European Review for Medical and Pharmacological Sciences 2019; 23: 9857-9862

Long non-coding RNA XIST promotes hepatocellular carcinoma progression by sponging miR-200b-3p

W.-G. LIU[1,2], Q. XU[1,2]

[1]Department of General Surgery, Beijing Bo'ai Hospital, China Rehabilitation Research Center, Beijing, China
[2]School of Rehabilitation, Capital Medical University, Beijing, China

Abstract. – OBJECTIVE: Recent studies have proved that long noncoding RNAs (lncRNAs) act as an important role in many diseases. In this research, lncRNA XIST was explored to identify how it functions in the development of hepatocellular carcinoma (HCC).

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was utilized to detect XIST expression in HCC patients. Then, we conducted Cell Counting Kit-8 (CCK-8) assay and colony formation assays in vitro. Furthermore, mechanism assays and the interaction between XIST and miR-200b-3p were conducted.

RESULTS: By comparing with XIST expression in adjacent tissues, the XIST expression level was significantly higher in HCC samples. Moreover, functional assays showed that the cell growth ability of HCC cells was inhibited after XIST was silenced in vitro, and tumor formation was inhibited after XIST was silenced in vivo. Further experiments showed that miR-200b-3p was directly targeted by XIST.

CONCLUSIONS: Above results suggest that XIST could enhance the cell growth ability of HCC by targeting miR-200b-3p, which suggest that XIST may be a potential therapeutic target in HCC.

Key Words:
Long noncoding RNA, XIST, Hepatocellular carcinoma, MiR-200b-3p.

Introduction

Hepatocellular carcinoma (HCC) is one of the primary liver cancers and accounts for nearly 90% of all primary liver neoplasms. Moreover, HCC-related mortality is significantly higher in developing countries, especially in China, where hepatitis B virus (HBV) is particularly prevalent[1,2]. Surgical resection is the main intervention for HCC patients who are diagnosed in the early stages. However, most of HCC cases are diagnosed at an advanced stage, with less opportunity to take curable surgery, thus contributing to the poor survival rate. Therefore, understanding the underlying molecular mechanism of HCC is urgent and could be an improvement for the diagnosis, management, and prognosis of HCC patients.

Long non-coding RNAs (lncRNAs) are a cluster of transcripts longer than 200 nucleotides. Some studies have revealed that lncRNAs participated in the biological behaviors and can regulate complicated networks in tumorigenesis. For example, lncRNA FAL1 is upregulated in colon cancer tissues and inhibits cell apoptosis *in vitro*[3]. LncRNA XIST promotes cell growth, migration, and invasion through the regulation of miR-124 in bladder cancer[4]. By sponging miR-335, lncRNA MSTO2P facilitates cell proliferation and colony formation in gastric cancer[5]. By suppressing the p53 signal pathway, lncRNA ROR facilitates cell proliferation, cell invasion, and chemoresistance in nasopharyngeal carcinoma[6]. Moreover, the knockdown of lncRNA CRNDE-h is reported to be associated with a poor prognosis of colorectal cancer, which might be used as a novel serum-based biomarker for the diagnosis of colorectal cancer[7].

Previous researches have suggested that lncRNA XIST plays an important role in tumor tumorigenesis and tumor development. However, the function of lncRNA XIST in HCC and the potential molecular mechanism have not been studied so far.

Our research found out that XIST expression was significantly higher in HCC tissues, and that the knockdown of XIST inhibited HCC cell pro-

Corresponding Author: Qing Xu, MD; e-mail: tsingcrrc@sina.cn

liferation *in vitro* and *in vivo*. Moreover, our further experiments have explored the underlying mechanism of how XIST functioned in the HCC development.

Patients and Methods

Tissue Samples

A total of 55 HCC patients were obtained from HCC patients who underwent surgery at the Beijing Bo'ai Hospital. Neither radiotherapy nor chemotherapy were performed before the surgery. All fresh tissues got from the surgery were stored immediately at –80°C. This investigation was approved by the Ethics Committee of Beijing Bo'ai Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human HCC cell lines (Bel-7402 and HepG2) and a normal liver epithelial cell (L02) were got from the American Type Culture Collection (ATCC; Manassas, VA, USA). The culture medium consisted of 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA), Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) as well as penicillin. Besides, cells were cultured in an incubator containing 5% of CO_2 at 37°C.

