover, high H3K4me3 or H3K27ac enrichment was strongly positively correlated with LINC01559 expression in TNBC tissues. These data suggest that upregulated LINC01559 in TNBC is mainly caused by gain of H3K4me3 and H3K27ac on its promoter region.

LncRNA has a variety of modes of action, among which it has been widely verified as miRNA molecular sponge, effectively inhibiting the interaction between miRNA and target mRNA, elevating gene expression [35]. miRNA is
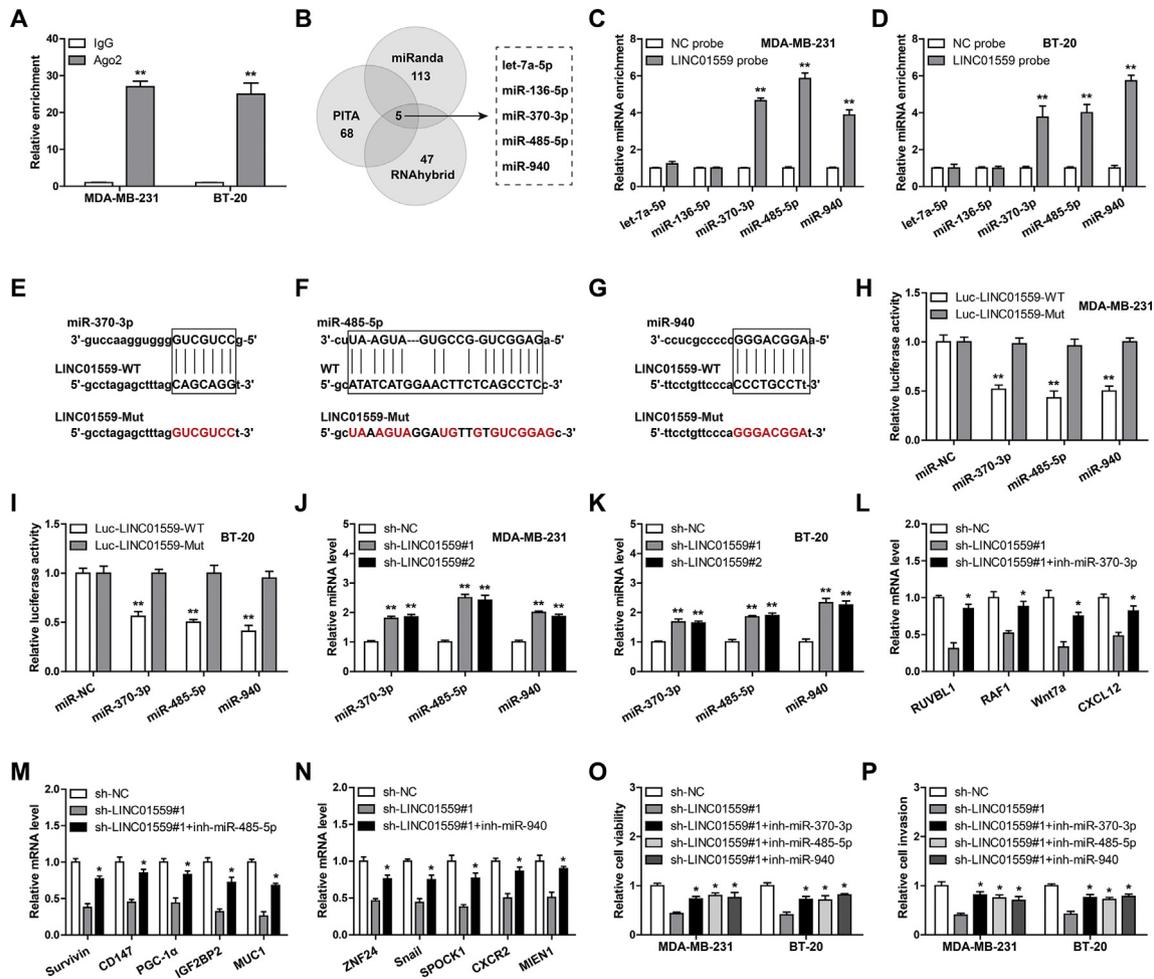
Fig. 4 LINC01559 is a ceRNA sponging miRNAs. (A) RIP assay detecting the enrichment of LINC01559 by AGO2. (B) Three online tools predicting the miRNAs interacting with LINC01559. (C, D) RNA pull-down assay detecting miRNA enrichment by LINC01559 probe. (E-G) The wild-type and mutant binding sequences between LINC01559 and the indicated three miRNAs. (H, I) Luciferase reporter assay in MDA-MB-231 and BT-20 cells testing the relationships between LINC01559 and the indicated three miRNAs, respectively. (J, K) qRT-PCR analysis of miRNA levels after LINC01559 knockdown. (L-N) qRT-PCR analysis of mRNA levels in LINC01559-depleted TNBC cells after silencing of the indicated miRNA. (O, P) CCK-8 and Transwell assays testing cell viability and invasion in LINC01559-depleted TNBC cells after silencing of the indicated miRNA. Data was the mean ± standard deviation (SD) of at least three independent experiments carried out in triplicate. The relative level was compared between the experimental group and the negative control (NC) group. *$p < 0.05$, **$p < 0.01$.

