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Upregulated expression of HOXC8 is associated with poor prognosis of cervical cancer

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Abstract. Homeobox C8 (HOXC8) is a transcription factor that has been reported to regulate numerous genes associated with tumor progression. However, its function in cervical cancer (CC) remains to be elucidated. In the present study, the expression level of HOXC8 was examined in CC tissues and cell lines using reverse transcription-quantitative polymerase chain reaction and western blot analysis. Additionally, CC cell lines were transfected with small interfering RNAs (siRNAs) to downregulate the expression of HOX8 and assess cell proliferation using Cell Counting Kit-8. The results demonstrated a significantly increased expression of HOXC8 in CC tissues and cell lines compared with non-tumor tissues, and a normal cervical cell line, respectively. Additionally, the downregulation of HOXC8, which was achieved by siRNA transfection, significantly inhibited the proliferation rate of CC cell lines. Kaplan-Meier curves demonstrated that the increased expression of HOXC8 was associated with poor prognosis of patients with CC. Additionally, univariate and multivariate analysis revealed that HOXC8 was a significant and independent predictor for overall survival of patients with CC. In conclusion, the results of the present study suggest that HOXC8 may be involved in the progression of CC and may serve as a therapeutic target for CC.

Introduction

Cervical cancer (CC) is the third most common type of cancer among females and the second leading cause of cancer-associated mortality worldwide (1). The incidence and mortality for CC are relatively low in developed countries compared with that in developing or undeveloped countries (2). Although the methods for detection and treatment for CC have been significantly improved, the mortality rate of patients with malignant CC remains high (3,4). Additionally, the molecular mechanisms underlying the development of CC remain poorly understood. Previous studies have demonstrated that aberrant expression of several proteins is associated with the proliferation and apoptosis of CC cell lines (5,6). Therefore, the identification of genetic alterations that may be associated with the progression of CC may provide insights for the development of novel therapeutic strategies.

The homeobox (HOX) gene family, which contains a total of 39 members, encode homeodomain-containing transcription factors that serve essential functions in cellular development and differentiation (7,8). Aberrant expression of HOX genes, including HOXA9, HOXB4, and HOXC10 in CC has been reported in previous studies (9-11), suggesting that HOX genes may serve an important function in tumorigenesis. HOXC8 is one of the 39 members of the HOX family proteins and is overexpressed in several types of human cancer, including colon (12), lung (13), prostate (14), and breast cancer (15). Several studies investigated the molecular mechanisms by which HOXC8 contributes to tumorigenesis. HOXC8 may exert its oncogenic function through regulating the expression of other cancer-associated genes. For example, HOXC8 may promote tumorigenesis by regulating the expression of cadherin-11 in breast cancer (15). Additionally, the expression of HOXC8 may be regulated by microRNA (miR)-196a (16). To the best of our knowledge, the association between the expression of HOXC8 and the progression of CC has not yet been investigated. Therefore, the present study aimed to examine the expression of HOXC8 and the clinical significance of HOXC8 expression in CC.

In the present study, the expression of HOXC8 was upregulated in CC tissues and cell lines. The association between the expression of HOXC8 and clinicopathological features was examined. Furthermore, HOXC8 expression may be used as an independent predictor for the overall survival of patients with CC. Additionally, downregulation of HOX8 decreased the proliferation rate of CC cells. Therefore, HOXC8 may be used as a potential therapeutic target for CC.

Materials and methods

Patients. A total of 36 patients (mean age, 56.7 years; range, 39-71 years) diagnosed with CC who underwent resection in the Department of Gynecology, the First Affiliated Hospital of Fujian Medical University (Fujian, China) were recruited

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between February 2006 and November 2011. None of the patients received anticancer treatment including radiotherapy or immunotherapy. Tumor tissues were obtained from patients with CC. Adjacent normal epithelial tissues were used as controls. The tissues were frozen in liquid nitrogen following surgery and stored at -80˚C. The clinicopathological features of patients with CC are presented in Table I. The study was conducted according to the declaration of Helsinki. The study was approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University (Fujian, China). Written informed consent was obtained from all patients. Overall survival was defined as the period of time between surgery and mortality.

