ABSTRACT
Long non-coding RNAs (lncRNAs) have been reported to be involved in the pathogenesis of a variety of malignancies, including oesophageal cancer. Alterations of the lncRNA focally amplified IncRNA on chromosome 1 (FAL1) are present in epithelial tumours. However, its expression pattern and function in oesophageal cancer are poorly addressed. In the current study, we reported that FAL1 is upregulated in oesophageal cancer tissues and is positively correlated with outcomes in oesophageal squamous cell carcinoma (OSCC). Consecutive experiments revealed that the expression level of FAL1 is higher in OSCC cell lines than in human normal oesophageal epithelium cell line HEEpiCs. Importantly, Knockdown of FAL1 suppressed cell proliferation, increased cell cycle arrest, inhibited cell invasion and epithelial-mesenchymal transition (EMT) by affected related genes. In contrast, overexpression of FAL1 has the opposite effects. Our findings underline a novel biological mechanism in which FAL1 acts as a regulator of oesophageal cancer cells and may provide insights into novel therapeutic strategies for oesophageal cancer.

Introduction
Oesophageal cancer is one of the most prevalent cancer type and a leading lethal cancer worldwide [1]. Oesophageal squamous cell carcinoma (OSCC) and oesophageal adenocarcinoma (EAC) are the two main types of oesophageal cancers [2]. OSCC is the predominant form of oesophageal cancer in China with a high incident rate [3]. Radiochemotherapy and surgical resection have been used to treat OSCC in the past decades, however, the prognosis remains disappointing due to the late diagnosis and rapid progression of the disease [4]. Clinical studies have shown that hundreds of gene expression changes have been associated with disease metastasis and patient survival during the progression of oesophageal cancer [5]. Additionally, in vitro and animal studies also demonstrate that the progressive accumulation of genetic changes plays a causal role in regulating cell proliferation, metastasis and rapid progression [6]. Therefore, a better understanding of the genetic and molecular mechanism and identification of new biomarkers and therapeutic targets of OSCC development are beneficial for the disease diagnosis and treatment.

The human genome contains a huge number of non-coding transcripts, long non-coding RNAs (lncRNAs) refer to RNA transcripts with larger than 200 nucleotides (nts), but lack of protein-coding capacity [7]. lncRNAs participate in plenty of fundamental biological processes, such as inflammation, cell proliferation, immune response and cancer [8]. Aberrant expression of lncRNAs has been associated with the development and pathological progression of oesophageal cancers [9]. The IncRNA focally amplified IncRNA on chromosome 1 (FAL1) has displayed striking oncogenic activity in multiple functional experiments. Increased expression of FAL1 has been found in several types of cancers, including papillary thyroid cancer (PTC). Upregulation of FAL1 promoted the expression of cell cycle-related proteins such as the E2F transcription factors 1 and 2, and cyclin D1 [10]. Increased expression and activity of FAL1 result in the repression of p21 expression in different human cancers such as ovarian cancers [11,12]. However, little information regarding the biological roles of FAL1 in oesophageal cancer has been reported before. In the present study, we evaluated FAL1 expression in oesophageal cancer and investigated the underlying molecular mechanisms.

Materials and methods
Clinical specimens
Experimental protocol in the current study was approved by the ethics committee of Heze municipal hospital. All the participants have signed written informed consent. All of the 88 paired tumour and the corresponding non-tumour tissue samples were provided by the second affiliated hospital of Heze municipal hospital. Samples were immediately frozen in liquid nitrogen and stored at −80 °C after resection. All of
the clinical characteristics of the patients have been recorded and analyzed.

**Cell culture and transfection**

Six OSCC cell lines (EC109, Eca9706, KYSE450, KYSE150, TE-1 and TE-2) and the human normal oesophageal epithelium cell line HEEpiC were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 medium (Hyclone, USA) supplemented with 10% foetal bovine serum (FBS) [13]. To knock down the expression of FAL1, TE-1 and Eca9706 cells were transfected with either siRNAs targeting FAL1 (siFAL1) or scrambled negative controls (NS) for 24 h using Lipofectamine 2000 (Life technologies, USA). To overexpress FAL1, TE-1 and Eca9706 cells were transduced with lentiviral-FAL1 (LV-FAL1) or empty vector (LV-vector) using polybrene (Life technologies, USA).

