High Expression of Long Non-Coding RNA *AFAP1-AS1* Predicts Chemoradioresistance and Poor Prognosis in Patients With Esophageal Squamous Cell Carcinoma Treated With Definitive Chemoradiotherapy

Xi-Lei Zhou,[1] Wan-Wei Wang,[1] Wei-Guo Zhu,[1] Chang-Hua Yu,[1] Guang-Zhou Tao,[1] Qing-Quan Wu,[2] Ya-Qi Song,[1] Peng Pan,[1] and Yu-Suo Tong[1]*

[1]*Department of Radiation Oncology, Huai'an First People's Hospital, Nanjing Medical University, Huai'an, Jiangsu, China*
[2]*Department of Thoracic Surgery, Huai'an First People's Hospital, Nanjing Medical University, Huai'an, Jiangsu, China*

To evaluate the clinical significance of lncRNAs in the resistance to cisplatin-based chemoradiotherapy in esophageal squamous cell carcinoma (ESCC). We focused on lncRNAs which were frequently reported in ESCC or were involved in chemoradiotherapy resistance. LncRNA expressions were examined in paired cisplatin-resistant and parental ESCC cell lines. Dysregulated lncRNAs were further measured in 162 pretreatment biopsy specimens of ESCC who received definitive chemoradiotherapy (dCRT). Then the correlations between lncRNA expression and response to dCRT and prognosis were analyzed. Three lncRNAs (*AFAP1-AS1*, *UCA1*, *HOTAIR*) were found to be deregulated in cisplatin-resistant cells compared with their parent cells. *AFAP1-AS1* was significantly up-regulated in tumor tissues compared with adjacent normal tissues ($P = 0.006$). Furthermore, overexpression of *AFAP1-AS1* was closely associated with lymph node metastasis ($P < 0.001$), distant metastasis ($P = 0.016$), advanced clinical stage ($P = 0.002$), and response to dCRT ($P < 0.001$). Kaplan–Meier survival analysis revealed that high expression of *AFAP1-AS1* was significantly associated with shorter progression free survival (PFS) (median, 15 months vs. 27 months, $P < 0.001$) and overall survival (OS) (median, 29 months vs. 42 months, $P < 0.001$). In the multivariate analysis, high expression of *AFAP1-AS1* was found to be an independent risk factor to predict poor PFS (HR, 1.626; $P = 0.027$) and OS (HR, 1.888; $P = 0.004$). Thus, high expression of *AFAP1-AS1* could serve as a potential biomarker to predict tumor response and survival. Determination of this lncRNA expression might be useful for selection ESCC patients for dCRT.

Key words: esophageal neoplasms; long non-coding RNA; *AFAP1-AS*1; chemoradiotherapy

INTRODUCTION

Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer-related death worldwide [1]. Esophageal squamous cell carcinoma (ESCC) remains the predominant histological type of esophageal cancer in East Asia and China [2]. Recently, despite remarkable improvements have been made in ESCC treatment and diagnosis, advanced stages of the disease are still difficult to manage [3]. This malignancy is usually diagnosed at a locally advanced stage with obvious enlargement node, long lesion, and/or serious esophageal invasion [4]. For these patients, the current standard treatment is surgical resection or concurrent definitive chemoradiotherapy (dCRT), or a combination of both [5,6]. Complete response (CR): defined by clinical disappearance of tumor, is used as indicator good response to dCRT. However, the combination of radiotherapy and concurrent chemotherapy has led to long-term survival in only 25% of patients. [7]. Thus, the emergence of chemoradiotherapy resistance is one of major obstacle in the management of ESCC patients. Therefore, the significance of detecting predictive biomarkers of therapeutic response should be emphasized.

A new insight into cancer pathogenesis emerged with the discovery of long non-coding RNAs (lncRNAs), which are longer than 200 nt with no protein-coding abilities but regulate expression of protein-coding genes [8]. To date, thousands of lncRNAs have been identified to have functional roles in a diverse range of cellular processes such as development, cell growth and apoptosis, and cancer

Abbreviations: lncRNA, long non-coding RNA; ESCC, esophageal squamous cell carcinoma; dCRT, definitive chemoradiotherapy; OS, overall survival; PFS, progression free survival; qRT-PCR, quantitative real-time polymerase chain reaction; TNM, tumor-node-metastasis; 5-FU, 5-fluorouracial; PTX, paclitaxel; CR, complete response; PR, partial response; PD, progressive disease; NC, no changed; IC50, 50% inhibition concentration; ROC, receiver operating characteristic curve; AUC, area under the ROC curve.

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*Correspondence to: Department of Radiation Oncology, Huai'an First People's Hospital, Nanjing Medical University, Huai'an, Jiangsu 223300, China.

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metastasis [9]. In addition, mounting evidence indicates that lncRNAs are frequently aberrant expression in numerous cancer types and some of them have been implicated in diagnosis and prognostication [10]. Examples include lncRNA *MALAT1* in prostate cancer, *MVIH* in hepatocellular carcinoma and *FENDRR* in gastric cancer, suggesting that lncRNAs could serve as a diagnostic and prognostic biomarkers for human malignancies [11–13]. Currently, the mechanisms underlying resistance development to chemotherapeutic agents are still not fully understood. Recently, several studies have suggested that lncRNAs are likely to play crucial roles in the development of chemotherapy resistance in cancer [14]. For example, W.P. Tsang demonstrated that lncRNA *H19* could induce P-glycoprotein expression and MDR-1 associated drug resistance in liver cancer cells through regulation of MDR-1 promoter methylation [15]. Considering their critical roles in cancer, we hypothesized that the expression levels of lncRNAs in tumor could be associated with chemoradiotherapy resistance in patients with ESCC.