Cell lines and culture. Human CC cell lines SiHa and HT-3 and normal cervical cell line Crl-2614 were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.). All cell lines were incubated at 37˚C in a humidified atmosphere containing of 5% CO_2.

Transient transfection. In order to downregulate the expression of HOXC8 in CC cell lines, a siRNA targeting HOX8 was used in the present study, which were synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China). The sequences used were as follows: HOXC8-siRNA, 5'-CAACAC TAACAGTAGCGAA-3'; negative control-siRNA: 5'-UGC AACAUCACGGAAUCAUTT-3'. For transfection, a total of $1x10^5$ cells/well were seeded into six-well plates and 2.5 nmol siRNAs were added to each well. Transfection was conducted using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 48 h of culture, cells were used for subsequent experiments.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the tissues and cell lines using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was quantified using the Nanodrop ND-2000 (Thermo Fisher Scientific, Inc.). RNA was reverse-transcribed into cDNA using the First Strand cDNA Synthesis kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. The primer sequences used in the present study were as follows: HOXC8 forward, 5'-CACGTCCAAGACTTCTTCCACCAC GGC-3' and reverse, 5'-CACTTCATCCTTCGATTCTGG AACC-3'; GAPDH forward, 5'-TGATGACATCAAGAAGGT GGTGAAG-3' and reverse, 5'-TCCTTGGAGGCCATGTGG GCCAT-3'. RT-qPCR was performed using the StepOne Plus Real-Time PCR system (Thermo Fisher Scientific, Inc.) with SYBR Premix Ex Taq™ II (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. The thermocycling conditions were as follows: Initial denaturing step at 94˚C for 5 min, followed by 30 cycles at 94˚C for 45 sec and final extension at 56˚C for 45 sec. The expression of HOXC8 was normalized to GAPDH and evaluated using the $2^{-\Delta\Delta Cq}$ method (17). The 75th percentile of HOXC8 expression level was used as cut-off point (1.14) to stratify patients into high or low HOXC8 expression group.

Table I. Association between HOXC8 expression and clinicopathological features of patients with CC.

		HOXC8 expression level		
Variable	Cases	High	Low	P-value
Age, years				
≥50	21	15	6	0.121
<50	15	9	6	
Lymph node metastasis				
Negative	16	9	7	0.074
Positive	20	15	5	
Tumor differentiation				
Poor	23	16	7	0.033
Well/moderate	13	8	8	
Tumor stage				
I-II	17	13	4	0.038
III	19	11	8	

HOXC8, Homeobox C8; CC, cervical cancer.

Western blot analysis. Total protein samples were extracted from the tissues and cell lines using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Total protein was quantified using a bicinchoninic acid assay. Equal amount of protein (50 μg) was separated using SDS-PAGE (10% gel) and transferred onto polyvinylidine diflouride membranes (EMD Millipore, Billerica, MA, USA). Membranes were then blocked with 5% fat-free milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) at room temperature for 1 h and incubated with the following primary antibodies: mouse Anti-HOXC8 monoclonal antibody (1:1,000; cat. no. sc-517007; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse anti-GAPDH monoclonal antibody (1:1,000; cat. no. sc-47724; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. Membranes were washed three times with TBST. Following the primary incubation, membranes were incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature, according to the manufacturer's protocol. Protein bands were visualized using enhanced chemiluminescence reagent (Beyotime Institute of Biotechnology). Densiometric analysis of the bands was performed using ImageJ software (version 1.43; National Institutes of Health, Bethesda, MD, USA).

Cell Counting Kit-8 (CCK-8). Cell proliferation was evaluated using a CCK-8 assay. Cells transfected with HOXC8-siRNA or negative control-siRNA were seeded into 96-well plates at a density of ~$2x10^3$ cells/well in a volume of 100 μl for 0, 24,

