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HOTAIR, a prognostic factor in esophageal squamous cell carcinoma, inhibits WIF-1 expression and activates Wnt pathway

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Long non-coding RNAs (LncRNAs) have been recently found to be pervasively transcribed in the genome and critical regulators of the epigenome. HOTAIR, as a well-known LncRNA, has been found to play important roles in several tumors. Herein, the clinical application value and biological functions of HOTAIR were focused and explored in esophageal squamous cell carcinoma (ESCC). It was found that there was a great upregulation of HOTAIR in ESCC compared to their adjacent normal esophageal tissues. Meanwhile, patients with high HOTAIR expression have a significantly poorer prognosis than those with low expression. Moreover, HOTAIR was further validated to promote migration and invasion of ESCC cells *in vitro*. Then some specific molecules with great significance were investigated after HOTAIR overexpression using microarray and quantitative real time-polymerase chain reaction (qPCR). WIF-1 playing an important role in Wnt/β-catenin signaling pathway was selected and further tested by immunehistochemistry. Generally, inverse correlation between HOTAIR and WIF-1 expression was demonstrated both in ESCC cells and tissues. Mechanistically, HOTAIR directly decreased WIF-1 expression by promoting its histone H3K27 methylation in the promoter region and then activated the Wnt/β-catenin signaling pathway. This newly identified HOTAIR/WIF-1 axis clarified the molecular mechanism of ESCC cell metastasis and represented a novel therapeutic target in patients with ESCC. (*Cancer Sci* 2013; 104: 1675–1682)

Esophageal cancer is one of the most common cancers worldwide.[1,2] Esophageal squamous cell carcinoma (ESCC) is the most prevalent type in eastern countries, including China.[3] Despite the wide application of radical esophagectomy and systemic chemo-radiotherapy, the overall 5-year survival rate of patients with ESCC remains extremely low.[4] Therefore, it is essential not only to quickly identify clinically applicable biomarkers for ESCC prognosis but also to determine the crucial molecular mechanisms associated with this disease.

Long non-coding RNAs (LncRNAs) are a new class of transcripts recently discovered to be pervasively transcribed in the genome and are critical regulators of the epigenome.[5] As with microRNAs, LncRNAs may be useful in predicting tumor prognosis and in regulating tumorigenesis.[6] As a result, LncRNAs have gained attention worldwide.

HOTAIR, a widely focused LncRNA, was initially proposed to be involved in primary breast cancer and breast cancer metastasis.[7] HOTAIR overexpression induces genome-wide targeting of the polycomb repressive complex 2 (PRC2), leading to an altered methylation of histone H3 lysine 27 (H3K27) and gene expression. Clinical studies demonstrated that HOTAIR overexpression is a potential candidate biomarker for predicting tumor recurrence in hepatocellular carcinoma patients who have undergone liver transplant therapy and may be a potential therapeutic target.[8] Consistent with the role in breast tumors, HOTAIR upregulation was also discovered to be a critical element in metastatic progression in colorectal cancer (CRC).[9] Furthermore, frequent HOTAIR upregulation was discovered to be associated with the malignant behavior of gastrointestinal stromal tumors.[10] Overall, numerous studies have clearly demonstrated the importance of HOTAIR in tumors.[7–13] HOTAIR overexpression correlates with poor prognosis and promotes metastasis of the ESCC cell line.[14] However, few studies have examined in detail the molecular mechanisms of HOTAIR in ESCC.

The Wnt/β-catenin signaling pathway is an evolutionarily conserved pathway required for adult tissue maintenance in bone, heart, muscle, and other tissues. In addition, the pathway plays an important role in regulating cell proliferation and migration and in controlling tumor progression. Aberrant activation of Wnt/β-catenin signaling, generally caused by genetic and epigenetic alterations, has been linked to several types of tumors, including ESCC.[15,16] Common epigenetic alterations include DNA hypermethylation in the promoter region of APC, Axin2, SFRPs, Wnt inhibitory factor 1 (WIF-1), etc.[17] WIF-1, as a key inhibitor of the Wnt/β-catenin signaling pathway, binds directly to extracellular Wnt ligands, preventing their interaction with the receptors and leading to degradation of cytosolic β-catenin by the APC/Axin1 destruction complex.[18] Previous studies have determined that the epigenetic silence of WIF-1 due to promoter hypermethylation is a frequent mechanism that causes aberrant activation of the Wnt/β-catenin pathway in several human cancers, as well as in ESCC.[19,20] Generally, WIF-1 downregulation is a prominent characteristic of tumor progressions. However, the epigenetic regulator of WIF-1 and the regulatory mechanism is poorly understood.

In this study, the prognosis value of HOTAIR in ESCC was further measured in a larger clinical cohort. More importantly, it was observed that altered HOTAIR expression was involved in repressing the transcription of WIF-1, thus activating the Wnt/β-catenin signaling pathway. This is the first report that HOTAIR increases the H3K27 methylation in the WIF-1 promoter and induces its silence. Apart from the DNA hypermethylation in the WIF-1 promoter region, trimethylation of H3K27 represented a novel mechanism for epigenetic regulation of WIF-1. This regulatory mode mediated by LncRNA may illustrate the epigenetic alteration of other key molecules.

