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Original Article

A novel long non-coding RNA FOXCUT and mRNA FOXC1 pair promote progression and predict poor prognosis in esophageal squamous cell carcinoma

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Abstract: Accumulating evidences demonstrated that many long non-coding RNAs (lncRNAs) can cooperate with the adjacent coding genes, forming into "lncRNA-mRNA gene pairs" in multiple biological cellular processes. Here, we showed that a novel long non-coding RNA FOXCUT (FOXC1 promoter upstream transcript) and its neighboring gene FOXC1 played a similar important role in the oncogenesis and progression of esophageal squamous cell carcinoma (ESCC). In this study, the expression of FOXCUT/FOXC1 was measured in 82 ESCC tissues and adjacent noncancerous tissues by real-time quantitative PCR (qPCR). The prognostic significance of the lncRNA-mRNA gene pair was evaluated using Kaplan-Meier survival analysis and log-rank test. Cell biological experiments were performed in ESCC cell lines to explore their functions in tumor progression. Notably elevated FOXCUT and FOXC1 expression levels were observed in cancerous tissues compared to adjacent noncancerous tissues (86.6% and 84.1%, respectively; $P < 0.01$), showing strong correlations with poor differentiation, advanced lymph node classification and metastasis ($P < 0.05$). Moreover, patients with upregulated FOXCUT or FOXC1 experienced a significantly worse prognosis than those with downregulated FOXCUT or FOXC1 ($P < 0.001$ and $P = 0.014$, respectively). In addition, the expression of FOXCUT was positively correlated with expression of FOXC1 in ESCC specimens. And the expression of FOXC1 was also decreased as the FOXCUT expression was silenced by siRNA. Assays in vitro demonstrated that knockdown of either FOXCUT or FOXC1 remarkably inhibited cell proliferation, colony formation, migration, invasion in ESCC cells. In conclusion, FOXCUT may be functionally involved in the tumor progression and survival of ESCC patients, at least in part, by modulating FOXC1. FOXCUT and FOXC1 may function as a lncRNA-mRNA gene pair, which may represent a potential prognostic biomarker and therapeutic target for ESCC patients.

Keywords: ESCC, lncRNA, FOXC1, FOXCUT, progression, prognosis

Introduction

Esophageal cancer (EC) is one of the most aggressive malignant tumors, ranking eighth by global morbidity and sixth by global mortality rate among all types of cancers [1, 2]. Histologically, the two main types of esophageal cancer, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), exhibit different etiologic and pathologic characteristics. In China, ESCC is the predominant subtype and contributes to nearly 90% of all ECs [3, 4]. Despite recent considerable advances in diagnosis and treatment, the overall prognosis for ESCC remains still poor, with 5-year survival rate of 5-45% [5-8]. The treatment failure can be attributed to the extensive local invasion and regional lymph node metastasis [9]. Therefore, to identify more accurate biomarkers for early diagnosis, therapeutic strategies and prognosis of ESCC is urgently needed.

Recently, accumulating evidences demonstrated that long non-coding RNAs (lncRNAs), the largest transcript class in human genome, may play an important role in the tumorigenesis and tumor progression [10-13]. LncRNAs are defined as transcribed RNA molecules that are longer than 200 nucleotides, possessing no potential protein-coding capacity [14, 15]. With advances in technologies, lncRNAs are found to be as important regulators involved in various

molecular mechanisms in gene networks [16, 17]. Studies showed that a large number of lncRNAs can functionally contact with their adjacent mRNAs developing into a new form of "lncRNA-mRNA pairs" in the regulatory networks. This new model indicated that transcription of lncRNAs may often be co-regulated with the adjacent protein-coding gene [18, 19].

Here, with bioinformatics analysis, we found a novel lncRNA TCONS_00011636 (http://genome.ucsc.edu/), which is situated at chromosome 6p25 and transcribed from the upstream side of FOXC1 promoter. So, we denominated it as "FOXC1 promoter upstream transcript, FOXCUT". And the lncRNA-mRNA pair (FOXCUT and FOXC1) may be a new functional form. FOXC1, a member of the Forkhead Box, is featured as a conserved 110 amino-acid DNA-binding domain. FOXC1 proteins are key regulators of diverse biological processes including the development of many organ systems [20], embryogenesis, tumorigenesis, and tumor progression [21-23]. Recent studies have demonstrated that FOXC1 was overexpressed in many kinds of cancer tissues and the high expression level of FOXC1 had a strong association with the poor prognosis in patients of multiple malignant cancers, such as gastric cancer, breast cancer, hepatocellular carcinoma, non-small cell lung cancer, pancreatic ductal adenocarcinoma [22, 24-27]. However, it hasn't been reported in ESCC yet. Considering the extensive clinical value of FOXC1, we speculated FOXCUT, the other part of lncRNA-mRNA pair, may be functionally involved in the tumor progression and survival of ESCC patients with FOXC1 together.

In the present study, we reported the expression patterns of FOXC1 and FOXCUT in ESCC tissues and adjacent non-cancerous tissues and analyzed the correlation between FOXCUT/FOXC1 and clinicopathological characteristics for the first time. Then, we explored their functional role in ESCC cells. In all, this study was to offer a new functional lncRNA-mRNA pair in ESCC and to evaluate the lncRNA-mRNA pair as a new biomarker that predicts poor prognosis in ESCC patients.