**Cell cycle analysis**

The cell cycle of TE-1 and Eca9706 cells was analyzed using flow cytometric analysis [14]. After necessary transfection, cells were harvested and washed twice with PBS, followed by fixation with 70% ethanol in a cold room for 24 h. Then cells were stained with propidium iodide using the cell cycle and apoptosis analysis kit (Beyotime, China). Cell cycle distribution was then analyzed on a FACS Calibur Cytometer (BD Biosciences, USA).

**Cell invasion assays**

Patterns of cell invasion were determined by matrigel with transwell inserts with an 8.0 mm pore size polyethylene terephthalate membrane. Cells were seeded in the upper chamber containing 200 μl of serum-free DMEM. The lower chambers contained RPMI1640 supplemented with 10% FBS. 24 h later, cells that invaded into the lower side of the membrane were fixed with methanol, stained with 0.1% crystal violet, and photographed with a digital microscope.

**Cell proliferation assay**

After necessary transfection or transduction, a commercial cell counting kit-8 (CCK-8) was used to determine cell proliferation of TE-1 and Eca9706 cells. Briefly, TE-1 and Eca9706 cells were seeded in 96-well plates at the density of $3 \times 10^3$ per well and incubated for 1, 2 and 3 days. The CCK-8 reagent was added and incubated for 1 h. OD value was recorded at 450 nm.

**Real-time polymerase chain reaction (PCR)**

Total RNA was extracted from TE-1 and Eca9706 cells using the Trizol reagent (Life technologies, USA). Abundance of intracellular RNA was determined by using a NanoDrop apparatus. Intracellular RNA was reverse transcribed into cDNA with the iScript cDNA synthesis kit (Bio-Rad, USA). Expression levels of target genes were analyzed with real-time PCR on an ABI7500 real-time PCR system with the SYBR Green qPCR Master Mix (Thermo fisher scientific, USA). Expressions of target gene were normalized to GAPDH using the $2^{-\Delta\Delta C_{t}}$ method [15].

**Western blot analysis**

Protein was extracted from TE-1 and Eca9706 cells using RIPA buffer (Bio-Rad, USA) containing the cocktail protease inhibitor. Protein concentration was determined by a BCA assay. Extracted proteins were denatured by boiling at 100°C for 5 min. Samples were run on an electrophoresis using 4–12% Bis-Tris gels (Novex, USA) and blotted onto polyvinylidene fluoride (PVDF) membranes using the iBlot transfer system (Life technologies, USA). Membranes were then blocked with blocking solution (Roche Applied Science, USA) [16]. Blots were sequentially incubated with primary antibodies and horseradish peroxidase (HRP)-linked anti-rabbit (7074) or antimouse (7076) secondary antibodies (Cell Signaling Technology, USA). Blots were detected using chemiluminescence (Sigma-aldrich, USA). The following antibodies were used in this study: Rabbit monoclonal antibody (mAb) against cyclin D1 (1: 2000, #ab134175, Abcam, USA); Rabbit mAb against Cyclin E (1:2000, #ab135380, Abcam, USA); Rabbit mAb against c-Myc (1: 2000, #ab32072, Abcam, USA); Mouse mAb against ZO-1 (1: 2000, #ab61357, Abcam, USA); Mouse mAb against N-Cadherin (1: 2000, #ab98952, Abcam, USA); Rabbit mAb against β-Actin (1: 5000, #4970, Cell Signaling Technology, USA); HRP-linked anti-rabbit IgG antibody (1: 2000, #7074, Cell Signaling Technology, USA); HRP-linked anti-mouse IgG antibody(1: 2000, #7076, Cell Signaling Technology, USA).

**Statistical analysis**

Experimental results are expressed as mean ± SEM. Statistical analysis was performed using student t-test or one-way or two-way analysis of variance (ANOVA) where appropriate. p-values of less than .05 was considered statistically significant.