Cell Transfection

The cDNA oligonucleotides specifically targeting XIST (sh-XIST) was synthesized by GenePharma (Shanghai, China) and was inserted into the shRNA expression vector pGPH1/Neo. sh-XIST was then used for transfection in Bel-7402 HCC cells. Lipofectamine 2000 was used to carry out the transfection of cells. 48 h later, Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was used to monitor the transfection efficiency.

Cell Counting Kit-8 (CCK-8) Assay

Following the protocol of the CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan), the cell growth ability of transfected cells in 96-well plates was assessed at 24, 48, and 72 h. The spectrophotometer (Thermo Scientific, Waltham, MA, USA) was utilized to measure the absorbance at 450 nm.

Colony Formation Assay

Bel-7402 cells were placed in a 6-well plate for 10 d. Then, colonies were treated with 10% of formaldehyde for 30 min, and stained for 5 min with 0.5% of crystal violet. The Image-Pro Plus 6.0 (Silver Springs, MD, USA) was used for data analysis.

RNA Extraction and RT-qPCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was utilized to separate the total RNA. Through the reverse Transcription Kit (TaKaRa Biotechnology Co. Ltd., Dalian, China), the total RNA was reverse-transcribed to complementary deoxyribose nucleic acids (cDNAs). The primers used for RT-qPCR are the following: XIST primers forward: 5′-CAGACGTGTGCTCTTC-3′, reverse: 5′-CGATCTGTAAGTCCACCA-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward: 5′-CCAAAATCAGATGGGGCAATGCTGG-3′ and reverse 5′-TGATGGCATGGACTGTGGTCATTCA-3′. The thermal cycle was as follows: 30 s at 95°C, 5 s for 40 cycles at 95°C, 35 s at 60°C.

Luciferase Assays and RNA Immunoprecipitation Assay (RIP)

Firstly, the 3′-UTR of XIST was cloned into the pGL3 vector (Promega, Madison, WI, USA). Site-directed mutagenesis of the miR-200b-3p binding site in XIST 3′-UTR was performed using the Quick-Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Cells were transfected with XIST WT-3′-UTR or XIST MUT-3′-UTR and miR-ctrl or miR-200b-3p for 48 h. Then, the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was utilized for luciferase assays.

For the RIP assay, Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was used according to protocol. RT-qPCR was used to monitor the co-precipitated RNAs.

Xenograft Model

After transfected, Bel-7402 cells (6×10^5/mL) were replaced into NOD/SCID mice (6 weeks old) subcutaneously. Tumor diameters were detected every 5 d. Tumor volume was calculated as the formula (volume = length × width^2 × 1/2). Tumors were extracted after 4 weeks. This investigation was approved by the Animal Ethics Committee of Capital Medical University.

Statistical Analysis

Statistical analysis was conducted by Statistical Product and Service Solutions (SPSS) 17.0

