Deregulated expression and subcellular localization of CPSF6, a circRNA-binding protein, promote malignant development of esophageal squamous cell carcinoma

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Abstract

Objective: Cleavage and polyadenylation specific factor 6 (CPSF6) has been documented as an oncoprotein in different types of cancer. However, functions of CPSF6 have not been investigated yet in esophageal squamous cell carcinoma (ESCC). Here, we aimed to investigate the potential clinical values and biological functions of CPSF6 in ESCC.

Methods: For determining the expression level of CPSF6 in ESCC patients, we analyzed published data, performed quantitative real-time polymerase chain reaction (RT-qPCR) and immunohistochemistry assays. Kaplan-Meier curves and log-rank tests were used for survival analyses. GO and KEGG analyses were done for CPSF6-related genes. Cell proliferation, colony formation and xenograft assays were conducted to verify the effects of CPSF6 on ESCC. In addition, cell cycle and apoptosis assays were also performed to manifest the functions of CPSF6 and circCPSF6. RNA pulldown and radioimmunoprecipitation (RIP) assays were used for confirming the interaction between circCPSF6 (hsa_circ_0000417) and CPSF6 protein. The regulatory relationship between CPSF6 protein and circCPSF6 was determined by RT-qPCR.

Results: We found that CPSF6 was upregulated in ESCC tissues and overexpression of cytoplasmic CPSF6 was associated with poor prognosis. GO and KEGG analyses suggested that CPSF6 could mainly affect cell division in ESCC. Further experiments manifested that CPSF6 promoted cell proliferation and colony formation in vitro. Xenograft assay showed that knockdown of CPSF6 significantly decreased tumor growth rate in vivo. Subsequently, we verified that depletion of CPSF6 led to cell cycle arrest and apoptosis. Finally, we validated that CPSF6, as a circRNA-binding protein, interacted with and regulated its circular isoform circCPSF6 (hsa_circ_0000417), of which depletion also resulted in cell cycle arrest and cell apoptosis in ESCC.

Conclusions: These findings gave us insight that overexpression of cytoplasmic CPSF6 protein is associated with poor prognosis in ESCC and CPSF6 may function as an oncoprotein, at least in part, through regulating circCPSF6 expression.

Keywords: CPSF6; esophageal squamous cell carcinoma; prognosis; cell proliferation; circRNA-binding protein; circCPSF6

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Introduction

The incidence and mortality rate of esophageal cancer are the seventh and sixth worldwide, respectively (1). Esophageal cancer is a highly malignant tumor with a median survival of less than one year and a five-year survival rate of less than 30% for patients with intermediate to advanced disease (2-4). According to the pathological classification, esophageal cancer is divided into esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). China has a high incidence of esophageal cancer. More than 50% of new patients worldwide are found in China, and more than 90% of them are ESCC (5). Despite the availability of more treatment strategies, the prognosis of ESCC patients is not promising due to its complexity. Biologically, ESCC shares some common pathogenic characteristics and mechanisms with head and neck squamous cell carcinoma (5,6). In addition, increasingly novel biological markers and potential therapeutic targets for ESCC were unveiled in recent years (7-9). However, the underlying mechanisms of tumorigenesis and progression of ESCC still need to be further unclosed.

Cleavage factor Im (CFIm) complex functions as a key regulator of alternative polyadenylation (APA), an RNA post-transcriptional mechanism for generating variable 3’ untranslated regions (3’ UTRs), raising transcriptome complexity of cells (10,11). Cleavage and polyadenylation specific factor 6 (CPSF6), also known as CFIm68, is one of CFIm complex subunits required for RNA cleavage and polyadenylation processing. Several independent research groups have found that CPSF6 is related to tumorigenesis and progression. For instance, CPSF6 is highly expressed in aggressive breast cancer and is a clinically relevant marker of poor patient outcomes (12). In hepatocellular carcinoma, overexpression of CPSF6 increases cell proliferation, migration and invasion both in vitro and in vivo (13). Whereas, there are no studies concerning the biological roles of CPSF6 in ESCC.

