

# Overexpression of long noncoding RNA HOTAIR predicts a poor prognosis in patients with cervical cancer

Long Huang · Ling-Min Liao · An-Wen Liu ·  
Jian-Bing Wu · Xiao-Ling Cheng · Jia-Xin Lin ·  
Min Zheng

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## Abstract

**Purpose** The long noncoding RNA HOTAIR has been reported to be a good biomarker for poor prognosis in a variety of human cancers. However, whether HOTAIR could serve as novel biomarker to predict prognosis in cervical cancer or not is unknown. The aim of the present study was to examine the expression of HOTAIR in cervical cancers and to investigate the relationship between this lncRNA expression levels and existing clinicopathological factors and patient survival.

**Methods** We examined the expression of HOTAIR in 218 cervical cancer tissues and matched 218 adjacent normal tissues using quantitative real-time RT-PCR and analyzed its correlation with the clinical parameters.

**Results** The results showed that HOTAIR expression in cervical cancer tissues was significantly upregulated compared with the matched nontumorous tissues ( $P < 0.0001$ ). Increased HOTAIR expression was significantly correlated with FIGO stage ( $P < 0.0001$ ), lymph node metastasis ( $P <$

$0.0001$ ), depth of cervical invasion ( $P < 0.0001$ ), tumor size ( $P = 0.006$ ) and age ( $P = 0.020$ ), but not other clinical characteristics. Moreover, cervical cancer patients with HOTAIR higher expression have shown significantly poorer overall survival ( $P < 0.0001$ ) and disease-free survival ( $P < 0.0001$ ) than those with lower HOTAIR expression. Univariate ( $P < 0.0001$ , HR = 4.566, 95 % CI 2.122–9.825) and multivariate ( $P = 0.012$ , HR = 2.863, 95 % CI 1.263–76.490). Cox regression analyses showed that HOTAIR expression served as an independent predictor for overall survival.

**Conclusions** our data indicate that high expression of HOTAIR is involved in cervical cancer progression and could be a potential target for diagnosis and gene therapy.

**Keywords** Cervical cancer · Long noncoding RNA · HOTAIR · Prognosis

## Introduction

Although the incidence of cervical cancer has been greatly reduced with the application of pap smear screening, cervical cancer is still the third most commonly diagnosed cancer in women worldwide, with estimated 529,800 new cases diagnosed annually [1]. Moreover, higher incidence rate and mortality rate observed in developing countries versus developed countries [2]. Therefore, it is still urgently for us to find new and effective therapeutic strategies for cervical cancer.

Recent genome sequencing studies have revealed that the human genome is comprised of less than 2 % protein coding genes and more than 90 % of the genome is transcribed as noncoding RNAs [3, 4]. These ncRNAs are classified into two groups depending on the nucleotide size. Those under 200 nucleotides in length are referred to as small ncRNAs, including the microRNAs (approximately

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L. Huang (✉) · A.-W. Liu · J.-B. Wu  
Department of Oncology, The Second Affiliated Hospital  
of Nanchang University, 1 Minde Road, Nanchang,  
Jiangxi, China  
e-mail: huanglong914@gmail.com

L.-M. Liao  
Department of Ultrasound, The Second Affiliated Hospital  
of Nanchang University, Nanchang, China

X.-L. Cheng  
Department of Medical Imaging, Women and Children Health  
Institute Futian, Shenzhen, China

J.-X. Lin · M. Zheng  
Department of Gynecology, Cancer Center, Sun Yat-sen  
University, Guangzhou, China

18–25 nucleotides in length) which have been intensively studied [5]. However, the long noncoding RNAs consist of more than 200 nucleotides have relevant limited investigations [6].

HOTAIR (Hox transcript antisense intergenic RNA) is a 2,158 bp lncRNA localized to a boundary in the HOXC gene cluster. Recently, HOTAIR has been identified to overexpression in a variety of cancers such as breast, colon, pancreatic and lung cancer [7–10] and enhanced HOTAIR expression in patients has been correlated with breast and colon cancer metastasis [11–13]. Meanwhile, knockdown of HOTAIR study showed that HOTAIR can inhibit cell invasion, cell proliferation, modulate of cell cycle progression and induce apoptosis, indicating that HOTAIR can play a direct role in the modulation of cancer progression [7–9, 13]. However, little is known about the expression and the impact of HOTAIR in cervical cancer development.