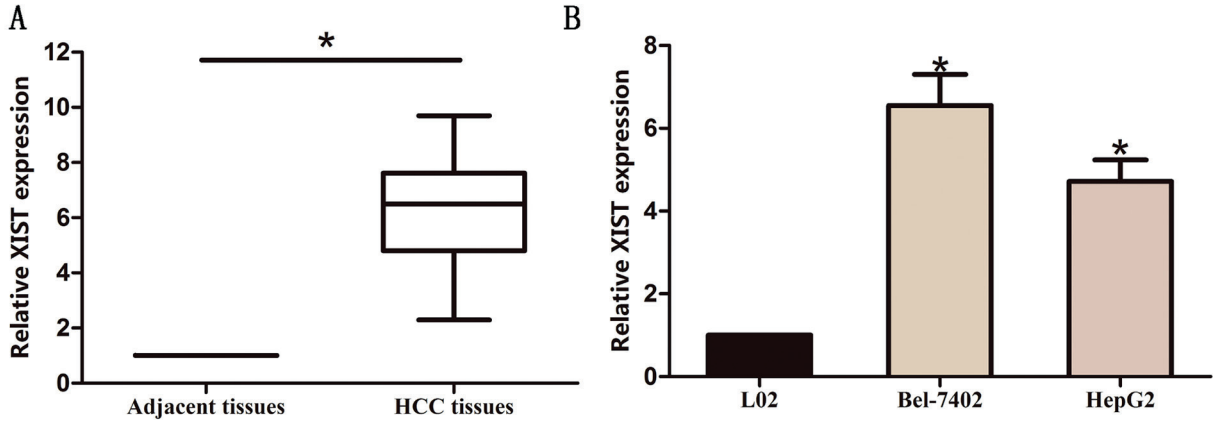

Figure 1. Expression levels of XIST were increased in HCC tissues and cell lines. **A,** XIST expression was significantly increased in HCC tissues compared with adjacent tissues. **B,** Expression levels of XIST relative to GAPDH were determined in human HCC cell lines and 16HBE (normal human bronchial epithelial cell) by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

(SPSS Inc., Chicago, IL, USA). The Student *t*-test method was performed on the data. Data were presented as mean ± SD (Standard Deviation). $p<0.05$ was considered of statistical significance.

Results

XIST Expression Level in HCC Tissues and Cells

RT-qPCR was used to detect XIST expression in 55 HCC patients' tissues and 2 HCC cell lines. Results showed that XIST was significantly up-regulated in tumor tissue samples (Figure 1A). The expression level of XIST in HCC cells was higher than that of L02 (Figure 1B).

Silence of XIST Suppressed Cell Proliferation of HCC Cells

In this work, Bel-7402 HCC cell line was chosen for the silence of XIST. Then, XIST expression was detected by RT-qPCR (Figure 2A). CCK8 assay showed that knockdown of XIST repressed the cell growth ability of Bel-7402 cells (Figure 2B). Colony formation assay also revealed that the number of colonies was remarkably reduced after XIST was knocked down in Bel-7402 cells (Figure 2C and 2D).

XIST Knockdown Inhibited Tumor Formation in Vivo

The ability of XIST in tumor formation was detected *in vivo*. The tumor size in sh-XIST group was smaller compared with sh-ctrl group (Figure 3A). Moreover, the expression level of XIST in tumor tissues was detected by RT-qPCR. Results showed that XIST was lower-expressed in sh-XIST group compared with sh-ctrl group (Figure 3B).

The Interaction Between MiR-200b-3p and XIST in HCC

DIANA LncBASE Predicted v.2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.phpr=lncbasev2%2Findex-predicted) was used to predict the possible miRNAs targeted by XIST. As miR-200b-3p was able to suppress cancer cell proliferation, miR-200b-3p was chosen and the binding site in XIST was shown in Figure 4A. The RT-qPCR assay showed that the expression of miR-200b-3p was higher in sh-XIST group compared with that in sh-ctrl group (Figure 4B). Moreover, the luciferase assay revealed that the luciferase activity was significantly inhibited *via* co-transfection of XIST-WT and miR-200b-3p (Figure 4C). RIP assay results showed that miR-200b-3p was enriched in the XIST group when compared with control group (Figure 4D). Above results showed that XIST might work as a miR-200b-3p sponge.

Discussion

Liver cancer has become one of the most common cancers among which HCC has the second highest cancer-related mortality[8]. Despite advances have been made in diagnosis and management of HCC in the past years, the morbidity and mor-