Materials and methods

Patient samples

A total of 82 fresh ESCC tissue samples and paired adjacent noncancerous tissue samples (> 1.5 cm away from cancer) were collected from patients who underwent surgery at Chinese PLA General Hospital (Beijing, China) between 2007 and 2012. The ESCC diagnosis was histopathologically confirmed. None of the patients received preoperative therapy such as radiotherapy or chemotherapy before surgical resection. All tissue specimens were immediately frozen and stored in liquid nitrogen after surgery until the extraction of total RNA. The data from all subjects were obtained from medical records, pathology reports and personal interviews. The acquired clinical information for all of the samples is summarized in **Table 1**. Follow-up periods ranged from 1 month to 72 months, and the result of patients who were lost to follow-up or died from other etiology instead of ESCC were regarded as censored data. The research was approved by the ethical committee of PLA General Hospital. Written informed consent was signed by all participants.

Cell line and cell culture

Human ESCC cell lines (KYSE30, KYSE70, KYSE140, KYSE150, and KYSE180) were cultured at 37°C with 5% CO_2 in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Hyclone, Logan, UT). One normal esophageal cell line Het-1A was cultured in LHC-9 medium supplemented with 2% fetal bovine serum (Hyclone, Logan, UT).

RNA extraction and real-time quantitative PCR

Total RNA was extracted from ESCC cancerous, matched adjacent noncancerous specimens and ESCC cells using the Trizol Total RNA Reagent (Invitrogen, Carlsbad CA). The concentration and A260/280 ratio were measured by NanoVue Plus (GE Healthcare, USA). The quality assessment of RNA was evaluated in the 28S and 18 S bands using agarose gel electrophoresis. 1 μg RNA was reversely transcribed into cDNAs using the PrimeScript® RT reagent Kit (TAKARA, Dalian, China), according to the manufacturer's protocol. Real-time quantitative PCR (qPCR) was performed using the SYBR® Premix Ex Taq™ (Takara, Dalian, China), in an Applied Biosystems 7500 Fluorescent Quantitative PCR System (Applied Biosystems, Foster City, CA). The reaction mixtures were incubated at 95°C for 30 s, followed by 40 amplification cycles of 95°C for 5 s and 60°C for 34 s. The comparative CT method was app-

Table 1. Correlations of FOXCUT and FOXC1 expression with clinicopathological characteristics in ESCC patients

Characteristics	FOXCUT expression		*P* value	FOXC1 expression		*P* value
	Low expression	High expression		Low expression	High expression	
Age (mean = 59.48)			0.022*			0.246
< 60	25	19		23	21	
≥ 60	12	26		15	23	
Gender			0.864			0.158
Male	24	30		22	32	
Female	13	15		16	12	
Tumor location			0.164			0.23
Upper	0	2		0	2	
Middle	10	18		11	17	
Lower	27	25		27	25	
Differentiation			0.001*			0.001*
Well	2	23		3	22	
Moderate	19	19		21	17	
Poor	16	3		14	5	
T classification			0.259			0.839
T1-2	27	26		25	28	
T3-4	11	18		13	16	
N classification			0.007*			0.045*
N0	12	4		11	5	
N1-2	25	41		27	39	
Metastasis			0.001*			0.001*
M0	31	15		30	16	
M1	6	30		8	28	
Clinical Stage			0.120			0.376
I-II	22	19		21	20	
III-IV	15	26		17	24	

P-values were two-tailed and based on the Pearson χ^2 test; *Statistically significant.

lied to quantify relative expression of mRNA and lncRNA. Results were normalized to the expression of house-keeping gene GAPDH. The primers used in this study were as follows: FOXC1 (Forward) 5′- GGCGAGCAGAGCTACTACC -3′, (Reverse) 5′- TGCGAGTACACGCTCATGG -3′; FOXCUT (Forward) 5′- GTCGCACCGATGACTAACG -3′, (Reverse) 5′- GCCCTGAAAGCCGAACTG -3′.

Transfection of siRNA

The siRNA sequences were designed by us and synthesised by GenePharma (Shanghai, China), including one negative control siRNA (NC siRNA) sequence, two FOXCUT siRNA sequences and two FOXC1 sequence. The sequences were as follows: si-FOXC1-1 sense strand 5'rCrCrArGrArUrArArCrArCrGrUrArArGrUrUrUrCrUrUrCTT, antisense strand 5'rArArGrArArGrArArArCrUrUrArArCrGrUrGrUrUrArUrCrUrGrGrArG; si-FOXC1-2 sense strand 5'rCrGrUrUrArArArUrUrGrCrCrUrGrArArArCrUrUrUrArAAT, antisense strand 5'rArUrUrUrArArArGrUrUrUrCrArGrGrCrArArUrUrUrArArCrGrUrC. si-FOXCUT-1 sense strand 5'rGrArArUrGrGrArGrArArCrUrArArGrArCrArArUrUrArUCT, antisense strand 5'rArGrArUrArArUrUrGrUrCrUrUrArGrUrUrCrUrCrCrArUrUrCrGrG; si-FOXCUT-2 sense strand 5'rCrArGrCrCrUrCrCrCrUrCrCrUrGrUrGrUrGrUrGrCrArGAG, antisense strand 5'rCrUrCrUrGrCrArCrArCrArCrArGrGrArGrGrGrArGrGrCrUrGrCrA. Transfection of siRNA was conducted by X-tremeGENE transfection reagent (Roche) according to the manufacturer's instructions. After transfection, total cells were collected for RNA isolation, cell proliferation assay, colony formation assay and scratch wound healing assay.

