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Long noncoding RNA HOXD-AS1 regulates proliferation of cervical cancer cells by activating Ras/ERK signaling pathway

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Abstract. – OBJECTIVE: To investigate the effects of HOXD cluster antisense RNA 1 (HOXD-AS1) in cervical cancer and its underlying mechanism.

PATIENTS AND METHODS: Real-time quantitative polymerase chain reaction (RT-qPCR) was used to examine the expression of HOXD-AS1 in human cervical cancer tissues. x2-test was used for analyzing the association of HOXD-AS1 expression and clinical parameters. Cell viability, colony formation capacity, and phosphorylation of extracellular regulated protein kinases 1/2 (ERK1/2) in treated HeLa and CaSki cells were detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay, colony formation assay, and Western blot analysis, respectively.

RESULTS: The results indicated that HOXD-AS1 was upregulated in cervical cancer cells significantly. Meanwhile, HOXD-AS1 expression was involved in tumor-node-metastasis stages, lymphovascular invasion, lymph node metastasis, as well as recurrence. HOXD-AS1 knockdown remarkably suppressed cervical cancer cell proliferation, colony formation capacity, and the Ras/ERK signaling pathway *in vitro*. Furthermore, xenograft assays confirmed the results *in vivo*.

CCONCLUSIONS: Our data elucidate that silencing HOXD-AS1 remarkably suppresses cell growth by inactivating the Ras/ERK pathway in cervical cancer, providing a more detailed understanding of cervical cancer pathogenesis and providing a possible theoretical foundation for long non-coding RNA for the diagnosis and therapy for cervical cancer.

Key Words:
Cervical cancer, Long noncoding RNA, HOXD-AS1, Ras/ERK signaling pathway.

Introduction

All over the world, cervical cancer has reported to be the third most popular cancer, as well as the fourth leading cause of malignancy related mortality in females[1]. Resulting from the progression of cervical screening programs, the morbidity and mortality of cervical cancer has reduced significantly, but it still remains to be a major problem for women health, especially in advanced patients[2,3]. Nowadays, as cell migration and invasion are important for the progression of cervical cancer, a majority of studies focus on exploring tumor-specific markers which can predict the biological behavior of cancer cells, because cell migration and invasion abilities are important in the progression of cancer[4]. To seek for better diagnosis and therapy methods, more researches are needed for the understanding of the molecular mechanisms involved in the development and progression of this disease.

Of late years, little non-coding RNAs, such as microRNAs, the small interfering RNAs (siRNA) and PIWI-interacting RNAs (piRNAs), have drawn a lot of attention of researchers. Among these, microRNAs have played a very important role via degrading mRNA or regulating gene expression[5]. Along with deep investigations into miRNAs and development of human genomics, various long non-coding RNAs have been explored and revealed. Long non-coding RNAs (lncRNAs) is a kind of cellular inner RNA, which cannot encode proteins, but play important roles in regulating various

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cellular activities, such as chromosome silence, genome imprinting, chromatin modification, transcriptional activation, post-transcriptional regulation and protein regulation[6-8]. According to published papers, lncRNAs have played vital roles in progression and development of various human cancers[9-11].

The homeotic (HOX) genes are the key developmental regulators in numerous processes, including apoptosis, receptor signaling, and differentiation[12,13]. Dysregulation of HOX genes is frequently related to malignancy and plays important roles in oncogenesis and tumor suppression[13]. LncRNA HOXD cluster antisense RNA 1 (HOXD-AS1) is transcribed from the HOXD cluster on the chromosome 1q31.2, which is one member of HOX gene clusters[14]. As reported in various cancers like neuroblastoma, adenocarcinoma, as well as breast cancer, the expression of HOXD-AS1 is higher than normal tissues and it is closely related to cancer progression and prognosis of patients[14]. Nevertheless, little is reported about how HOXD-AS1 works in cervical cancer and its underlying mechanism.