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Materials and Methods

ESCC samples and cell lines. A total of 137 ESCC tumor tissues and matched adjacent normal esophageal tissues were obtained from patients at the Sun Yat-Sen University Cancer Center between the years 2004 and 2007. The tumor stage was classified according to the tumor node metastasis (TNM) classification of the 6th edition The American Joint Committee on Cancer. The patients included 94 men and 43 women with age ranging from 34 to 80 (mean age: 56 years). All patients recruited to this study did not receive any pre-operative treatments. This study was approved by the Human Ethics Committee of the Sun Yat-Sen University Cancer Center and, all patients signed an informed consent form.

The ESCC cell lines KYSE30, KYSE140, KYSE180, KYSE410 and KYSE510 were obtained from DSMZ, the German Resource Center for Biological Material and grown using standard condition (Data S1).

RNA preparation, reverse transcription and qPCR. Total RNA was extracted from the cell lines and frozen ESCC samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China) and SYBR Premix Ex TaqII kit (Takara) were used to perform reverse transcription and qPCR (Data S1).

RNAi and overexpression HOTAIR, migration and invasion assay. siRNA oligonucleotides targeting HOTAIR or the negative control were transfected into KYSE180 and KYSE140 cells, respectively, as described in the Data S1. Overexpression HOTAIR in KYSE180 and KYSE410 cells was performed by retrovirus mediated gene transfer. For migration and invasion assay, Chamber from Becton Dickson (Bedford, MA, USA) (8 μm pore size) with or without matrigel were used (Data S1).

cDNA microarray and bioinformatic analysis. The cDNA was labeled and hybridized to the 4 × 44K human gene expression microarray (Agilent Technologies, Santa Clara, CA, USA). The threshold set for up- and downregulated genes was a 2.0-fold change. The microarray results are indicated in Table S1. A gene ontology (GO) annotation for Biological Process and Pathway analysis was conducted using DAVID online tools (http://david.abcc.ncifcrf.gov/).[21] A selection of the top five enriched the GO biological process for genes with a *P*-value lower than 0.05. The data were sorted by the number of genes that associated with each GO term and pathway.

Nuclear protein extraction, Western blot, and chromatin immunoprecipitationassay. The nuclear protein fraction was extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit (78835; Pierce Biotechnology, Rockford, IL, USA) from KYSE180-vector cells and KYSE180-HOTAIR cells, according to the manufacturer's instructions. For Western blot, antibodies against WIF-1 (sc-373780; Santa Cruz, CA, USA), β-catenin (06-734; Millipore, Bedford, MA, USA) and GAPDH (D16H11, CST) were used. A chromatin immunoprecipitation (CHIP) assay was conducted using EZ-ChIP (17-371; Millipore), according to the manufacturer's instruction. Retrieved DNA was detected by PCR reaction. The primers for the WIF-1 promoter region are listed in Data S1.

Tissue microarray construction and immunohistochemistry (IHC). The tissue samples (98 of the 137 ESCC tissues that mentioned before) were collected, fixed in formalin and embedded in paraffin. The HE-stained sections from a single random block of each specimen were reviewed by a senior pathologist to define the representative tumor. Two cores of each sample were obtained using a tissue array instrument (ALPHELYS, Plaisir, France). Immunohistochemistry (IHC) was performed based on a previously described method.[22]

Immunofluorescent confocal analysis. The cells were probed using a β-catenin primary antibody (06-734; Millipore) and Alexa Fluor 488 secondary antibodies (Invitrogen). Nuclear DNA were stained with DAPI. Confocal fluorescent images were captured using an Olympus FV500 microscope under a 60× oil objective.

Statistical analysis. Statistical analysis was performed using the Stata 10.0 statistical software package (Stata; College Station, TX, USA). Receiver operating characteristic (ROC) curves were used to determine the cutoff value for the HOTAIR high and HOTAIR low groups in this study. The gene expression levels of HOTAIR in tumors were compared with normal adjacent mucosa using the Wilcoxon test, whereas the associations between HOTAIR expression and clinical characteristics were evaluated using the chi-square test. Survival curves were estimated using the Kaplan–Meier method. The log-rank test was used to estimate the significant differences between the survival curves. A 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. In addition, a multivariate Cox regression was performed to adjust for other covariates. A two-tailed *P*-value of 0.05 or less was considered to be statistically significant.

