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Original Article

Overexpression of long non-coding RNA UCA1 predicts a poor prognosis in patients with esophageal squamous cell carcinoma

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Abstract: Introduction: Long non-coding RNAs (lncRNAs) have been shown to have important regulatory roles in cancer biology, and the lncRNA UCA1 is upregulated in several cancers such as bladder cancer, breast cancer and colorectal cancer, however, the contributions of UCA1 to esophageal cancer remain largely unknown. Methods: Expression levels of lncRNA UCA1 in esophageal squamous cell carcinoma (ESCC) patients and esophageal cancer cell lines were evaluated by quantitative real-time PCR (qRT-PCR), and its association with overall survival of patients was analyzed by statistical analysis. Small interfering RNA was used to suppress UCA1 expression in esophageal cancer cell line. In vitro assays were conducted to further explore its underlying roles in tumor progression. Results: The relative level of UCA1 was significantly higher in ESCC tissues compared to the adjacent non-tumor tissues, and remarkably higher expression of UCA1 was found in esophageal cancer cell lines compared with the immortalized esophageal epithelial cell line NE1. The ESCC patients with higher UCA1 expression had an advanced clinical stage and a poorer prognosis than those with lower expression. In vitro assays, our data indicated that downregulation of UCA1 decrease cell proliferation, migration, and invasion ability. Conclusions: lncRNA UCA1 might be considered as a novel molecule involved in ESCC progression, which provides a potential prognostic biomarker and therapeutic target.

Keywords: Long non-coding RNAs, UCA1, esophageal squamous cell carcinoma

Introduction

Esophageal cancer is the eighth most common cancer worldwide and the sixth most common cause of death from cancer [1]. Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EA) are the main types of esophageal carcinoma, however, in China, 90% of cases involve squamous cell carcinomas [2]. It is difficult to diagnose ESCC at early stages of disease development, and advanced ESCC frequently presents with extensive local invasion or regional lymph node metastasis [3]. The 5-year survival rate worldwide for ESCC remains below 40% [4]. Therefore, it is imperative to identify effective biomarkers for early-stage diagnosis and potential targets for therapy.

Long non-coding RNAs (lncRNAs) are evolutionarily conserved non-coding RNAs that are more than 200 nucleotides in length with no protein coding capacity [5]. Recent studies showed that lncRNAs play key roles in diverse biological processes, such as embryonic development, cell growth and tumorigenesis by regulating gene expression at the transcriptional and post-transcriptional levels [6-8]. For example, The HOTAIR lncRNA, which was known to regulate the expression of HOX gene clusters, was highly induced in bladder cancer samples and its elevated expression has been correlated with tumor recurrence [9]. Tu et al found that lncRNA GAS5 expression was decreased in human hepatocellular carcinoma and was associated with advanced tumor progression. And GAS5 expression was demonstrated to be an independent marker for predicting the clinical outcome of patients with hepatocellular carcinoma [10]. Ren et al demonstrated that lncRNA MALAT-1 was increased in prostate cancer and higher MALAT-1 expression was correlated with Gleason score, prostate specific antigen, tumor stage and castration resistant prostate cancer,

Table 1. UCA1 expression and clinicopathological factors

Parameters	Group	Total	UCA1 expression		*P* value
			High	Low	
Gender	Male	50	24	26	0.603
	Female	40	17	23	
Age (years)	< 60	49	21	28	0.574
	≥ 60	41	20	21	
Tumor location	Upper	18	8	10	
	Middle	38	18	20	0.831
	Lower	34	15	19	
Differentiation grade	G1	28	5	23	0.001
	G2+G3	62	36	26	
Lymph nodes metastasis	Absence	56	19	37	0.004
	Presence	34	22	12	
Clinical stage	I + II	39	11	28	0.004
	III + IV	51	30	21	

moreover downregulation of MALAT-1 could inhibit prostate cancer cell growth, invasion and migration [11]. Xie et al showed that ESCC patients with higher SPRY4-IT1 expression had an advanced clinical stage and poorer prognosis than those with lower SPRY4-IT1 expression, and knockdown of SPRY4-IT1 reduced cell proliferation, invasion, and migration [12]. However, to our knowledge, research of lncRNAs in ESCC remains largely unknown.