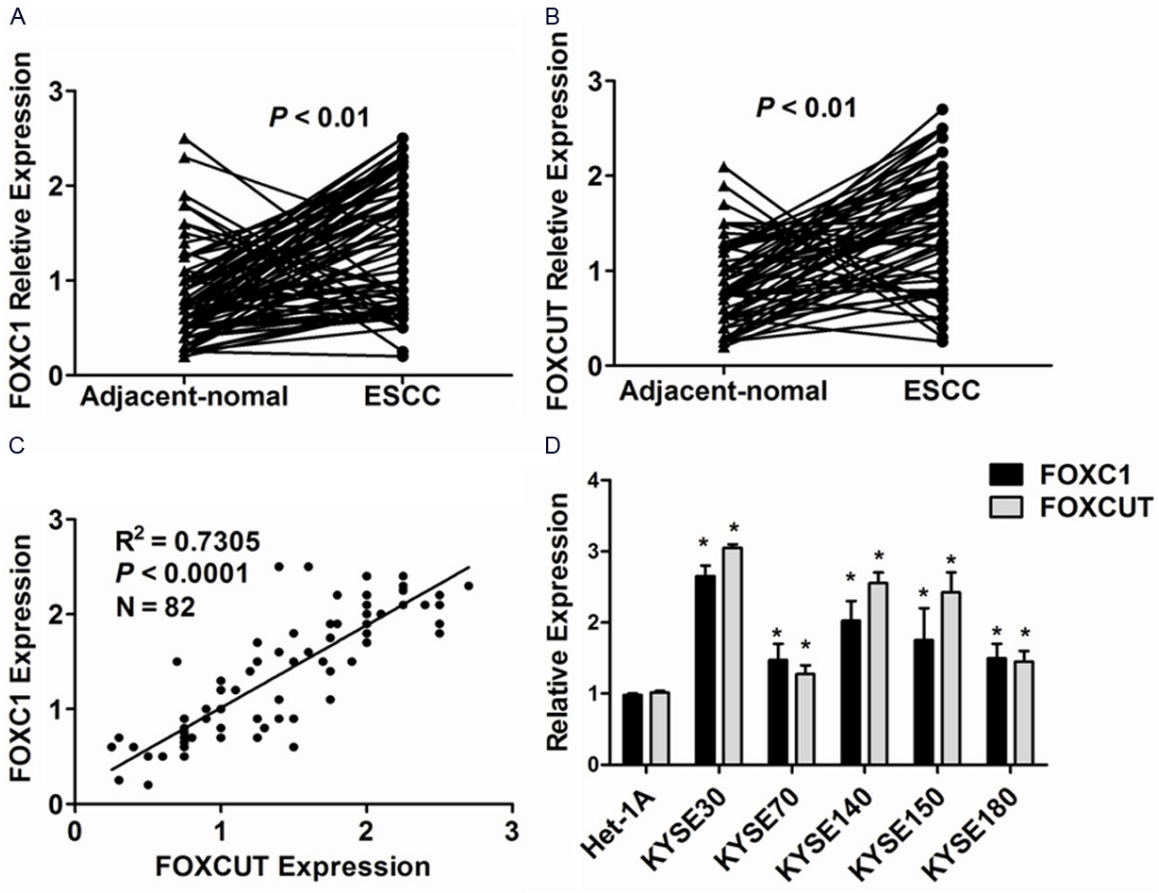

Figure 1. FOXC1 and lncRNA-FOXCUT expression levels were analyzed by qPCR in 82 ESCC tissue samples and ESCC cell lines. A: The expression level of FOXC1 in ESCC cancerous tissues was remarkably higher than those in adjacent noncancerous tissues ($P < 0.01$). B: The expression level of lncRNA-FOXCUT in ESCC cancerous tissues was also significantly higher than those in adjacent noncancerous tissues ($P < 0.01$). C: Linear Regression analysis was performed on FOXC1 and lncRNA-FOXCUT expression levels in 82 ESCC tissue samples ($R^2 = 0.7305$, $P < 0.0001$). D: The FOXC1 and lncRNA-FOXCUT expression levels were evidently higher in ESCC cell lines compared to normal esophageal cell line Het-1A ($P < 0.05$, the symbol * indicates statistically significant).

Cell proliferation assay

After 24 hours (h) of transfection, cell proliferation was measured by MTS assay (Promega) following the manufacturer's protocol. KYSE30 (1,000 cells per well) were seeded in 96-well plates. The cells were incubated for 0, 1, 2, 3 or 4 days, respectively. And 20 μl of the MTS reagent was added to each well containing 100 μl culture medium. The plate was incubated for 2 h at 37°C in a humid, 5% CO_2 atmosphere. The absorbance values of each well were detected with a universal microplate reader at the wavelength of 490 nm.

Colony formation assay

After 24 h of transfection, the cells (KYSE30) were reseeded into 6-well plates at 1000 cells per well. The culture medium was replaced every 5 days. Cells were stopped after 10 days' incubation at 37°C, and were washed twice with PBS, fixed and stained with 0.5% crystal violet. Colonies were counted by under an optical microscope.

Scratch wound healing assay

KYSE30 were seeded on plastic 6-well plates. When cell confluence reached approximately 90% at 24 h of transfection, wounds were made in confluent cells using a 10 μl pipette tip. Wound healing was observed at 0 h and 48 h respectively under optical microscope. Duplicate wells for each condition were examined, and each experiment was repeated in triplicate.