In the current paper, results demonstrated that lncRNA HOXD-AS1 expression was markedly increased in cervical cancer cell lines. Moreover, we discovered that silencing HOXD-AS1 inhibited both cervical cancer cell proliferation and colony formation. In addition, this study showed that HOXD-AS1 knockdown dramatically inhibited the Ras/ERK signaling pathway in. The studies further revealed that HOXD-AS1 knockdown inhibited tumorigenesis of cervical cancer cells *in vivo*. Our results provided a supplement on the possible function of lncRNA HOXD-AS1 in cervical cancer cells, as well as its involvement in regulating the Ras/ERK pathway, indicating the potential application of HOXD-AS1 to conquer cervical cancer.

Patients and Methods

Patients and Tissues

Altogether 122 biopsy samples of cervical cancer were collected from Navy General Hospital of People's Liberation Army. All tissues were verified by two independent clinical pathologists. The characteristics of patients with cervical cancer are presented in Table I. The tissues were obtained under the condition of signed informed consents from all patients. At the same time, this procedure was performed with approval of the Ethics Committee of Navy General Hospital of People's Liberation Army.

Cell Culture

Six human cervical cancer cell lines (including HeLa, CaSki, ME-100, C33A and SiHa), as well as normal human ovarian cell line HOSE were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All of the cells used in the study were grown and maintained in Roswell Park Memorial Institute 1640 (RPMI-1640) Gibco (Grand Island, NY, USA) with 10% fetal bovine serum (FBS) Gibco (Grand Island, NY, USA) under the condition of atmosphere containing 5% CO_2 at 37°C.

Cell Transfection

siRNAs specially targeting HOXD-AS1 (HOXD-AS1 siRNA1 and HOXD-AS1 siRNA2) and negative control siRNA (control siRNA) were obtained from GenePharma Co. Ltd. (Shanghai, China). After cell adherence on the 6-well plates, HeLa and CaSki cells were transiently transfected with HOXD-AS1 siRNAs or matched controls by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The subsequent experiments were carried out 48 h after transfection.

RT-qPCR

TRIzol reagent (TaKaRa, Dalian, Liaoning, China) was used to obtain total RNA of tissues and cells. After testing the quantity if RNA using an ultraviolet spectrophotometer, RT-PCR kit (TaKaRa, Dalian, Liaoning, China) was used for the reverse transcription reactions. The messenger RNA (mRNA) expression analysis of HOXD-AS1 and U6 were performed via RT-qPCR using a standard SYBR Green PCR kit (TaKaRa, Dalian, Liaoning, China) according to the manufactures' protocol. Primes of HOXD-AS1 and U6 were obtained from TaKaRa (Dalian, Liaoning, China), and U6 was used as an internal control.

Colony Formation Assay

Transfected or non-transfected HeLa and CaSki cells (500 cells/well) were evenly put in six-well plates to allow culturing for 14 days. Afterwards, cells were fixed using 4% paraformaldehyde (Sigma-Aldrich, Springfield, MO, USA) for 15 min. Later on, cells were stained using Giemsa (Sigma-Aldrich, Springfield, MO, USA) for another 30 minutes, and pictures were taken via an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Western Blot Analysis

RIPA lysis buffer (Beyotime, Shanghai, China) was applied to extract the total proteins from cells. Protein concentration was tested via a bicinchoninic acid assay (BCA) Protein Assay Reagent Kit (Beyotime, Shanghai, China). Afterwards, 40 ug proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to the polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk for 1 h at room temperature, the PVDF membranes were incubated with the primary antibodies against Ras, p-ERK1/2, t-ERK1/2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 4°C. After incubation overnight, the PVDF membranes were washed with PBST and the incubated in secondary antibodies for 1 hour at room temperature. Later on, phosphate-buffer solution-tween-20 (PBST) was used to wash the PVDF membranes again, and then electrochemiluminescence was used to visualize the protein bands. GAPDH was used as an internal control. The primary antibodies against Ras, p-ERK1/2, t-ERK1/2 and GAPDH were obtained from CST Technology (Danvers, MA, USA).