Results

HOTAIR expression and clinicopathologic factors in ESCC. Upregulation of HOTAIR was frequently detected in ESCC tissues (Fig. 1a). Approximately 79% (108/137) of the tumor tissue expression of HOTAIR was >1.5-fold higher than the corresponding normal tissues. There was no significant association of the expression with gender, age, tumor location (upper/middle/lower), T status as indicated in Table 1. However, an association between HOTAIR expression and histologic grade (G1/G2/G3) or Nodal status approached statistical significance ($P = 0.080$ and $P = 0.074$ for histologic grade and Nodal status, respectively; Table 1). The 137 patients were then divided into HOTAIR-high ($n = 90$) and low groups ($n = 47$),

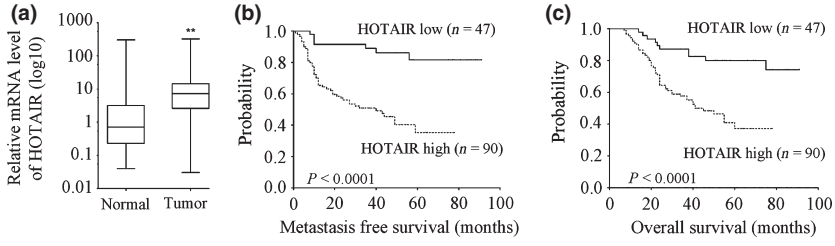

Fig. 1. HOTAIR is upregulated in esophageal squamous cell carcinoma (ESCC) tissues and have prognostic value for metastasis and death. (a) Box plot analysis, based on quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis expression of HOTAIR in 137 paired ESCC tumor samples and corresponding normal esophageal tissues. Kaplan–Meier curves for metastasis-free survival (b) or overall survival (c) of the same 137 ESCC tissues measured in (a). $^{**}P < 0.005$.

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Table 1. HOTAIR expression and clinicopathological characteristics in esophageal squamous cell carcinoma (ESCC)

Characteristics	Case number	HOTAIR expression		*P*-value
		High	Low	
Gender				
Male	94	70	24	0.497
Female	43	33	10	
Age				
≤56	94	72	22	0.324
>56	43	31	12	
T status				
T1-2	33	26	7	0.775
T3-4	104	77	27	
N status				
N0	73	52	21	0.074
N1/2/3	64	51	13	
Histologic grade				
G1	39	17	22	0.080
G2	66	24	42	
G3	32	6	26	
Tumor location				
Upper	13	8	5	0.843
Middle	89	60	29	
Lower	35	22	13	

Table 2. Multivariate analysis of risk factor for death and metastasis as the first recurrence event in esophageal squamous cell carcinoma (ESCC; Cox proportional hazards regression model)

Risk factors	Death			Metastasis		
	HR	*P*-value	95% CI	HR	*P*-value	95% CI
HOTAIR expression (low/high)	3.16	0.002	1.53–6.52	4.47	<0.001	1.99–10.06
Age (≤56/>56)	1.43	0.201	0.83–2.48	1.17	0.58	0.67–2.06
Gender (male/female)	0.98	0.955	0.55–1.75	0.80	0.47	0.44–1.47
Histologic grade (G1/G2/G3)	1.07	0.732	0.74–1.53	1.24	0.26	0.85–1.79
Tumor location	0.78	0.319	0.47–1.28	1.04	0.89	0.62–1.73
T status (T1-2/T3-4)	0.90	0.728	0.50–1.64	0.89	0.73	0.47–1.69
N status (N0/N1,2,3)	3.18	<0.001	1.76–5.77	2.19	0.008	1.23–3.93

HR, hazards ratio; 95% CI, 95% confidence interval.

according to the ROC curve method. A high expression level of HOTAIR is a significant predictor of subsequent metastasis and death ($P < 0.0001$ for both metastasis and death, Fig. 1b, c). Compared with the low expression group, the 5-year overall survival rates were 46% in the high expression group and 81% in the low expression group. Moreover, multivariate analysis indicated that HOTAIR expression was an independent prognostic indicator for metastasis and death ($P < 0.001$ and $P = 0.002$ for metastasis and death, respectively, Table 2).

HOTAIR promoted the migration and invasion of ESCC cell lines *in vitro*. Given upregulation of HOTAIR was significantly associated with distant metastasis in patients with ESCC, we inhibited HOTAIR function via small interfering RNAs (siRNAs) in KYSE180 and KYSE140, two cell lines that express HOTAIR (Fig. S1). HOTAIR siRNAs decreased their migration and invasion ability (Fig. 2a,c). Conversely, the ESCC cell lines KYSE180 and KYSE410 (KYSE410 cells has relative low HOTAIR expression, Fig. S1) with stable HOTAIR expression demonstrated greater cancer cell migration and invasion (Fig. 2d–h) without affecting the cell proliferation of both cell lines (Fig. S2). Based on our results, the overexpression of HOTAIR promoted the migration and invasion of ESCC cells *in vitro*.

