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Long noncoding RNA CASC2 alleviates the growth, migration and invasion of oral squamous cell carcinoma *via* downregulating CDK1

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Abstract. – OBJECTIVE: Recent studies have revealed the important role of long noncoding RNAs (lncRNAs) in regulating the progression of tumorigenesis. Oral squamous cell carcinoma (OSCC) is a prevalent tumor in the world. This study aims to identify the specific mechanism of lncRNA CASC2 in alleviating th gression of OSCC.

PATIENTS AND METHODS: CASC2 e s- sion in OSCC cell lines and 40 paired CC samples were detected by quantitative al-time polymerase chain reaction (qRT-PC Moreover, in vitro functions o in regu lating the cellular behaviors lls were identified by performing tr ell as wound healing assay and prolif on ass un- derlying mechanism of in progression of OSCC as y qRT-PCR and Western blot.

RESULTS: CAS xpression w arkably downregulated i C tissues co d with that in adjace h mples. More ver, pro- liferation, inv on and tion were inhibited in OSCC ls overexpre CASC2. CASC2 overexpr on in OSCC ce ownregulated CDK1 th mRNA and prote levels *in vitro*. Besi the e ssion of CDK1 in OSCC tis- su s ne ely co elated to the expres- sion

CON NS: C 2 suppresses the mi- n, inv a oliferation of OSCC cells dow ng CDK1, which may of- fer a w therap c intervention for OSCC pa- tien

Words:
noncoding RNA, CASC2, Oral squamous cell ca , CDK1.

Introduction

al cancer is sixth most common cancer i world, a s incidence ranks the third in the untries[1]. As the major subtype of oral cancer, oral squamous cell carcinoma (OSCC), which contributes to almost 3% of the gnosed cancer cases, has the highest orbi y in the head and neck cancers. Although corresponding diagnostic and therapeutic strategies for OSCC have been made strides in the past decades, the survival rate of OSCC patients is still lower than 50%[2]. Therefore, it is urgent to clarify the underlying molecular mechanism of OSCC development and search for new therapeutic strategies, so as to improve the treatment efficacy and the prognosis of OSCC.

Long non-coding RNA (lncRNA) is a cluster of non-coding RNA transcripts longer than 200 nt. Evidence indicated the important role of lncRNAs in many biological behaviors, including carcinogenesis, cell apoptosis, proliferation and metastasis in cancer. Therefore, lncRNAs are often used as biomarkers for predicting the progression of tumorigenesis[3,4]. For example, lncRNA MSTO2P facilitates cell growth and colony formation in gastric cancer by regulating the expression of miR-335[5]. Through binding to SRSF6, LINC01133 functions as a tumor-suppressor gene in colorectal cancer by inhibiting epithelial-mesenchymal transition and cell metastasis[6]. LncRNA OR3A4 is upregulated in breast cancer, which may be a novel prognostic marker and therapeutic target[7]. In addition, it is found that overexpression of lncRNA GHET1

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promotes cell proliferation of pancreatic cancer, and its expression level is associated with TNM staging and prognosis[8]. However, the specific function of lncRNA CASC2 in the proliferation of OSCC remains unclear. In the present study, it was revealed that the expression level of lncRNA CASC2 was remarkably downregulated in OSCC samples. Moreover, *in vitro* experiments revealed that CASC2 suppressed the proliferation, invasion and migration of OSCC cells. Furthermore, we discovered that lncRNA CASC2 exerted its functional roles in the progression of OSCC by downregulating CDK1.

Patients and Methods

Cell Culture and Cell Transfection

OSCC cell lines (Tca8113, TSCCA, CAL-27, SCC-9) and the normal human oral keratinocytes (NHOK) were provided by Chinese Type Culture Collection, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, USA) and 1% penicillin, in a 5% CO_2 incubator at 37°C. Lentiviral virus targeting CASC2 was compounded and cloned into pLenti-EF1a-EGFP-F2A-Puro vector (Biosettia Inc., San Diego, CA, USA). Empty vector and the CASC2 lentiviruses (CASC2) were packaged in 293T cells.