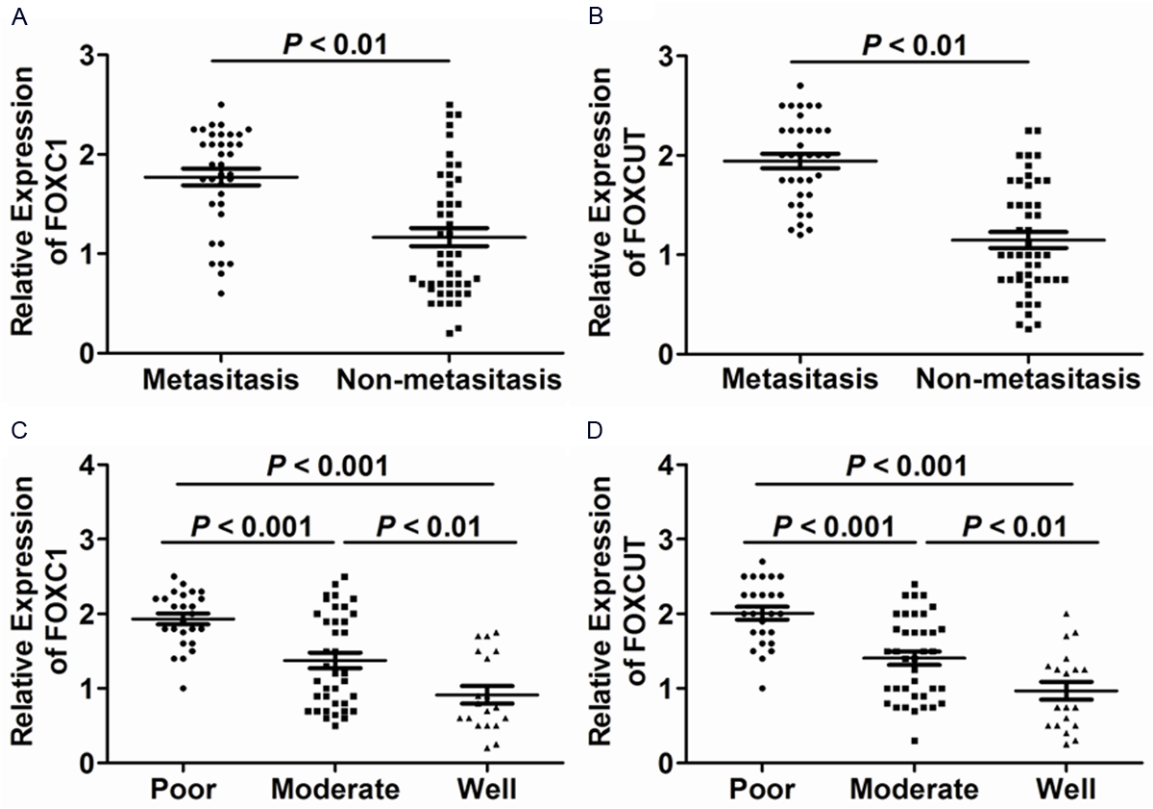

Figure 2. FOXC1 and lncRNA-FOXCUT expression levels were elevated in metastatic ESCC and poorly differentiated tumor tissues. (A) The expression levels of FOXC1 and (B) lncRNA-FOXCUT were higher in metastatic tumor tissues (n = 36) compared to non-metastatic tumor tissues (n = 46) ($P < 0.01$). (C) The expression levels of FOXC1 and (D) lncRNA-FOXCUT were highest in poorly differentiated tumor tissues (n = 25, $P < 0.001$) and were elevated in moderately differentiated tumor tissues (n = 38) compared to well differentiated tumor tissues (n = 19) ($P < 0.01$).

Migration and invasion assay

The cell migration assay was carried out by using Transwell® Permeable Supports with 8 mm pores in 24-well tissue culture plates (Corning, USA). A cell invasion assay was performed using modified BD BioCoat™ Matrigel™ Invasion Chamber with 8 mm pores in 24-well tissue culture plates (BD, USA). 1 x 10^5 cells in 200 μl serum-free RPMI 1640 medium were added to the upper chambers of the inserts of a 24-well culture plate. In contrast, culture medium containing 20% fetal bovine serum in the lower chamber served as the chemoattractant. The cells that had migrated through the filter to the lower sides of the chambers were stained with crystal violet, air-dried, photographed and counted.

Statistical analysis

All statistical analyses were performed by using SPSS version 18.0 (SPSS, Chicago, IL). Differences between groups were analyzed using Student's t test, one-way ANOVA, chi-square test. Correlation between gene expressions was analyzed by using Pearson's correlation coefficient. Oveall survival probability was calculated by the Kaplan-Meier methods and was evaluated by log-rank test. For all statistical analyses, $P < 0.05$ was considered statistically significant.

Results

FOXC1 and FOXCUT were co-overexpressed in ESCC tissue specimens and ESCC cell lines

The FOXC1 mRNA and FOXCUT lncRNA expression levels were detected in a total of 82 paired ESCC cancerous and adjacent noncancerous tissues from ESCC patients by qPCR. Using GAPDH as the normalization control, 69 of the 82 ESCC patients (84.1%, $P < 0.01$) exhibited remarkably higher expression of FOXC1 mRNA in cancerous tissues than in noncancerous tis-

sues (**Figure 1A**) and 71 of the 82 ESCC patients (86.6%, $P < 0.01$) showed significantly higher expression FOXCUT lncRNA in cancerous tissues compared to the levels in noncancerous tissues (**Figure 1B**). In particular, the relative expression of FOXC1 was positively correlated with that of FOXCUT in ESCC tissue specimens ($R^2 = 0.7305$, $P < 0.0001$, **Figure 1C**). Then, the expression of FOXC1 and FOXCUT were assessed in ESCC cell lines, including KYSE30, KYSE70, KYSE140, KYSE150, and KYSE180 and in the normal esophageal cell line Het-1A. The expression of FOXC1 and FOXCUT were remarkably higher in these ESCC cell lines than Het-1A. Of the five ESCC cell lines, KYSE30 cell lines expressed the highest levels of FOXC1 and FOXCUT ($P < 0.05$, **Figure 1D**).