Tumor Xenografts of Nude Mice

6-week old female BALB/c nude mice obtained from SLRC Company (Shanghai, China) were randomly divided into two groups. A volume of 3×10^6 HeLa cells (in 100 μL PBS) transfected with HOXD-AS1 siRNA1 or control siRNA were prepared and subcutaneously inoculated into the nude mice. Seven days later, size of tumor xenografts was estimated for every 7 days using a caliper. After 21 days, the tumor-bearing mice were sacrificed and weighted. Subsequently, the expressions of HOXD-AS1, Ras, p-ERK1/2 and ERK1/2 in excised tumor samples were detected by RT-qPCR and Western blot analysis.

Statistical Analysis

SPSS 18.0 (SPSS Inc., Chicago, IL, USA) software was used for statistical analyses. All data are listed as mean ± standard deviation (SD) from individual experiments. *t*-test for two groups and ANOVA (followed by Least Significant Difference as its Post Hoc Test) for more than two groups were used for calculation. Meanwhile, x^2-test was used to examine the association of HOXD-AS1 expression and clinical parameters. A $p<0.05$ was thought as statistical significance.

Table I. Association between HOXD-AS1 expression and clinical parameters of patients with cervical cancer.

Clinical parameters	HOXD-AS1		*p*-value
	Low expression (n = 55)	High expression (n = 67)	
Age (years)			0.363
≤60	25	36	
>60	30	31	
Stage			0.001
I	25	12	
II	16	15	
III	8	17	
IV	6	23	
Histology			0.511
Squamous cell	15	13	
Adenomatous	16	17	
Mixed	14	18	
Small cell	10	19	
Lymphovascular invasion		0.004	
YES	20	42	
NO	35	25	
Lymph node metastasis			0.013
YES	5	18	
NO	50	49	
Recurrence			
YES	3	16	0.005
NO	52	51	

Results

Association Between HOXD-AS1 Expression and Clinical Parameters of Cervical Cancer Patients

For the purpose of finding the role of HOXD-AS1 in cervical cancer progression, the expression of HOXD-AS1 in 122 cervical cancer tissues were examined first via RT-qPCR. Afterwards, x^2-test was applied to evaluate the association between HOXD-AS1 expression and clinical parameters of the patients (Table I). Statistical analysis showed that HOXD-AS1 expression in cervical cancer patients was greatly correlated with tumor-node-metastasis (TNM) stages, lymphovascular invasion, lymph node metastasis, as well as tumor recurrence ($p<0.05$). However, no significant difference was seen in the rest parameters: age and histologic differentiation ($p>0.05$).

HOXD-AS1 Knockdown Suppresses Cervical Cancer Cell Growth in vitro

In order to get more detailed understanding of the biological significance of HOXD-AS1 in the tumorigenesis of cervical cancer, we also performed RT-qPCR assays on HOXA11-AS expression levels in six different cell lines, one of which was derived from human normal ovarian cells (HOSE), and five of which were derived from human cervical cancers. We found that the level of HOXA11-AS was higher in epithelioid cervical carcinoma (HeLa), epidermoid cervical carcinoma established from a metastasis in the small bowel mesentery (CaSki), and squamous cervical carcinoma (SiHa) cells than in epidermoid cervical carcinoma (ME-180) and HPV negative cervical carcinoma (C33A) cells (Figure 1A). Consequently, HeLa and CaSki cells were selected for the follow-up experiments. As a result of high expression of HOXD-AS in cervical cancer cells, siRNA mediated knockdown of HOXD-AS1 in HeLa and CaSki cells was performed to examine the impact ofHOXD-AS1 on cell viability and colony formation ability. As shown in Figure 1B and 1C, HOXD-AS1 siRNA1 and HOXD-AS1 siRNA2 significantly downregulatedHOXD-AS1 expression in HeLa and CaSki cells compared with control siRNA group, confirming the knockdown efficiency of siRNAs. Besides, a significant decrease in cell viability at 48 and 72 h was observed inHOXD-AS1 siRNA-treated HeLa (Figure 1D) and CaSki (Figure 1E) cells compared with the control siRNA-transfected cells. Furthermore, HOXD-AS1knockdown remarkably reduced colony numbers of HeLa (Figure 1F) and CaSki (Figure 1G) cells compared with control siRNA group. These data suggested that the HOXD-AS1 knockdown suppresses cervical cancer cell growth *in vitro*.