To test the hypothesis, 18 lncRNAs, which were frequently reported in esophageal cancer [16–24] or were involved in chemoradiotherapy resistance [8,14,15,25–30], were selected as candidates. They were examined in cisplatin resistant ESCC cell lines and patients treated with dCRT. Subsequently, the correlation between lncRNA (particularly lncRNA *AFAP1-AS1*) expressions and patient clinical/prognostic factors were assessed to determine whether lncRNA expressions have predictive value of dCRT response and clinical outcome in patients with ESCC.

MATERIALS AND METHODS

Criteria for reporting recommendations for tumor markers in prognosis study (REMARK) were followed wherever possible.

Patient Information and Tissue Specimens

A total of 204 ESCC patients treated with dCRT between January 2008 and December 2009 in our hospital (Huai'an First Hospital, Nanjing Medical University, Jiangsu, China) were collected in the present study for survival analysis. Tumor tissue specimens and the matched normal esophageal mucosa tissues were obtained from patients by endoscopy before dCRT. And another 48 fresh ESCC tissues and paired adjacent normal tissues were obtained from patients undergoing surgery at Department of Thoracic Surgery between January 1 and May 30, 2014. These tissues were selected for qRT-PCR analysis. All cases selected were based on the following criteria: histologically confirmed primary ESCC by available biopsy specimens; no previous local or systemic treatment; age less than 75 years; karnofsky$\geq$70; adequate bone marrow, renal, pulmonary, and hepatic function; no significant medical disease. All biopsy tissue samples were snap frozen in liquid nitrogen and then stored at -80°C until RNA extraction. Tumor staging were determined according to the sixth edition of tumor-node-metastasis (TNM) classification for esophageal carcinoma (UICC, 2002).

The study was approved by Research Ethics Committee, Nanjing Medical University Huai'an First Hospital, and written informed consents were obtained from all patients.

Definitive Chemoradiotherapy

All the 204 patients treated with the same dCRT which included 5-fluorouracial and cisplatin (FP) based regimens. Specifically, cisplatin was administered at 80 mg/m^2 by intravenous infusion on day 1; and 5-fluorouracial (5-FU) 1000 mg/m^2 was administered by continuous infusion for 24 h on days 1–4. Two courses of chemotherapy were used during radiotherapy at 4-week intervals. Radiotherapy was initiated on day 1 of chemotherapy. All patients received external beam radiotherapy using 6 or 15Me LINAC (Siemens ONCOR). A total radiation dose of 60–70 Gy (1.8–2.0 Gy per day, 5 days per week) was delivered with 3- or 4-field technique.

Clinical Response Evaluation and Follow-Up

Four weeks after completion of dCRT, tumor responses to chemoradiotherapy were evaluated through endoscopy and CT scan. Briefly, the clinical responses were categorized as follows: complete response (CR) was defined as total regression of all assessable lesions; partial response (PR) was defined as more than 50% reduction in primary tumor size or more of the sum of the lesions and no progression of assessable lesions; progressive disease (PD) was defined as more than a 25% increase in primary tumor volume or appearance of new lesions; the remaining patients which did not meet the criteria of PD or PR were categorized as no changed (NC). Patients who were evaluated as CR and PR were included in the effective group; and the remaining patients were designated as the resistant group (NC + PD).

Post-treatment follow-up was performed 1 month after dCRT, followed by every 3 month during the first year, and then every 6 month for the second year, and finally annually until 5 yr after treatment.

Cell Lines and Cell Culture

The normal esophageal epithelial cell (Het-1A) and human ESCC cells (KYSE30, KYSE70, KYSE150, KYSE450, KYSE510, and TE10) were maintained in RPMI-1640 medium (Invitrogen, Carlsbad CA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) at 37°C with 5% CO_2. All cells were kind gifts from Prof. Zhi-hua Liu (the State Key Laboratory of Molecular Oncology,

Cancer Institute, Chinese Academy of Medical Sciences, Beijing, China).

Establishment of Cisplatin-Resistant Cell Lines

A cisplatin-resistant ESCC cell line, KYSE30-R, was established from KYSE30 cell line by exposure to gradually increasing concentrations of cisplatin (from 0.2 to 10 μmol/L) over a period of 5 months. Briefly, KYSE30 was exposed to an initial cisplatin concentration of 0.2 μmol/L in RPMI-1640 plus 10% FBS. After 48 h, the treated cells were then washed three times with phosphate buffered saline and cultured in cisplatin-free medium. Upon reaching of 70–80% confluence, the cells were grown in a higher drug concentration (10–20% increase per passage). The above treatment was then repeated until it reached a concentration of 10 μmol/L. We established cisplatin–resistant ESCC cell line in KYSE30, because the expressions of the majority of the lncRNAs were lower in KYSE30 when compared with other ESCC cell lines.

MTT Assay

The MTT assay was used to calculate the 50% inhibition concentration (IC_{50}) for cisplatin and other anticancer agents. Briefly, cells (5×10^3/well) in 100 μl RPMI-1640 with 10% FBS were plated into 96-well plates in quadruplicate. After incubation overnight, they were treated with cisplatin at a concentration range of 0.3125–50 μmol/L (0.3125, 0.625, 1.25, 2.5, 5, 10, 25, and 50 μmol/L) for 6 h. Then the medium was removed, and 100 μl cisplatin-free medium was added. After an additional 48 h, MTT solution (10 μl/well) was added, and the plate was incubated for 4 h. The blue dye taken up by cells was dissolved with dimethyl sulfoxide (100 μl /well), and the absorbance at 490 nm was measured using a microplate reader (Bio-Rad Laboratories, California, USA). The IC_{50} of each anticancer drug was estimated by the dose–response curve.

Total RNA Extraction, Reverse Transcription and Quantitative Real-Time PCR

Total RNA extraction was performed using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA concentration and purity was assessed by measuring absorption (A260/A280) on spectrophotometry. Only samples with an A260/A280 ratio between 1.8 and 2.1 were considered for further experiments.

