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LncRNA HOTAIR participates in the development and progression of adrenocortical carcinoma via regulating cell cycle

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Abstract. – OBJECTIVE: To explore the role of HOTAIR in the pathogenesis of adrenocortical carcinoma (ACC) and its underlying mechanism.

PATIENTS AND METHODS: Differentially expressed lncRNA (HOTAIR) in ACC was screened out from the GEO database. The survival analysis and ROC curve were performed according to HOTAIR expressions in ACC patients. The correlation between HOTAIR expression and clinical information of ACC patients was analyzed by chi-square test. The univariate and multivariate COX regression analysis was carried out to analyze the relationship between HOTAIR expression, disease-free survival (DFS) and overall survival (OS) of ACC patients. We then detected HOTAIR expression in 77 ACC tissues and 30 normal tissues by qRT-PCR (quantitative Real-time polymerase chain reaction). ACC cell lines were further screened out for the following in vitro experiments. After altering HOTAIR expression in ACC cells by plasmid transfection, proliferation and cell cycle were detected by Cell Counting Kit-8 (CCK-8) and colony formation assay, respectively. Finally, Western blot was utilized to detect expressions of cell cycle-related genes in ACC cells.

RESULTS: HOTAIR was overexpressed in ACC tissues than that of normal tissues. HOTAIR expression was remarkably increased in ACC with T3 and T4 stage than that of T1 and T2 stage. Moreover, HOTAIR expression was remarkably increased in ACC with stage III and IV than that of stage I and II. HOTAIR was an independent prognostic factor for DFS and OS of ACC patients. For in vitro experiments, inhibited proliferation and arrested cell cycle were observed in H295R cells transfected with si-HOTAIR. Opposite results were obtained after SW-13 cells were transfected with HOTAIR overexpression plasmid. Furthermore, expressions of cell cycle-related genes, including Cyclin D1, p-Rb and p-GSK3β were remarkably decreased after HOTAIR knockdown.

CONCLUSIONS: We demonstrated for the first time that HOTAIR is overexpressed in ACC and is a prognostic risk factor in ACC patients. HOTAIR participates in the development and progression of ACC via shortening cell cycle and promoting proliferation of ACC cells.

Key Words:
Adrenocortical carcinoma, HOTAIR, Cell proliferation, Cell cycle.

Introduction

Adrenocortical carcinoma (ACC) is a rare malignancy that originates in adrenocortical cells. The incidence of ACC is about 0.7/10000 to 2/10000, which frequently affects children younger than 5 years and adults in 40-50 years[1,2]. ACC is characterized as high malignancy and recurrence rate, strong invasiveness and poor prognosis. More seriously, the 5-year overall survival rate of ACC is only 16-38%[3]. Higher malignancy was found in adult ACC patients than that in children[4]. However, the specific molecular mechanism of ACC is still not fully elucidated[5].

Recent works[6,7] have shown that over 90% of the human genomes can be transcribed into non-coding RNAs (ncRNAs), which present limited function or even without protein-coding function. In-depth studies have demonstrated that lncRNA participates in multiple biological processes at different transcriptional levels. Dysfunctional lncRNAs are proved to be related to different diseases, including cancer[8-10]. Moreover, lncRNAs may affect epigenetic regulation through chromatin-modifying complexes, thus leading to the altered phenotypes required for tumor progression and metastasis[11-13]. Therefore, recognizing cancer-associated lncRNAs and exploring their interaction with protein-coding

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genes are essential to inhibit cancer development. Among them, Hox transcript antisense intergenic RNA (HOTAIR) is one of the most studied lncRNAs. HOTAIR exerts its biological function in a trans-silencing manner with 2158 bp in length[14]. Great progress has been made in exploration of the effects of HOTAIR on breast cancer, colon cancer, adrenocortical carcinoma and pancreatic cancer, suggesting that HOTAIR may play a direct role in regulating cancer progression[15-18]. However, the underlying mechanism of HOTAIR in ACC is still poorly understood. This study aims to investigate the specific effect of HOTAIR on ACC, so as to provide new directions in better improving treatment efficacy.

Patients and Methods

Patients

GEO (Gene Expression Omnibus) database is a public database that stores microarrays and sequencing data. We first downloaded GSE33371 dataset from GPL570 platform, containing 10 normal adrenal cortex tissues and 33 ACC tissues. Clinical data of 92 ACC cases and their corresponding genome-wide expression profiles were downloaded from https://cancergenome.nih.gov. Limma package was used to calculate differentially expressed genes in GSE33371. TCGA (The Cancer Genome Atlas) data were standardized in the MD Anderson Cancer Center.

Sample Collection

Adrenal gland tissues surgically resected in Urology Surgery Department, Dongying People's Hospital from July 2012 to July 2017 were collected, including 77 ACC tissues and 30 normal adrenal tissues. All the samples have been pathologically diagnosed as ACC. Samples were immediately frozen in liquid nitrogen and stored at -80°C for the following experiments. Enrolled patients did not receive any preoperative radiotherapy and chemotherapy. The study was approved by the Hospital Ethics Committee and patients were all informed consent. Each patient received complete follow-up. The overall survival was calculated from the day of the first surgery to the death or the last day of follow-up.

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

The total RNA was extracted from tissue samples by TRIzol method (Beyotime, Shanghai, China) and then transcribed into complementary Deoxyribose Nucleic Acid (cDNA) according to the instructions of PrimeScript RT Master Mix (Invitrogen, Carlsbad, CA, USA). QRT-PCR was then performed following the instructions of SYBR® Green Master Mix (TaKaRa, Otsu, Shiga, Japan). Primer sequences used in this study were as follows: GAPDH, F: 5′-CACCCACTCCTCCACCTTTG-3′, R: 5′-CCACCACCCTGTTGCTGTAG-3′; HOTAIR, F: 5'-ATAGGCAAATGTCAGAGGGTT-3', R: 5'-ATTCTTAAATTGGGCTGGGTC-3'.