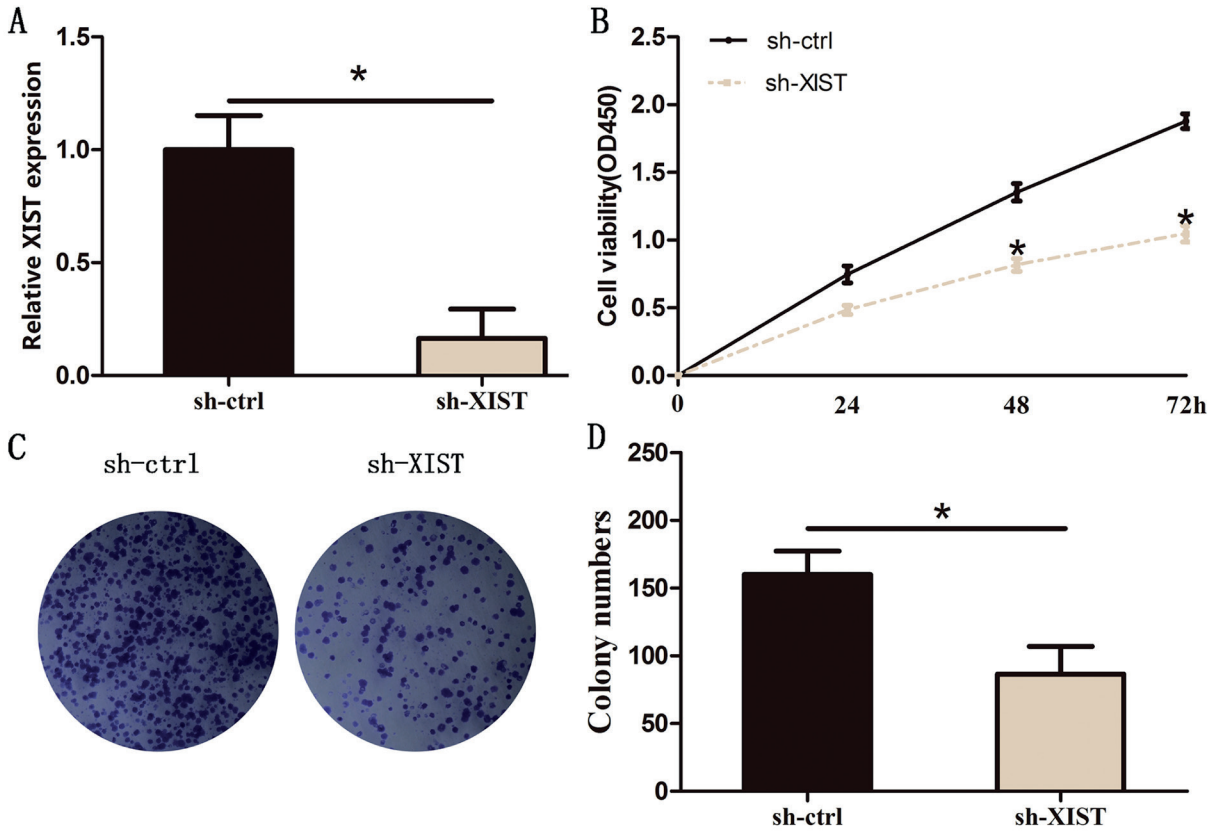

Figure 2. Knockdown of XIST inhibited Bel-7402 HCC cell proliferation. **A,** XIST expression in Bel-7402 HCC cells transfected with sh-XIST and sh-ctrl was detected by RT-qPCR. GAPDH was used as an internal control. **B,** CCK8 assay showed that silence of XIST significantly repressed cell proliferation in Bel-7402 HCC cells. **C,** Representative pictures of colony formation assay in sh-XIST and sh-ctrl group (magnification: 40×). **D,** Colony formation assay showed that the number of colonies was significantly decreased *via* silence of XIST in Bel-7402 HCC cells. The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