The reverse transcription reaction was performed with PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Dalian, China) in a 20 μl reaction volume.

SYBR® Premix Ex Tag™ II (Takara, Dalian, China) was used to examine the expression level of lncRNAs in tissue samples and cultured cells. All reactions were examined in triplicate and the specificity of each PCR reaction was confirmed by melt curve analyses. The expression levels of lncRNAs were calculated using △Ct method, where $\triangle Ct = Ct_{target} - Ct_{reference}$, smaller △Ct value indicates higher expression. Relative expression of lncRNAs was analyzed using $2^{-\Delta\Delta CT}$ method with *GAPDH* as the endogenous control to

Table 1. lncRNA *AFAP1-AS1* Expression and Clinicopathologic Characteristics

Characteristics	[a]*AFAP1-AS1* expression (%)		Chi-squared test
	High	Low	*P*-value
Age (year)			0.641
≤55	9 (11.1)	12 (14.8)	
>55	72 (88.9)	69 (85.2)	
Gender			0.463
Male	64 (79.0)	59 (72.8)	
Female	17 (21.0)	22 (27.2)	
Tumor location			1.000
Proximal third	7 (8.6)	8 (9.9)	
Middle/distal third	74 (91.4)	73 (90.1)	
Tobacco use			0.107
Never	44 (54.3)	55 (67.9)	
Ever	37 (45.7)	26 (32.1)	
Alcohol use			1.000
Never	60 (74.1)	61 (75.3)	
Ever	21 (25.9)	20 (24.7)	
Primary tumor length (cm)			0.738
≤5	53 (65.4)	56 (69.1)	
>5	28 (24.6)	25 (30.9)	
Histological differentiation			0.890
Well	9 (11.1)	11 (13.6)	
Moderate	49 (60.5)	48 (59.3)	
Poor	23 (28.4)	22 (27.1)	
Tumor depth			0.185
T1/T2	14 (17.2)	22 (27.1)	
T3/T4	67 (82.8)	59 (72.9)	
Lymph node metastasis			<0.001
Node negative	26 (32.1)	49 (60.5)	
Node positive	55 (67.9)	32 (39.5)	
Distant metastasis			0.016
M0	59 (72.8)	72 (88.9)	
[b]M1	22 (27.2)	9 (11.1)	
TNM stage			0.002
I	8 (9.9)	18 (22.2)	
II	20 (24.7)	33 (40.8)	
III	31 (38.3)	21 (25.9)	
IV	22 (27.1)	9 (11.1)	
dCRT response			<0.001
Effective (CR + PR)	30 (37.0)	68 (84.0)	
Resistant (CD + PD)	51 (63.0)	13 (16.0)	

CR, complete response; PR, partial response; PD, progressive disease; NC, no changed.
[a]Median expression level was used as a cut-off to divide the 162 patients into *AFAP1-AS1*-high ($n = 81$) and *AFAP1-AS1*-low group ($n = 81$).
[b]M1, there were 15 patients with cervical node metastasis, 9 patients with abdominal nodes, and 7 with metastasis in both nodes.

Table 2. List of lncRNAs Expression in KYSE30-R Versus KYSE30 Cell

lncRNA	Average fold change
AFAP1-AS1	2.75
UCA-1	3.03
HOTAIR	2.23
POU3F3	1.17
HNF1A-AS1	1.14
SPRY4-IT1	0.96
PlncRNA1	1.21
ENST00000435885.1	1.05
ENST00000547963.1	1.12
XLOC_013104	1.24
91H	0.97
LOC285194	0.92
ARA	1.31
CCAT2	1.26
AC006050.3-003	1.19
GAS5	1.22
H19	1.09
AK294004	1.21

Average fold was calculated from the delta-delta Ct value (KYSE30-R cell vs. KYSE30 cell) of 3 expts.

normalize the data. The primers used in this study are listed in Supplementary Table S1.

Statistical Analysis

The statistical significance of tissue and cell lncRNA levels between cancer and normal group was analyzed by Mann–Whitney or Student's *t*-test. The Shapiro–Wilk test was used to verify if *AFAP1-AS1* expression follows a normal distribution. Correlations between *AFAP1-AS1* expression and various clinicopathological factors were evaluated by the χ^2 test. Overall survival (OS) and progression free survival (PFS) were analyzed with the Kaplan–Meier method. OS and PFS were defined as the time from start of treatment to death and tumor progression. The cox proportional hazards regression model was used to test the prognostic values of clinical and biological variables. Statistical analyses were performed using SPSS software (version 20.0). All tests were two-sided and *P*-value less than 0.05 was considered significant.

Table 3. Multidrug Resistant Phenotype of KYSE30-R Cells Compared to Its Parental KYSE30 Cells

	IC50 values (mean ± SD, μM)[a]		
Drugs	KYSE30	KYSE30-R	*P-value
Cisplatin	0.934 ± 0.0277	13.063 ± 0.395	<0.001
5-FU	10.717 ± 0.476	27.307 ± 0.272	<0.001
PTX	0.217 ± 0.029	1.099 ± 0.089	=0.001

IC50, 50% inhibition concentration; 5-FU, 5-fluorouracil; PTX, paclitaxel.
[a]IC50 values were calculated by MTT assays as described in the materials and methods.
*P-value was determined by Student's *t*-test.

RESULTS

Patient Characteristics

Of the 204 patients collected for survival analysis, 42 patients were excluded from the present study for the following reasons: lost to follow-up (28 patients); fail to complete the dCRT (9 patients), and previous history of cancer (5 patients). Consequently, a total of 162 patients were selected for further investigation.

Of the 162 eligible patients, clinical stage included: stage I in 26 cases, stage II in 53 cases, stage III in 52 cases, and stage IV in 31 cases. The detailed information was listed in Table 1.

