European Review for Medical and Pharmacological Sciences 2017; 21: 4584-4590

Long noncoding RNA CASC2 inhibits metastasis and epithelial to mesenchymal transition of lung adenocarcinoma via suppressing SOX4

D. WANG[1], Z.-M. GAO[2], L.-G. HAN[3], F. XU[1], K. LIU[1], Y. SHEN[1]

[1]Department of Thoracic Surgery, Affiliated Hospital of Qingdao University, Qingdao, Shandong Province, China
[2]Department of Thoracic Surgery, Binzhou People's Hospital, Binzhou, Shandong Province, China
[3]Department of Thoracic Surgery, Qingdao Central Hospital, Qingdao, Shandong Province, China

Abstract. – OBJECTIVE: Recently, long non-coding RNAs (lncRNAs) have caught more attention for their role in tumor progression. Lung adenocarcinoma (LAC) is one of these ordinary malignant tumors. This study aims to identify whether lncRNA CASC2 (cancer susceptibility candidate 2) can regulate the metastasis of LAC, and find out its potential mechanism.

PATIENTS AND METHODS: RT-qPCR was conducted to detect CASC2 expression level in 63 LAC tissues and 4 LAC cells. Besides, statistical methods were applied to analyze clinical data and prognosis in the 63 patients. Furthermore, function experiments were performed to determine the effect of CASC2 on LAC metastasis in vitro. The potential mechanism was further explored by RT-qPCR and Western blot assay.

RESULTS: In our study, CASC2 expression level was lower in LAC tissues than that in corresponding tissues. CASC2 expression was associated with lymph node metastasis, TNM stage and survival time of these patients. Moreover, overexpression of CASC2 inhibited migrated and invaded ability of LAC cells. Meanwhile, epithelial to mesenchymal transition process of LAC cells and SOX4 expression was suppressed by upregulating CASC2.

CONCLUSIONS: These results indicate that CASC2 could inhibit metastasis and EMT of LAC via suppressing SOX4, and may offer a new vision for interpreting the mechanism of LAC metastasis.

Key Words:
LncRNA, CASC2, Lung adenocarcinoma, SOX4, EMT.

Introduction

Lung cancer (LC) ranks the second common cancer among human malignant tumors. 80% of LC is non-small-cell lung cancer (NSCLC) worldwide, of which lung adenocarcinoma (LAC) is the main subtype. The incidence of liver cancer in China accounts for more than half of the whole world. Although molecular targeted therapy is available for LAC patients, only a small part of patients benefits from the discovered driver mutations. Therefore, it is urgent to find more potential regulators and targets for treatment of LAC. Recently, a number of long non-coding RNAs (lncRNAs) expression identified in most solid cancers closely associated with patients' prognosis. Moreover, mounting evidence indicates that lncRNAs contribute to regulating oncogene expression, which further acts on progression of malignant cancers, including LAC[1,2]. LncRNA CASC2, a novel tumor suppressor, has been downregulated in many human cancers, such as glioma, hepatocellular carcinoma, colorectal cancer, and thyroid carcinoma[3-7]. Accumulating evidence demonstrates that CASC2 participates in tumor carcinogenesis. For instance, CASC2 knockdown promotes cell metastasis *via* activating Wnt signaling pathway in bladder cancer[8]. By targeting MAPK signaling pathway, CASC2 functions as a tumor suppressor and inhibits cell growth in gastric cancer[9]. Moreover, miR-21 can downregulate CASC2 and further promote cell growth and migration in renal cell carcinoma[10]. CASC2 contributes to enhancing sensitivity of cells to cisplatin in cervical carcinoma[11]. He et al[12] detected aberrant CASC2 expression in NSCLC, and revealed the association between CASC2 expression level and postoperative prognosis.