A wide range of gene expressions was altered after HOTAIR overexpression. Gene expression profiling of HOTAIR overexpression in KYSE180 cells on cDNA microarrays (44K human microarrays; Agilent Technologies) indicated that HOTAIR modulates the transcriptional regulation of 847 genes (427 upregulated, 420 downregulated; Table S1). Gene ontology analysis indicated that significant numbers of upregulated genes were associated with biological processes such as intracellular signaling and cell adhesion, whereas downregulated genes were clustered in biological processes such as ion transport, homeostatic processes, immune response, response to organic substance and cell proliferation (Fig. 3a). Consistently, pathway analysis revealed that 11 genes were enriched in the WNT/β-catenin signaling pathway, which is usually activated and promotes metastasis of ESCC (Fig. 3b). To further identify the possible target genes of HOTAIR, we downloaded and analyzed microarray data collected for HOTAIR RNAi gastrointestinal stromal tumor (GIST) cells (GIST-T1) from Gene Expression Omnibus (GEO). Forty-three potential target genes overlapped in the two microarray data (Fig. 3c), among which WNT5B and WIF-1 are important regulatory molecules in the WNT/β-catenin signaling pathway. In addition, seven genes, which have been reported to be related to ESCC, were also validated by qRT-PCR. WIF-1 had the most significance difference (fold change 13.9, $P < 0.005$) and the only one that expression been suppressed by HOTAIR overexpression (Fig. S3).

HOTAIR promoted H3K27 trimethylation in the WIF-1 promoter region and was inversely correlated with WIF-1 expression. Consistent with the qRT-PCR results, the protein level of WIF-1 was greatly decreased after HOTAIR overexpression (Fig. 4a). Conversely, the knock down of HOTAIR by siRNA restored the mRNA level of WIF-1; however, the protein level slightly changed (Fig. 4b). A Dual Luciferase reporter assay was performed to further demonstrate the inverse correlation between HOTAIR and WIF-1 expression. Compared to the KYSE180-vector group, a significant decrease of luciferase fluorescence intensity was observed in the KYSE180-HOTAIR group (Fig. S4). Besides, it has been reported that EZH2 was the main component of PRC2, and HOTAIR exerting its function was PRC2 dependent. After PRC2 depletion, there was a great increase of WIF1 mRNA expression. Correspondingly, migration ability was greatly decreased (Fig. S5). After adding recombinant human WIF-1, it could significantly inhibit HOTAIR-induced migration and invasion. These results strongly indicated that HOTAIR-induced activity was WIF-1 dependent (Fig. S6). The inverse correlation between HOTAIR and WIF-1 expression was further validated in 16 ESCC clinical samples using real time qRT-PCR (Fig. 4c). Furthermore, immunohistochemistry analysis of WIF-1 expression in 98 ESCC tissues revealed that WIF-1 expression was significantly inversely correlated with HOTAIR expression (Fig. 4d,e). Spearman correlation analysis indicated there was a negative correlation between HOTAIR expression and WIF-1 mRNA levels ($r = -0.365$, $P < 0.001$). Based on the regulatory mechanism in breast and colorectal cancer, the levels of histone H3K27 trimethylation in KYSE180-Vector and

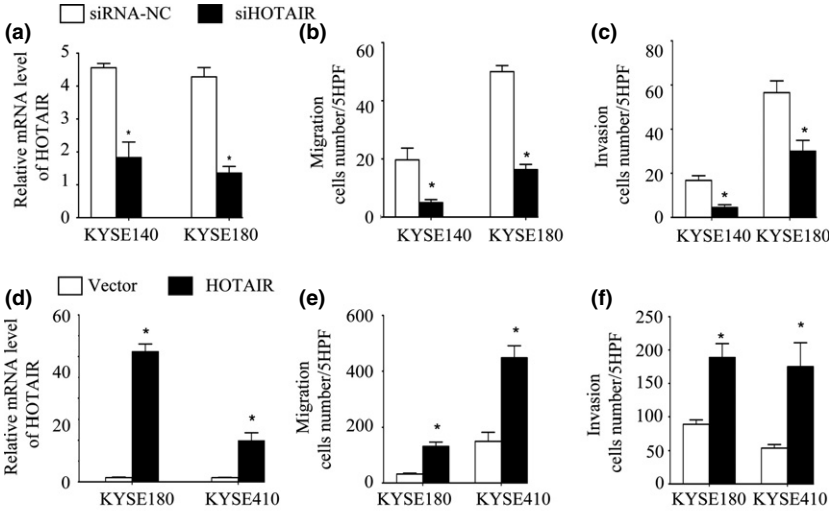

Fig. 2. HOTAIR promotes migration and invasion of esophageal squamous cell carcinoma (ESCC) cells *in vitro* (a), siRNA mediated knock down of HOTAIR in KYSE140 and KYSE180 cells transfected with negative control (siRNA-NC) or siRNA target HOTAIR (siRNA-HOTAIR) (error bars = SD, $n = 3$). The relative mRNA level was normalized to the endogenous genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (b, c) Migration (b) and invasion (c) assay in the KYSE140 and KYSE180 cells that transfected with siRNA in (a) (error bars = SD, $n = 3$). HPF, high power field. (d) qPCR of relative mRNA level of HOTAIR in retrovirus infected KYSE180 cells and KYSE410 cells. (error bars = SD, $n = 3$). (e–h). Migration (e, g) and invasion (f, h) assay after enforced HOTAIR expression in KYSE180 and KYSE410 cells (error bars = SD, $n = 3$). $^*P < 0.05$.