To better understand the role of HOTAIR in cervical cancer development, we investigated the expression of HOTAIR in cervical cancer tissues and analyzed the relationship between this HOTAIR expression levels and existing clinicopathological factors and patient survival.

## Materials and methods

### Patients and sample collection

Two hundred and eighteen cervical cancer tumors and 218 matched adjacent normal tissues were obtained from Sun Yat-sen University Cancer Center (SYSUCC) (Guangzhou, China) between January 2005 and October 2009. All patients recruited in this study were not subjected to preoperative radiotherapy and/or chemotherapy and were diagnosed as infiltrating carcinoma by pathology. The tumor stage was classified by two experienced gynecological oncologists according to the International Federation of Gynecology and Obstetrics (FIGO) staging system for cervical cancer. Clinical and pathological variables analyzed are shown in Table 1. All patients were regularly followed up, with a mean observation period of 42 months (range 2–55 months). Tissue specimens were immediately stored at 4 °C for 24 h in RNA Later (Ambion Inc.), then at –80 °C liquid nitrogen until the extraction of total RNA. Total RNA from fresh cervical cancer tissues was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The study was approved by the Medical Ethics Committee of Sun Yat-sen University Cancer Center.

### Quantitative real-time reverse transcriptase PCR

The quality and concentration of RNA were determined using a Nanodrop 1000 (Thermo, Wilmington, DE, USA).

**Table 1** Association of HOTAIR expression with clinicopathological features in cervical cancer

Clinicopathological	HOTAIR expression		P value
	Low (%)	High (%)	
Age			
≤35	54 (24.8)	37 (17.0)	
>35	55 (25.2)	72 (33.0)	0.020*
Histology			
Squamous	94 (43.1)	96 (44.0)	
Adenocarcinoma	15 (6.9)	13 (6.0)	0.686
FIGO stage			
I B1	96 (44.0)	59 (27.1)	
I B2–II A	11 (5.1)	31 (14.2)	
II b	2 (0.9)	19 (8.7)	<0.0001*
Differentiation			
Well	4 (1.9)	7 (3.3)	
Moderate	70 (32.5)	64 (29.8)	
Poor	33 (15.3)	37 (17.2)	0.519
SCC-Ag (μg/L)			
≤4	87 (45.1)	81 (42.0)	
>4	12 (6.2)	13 (6.7)	0.724
Tumor diameter (cm)			
≤4	88 (40.4)	70 (32.1)	
>4	21 (9.6)	39 (17.9)	0.006*
Depth of cervical invasion			
<2/3	71 (33.5)	40 (18.9)	
≥2/3	35 (16.5)	66 (31.1)	<0.0001*
Uterine corpus invasion			
Negative	75 (34.9)	71 (33.0)	
Positive	32 (14.9)	37 (17.2)	0.494
Lymphoid node			
Negative	103 (47.2)	68 (31.2)	
Positive	6 (2.8)	41 (18.8)	<0.0001*

Pearson Chi-square test

\* Significant values

Then, 1 μg total RNA reversely transcribed from each sample to synthesis cDNA by the RT reagent Kit (Promega, USA, A3500) according to the manufacturer's instructions with minor modification. Real-time PCR was performed to determine the relative expression level of target genes by the SYBR Green qRT-PCR kit (Promega, USA, A6001) on the CFX96 Real-Time PCR Detection System (Bio-Rad). The level of HOTAIR expression in each sample was normalized to the respective GAPDH expression level. The primers used were as follows: HOTAIR sense, 5'-CAGTGGGGAAGTCTGACTCG-3' and antisense, 5'-GTGCCTG GTGCTCTCTTACC-3'; GAPDH sense, 5'-CTCCTCCTGTTTCGACAGTCAGC-3' and antisense, 5'-CCCAATACGACCAAATCCGTT-3'. The amplification

profile was 95 °C for 5 min, followed by 42 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 60 s. The specificity of each PCR was confirmed by melting curve analyses. The comparative *Ct* method ( $\Delta\Delta C_t$ ) was used for quantification of the gene expression. The relative expression amount of HOTAIR to GAPDH was calculated using the equation  $2^{-\Delta\Delta C_t}$ , where  $\Delta C_t = C_t \text{ HOTAIR} - C_t \text{ GAPDH}$ . The gene expression levels of HOTAIR in tumor were compared with normal tissues. To minimize experimental variability, each sample was analyzed in triplicate and the mean expression level was calculated.