At the evaluation time, CR was achieved in 32 cases (19.8%), PR in 66 cases (40.7%), NC in 61 cases (37.7%), and PD in 3 cases (1.8%), respectively. After dCRT, 7 patients underwent esophagectomy, and 41 patients received adjuvant chemotherapy.

With a median follow-up of 31 months (range 6–72 months), we identified 118 (73%) tumor progression and 122 (75%) deaths. Among the 122 dead patients, 110 (90%) died from tumor recurrence or distant metastasis, and 12 (10%) from other causes (three from heart disease, two from cirrhosis, two from kidney failure, two from bacterial pneumonia, two from second primary tumor, and one from cerebral hemorrhage). The 3- and 5-yr OS rate was 48.1% and 24.7%, respectively.

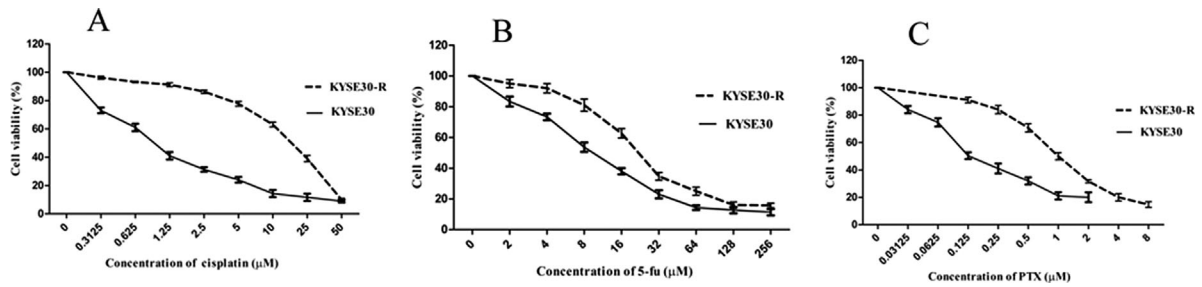

Figure 1. Dose response curves of KYSE30 and KYSE30-R cells to cisplatin, 5-fuorouracil (5-fu), and paclitaxel (PTX). Cell viability was evaluated by MTT assay. The IC_{50} values to each drugs were calculated by SPSS 20.0. KYSE30-R cells were more resistant to cisplatin (mean IC_{50}, 13.063 vs. 0.934 μM, A), 5-fu (mean IC_{50}, 27.307 versus 10.717 μM, B), and PTX (mean IC_{50}, 1.099 vs. 0.217 μM, C) than KYSE30 cells. Data are expressed as the mean ± SD.

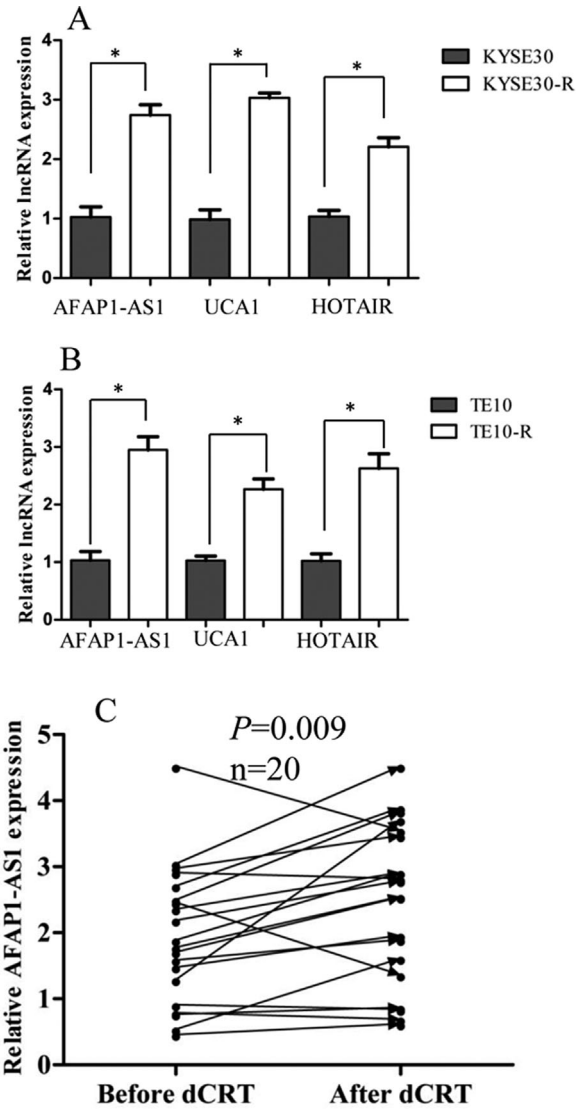

Figure 2. Cisplatin treatment increases lncRNA *AFAP1-AS1*, *UCA1*, and *HOTAIR* expression. The expression levels of the three lncRNAs were significantly increased in cisplatin-resistant sublines KYSE30-R (A) and TE10-R (B) compared with their parent cells. Data are presented mean ± SD. The Student's *t*-test was used to compared the gene expression between the parental and cisplatin-resistant cell lines (*$P < 0.05$). (C) *AFAP1-AS1* expression was up-regulated in patients with local recurrence after definitive chemoradiotherapy ($n = 20$, $P = 0.009$). Statistical differences were analyzed using paired *t* test.