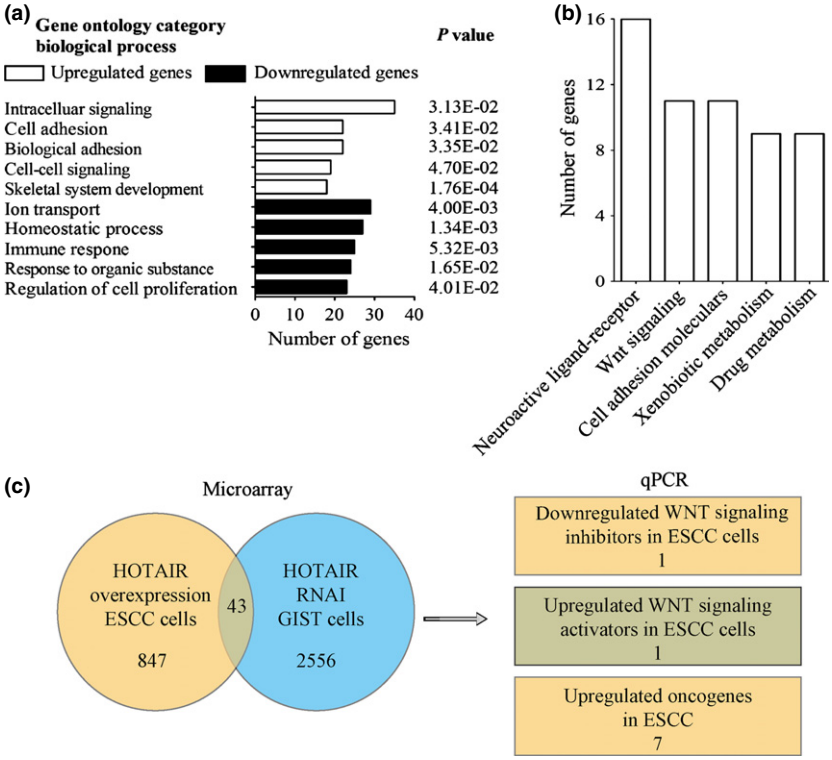

Fig. 3. The expression of target genes regulated by HOTAIR. (a) Gene ontology analysis of HOTAIR overexpression microarray data in KYSE180 Cells using the DAVID program. (b) Pathway analysis of HOTAIR overexpression microarray data in KYSE180 Cells using the DAVID program. (c) Schematic depicting integrative approach that led to identification of putative HOTAIR target genes. Transcriptomic profiling of genes upregulated by greater than twofold upon HOTAIR overexpression in KYSE180 cells were overlapped with genes down-regulated by more than twofold upon HOTAIR RNAI in GIST cells.

KYSE180-HOTAIR cells were measured. Chromatin immunoprecipitation (CHIP) clearly demonstrated that there was a significant increase in H3K27 trimethylation in the WIF-1 promoter (Fig. 4f).

HOTAIR, targeting WIF-1, activated Wnt/β-catenin signaling pathway. After HOTAIR overexpression, the presence of β-catenin was reduced on cell membrane and increase accumulation in the cell nucleus (Fig. 5a). The concentration of β-catenin in the cell nucleus and cytoplasma plus cell membrane were measured, and increased β-catenin expression was observed in the nucleus after HOTAIR overexpression by Western blot analysis (Fig. 5b). Accumulation of β-catenin in cell nucleus indicated the activation of canonical Wnt/β-catenin pathway. Then expressions of some downstream target