Cell culture and Transfection

Human adrenal normal cell line (Y1) and ACC cell lines (SW-13, H295R) were purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin (HyClone, South Logan, UT, USA), and inserted in a 5% CO_2 incubator at 37°C.

Cells in good growth condition were selected and seeded in the 6-well plates. Cell transfection was performed when the cell confluence was up to 50%-60% according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The sequence of siRNA was as follows: sense: 5'-CATGGATCCACATTCTGCCCTGATTTCCGGAACC-3'; antisense: 5'-ACTCTCGAGCCACCACACACACACAACCTACAC-3'.

Cell Counting Kit-8 (CCK-8) assay

Transfected cellswere seeded into 96-well plates with 2×10^3 per well, with 6 replicates in each group. 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) were added in each well after cell culture for 6, 24, 48, 72 and 96 h, respectively. The absorbance at 450 nm of each sample was measure by a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

Cells in the logarithmic growth phase were seeded into dishes containing 10 ml of pre-warmed DMEM at a density of 50, 100 and 200 cells per dish, respectively. Cell culture was terminated when macroscopic colonies were formed in the dish. Colonies were then washed with PBS (phosphate-buffered saline), fixed with 4% paraformaldehyde and stained with Gimsa solution for 10-30 min. The number of colonies with over 10 cells were counted with a microscope at low magnification.

Cell Cycle Detection

Cells in logarithmic growth phase were prepared into single cell suspension, followed by centrifugation at 1000 rpm for 5 min. Cells were washed with PBS and fixed with 5 mL of 70% pre-cooled ethanol. For cell cycle detection, 5 μL of Rnase (10 mg/mL) were added and incubated at 37°C for 1 h. After that, 100 μg/mL PI (propidium iodide) were used to stain in the dark for 30 min. Finally, 10,000 cells were counted and the cell cycle was measured by flow cytometry (Partec AG, Arlesheim, Switzerland).

Western Blot

Total protein was extracted from treated cells by radioimmunoprecipitation assay (RIPA) solution (Invitrogen, Carlsbad, CA, USA). Protein sample was separated by electrophoresis on 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and then transferred to PVDF (polyvinylidene difluoride) membrane (Roche, Basel, Switzerland). After membranes were blocked with skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed with TBST (Tris-buffered saline with Tween 20) and followed by the incubation of secondary antibody. The protein blot on the membrane was exposed by chemiluminescence.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) were used for data analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$) and compared using the *t*-test. x^2-test was performed to test the classification data. Kaplan-Meier survival curve was used for survival analysis, and those indicators with significant differences in survival were included into the COX regression analysis. $p<0.05$ considered the difference was statistically significant.

Results

HOTAIR was Overexpressed in ACC Tissues Analyzed from GEO Database

LncRNA expression profiles of ACC were downloaded from the GEO database. Differentially expressed lncRNAs in 10 normal tissues and 33 ACC tissues in the GSE33371 data were analyzed by the Limma package. Totally, there were 248 upregulated lncRNAs and 127 downregulated lncRNAs. Among them, HOTAIR was the significantly upregulated lncRNA in ACC (Figure 1A and 1B).

Clinical information and lncRNA expressions of ACC patients were downloaded from the TCGA database. The results showed no difference in HOTAIR expression between ACC patients older than 50 years and those younger than 50 years (Figure 2A). Higher HOTAIR expression was found in the distant metastasis group, lymph node metastasis group, residual tumor group, advanced tumor group and local tumor infiltration group, respectively (Figure 2B-2F). To investigate the relationship between HOTAIR expression and clinical data, ACC patients were further assigned into high-expression and low-expression group according to their expression levels of HOTAIR. Specifically, higher tumor stage, deeper tumor infiltration and more residual tumors were found in ACC patients with higher expression of HOTAIR. However, HOTAIR expression was not associated with age, gender, lymph node metastasis and distant metastasis of ACC patients (Table I).

We next detected the correlation between HOTAIR expression, disease-free survival (DFS)

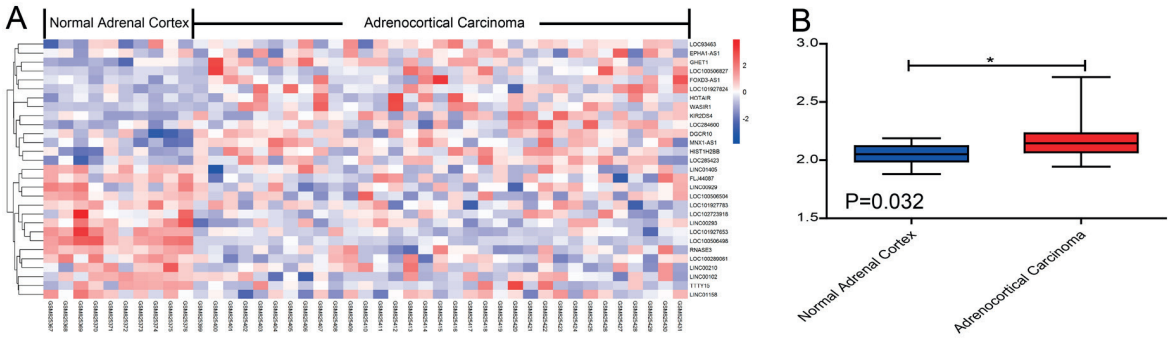

Figure 1. HOTAIR was overexpressed in ACC. ***A-B,*** HOTAIR was significantly upregulated inGSE33371.