FOXC1 and FOXCUT were correlated respectively with clinicopathological characteristics in ESCC

According to the mean value of relative FOXC1 and FOXCUT expression (1.438 and 1.488, respectively) in tumor tissues, the 82 ESCC patients were divided into two groups including the high expression of FOXC1 (n = 44)/FOXCUT (n = 45) and the low expression of FOXC1 (n = 38)/FOXCUT (n = 37). We then evaluated the correlation of FOXC1 and FOXCUT expression levels with clinicopathological characteristics in ESCC patients (**Table 1**). FOXC1 upregulation was correlated with poor differentiation ($P = 0.001$, **Table 1**), advanced lymph node classification ($P = 0.045$, **Table 1**) and metastasis ($P = 0.001$, **Table 1**), however, statistical analyses showed no correlation of FOXC1 with age, gender, tumor location, tumor size, and clinical stage. Similarly, high expression of FOXCUT was correlated with age ($P = 0.022$), poor differentiation ($P = 0.001$), advanced lymph node classification ($P = 0.007$) and metastasis ($P = 0.001$) and has no association with gender, tumor location, tumor classification, and clinical stage. Furthermore, we discovered that FOXC1 and FOXCUT expression levels were remarkably higher in metastatic ESCC tumor tissues (n = 36) than in non-metastatic ESCC tumor tissues (n = 46) ($P < 0.01$, **Figure 2A**, **2B**). And FOXC1 and FOXCUT expression levels were significantly elevated in poorly differentiated tumor tissues ($P < 0.01$, **Figure 2C**, **2D**). Combined with all these above results, it showed that both of the elevated expression levels of FOXC1 and FOXCUT were related to the progression of ESCC respectively.

Upregulation of FOXC1 and FOXCUT were correlated with poor prognosis in ESCC patients

Kaplan-Meier survival analysis and log-rank tests were conducted to further evaluate the relationship between FOXC1/FOXCUT and prognosis of ESCC patients. From the Kaplan-Meier survival curve, we found that the median survival time of patients with high and low expression levels of FOXC1 were 20 months and 32 months, respectively. The five-year survival rate of high expression group (15.2%) was remarkably lower than that of low expression group (33.3%). The patients with upregulation of FOXC1 (n = 44) had significantly shorter survival time than those with downregulation of FOXC1 ($P = 0.014$, **Figure 3A**). Similarly, the median survival time of patients with high and low expression levels of FOXCUT were 20 months and 48 months, respectively. The five-year survival rate of high expression group (11.10%) was significantly lower than that of low expression group (39.0%). The patients with upregulation of FOXCUT (n = 45) had remarkably shorter survival time than those with downregulation of FOXCUT ($P < 0.001$, **Figure 3B**). These findings supported that upregulation of FOXC1 and FOXCUT were correlated with poor prognosis in ESCC patients.

FOXC1 expression in KYSE30 cells was suppressed by FOXC1 siRNA and FOXCUT siRNA

In ESCC cell line KYSE30, RNAi technique was executed to further demonstrate the correlation between the expression of lncRNA-FOXCUT and mRNA FOXC1. The results proved that the FOXC1 expression level was apparently knocked down by two kinds of FOXC1 siRNAs ($P < 0.05$, **Figure 4A**). And si-FOXC1-2 played more significant effect than si-FOXC1-1 (**Figure 4A**). Moreover, the FOXC1 expression level was also down-regulated in both FOXCUT siRNAs compared with the control siRNA (**Figure 4B**). Particularly, as the lncRNA-FOXCUT expression was knocked down up to 86% by si-FOXCUT-1, the FOXC1 mRNA expression was co-suppressed by 64% (**Figure 4B**). Whereas, the FOXCUT expression levels did not decrease together with the FOXC1 downregulation caused by FOXC1 siRNAs (**Figure 4A**). Considering these findings, it indicated that the expres-

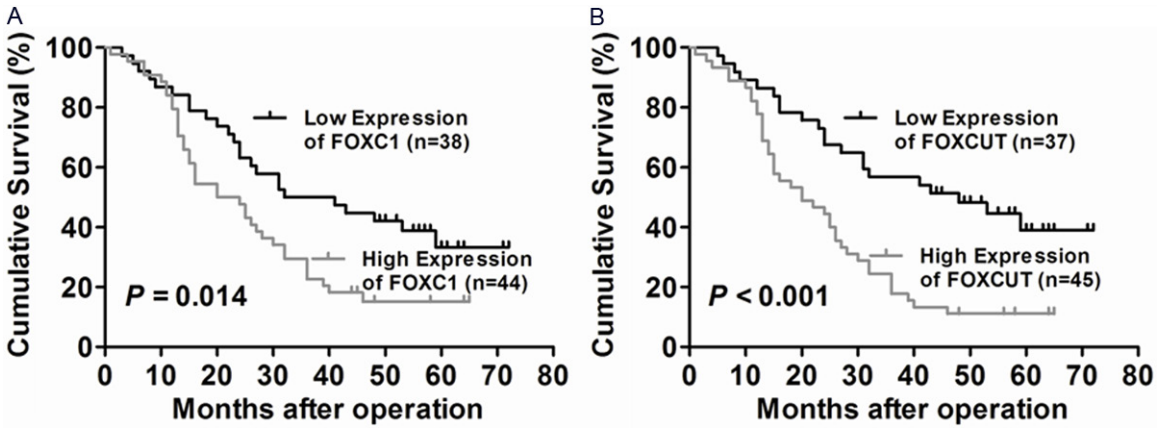

Figure 3. Kaplan-Meier overall survival curve for ESCC patients (n = 82) with different FOXC1 and FOXCUT expression levels. A: Difference in overall survival for ESCC patients with high expression and low expression of FOXC1 was analyzed by log-rank test ($P = 0.014$). Patients with high expression of FOXC1 had a significantly worse prognosis than those with low expression of FOXC1. B: Difference in overall survival for ESCC patients with high expression and low expression of FOXCUT was analyzed by log-rank test ($P < 0.001$). Patients with high expression of FOXCUT had a remarkably worse prognosis than those with low expression of FOXCUT.