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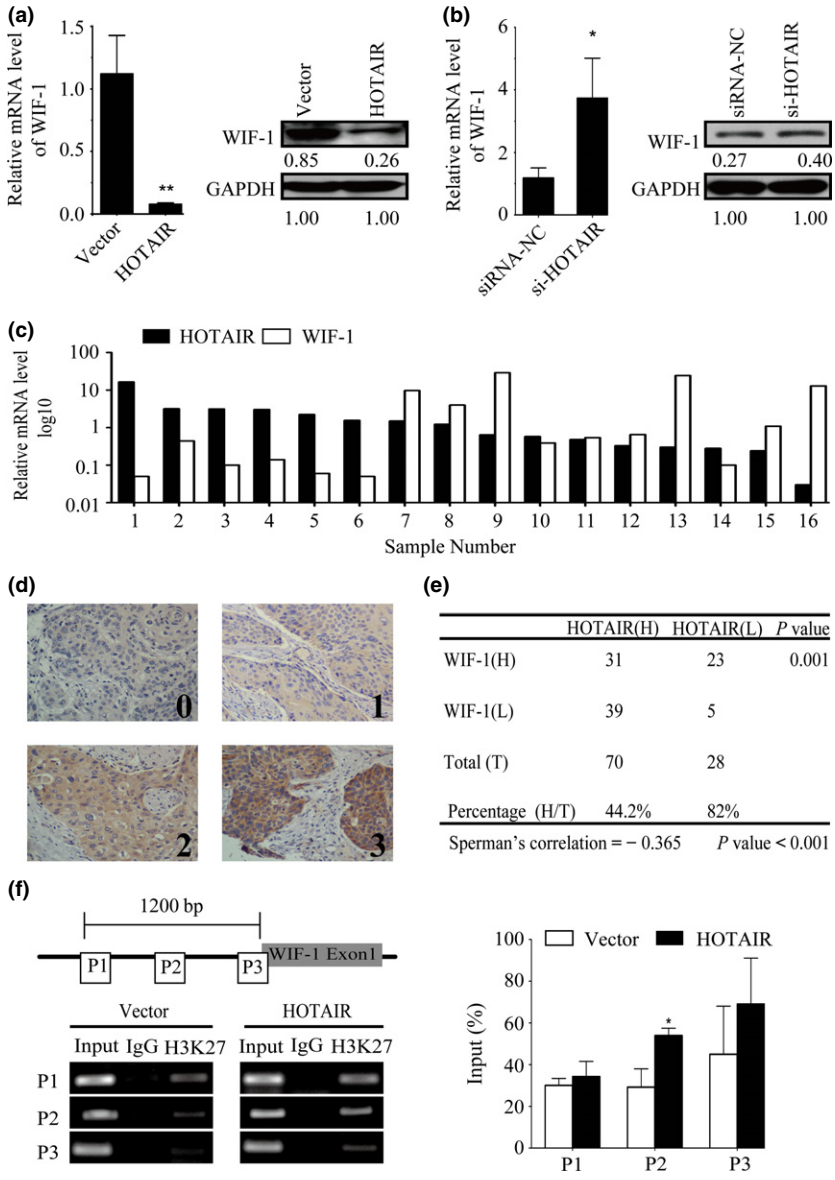

	HOTAIR(H)	HOTAIR(L)	*P* value
WIF-1(H)	31	23	0.001
WIF-1(L)	39	5	
Total (T)	70	28	
Percentage (H/T)	44.2%	82%	
Sperman's correlation = − 0.365		*P* value < 0.001	

Fig. 4. The mechanism of HOTAIR that promotes cell migration and invasion (a, b) Analysis mRNA level of WIF-1 by qRT-PCR and protein level by Western blot in HOTAIR overexpression KYSE180 cells (a) and HOTAIR RNAI KYSE180 cells (b) (error bars = SD, $n = 3$). (c) Analysis mRNA level of both WIF-1 and HOTAIR by qRT-PCR in 16 esophageal squamous cell carcinoma (ESCC) tumor tissues. The Relative mRNA level was normalized to the endogenous genes GAPDH. (d) WIF-1expression by immunohistochemical staining (IHC). The images show four different tumor samples with different cytoplasmatic staining intensities gainst the WIF-1 protein. The IHC-score of 0, 1, 2 or 3 is shown at the lower right corner of ach image. The examples are representative for the whole set of samples (magnification: 200). (e) The results were calculated on the basis of analyses performed using 98 ESCC issue samples. The relationship between WIF-1 and HOTAIR expression was compared sing Spearman's correlation coefficient. (f) CHIP assay in KYSE180-Vector or HOTAIR ells. Primers locations are indicated in the WIF-1 schematic. The PCR products were analyzed by gel electrophoresis and quantitated by Gel Analyzer (error bars = SD, $n = 3$). *$P < 0.05$, **$P < 0.005$.

genes such as ZEB1, SNAIL and MMP13, playing important roles in tumor progression, were all increased in the HOTAIR overexpressed cells. These results further demonstrated the activation of Wnt/β-catenin pathway (Fig. 5c). Based on our results, it was suggested that HOTAIR, along with PRC2, inhibited WIF-1 expression by increase H3K27 trimethylation in the WIF-1 promoter and then activated Wnt/β-catenin signaling pathway (Fig. 5d).

Discussion

In this study consisting of 137 patients and in a previous study consisting of 78 patients,[14] HOTAIR was demonstrated as an independent prognostic indicator of ESCC (Fig. 1). Compared with the previous study, evaluating HOTAIR in another clinical cohort in our study will greatly increase its value in clinical application. In fact, HOTAIR overexpression was also discovered to be associated with poor prognosis in other cancers.[7–13] It was believed that this tendency would be demonstrated in more cancers. This common feature strengthened the clinical value of HOTAIR.