widely distributed in eukaryotes with a length of 18—25 nt, it regulates about 1/3 of human genes [36]. Most mature miR-NAs bind to human protein AGO2 and generate a gene silencing complex (RNA-induced silencing complex, RISC), and then bind specifically to mRNA 3′-UTR, preventing the translation of target genes or directly degrade them. Herein, by performing RIP, RNA pull-down and luciferase reporter assays, we found that LINC01559 was abundantly enriched by AGO2 protein and could concurrently sponge three tumor-suppressive miRNAs, namely miR-370-3p, miR-485-5p and miR-940. Consistently, the respective target onco-genes of above miRNAs were uniformly downregulated after LINC01559 knockdown, and silencing of these miRNAs alone

could significantly rescue the decreased malignant pheno-type of TNBC cells caused by depletion of LINC01559, sug-gesting that the ceRNA axis of LINC01559/miR-370-3p/miR-485-5p/miR-940 does exist in TNBC cells. Of note, lncRNA plays different roles or even opposite roles in different con-texts, some studies showed that LINC01559 sponged miR-1343-3p and bound to EZH2 in gastric cancer [16], and absorbed miR-6783-3p [17] and miR-607 [19] in hepatocellular carcinoma and pancreatic cancer, respectively. However, we did not observe above phenomena in TNBC (data not shown). It is of great interest to elucidate the mechanism of this functional variation. Moreover, further studies are needed to explore the expression and function mechanism of
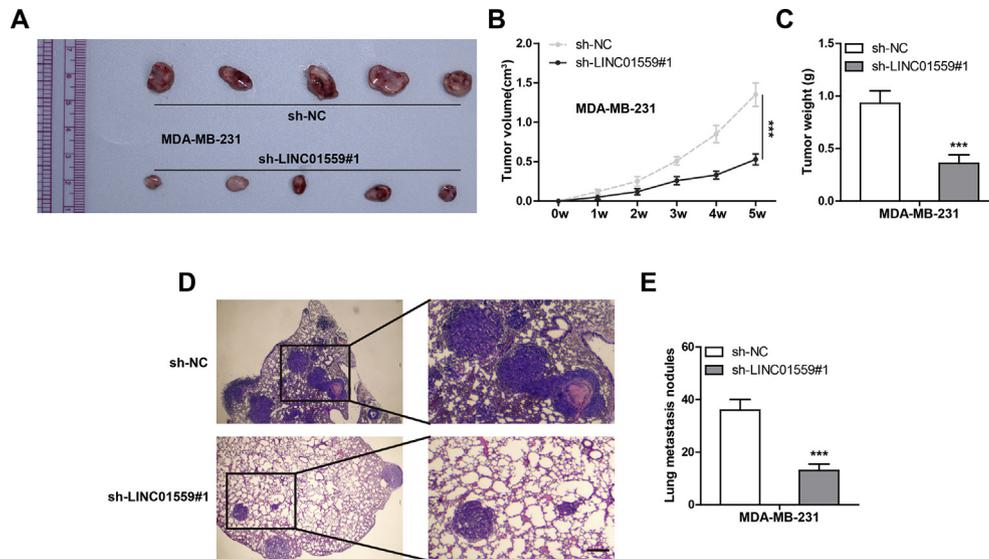
Fig. 5 LINC01559 knockdown inhibits TNBC growth and metastasis. (A) The image of subcutaneous tumor of nude mice injected with control and LINC01559 knockdown MDA-MB-231 cells. (B, C) Tumor volume and weight of control and LINC01559 groups. (D, E) The representative lung metastasis nodules stained by H&E staining in control and LINC01559 groups. Scale bar = 50 μm, ***$p < 0.001$.