Epithelial to mesenchymal transition (EMT) has been identified as a key step of metastasis in epithelial cancers. More evidence suggests that lncRNAs could affect the migrated and

Corresponding Author: Yi Shen, MD; e-mail: qdsheny163.com

invaded ability of cancer cells through regulating EMT[13-15]. However, the role of CASC2 in the metastasis of LAC remains unknown. Our present study firstly revealed that CASC2 was downregulated in clinical LAC samples, and its expression was correlated with clinicopathological features and prognosis. Besides, overexpressed CASC2 inhibited cell migration, and invasion of LAC, as well as EMT *in vitro*. Further study showed that SOX4 might serve as a potential target of CASC2.

Patients and Methods

Clinical Samples and Cell Lines

Cancer tissue specimens and corresponding tissues were acquired from 63 LAC patients who received surgery at the Affiliated Hospital of Qingdao University. No patients received radiotherapy or chemotherapy before operation. All LAC tissues were stored at -80°C. An experienced pathologist assessed the clinical data. The written informed consent was available for this study. The study conformed to require of the Ethics Committee of the Affiliated tal of Qingdao University. The Institute o chemistry and Cell Biology, Chinese Acad of Science (Shanghai, China) provided us 4 LA cell lines including A549, SPCA1 1975 and PC-9, and 16 HBE (human bro thelial cell). Culture medium was co ed c ipicil-lin, 10% fetal bovine serum S), w purchased by Invitrogen Life s (Car lsdab, CA, USA), and Du ecco ed Eagle's medium (DMEM) ich was by Thermo Fisher Scienti Waltham, MA, A). Besides, cells were c humidified incubator containing 5% O_2 a

Plasmid Cons tion

Ribobio (G gzhou China) helped us synthesizing CA 2 seq e. Then, sequence was sub-cloned cD 3.1. transfection in LAC cells, p ASC2 companied with Lipofectamine 20 en, Carlsbad, CA, USA) d. Besi mpty pcDNA vector (EV as control. 48 h later, detection of CA 2 exp in these cells was con-d si

Quant RT-PCR

Firstly, t A was separated using TRIzol reagent (In itrogen, Carlsbad, CA, USA) following manufacturer's i Next, RNA was reverse-transcr to c via reverse Transcription K TaKaRa L ch-nology Co., Dalian,) qRT-PC as performed on ABI 75 CR d tion system (Applied Bi ystems, CA, USA). Following the prim g for qRT-PCR: CASC forw 5'-GC CATTG-GACGGTGTTT -3' rever 5'-CCCA-GTCCTTCAC 3'; S 4, forward 5'-GGTCTCTAG CAC TC-3' and reverse 5 GGAAT TAAGGAG-3'; GAPDH ward 5'-AC ACATCGCTCA-GACAC everse 5'-GCCCAATACGAC-CAA C thermal cycle was as follows: 3 s at 95 95°C for 40 cycles, 35 s C.

estern Blot

Reagent ra mmunoprecipitation assay A) (Beyot , Shanghai, China) was utilized otein from cells. Bicinchoninic acid otein assay kit (TaKaRa, Dalian, China) was chosen for quantifying protein concentrations. The target proteins were separated dodecyl sulphate-polyacrylamide gel oresis (SDS-PAGE). Next, they were placed to the polyvinylidene fluoride (PVDF) membrane, which was incubated with antibodies. Cell Signaling Technology (CST, Danvers, MA, SA) provided us rabbit anti-E-cadherin, rabbit anti-N-cadherin, rabbit anti-SOX4 and rabbit anti-GAPDH, as well as goat anti-rabbit secondary antibody. Chemiluminescent film was applied for assessment of protein expression with ImageJ software.

Wound Healing Assay

Cells were transferred into 6-well plates, were cultured in DMEM overnight. After they were scratched with a plastic tip, cells were cultured in serum-free DMEM. Wound closure was viewed at different time points. Each assay was independently repeated in triplicate.