### Statistical analysis

Statistical analysis was carried out with SPSS software package (IBM, standard version 18.0). The relationship between HOTAIR expression and clinicopathological characteristics was assessed using Pearson's  $\chi^2$  test. Survival curves were estimated by the Kaplan–Meier method. The log-rank test was used to estimate the statistical differences between survival curves. Cox proportional hazards analysis was performed to calculate the hazard ratio (HR) and the 95 % confidence interval (CI) to evaluate the association between HOTAIR expression and survival. Multivariate survival analysis was carried out for all of the parameters that were significant in the univariate analysis using the Cox regression model. A two-sided *P* value of <0.05 was considered statistically significant.

## Results

### HOTAIR was overexpressed in cervical cancer

The HOTAIR expression levels were detected in 218 cervical cancer samples and adjacent, histologically normal tissues by qRT-PCR. HOTAIR expression was significantly upregulated in cancerous tissues compared with its own surrounding tissues ( $2.383 \pm 0.152$ ,  $P < 0.0001$ ) (Fig. 1a). Furthermore, the results revealed a significant association between HOTAIR upregulation and advanced FIGO stage (IB1 vs. IB2-IIA vs. IIB,  $P < 0.001$ ) (Fig. 1b) and lymph node metastasis (metastasis vs. not metastasis,  $P = 0.012$ ; number of positive lymph node = 0 vs. number of positive lymph node = 1 vs. number of positive lymph node  $\geq 2$ ,  $P = 0.018$ ) (Fig. 1c, d).

### Correlations between the expression of HOTAIR and the clinicopathological factors in cervical cancer

To identify the clinical relevance of HOTAIR expression in cervical cancer, correlation between HOTAIR expression

and clinicopathological parameters such as age, histology, FIGO stage, tumor differentiation, SCC-Ag, tumor size, depth of cervical invasion, uterine corpus invasion and lymph node metastasis was examined. Of the 218 human cervical cancer tissues were further classified into the high-HOTAIR group ( $n = 109$ ) and low-HOTAIR group ( $n = 109$ ) using the median expression value of HOTAIR as the cutoff point. The results showed that increased HOTAIR expression was significantly correlated with FIGO stage ( $P < 0.0001$ ), lymph node metastasis ( $P < 0.0001$ ), depth of cervical invasion ( $P < 0.0001$ ), tumor size ( $P = 0.006$ ) and age ( $P = 0.020$ ), but not other clinical characteristics (Table 1).