HOXD-AS1 Knockdown Repressed Cervical Cancer Cell Growth by Inactivating the Ras/ERK Pathway in vivo

HeLa cells transfected with HOXD-AS1 siRNA1 or control siRNA were subcutaneously injected into nude mice for xenografts to further examine the tumor-suppressing effect of HOXD-AS1 siRNA in cervical cancer *in vivo*. The results of *in vivo* xenograft assays showed that HOXD-AS1 knockdown significantly inhibited xenograft tumor volume at the indicated time (Figure 2A) and xenograft tumor weight (Figure 2B) in comparison with that of control siRNA group. Besides, the expressions of HOXD-AS1 (Figure 2C), Ras and phosphorylation of ERK1/2 (Figure 2D) in excised tumor samples were significantly reduced by HOXD-AS1 knockdown. These data indicated that HOXD-AS1 knockdown repressed cervical cancer cell growth by inactivating the Ras/ERK signaling pathway *in vivo*.

Discussion

Long noncoding RNAs have attracted more and more attention in recent years. They have been reported to play a vital role in cancer, as numerous papers have conformed their role in tumor progression, metastasis and drug resistance[15-17]. Long noncoding RNAs are a kind of noncoding RNAs that have no capacity in coding proteins, and they are transcripts of at least 200 nucleotides. Till now, there are limited reports about the regulatory functions of lncRNAs in human diseases, not like miRNAs. Many lncRNAs are capped, spliced, and polyadenylated, like their protein coding counterparts[18]. The expression of long noncoding RNAs are different in different tissues. In recent years, more and more researches have focused on the biological function of lncRNAs, and results suggest that it plays a critical role in numerous physiological processes[19]. As a result, lncRNAs may serve as biomarkers and targets for various cancers, and regulating the expression of lncRNAs may influence development and progression of cancer[20].

HOXD-AS1 is a kind of lncRNA, which has been reported to be related to the development