Table I. Correlation between HOTAIR expression and clinicopathological characteristics of ACC.

Variables	Number of cases	Expression level		*p*-value
		Low	High	
Age				0.9056
<50	40	20	20	
≥50	37	18	19	
Gender				
Female	29	16	13	0.4271
Male	48	22	26	
Residual tumor				
No	55	32	23	0.0087
Yes	15	3	12	
Stage				
I/II	46	29	17	0.0034
III/IV	31	9	22	
Distant metastasis				
No	62	34	28	0.0502
Yes	15	4	11	
Lymph node metastasis				
No	68	35	33	0.3064
Yes	9	3	6	
Depth of invasion				
T1+T2	51	31	20	0.0049
T3+T4	26	7	19	

and overall survival (OS) in ACC patients through Kaplan-Meier and log-rank analysis. The data illustrated that upregulated HOTAIR was negatively correlated with DFS (Figure3A and 3B) and OS (Figure3C and 3D). Furthermore, HOTAIR was proved to be an independent prognostic factor in OS and DFS of ACC patients through COX regression analysis, suggesting that overexpressed HOTAIR indicates a worse prognosis of ACC patients (Table III).

HOTAIR was Upregulated in Human ACC Samples

We detected mRNA expressions of HOTAIR in 77 ACC tissues and 30 normal adrenal gland tissues by qRT-PCR. The results showed a higher expression of HOTAIR in ACC tissues than that of normal adrenal gland tissues (Figure 4A). By analyzing the infiltrative depth in ACC, we found higher expression of HOTAIR in T1 and T2 stage than that of T3 and T4 stage (Figure 4B). TNM (tumor, node, metastasis) staging showed that higher expression of HOTAIR was found in ACC with stage III and IV than that of stage I and II (Figure 4C). After ACC patients were further assigned into high-expression and low-expression of HOTAIR, DFS was remarkably decreased in high-expression group (p=0.0020, Figure 4D). These results indicated that HOTAIR may be involved in ACC development.

Table II. Univariate and multivariate Cox regression analyses HOTAIR for DFS of patients in study cohort.

Variables	Univariate analysis			Multivariate analysis		
	p-value	HR	95% CI	*p*-value	HR	95% CI
Age	0.993	1.000	0.978-1.022	0.687	1.005	0.981-1.029
Gender	0.177	1.805	0.766-4.250	0.09	2.236	0.881-5.675
Residual	0.000	2.480	1.549-3.969	0.524	1.285	0.595-2.776
Stage	0.000	2.462	1.636-3.707	0.000	2.201	1.416-3.419
M	0.000	4.932	2.089-11.645	0.635	0.673	0.131-3.458
N	0.040	2.905	1.049-8.046	0.472	0.655	0.207-2.076
T	0.000	2.183	1.489-3.201	0.906	0.943	0.359-2.478
HOTAIR	0.000	1.547	0.321-1.812	0.000	1.521	1.291-1.792

Table III. Univariate and multivariate Cox regression analyses HOTAIR for OS of patients in study cohort.

	Univariate analysis			Multivariate analysis		
Variables	*p*-value	HR	95% CI	*p*-value	HR	95% CI
Age	0.131	1.019	0.994-1.045	0.228	1.017	0.990-1.044
Gender	0.115	0.514	0.225-1.176	0.118	0.513	0.222-1.185
Residual	0.243	1.347	0.817-2.222	0.027	1.991	1.079-3.673
Stage	0.469	1.171	0.763-1797	0.804	1.372	0.113-16.641
M	0.303	1.615	0.648-4.022	0.862	0.852	0.141-5.170
N	0.261	1.888	0.624-5.712	0.457	1.649	0.441-6.159
T	0.803	1.090	0.555-2.139	0.929	0.958	0.374-2.453
HOTAIR	0.365	0.923	0.775-1.098	0.054	0.800	0.638-1.004

HOTAIR Promoted Proliferation of ACC Cells

To investigate the effect of HOTAIR on ACC cells, we first detected HOTAIR expressions in normal human adrenal cell line (Y1) and ACC cell lines (SW-13 and H295R) by qRT-PCR. HOTAIR was found to be overexpressed in ACC cells (Figure 5A). Transfection efficacy of HOTAIR overexpression plasmid and si-HOTAIR were then verified, respectively (Figure 5B and 5C). For *in vitro* experiments, viability of ACC cells was remarkably reduced after HOTAIR knockdown (Figure 5D). Colony formation obtained similar results as well (Figure 5E). We then transfected with HO-