**Results**

Firstly, we detected the level of FAL1 in 88-paired OSCC tissues and adjacent normal tissues by real-time PCR. Results in Figure 1(A) indicate that FAL1 expression was significantly higher in OSCC tissues than adjacent normal tissues. We then investigated the relationship between FAL1 expression and the clinic-pathological progression of OSCC patients. We found that the expression of FAL1 in patients with advanced TNM stage (III/IV) was significantly higher than the levels of which in patients with local TNM stage (I/II) (Figure 1(B)). Additionally, the expression of FAL1 in patients with positive lymph node metastasis was significantly higher than those in patients with negative lymph node metastasis (Figure 1(C)). The influence of FAL1 expression on clinical outcomes in OSCC patients was analyzed using the Kaplan–Meier analysis. Results in Figure 2 indicate that the
The survival time for patients with low FAL1 expression was significantly longer than patients with high FAL1 expression. These results suggested that high FAL1 expression predicted a poor prognosis in OSCC patients.

We next compared the expression level of FAL1 in six OSCC cell lines (EC109, Eca9706, KYSE450, KYSE150, TE-1 and TE-2) and human normal oesophageal epithelium cell line HEEpiCs by real-time PCR analysis. Results in Figure 3 indicate the results.

OSCC cells displayed a significantly higher FAL1 expression status, especially in TE-1 and Eca9706 cell lines (Figure 3). To assess the biological functions of FAL1 in OSCC, the expression of FAL1 was knocked down by transfection with FAL1 siRNA in both TE-1 and Eca9706 cell lines. Cell growth was evaluated using the CCK-8 assay. Results in Figure 4(A) and Figure 4(B) indicate that silencing of FAL1 resulted in a significant decrease in cell growth relative to negative control at day 4 in both cell lines, respectively. In contrast, overexpression of FAL1 in TE-1 and Eca9706 cell lines significantly promoted cell growth (Figure 5(A,B)).

We then set out to investigate the effects of FAL1 in the cell cycle. Cells were synchronized at G0/G1 stage by serum starvation (5S) for 24 h. The arrested cells were re-stimulated to enter S phase [17]. Cell cycle was monitored by flow cytometric analysis. Importantly, we found that serum starvation caused arrest at G0/G1 stage resulted in a significant decrease in the expression of FAL1. As expected, re-enter of S stage restored the reduction of FAL1 caused by serum starvation in both the TE-1 and Eca9706 cells (Figure 6 (A, B)). These results suggested that FAL1 might play an important role in regulating the cell cycle. Results in Figure 7(A,B) indicate that knockdown of FAL1 resulted in a significant arrest of cells at G0/G1 phase and a significant decrease in S phase in both the TE-1 and Eca9706 cells. In contrast, overexpression of FAL1 caused a significant increase of cells in S-phase but a decrease of cells in G0/G1 phase in both TE-1 (Figure 7(C)) and Eca9706 cells (Figure 7(D)). In addition, the roles of FAL1 on cell cycle protein expressions were then evaluated. Similarly, western blot analysis showed that knockdown of FAL1 significantly decreased the expression of cycline D1, cyclin E and c-Myc in both the TE-1 (Figure 8(A)) and Eca9706 cells (Figure 8(B)). In contrast, overexpression of FAL1 increased the expression of these cell cycle proteins in both the TE-1 (Figure 8(C)) and Eca9706 cells (Figure 8(D)).

We then investigated the effects of FAL1 on invasiveness of OSCC cells. Knockdown of FAL1 significantly reduced the
invasion of TE-1 (Figure 9(A)) and Eca9706 cells (Figure 9(B)). In contrast, overexpression of FAL1 stimulated the invasion of TE-1 (Figure 9(C)) and Eca9706 cells (Figure 9(D)). Epithelial-mesenchymal transition (EMT) is the remarkable presentation of cell invasion and tumour metastasis. In the physiological process of EMT, cells sacrifice their cell epithelial properties, acquire mesenchymal capacities, and enhance the ability of migration and invasion. Decreased level of the epithelial marker ZO-1 and increased expression of the mesenchymal marker N-cadherin have been associated with tumour metastasis. Therefore, the expressions of EMT markers ZO-1 and N-cadherin were evaluated. Western blot analysis displayed that knockdown of FAL1 obviously elevated the levels of ZO-1, but reduced the levels of N-cadherin in both TE-1 (Figure 10(A)) and Eca9706 cells (Figure 10(B)). In contrast, overexpression of FAL1 reduced the levels of ZO-1, but increased the levels of N-cadherin in both TE-1 (Figure 10(C)) and Eca9706 cells (Figure 10(D)). These findings suggest that FAL1 might have an impact on OSCC cells metastasis by regulating EMT.

