The long non-coding RNA *HOTAIR* is upregulated in endometrial carcinoma and correlates with poor prognosis

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Abstract. Long non-coding RNAs (lncRNAs) are emerging as key molecules in human cancer. Homeobox (HOX) transcript antisense intergenic RNA (*HOTAIR*), a long non-coding RNA (lncRNA), is associated with a variety of human cancers, such as breast, liver and lung cancer. However, whether *HOTAIR* can function as a molecular marker in endometrial carcinoma (EC) remains unknown. In the present study, the expression of *HOTAIR* in 66 EC tissues from patients with EC and 30 normal tissues from healthy age-matched control subjects was determined using quantitative reverse transcription PCR. Furthermore, using *in situ* hybridization, we measured *HOTAIR* expression in 129 formalin-fixed paraffin-embedded (FFPE) tissue sections, which included 96 tissues that matched the frozen cases, 21 other EC tissues and 12 atypical hyperplasia tissues. Correlations between *HOTAIR* expression and the clinicopathological characteristics of patients were analyzed. Our results revealed that *HOTAIR* expression in the EC tissues was significantly upregulated compared with normal tissues (p<0.001). In addition, we observed a significant association between *HOTAIR* expression and the EC grade (p<0.05) and lymph node metastasis (p<0.05). Moreover, in the FFPE tissues, but not the frozen tissues, we found that a higher *HOTAIR* expression also correlated with the depth of myometrial invasion (p=0.019) and lymphovascular space invasion (p=0.015). More importantly, patients with a higher *HOTAIR* expression showed significantly poorer overall survival than those with lower *HOTAIR* expression (p<0.05). In conclusion, our results suggest that a high expression of *HOTAIR* is involved in the progression of cancer and may be a novel biomarker of poor prognosis in patients with EC.

Introduction

Endometrial carcinoma (EC) is one of the most common malignancies of the female reproductive system in Western countries. In 2013, an estimated 49,500 new cases and 8,200 deaths due to EC are expected in the USA (1). With the increase in obesity and the decrease in physical activity, the incidence of EC is rising and shows a trend in younger women (2). EC is usually classified into two types to determine the risk of metastasis and recurrence (3). Generally, type I endometrioid endometrial carcinomas (EECs) have a good prognosis and account for 80-85% of the total cases of EC. By contrast, type II non-EECs are often associated with a worse outcome (3,4). However, the prognostic value of this classification is unsatisfactory, as approximately 20% of type I tumors recur, whereas 50% of type II tumors recur (5). A number of previous studies have demonstrated the utility of molecular alterations as prognostic markers, including p53 (6), phosphatase and tensin homolog (PTEN) (7) and Ki-67 (8); however, their value is limited (9). Thus, a deeper understanding of the molecular mechanisms responsible for EC is required for risk stratification and a clinical decision regarding individualized treatment strategies.

Recent studies have indicated that only 2% of transcripts are protein-coding RNAs, and up to 90% of the genomic DNA in eukaryotic genomes produces non-coding RNA (10). Initially, non-coding RNAs were regarded as 'junk'; however, numerous studies have confirmed that non-coding RNAs, including microRNAs and long non-coding RNAs (lncRNAs) participate in diverse biological processes (11). Moreover, lncRNAs are regarded as drivers of tumor suppressive and oncogenic functions in various types of cancer (12,13). For example, growth arrest-specific 5 (*GAS5*) is a tumor suppressor lncRNA; *GAS5* induces growth arrest and apoptosis in several mammalian cell lines, whereas the loss of *GAS5* is associated with tumor formation (14). Several studies have indicated that lncRNAs contribute to oncogenesis. Certain lncRNAs, such as *H19*, metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*), homeobox (HOX) transcript antisense intergenic RNA (*HOTAIR*), steroid receptor RNA activator (*SRA*) and

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prostate cancer-associated ncRNA transcript-1 (*PCAT-1*) are overexpressed in a variety of human cancers, suggesting their involvement in tumorigenesis (15-19). However, the current knowledge is only the first step toward understanding the biological role of lncRNAs in cancer. Eventually, new discoveries will be used to design novel therapeutic agents (20), particularly for the treatment of patients with recurring EC.

