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

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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 = 0.001) 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. © 2016 The Authors. Molecular Carcinogenesis published by Wiley Periodicals, Inc.

Key words: esophageal neoplasms; long non-coding RNA; AFAP1-AS1; 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...
metastasis [9]. In addition, mounting evidence indicates that IncRNAs are frequently aberrant expression in numerous cancer types and some of them have been implicated in diagnosis and prognosis testing [10]. Examples include IncRNA MALAT1 in prostate cancer, MVIH in hepatocellular carcinoma and FENDRR in gastric cancer, suggesting that IncRNAs 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 IncRNAs are likely to play crucial roles in the development of chemotherapy resistance in cancer [14]. For example, W.P. Tsang demonstrated that IncRNA 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 IncRNAs in tumor could be associated with chemoradiotherapy resistance in patients with ESCC.

To test the hypothesis, 18 IncRNAs, 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 IncRNA (particularly IncRNA AFAP1-AS1) expressions and patient clinical/prognostic factors were assessed to determine whether IncRNA 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 ≥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-fluorouracil and cisplatin (FP) based regimens. Specifically, cisplatin was administered at 80 mg/m² by intravenous infusion on day 1; and 5-fluorouracil (5-FU) 1000 mg/m² 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% CO₂. All cells were kind gifts from Prof. Zhi-hua Liu (the State Key Laboratory of Molecular Oncology, Molecular Carcinogenesis
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 (IC50) 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 IC50 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 A280/A260 ratio between 1.8 and 2.1 were considered further experiments.

The reverse transcription reaction was performed with PrimeScriptTM 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 the 2^−ΔΔCt method with GAPDH as the endogenous control to

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

| Characteristics | High   | Low    | P-value |
|-----------------|--------|--------|---------|
| Age (year)      |        |        |         |
| ≤55             | 9 (11.1) | 12 (14.8) |       |
| >55             | 72 (88.9) | 69 (85.2) | 0.641  |
| Gender          |        |        |         |
| Male            | 64 (79.0) | 59 (72.8) | 0.463  |
| 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) |         |
| 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       | 30 (37.0) | 68 (84.0) |         |
| Resistant       | 51 (63.0) | 13 (16.0) |         |
| (CD + PD)       |        |        |         |

CR, complete response; PR, partial response; PD, progressive disease; NC, no changed.

*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).

**M1, there were 15 patients with cervical node metastasis, 9 patients with abdominal nodes, and 7 with metastasis in both nodes.

ΔCt method, where ΔCt = Ct_target − Ct_reference, smaller ΔCt value indicates higher expression. Relative expression of lncRNAs was analyzed using 2^−ΔΔCt method with GAPDH as the endogenous control to
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 x² 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.

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.

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

| IncRNA     | Average fold change |
|------------|---------------------|
| AFAP1-AS1  | 2.75                |
| UCA-1      | 3.03                |
| HOTAIR     | 2.23                |
| POU5F3     | 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.

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

| Drugs     | IC50 values (mean ± SD, μM) | 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. |
| P-value was determined by Student’s t-test. |

Figure 1. Dose response curves of KYSE30 and KYSE30-R cells to cisplatin, 5-fluorouracil (5-fu), and paclitaxel (PTX). Cell viability was evaluated by MTT assay. The IC50 values to each drugs were calculated by SPSS 20.0. KYSE30-R cells were more resistant to cisplatin (mean IC50, 13.063 vs. 0.934 μM, A), 5-fu (mean IC50, 27.307 vs. 10.717 μM, B), and PTX (mean IC50, 1.099 vs. 0.217 μM, C) than KYSE30 cells. Data are expressed as the mean ± SD.
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\textsubscript{50} values for KYSE30 and KYSE30-R were 0.934 and 13.063 \mu mol/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, UCA1, HOTAIR) were deregulated more than twofold in the paired cell lines (Figure 2A). To further identify changes in lncRNA expression associated with cisplatin resistance, another pair of parental and cisplatin-resistant ESCC cell line models (TE10 and TE10-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’s 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).
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 IncRNA 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 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 cases (32.1%), PR in 17 cases (21%), NC in 30 cases (37.0%), and PD in 8 cases (10.0%), respectively. Statistical differences were analyzed using Student’s $t$-test. The AUC to detect early ESCC was 0.805.

Figure 3. qRT-PCR analysis of IncRNA $AFAP1$-AS1 expression in ESCC. (A) IncRNA $AFAP1$-AS1 was significantly up-regulated in 48 ESCC tumor samples compared with corresponding normal esophageal mucosa tissues ($P = 0.006$). $ΔCt$ method was used to measure the $AFAP1$-AS1 expression, which was normalized to GAPDH. Smaller $ΔCt$ 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 ($P < 0.05$).

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 $ΔCt$ 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 0.805.
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 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’s esophagus and esophageal adenocarcinoma, was significantly increased in cisplatin-treated KYSE30-R 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.
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

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

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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 ESCC 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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