lncRNA Expression Is Altered in Cisplatin-Resistant ECSS Cell Lines

On the basis of previous studies, 18 lncRNAs (Table 2), which were frequently reported in esophageal cancer or were involved in chemoradiotherapy resistance, were selected in this study. To determine whether these lncRNAs are involved in the development of resistance to cisplatin in ESCC cells, we used qRT-PCR to examine the expressions of the 18 lncRNAs in cisplatin–resistant ESCC cell line KYSE30-R and its parental cell line KYSE30. The former was able to tolerate much higher concentrations (14-fold) of cisplatin than its parental cell line (Table 3 and Figure 1). The IC_{50} values for KYSE30 and KYSE30-R were 0.934 and 13.063 μmol/L, respectively. Simultaneously, KYSE30-R also exhibited cross-resistance to 5-FU (∼threefold) and paclitaxel (∼fivefold) (Table 3 and Figure 1), two anticancer drugs that are widely used in combination with cisplatin for the treatment of ESCC. It was shown that the expressions of the majority of the lncRNAs remained unchanged (Table 2), three lncRNAs (*AFAP1-AS1*, *UCAT-1*, *HOTAIR*) were deregulated more than twofold in the paired cell lines (Figure 2A). To further identify changes in lncRNAs expression associated with cisplatin resistance, another pair of parental and cisplatin-resistant ESCC cell line models (TE10 and T E10-R) was also evaluated. As shown in Figure 2B, a similar result was also observed between TE10-R and TE10. Of the three lncRNAs, *AFAP1-AS1* was chosen for further investigation because we found that its expression was up-regulated in patients with local recurrence after dCRT ($P = 0.009$, Figure 2C).

AFAP1-AS1 Is Overexpressed in ESCC

Previous study has shown that *AFAP1-AS1* is overexpressed in barrett' esophagus and esophageal adenocarcinoma. In this study, We measured the expression levels of *AFAP1-AS1* by qRT-PCR in 48 pairs ESCC samples and adjacent normal tissues (The detailed information was listed in Suppl. Table S2), and detected significantly higher expression of *AFAP1-AS1* in tumor specimens (39/48, 81%) compared to normal specimens ($P = 0.006$, Figure 3A). Moreover, we also examined the levels of *AFAP1-AS1* in ESCC cell lines, including KYSE30, KYSE70, KYSE150, KYSE450, KYSE510, TE10 cells and normal esophageal mucosa cell Het-1A. *AFAP1-AS1* was up-regulated in all of the six analyzed ESCC cell lines by 2.2- to 15-fold (Figure 3B).

Correlation Between *AFAP1-AS1* Expression and Clinicopathological Variables

In light of these findings, we then determined whether there was a correlation between *AFAP1-AS1* expression and clinicopathological characteristics. We examined *AFAP1-AS1* expression in cancer tissues from another 162 ESCC patients independent of the 48 ESCC patients from cohort 1. According to the median value of relative *AFAP1-AS1* expression level (2.6-fold, tumor/noncancerous tissues), the 162 patients were divided into two groups: *AFAP1-AS1*-high group (≥median, $n = 81$) and *AFAP1-AS1*-low group (<median, $n = 81$). As shown in Table 1, *AFAP1-AS1* up-regulation was closely associated lymph node metastasis ($P < 0.001$), distant metastasis ($P = 0.016$), advanced clinical stage ($P = 0.002$), and lack of response to dCRT ($P < 0.001$, Table 1). However, there was no significant correlation between *AFAP1-AS1* expression and other clinicopathological variables ($P > 0.05$, Table 1).

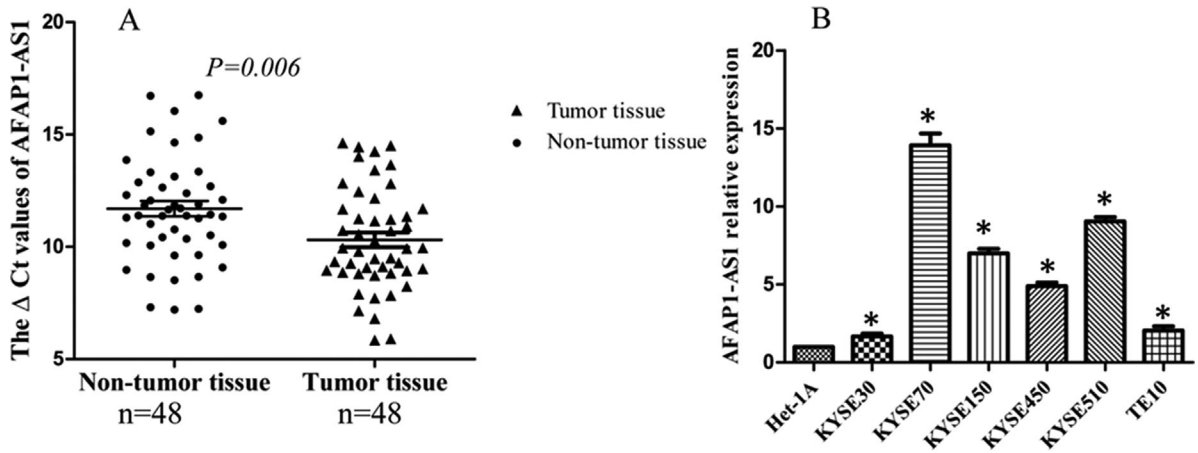

Figure 3. qRT-PCR analysis of lncRNA *AFAP1-AS1* expression in ESCC. (A) lncRNA *AFAP1-AS1* was significantly up-regulated in 48 ESCC tumor samples compared with corresponding normal esophageal mucosa tissues ($P = 0.006$). △Ct method was used to measure the *AFAP1-AS1* expression, which was normalized to GAPDH. Smaller △Ct value indicates higher expression. Horizontal bars indicate median and interquartile range. The Student's *t*-test was used to determine the significance of differences between groups. (B) *AFAP1-AS1* was up-regulated in all of the six analyzed ESCC cell lines compared with normal esophageal mucosa cell Het-1A. All data analyzed using Student's *t*-test. *Significantly different from control ($P < 0.05$).

Diagnostic Utility of *AFAP1-AS1*

Analysis of the levels of *AFAP1-AS1* shows that it could be utilized to distinguish tumor samples from normal esophageal mucosa (Figure 3A). We therefore examined the diagnostic performance of *AFAP1-AS1*. As shown in Figure 4A, *AFAP1-AS1* yield an area under curve (AUC) of 0.802 (95%CI: 0.765–849; $P < 0.001$) with 79.4% specificity and 73.3% sensitivity for distinguishing ESCC samples from normal esophageal mucosa.