HOTAIR, a well-characterized lncRNA, has gained attention as it has been investigated in numerous solid tumors, including breast (17,21), liver (22), colorectal (23) and lung cancers (24). In these tumors, *HOTAIR* is upregulated and correlates with invasiveness and metastasis. *HOTAIR* has been identified as a predictor of overall survival and progression-free survival in several types of cancer. These studies have also indicated that *HOTAIR* serves as a modular scaffold. *HOTAIR* has two important domains, with polycomb repressive complex 2 (PRC2) binding to the 5' end and the complex of lysine-specific demethylase 1 (LSD1) with its co-repressor protein, CoREST1, binding to the 3' end (25). Thus, *HOTAIR* can coordinate two complexes to control the state of chromatin. In this way, *HOTAIR* epigenetically regulates the expression of the *HOXD* gene cluster, as well as other genes. However, to the best of our knowledge, no previous study has reported the role of *HOTAIR* in EC.

In the present study, we investigated the expression pattern of *HOTAIR* in EC and investigated the association between *HOTAIR* expression and clinicopathological characteristics. Our results demonstrated that *HOTAIR* was overexpressed in EC and in particular, was highly upregulated in EC that subsequently metastasized and had an unfavorable prognosis. Thus, we evaluated *HOTAIR* as a possible biomarker for the prognosis of EC.

Materials and methods

Patients and samples. Sixty-six fresh-frozen samples were obtained from patients pathologically diagnosed with EC who underwent an initial hysterectomy at International Peace Maternity and Child Health Hospital of the China Welfare Institute, affiliated to Shanghai Jiao Tong University School of Medicine (Shanghai, China) between 2008 and 2012. Thirty normal, age-matched endometrial tissues were obtained from patients who underwent hysterectomy due to benign diseases, such as myoma or adenomyosis. Following surgical excision, the tissue specimens were immediately frozen in liquid nitrogen and stored until total RNA extraction. A total of 129 formalin-fixed paraffin-embedded (FFPE) tissues were collected for *in situ* hybridization (ISH) and immunohistochemical analysis. Apart from the 96 matched frozen cases, the FFPE specimens also included another 21 EC tissues and 12 atypical hyperplasia tissues. The details regarding the patients are presented in Tables I and II. Pathological diagnoses were performed by a gynecologic pathologist according to the classification system of the International Federation of Gynecology and Obstetrics (FIGO). All frozen and FFPE tissues were subjected to hematoxylin and eosin staining to confirm the histological composition. None of the patients enrolled in this study received hormone therapy, radiotherapy, or chemotherapy prior to surgery. This study was approved by the medical Ethics Committee of the International Peace Maternity and Child Health Hospital. Informed consent was obtained from each patient.

Quantitative reverse transcription PCR (qRT-PCR). After grinding the tissues, total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Dalian, China). Quantitative PCR was employed to detect the relative expression levels of target genes using the SYBR Premix ExTaq II kit (Takara) on the Mastercycler ep realplex (Eppendorf, Hamburg, Germany). Each reaction was performed in triplicate using *HOTAIR* primers (forward, 5'-GGTAGAAAAAGCAACCACGAAGC-3' and reverse, 5'-ACATAAACCTCTGTCTGTGAGTGCC-3'). The amplification program was as follows: 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing at 60°C for 34 sec. GAPDH was used as an internal control. The GAPDH primers were as follows: forward, 5'-CGGATTTGG TCGTATTGGG-3' and reverse, 5'-CTGGAAGATGGTGAT GGGATT-3'. Gene expression was calculated using the $2^{-\Delta Ct}$ method.

ISH. ISH was employed to analyze *HOTAIR* expression in the FFPE tissue sections. In brief, the sections were dewaxed, rehydrated and digested with proteinase K. The 5'-digoxigenin-labeled locked nucleic acid-modified *HOTAIR* probe (Exiqon, Vedbaek, Denmark) was diluted in hybridization solution to 30 nM and incubated with a coverslip at 38°C overnight. The following day, after washing, the sections were incubated for 1 h at 37°C with anti-digoxin monoclonal antibody (Boster, Wuhan, China). The sections were then stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolylpho sphate in the dark. After terminating the reaction, the sections were scored. Positive staining for *HOTAIR* expression (in blue) was specifically observed in the cytoplasm. The staining scores were evaluated by the intensity and the proportion of *HOTAIR*-positive cells in 10 random fields under 40 multiple objectives. Positively stained tumor cells were classified as follows: 0, no positive cells; 1, <10%; 2, 10-50%; and 3, >50%. The staining intensity was scored on a scale of 0, no staining; 1, light blue; 2, blue; and 3, dark blue. The staining index (SI) was calculated according to the following formula: SI = staining intensity x proportion of positively stained cells. Using this method, *HOTAIR* expression was evaluated using the SI. An SI score of 6 was used as a cut-off value, as previously described (26). The slides were scored independently by two gynecologic pathologists.