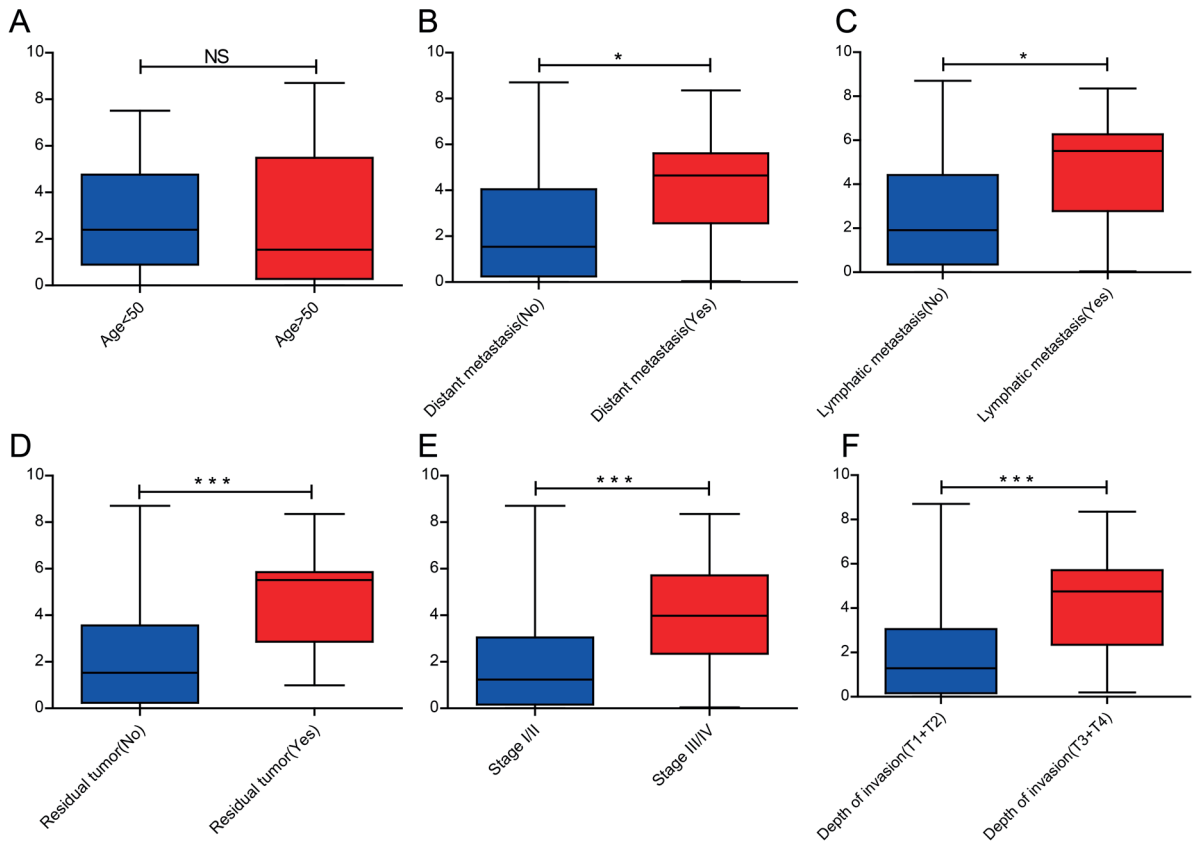

Figure 2. The relationship between HOTAIR expression and clinical data of ACC patients. ***A,*** No difference in HOTAIR expression was found between ACC patients who were older than 50 years and those younger than 50 years. ***B-F,*** Higher HOTAIR expression was found in the distant metastasis group (***B***), lymph node metastasis group (***C***), residual tumor group (***D***), advanced tumor group (***E***) and local tumor infiltration group (***F***).

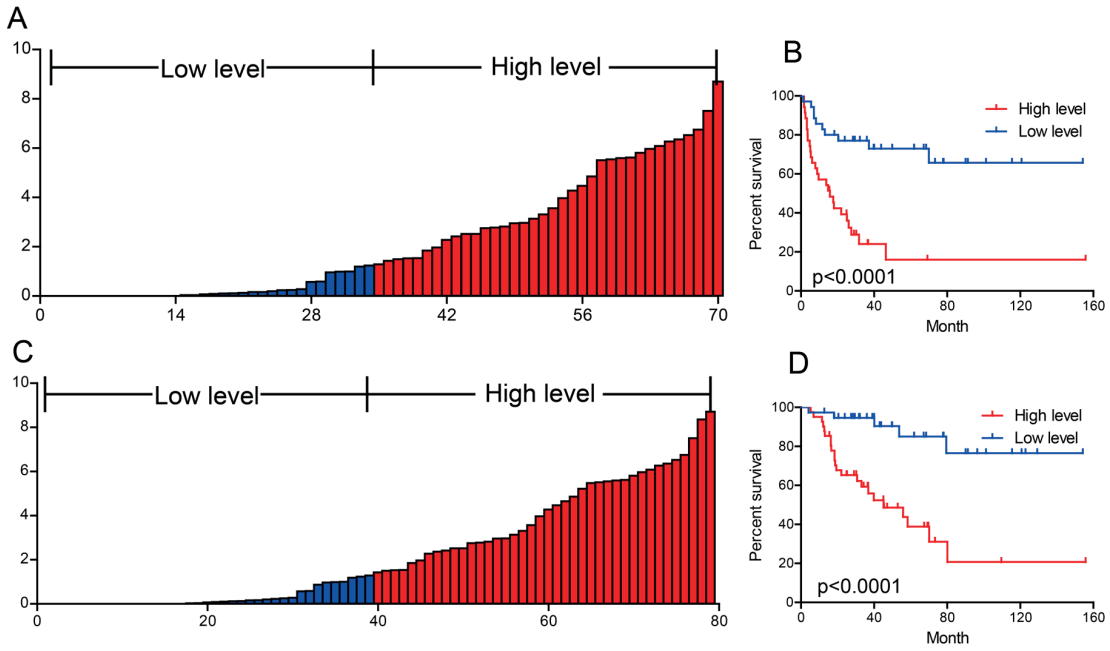

Figure 3. HOTAIR expression was negatively correlated with DFS and OS. ***A-B,*** DFS was decreased in ACC patients with higher expression of HOTAIR. ***C-D,*** OS was decreased in ACC patients with higher expression of HOTAIR.