Clinical Samples

40 paired OSCC tissues and adjacent normal tissues were surgically resected from OSCC patients admitted to Weihai Municipal Hospital from July 2016 to December 2017. All tissues were kept at -80°C. An informed consent was offered by each patient before the surgery. This study was approved by the Ethics Committee of Weihai Municipal Hospital.

RNA Extraction and Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) was utilized to reverse transcribe the total RNAs to complementary deoxynucleic acids (cDNAs). QRT-PCR was conducted on ABI 7500 system (Applied Biosystems, Foster City, CA, USA) using the SYBR Green Real-time PCR kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Primers used for CASC2 were as follows: CASC2, forward: 5'-GCCATTGGACGGTGTTTCC-3', reverse: 5'-CCCAGTCCTTCACAGGTCAC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5'-CCAAAATCAGATGGCCAATG-3', reverse: 5'-TGATGGCAGACTGTGGTCA-3'. The thermal cycle was as follows: 95°C for 30 s, at 95°C, 5 s at 95°C, 35 s at 60°C, for a total of 40 cycles.

Colony Formation Assay

Cells were seeded in a 6-well plate with 5×10^2 cells per well and cultured for 2 weeks. Subsequently, cells were fixed with methanol and stained by 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). The growing colonies (>50 cells per colony) were finally counted under a microscope.

Cell Proliferation Assay

Cells were seeded in a 96-well plate and the proliferation ability was determined every 24 h following the protocol of cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, medium containing 10% CCK-8 reagent (10 µl) was applied in each well. Spectrophotometer (Thermo Scientific, Rockford, IL, USA) was utilized to measure the absorbance at 450 nm after 2-h incubation.

Wound Healing Assay

Cells seeded into 6-well plates were cultured in DMEM overnight. After scratching a line in the bottom with a plastic tip, cells were cultured in serum-free DMEM. Wound closure was viewed at the appointed time points. Each assay was independently repeated for three times.

Transwell Assay

5×10^4 cells suspended in 200 µL of serum-free DMEM were applied on the top side of the transwell chamber (8 µm in pore size, Millipore, Billerica, MA, USA) pre-coated with 50 µg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). DMEM containing 10% fetal bovine serum (FBS) was added on the bottom chamber. 48 h later, unpenetrating cells on the top side were cleaned with the cotton swab and penetrating cells on the bottom side were immersed in pre-cooled methanol for 10 min. After staining with crystal violet for 30 min, invasive cells were counted in 3 randomly selected fields per sample.

Western Blot

Total protein was extracted from cells using the reagent radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) and quantified using the Bicinchoninic acid (BCA) protein assay kit (TaKaRa, Dalian, China). The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on the polyvinylidene difluoride (PVDF) membrane. Membranes were then incubated with primary (rabbit anti-GAPDH and rabbit anti-CDK1) and goat anti-rabbit secondary antibody provided by Cell Signaling Technology (CST, Danvers, MA, USA). Chemiluminescent film was applied for assessment of protein expression with Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical analysis was conducted by Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA). Categorical data and measurement data were analyzed by Chi-square test and Student *t*-test, respectively. Data were presented by mean ± SD (Standard deviation). $p<0.05$ was considered as statistically significant.

Results

Expression Level of CASC2 in OSCC Tissues

First, qRT-PCR was conducted for detecting CASC2 expression in 40 pairs of OSCC tissues and adjacent normal tissues. As the results revealed, CASC2 was significantly downregulated in tumor tissue samples than in adjacent tissues (Figure 1).

CASC2 Overexpression Suppressed Proliferative Capacity of OSCC Cells

Compared with the expression in normal human oral keratinocyte line HOK, CASC2 level was significantly downregulated in OSCC cell lines (Figure 2A). According to CASC2 expression in different cells, CAL-27 cells were selected for overexpression of CASC2. The CASC2 lentivirus (CASC2) and empty vector were synthetized and transfected into CAL-27 cells. CASC2 expression was determined by qRT-PCR to verify transfection efficacy (Figure 2B). Subsequently, colony formation assay revealed that overexpression of CASC2 inhibited colony formation capacity of OSCC cells (Figure 2C). Furthermore, CCK-8 assay showed that proliferative ability of OSCC cells was suppressed after CASC2 overexpression (Figure 2D).