Matrigel Assay

To detect the invasion of LAC cells, 5 $\times 10^4$ cells in 200 µL serum-free DMEM were transformed to top chamber of an insert (8 µm pore size; Millipore, Billerica, MA, USA) coated with 50 µg Matrigel (BD Biosciences, San Jose, CA, USA). The bottom chamber was added with DMEM containing 10% FBS. After the top surface of chambers was cultured for

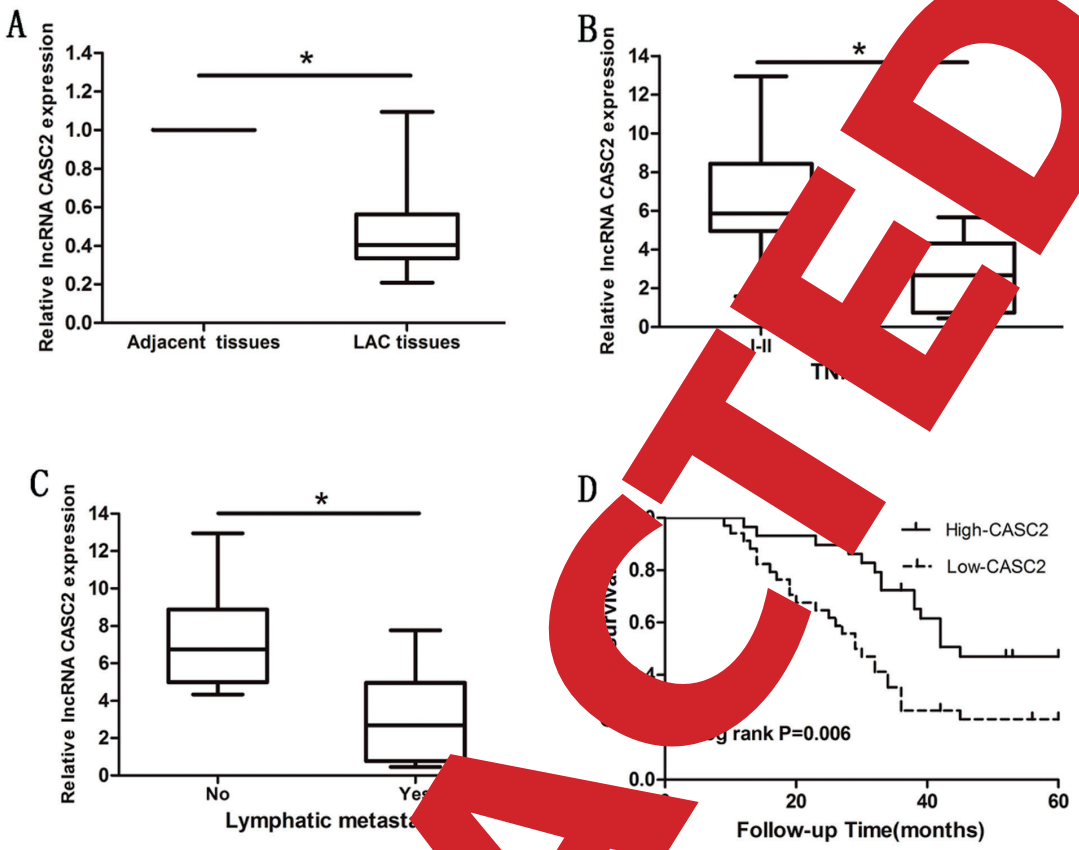


Figure 1. Expression levels of CASC2 were decrease issues. *(A)* CASC2 expression was significantly decreased in the LAC tissues compared with adjacent tissues. *(B)* C xpression was significantly lower in patients with advanced clinical stage. *(C)* CASC2 expression was si antly lowe ients with lymph nodes metastasis. *(D)* Kaplan–Meier overall survival curves showed overall sur -CASC2 g n=34) was significantly shorter than that of high-CASC2 group (n=29). (p<0.001, log-rank test) 0.01.

48 h, it was wiped by co on sw immersed for 10 min with pr bling me The followings were stain crystal viole 30 min. The data for i as counted from three fields per me anc

Statistical An sis

Statistical a ysis w s performed with SPSS 17.0 (SPSS I Chic , IL, USA). x^2 test, Student t-text -Meie ethod were selected when te. R s were presented as mean + SD. p< sidered statistically signif

ults

Do lated CASC2 is Detected in Huma issues

First, R R was conducted for detecting CASC2 expression in 63 pairs of LAC tissues and corresponding tissues. As the result, CASC2 was significantly downregulated in LAC tissue samples (Figure 1A). Clinicopathological features in those LAC patients were presented in Table I. Results revealed that downregulated CASC2 was related to lymph node metastasis and clinical stage (Figure 1B-C).