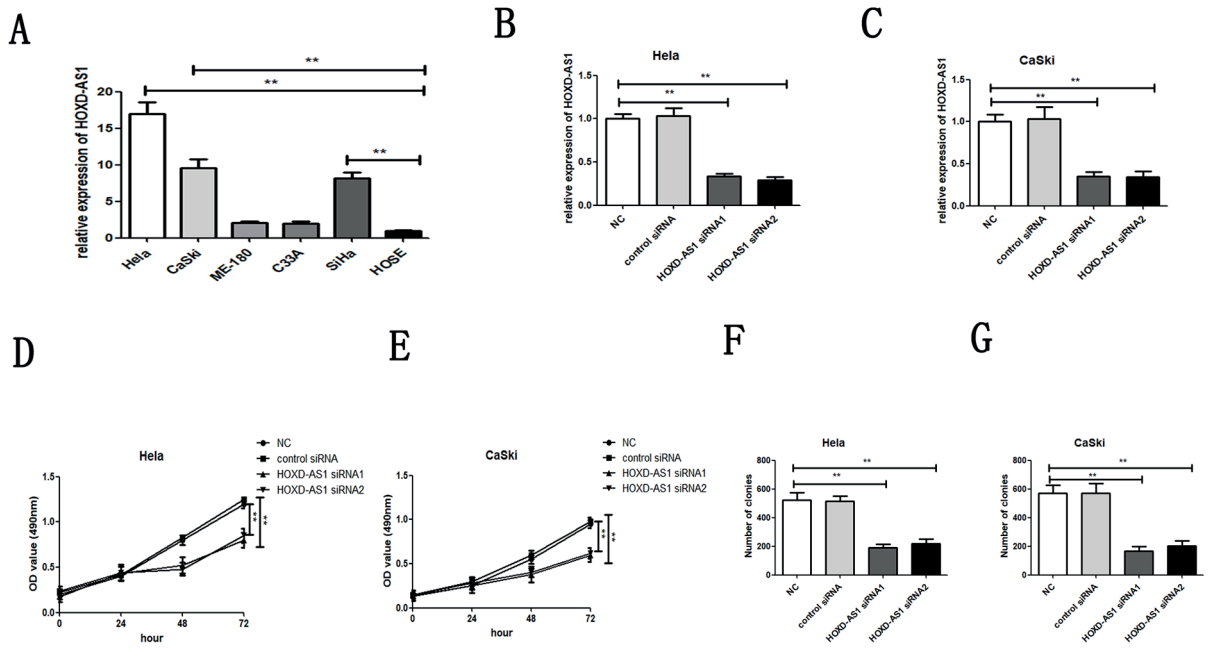

Figure 1. The relative expression of HOXD-AS1 in cervical cancer cell lines and its role in cervical cancer cell growth *in vitro*. ***(A)*** The relative expression of HOXD-AS1 in cervical cancer cell lines (HeLa, CaSki, Me-180, C33A, and SiHa) and normal human ovary cell line HOSE was evaluated by RT-qPCR analysis. The expression of HOXD-AS1 in ***(B)*** HeLa and ***(C)*** CaSki cells transfected with HOXD-AS1 siRNA1, HOXD-AS1 siRNA2, or control siRNA was determined by RT-qPCR, and U6 was used as the endogenous control. Cell viability in HOXD-AS1 siRNA-treated ***(D)*** HeLa and ***(E)*** CaSki cells was examined by MTT assay at 24, 48, and 72 h post transfection. Colony formation ability in HOXD-AS1 siRNA-treated (F) HeLa and ***(G)*** CaSki cells was assessed by colony formation assay. Data are presented as the mean ± SD (n = 3; $^{*}p<0.05$ and $^{**}p<0.01$).

and progression of numerous cancers. For example, Li et al[14] reported that HOXD-AS1 overexpression was related to the bladder cancer via inhibiting the phenotypes, as well as regulating endogenous cancer-related signaling pathway. Via comprehensive analysis of non-coding transcriptome, Yarmishyn et al[12] found HOXD-AS1 may work as a molecular marker of the progression of neuroblastoma. Till now, there were few reports about the biological functions of HOXD-AS1 in cervical cancer and the underlying mechanisms, and that's which we want to explore in the current study.

In this paper, we explored the clinical role of HOXD-AS1 in human cervical cancer tissues. Results suggested that high expression of HOXD-AS1 was clinically related to tumor-node-metastasis (TNM) stages, lymphovascular invasion, lymph node metastasis, as well as recurrence in cervical cancer patients. Moreover, we detected the influence of HOXD-AS1 on cervical cancer both in cervical cancer cells, as well as xenograft mice models. Results proved that HOXD-AS1 expression was involved in cancer metastasis and recurrence. In HeLa and CaSki cells, when down-regulating the expression of HOXD-AS1 using siRNA, cell viability and colony formation ability were significantly repressed. Furthermore, results demonstrated that the promoting role of HOXD-AS1 in cancer cells may be involved in the Ras/ERK signaling pathway. Later on, we confirmed the hypothesis in the xenograft models. From all the above results, we concluded that HOXD-AS1 may work as a promising biomarker and target for cervical cancer.

Ras signaling pathway plays an important part in cell growth, as well as cell differentiation. Active Ras-GTP can subsequently regulate the serine/threonine kinase Raf, and then activates the protein kinases MEK1/2. Afterwards, p44/p42 (ERK1/2) can be phosphorylated/activated[21]. From this study, we may draw a conclusion that the Ras/ERK signaling pathway may be a targeted molecular pathway of HOXD-AS1 in regulating carcinogenesis and development of cervical cancer.