LINC01559 in other malignant tumors, so as to reveal whether it is a pan-oncogene.

Certainly, there are some limitations in our study. For example, there are some off-target and incomplete interference effects of shRNA, and the results will be more convincing if LINC01559 is completely knocked out by gene editing technology, such as CRISPR/Cas9 method. In addition, the samples are all retrospective studies, which can not avoid the potential for bias between different treatments.

Conclusion

In this study, we for the first time show that abnormal histone modification-mediated activation of LINC01559 enhances TNBC growth and metastasis via acting as a ceRNA and concurrently sponging three miRNAs, which provides evidence for the use of LINC01559 as a promising prognostic biomarker and treatment for TNBC patients.

Conflicts of interest

The authors declare no competing interests.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bj.2021.05.002.

REFERENCES

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68:394−424.

[2] Navrátil J, Fabian P, Palácová M, Petráková K, Vyzula R, Svoboda M. Triple negative breast cancer. Klin Onkol 2015;28:405−15.

[3] Kumar P, Aggarwal R. An overview of triple-negative breast cancer. Arch Gynecol Obstet 2016;293:247−69.

[4] Lebert JM, Lester R, Powell E, Seal M, McCarthy J. Advances in the systemic treatment of triple-negative breast cancer. Curr Oncol 2018;25:S142−50.

[5] Lyons TG. Targeted therapies for triple-negative breast cancer. Curr Treat Options Oncol 2019;20:82.

[6] Gutschner T, Diederichs S. The hallmarks of cancer: a long non-coding RNA point of view. RNA Biol 2012;9:703−19.

[7] Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function. Nat Rev Genet 2016;17:47−62.

[8] Cabili MN, Trapnell C, Goff L, Koziol M, Tazon-Vega B, Regev A, et al. Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. Genes Dev 2011;25:1915−27.

[9] Guttman M, Rinn JL. Modular regulatory principles of large non-coding RNAs. Nature 2012;482:339−46.

[10] Gao F, Cai Y, Kapranov P, Xu D. Reverse-genetics studies of lncRNAs-what we have learnt and paths forward. Genome Biol 2020;21:93.

[11] Jiang MC, Ni JJ, Cui WY, Wang BY, Zhuo W. Emerging roles of lncRNA in cancer and therapeutic opportunities. Am J Cancer Res 2019;9:1354–66.

[12] Kumar S, Gonzalez EA, Rameshwar P, Etchegaray JP. Non-coding RNAs as mediators of epigenetic changes in malignancies. Cancers 2020;12:3657.

[13] Du XH, Wei H, Qu GX, Tian ZC, Yao WT, Cai QQ. Gene expression regulations by long noncoding RNAs and their roles in cancer. Pathol Res Pract 2020;216:152983.

[14] Paraskevopoulou MD, Hatzigeorgiou AG. Analyzing MiRNA-LncRNA interactions. Methods Mol Biol 2016;1402:271–86.

[15] Li C, Zhao W, Pan X, Li X, Yan F, Liu S, et al. LncRNA KTN1-AS1 promotes the progression of non-small cell lung cancer via sponging of miR-130a-5p and activation of PDPK1. Oncogene 2020;39:6157–71.