Here, we reported that CPSF6 was up-regulated in ESCC and the augmented expression level of cytoplasmic CPSF6 protein was associated with poor prognostic outcomes in ESCC. Functionally, we discovered that CPSF6 enhanced the malignant phenotypes of ESCC cells in vitro and in vivo. Moreover, CPSF6 protein could interact with circCPSF6 and impact its expression. We also manifested that depletion of circCPSF6 raised cell cycle arrest at G0/G1 phase and promoted cell apoptosis in ESCC, which as well arose in CPSF6-delpeted cells. Generally speaking, we conclude that CPSF6 functions as an oncoprotein and may be a potential prognostic indicator and therapeutic target for ESCC.

Materials and methods

Data collection and processing

We downloaded the GSE53624 dataset, which included CPSF6 mRNA expression data of paired tumor and normal tissues and relevant clinical information in 119 ESCC patients, from Gene Expression Omnibus (GEO) database (14). The probes were subsequently re-annotated using methods reported by our previous study (15). Additionally, we obtained the gene expression fragments per kilobase million (FPKM) values of The Cancer Genome Atlas (TCGA) ESCC cohort from TCGA Genomic Data Commons (GDC) portal, and then log2 transformed values were used to analyze CPSF6 expression. We divided the patients into “CPSF6 high” group and “CPSF6 low” group according to the Jorden index. To investigate the potential biological function of CPSF6, we identified CPSF6-related genes in GSE53624 dataset with adjusted P<0.05 and |Pearson correlation coefficient| ≥0.5. The CPSF6-binding RNAs were grabbed from the Encyclopedia of RNA Interactomes (ENCORI) database (16) and organized artificially. R Bioconductor package “clusterProfiler” was used for GO and KEGG enrichment analyses (17).

ESCC tissue microarray and immunohistochemistry (IHC)

One microarray with ESCC tissues (n=55) and adjacent normal tissues (n=49) (HEsoS105Su01) was obtained from OUTDO Biotech (Shanghai, China), and the IHC detection of CPSF6 was performed by OUTDO Biotech according to standard procedures. The antibody of CPSF6 (#A5963, ABclonal, Wuhan, China) was diluted in 1:1,000 during this experiment. We defined the expression level (index) of CPSF6 by multiplying the intensity of the
staining and the frequency of positive cells. The CPSF6 expression indexes were used for paired-sample t-test analysis. In addition, the intensity of the staining was scored as follows: no staining, 0; light staining, 1; intermediate staining, 2; and dark staining, 3. The frequency of CPSF6-positive cells was scored as follows: less than 5%, 0; 5%−25%, 1; 26%−50%, 2; 51%−75%, 3; and 76%−100%, 4. So the CPSF6 staining strength was valued from 0 to 12 by multiplying intensity scores and frequency scores: 0−4 were considered as weak grades, 5−8 as moderate grades, and 9−12 as strong grades. Wilcoxon rank-sum test was used to analyze the frequency distribution of different IHC grades in ESCC tissues and adjacent normal tissues. We divided the patients into “CPSF6 high” group and “CPSF6 low” group according to the median value of CPSF6 expression. The details of clinical information of ESCC patients and IHC data are gathered in Supplementary Table S1.

**Cell lines and cell culture**

NE2 and NE3, the two immortalized human esophageal epithelial cell lines, were cultured in medium with 1:1 mixture of Defined Keratinocyte SFM and EpiLife added EpiLife Defined Growth Supplement (Gibco, California, USA). Human ESCC cell lines (YES2, KYSE30, KYSE70, KYSE140, KYSE150, KYSE180, KYSE410, KYSE450, KYSE510 and COLO680N) were cultured in RPMI 1640 (Gibco, California, USA) supplemented with 10% fetal bovine serum (FBS). All above cell lines were incubated at 37 °C in humidified air with 5% CO2.