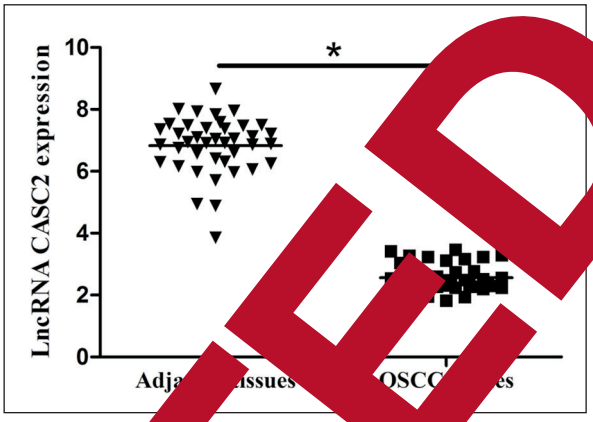

Figure 1. Expression level of CASC2 was significantly downregulated in OSCC tissues. CASC2 expression was significantly downregulated in the OSCC tissues compared with adjacent tissues. Data were presented as the mean ± standard error of the mean. $p<0.05$.

CASC2 Overexpression Suppressed Cell Migration and Invasion In Vitro

Wound healing assay found that overexpression of CASC2 inhibited migration of OSCC cells (Figure 3A). Furthermore, transwell assay showed that invasive capacity of OSCC cells was inhibited after CASC2 overexpression (Figure 3B).

CASC2 Inhibited the Tumorigenesis of OSCC via Mediating CDK1

Many studies verified that CDK1 acts as an oncogene in various cancers including OSCC. We then explored the interaction between CDK1 and CASC2 in OSCC. Expression level of CDK1 was detected in OSCC tissues. CDK1 expression was upregulated in OSCC tissues compared with that in adjacent tissues (Figure 4A). QRT-PCR results further demonstrated that the mRNA expression of CDK1 was downregulated in OSCC cells transfected with CASC2 lentivirus (Figure 4B). Western blot obtained the same result at protein level (Figure 4C). The results of linear correlation analysis showed that in OSCC tissues, the expression of CDK1 was negatively correlated to CASC2 expression (Figure 4D).