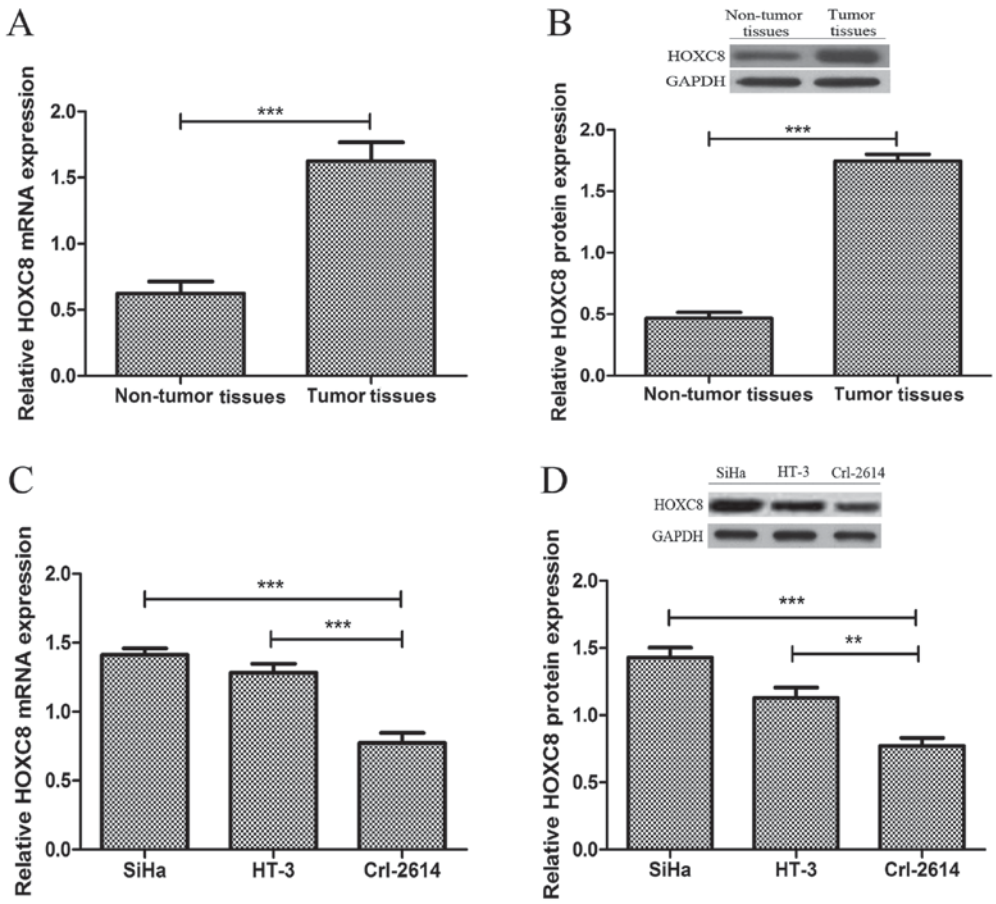

Figure 1. HOXC8 is upregulated in cervical cancer. (A) The expression of HOXC8 in cervical cancer tissues was evaluated using RT-qPCR. (B) The expression of HOXC8 in cervical cancer tissues was evaluated using western blot analysis. (C) The expression of HOXC8 in cervical cancer cell lines (SiHa and HT-3) and normal cervical cell line (Crl-2614) was evaluated using RT-qPCR. (D) The expression of HOXC8 in cervical cancer cell lines (SiHa and HT-3) and normal cervical cell line (Crl-2614) was evaluated using western blot analysis. **P<0.01; ***P<0.001. HOXC8, Homeobox C8; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

48, and 72 h and cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere containing of 5% CO_2. At indicated timepoints, 10 μl CCK-8 solution (Beyotime Institute of Biotechnology) was added to each well and incubated for 1 h. The absorbance of each well was measured using a microplate reader at 450 nm. Three individual experiments were performed.

Statistical analysis. Data were analyzed using SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA. The relevant data are expressed as the mean ± standard deviation. Statistical significance between two groups was determined using a Student's t-test. One-way analysis of variance followed by post hoc analysis with Tukey's test was used to examine differences among multiple groups. The chi-square test was used to analyze the associations between HOXC8 expression and clinicopathological features. Overall survival of patients with CC was evaluated using Kaplan-Meier estimator analysis and log-rank test. Univariate and multivariate Cox regression models were used to identify independent prognostic factors associated with the overall survival of patients with CC. Three individual experiments were performed. P<0.05 was considered to indicate a statistically significant difference.