The level of PTEN and its downstream AKT activity have been associated with EMT. Therefore, we investigated the effects of FAL1 on the expression of PTEN and AKT phosphorylation. Interestingly, we found that knockdown of FAL1 increased the expression of PTEN and reduced the phosphorylation of AKT in both TE-1 (Figure 11(A)) and Eca9706 cells (Figure 11(B)). However, the total level of AKT remains consistent. In contrast, overexpression of FAL1 reduced the expression of PTEN and increased the phosphorylation of AKT in both TE-1 (Figure 11(C)) and Eca9706 cells (Figure 11(D)). Our results were consistent with a previous study showing that FAL1 may promote tumourigenesis and progression of NSCLC through the PTEN/AKT pathway [18].
Figure 7. FAL1 regulated cell cycle in OSCC TE-1 and Eca9706 cells. (A,B) Flow cytometry analysis demonstrates that knockdown of FAL1 resulted in a significant arrest of cells at G0/G1 phase and a significant decrease in S phase in both the TE-1 and Eca9706 cells (**p < .01 vs. NS group); (C,D) Flow cytometry analysis demonstrates that overexpression of FAL1 resulted in a significant increase of cells in S-phase but a decrease of cells in G0/G1 phase (**p < .01 vs. EV group).

Figure 8. FAL1 regulated cell cycle protein expression in OSCC TE-1 and Eca9706 cells. (A,B) Western blot analysis demonstrates that knockdown of FAL1 decreased the expression of cycline D1, cyclin E, and c-Myc in both the TE-1 and Eca9706 cells (**p < .01 vs. NS group); (C,D) Western blot analysis demonstrates that overexpression of FAL1 increased the expression of cycline D1, cyclin E, and c-Myc in both the TE-1 and Eca9706 cells (**p < .01 vs. EV group).

Figure 9. FAL1 regulated cell invasion in OSCC TE-1 and Eca9706 cells. (A,B) Inhibition of FAL1 reduced the invasion of TE-1 cells and Eca9706 cells (**p < .01 vs. NS group); (C,D) Overexpression of FAL1 promoted the invasion of TE-1 cells and Eca9706 cells (**p < .01 vs. EV group).
Discussion