Urothelial carcinoma associated 1 (UCA1) is an lncRNA originally identified in bladder transitional cell carcinoma. The entire sequence consists of three exons with 1.4 kb in length. As it is highly expressed in bladder transitional cell carcinoma, it was suggested to serve as a biomarker for the diagnosis of bladder cancer [13]. Fan et al demonstrated that UCA1 increases the cisplatin resistance of bladder cancer cells by enhancing the expression of Wnt6, and thus represents a potential target to overcome chemoresistance in bladder cancer [14]. Huang et al showed that lncRNA UCA1 has an oncogenic role in breast cancer and promotes breast tumor growth by suppression of p27 [15]. Han et al found UCA1 levels were markedly increased in colorectal cancer tissues and cells, and influence the proliferation, apoptosis and cell cycle progression of colorectal cancer cells [16]. However, the lncRNA UCA1 expression in ESCC and underlying mechanism is still unknown.

In the present study, we investigated the expression of UCA1 in ESCC tissues and cell lines. We then examined the relationships between UCA1 levels in tumor tissues and the clinicopathological features of ESCC. Finally, we conducted in vitro assays to demonstrate the biological functions of UCA1 in ESCC development and progression. Our research revealed that lncRNA UCA1 involved in the progression of ESCC.

Materials and methods

Patients and specimens

Ninety ESCC samples and matched adjacent non-tumor tissues were obtained from patients undergoing surgery at Department of Thoracic Surgery, the First Affiliated Hospital of Henan University of Science and Technology between 2007 and 2010. None of the patients had received local or systemic therapy prior to surgery. After surgical resection, tumor specimens and adjacent non-tumor tissues were collected and stored in liquid nitrogen until use. Clinicopathological characteristics in our study are presented in **Table 1**. All patients were followed up until March 2012 with a median observation time of 43 months. Permission to use the tissue sections for research purposes was obtained and approved by the Ethics Committee of the First Affiliated Hospital of Henan University of Science and Technology

Cell culture and treatment

ESCC cell lines EC109, EC9706, KYSE150, and KYSE510 and immortalized esophageal epithelial cell line NE1 were purchased from the American Type Culture Collection (ATCC, USA). The immortalized esophageal epithelial cell lines were cultured in a 1:1 mixture of defined keratinocyte serum free medium (Gibco) and EpiLife medium (Cascade Biologics). The ESCC cells were cultured in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (Gibco), 50 U/ml of penicillin and 50 μg/ml of streptomycin. All cells were cultured in a sterile incubator maintained at 37°C with 5% CO_2.

Small interfering RNA that targeted UCA1 RNA (si-UCA1) and a scrambled negative control (si-