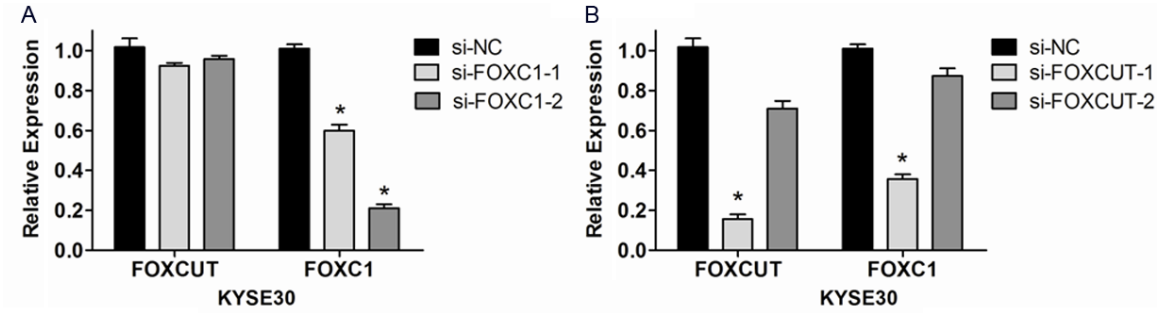

Figure 4. The expression levels of FOXC1 mRNA and FOXCUT lncRNA in KYSE30 cells after siRNA transfection. A: The expression level of FOXC1 in si-FOXC1-2 group was significantly knocked down in KYSE30 cells ($P < 0.05$, the symbol * indicates statistically significant). B: The expression levels of both FOXCUT and FOXC1 were significantly knocked down in si-FOXCUT-1 group ($P < 0.05$, the symbol * indicates statistically significant).

sion of mRNA FOXC1 might be regulated by lncRNA FOXCUT.

Knockdown of FOXC1 inhibited cell proliferation, migration, invasion abilities in KYSE30

Cell proliferation, migration, invasion abilities were important aspects of cancer progression. To clarify whether FOXC1 has a functional role in facilitating ESCC cell progression, a series of cell function experiments were conducted in KYSE30 after siRNA transfection. MTS assay and colony formation assay showed that suppression of FOXC1 notably repressed the cell proliferation of KYSE30 in contrast with the negative control (**Figure 5A**) and the number of cell colonies in the knockdown of FOXC1 groups was also significantly reduced compared with the NC siRNA group (**Figure 5B**). And si-FOXC1-2 played more significant value than si-FOXC1-1 in KYSE30 which was in accordance with down-regulation of FOXC1 by the two FOXC1 siRNAs (**Figures 4A**, **5A** and **5B**). Moreover, both scratch wound healing assay and migration assay proved that knockdown of FOXC1 inhibited cell migration by 52% and 54% respectively (**Figure 5C**, **5D**). In addition, the matrigel invasion assay similarly indicated the silence of FOXC1 in KYSE30 cells declined cell invasion in the Matrigel substrate by 56% (**Figure 5E**). These data demonstrated that FOXC1 promoted cell proliferation, enhanced cell migration, invasion abilities in KYSE30.

Knockdown of FOXCUT inhibited cell proliferation, migration and invasion abilities in KYSE30

To further testify the function of FOXCUT on the growth characteristics of the ESCC cell lines,