Low Expression of CASC2 is Correlated with Postoperative Prognosis in LAC Patients

Patients' survival after surgery was analyzed through Kaplan-Meier method. We divided 63 LAC patients into two groups, high-CASC2 and low-CASC2, via median expression. Results showed that patients in high-CASC2 group had better survival than those in low-CASC2 group (Figure 1D).

Table I. Correlation between lncRNA CASC2 expression and clinicopathological characteristics in LA

Characteristics	Patients	Expression of lncRNA CASC		p-va
		High-CASC2	Lo	
Total	63	29	34	
Age (years)				
≤50	28	15	13	
>50	35	14		
Gender				0.36019
Male	30	12		
Female	33	17		
TNM stage				0.031
I-II	32	19		
III-IV	31	10	21	
Tumor size				0.360
<3 cm	33	17	16	
>3 cm	30	12	18	
Lymphatic metastasis				0.038
No	26		10	
Yes	37		24	

$p<0.05$ is considered as statistically significant.

CASC2 Suppresses Cell Migration and Invasion of LAC Cells

According to CASC2 expression in LA ls (Figure 2A), we chose A549 cells for len rus transfection. The CASC2 expression in ce transfected with pcDNA-CASC2 a mpty pc- DNA vector (EV) was determin T-PCR (Figure 2B). The migration i DN ASC2 cells was decreased compare h EV gure 2C). Besides, Matrige strated that numbers of invading lls ced in pcDNA-CASC2 cells c ared wi lls (Figure 2D).

Overexpression LAS bits EMT in LAC cells

To determine function of CA 2 on EMT process, West blot s conducted and results showed t N-cad n was downregulated and E-cadh as egula at protein level after CASC2 rexpr d in A549 cells (Figure 3).

Co Between Expression of SOX4 a LASC

ion was detected in LAC cel ue samples. SOX4 was remarkably highly in LAC tissue specimens compared with ponding samples (Figure 4A). SOX4 was upregulated in LAC cells (Figure 4B). Furthermore, expression of CASC2 was negatiiated with SOX4 expression in LAC igure 4C). Western blot was conducted d results showed that SOX4 was downregulaed at protein level after CASC2 was overexpresed in A549 cells (Figure 4D).

Discussion

In our investigation, CASC2 was found downregulated in tissue samples and cells of LAC, and significant correlation was seen between clinical stage, lymph node metastasis and patients' prognosis. Furthermore, the migrated and invaded ability was inhibited in LAC cells after CASC2 was overexpressed. Data above suggests that CASC2 serves as a tumor suppressor and inhibits the aggressiveness of LAC.

Then, we further explored whether CASC2 could regulate EMT of LAC cells. Moreover, results of Western blot assay demonstrated that level of N-cadherin was decreased and that of E-cadherin was increased once CASC2 was overexpressed. Indeed, N-cadherin is one of the mesenchymal phenotype cell biomarkers, while E-cadherin is one of the epithelial phenotype cell biomarkers. These data indicated that CASC2 made effect in LAC metastasis via re-