It was reported that HOTAIR, in collaboration with PRC2, reprogrammed the chromatin state and regulated the expression of hundreds of genes by epigenetic regulation to promote cancer metastasis. Considering the regulatory complexity and diversity in different types of tumors, it was necessary to conduct a systematic study in ESCC. Consistent with the previous report in ESCC, HOTAIR overexpression promotes migration and invasion in ESCC cell lines (KYSE180 and KYSE410). Compared with the single knockdown experiment, stable overexpression and a corresponding knockdown experiment in this study fully validated this phenomenon (Fig. 2).

The expression of 847 genes changed after HOTAIR overexpression in ESCC cells (Fig. 3a). Pathway analysis based on these genes by DAVID online tools revealed 11 genes were involved in WNT signaling (Fig. 3b). Alteration of WNT signaling regulators including WIF-1 and WNT5B were both demonstrated by microarray and qRT-PCR (Figs 3c,S3). These results suggest that HOTAIR may affect the Wnt/β-catenin signaling cascade by epigenetic regulation. In addition, FOXQ1, FGF12 and CXCR7, which contribute to ESCC progression, were also detected and validated in our study.

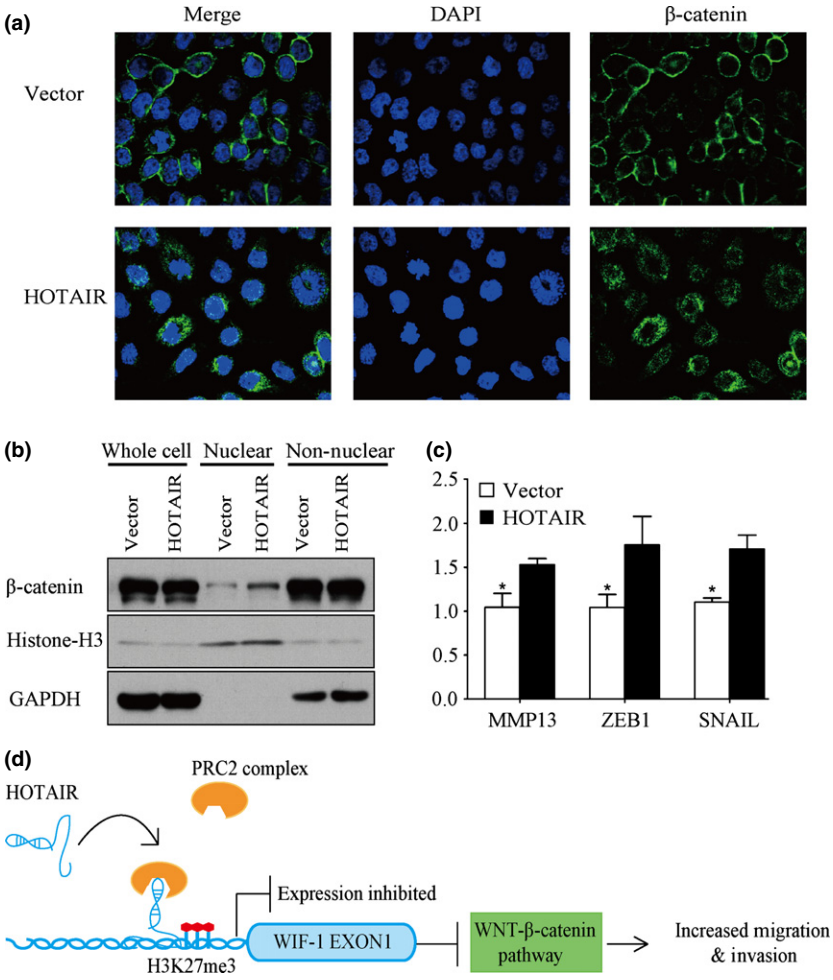

Fig. 5. HOTAIR promoted accumulation of β-catenin in cell nucleus. (a) Immunofluorescence staining of β-catenin in KYSE180-Vector cells and KYSE180-HOTAIR cells. Blue, DAPI; green, β-catenin; ×60. (b) Western-blot analysis of β-catenin in cell nucleus and cytoplasma plus cell membrane in KYSE180-Vector cells and KYSE180-HOTAIR cells. GAPDH was used as the control in cytoplasm and histone-H3 was used as the control in nucleus. Results in three experiments are similar. (c) Expressions of some downstream target genes such as ZEB1, SNAIL and MMP13 were measured by Q-PCR. (d) Proposed model illustrating the effect of HOTAIR in the esophageal squamous cell carcinoma (ESCC).