Next, to explore the potential role of this lncRNA as a marker for early detection of ESCC, the *AFAP1-AS1* expression levels in tumor samples from early ESCC (stage I + II, $n = 79$) and paired normal tissues were then analyzed. As shown in Figure 4B, *AFAP1-AS1* expression in patients with early ESCC was significantly higher than those of paired normal tissues ($P < 0.001$). Figure 4C shows the diagnostic power of *AFAP1-AS1*, the value of AUC used to detect early ESCC was 0.803 (95%CI, 0.735–870; $P < 0.001$). The diagnostic sensitivity and specificity were 44.6% and 92.3%. Our data provided evidence that *AFAP1-AS1* has great potential as a biomarker for early detection of ESCC.

Correlation Between Clinicopathological Parameters, *AFAP1-AS1* Expression and dCRT Response

Patients who were *AFAP1-AS1*-high on pretreatment cancer biopsies ($n = 81$), CR was achieved in 6 cases (7.4%), PR in 24 cases (29.6%), NC in 48 cases (59.3%), and PD in 3 cases (3.7%), respectively. In contrast, of the 81 patients with *AFAP1-AS1*-low expression, CR, PR, NC, and PD was achieved in 26

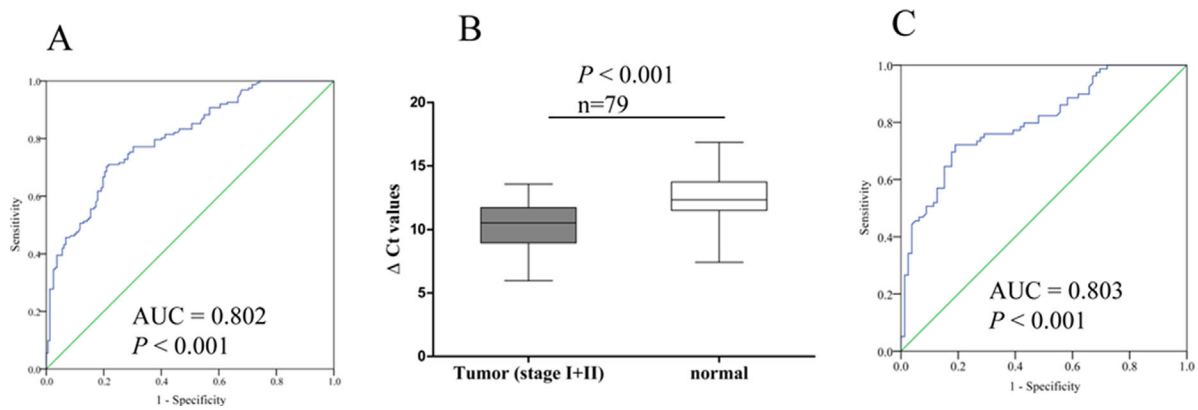

Figure 4. Tissue *AFAP1-AS1* may be a potential biomarker of ESCC. (A) *AFAP1-AS1* can clearly distinguish tumor samples from normal esophageal mucosa. The value of area under curve (AUC) to detect ESCC was 0.802 ($P < 0.001$). (B) *AFAP1-AS1* expression in patients with early ESCC (stage I + II, $n = 79$) was significantly higher than those of paired normal tissues ($P < 0.001$). *AFAP1-AS1* expression levels were calculated by △Ct method, and smaller ΔCt value indicates higher expression. Horizontal lines inside the box plots represent the median, boxes represent the interquartile range, and error bars represent 97.5th and 2.5th percentiles. Statistical differences were analyzed using Student's *t*-test. (C) The value of AUC to detect early ESCC was 0.803.

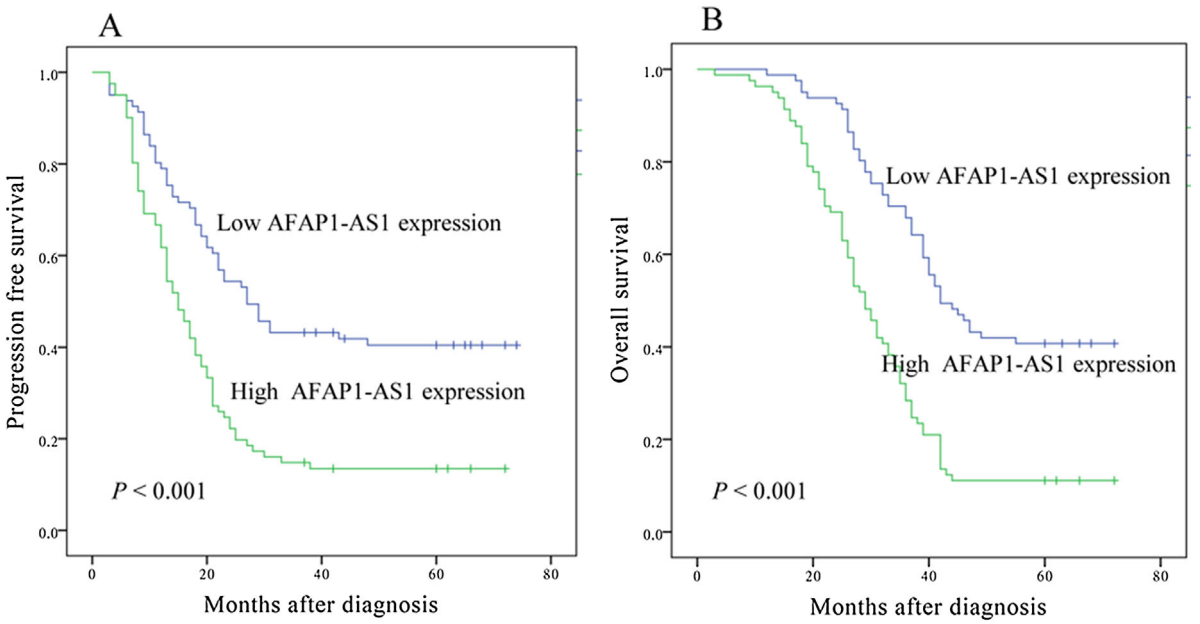

Figure 5. Kaplan–Meier survival analysis of progression free survival (A) and overall survival (B) based on *AFAP1-AS1* expression in all 162 ESCC patients treated with definitive chemoradiotherapy ($P < 0.001$).