### Association between HOTAIR expression and prognosis of cervical cancer patients

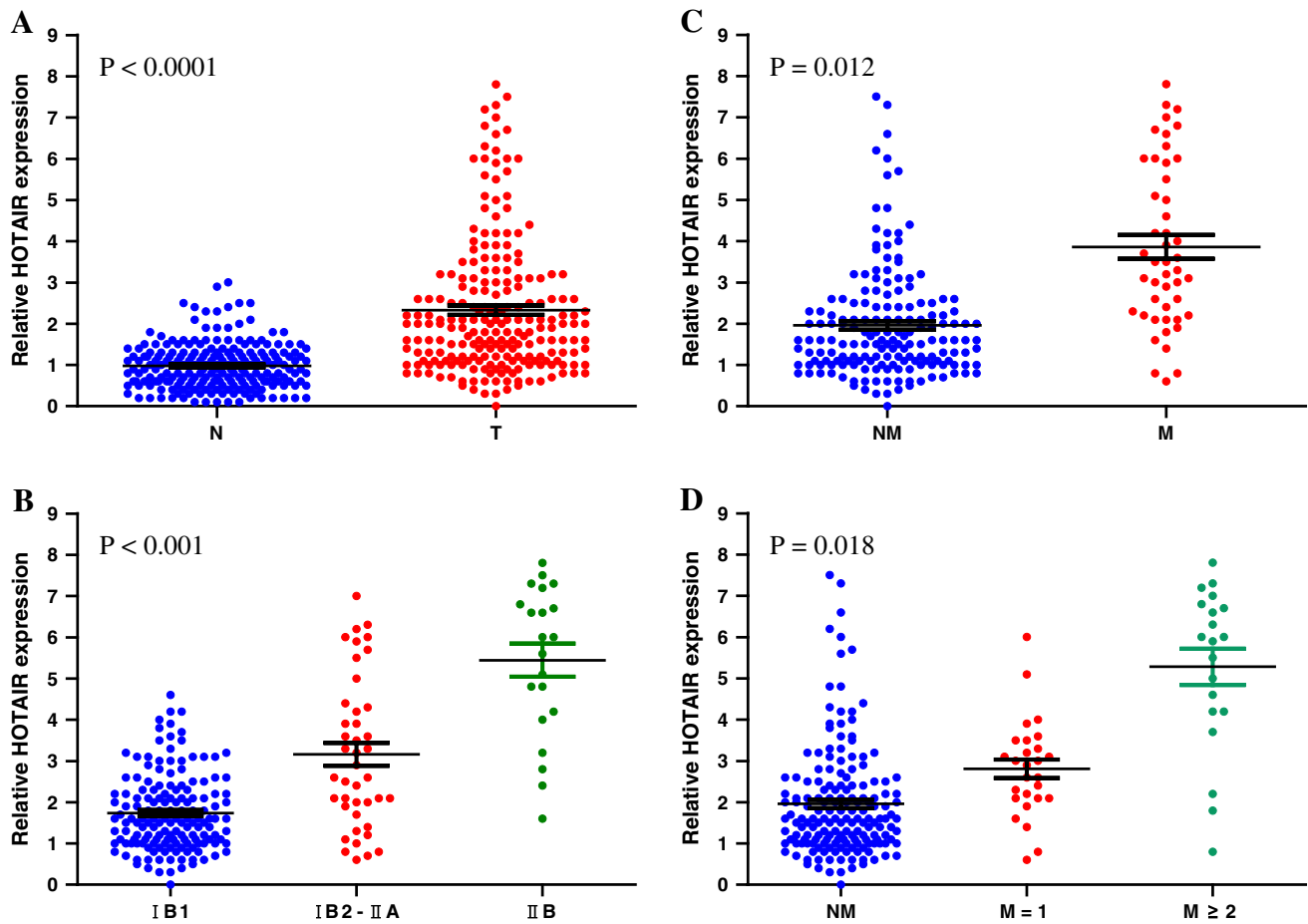
Overall survival curves and disease-free survival curves in different FIGO stage group are shown in Fig. 2a, b. As was expected, patients with advanced FIGO stage have shown significantly poorer overall survival ( $P < 0.0001$ ) and disease-free survival ( $P < 0.0001$ ). Overall survival curves and disease-free survival curves in high-HOTAIR group and low-HOTAIR group are shown in Fig. 2c, d. Patients with high HOTAIR expression have shown significantly poorer overall survival ( $P < 0.0001$ ) and disease-free survival ( $P < 0.0001$ ) than those with low HOTAIR expression.

### Univariate and multivariate analyses show HOTAIR expression is an independent predictor for overall survival

As shown in Table 2, univariate analysis identified four prognostic factors: FIGO stage (IB2-IIB vs. IB1), depth of cervical invasion ( $\geq 2/3$  vs.  $< 2/3$ ), lymph node (positive vs. negative) and HOTAIR expression (high vs. low). The other clinicopathological characteristics, such as age, histology, differentiation, SCC-Ag, tumor size and uterine corpus invasion, were not statistically significant prognosis factors. Parameters that were significantly related to survival in univariate analysis were entered into the multivariate analysis. When it comes to multivariate, Cox regression model revealed that only FIGO stage ( $P < 0.0001$ , HR = 1.994, 95 % CI 1.359–2.927), lymph node metastasis ( $P = 0.005$ , HR = 2.636, 95 % CI 1.348–5.156) and HOTAIR expression level ( $P = 0.012$ , HR = 2.863, 95 % CI 1.263–6.490) served as independent prognostic factors for poor overall survival.