TAIR overexpression plasmid in SW-13 cells, and both viability and colony formation abilities were remarkably elevated (Figure 5F and 5G). The above data suggested that upregulated HOTAIR could promote proliferation of ACC cells.

HOTAIR Participated in the Development and Progression of ACC via Regulating Cell Cycle

To further explore the biological function of HOTAIR in ACC cells, we detected cell cycle after altering HOTAIR expression. The results showed that the ratio of G0/G1 was elevated after HOTAIR knockdown (Figure 6A). Opposite results were obtained after overexpressing HOTAIR in SW-13 cells (Figure 6B). Cell cycle-related genes, including Cyclin D1, p-Rb and GSK3β were found to be downregulated in ACC cells after HOTAIR knockdown (Figure 6C and 6D), indicating that HOTAIR participates in the development and progression of ACC *via* regulating cell cycle.

Discussion

ACC is a malignant tumor that originates in adrenal cortical cells, which is manifested as lumbar masses, lumbar pain, fatigue and emaciation[1]. At present, the molecular mechanism of ACC development has not yet been elucidated, which brings great challenges to the clinical diagnosis and treatment of ACC. Human genome project has revealed that only about 2% of genes could encode proteins, which are called non-coding RNAs (ncRNAs)[19]. Based on the molecular size and biological function, ncRNAs are further divided into small interfering RNAs (siRNAs), microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs) and long non-coding RNAs (lncRNAs)[20]. Multiple studies have shown that ncRNA exerts a crucial role in tumor progression[21]. HOTAIR is the first discovered lncRNA only expressed in mammals, which regulates gene expressions at trans-transcriptional level. Human HOTAIR is located between HOXC11 and HOXC12 on chromosome 12q13.13 and includes 6 exons[22]. Scholars have shown that HOTAIR has significant effects on the proliferation and apoptosis of breast cancer, liver cancer, esophageal cancer and lung cancer[15, 16, 23].

Previous investigations[24] have demonstrated that HOTAIR can bind to mammalian polycomb repressive complex 2 (PRC2) and histone demethylase complex (LSD1/REST), so as to exert scaffolding function. EZH2 subunit that exerts the major role in PRC2 catalyzes the methylation of histone H3 lysine K27 (H3K27) to generate H3K27me3 (a transcriptional repression mar-