Immunohistochemistry. The deparaffinized sections were rehydrated in an ethanol series using a routine method (27). Briefly, the sections were incubated in three washes of xylene for 5 min each and then incubated in two washes of 75% ethanol for 10 min each. This was followed by incubation in two washes of 95% ethanol for 10 min each and further incubation in two washes of 100% ethanol for 10 min. Heat-mediated antigen retrieval was then performed in EDTA buffer (pH 9.0, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) using microwave treatment for 20 min. Endogenous peroxidase was quenched with 3% hydrogen peroxide and 10% goat serum. After the primary antibody was applied, the

Table I. Expression of *HOTAIR* in different endometrial tissues.

| Group | | *HOTAIR in situ* hybridization | | | |
	n	+	−	Positive rate (%)	p-value
Normal endometrium	30	4	26	13.3	<0.001
Atypical hyperplasia	12	5	7	41.7	
Endometrial carcinoma	87	63	24	72.4	

Table II. Correlation between *HOTAIR* expression and clinicopathological parameters in endometrial carcinoma.

| Clinicopathological parameters | | *HOTAIR in situ* hybridization | | | |
	n	+	−	Positive rate (%)	p-value
Age					
≥55 years	55	40	15	72.7	0.932
<55 years	32	23	9	71.9	
FIGO stage					
I–II	74	51	23	68.9	0.102
III–IV	13	12	1	92.3	
Pathological type					
Endometrioid	80	58	22	72.5	1.000
Non-endometrioid	7	5	2	71.4	
Histological grade					
Grade 1	43	27	16	62.8	0.045[a]
Grade 2	27	21	6	77.8	
Grade 3	10	10	0	100.0	
LNM					
Positive	11	11	0	100.0	0.031[a]
Negative	66	52	24	78.9	
LVS					
Positive	24	22	2	91.7	0.015[a]
Negative	63	41	22	65.1	
Depth of myometrial invasion					
≤1/2	69	46	23	66.7	0.019[a]
>1/2	18	17	1	94.4	
ER expression					
Positive	71	49	22	69.0	0.216
Negative	16	14	2	87.5	
PR expression					
Positive	69	47	22	68.1	0.136
Negative	18	16	2	88.9	
p53 expression					
Positive	15	13	2	100.0	0.219
Negative	72	50	22	70.7	

[a]Statistically significant (p<0.05). FIGO, International Federation of Gynecology and Obstetrics; LNM, lymph node metastasis; LVS, lymphovascular space invasion; ER, estrogen receptor; PR, progesterone receptor.