### Receiver operating characteristic (ROC) curve analysis

ROC curve analysis showed that the HOTAIR expression is a good candidate to discriminate tumor tissues from non-tumorous tissues (sensitivity: 60.6 %, specificity: 87.2 %)



**Fig. 1** HOTAIR expression levels were detected in 218 cervical cancer samples and adjacent normal tissues by qRT-PCR. **a** HOTAIR expression was significantly upregulated in cancerous tissues compared with corresponding normal tissues. **b** HOTAIR expression was significantly higher in tumors of higher p FIGO stage than that in tumors of lower FIGO stage. The expression of HOTAIR increased

along with the number of lymph node metastasis, **c** metastasis (M) vs. not metastasis (NM); **d** number of positive lymph node = 0 vs. number of positive lymph node = 1 vs. number of positive lymph node  $\geq 2$ ). Expression levels are normalized to GAPDH. The relation expression of HOTAIR was compared with the median expression value of HOTAIR of normal tissues. Error bars represent SE

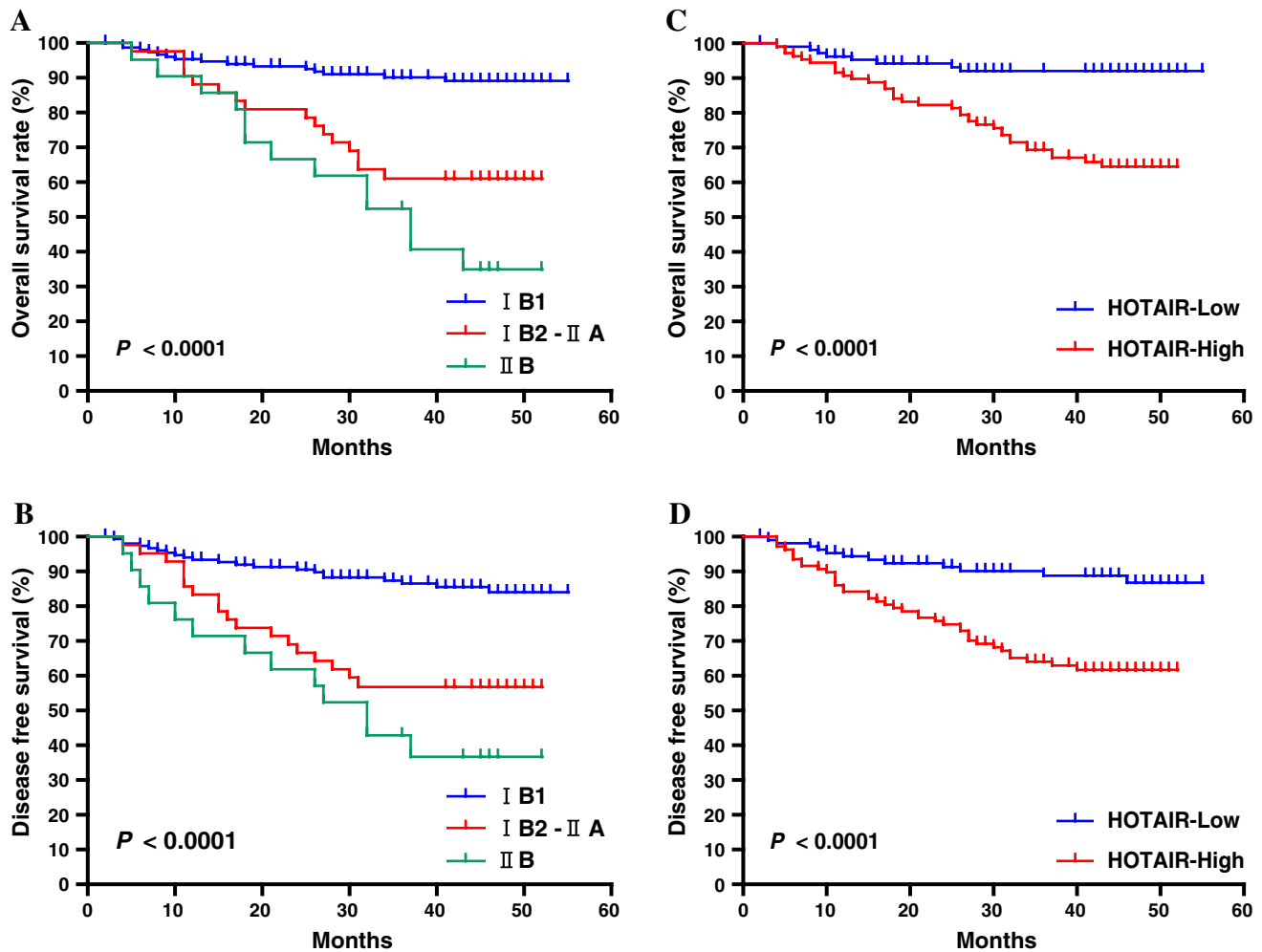
and the presence or absence of lymph node metastasis (sensitivity: 85.1 %, specificity: 64.9 %). The areas under the ROC curve (AUC) are 0.803 (95 % CI 0.762–0.839,  $P < 0.0001$ ) and 0.802 (95 % CI 0.742–0.852,  $P < 0.0001$ ), respectively (Fig. 3).