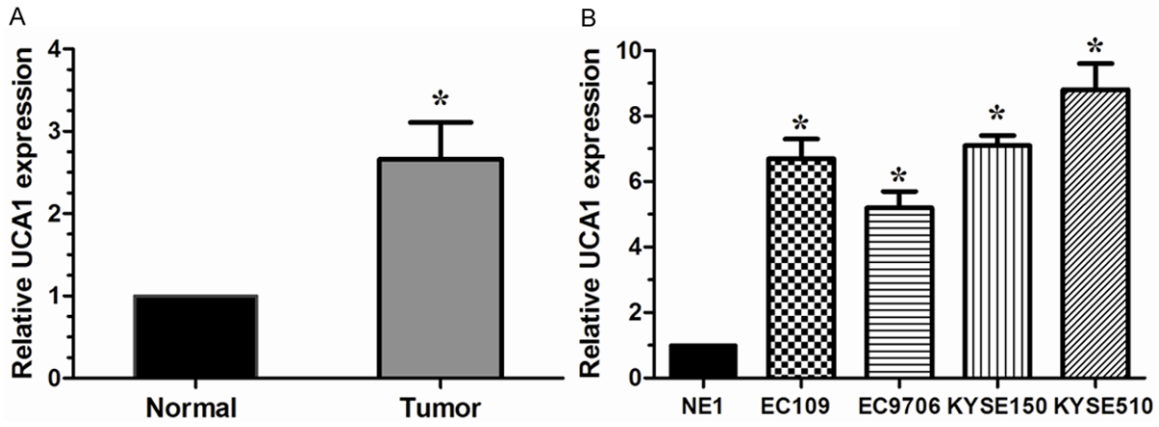

Figure 1. LncRNA UCA1 expression levels in ESCC tissues and esophageal cancer cells. The levels of UCA1 were assessed by qRT-PCR, using GAPDH as a normalisation control. A: The UCA1 expression levels in ESCC samples were significantly higher than those in adjacent non-tumor tissues. B: Higher expression levels of UCA1 were detected in 4 esophageal cancer cell lines than in immortalized esophageal epithelial cell line NE1. Results were expressed as mean ± SD for three replicate determination $*P < 0.05$.

NC) were generously provided by Life Technologies. The sequences of si-UCA1 were as follows: si-UCA1-1: 5'-GTTAATCCAGGAGACAAAGA-3', si-UCA-2: 5'-TCTTTGTCTCCTGGATTAAC-3'. Transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. After 48 hours, cells transfected with siRNA were harvested for qRT-PCR to determine the transfection efficiency.

Quantitative real-time PCR assay

Total RNA was isolated from tissues or cells using TRIZOL reagent according to the manufacturer's protocol (Invitrogen). RNA was reverse transcribed using SuperScript First Strand cDNA System (Invitrogen) according to the manufacturer's instructions. The PCR amplification were performed for 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, on a Applied Biosystems 7900HT (Applied Biosystems) with 1.0 μl of cDNA and SYBR Green Real-time PCR Master Mix (Takara). Data was collected and analyzed by SDS2.3 software (Applied Biosystems). The expression level of each candidate gene was internally normalized against that of the GAPDH. The relative quantitative value was expressed by the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicates and repeated three times.

Cell proliferation assay

The proliferation of KYSE510 cells was evaluated using the MTT assay according to the manufacturer's instructions. KYSE510 cells that had been transfected with either si-UCA1 or si-NC for 48 hours were reseeded into 96-well plates. Cell density was adjusted to 5×10^3/well, and the final volume was 150 μl/well. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution (20 μl) was added to the plates 24, 48, 72, and 96 hours later. The cells were cultured for 4 hours at 37°C. Then, the medium was discarded and 150 μl DMSO was added and oscillated for 15 min. The absorbance was measured at 490 nm using an enzyme-labeled analyzer. Three independent experiments were performed.

Cell wound healing assay

To determine cell migration, KYSE510 cells transfected with either si-UCA1 or si-NC were seeded into 6-well plates, incubated in their respective complete culture medium and grown to confluence over-night. Wounds were created by scratching cell monolayers with a sterile 200 μl plastic pipette tip, and the debris was rinsed with PBS, photographs were obtained at 0, and 24 hours using a phase contrast microscope (Nikon). Three independent experiments were carried out.

Transwell invasion assays

Cell invasion assays were performed using a 24-well chamber containing a matrigel-coated membrane of 8 μm pore size (Costar). KYSE510 cells transfected with either si-UCA1 or si-NC were collected and resuspended in serum-free

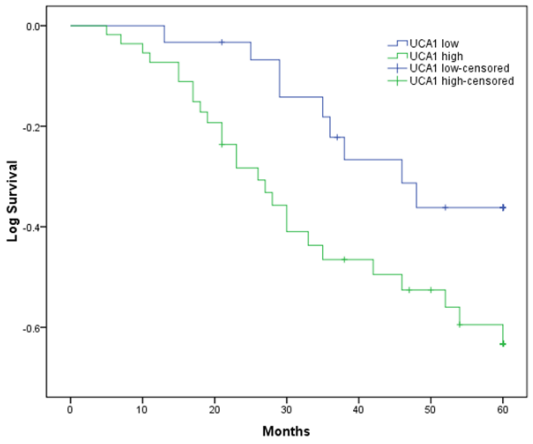

Figure 2. Kaplan-Meier overall survival curves according to the UCA1 expression level. ESCC patients with high UCA1 expression showed a significantly poorer prognosis than those with low UCA1 expression (log-rank test, $P < 0.05$).

medium at a concentration of 1×10^5 cells/ml, respectively. Then, 200 μl cell suspensions were added into the upper chamber, and the bottom chamber was filled with 500 μl culture medium containing 10% FBS. After incubation for 48 hours at 37°C, 5% CO_2. After incubation, the non-invaded cells on the upper membrane surface were removed with a cotton tip, and the cells that passed through the filter were fixed and stained using 0.1% crystal violet. The numbers of invaded cells were counted in 3 randomly selected high power fields under a microscope (Nikon).