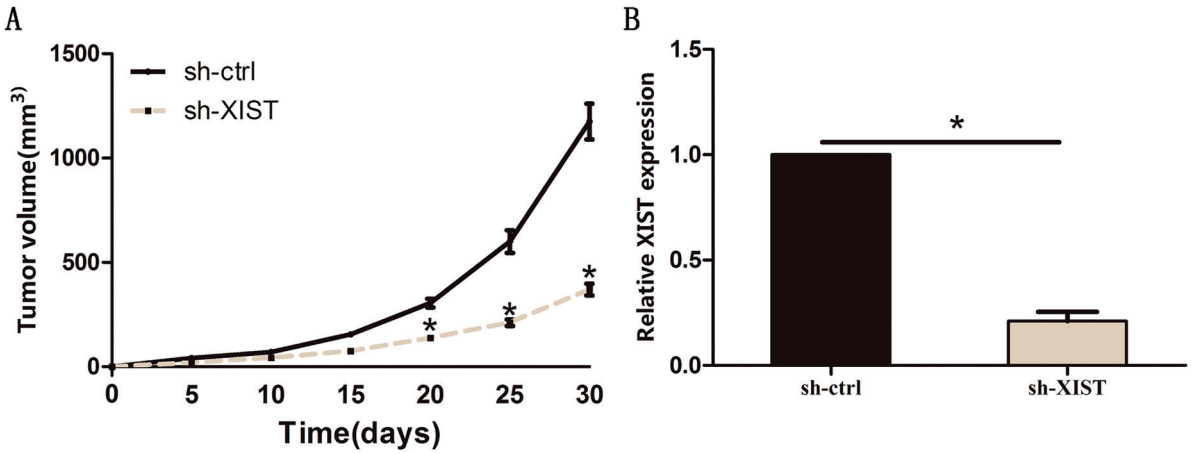

Figure 3. Knockdown of XIST inhibited tumor formation *in vivo*. **A,** After tumor extraction, tumor volume was calculated respectively in sh-ctrl or sh-XIST group and made into a graph. **B,** The relative expression of XIST in tumors was were examined by RT-qPCR. Data are presented as the mean ± SD of three independent experiments. *p<0.05.

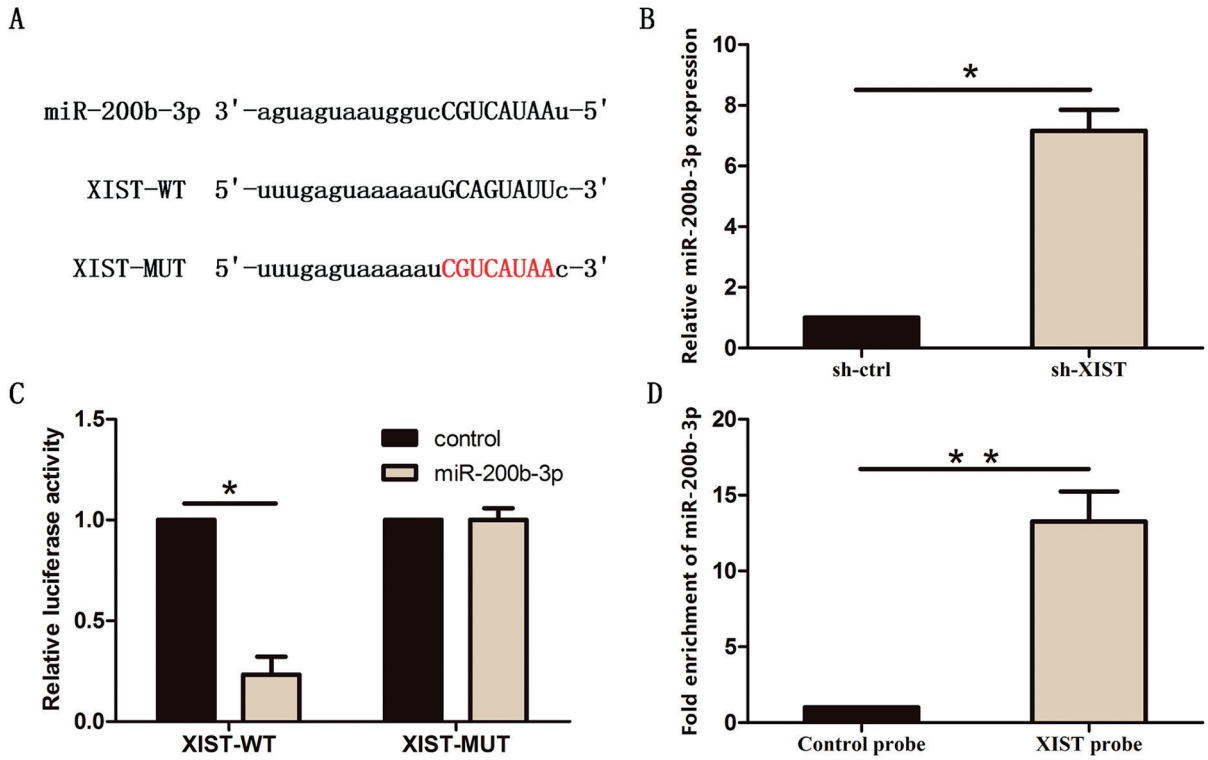

Figure 4. Reciprocal repression between XIST and miR-200b-3p. **A,** Binding sites of miR-200b-3p on XIST. **B,** MiR-200b-3p expression was increased in sh-XIST group compared with control group. **C,** Co-transfection of miR-200b-3p and XIST-WT strongly decreased luciferase activity, while co-transfection of mir-control and XIST-WT did not change luciferase activity, and co-transfection of miR-200b-3p and XIST-MUT did not change luciferase activity either. **D,** RIP assay results demonstrated that miR-200b-3p could be remarkably enriched in the XIST group compared with control group. Results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