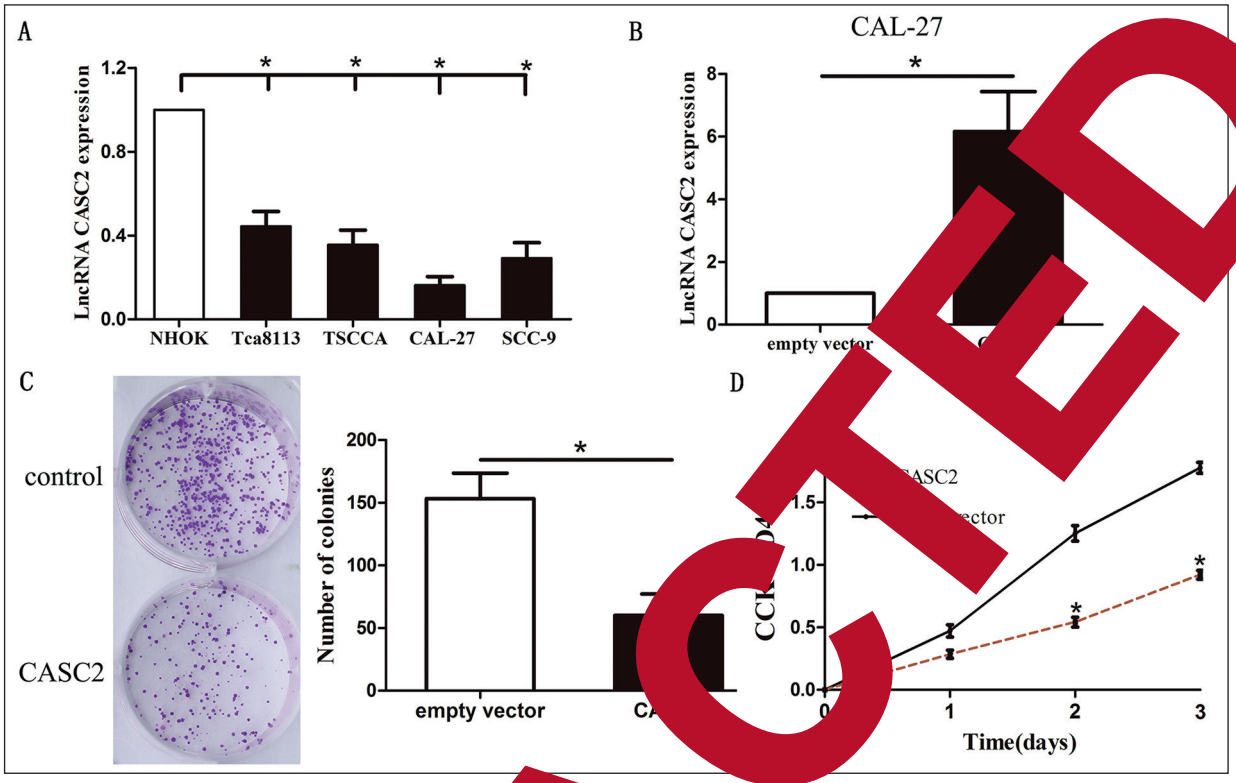

Figure 2. Overexpression of CASC2 inhibited t of OSCC cells. ***A,*** Expression levels of CASC2 relative to GAPDH were determined in the human-derived C mal ovarian cell (NHOK) by qRT-PCR. ***B,*** CASC2 expression in CAL-27 cells transfected with empty or CA (CASC2) was detected by qRT-PCR. GAPDH was used as an internal control. ***C,*** Colony formati ed that number of colonies in CASC2 lentivirus group obviously decreased compared with empty vector gro -27 cells. ***D,*** CCK-8 assay showed that overexpression of CASC2 significantly suppressed the proliferation of CA lls. The results represented the average of three independent experiments (mean ± standard error o). *$p<0.05$, mpared with the control cells.

Discu n

Numerous resea have strated the significant roles ncRNAs in iety of biological beha ing the prog sion of OSCC. For in ce, l A MEG3 acts as a tumor-suppr or gene and s cell growth and metastasi OSCC through ively regulating W -catenin signaling pathway[9]. LncRNA HOT facili cell invasion and metastasis in by i ectly r uiting EZH2 and inhibiti erin[10]. NA AC132217.4 promotes ce astas OSCC *via* regulating media expression [11]. Knockdown of l NA CC alleviates the malignancy of OSC hrough Wnt/beta-catenin pathway[12]. In ad A FTH1P3 promotes the development of OSCC by modulating the expression zzled 5[13]. LncRNA CASC2 is a newly discovered tumor suppressor that plays a crucial role in tu carcinogenesis. For example, lncRNA CASC2 suppresses cell metastasis and epithelial-mesenchymal transition of lung cancer by downregulating SOX4[14]. LncRNA CASC2 serves as an important part in regulating DDP-sensitivity in the cervical cancer, which may serve as a potential therapeutic target for cervical cancer[15]. By inhibiting Wnt/beta-catenin signaling pathway, lncRNA CASC2 acts as a tumor suppressor in gliomas and predicts the prognosis of glioma patients[16]. Moreover, lncRNA CASC2 facilitates the tumorigenesis of thyroid carcinoma and indicates a poor prognosis of patients with low-level CASC2[17]. LncRNA CASC2 was found to be downregulated in both OSCC tissues and cells in the present study. Furthermore, CASC2 overexpression suppressed the abilities of cell growth, migration and invasion. These data indicated that CASC2 functioned as a tumor suppressor and inhibited the tumorigenesis of OSCC. Cyclin-dependent kinase 1 (CDK1) has been reported to participate in cell cycle progression, which has