sections were incubated overnight at 4˚C in a humidified box. The immunohistochemistry reaction was visualized using a Histostain-Plus IHC kit (MingRui-Bio Co., Shanghai, China). Primary antibodies used in this study were anti-estrogen
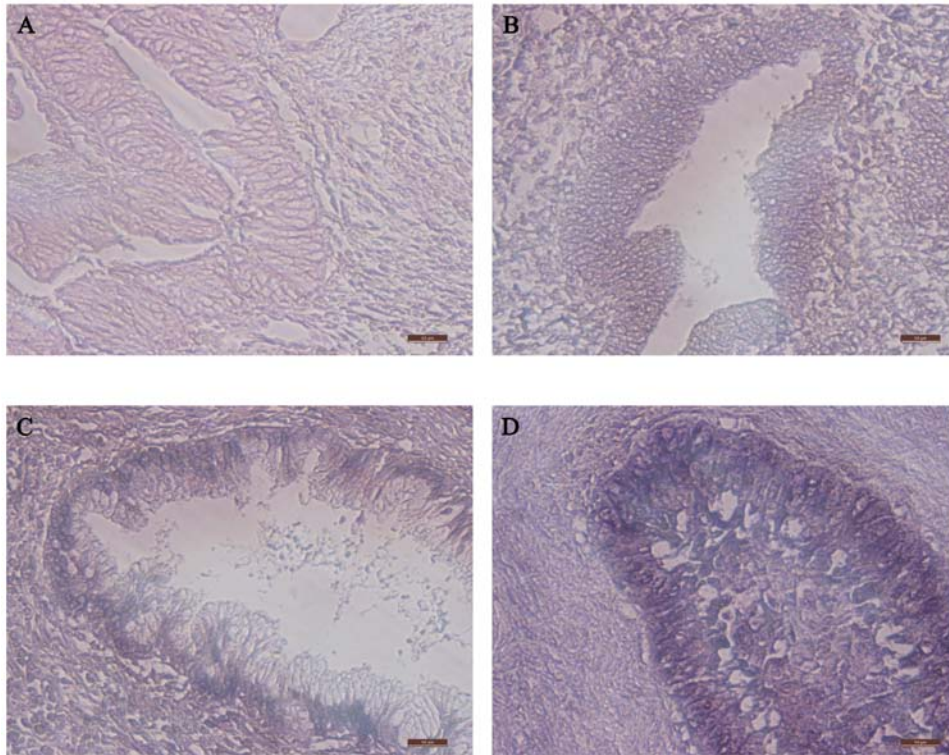
Figure 1. *In situ* hybridization staining of *HOTAIR* expression in (A) normal endometrial, (B) atypical, (C) serous endometrial carcinoma and (D) endometrioid carcinoma tissues.

receptor (ER) (diluted 1:100; Cell Signaling Technology, Danvers, MA, USA), anti-progesterone receptor (PR) (diluted 1:100; Cell Signaling Technology) and anti-p53 (diluted 1:150; Cell Signaling Technology). The positively stained tumor cells were classified as follows: no expression, 0-1%; 1, ≤25%; 2, ≤50%; and 3, >50%. Scores ≥2 were regarded as positive. All slides were visually scored separately scored by two gynecologic pathologists.

lncRNA data mining. An integrated genomic characterization of EC was conducted by The Cancer Genome Atlas (TCGA) network (28). We downloaded Uterine Corpus Endometrial Carcinoma (UCEC) RNA sequencing data from the TCGA website (http://cancergenome.nih.gov/). The clinical information was downloaded from the supplementary file of the TCGA UCEC study (28). The chromosomal locations were obtained from the lncRNA chip annotation file, and the *HOTAIR* probe termed ASHG19A3A000361 was searched. The relative abundance of lncRNAs was calculated from the raw exon call file using the following formula:

$$A = \frac{\sum_{i=1}^{n}(C_i \times L_i)}{\sum_{j=1}^{m}(C_j \times L_j)}$$

Given that there are *m* total exons detected in the given sample, C_j is the read count of the *j* exon and L_j is the length (bp) of this exon. Given that there are *n* exons detected within the boundary of defined lncRNAs (i.e., exons between txStart and txEnd of the above lncRNAs according to the lncRNA annotation files) and in the same strand, C_i is the read count

of the *i* lncRNA-exon and L_i is the length (bp) of this exon. *A* is the abundance of the given lncRNA. To ensure the quality, we only used patient data for which at least one raw lncRNA exon read was detected with RNA sequencing. We found 145 patients that expressed *HOTAIR* from a larger group of patieints with EC with complete clinical data. We then stratified these patients into two groups according to the *HOTAIR* expression level using the median lncRNA abundance as the threshold (low expression, n=83; high expression, n=62).

Statistical analyses. All values are expressed as the means ± SE. All statistical analyses were performed using the SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA). The differences in *HOTAIR* expression were evaluated using one-way ANOVA for three-group comparisons and Student's t-tests for two-group comparisons, whereas the associations between *HOTAIR* expression and clinicopathological characteristics were evaluated with the χ^2 test or Fisher's exact test. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Differences were considered statistically significant at a level of p<0.05.