tality of HCC remain high and the 5-year overall survival rate is less than 20%[9,10]. Therefore, it's crucial to find the biomarkers and targets for HCC and to improve the poor prognosis of patients with HCC.

Recently, XIST, located in the X chromosome, is found upregulated in several malignant tumors and acts as an oncogene. For example, XIST enhances proliferation and invasion in the non-small cell lung cancer[11]. Besides, XIST is significantly higher-expressed in colorectal cancer tissues when compared to the paired adjacent normal tissues[12]. Ma et al[13] have shown that XIST is upregulated in HCC tissues and is associated with HCC patients' prognosis. In this investigation, XIST was upregulated in the HCC samples. Besides, the silence of XIST inhibited cell growth ability of HCC cells. The tumorigenesis assay also revealed that knockdown of XIST could inhibit the tumor formation *in vivo*. Above results indicated that XIST promotes the tumorigenesis of HCC and might act as an oncogene.

Recently, the interaction between lncRNAs and microRNAs has caught more and more attention in many diseases. Moreover, microRNAs sponged by lncRNAs participate in tumor progression and metastasis[14-16]. Some studies suggest that miR-200b-3p can inhibit both the development and the progression of tumors. For example, miR-200b-3p inhibits cell proliferation of glioma *via* targeting ERK5[17]. MiR-200b-3p suppressed metastasis of breast cancer through the LIMK1/CFL1 pathway[18]. Moreover, TIMP4 is a direct target of miR-200b-3p and functions in the progression of prostate cancer[19]. Tsai et al[20] discover that miR-200b-3p is down-regulated in HCC and its expression is associated with patients' prognosis. Our research firstly uncovered the interaction between miR-200b-3p and XIST. We found that miR-200b-3p could be directly targeted by XIST through a luciferase assay. Moreover, the miR-200b-3p expression could be promoted through the knockdown of XIST. Furthermore, miR-200b-3p was markedly enriched by the XIST RIP assay.