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software. Data were analyzed using independent two-tailed t test. Categorical data were analyzed using the two-side chi-square test. Overall survival was estimated by using Kaplan-Meier method, and univariate analysis was conducted by log-rank test. The Cox proportional hazards model was used in the multivariate analysis. Values of $P < 0.05$ were considered statistically significant.

Results

LncRNA UCA1 is upregulated in ESCC tissues and cell lines

qRT-PCR was used to measure UCA1 expression levels in a total of 90 patients with ESCC: LncRNA UCA1 expression was significantly upregulated in clinical ESCC specimens compared to adjacent non-tumor tissues ($P < 0.05$, **Figure 1A**). Expression was also examined by qRT-PCR in four esophageal cancer cell lines and an immortalized esophageal epithelial cell line NE1. This experiment showed that UCA1 expression was higher in esophageal cancer cell lines than in immortalized esophageal epithelial cell line NE1 ($P < 0.05$, **Figure 1B**).

To assess the correlation between UCA1 expression and clinicopathological data, we divided the patients with ESCC into a high UCA1 expression group and a low expression group according to the mean UCA1 expression level in the tumor tissues. As shown in **Table 1**, UCA1 upregulation was correlated with tumor differentiation, clinical stage, and lymph node metastasis but not correlated with patient's age, gender, and tumor location ($P > 0.05$). Taken together, these observations indicated that increased UCA1 expression is associated with the progression and development of ESCC.

High UCA1 expression levels correlate with ESCC patients' poor prognosis

As is shown in **Figure 2**, patients with high UCA1 expression had a significantly poorer prognosis than those with low expression ($P < 0.05$, **Figure 2**). Univariate analysis showed that the relative level of UCA1 expression, tumor differentiation, clinical stage, and lymph node metastasis were correlated with overall survival rate of patients with ESCC ($P < 0.05$, **Table 2**). Multivariate analysis indicated that UCA1 expression level, tumor differentiation, clinical stage, and lymph node metastasis were independent prognostic indicators for the overall survival of patients with ESCC ($P < 0.05$, **Table 2**). The results suggested that expression of UCA1 in ESCC patients can be developed as a powerful independent prognostic factor.

si-UCA1 significantly decrease the expression of UCA1 in KYSE150 cells

To further investigate the role of UCA1 in human esophageal cancer cells, UCA1 specific siRNA (si-UCA1) was transfected into KYSE510 cells, respectively. Nonspecific siRNA was used as a negative control (si-NC). As shown in **Figure 3A**, cells transfection with si-UCA1 showed a significant decreased mRNA expression of UCA1

Table 2. Prognostic factors in Cox proportional hazards model

Variable	Univariate analysis			Multivariate analysis		
	Risk ratio	95% CI	*P*	Risk ratio	95% CI	*P*
Gender	1.317	0.517-2.172	0.328			
Male vs Female						
Age (years)	1.977	0.296-2.328	0.271			
≥ 60 vs < 60						
Tumor location	1.327	0.748-1.996	0.402			
Upper vs (Middle + Lower)						
Differentiation grade	3.617	1.947-7.283	0.029	2.794	2.116-5.946	0.013
G2 + G3 vs G1						
Lymph node	3.374	2.283-6.795	0.011	2.617	2.039-5.117	0.008
Presence vs Absence						
Clinical stage	4.163	2.914-8.719	0.005	3.783	2.179-6.882	0.015
III + IV vs I + II						
UCA1	2.931	1.72-6.214	0.006	2.627	1.416-5.874	< 0.001
High vs Low						

compared to the si-NC group ($P < 0.05$). Our data demonstrated that we successfully downregulated the UCA1 expression in human esophageal cancer KYSE510 cells.