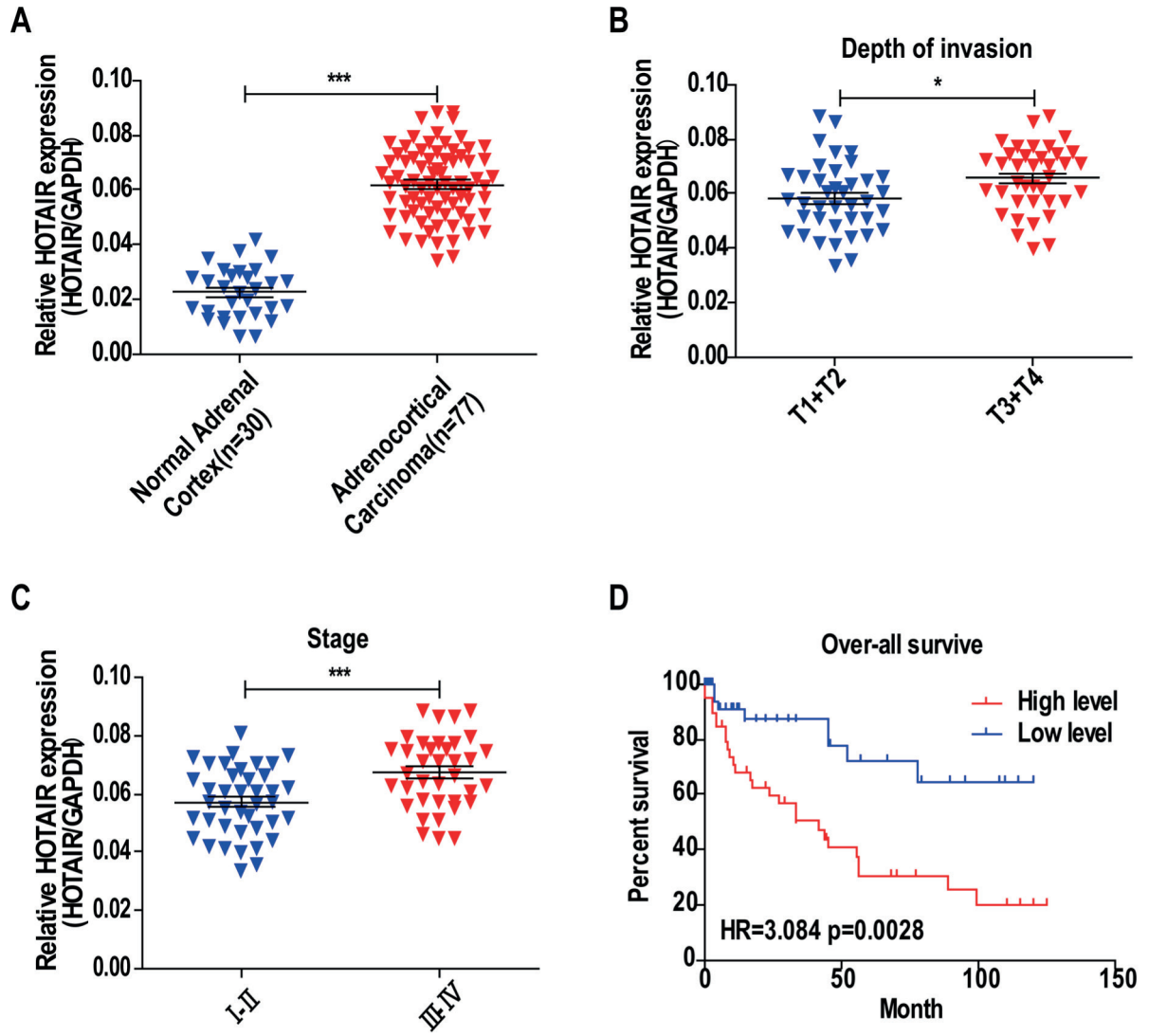

Figure 4. HOTAIR was upregulated in ACC tissues. ***A,*** Higher expression of HOTAIR in ACC tissues than that of normal adrenal gland tissues. ***B,*** Higher expression of HOTAIR was found in T1 and T2 stage than that of T3 and T4. ***C,*** Higher expression of HOTAIR was found in ACC with stage III and IV than that of stage I and II. ***D,*** DFS was remarkably decreased in high-expression group. ***E,*** ROC curve between HOTAIR expression and sensitivity and specificity of HOTAIR in diagnosing ACC.

ker). H3K27me3 further recruits PRC2 to specific sites to silence the HOXD locus and related transcriptional genes, such as HOXD10, JAM2, PCDH10, etc., thereby leading to the metastasis or recurrence of malignant tumors[17]. In the present work, HOTAIR was overexpressed in ACC by analyzing GSE33371. HOTAIR was remarkably increased in the distant metastasis group, lymph node metastasis group, residual tumor group, advanced tumor group, and local tumor infiltration group. Moreover, HOTAIR was negatively correlated with the prognosis, but positively correlated with tumor grade, local tumor invasion and tumor remnants of ACC. HOTAIR was proved to be an independent prognostic risk factor for DFS and OS in ACC patients by univariate and multivariate COX regression analysis. Cell cycle is the series of events that take place in a cell leading to its division and duplication of its DNA (DNA replication) to produce two daughter cells. Abnormal cell cycle is frequently seen in cancer cells[25]. Therefore, regulation of tumor cell cycle is an important strategy for cancer therapy. Cyclins are capable of inducing cell cycle progression and activating corresponding downstream ligands, thereafter leading to the abnormal separation of each cell cycle phase. In particular, Cyclin D1 is a key checkpoint protein