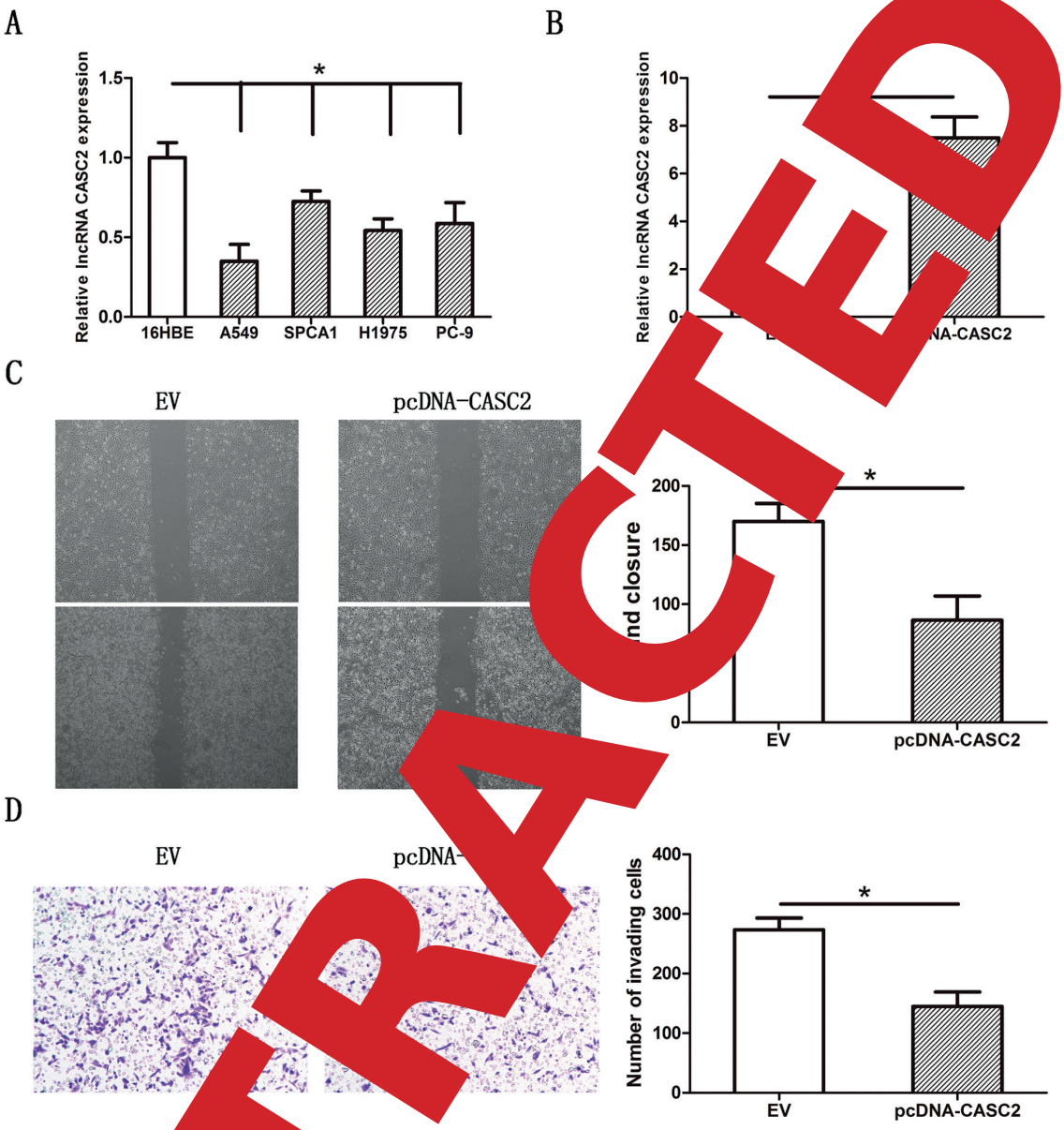


Figure 2. Overexpr[illegible] of C[illegible]ibited migration and invasion of LAC cells. ***(A)*** Expression levels of CASC2 were determined in the human LAC[illegible]nd human bronchial epithelial cell (16HBE) by RT-qPCR. ***(B)*** The expression of CASC2 in L[illegible] cells transduced w[illegible]mpty pcDNA vector (EV) or pcDNA-CASC2 was detected by RT-qPCR. ***(C)*** Wound healing a[illegible]showed that the migration of cells transfected with pcDNA-CASC2 was decreased compared with those transfected with [illegible]ty pcD[illegible]vector (EV). ***(D)*** Matrigel assay demonstrated that numbers of invading cells were reduced in pcDNA-CASC[illegible]ls co[illegible]d with EV cells. The results represent the average of three independent experiments (mean ± standard err[illegible] $p<0.0$[illegible]

gulat[illegible]T. Howe[illegible]remained unknown thro[illegible]mechanism CASC2 influenced th[illegible]ocess[illegible]