Results

HOTAIR is overexpressed in EC. To determine the role of *HOTAIR* in the progression of EC, we first detected *HOTAIR* expression in EC samples, atypical endometrial hyperplasia tissues and normal endometrial tissues using ISH (Fig. 1). Almost three quarters of the tissue samples from the patients with EC (63/87; 72.4%) showed postive staining for *HOTAIR* in the cytoplasm of the cancer cells, and almost half of the tissue
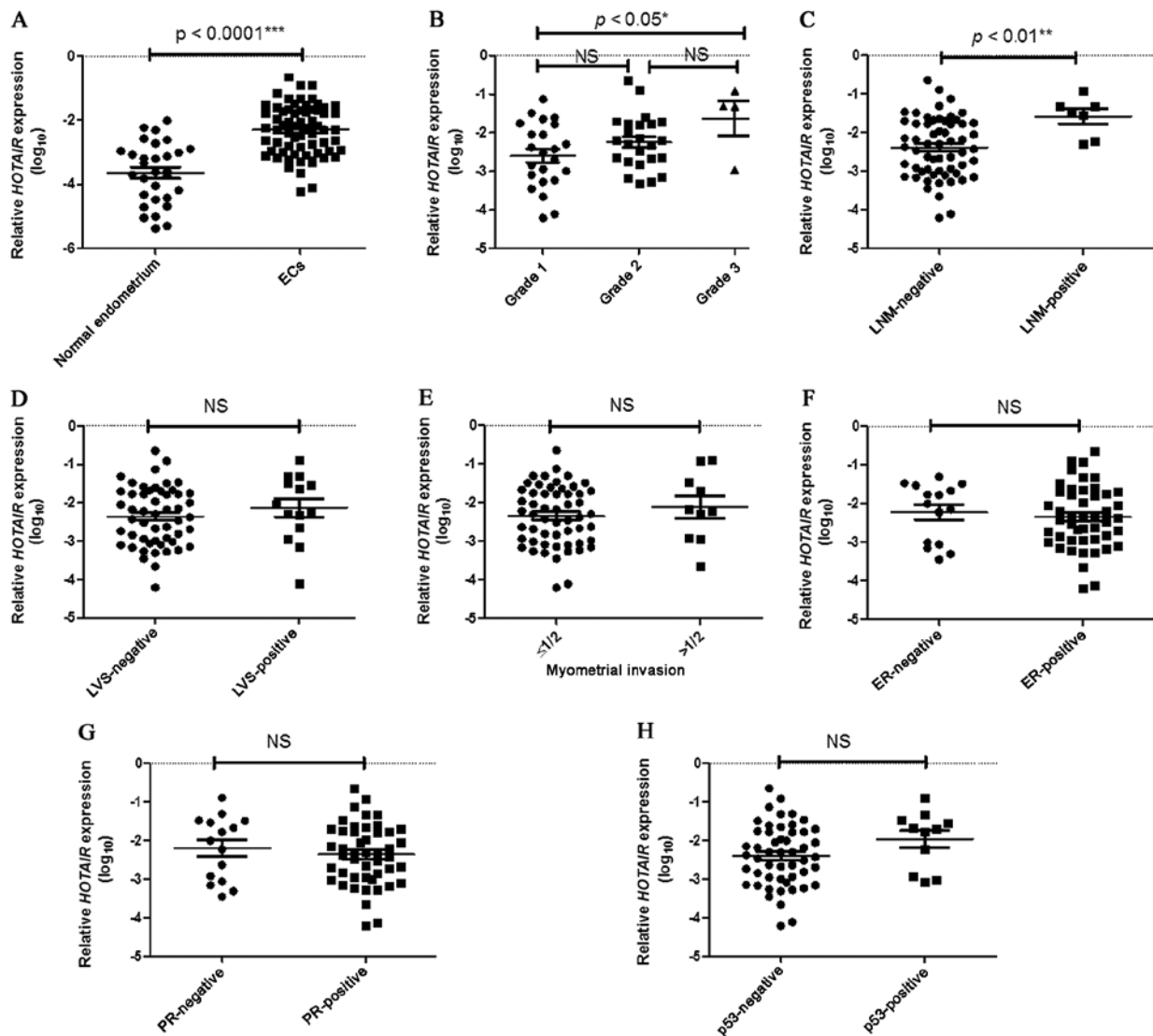
Figure 2. *HOTAIR* expression and clinicopathological characteristics. The expression of *HOTAIR* in clinical samples was analyzed using qRT-PCR. *HOTAIR* expression was compared between (A) normal endometrial and endometrial carcinoma (EC) tissues, (B) histological grade, (C) lymph node metastasis (LNM) status, (D) lymphovascular space invasion (LVS) status, (E) depth of myometrial invasion, (F) estrogen receptor ER expression status, (G) progesterone receptor (PR) expression status and (H) p53 expression status. The relative amount of *HOTAIR* expressino was calculated using the following equation: $\log_{10}[2^{-\Delta Ct}]$, where $\Delta CT = CT_{HOTAIR} - CT_{GAPDH}$. NS, not significant.