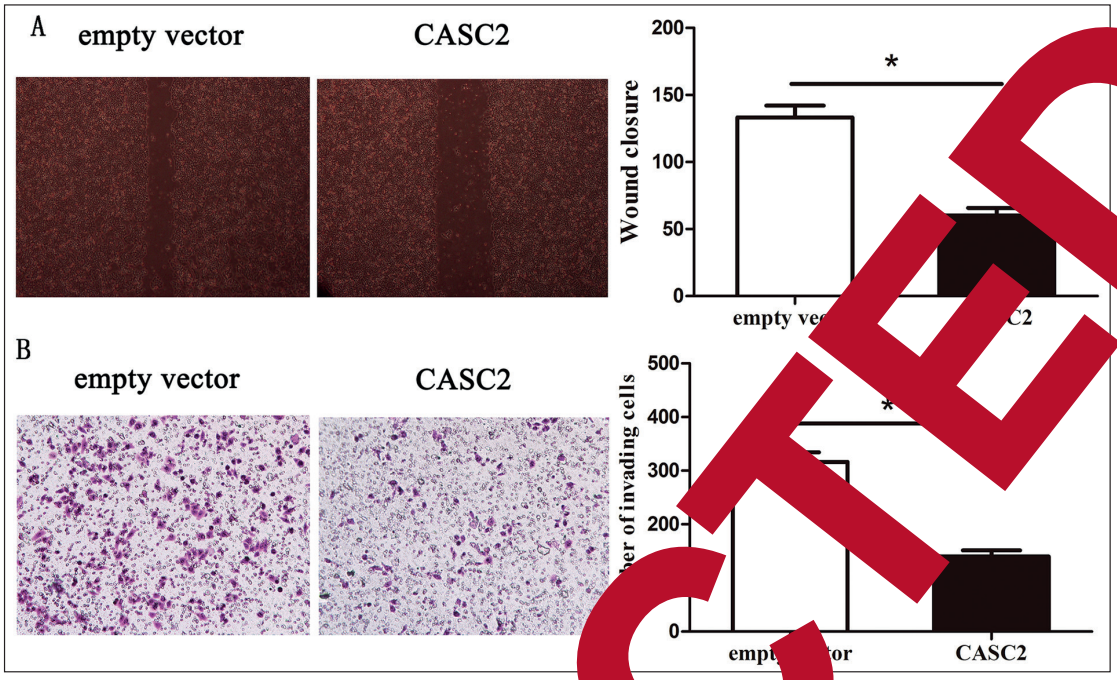

Figure 3. Overexpression of CASC2 suppressed migration and i[illegible] of OSCC cel[illegible] Wound-healing assay showed that the wound closure of cells in CASC2 lentivirus group significantly [illegible]ed compa[illegible]ith empty control group in CAL-27 cells. ***B,*** Transwell assay showed that the migrated cells in CASC2 lenti[illegible]viously reduced compared with empty control group in CAL-27 cells. ***C,*** Transwell assay sho[illegible]ad that overexpressio[illegible]2 significantly suppressed invasion ability of CAL-27 cells. The results represented the avera[illegible] independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control cells.