[illegible]m[illegible]ng[illegible]ence reveals that SOX4 act[illegible]ncogene and induces EMT process of canc[illegible]nstance, the expression level of SOX4 is co[illegible]d with poor prognosis of prostate cancer[16]. Upregulated SOX4 promotes cell growth, and invasion of colorectal cancer[17]. Moreover, SOX4 acts as a critical regulator of EMT and is associated with epigenetic reprogramming[18]. In breast cancer, EMT induced by SOX4 promotes tumor progression *in vivo*[19]. Several studies reveal that SOX4 emerges as a potential target, which can be regulated by non-coding RNA. SOX4 is the direct target of miR-338-3p,

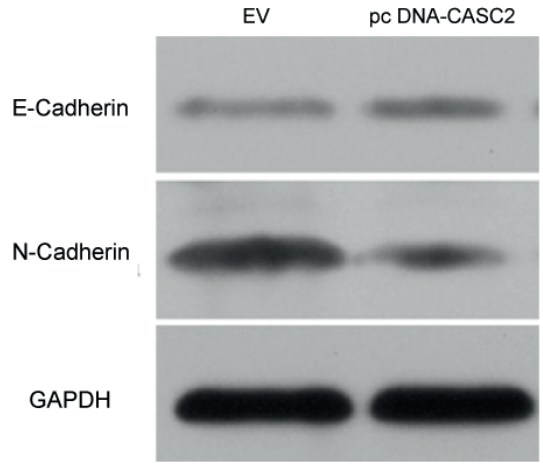


Figure 3. Overexpression of CASC2 suppressed EMT process of LAC cells. The level of EMT-related protein was significantly changed by upregulating CASC2 in A549 cells. *p<0.05.

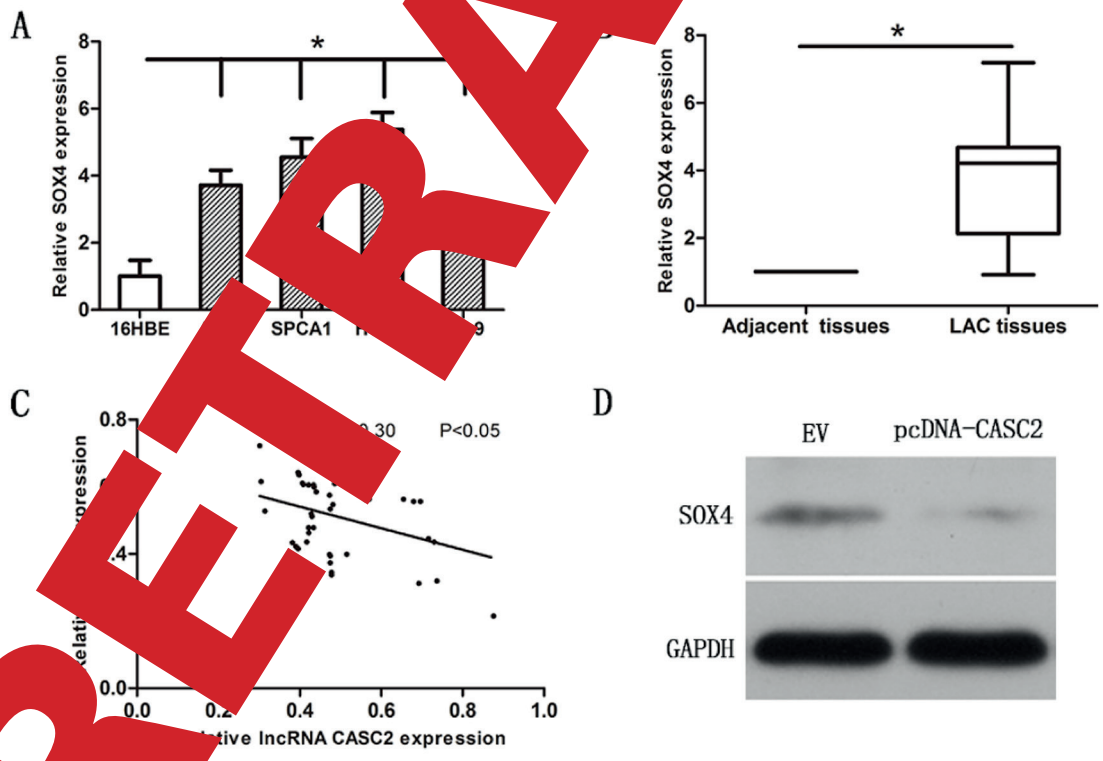
which suppresses metastasis of breast cancer *in vivo*[20]. Besides, lncRNA UCA1, as a ceRNA of SOX4, promotes cell growth in esophageal cancer[21]. Wang et al[22] show t… highly expressed in NSCLC tissu… and is … ted with overall survival of … LC patie… In our study, overexpressi… CASC2 in… ed the expression of SOX… over, …C2 expression was n…atively … with SOX4 expression i… AC tissues.