Oesophageal cancer is one of the most common and fatal malignancies in the world [19]. OSCC is the major subtype of oesophageal cancer, arising from oesophageal epithelial cells [20]. However, the aetiology has not been clearly elucidated. A better understanding of the molecular and genetic basis of OSCC development and progression is beneficial for exploring efficient therapeutic strategy. Additionally, successful identification of novel biomarkers and therapeutic targets are important for improving OSCC diagnosis and treatment. In the past decades, lncRNAs have attracted more and more attention and have been associated with a variety of important cancer phenotypes. Aberrant expression of lncRNAs has been found in various cancers [21]. Multiple lines of evidence have shown that lncRNAs regulate the proliferation, metastasis, invasion, migration and apoptosis in human cancer cells [22]. It is noted that the importance of lncRNAs in OSCC carcinogenesis has been gradually recognized. However, only a small percentage of functional lncRNAs have been well characterized in the pathological progression of OSCC carcinogenesis [23]. In the current study, we analyzed the expression level of lncRNA FAL1 and explored its clinical significance in OSCC.
Oncogenic properties of FAL1 have been reported in previous studies [24]. However, little information regarding the effects of FAL1 in the pathological progression in OSCC has been reported. Here, we compared FAL1 expression in an independent cohort of OSCC tissues and normal tissues. We found that the expression of FAL1 was higher in OSCC tissues. Clinical analysis demonstrated that FAL1 expression was negatively correlated with a cumulative survival rate in patients with OSCC. OSCC patients with higher FAL1 expression tend to have advanced TNM stage. We also found that FAL1 expression is elevated in OSCC cell lines. Knocking down of FAL1 inhibited cell proliferation of OSCC cells but overexpression of FAL1 promoted cell proliferation of OSCC cells. Consistently, inhibition of FAL1 caused retardation of cell proliferation rates and cellular senescence in different types of tumour cell lines [25]. Another important finding in the current study is that knocking down of FAL1 caused an arrest of cells at G0/G1 phase and a significant decrease in S phase in both the TE-1 and Eca9706 OSCC cell lines. Similarly, it has been recently shown that knocking down of FAL1 expression resulted in cell cycle arrest in both A2780 and MCF7 cell lines in ovarian tumours [12]. Previous studies have proved that numerous lncRNAs are associated with cell cycle arrest [26]. For example, the lncRNA CCAT2 has been shown to affect tumour growth by regulating cell cycle arrest [27]. FAL1 dramatically promoted malignant transformation in primary epithelial cells by combining with other well-known oncogenes, such as RAS and MYC [28]. Here, for the first time, we report that FAL1 could accelerate the invasion activity of OSCC cell lines. EMT is an essential mechanism of tumour metastasis in OSCC [20]. During the process of EMT, epithelial cells temporarily lose their own characteristics and develop characteristics of interstitial cells, which can invade and migrate through the body. Expression of the epithelial markers such as E-cadherin and ZO-1 is reduced. In contrast, mesenchymal markers, such as Vimentin and N-cadherin are increased [29]. In the present study, we confirmed that FAL1 could accelerate EMT of OSCC cell lines by regulating the expression of ZO-1 and N-cadherin in both TE-1 and Eca9706 cells.

To sum up, our current study indicates that elevation of FAL1 is associated with OSCC progression. Our findings provide new insights into the biological functions of lncRNAs in the pathogenesis of OSCC and implicate that FAL1 might be a novel biomarker and a therapeutic target for OSCC.

**Disclosure statement**

Authors of this article declare they don’t have any conflict interest that needs to be disclosed.

**References**

[1] Smyth EC, Lagergren J, Fitzgerald RC, et al. Oesophageal cancer. Nat Rev Dis Primers. 2017;3:17048.

[2] Lagergren J, Smyth E, Cunningham D, et al. Oesophageal cancer. Lancet. 2017;390:2383–2396.

[3] Samson P, Lockhart AC. Biologic therapy in esophageal and gastric malignancies: current therapies and future directions. J Gastrointest Oncol. 2017;8:418–429.

[4] Kikuchi O, Ohashi S, Nakai Y, et al. Novel 5-fuorouracil-resistant human esophageal squamous cell carcinoma cells with dihydropteridine dehydrogenase overexpression. Am J Cancer Res. 2015;5:2431–2440.

[5] Guo W, Jiang YG. Current gene expression studies in esophageal carcinoma. Curr Genomics 2009;10:534–539.

[6] Kaz AM, Grady WM. Epigenetic biomarkers in esophageal cancer. Cancer Lett. 2014;342:193–199.

[7] Bunch H. Gene regulation of mammalian long non-coding RNA. Mol Genet Genomics. 2018;293:1–15.

[8] Weidie UH, Bizele F, Kollmorgen G, et al. Long non-coding RNAs and their Role in Metastasis. Cancer Genomics Proteomics. 2017;14:143–160.

[9] Hou X, Wen J, Ren Z, et al. Non-coding RNAs: new biomarkers and therapeutic targets for esophageal cancer. Oncotarget. 2017;8:43571–43578.

[10] Jeong I, Lee J, Kim D, et al. Relationship of Focally Amplified Long Noncoding on Chromosome 1 (FAL1) lncRNA with EZF Transcription Factors in Thyroid Cancer. Medicine (Baltimore). 2016;95:e2392.