Results

HOXC8 is highly expressed in CC tissues and cell lines. The expression of HOXC8 in CC tissues and adjacent non-tumor tissues was evaluated using RT-qPCR, and western blot analysis. As presented in Fig. 1A, mRNA expression levels of HOXC8 was significantly increased in CC tissues compared with that in adjacent non-tumor tissues (P<0.001). Western blot analysis confirmed that the expression level of HOXC8 was also significantly increased in CC tissues compared with that adjacent non-tumor tissues (P<0.001; Fig. 1B). Patients were divided into two groups based on the expression levels of HOXC8: HOX8 high (n=24) and low (n=12) groups. The expression of HOXC8 was also evaluated in CC cell lines, HiHa and HT-3, and normal cervical cell line Crl-2614. As presented Fig. 1C and D, the mRNA and protein expression of HOXC8 was increased significantly in both CC cell lines compared with that in normal cervical cell line. These results suggest that HOX8 may serve an important function in CC.

Effects of HOXC8 expression on cell proliferation. In order to assess the effect of the expression of HOXC8 on cell proliferation, SiHa and HT-3 cells were transfected with HOXC8-specific and negative control-siRNAs. The cell proliferation rate was evaluated using a CCK-8 assay.

Table II. Univariate and multivariate analyses of overall survival rate.

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
HOXC8	2.453	1.053-5.176	0.038	2.534	1.107-5.796	0.028
Age	2.119	0.836-5.370	0.114	-	-	-
Lymph node metastasis	2.213	0.894-5.478	0.086	-	-	-
Tumor differentiation	2.249	1.015-4.985	0.046	2.446	1.050-5.701	0.038
Tumor stage	2.313	1.059-5.052	0.035	2.578	1.144-5.807	0.022

HR, hazard ratio; CI, confidence interval; HOXC8, homeobox C8.