Suppressing UCA1 expression decreases cell proliferation, migration and invasion in vitro

MTT assays showed that the cell growth rate was downregulated in the si-UCA1 transfected KYSE510 cells compared with the si-NC group ($P < 0.05$, **Figure 3B**). Furthermore, to analyze the role of lncRNA UCA1 in cell migration and invasion, wound healing assays and transwell invasion assays were performed with KYSE510 cells. Wound healing assays showed that cells transfection with si-UCA1 were distinctively less migratory than the cells transfected with si-NC ($P < 0.05$, **Figure 3C**). Transwell invasion assays indicated that the invasiveness of the cells that were transfected with si-UCA1 was dramatically decreased compared with the cells transfected with si-NC ($P < 0.05$, **Figure 3D**). Taken together, these data demonstrated that downregulation of lncRNA UCA1 expression can inhibit cell proliferation, migration and invasion of esophageal cancer cells in vitro.

Discussion

Esophageal squamous cell carcinoma is one of the most common cancers and a leading cause of cancer-related death all over the world. Because most patients are diagnosed at an advanced stage, and treatments are less effective, the prognosis of ESCC patients is still quite poor [17]. Therefore a better understanding of the molecular events associated with ESCC is of great importance to improve the clinical strategies and outcomes of patients with ESCC.

It has been previously demonstrated that protein-coding genes account for only 2% of the total genome, whereas the vast majority of the human genome can be transcripted into non-coding RNAs [18]. Among these, long non-coding RNAs (lncRNAs) have been certified as important biological RNAs in the post-transcriptional regulation of the target genes [19]. In recent years, more and more evidences revealed the contribution of lncRNAs as having oncogenic or tumor suppressor roles in tumorigenesis [20]. In this study, we tried to investigate the role of lncRNA UCA1 in the development of ESCC.

In the study, we investigated the clinical significance of UCA1 in ESCC patients for the first time. By using qRT-PCR, we found that lncRNA UCA1 was upregulated in ESCC tissues and esophageal cancer cell lines to a greater extent than in corresponding non-tumor tissues and immortalized esophageal epithelial cell line NE1. Our results showed that the expression level of UCA1 was associated with tumor differentiation, clinical stage, and lymph node metastasis of ESCC patients. However, lncRNA UCA1 expression was not correlated with patient's age, gender, and tumor location. In addition,