Conclusions

In this study, XIST was remarkably upregulated in HCC tissues and could promote the cell growth ability in HCC through sponging miR-200b-3p, thus becoming a potential biomarker for HCC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Venook AP, Papandreou C, Furuse J, de Guevara LL. The incidence and epidemiology of hepatocellular carcinoma: a global and regional perspective. Oncologist 2010; 15 Suppl 4: 5-13.
2) Liu ZH, Zhang YF, Xu ZD. UNC119 promoted cell growth and migration by Wnt/β-catenin signal and TGF-β/EMT signal pathway in hepatocellular carcinoma. J BUON 2018; 23: 1717-1724.
3) Wu K, Zhang N, Ma J, Huang J, Chen J, Wang L, Zhang J. Long noncoding RNA FAL1 promotes proliferation and inhibits apoptosis of human colon cancer cells. IUBMB Life 2018; 70: 1093-1100.
4) Xiong Y, Wang L, Li Y, Chen M, He W, Qi L. The long non-coding RNA XIST interacted with miR-124 to modulate bladder cancer growth, invasion and migration by targeting androgen receptor (AR). Cell Physiol Biochem 2017; 43: 405-418.
5) Li H, Zhu H, Zhou Y, Wang H, Niu Z, Shen Y, Lv L. Long non-coding RNA MSTO2P promotes the proliferation and colony formation in gastric cancer by indirectly regulating miR-335 expression. Tumour Biol 2017; 39: 1010428317705506. doi: 10.1177/1010428317705506.
6) Li L, Gu M, You B, Shi S, Shan Y, Bao L, You Y. Long non-coding RNA ROR promotes proliferation, migration and chemoresistance of nasopharyngeal carcinoma. Cancer Sci 2016; 107: 1215-1222.
7) Liu T, Zhang X, Gao S, Jing F, Yang Y, Du L, Zheng G, Li P, Li C, Wang C. Exosomal long noncoding RNA CRNDE-h as a novel serum-based biomarker for diagnosis and prognosis of colorectal cancer. Oncotarget 2016; 7: 85551-85563.
8) Jin XL, Lian JR, Guan YH. Overexpression of long non-coding RNA MINCR contributes to progressive clinicopathological features and poor prognosis of human hepatocellular carcinoma. Eur Rev Med Pharmacol Sci 2018; 22: 8197-8202.
9) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin 2018; 68: 7-30.
10) Allemani C, Weir HK, Carreira H, Harewood R, Spika D, Wang XS, Bannon F, Ahn JV, Johnson CJ, Bonaventure A, Marcos-Gragera R, Stiller C, Azevedo e Silva G ESG, Chen WQ, Ogunbiyi OJ, Rachet B, Soeberg MJ, You H, Matsuda T, Bielska-Lasota M, Storm H, Tucker TC, Coleman MP; CONCORD Working Group. Global surveillance of cancer survival 1995-2009: analysis of individual data for 25,676,887 patients from 279 population-based registries in 67 countries (CONCORD-2). Lancet 2015; 385: 977-1010.
11) Fang J, Sun CC, Gong C. Long noncoding RNA XIST acts as an oncogene in non-small cell lung cancer by epigenetically repressing KLF2 expression. Biochem Biophys Res Commun 2016; 478: 811-817.
12) Kara M, Yumrutas O, Ozcan O, Celik OI, Bozgeyik E, Bozgeyik I, Tasdemir S. Differential expressions of cancer-associated genes and their regulatory miRNAs in colorectal carcinoma. Gene 2015; 567: 81-86.
13) Ma W, Wang H, Jing W, Zhou F, Chang L, Hong Z, Liu H, Liu Z, Yuan Y. Downregulation of long non-coding RNAs JPX and XIST is associated with the prognosis of hepatocellular carcinoma. Clin Res Hepatol Gastroenterol 2017; 41: 163-170.
14) Li Y, Chen D, Gao X, Li X, Shi G. LncRNA NEAT1 regulates cell viability and invasion in esophageal squamous cell carcinoma through the miR-129/CTBP2 axis. Dis Markers 2017; 2017: 5314649.
15) Yang N, Chen J, Zhang H, Wang X, Yao H, Peng Y, Zhang W. LncRNA OIP5-AS1 loss-induced microRNA-410 accumulation regulates cell proliferation and apoptosis by targeting KLF10 via activating PTEN/PI3K/AKT pathway in multiple myeloma. Cell Death Dis 2017; 8: e2975.
16) Zhao L, Han T, Li Y, Sun J, Zhang S, Liu Y, Shan B, Zheng D, Shi J. The lncRNA SNHG5/miR-32 axis regulates gastric cancer cell proliferation and migration by targeting KLF4. FASEB J 2017; 31: 893-903.
17) Wu J, Cui H, Zhu Z, Wang L. MicroRNA-200b-3p suppresses epithelial-mesenchymal transition and inhibits tumor growth of glioma through down-regulation of ERK5. Biochem Biophys Res Commun 2016; 478: 1158-1164.
18) Li D, Wang H, Song H, Xu H, Zhao B, Wu C, Hu J, Wu T, Xie D, Zhao J, Shen Q, Fang L. The microRNAs miR-200b-3p and miR-429-5p target the LIMK1/CFL1 pathway to inhibit growth and motility of breast cancer cells. Oncotarget 2017; 8: 85276-85289.
19) Janiak M, Paskal W, Rak B, Garbicz F, Jarema R, Sikora K, Wlodarski P. TIMP4 expression is regulated by miR-200b-3p in prostate cancer cells. APMIS 2017; 125: 101-105.
20) Tsai SC, Lin CC, Shih TC, Tseng RJ, Yu MC, Lin YJ, Hsieh SY. The miR-200b-ZEB1 circuit regulates diverse stemness of human hepatocellular carcinoma. Mol Carcinog 2017; 56: 2035-2047.