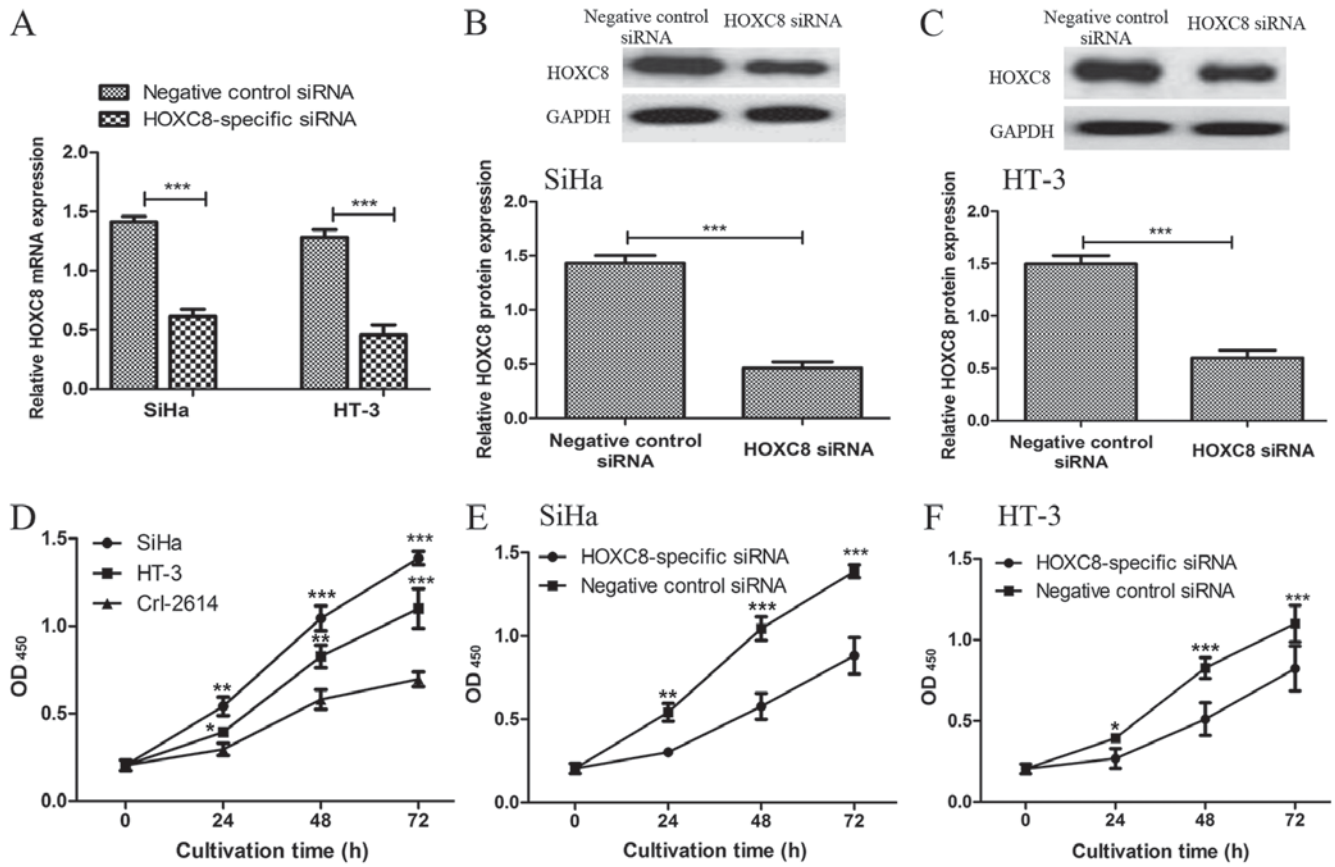

Figure 2. Downregulation of HOXC8 decreases cervical cancer cell proliferation *in vitro*. (A) Downregulation of HOXC8 in cervical cancer cell lines (achieved by using HOXC8-specific siRNA) was confirmed using RT-qPCR. (B) Downregulation of HOXC8 in SiHa cell line (achieved by using HOXC8-specific siRNA) was confirmed using western blot analysis. (C) Downregulation of HOXC8 in HT-3 cell line (achieved by using HOXC8-specific siRNA) was confirmed using western blot analysis. (D) Cell Counting Kit-8 assay was used to determine the cell proliferation of cervical cancer cell lines (SiHa and HT-3) and normal cervical cell line (Crl-2614). (E) Cell Counting Kit-8 assay was used to determine the cell proliferation of cervical cancer cell line SiHa following transfection with HOXC8-specific and negative control-siRNAs. (F) Cell Counting Kit-8 assay was used to determine the cell proliferation of cervical cancer cell line HT-3 following transfection with HOXC8-specific and negative control-siRNAs. *P<0.05, **P<0.01, ***P<0.001. HOXC8, homeobox C8; siRNA, small-interfering RNA; OD, optical density; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Downregulation of HOXC8 (achieved by transfection with HOXC8-specific siRNA) in CC cell lines was confirmed using RT-qPCR (Fig. 2A) and western blot analysis (Fig. 2B and C). The results demonstrated that the proliferation rates of SiHa and HT-3 cells were significantly increased compared with that in the normal cervical cell line at the time points investigated (Fig. 2D). Additionally, transfection using HOXC8-specific siRNA significantly downregulated the proliferation rate of CC cell lines at the time points investigated (Fig. 2E and F). These results suggest that HOXC8 may be involved in the progression of CC by regulating cell proliferation.

Association between the expression of HOXC8 and clinicopathological features of patients with CC. The clinicopathological features of all patients are summarized in Table I. The results demonstrated that increased expression of HOXC8

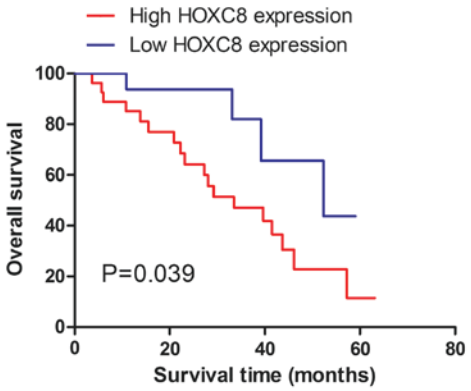

Figure 3. Overall survival rate of patients with cervical cancer. Patients in the high HOXC8 expression group (n=24) exhibited a shorter 5-year survival rate compared with those in the low HOXC8 expression group (n=12) as assessed using Kaplan-Meier curves. HOXC8, homeobox C8.

in CC was significantly associated with tumor differentiation (P=0.033) and tumor stage (P=0.038; Table I). However, no significant association was identified between increased expression of HOXC8 and other parameters, including age and lymph node metastasis (P>0.05; Table I).