samples from patients with atypical hyperplasia were shown to express *HOTAIR* (5/12; 41.7%); however, the majority of the normal endometrial samples did not show postive staining for *HOTAIR* (26/30; 86.7%). *HOTAIR* expression was increased in the atypical tissues and was even higher in the EC samples (p<0.01; Table I). In addition, qRT-PCR verified that *HOTAIR* was significantly upregulated in the majority of the EC tissues, consistent with the results from ISH (Fig. 2).

Correlation between HOTAIR expression and clinicopathological characteristics. To assess the clinical relevance of *HOTAIR* expression in EC, we examined the correlation between *HOTAIR* expression in the FFPE tissues as judged by ISH and clinicopathological parameters (Table II). *HOTAIR* overexpression was markedly associated with tumor grade (p=0.045), lymph node metastasis (LNM) (p=0.031), lymphovascular space invasion (LVS) (p=0.015) and the depth of myometrial invasion (p=0.019), but not with age, FIGO stage,

or pathological type (Table II). ISH also revealed that *HOTAIR* overexpression was clearly linked to the different degrees of differentiation in the EC tissues (Fig. 3). We further evaluated the correlation between *HOTAIR* expression levels as judged by qRT-PCR in fresh-frozen tissues and the clinicopathological characteristics (Fig. 2). Our results indicated that *HOTAIR* expression was higher in grade 3 compared with grade 1 tissues (p<0.05), and *HOTAIR* was more highly expressed in the LNM metastasis group (p<0.01). These data indicate that upregulated *HOTAIR* expression is associated with metastasis in EC. We also examined the correlation between *HOTAIR* expression and immunohistochemical parameters. The immunohistochemical staining revealed no correlation between *HOTAIR* and the expression status of ER (p=0.216), PR (p=0.136), or p53 (p=0.219) (Table II).