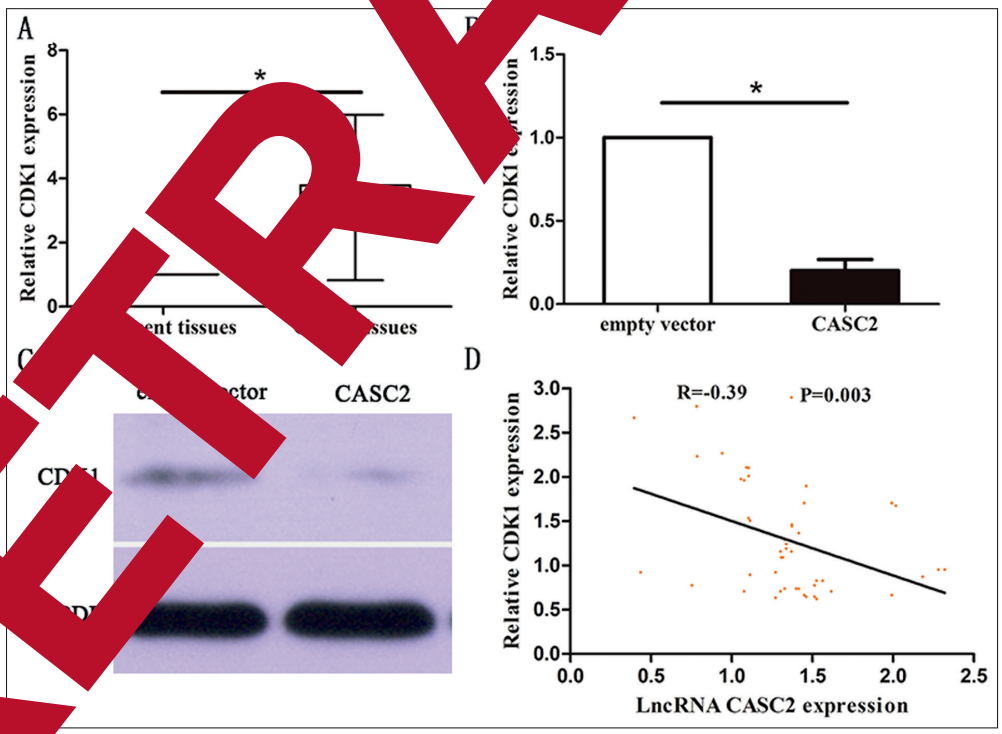

Figure 4. Interaction between CDK1 and CASC2 in OSCC. ***A,*** CDK1 was significantly upregulated in OSCC tissues compared [illegible] adjacent tissues. ***B,*** The mRNA expression level of CDK1 in CASC2 cells was significantly downregulated compared with [illegible]ontrol cells in CAL-27 cells. ***C,*** Protein expression of CDK1 was downregulated after overexpression of CASC2 in CAL-27 [illegible]. The linear correlation between the expression levels of CDK1 and CASC2 in OSCC tissues. The results represented the av[illegible]e of three independent experiments Data were presented as the mean ± standard error of the mean. *$p<0.05$.

been regarded as a potential prognostic and therapeutic marker in a variety of cancers. Inhibition of CDK1 shortens survival time and induces cell apoptosis. For example, upregulated CDK1 in colorectal cancer affects cell proliferation and apoptosis *via* p53 apoptosis pathway[18]. CDK1 is positively correlated with the malignant degree of tumor and CDK1 deficiency can act as a potential biomarker of treatment efficacy in breast cancer[19]. CDK1 mediates cell apoptosis and proliferation in ovarian cancer by activating Chk1-CDC25C and P53-P21WAF1 signaling pathway[20]. In our study, CDK1 was downregulated after CASC2 overexpression *in vitro*. Moreover, CDK1 was remarkably upregulated in OSCC samples compared with that in adjacent tissues. A negative correlation was discovered between CDK1 and CASC2 expression in OSCC tissues. Our results revealed that CASC2 may exert its function *via* regulating CDK1.

Conclusions

We detected that lncRNA CASC2 is a vital regulator in the carcinogenesis of OSCC. CASC2 may be served as a promising hallmark for OSCC.

Conflict of Interest

The Authors declare that they have no conflicts of interests.

References

1) Rosebush MS, [illegible] K, Samant S, [illegible] Handorf CR, Pfeffer L[illegible] CA. Oral cancer in children during characteris[illegible]ging trends. J Penn Dent Assoc 20[illegible]1: 24-2[illegible]

2) Brockl[illegible]t PR, Baker SR, [illegible] PM. Oral cancer scre[illegible]g: what have we lea[illegible]d what is there sti[illegible]achieve Future Oncol 2010; 6: 299-304.

3) [illegible]EA, Br[illegible]J, Lam WL. The functional role of non-[illegible]g RNA [illegible]human carcinomas. Mol C[illegible]; 10: 38[illegible]

4) Gutsc[illegible] Diede[illegible]S. The hallmarks of cancer: long n[illegible]NA point of view. RNA Biol [illegible]2; 9: 70[illegible]

5) [illegible], Zhu H, Zhou Y, Wang H, Niu Z, Shen Y, Lv L. [illegible]ting RNA MSTO2P promotes the [illegible]and colony formation in gastric cancer by indirectly regulating miR-335 expression. Tumour Biol 2017; 39: 1393384158.

[illegible] J, Sun W, Li C, Wan L, Wang S, Wu Y, [illegible]E, Zhang H, Lai M. Long non-coding RNA LINC01133 inhibits epithelial-mesenchymal transition and metastasis in colorectal ca[illegible] teracting with SRSF6. Cancer Le[illegible] 476-484.