cases (32.1%), 42 cases (51.9%), 13 cases (16.0%), 0 cases (0%), respectively. *AFAP1-AS1* expression was the only factor that showed a significant association with CRT response ($P < 0.001$, Table 1). Unexpectedly, no clinicopathological parameter was detected to be correlated with dCRT response ($P > 0.05$, Suppl. Table S3).

AFAP1-AS1 Expression and Clinical Outcomes

From Kaplan–Meier survival analysis, we found that high expression of *AFAP1-AS1* was significantly correlated with shorter PFS ($P < 0.001$, Figure 5A). The median PFS for patients with *AFAP1-AS1*-high tumor was 15 months compared with 27 months for patients with *AFAP1-AS1*-low expression. On univariate analysis, factor associated with PFS were: tumor depth ($P = 0.005$), lymphatic metastasis ($P = 0.014$), TNM stage ($P = 0.008$), dCRT response ($P < 0.001$), and *AFAP1-AS1* expression ($P < 0.001$), (Table 4). After adjustment for tumor depth, lymphatic metastasis, distant metastasis, TNM stage, and dCRT response, *AFAP1-AS1* expression remained an independent prognostic factor of PFS (HR, 1.626; 95%CI, 1.057–2.501; $P = 0.027$, Table 5). Furthermore, our multivariate analysis showed that tumor depth (HR, 1.942; 95%CI, 1.066–3.535; $P = 0.030$) and dCRT response (HR, 1.744; 95%CI, 1.160–2.624; $P = 0.008$) were also significantly independent prognostic factors for PFS (Table 5).

Similarly, patients with higher *AFAP1-AS1* expression had a poorer OS ($P < 0.001$, Figure 5B). The median OS for patients with *AFAP1-AS1*-high tumor was 29 months, compared with 42 months for those patients who had *AFAP1-AS1*-low expression. In univariate analysis, factors associated with OS were: tumor depth ($P = 0.012$), lymphatic metastasis ($P = 0.002$), distant metastasis ($P = 0.046$), TNM stage ($P = 0.002$), dCRT response ($P < 0.001$)), and *AFAP1-AS1* expression ($P < 0.001$), (Table 4). All the six clinicopathological characteristics were further applied for the multiple analyses. The multivariate cox proportional hazard regression analysis indicated that high expression of *AFAP1-AS1* was the most significantly unfavorable prognostic factor of OS (HR, 1.888; 95%CI, 1.223–2.915; $P = 0.004$) followed by lack of dCRT response (HR, 1.672; 95%CI, 1.103–2.538; $P = 0.015$, Table 5).

DISCUSSION

Recent studies have shown that certain lncRNAs (e.g., *PVT1, MEG3*, and *HOTAIR*) are involved in the resistance to cytotoxic drugs and ionizing radiation [31–33]. In the present study, we identified alterations in lncRNAs expression during the development of cisplatin resistance on KYSE30-R and its parental cell line. Among the 18 lncRNAs investigated, *AFAP1-AS1*, previously reported as having oncogenic roles in Barrett' esophagus and esophageal adenocarcinoma, was significantly increased in cisplatin-treated KYSE30-R cell line compared its parent cell line. Furthermore, we also observed that its expression was up-regulated in patients with local recurrence after dCRT. *AFAP1-AS1* is an lncRNA which was extremely hypomethylated and overexpressed in esophageal adenocarcinoma. Its silencing by small interfering RNA inhibited proliferation, induced apoptosis, and reduced tumor cells migration and invasion [16]. In view of these findings, *AFAP1-AS1* was chosen for further investigation.

Table 4. Univariate Cox Regression Analysis of PFS and OS

		PFS			OS		
Prognostic factors	Case	HR	95%CI	*P-value	HR	95%CI	*P-value
Age (year)		1.062	0.618–1.827	0.827	1.288	0.703–2.145	0.470
≤55	21						
>55	141						
Gender		0.925	0.602–1.421	0.721	0.936	0.616–1.422	0.756
Male	123						
Female	39						
Tumor location		1.033	0.555–1.922	0.918	0.901	0.484–1.675	0.741
Proximal third	15						
Middle/distal third	147						
Tobacco use		1.144	0.791–1.653	0.476	1.155	0.801–1.664	0.441
Never	99						
Ever	63						
Alcohol use		0.886	0.583–1.348	0.572	0.988	0.654–1.494	0.954
Never	121						
Ever	41						
Primary tumor length (cm)		1.051	0.716–1.542	0.801	1.234	0.846–1.800	0.276
≤5	109						
>5	53						
Histological differentiation		1.188	0.882–1.600	0.257	1.137	0.848–1.523	0.391
Well	20						
Moderate	97						
Poor	45						
Tumor depth		2.052	1.240–3.395	0.005	1.881	1.150–3.075	0.012
T1/T2	36						
T3/T4	126						
Lymph node metastasis		1.582	1.096–2.285	0.014	1.779	1.231–2.571	0.002
Node negative	75						
Node positive	87						
Distant metastasis		1.325	0.839–2.092	0.227	1.567	1.007–2.437	0.046
M0	131						
M1	31						
TNM stage		1.645	1.141–2.371	0.008	1.796	1.247–2.586	0.002
I + II	79						
III + IV	83						
dCRT response		2.117	1.470–3.051	<0.001	2.170	1.511–3.117	<0.001
Effective (CR + PR)	98						
Resistant (CD + PD)	64						
Adjuvant chemotherapy		0.704	0.472–1.050	0.085	0.834	0.560–1.242	0.371
Yes	41						
No	121						
Total radiotherapy dose (Gy)		1.031	0.692–1.535	0.882	0.877	0.587–1.309	0.520
60	116						
>60	46						
AFAP1-AS1 expression		2.242	1.545–3.255	<0.001	2.665	1.838–3.865	<0.001
Low	81						
High	81						

PFS, progression free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; CR, complete response; PR, partial response; PD, progressive disease; NC, no changed.
*P log-rank test.