Association of HOTAIR with overall survival of patients with EC. After downloading the UCEC RNA sequence data from
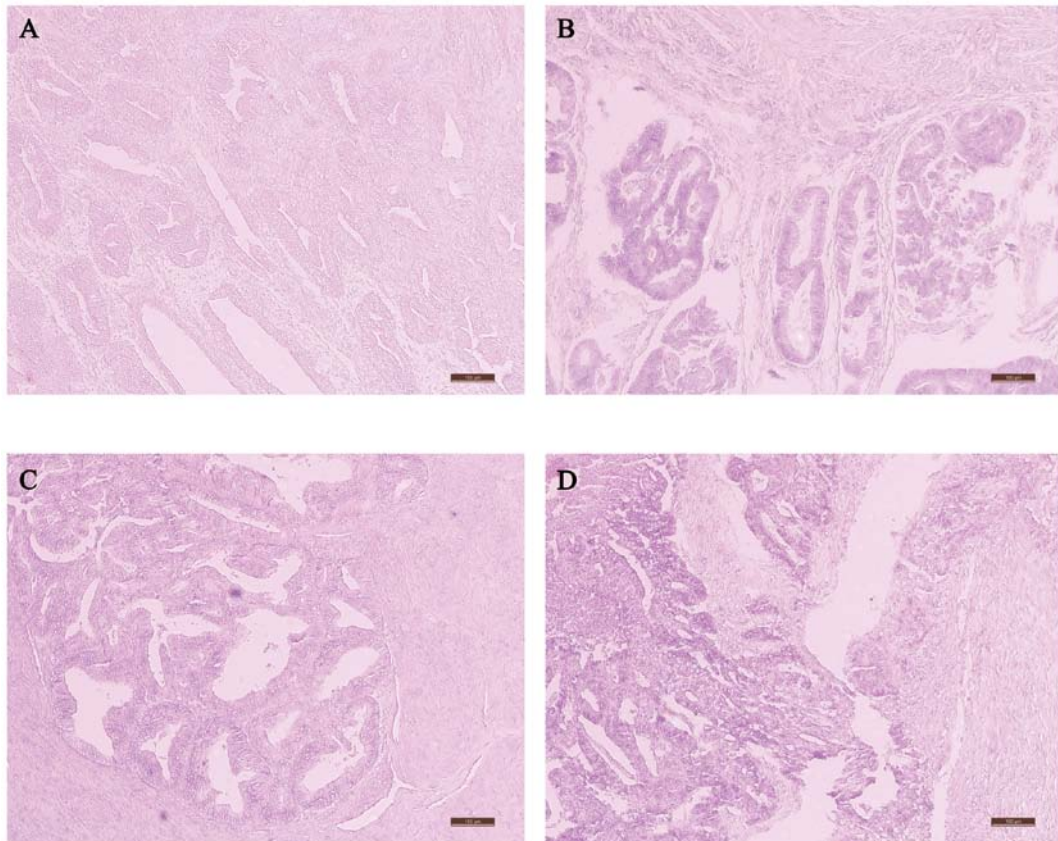
Figure 3. Representative images of *HOTAIR* expression determined by *in situ* hybridization of paraffin-embedded tissues with varying degrees of differentiation. (A) Normal endometrial, (B) grade 1, (C) grade 2 and (D) grade 3 tissue.
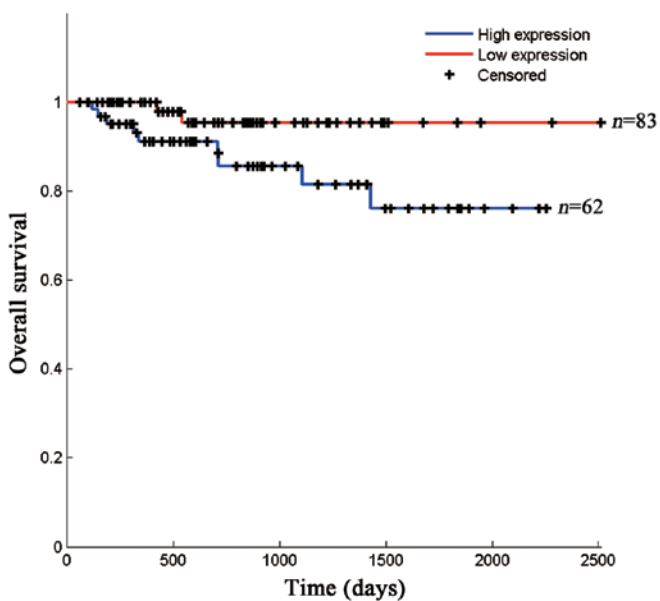

Figure 4. *HOTAIR* levels are associated with the prognosis of endometrial carcinoma (EC). The Kaplan-Meier curves show that high *HOTAIR* expression levels correlated with poor survival in the 145 patients (data obtained from TCGA; 141 endometrioid endometrial carcinomas and 4 mixed serous and endometrioid carcinomas). p=0.034.

the TCGA dataset, we calculated the relative expression abundance of *HOTAIR*. The data of patients from TCGA (n=145)

were stratified according to the median *HOTAIR* abundance into a low expression group (n=83) and a high expression group (n=62). These patients included 141 endometrioid endometrial adenocarcinomas and 4 mixed serous and endometrioid carcinomas. The overall survival curves in the low and high *HOTAIR* mRNA expression groups are shown in Fig. 4. The overall survival was significantly lower in patients with a high *HOTAIR* expression compared with the patients with a low expression (p<0.05).

Discussion

Although the majority of EC cases are treated at an early stage, the disease is still associated with a significantly high mortality rate (29). Thus, further investigation of the pathogenesis of the disease and identification of meaningful prognostic markers is critical. In this study, we demonstrated that *HOTAIR* was more highly expressed in EC tissues than in normal tissues. Moreover, a higher *HOTAIR* expression in EC tissues was associated with metastasis and shorter overall survival. Of note, the level of *HOTAIR* expression estimated using ISH significantly correlated with myometrial invasion and LVS, whereas this correlation was not observed wehn *HOTAIR* expression as estimated using qRT-PCR. We suspect that the reason for this result may be the number of specimens from the different groups. A previous study suggested that FFPE tissues are a valuable resource for RNA studies, including lncRNA studies, as they can be easily stored and are easily obtained (21).

Certainly, compared to PCR, the accuracy of the ISH data is poor. Although the significance of the above data is limited by the shortage of PCR samples and the qualitative accuracy of ISH, we confirmed the trend of a high *HOTAIR* expression in patients with high-grade EC and LNM-positive patients.