7) Liu G, Hu X, Zhou G. Long[illegible]-coding RNA OR3A4 promotes proliferati[illegible]migration in breast cancer. Biomed Pharma[illegible]2017; 96: 426-433.

8) Zhou HY, Zhu H, Wu [illegible]hen XD, Qiao X, Yao XM, Tang JH[illegible]ressio[illegible]nd clinica[illegible]nificance of long-n[illegible]oding R[illegible]GHET1 in pancreatic cancer. E[illegible] Med[illegible]macol [illegible] 2017; 21: 5081-5088[illegible]

9) Liu Z, Wu C[illegible]N, Wan[illegible]g no[illegible]ing RNA MEG3 inh[illegible]the prolifer[illegible]tastasis of oral sq[illegible]cell carcinom[illegible]egulating the WNT/[illegible] signaling pat[illegible]ay. Oncol Lett 201[illegible]405[illegible]

10) Wu Y, Zhang L, Z[illegible] Wang Y, Li H, Ren X, [illegible]u W, Liu T, W[illegible]Zhou X, Yu J, Hao X. [illegible]non-coding RNA [illegible]OTAIR promotes tumor cell invasion and metastasis by recruiting EZH2 and repress[illegible]E-cadherin in oral squamous cell carcinom[illegible]Int J Oncol 2015; 46: 2586-2594.

11) [illegible]Ma C[illegible]L, Li N, Zhang X, He J, He R, Shao [illegible]ng L, Han C. LncRNAAC132217.4, a KLF[illegible]ated long non-coding RNA, facilitates oral squamous cell carcinoma metastasis by up-[illegible]gulating IGF2 expression. Cancer Lett 2017; [illegible]-56.

12) Ma Y, Hu X, Shang C, Zhong M, Guo Y. Silencing of long non-coding RNA CCAT2 depressed malignancy of oral squamous cell carcinoma via Wnt/beta-catenin pathway. Tumour Biol 2017; 39: 1393371994.

13) Zhang CZ. Long non-coding RNA FTH1P3 facilitates oral squamous cell carcinoma progression by acting as a molecular sponge of miR-224-5p to modulate fizzled 5 expression. Gene 2017; 607: 47-55.

14) Wang D, Gao ZM, Han LG, Xu F, Liu K, Shen Y. Long noncoding RNA CASC2 inhibits metastasis and epithelial to mesenchymal transition of lung adenocarcinoma via suppressing SOX4. Eur Rev Med Pharmacol Sci 2017; 21: 4584-4590.

15) Feng Y, Zou W, Hu C, Li G, Zhou S, He Y, Ma F, Deng C, Sun L. Modulation of CASC2/miR-21/PTEN pathway sensitizes cervical cancer to cisplatin. Arch Biochem Biophys 2017; 624: 20-30.

16) Wang R, Li Y, Zhu G, Tian B, Zeng W, Yang Y, Li Z. Long noncoding RNA CASC2 predicts the prognosis of glioma patients and functions as a suppressor for gliomas by suppressing Wnt/beta-catenin signaling pathway. Neuropsychiatr Dis Treat 2017; 13: 1805-1813.

17) Xiong X, Zhu H, Chen X. Low expression of long noncoding RNA CASC2 indicates a poor prognosis and promotes tumorigenesis in thyroid carcinoma. Biomed Pharmacother 2017; 93: 391-397.

18) Gan W, Zhao H, Li T, Liu K, Huang J. CDK1 interacts with iASPP to regulate colorectal cancer cell proliferation through p53 pathway. Oncotarget 2017; 8: 71618-71629.

19) Galindo-Moreno M, Giraldez S, Saez C, Japon MA, Tortolero M, Romero F. Both p62/SQSTM1-HDAC6-dependent autophagy and the aggresome pathway mediate CDK1 degradation in human breast cancer. Sci Rep 2017.

20) Zhang R, Shi H, Ren F, Zhang M, Ji [illegible] C. The aberrant upstream pathw[illegible]gulations o CDK1 protein were implicated [illegible]e proliferation and apoptosis of ovarian can[illegible] J Ovarian Res 2017; 10: 60.