Association between the expression of HOXC8 and overall survival rate of patients with CC. The association between the expression of HOXC8 and overall survival of patients with CC was evaluated using Kaplan-Meier survival curves, and the log-rank test. The expression of HOXC8 was significantly associated with overall survival of patients with CC (P=0.039; Fig. 3), suggesting that patients with CC with increased expression of HOXC8 exhibited a poor 5-year overall survival compared with those with low expression of HOXC8. Additionally, independent predictor factors for the overall survival of CC patients with CC were examined using univariate and multivariate Cox regression models. The results demonstrated that the expression of HOXC8 (P=0.028), tumor differentiation (P=0.038) and tumor stage (P=0.022) were identified as independent prognostic factors for the overall survival of patients with CC (Table II).

Discussion

Previous studies have reported the upregulation of HOXC8 in various types of cancer suggesting a possible function in tumor progression (12-15). Additionally, the molecular mechanisms underlying HOXC8-mediated tumor progression have been investigated over the last decades (18-20). Emerging evidence suggested that silencing of HOXC8 in cancer cells may inhibit cell proliferation and migration *in vitro* (19,21). These findings suggest that HOXC8 may serve an essential function in the development and progression of cancer. A previous study has investigated the expression of HOXC8 in CC cell lines, but the clinical significance of HOXC8 in CC remains unknown (22).

In the present study, the expression of HOXC8 was examined in CC tissues and cell lines using RT-qPCR, and western blot analysis. The results demonstrated that the mRNA expression level of HOXC8 was significantly upregulated in CC tissues and cell lines compared with non-tumor tissue, and the normal cervical cell line. Additionally, the protein expression level of HOXC8 was examined using western blot analysis and was also identified to be significantly increased in CC tissues and cell lines. Therefore, the results demonstrated that HOXC8 may serve an important function in the development of CC. Additionally, overall survival of all enrolled patients with CC was examined using Kaplan-Meier method and log-rank test. The results demonstrated that patients with increased expression of HOXC8 exhibited significantly shorter overall survival compared with those with low expression of HOXC8. Additionally, the expression of HOXC8 was associated with clinicopathological features of patients with CC, including tumor differentiation and tumor stage, suggesting that HOXC8 may be involved in the progression of CC. Univariate and multivariate analyses were performed using Cox's proportional hazards model and revealed that HOXC8 may be an independent predictor for the overall survival of patients with CC, thus suggesting an important function of HOXC8 in CC.

The ability of HOXC8 to regulate the proliferation of CC cells was also examined. The results demonstrated that CC cell lines exhibited significantly increased proliferation rates compared with that in the normal cervical cell line. Additionally, downregulation of HOXC8, which was achieved by HOXC8-specific siRNA, significantly decreased the proliferation rate of CC cell lines. Therefore, these results indicated that HOXC8 may contribute to tumor progression by promoting cell proliferation. However, it would also be interesting to investigate whether the ectopic expression of HOXC8 serves a role in normal cervical cell lines, to examine whether or not HOXC8 can promote tumor initiation.

The results of the present study demonstrated that HOXC8 was significantly overexpressed in CC tissues. In addition, the overexpression of HOXC8 was identified to be associated with tumor differentiation and tumor stage, and indicated poor prognosis for CC. Therefore, HOXC8 may be used as a potential therapeutic target in CC. However, a limitation of the present study was the small cohort size used. Therefore, further large-scale studies are required to confirm these conclusions and investigate the underlying molecular mechanisms of HOXC8 in CC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YH designed the study. YH, LC and AG performed all experiments. YH and AG collected and interpreted the data. LC and AG wrote the manuscript, and YH revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University (Fujian, China), and written informed consent was obtained from all patients.

Consent for publication

This manuscript does not contain any identifying information for the enrolled patients.

Competing interests

The authors declare that they have no competing interests.

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