To explore the prognostic value of *HOTAIR* in EC, the availability of a large multi-center population and long-term follow-up is essential. Existing company and university databases that are currently not being investigated should be explored. Although the lncRNA data profile is large, the mining approach has been demonstrated to be effective when using a proper algorithm (30). In addition, when using data mining for medical research, the dataset should be large and confirmed as reliable. Applying these considerations, we performed *HOTAIR* profiling of data from a large cohort of EC patients obtained from TCGA (28). These data were strong and well-documented. By analyzing the associations between *HOTAIR* expression and clinical outcomes of pateints with EC, we found that a high *HOTAIR* expression was significantly associated with a reduced overall survival. To the best of our knowledge, this is the first report that correlates *HOTAIR* expression with patient prognosis in EC. Furthermore, the current study also demonstrates that new results from data mining may prove useful in medical research and clinical practice.

Recent studies have suggested that lncRNAs play a major role in oncogenesis. *HOTAIR*, which is transcribed from the HOX genes, is upregulated in breast cancer (17), hepatocellular carcinoma (22), colorectal cancer (23), pancreatic cancer (31), laryngeal squamous cell carcinoma (32) and gastrointestinal stromal tumors (33). Furthermore, a higher *HOTAIR* expression has been shown to negatively regulate metastasis-related suppressor genes and promote tumor malignancy (17). By contrast, the depletion of *HOTAIR* has been shown to reduce the metastatic capacity of tumor cells *in vitro* (22). A previous study demonstrated that *HOTAIR* is also linked to chemotherapeutic sensitivity (22). These authors found that silencing *HOTAIR* expression in HepG2 cells contributes to apoptosis through the tumor necrosis factor-α pathway. Furthermore, they revealed that the knock down of *HOTAIR* expression sensitizes HepG2 cells to cisplatin and doxorubicin (22). These novel findings clearly indicate that *HOTAIR* is an important component of tumorigenesis and that the overexpression of *HOTAIR* is an independent predictor of metastasis and progression-free survival. Our data are consistent with those of previous studies, suggesting that *HOTAIR* promotes progression in various types of cancer.

HOTAIR expression is transcriptionally induced by estradiol (E2) and is potentially regulated by ERs in breast cancer (34). Bhan *et al* (34) found that the *HOTAIR* promoter contains multiple estrogen response elements. Following treatment with E2, *HOTAIR* transcription is activated, and this activation is coordinated by ERs and several ER co-regulators. A recent study by Chisholm *et al* (21) demonstrated that *HOTAIR* is associated with the ER status in breast cancer. However, in our study, significant correlations were not observed between *HOTAIR* and the ER status. Lu *et al* (35) detected *HOTAIR* expression using qRT-PCR in 336 breast cancer patients and found no significant associations with the ER status. Indeed, ER-negative tumors appear to have higher *HOTAIR* levels, although the difference is not statistically

significant (p=0.11) (35). In our study, the level of *HOTAIR* expression was significantly higher in ER-negative AN3CA cells than in ER-positive endometrial cells (data not shown). Thus, future studies are required to investigate the mechanisms of action of *HOTAIR* in EC cells and to determine whether hormones are involved in the expression of *HOTAIR*.

Previous studies (17,25) have demonstrated that *HOTAIR* promotes chromatin reprogramming and affects the expression of various target genes, including those involved in apoptosis, tumorigenesis and cell differentiation. In addition, the overexpression of *HOTAIR* is strongly associated with metastasis and unfavorable prognosis in multiple types of cancer. However, the relevance between the biochemical changes and biological behavior, including invasion and metastasis remains obscure. The processes of metastasis include cancer cell proliferation, local invasion, intravasation, extravasation and colonization. Metastasis is strongly associated with epithelial-to-mesenchymal transition, interaction with inflammatory cells and angiogenesis (36). However, whether *HOTAIR* is involved in the various processes of metastasis and its exact biological role remain unclear.

In conclusion, we found that *HOTAIR* was frequently upregulated in EC and was associated with poor differentiation and LNM. The data from 145 patients with EC were mined, and we demonstrated that *HOTAIR* may be a valuable prognostic molecular marker for EC. However, further studies are required to uncover the underlying mechanisms through which *HOTAIR* governs EC progression and metastasis. *HOTAIR* is a potential target for the development of novel therapies for EC.

Acknowledgements

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