[16] Wang L, Bo X, Yi X, Xiao X, Zheng Q, Ma L, et al. Exosome-transferred LINC01559 promotes the progression of gastric cancer via PI3K/AKT signaling pathway. Cell Death Dis 2020;11:723.

[17] Dong S, Fu Y, Yang K, Zhang X, Miao R, Long Y, et al. linc01559 served as a potential oncogene and promoted resistance of hepatocellular carcinoma to oxaliplatin by directly sponging miR-6783-3p. Anticancer Agents Med Chem 2021;21:278–86.

[18] Chen C, Wang J, Xie F, Mou T, Zhong P, Hua H, et al. Long noncoding RNA LINC01559 promotes pancreatic cancer progression by acting as a competing endogenous RNA of miR-1343-3p to upregulate RAF1 expression. Aging 2020;12:14452–66.

[19] Lou C, Zhao J, Gu Y, Li Q, Tang S, Wu Y, et al. LINC01559 accelerates pancreatic cancer cell proliferation and migration through YAP-mediated pathway. J Cell Physiol 2020;235:3928–38.

[20] Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, et al. The human genome browser at UCSC. Genome Res 2002;12:996–1006.

[21] Kruger J, Rehmsmeier M. RNAhybrid: microRNA target prediction easy, fast and flexible. Nucleic Acids Res 2006;34:W451–4.

[22] Huang H, Sabari BR, Garcia BA, Allis CD, Zhao Y. SnapShot: histone modifications. Cell 2014;159:458.

[23] Jenuwein T, Allis CD. Translating the histone code. Science 2001;293:1074–80.

[24] Audia JE, Campbell RM. Histone modifications and cancer. Cold Spring Harb Perspect Biol 2016;8:a19521.

[25] Ferrari P, Strubin M. Uncoupling histone turnover from transcription-associated histone H3 modifications. Nucleic Acids Res 2015;43:3972–85.

[26] Al Aboud NM, Tupper C, Jialal I. Genetics, epigenetic mechanism. 2021 Aug 11. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022.

[27] Longbotham JE, Zhang MY, Fujimori DG. Domain cross-talk in regulation of histone modifications: molecular mechanisms and targeting opportunities. Curr Opin Chem Biol 2020;57:105–13.

[28] Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, et al. Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 2000;406:593–9.

[29] Zhang E, He X, Zhang C, Su J, Lu X, Si X, et al. A novel long noncoding RNA HOXC-AS3 mediates tumorigenesis of gastric cancer by binding to YBX1. Genome Biol 2018;19:154.

[30] Xu M, Xu X, Pan B, Chen X, Lin K, Zeng K, et al. LncRNA SATB2-AS1 inhibits tumor metastasis and affects the tumor immune cell microenvironment in colorectal cancer by regulating SATB2. Mol Cancer 2019;18:135.

[31] Shao L, Sun W, Wang Z, Dong W, Qin Y. Long noncoding RNA SAMMSON promotes papillary thyroid carcinoma progression through p300/Sp1 axis and serves as a novel diagnostic and prognostic biomarker. IUBMB Life 2019.

[32] Guo X, Zhang Y, Mayakonda A, Madan V, Ding LW, Lin LH, et al. ARID1A and CEBPalpha cooperatively inhibit UCA1 transcription in breast cancer. Oncogene 2018;37:5939–51.

[33] Panda AC. Circular RNAs act as miRNA sponges. Adv Exp Med Biol 2018;1087:67–79.

[34] Jonas S, Izaurralde E. Towards a molecular understanding of microRNA-mediated gene silencing. Nat Rev Genet 2015;16:421–33.

[35] Achkar NP, Cambiagno DA, Manavella PA. miRNA biogenesis: a dynamic pathway. Trends Plant Sci 2016;21:1034–44.

[36] Batista PJ, Chang HY. Long noncoding RNAs: cellular address codes in development and disease. Cell 2013;152:1298–307.