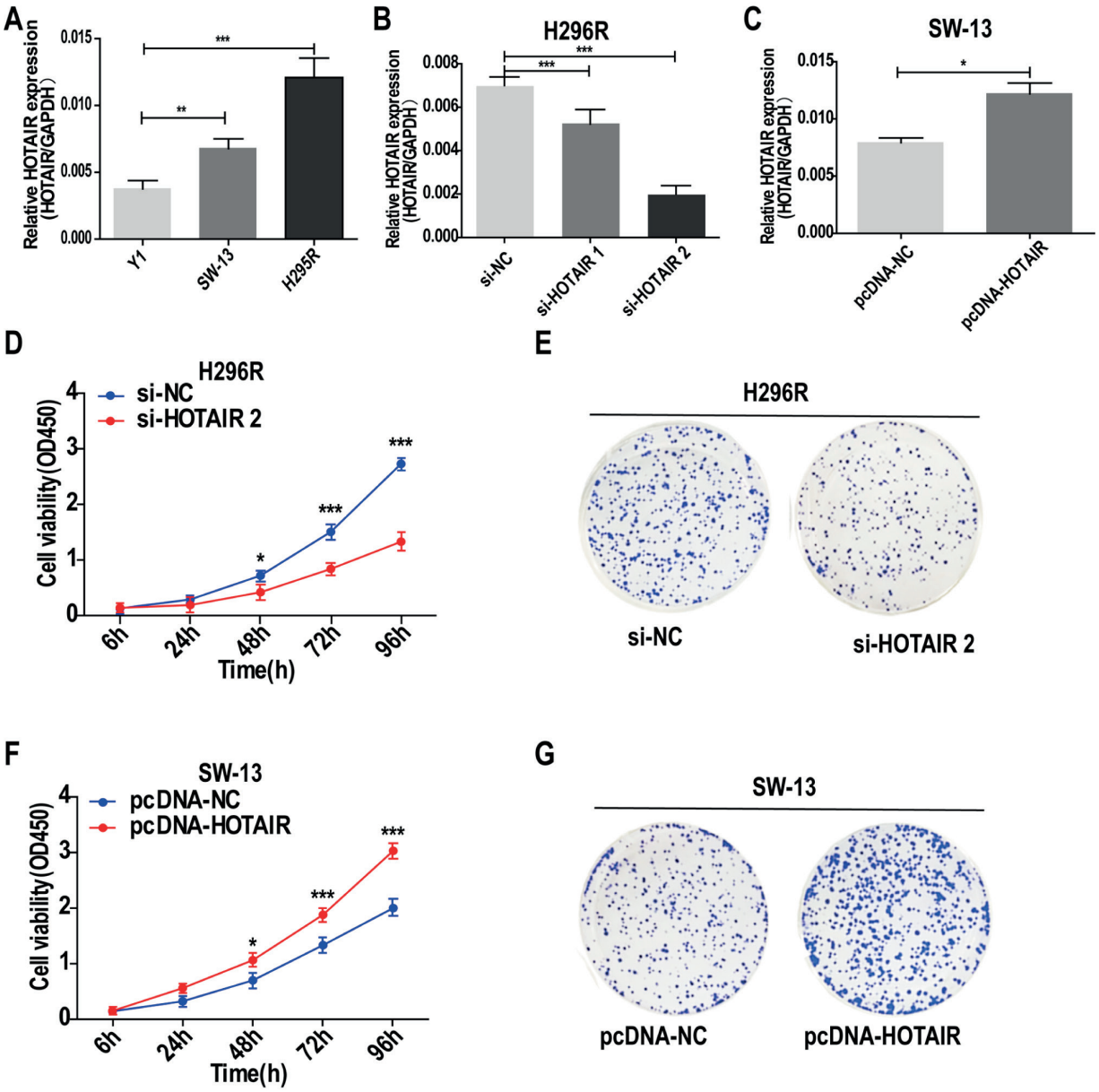

Figure 5. Upregulated HOTAIR promoted proliferation of ACC cells. ***A,*** HOTAIR expression in Y1, SW-13 and H295R cells. ***B,*** Transfection efficacy in H295R cells. ***C,*** Transfection efficacy in SW-13 cells. ***D,*** Decreased viability after HOTAIR knockdown. ***E,*** Decreased proliferation after HOTAIR knockdown. ***F,*** Increased viability after HOTAIR overexpression. ***G,*** Increased proliferation after HOTAIR overexpression.

responsible for cell cycle transformation from S phase to G1 phase. Functionally, Cyclin D1 mainly participates in transcriptional regulation and DNA repair in the cell cycle progression[26]. Our study found that Cyclin D1 expression was inhibited after HOTAIR knockdown, suggesting that downregulated HOTAIR remarkably arrests the cell cycle in G1 phase, thereby inhibiting cell cycle progression and cell proliferation of ACC cells. GSK3β is a highly conserved protein kinase that is inhibited by phosphorylated AKT. GSK3β controls cell cycle activity through phosphorylated substrates, including c-myc, Cyclin D1 and Cyclin E. In this study, p-GSK3β was downregulated in ACC cells after inhibition of HOTAIR, thereby prolonging the cell cycle. Rb gene is a tumor suppressor gene distributed in the nucleus, which can regulate cell proliferation, differentiation and apoptosis[27]. The phosphorylated and dephosphorylated forms of the

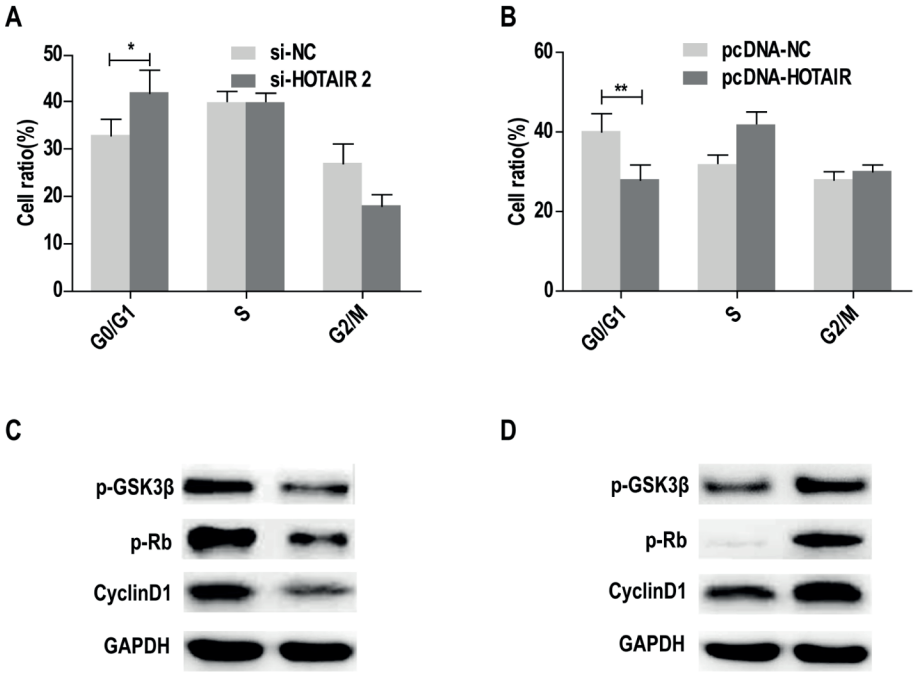

Figure 6. HOTAIR participates in the development and progression of ACC *via* cell cycle regulation. ***A*,** Cell cycle was arrested in H295R cells after HOTAIR knockdown. ***B*,** Cell cycle was promoted in SW-13 cells after HOTAIR overexpression. ***C*,** Expressions of p-GSK3β, p-Rb and CyclinD1 were decreased after HOTAIR knockdown. ***D*,** Expressions of p-GSK3β, p-Rb and CyclinD1 were increased after HOTAIR overexpression.

Rb protein regulate *in vitro* biological function[28]. Cyclic-dependent protein kinases (CDKs) could inactivate Rb into phosphorylation state[29]. Our study pointed out that phosphorylated Rb is downregulated after HOTAIR knockdown, thus blocking the cell cycle of ACC cells.