…ons

CASC2 … downreg… LAC tissue samples and … and is asso… with clinicopathologi… teristics and postoperative prognosi… esi… xpression of CASC2 could inhibit migration, … and EMT process of L… through regula… SOX4. These findings …ed that CASC2 could act as a prospective …rapeutic target for LAC.

…est

The … re no conflicts of interest.



Fig… …elation between SOX4 and CASC2 in LAC tissues and LAC cell lines. (A) SOX4 was significantly higher expres… tissues compared with corresponding normal tissues. (B) SOX4 was higher expressed in LAC cell lines compared … cells. (C) Correlation analysis revealed that CASC2 expression was negatively correlated with SOX4 expression in L… sues. (D) Western blot assay showed that SOX4 was downregulated at protein level after CASC2 was overexpressed in A…9 cells. * p<0.05.

References

1) Fu X, Li H, Liu C, Hu B, Li T, Wang Y. Long non-coding RNA AK126698 inhibits proliferation and migration of non-small cell lung cancer cells by targeting Frizzled-8 and suppressing Wnt/beta-catenin signaling pathway. Onco Targets Ther 2016; 9: 3815-3827.
2) Lv XB, Lian GY, Wang HR, Song E, Yao H, Wang MH. Long noncoding RNA HOTAIR is a prognostic marker for esophageal squamous cell carcinoma progression and survival. PLoS One 2013; 8: e63516.
3) Huang G, Wu X, Li S, Xu X, Zhu H, Chen X. The long noncoding RNA CASC2 functions as a competing endogenous RNA by sponging miR-18a in colorectal cancer. Sci Rep 2016; 6: 26524.
4) Gan Y, Han N, He X, Yu J, Zhang M, Zhou Y, Liang H, Deng J, Zheng Y, Ge W, Long Z, Xu X. Long non-coding RNA CASC2 regulates cell biological behaviour through the MAPK signalling pathway in hepatocellular carcinoma. Tumour Biol 2017; 39: 1393383435.
5) Wang P, Liu YH, Yao YL, Li Z, Li ZQ, Ma J, Xue YX. Long non-coding RNA CASC2 suppresses malignancy in human gliomas by miR-21. Cell Signal 2015; 27: 275-282.
6) Xiong X, Zhu H, Chen X. Low expression [illegible] noncoding RNA CASC2 indicates a poo[illegible] sis and promotes tumorigenesis in thyroi[illegible] noma. Biomed Pharmacother 2017; 93: 391-
7) Liao Y, Shen L, Zhao H, Liu Q, Fu J, Guo Y, Peng [illegible] Cheng L. LncRNA CASC2 interacts with miR-181 to modulate glioma growth and [illegible] to TMZ through PTEN pathway. J Cell [illegible] 17; 118: 1889-1899.
8) Pei Z, Du X, Song Y, Fan L, [illegible] Y, Li W, Zhou H, Yang Y, [illegible] of lncRNA CASC2 prom[illegible] metastasis of bladder [illegible] Wnt/beta-catenin sig[illegible] 2017; 8: 18145-181[illegible]
9) Li P, Xue WJ, Fe[illegible] RNA CASC2 sup[illegible] cancer cells b[illegible] thway. Am J[illegible] 3529.
10) Cao Y, Xu [illegible] tion of ln[illegible] the pro[illegible] noma c[illegible] Rep [illegible] 14: 1019-1025.