[11] Sati S, Ghosh S, Jain V, et al. Genome-wide analysis reveals distinct patterns of epigenetic features in long non-coding RNA loci. NucleicAcids Res. 2012;40:10018–10031.

[12] Hu X, Feng Y, Zhang D, et al. A functional genomic approach identifies FAL1 as an oncogenic long noncoding RNA that associates with BMI1 and represses p21 expression in cancer. Cancer Cell. 2014;26:344–357.

[13] Yu Y, Wang B, Zhang K, et al. High expression of lysine-specific demethylase 1 correlates with poor prognosis of patients with esophageal squamous cell carcinoma. Biochem Biophys Res Commun. 2013;437:192–198.

[14] Zhang Y, Ren S, Yuan F, et al. miR-135 promotes proliferation and stemness of esophageal squamous cell carcinoma by targeting RERG. Artif Cells Nanomed Biotechnol. 2018;2:1–10.

[15] Jafarloo M, Shamehbandi D, Dehghan P, et al. Enhancement of chemosensitivity by simultaneously silencing of Mcl-1 and Survivin genes using small interfering RNA in human myelomonocytic leukemia. Artif Cells Nanomed Biotechnol. 2018;46:1792–1798.

[16] Qin Z, Wei X, Jin N, et al. MiR-199a targeting ROCK1 to affect kidney cell proliferation, invasion and apoptosis. Artif Cells Nanomed Biotechnol 2018;46:1920–1925.

[17] Du X, Fu X, Yao K, et al. Bcl-2 delays cell cycle through mitochondrial ATP and ROS. Cell Cycle. 2017;16:707–713.

[18] Pan C, Yao G, Liu B, et al. Long noncoding RNA FAL1 promotes cell proliferation, invasion and epithelial-mesenchymal transition through the PTEN/akt signaling axis in non-small cell lung cancer. Cell Physiol Biochem. 2017;43:339–352.

[19] Rustgi AK, El-Serag HB. Esophageal carcinoma. N Engl J Med. 2010;362:1925–1937.

[20] Dehghan P, Shamehbandi D, Jafarloo M, et al. Enhancement of chemosensitivity by simultaneously silencing of Mcl-1 and Survivin genes using small interfering RNA in human myelomonocytic leukemia. Artif Cells Nanomed Biotechnol. 2018;46:1792–1798.

[21] Sun W, Yang Y, Xu C, et al. Regulatory mechanisms of long noncoding RNAs on gene expression in cancers. Cancer Genet. 2016;250:9–16.

[22] Sun W, Yang Y, Xu C, et al. Regulatory mechanisms of long noncoding RNAs on gene expression in cancers. Cancer Genet. 2017;250:9–16.

[23] Deng HY, Wang YC, Ni PZ, et al. Long noncoding RNAs are novel potential prognostic biomarkers for esophageal squamous cell carcinoma: an overview. J Thorac Dis. 2016;8:E653–E659.

[24] Yap KL, Li S, Munoz-Caballo AM, et al. Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Mol Cell. 2010;38:662–674.

[25] Zhong X, Hu X, Zhang L. Oncogenic long noncoding RNA FAL1 in human cancer. Mol Cell Oncol. 2015;2:e977154.
[26] Rafiee A, Riazi-Rad F, Havaskary M, et al. Long noncoding RNAs: regulation, function and cancer. Biotechnol Genet Eng Rev. 2018; 34:153–180.

[27] Wu ZJ, Li Y, Wu YZ, et al. Long non-coding RNA CCAT2 promotes the breast cancer growth and metastasis by regulating TGF-β signaling pathway. Eur Rev Med Pharmacol Sci. 2017;21:706–714.

[28] Murugan AK, Munirajan AK, Alzahrani AS. Long noncoding RNAs: emerging players in thyroid cancer pathogenesis. Endocr Relat Cancer 2017;25:R59–R82. ERC-17-0188.

[29] Hu L, Wu Y, Tan D, et al. Up-regulation of long noncoding RNA MALAT1 contributes to proliferation and metastasis in esophageal squamous cell carcinoma. J Exp Clin Cancer Res. 2015;34:7.