## Discussion

LncRNAs have been recently implicated as having oncogenic [14] and tumor suppressor roles [15]. In this study, we found enhanced expression of HOTAIR in cervical cancer tissues compared with adjacent normal tissues. We showed for the first time that HOTAIR was frequently up-regulated in cervical cancer. These findings are consistent with the recent study showing that upregulation of HOTAIR in a variety of cancers such as breast, colon,

pancreatic and lung cancer. Our results also revealed that the high level expression of HOTAIR was associated with lymph node metastasis. These findings are also consistent with a study showing that upregulation of HOTAIR is correlated with lymph node metastasis of patients with gastric cancer [16]. Moreover, the current study showed that HOTAIR knockdown can inhibit the invasion and metastasis of NSCLC in vitro and in vivo [17]. Therefore, we hypothesis HOTAIR may involved in the progression of cervical cancer through promoting the invasion metastasis.

Although evidence of carcinogenicity of HOTAIR in humans is strong, the molecular mechanism by which tumor development and metastasis are promoted is not fully understood. HOTAIR resided in the HOXC locus, but repressed transcription in the more distal HOXD locus in foreskin fibroblasts. It has been revealed that



**Fig. 2** Kaplan–Meier survival curve analysis shows that FIGO stage was significantly associated with **a** poor overall survival and **b** disease-free survival in 218 cervical cancer cases. Patients with higher

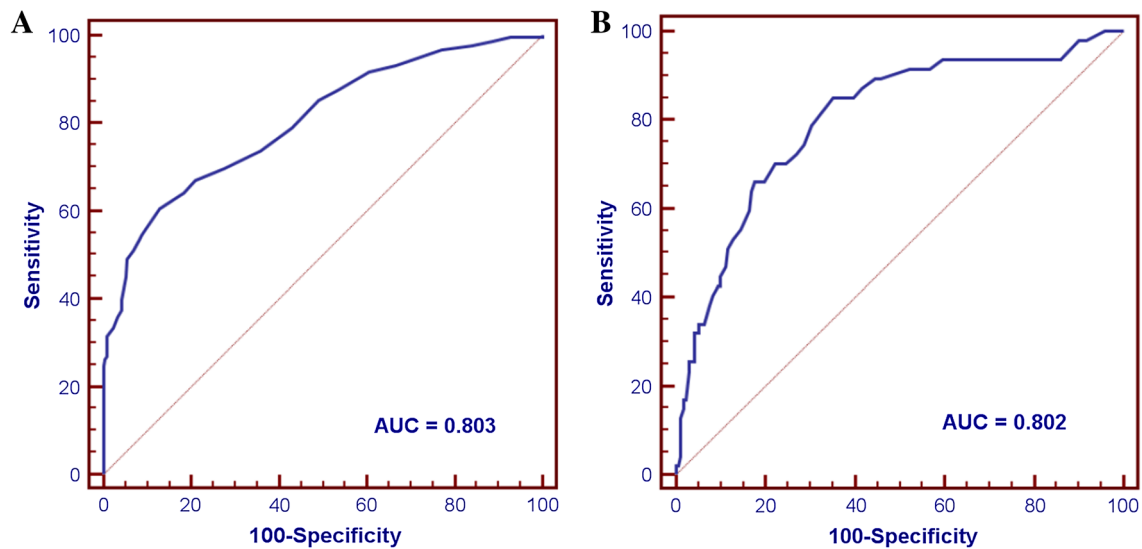
expression of HOTAIR showed decreased **c** overall survival and **d** disease-free survival compared with patients with lower expression of HOTAIR. *P* value was calculated by Log-rank test