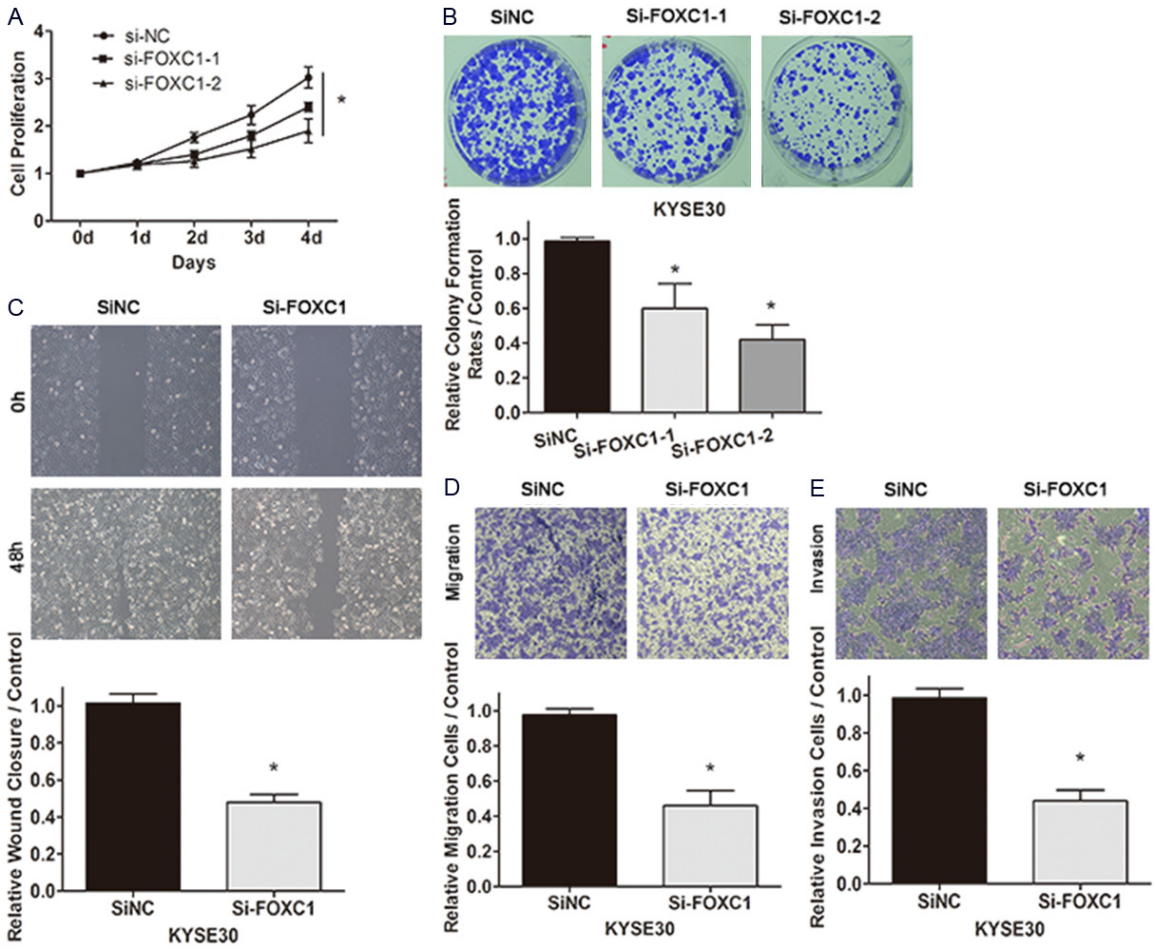

Figure 5. Knockdown of FOXC1 inhibited cell proliferation, migration, invasion abilities in KYSE30. A: Si-FOXC1-2 remarkably inhibited the KYSE30 cell proliferation compared with siNC in MTS assay ($P < 0.05$). B: The colony formation rate of si-FOXC1-2 was significantly decreased compared with siNC ($P < 0.05$). C, D: Both scratch wound healing assay and migration assay proved that knockdown of FOXC1 inhibited cell migration by 52% and 54% respectively. E: The silence of FOXC1 evidently impaired the capacity of cell invasion in the Matrigel substrate ($P < 0.05$).

the same series of cell function experiments were performed in KYSE30 after siRNA transfection. The results showed that cell proliferation ability of KYSE30 was also efficiently suppressed by si-FOXCUT (**Figure 6A**) and the number of cell colonies was apparently decreased through knockdown of FOXCUT compared with the NC siRNA (**Figure 6B**). Similarly, si-FOXCUT-1 played more vital part than si-FOXCUT-2 in KYSE30 corresponding to downregulation of FOXCUT by the two FOXCUT siRNAs (**Figures 4B**, **6A** and **6B**). Furthermore, silence of FOXCUT inhibited cell migration by 44% and 48% respectively (**Figure 6C**, **6D**) by scratch wound healing assay and migration assay. Besides, the matrigel invasion assay similarly proved the downregulation of FOXCUT in KYSE30 cells retarded cell invasion significantly in the Matrigel substrate by 43% (**Figure 6E**). Taking these remarkable results into account, FOXCUT may promote cell proliferation, enhance cell migration, invasion abilities in KYSE30.

Discussion

In the present study, we first identified a new lncRNA-mRNA pair, lncRNAFOXCUT and its adjacent mRNA FOXC1, as a new form of cancer-related gene compound correlated clinically with aggressive biological behaviors and poor survival in ESCC.

ESCC is a kind of tumor involved in complex dynamic biological processes and it initiates from multiple steps of genetic and epigenetic alterations [28]. Previous studies about ESCC