11) Feng Y, Zou [illegible] C[illegible] Modula[illegible] t[illegible] itizes cer[illegible] cancer to cisplatin. Arch [illegible] ophys 2017; 623-624: 20-30.
12) He X, Liu Z, Su J, Yang J, Yin [illegible] Guo R. Low expression of long [illegible] SC2 indicates a poor prognos[illegible] d regulate[illegible] pro-liferation in non-small[illegible] lung cancer.[illegible] our Biol 2016; 37: 9503-9[illegible]
13) Yao GL, Pan CF, Xu H, We[illegible] Zhai R[illegible] en YJ. Long noncoding [illegible] A RP11-[illegible] ons as a tumor suppre[illegible] by regulati[illegible] lial-mesenchymal tra[illegible] on in [illegible] sophagea[illegible] quamous cell carcinom[illegible] biome[illegible] armacother 2017; 88: 778-785.
14) He YX, Song X[illegible] Zhao [illegible] OXA13 upregulation in [illegible] tric [illegible] ted with enhanced ca[illegible] cell invas[illegible] thelial-to-mesenchym[illegible] ansition. Eur [illegible] ed Pharmacol Sci 201[illegible] 265.
15) Z[illegible] Y, Li XN, Yang Y, Liu DL, Zhao [illegible] hu DY, W[illegible] XD, Zhao S. Upregulation [illegible] long noncodi[illegible] SPRY4-IT1 promotes metastasis of esophage[illegible] squamous cell carcinoma via induction of epithelial-mesenchymal transition. Cell Biol Toxicol 2016; 32: 391-401.
Wang L, Zha[illegible] Yang X, Chang YW, Qi M, Zhou Z, Zhang J[illegible] B. SOX4 is associated with poor [illegible] prostate cancer and promotes epi-[illegible] nchymal transition in vitro. Prostate [illegible] Prostatic Dis 2013; 16: 301-307.
17) Wang B, Li Y, Tan F, Xiao Z. Increased expression [illegible] SOX4 is associated with colorectal cancer pro-[illegible] ion. Tumour Biol 2016; 37: 9131-9137.
Tiwari N, Tiwari VK, Waldmeier L, Balwierz PJ, Arnold P, Pachkov M, Meyer-Schaller N, Schubeler D, van Nimwegen E, Christofori G. Sox4 is a master regulator of epithelial-mesenchymal transition by controlling Ezh2 expression and epigenetic reprogramming. Cancer Cell 2013; 23: 768-783.
19) Zhang J, Liang Q, Lei Y, Yao M, Li L, Gao X, Feng J, Zhang Y, Gao H, Liu DX, Lu J, Huang B. SOX4 induces epithelial-mesenchymal transition and contributes to breast cancer progression. Cancer Res 2012; 72: 4597-4608.
20) Jin Y, Zhao M, Xie Q, Zhang H, Wang Q, Ma Q. MicroRNA-338-3p functions as tumor suppressor in breast cancer by targeting SOX4. Int J Oncol 2015; 47: 1594-1602.
21) Jiao C, Song Z, Chen J, Zhong J, Cai W, Tian S, Chen S, Yi Y, Xiao Y. LncRNA-UCA1 enhances cell proliferation through functioning as a ceRNA of Sox4 in esophageal cancer. Oncol Rep 2016; 36: 2960-2966.
22) Wang D, Hao T, Pan Y, Qian X, Zhou D. Increased expression of SOX4 is a biomarker for malignant status and poor prognosis in patients with non-small cell lung cancer. Mol Cell Biochem 2015; 402: 75-82.