Conclusions

We demonstrated for the first time that HOTAIR is overexpressed in ACC and is a prognostic risk factor in ACC patients. HOTAIR participates in the development and progression of ACC *via* shortening cell cycle and promoting proliferation of ACC cells.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Kebebew E, Reiff E, Duh QY, Clark OH, McMillan A. Extent of disease at presentation and outcome for adrenocortical carcinoma: have we made progress? World J Surg 2006; 30: 872-878.
2) Kerkhofs TM, Verhoeven RH, Van der Zwan JM, Dieleman J, Kerstens MN, Links TP, Van de Poll-Franse LV, Haak HR. Adrenocortical carcinoma: a population-based study on incidence and survival in the Netherlands since 1993. Eur J Cancer 2013; 49: 2579-2586.
3) Fassnacht M, Libe R, Kroiss M, Allolio B. Adrenocortical carcinoma: a clinician's update. Nat Rev Endocrinol 2011; 7: 323-335.
4) Walker K, Badawi N, Levison J, Halliday R, Holland AJ, Williams G, Shi E. Re: a population-based study of congenital diaphragmatic hernia outcome in New South Wales and the Australian Capital Territory, Australia, 1992-2001. J Pediatr Surg 2006; 41: 1942.
5) Volante M, Buttigliero C, Greco E, Berruti A, Papotti M. Pathological and molecular features of adrenocortical carcinoma: an update. J Clin Pathol 2008; 61: 787-793.
6) Wang PQ, Wu YX, Zhong XD, Liu B, Qiao G. Prognostic significance of overexpressed long non-coding RNA TUG1 in patients with clear cell renal cell carcinoma. Eur Rev Med Pharmacol Sci 2017; 21: 82-86.
7) Bertone P, Stolc V, Royce TE, Rozowsky JS, Urban AE, Zhu X, Rinn JL, Tongprasit W, Samanta M, Weissman S, Gerstein M, Snyder M. Global identification of human transcribed sequences with genome tiling arrays. Science 2004; 306: 2242-2246.

8) Louro R, Smirnova AS, Verjovski-Almeida S. Long intronic noncoding RNA transcription: expression noise or expression choice? Genomics 2009; 93: 291-298.

9) Nagano T, Fraser P. No-nonsense functions for long noncoding RNAs. Cell 2011; 145: 178-181.

10) Wapinski O, Chang HY. Long noncoding RNAs and human disease. Trends Cell Biol 2011; 21: 354-361.

11) Kotake Y, Nakagawa T, Kitagawa K, Suzuki S, Liu N, Kitagawa M, Xiong Y. Long non-coding RNA ANRIL is required for the PRC2 recruitment to and silencing of p15(INK4B) tumor suppressor gene. Oncogene 2011; 30: 1956-1962.

12) Wu HA, Bernstein E. Partners in imprinting: noncoding RNA and polycomb group proteins. Dev Cell 2008; 15: 637-638.

13) Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea MD, Thomas K, Presser A, Bernstein BE, van Oudenaarden A, Regev A, Lander ES, Rinn JL. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci U S A 2009; 106: 11667-11672.

14) Saxena A, Carninci P. Long non-coding RNA modifies chromatin: epigenetic silencing by long non-coding RNAs. Bioessays 2011; 33: 830-839.

15) Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL, Wang Y, Brzoska P, Kong B, Li R, West RB, van de Vijver MJ, Sukumar S, Chang HY. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 2010; 464: 1071-1076.

16) Yang Z, Zhou L, Wu LM, Lai MC, Xie HY, Zhang F, Zheng SS. Overexpression of long non-coding RNA HOTAIR predicts tumor recurrence in hepatocellular carcinoma patients following liver transplantation. Ann Surg Oncol 2011; 18: 1243-1250.

17) Kim K, Jutooru I, Chadalapaka G, Johnson G, Frank J, Burghardt R, Kim S, Safe S. HOTAIR is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer. Oncogene 2013; 32: 1616-1625.

18) Kogo R, Shimamura T, Mimori K, Kawahara K, Imoto S, Sudo T, Tanaka F, Shibata K, Suzuki A, Komune S, Miyano S, Mori M. Long noncoding RNA HOTAIR regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers. Cancer Res 2011; 71: 6320-6326.

19) Muers M. RNA: genome-wide views of long non-coding RNAs. Nat Rev Genet 2011; 12: 742.

20) Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet 2009; 10: 155-159.

21) Wang ZQ, Cai Q, Hu L, He CY, Li JF, Quan ZW, Liu BY, Li C, Zhu ZG. Long noncoding RNA UCA1 induced by SP1 promotes cell proliferation via recruiting EZH2 and activating AKT pathway in gastric cancer. Cell Death Dis 2017; 8: e2839.

22) He S, Liu S, Zhu H. The sequence, structure and evolutionary features of HOTAIR in mammals. BMC Evol Biol 2011; 11: 102.

23) Chen FJ, Sun M, Li SQ, Wu QQ, Ji L, Liu ZL, Zhou GZ, Cao G, Jin L, Xie HW, Wang CM, Lv J, De W, Wu M, Cao XF. Upregulation of the long non-coding RNA HOTAIR promotes esophageal squamous cell carcinoma metastasis and poor prognosis. Mol Carcinog 2013; 52: 908-915.

24) Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, Shi Y, Segal E, Chang HY. Long noncoding RNA as modular scaffold of histone modification complexes. Science 2010; 329: 689-693.

25) Mishra R. Cell cycle-regulatory cyclins and their deregulation in oral cancer. Oral Oncol 2013; 49: 475-481.

26) Fu M, Wang C, Li Z, Sakamaki T, Pestell RG. Minireview: cyclin D1: normal and abnormal functions. Endocrinology 2004; 145: 5439-5447.

27) Manning AL, Dyson NJ. RB: mitotic implications of a tumour suppressor. Nat Rev Cancer 2012; 12: 220-226.

28) Gordon GM, Du W. Conserved RB functions in development and tumor suppression. Protein Cell 2011; 2: 864-878.

29) Du W, Searle JS. The rb pathway and cancer therapeutics. Curr Drug Targets 2009; 10: 581-589.