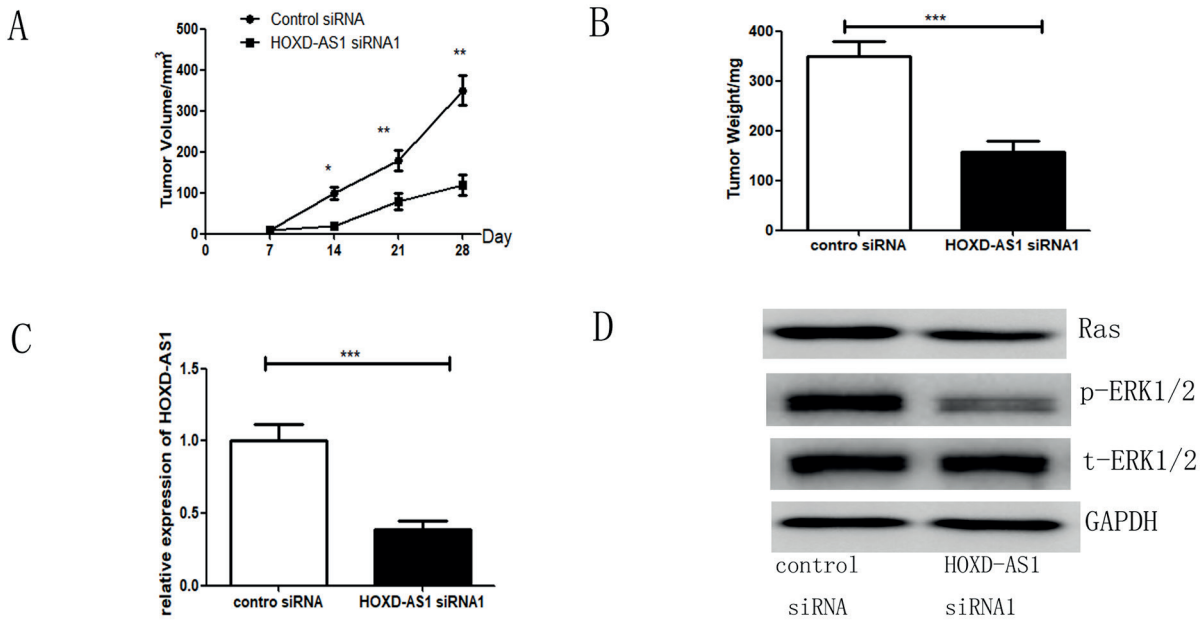

Figure 2. Effect of HOXD-AS1 knockdown on cervical cancer cell growth *in vivo* and its potential mechanism. HeLa cells transfected with HOXD-AS1 siRNA1 or control siRNA were subcutaneously injected into nude mice for xenograft. ***(A)*** Tumor volume of *in vivo* xenograft experiments was calculated for every 7 days from day 7 of injection. ***(B)*** Tumor weight of *in vivo* xenograft experiments was detected at 21 days after injection. ***(C)*** The expression of HOXD-AS1 in excised tumor samples was determined by RT-qPCR. ***(D)*** The levels of Ras, p-ERK1/2, and t-ERK1/2 in excised tumor samples were evaluated by Western blot analysis. Data are presented as the mean ± SD (n = 6; $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$).

Conclusions

We first explored the biological functions of HOXD-AS1 in carcinogenesis and development of cervical cancer and its underlying molecular mechanisms. Results in this study implied that HOXD-AS1 may work as an effective biology marker for cervical cancer via regulating the Ras/ERK pathway *in vitro* and *in vivo*. Therefore, HOXD-AS1 may serve as a new factor for predicting prognosis, as well as a promising therapeutic target to conquer cervical cancer.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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