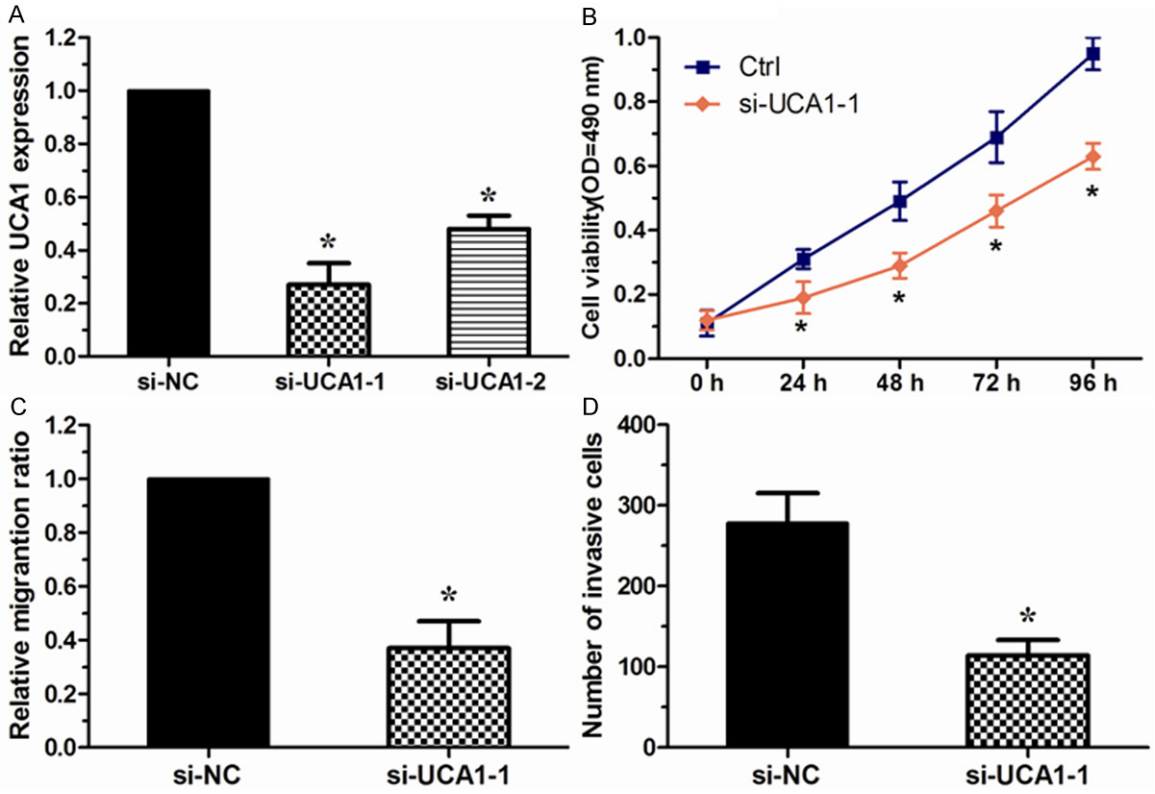

Figure 3. Evaluation of biological functions of UCA1 in esophageal cancer cells. A: Transfection efficiency of si-UCA1 in esophageal cancer cells was indicated by qRT-PCR. B: Si-UCA1 inhibited the proliferation of esophageal cancer cells. MTT assay was performed to investigate the effects of si-UCA1 on cell proliferation in KYSE510 cells. C: Si-UCA1 inhibited the migration of esophageal cancer cells. Wound healing assay was performed to investigate the effects of si-UCA1 on cell migration in KYSE510 cells. D: Si-UCA1 inhibited the invasiveness of esophageal cancer cells. Transwell invasion assay was performed to investigate the effects of si-UCA1 on cell invasion in KYSE510 cells. si-NC, cells transfected with nonspecific siRNA; si-UCA1, cells transfected with UCA1-specific siRNA. Results are expressed as means ± SD for three replicate determination $*P < 0.05$.

UCA1 overexpression was associated with poor survival rates and could be an independent prognostic factor in patients with ESCC. Taken together, these findings supported our previous hypothesis that lncRNA UCA1 might play an important role in development and progression of ESCC.

Fang et al indicated that the expression levels of lncRNA UCA1 were significantly elevated in tongue squamous cell carcinoma tissues and were correlated with lymph node metastasis. Over-expression of lncRNA UCA1 could promote metastatic ability of tongue squamous cell carcinoma cells [21]. From our clinical pathological data, we suggest that lncRNA UCA1 expression is closely correlated with tumor differentiation, clinical stage, and lymph node metastasis, thus we suppose UCA1 may also regulate the growth and metastasis of esophageal cancer cells. So, it is necessary to identify the biological function of UCA1 in esophageal cancer.

To further understand the underlying mechanism of UCA1 in esophageal cancer progression, in vitro experiments were conducted. siRNA-mediated knockdown of UCA1 significantly decreased proliferation, migration and invasion capability of esophageal cancer cells compared with control group, which indicated that down-regulation of UCA1 can suppress the development of esophageal cancer. Taken together, these results indicated that UCA1 could function as a tumor oncogenic via regulating cell proliferation, migration and invasion, and may be useful in the development of novel prognostic or progression markers for esophageal cancer.

In summary, our studies showed that lncRNA UCA1 is upregulated in ESCC tissues and is significantly associated with advanced tumor progression. Moreover, UCA1 expression was suggested to be an independent marker for predicting prognosis of patients with ESCC. The

upregulation of UCA1 play key roles in the development and progression of ESCC by regulating cell proliferation, migration and invasion. Our findings demonstrated that UCA1 is a potential diagnostic and therapeutic target in patients with ESCC in the future.

Disclosure of conflict of interest

None.

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