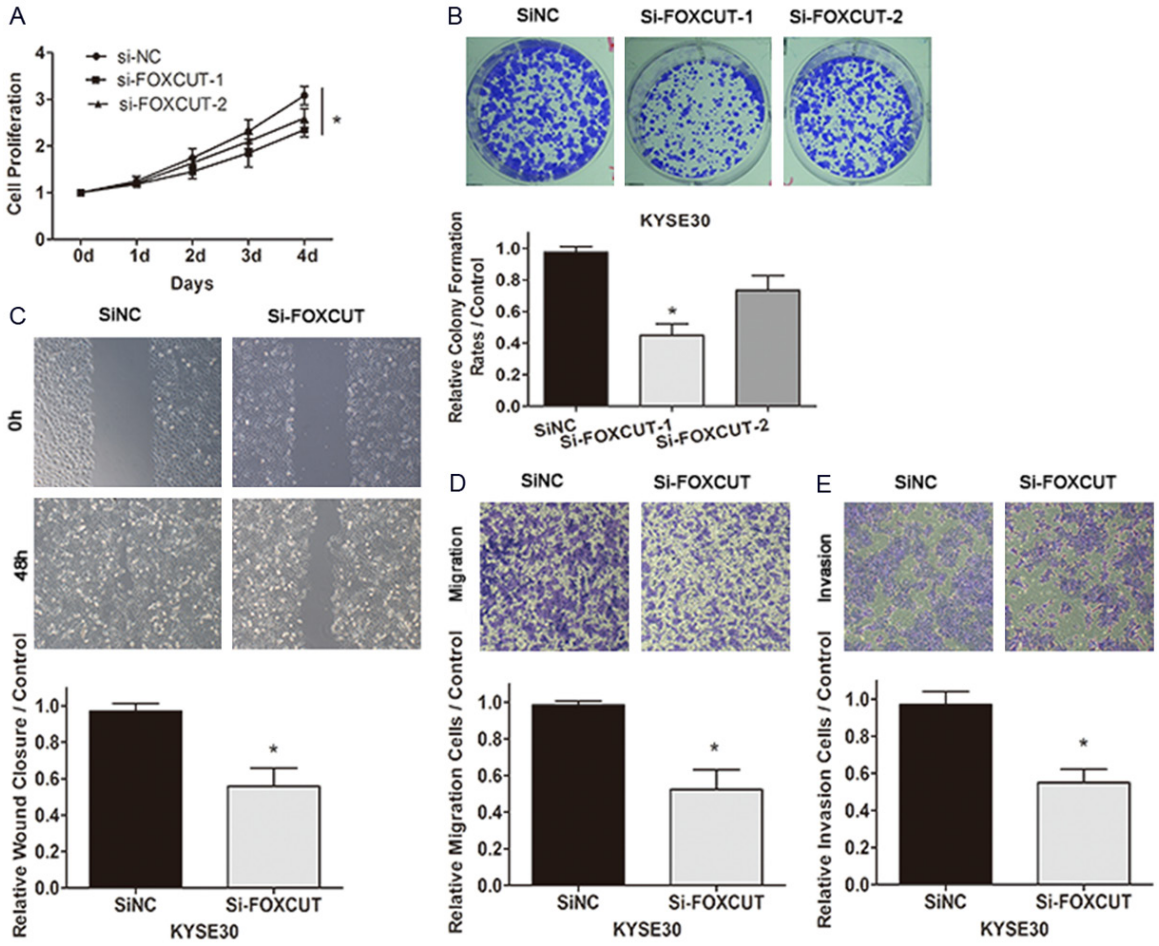

Figure 6. Knockdown of FOXCUT inhibited cell proliferation, migration, invasion abilities in KYSE30. A: Si-FOXCUT-1 remarkably inhibited the KYSE30 cell proliferation compared with siNC in MTS assay ($P < 0.05$). B: The colony formation rate of si-FOXCUT-1 was significantly decreased compared with siNC ($P < 0.05$). C, D: Both scratch wound healing assay and migration assay proved that knockdown of FOXCUT-1 inhibited cell migration by 44% and 48% respectively. E: The silence of FOXCUT-1 evidently impaired the capacity of cell invasion in the Matrigel substrate ($P < 0.05$).

related genes mainly focused on protein-coding genes. Recently, advances in high-throughput sequencing has helped revealing great functions of lncRNAs in cancers [18]. Numerous new lncRNA molecules have been proved to play significant roles in the tumorigenesis, progression of various malignant tumors [29-31]. In ESCC, several lncRNAs have been identified as new biomarkers and therapeutic targets such as PlncRNA-1, HOTAIR [32, 33]. However, the expression and functional roles of most lncRNAs are still unknown in ESCC.

Emerging evidences have demonstrated that FOXC1 was overexpressed in a wide variety of malignant cancers and it can promote tumorigenesis, and tumor progression [21-23]. In our current study, we found a new lncRNA FOXCUT transcribed from the upstream side of FOXC1 promoter through bioinformatics analysis. FOXCUT, an adjacent lncRNA of FOXC1, belongs to promoter upstream transcripts (PROMPTs) [34, 35]. The PROMPTs are often associated with the adjacent protein coding transcripts in their functions [19]. So, we speculated that lncRNA FOXCUT and mRNA FOXC1 may constitute into a new functional lncRNA-mRNA gene pair.

In our study, it is the first time to show that the expression of FOXCUT and FOXC1 (lncRNA-mRNA gene pair) were both identified to be upregulated in 82 ESCC tissues compared with adjacent noncancerous tissues. Linear regres-

sion analysis revealed that FOXCUT and FOXC1 had a positive correlation with each other in ESCC. Furthermore, the expression of FOXC1 was also decreased as the FOXCUT expression was downregulated by siRNA. Whereas, the silence of FOXC1 did not influence the expression of FOXCUT. It indicates that lncRNA FOXCUT may regulate the expression of FOXC1 by some specific mechanisms, which needs further researches to be elucidated completely.

Additionally, our study demonstrated that FOXC1 and FOXCUT expression levels were significantly elevated in tumor samples from patients with the presence of metastasis and poor differentiation. Upregulation of FOXC1 and FOXCUT were both correlated with aggressive clinicopathological characteristics, such as poor differentiation, advanced lymph node classification, metastasis. And, patients with high expression of FOXCUT or FOXC1 had a significantly poor prognosis. These data suggests that high expression of FOXCUT or FOXC1 might play an important role in the tumorigenesis, development, progression of ESCC, and both of them may serve as prognostic biomarkers for ESCC patients.

To further explore the functional role of this new lncRNA-mRNA gene pair in ESCC cells, we designed siRNAs to knock down the expression of FOXC1 or FOXCUT. Functional assays *in vitro* demonstrated that knockdown of FOXCUT or FOXC1 remarkably impeded cell proliferation, migration and invasion abilities in ESCC cells which were consistent with the reported functions of FOXC1 in other cancers [22]. The results indicate that this new lncRNA-mRNA gene pair is an important compound functioning in the ESCC cell aggressive biological behaviours, similar to the well-know lncRNA, HOTAIR in ESCC [6, 32].

In conclusion, FOXCUT and FOXC1, the new lncRNA-mRNA gene pair, are both novel upregulated functional molecules in ESCC. Upregulation of the lncRNA-mRNA pair may be a prognostic factor for ESCC patients, indicating short survival and high risk for metastasis and poor differentiation. FOXCUT may inhibit ESCC cell proliferation, migration and invasion abilities, partially by the regulation of FOXC1 expression. And both FOXCUT and FOXC1 may serve as potential diagnostic markers and therapeutic targets for future ESCC treatment.

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Disclosure of conflict of interest

None.

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