**Table 2** Univariate and multivariate analysis of clinicopathological factors for overall survival

Clinicopathological	Subset	Hazard ratio (95 % CI)	<i>P</i> value
Univariate analysis ( <i>n</i> = 218)			
Age	≤35 vs. >35	1.176 (0.641–2.158)	0.600
Histology	Adenocarcinoma vs. squamous	1.144 (0.483–2.707)	0.760
FIGO stage	I B2-II B vs. I B1	2.710 (1.913–3.839)	<0.0001*
Differentiation	Well vs. moderate, poor	0.795 (0.452–1.400)	0.427
SCC-Ag	>4 (μg/L) vs. ≤4 (μg/L)	1.758 (0.810–3.816)	0.154
Tumor diameter (cm)	>4 (cm) vs. ≤4 (cm)	1.227 (0.628–2.396)	0.550
Depth of cervical invasion	≥2/3 vs. <2/3	2.109 (1.010–4.404)	0.047*
Uterine corpus invasion	Positive vs. negative	1.991 (0.954–4.156)	0.067
Lymphoid node	Positive vs. negative	4.753 (2.624–8.608)	<0.0001*
HOTAIR expression	High vs. low	4.566 (2.122–9.825)	<0.0001*
Multivariate analysis ( <i>n</i> = 218)			
FIGO stage	I B2-II B vs. I B1	1.994 (1.359–2.927)	<0.0001*
Lymphoid node	Positive vs. negative	2.636 (1.348–5.156)	0.005*
HOTAIR expression	High vs. low	2.863 (1.263–6.490)	0.012*

FIGO International Federation of Gynecology and Obstetrics, CI confidence interval

\* Significant values



**Fig. 3** Receiver operating characteristic curve (ROC) analysis was employed to determine whether HOTAIR is really good candidates to discriminate (a) tumor tissues from nontumorous tissues and (b) the

presence or absence of lymph node metastasis. The areas under the ROC curve (AUC) are 0.803 (95 % CI 0.762–0.839,  $P < 0.0001$ ) and 0.802 (95 % CI 0.742–0.852,  $P < 0.0001$ ), respectively

HOTAIR interacted with the Polycomb Repressive Complex 2 (PRC2) complex, a methyl-transferase that trimethylates histone H3 at lysine 27 (K27) to repress transcription and up to 20–40 % known lncRNAs are associated with it [18–20] and was required for PRC2-dependent histone H3 lysine 27 trimethylation and gene silencing. Alteration of HOTAIR expression in cancer cells results in both enhanced and suppressed expression of genes, and a subset of genes repressed by HOTAIR was also coregulated by PRC2 [21, 22], suggesting that HOTAIR-mediated suppression of genes in cancer cells is PRC2 dependent.

In this study, GAPDH was selected to be the house-keeping gene. The expression data of HOTAIR were based on GAPDH. Although some articles reported that GAPDH has been shown to be upregulated in many cancers and downregulated by chemotherapeutic drugs [23, 24], we have confirmed that GAPDH is not itself differentially repressed in cervical cancer tissue in our previous study.

In conclusion, in our study, for the first time, we reported that HOTAIR was frequently upregulated in cervical cancer tissues, and as an independent prognostic factor in patients with cervical cancer. This new findings indicate that HOTAIR may be used as a potential target for diagnosis and gene therapy of cervical cancer. However, the precise molecular mechanisms of HOTAIR that involved in cervical cancer need to be further studied.

**Conflict of interest** The authors declare that they have no competing interests.

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