Epigenetic disruptions, including promoter CpG island methylation and histone modification of tumor-related genes, have been identified as key events in cancer development. So far, many critical genes silenced by promoter methylation in ESCC have been reported.[23] It is worth noting that the expression of WNT signaling-related genes such as APC, SFRP1/2, WNT5A and WIF-1 were all changed after promoter region methylation.[17,24–27] However, the mechanism leading to this abnormality is unclear. The inverse correlation between HOTAIR overexpression and decreased WIF-1 expression was validated in both ESCC cells and tissues (Fig. 4a–e). In this study, we further confirmed that HOTAIR overexpression promoted H3K27 methylation in promoter region of WIF-1 (Fig. 4f). H3K27 trimethylation is characteristic of Polycomb group (PcG) target genes and is associated with transcriptional repression.[28] The PcG protein such as PRC2 complex catalyzes H3K27 methylation.[29] We suggest that HOTAIR bound by PRC2 inhibits the expression of WIF-1. Although a direct interaction between HOTAIR and PRC2 in ESCC cells is not yet reported, the abnormal PRC2 expression has been associated with various cancers including ESCC,[30,31] and HOTAIR was proved to directly interact with PRC2 in types of tumors.[7,9,32] Consistently, a significant increase of WIF-1 mRNA expression and decrease of migration ability were observed in our experiment (Fig. S5). The HOTAIR/WIF-1 regulatory model identified in this study provides a novel view on WIF-1 regulation. In fact, WIF-1 downregulation has been reported to be involved in progression in types of tumors including ESCC.[27,33,34] Furthermore, Rubin *et al.*[35] have reported that inhibition of WIF-1 could trigger Wnt/β-catenin signaling and thereby promotes tumor invasion and migration. In our experiments, exogenously added recombinant human WIF-1 protein in HOTAIR overexpression ESCC cells inhibited the migration and invasion ability (Fig. S6). However, we did not find the correlation between WIF-1 protein level and prognosis of patients with ESCC. Based on this regulation model, further functional investigation is required for exploring the epigenetic alterations of other genes related to ESCC and exploring these genes in other HOTAIR-overexpression cancers.

β-catenin, as a significant transcription factor, acts as the main effector of the canonical WNT signaling cascade.[36] Cytoplasmic β-catenin is degraded by a multiprotein degradation complex when the WNT signaling pathway is inactive. Conversely, β-catenin evades degradation, accumulates in the cytoplasm and finally translocates to the nucleus during WNT signaling pathway activity, thus exerting its transcriptional activity. It was hypothesized that HOTAIR overexpression activated the WNT signaling pathway by decreasing WIF-1 expression. As expected, there was a great increase in the amount of β-catenin in the cell nucleus after HOTAIR overexpression (Fig. 5a,b). Increased expression of some target genes such as ZEB1, SNAIL and MMP13 further illustrated the activation of canonical WNT/β-catenin signal (Fig. 5c). Our results demonstrated the activation of the Wnt/β-catenin signaling pathway by inhibiting WIF-1 expression after HOTAIR overexpression. Despite limitations, our results provided convincing insight into the mechanisms underlying HOTAIR-promoted migration and invasion of ESCC cells (Fig. 5d). It had been reported that non-canonical Wnt

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signaling through Wnt5B promoted cell migration in a previous study.[37] Actually, we did observe that a significant increase of WNT5B expression after HOTAIR overexpression in KYSE180-HOTAIR cells (Fig. S3). However, further study showed that WNT5B depletion did not have an effect on cell migration (Fig. S7). Therefore, it was thought that HOTAIR induced cell metastasis was through canonical Wnt pathway.

Apart from promoting migration and invasion, increased levels of β-catenin can also initiate transcriptional activation of proteins such as cyclin D1 and c-myc, which control the G1 to S phase transition in the cell cycle. However, we did not observe a significant increase of proliferation after HOTAIR overexpression (Fig. S2). In mechanism, the activity of HOTAIR is at least in part due to interaction with PCR2, which enhances H3K27 trimethylation to decrease expression of multiple genes.[7] Previously, studies of HOTAIR in breast cancer, colon cancer and pancreatic cancer and so on indicated that HOTAIR-dependent gene regulation differed significantly among different cancer cells and was very complex. For example, an expanded function of HOTAIR in pancreatic cancer cells involving cell cycle progression was observed, compared to its function in breast cancer.[30] However, the regulation of HOTAIR on cell cycle in ESCC cells was not very clear. It was concluded that a balance underlying cell proliferation may not be broken. However, further functional investigation is needed to be conducted in the future.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Materials and methods.

Fig. S1. Relative expression of HOTAIR in esophageal squamous cell carcinoma (ESCC) cell lines detected by reverse transcription-polymerase chain reaction (RT-PCR).

Fig. S2. Effects of HOTAIR on the proliferation of KYSE180 and KYSE410 cells.

Fig. S3. HOTAIR induced gene expression changes in KYSE180 cells.

Fig. S4. Overexpression of HOTAIR inhibits WIF-1 promoter activity.

Fig. S5. WIF-1 de-repressed after concomitant EZH2 RNAi in KYSE180-HOTAIR cells.

Fig. S6. HOTAIR-induced migration and invasion were inhibited by exogenous recombinant human WIF-1.

Fig. S7. Concomitant WNT5B RNAi in KYSE180-HOTAIR cells.

Table S1. cDNA microarray data in KYSE180-vector cells and KYSE180-HOTAIR cells.

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