Recently, many biomarkers such as p53, Bax, hMLH1, EZH2 have been evaluated as possible predictive factors of tumor response to dCRT [34–37]. However, several of these data obtained by different studies is conflicting, such reliable tumor markers are still currently lacking. In this study, we observed that high expression of *AFAP1-AS1* was significantly correlated with poor response to dCRT in patients with ESCC. Several lncRNAs are known to be correlated with chemo-radiotherapy sensitivity phenotypes in cancers. For example, over-expression of *HOTAIR* could decrease the sensitivity of lung adenocarcinoma cells to cisplatin through regulation of p21 expression [27]. Fan Y and his colleagues demonstrated that lncRNA *UCA1* was up-regulated in patients with metastatic

Table 5. Multivariate Cox Regression Analysis of PFS and OS

Prognostic factors	PFS			OS		
	HR	95%CI	*P*-value	HR	95%CI	*P*-value
Tumor depth (T1/T2 vs. T3/T4)	1.942	1.066–3.535	0.030	1.661	0.919–3.003	0.093
Lymphatic metastasis (No vs. Yes)	1.474	0.632–3.438	0.370	1.413	0.587–3.401	0.441
Distant metastasis (M0 vs. M1)	1.039	0.622–1.736	0.883	1.257	0.758–2.084	0.375
TNM stage (I + II vs. III + IV)	0.728	0.291–1.824	0.499	0.843	0.326–2.174	0.723
dCRT response (Effective vs. Resistant)	1.744	1.160–2.624	0.008	1.673	1.103–2.538	0.015
Expression of *AFAP1-AS1*(Low vs. High)	1.626	1.057–2.501	0.027	1.888	1.223–2.915	0.004

PFS, progression free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval.

bladder cancer after cisplatin-based chemotherapy, and over-expression of *UCA1* significantly increased cell viability in cisplatin treatment by regulating wnt signaling [28]. To the best of our knowledge, the correlation between *AFAP1-AS1* expression and resistance to chemoradiotherapy for ESCC has not been analyzed previously. The present results revealed a close relationship between *AFAP1-AS1* expression and ESCC chemoradiotherapy response. In other words, patients with high expression of *AFAP1-AS1* suggested resistance to dCRT. These results suggested that down-regulation of *AFAP1-AS1* expression may have a new therapeutic application for ESCC patients in the future.

With a cohort of 162 randomly selected ESCC patients, we discovered that the *AFAP1-AS1* expression level was significantly up-regulated in tumor samples compared with adjacent normal tissues. ROC curve analysis further indicated that *AFAP1-AS1* might be a good tumor marker for ESCC diagnosis. In recent years, growing evidence has indicated that noncoding RNAs, predominantly lncRNAs, could distinguish tumor patients from normal controls. Many lncRNAs are expressed in tissue specific manner compared with protein-coding RNAs and have shown the feasibility of using them as molecular markers for diagnosis of cancer [38]. For example, the lncRNA *AC096655.1-002* was significantly down-regulated in gastric cancer tissues compared with paired adjacent normal tissues, and use of this lncRNA alone provided a remarkable improvement in the diagnosis of gastric cancer compared with classic tumor marker serum carcinoembryonic antigen [39]. In prostate cancer, *PCA3/DD3* was found to be upregulated more than sixty times in tumor tissue compared with normal prostate tissue. Such a large difference expressions in tumor compared to normal sample make *PCA3/DD3* a promising biomarker for prostate cancer diagnosis [40].

The most important finding of the current study was the prognostic value of *AFAP1-AS1* expression in ESCC. To date, clinical complete response to treatment and TNM stage have been reported as the most important predictors of outcomes for patients with ESCC treated with definitive chemoradiotherapy [41]. However, our multivariate analysis demonstrated that high expression of *AFAP1-AS1* was the most significantly unfavorable prognostic factor of OS surpassing the advanced TNM stage and the lack of primary CR. Consistently, similar results were also observed in pancreatic ductal adenocarcinoma [42]. In addition, we found that patients with higher *AFAP1-AS1* level had significantly poorer PFS after dCRT. Therefore, *AFAP1-AS1* expression could be used as an attractive biomarker, in addition to other clinical parameters, in identifying patients with ESCC who are at a higher risk of tumor progression.

Lastly, it is worth mentioning that there were several potential limitations in our study, First of all,

the present study was limited to retrospective assessments with a relatively small sample size. Second, we confirmed that *AFAP1-AS1* expression could predict resistance to chemoradiotherapy in patients with ESCC. However, the exact mechanisms were still unclear. Further functional experiments are thus required to elucidate which signaling pathway is involved between the high expression of *AFAP1-AS1* and chemoradiotherapy sensitivity in ESCC upon cellular level.

CONCLUSION

Our results demonstrated that *AFAP1-AS1* could represent a novel predictive biomarker of clinical outcomes (poorer PFS and OS) for ESSC patients treated with dCRT, for which overexpression of this lncRNA will indicate that these patients will not benefit from FP-based dCRT.

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