**Vector construction and siRNA synthesis**

CPSF6 fragments (1,731 bp) were amplified from cDNA of ESCC cells by Q5 Hot Start High-Fidelity 2× Master Mix (NEB, Massachusetts, USA). The sequences of primers were as follows: forward primer: TGAACCGTCAGATCCGCTAGCTAGTCCGCTGCTGCTG; reverse primer: GTCATCCTTATAATCACGATGACGATATTCCGCTAGCGCTAGATCCGCTGCTGCTG. The pEX3-flag vector, reformed from the pEX3 vector (GenePharma, Shanghai, China), was cut by Nhe I enzyme (NEB, Massachusetts, USA) at 37 °C for 2 h. Then, the PCR products and linear vectors were both separated by electrophoresis within a 1% GelRed-stained agarose gel, and purified using gel extraction kits (TIANGEN, Beijing, China). After ligating with pEASY-Basic Seamless Cloning and Assembly Kit (TransGen Biotech, Beijing, China), plasmids were transformed into competent cells (Escherichia coli, DH5α) by heat shock. Positive colonies were identified by performing Sanger sequencing serviced by SinoGenoMax (Beijing, China). Correct plasmids were used for subsequent experiments or long-term storage at −80 °C.

siRNAs were designed and synthesized by RiboBio (Guangzhou, China). si-NC: GGCTCTAGAAAAGCCTATGC; si-CPSF6: GATGTCGGCGAAGAGTTCA; si-circCPSF6: GTTGAAGGAAAGCTGATA.

**Transfection**

Before transfection, appropriate amounts of cells were seeded in 60-mm dishes and incubated for 24 h. siRNAs (a final concentration of about 100 nmol/L) and/or plasmids (4 μg) coated by lipofectamine 2000 reagent (Invitrogen, New York, USA) were transfected into cells following the protocol of manuals. The efficiency of transfection was determined by quantitative real-time polymerase chain reaction (RT-qPCR) and Western blot assays after 48 h of transfection.

**Lentiviral infection**

High titer lentiviruses were prepared by Genechem (Shanghai, China). Lentiviral infection procedure was referenced to manufacturer’s manuals. One day before lentiviral infection, 1.0×10⁵ cells were seeded into a well of a six-well plate so that about 20% confluence of cells was obtained after 24 h. On the second day, the spent medium was replaced by complete fresh medium with an appropriate volume of lentiviral particles to achieve a desired multiplicity of infection (MOI). The virus-contained medium was removed after infection for 16 h and new complete medium was added. The efficiency of CPSF6 depletion was observed and quantified with RT-qPCR and Western blot assays after 72 h of infection.

**Extraction of nuclear and cytoplasmic fractions**

The nuclear and cytoplasmic fractions of cells were extracted using the PARIS™ kit (AM1921, Invitrogen, New York, USA). Firstly, we collected 1.0×10⁶ fresh cultured cells after washing with cold phosphate buffer saline (PBS). Secondly, cells were resuspended in 300 μL cell fractionation buffer by pipetting gently and centrifuged 5 min at 4 °C and 500×g after incubating on ice for 5−10
min. Supernatant with cytoplasmic fraction was collected carefully into a new nuclease-free microtube on ice for RNA and protein isolation later on. Thirdly, the same volume of cell fractionation buffer was added to wash the nuclear pellet to avoid contamination of the nuclear fraction with portions of the cytoplasmic fraction. Finally, the nuclei were re-pelleted and lysed in 300 μL cell disruption buffer by vortexing until the lysate was homogenous. The nuclear and cytoplasmic lysates were used to isolate RNA and protein.

**RNA extraction, RNase R treatment and RT-qPCR analysis**

Total RNAs were extracted according to the manual of the RNAExpress Total RNA Kit (NCM biotech, Suzhou, China), followed by first-strand cDNA synthesis using the M-MLV reverse transcription kit (Promega, Wisconsin, USA). For circRNA enrichment, 1 μg total RNAs were treated with 1 U RNase R (Geneseed, Guangzhou, China) at 37 °C for 15 min before reverse transcription. cDNA was amplified with PerfectStart Green qPCR SuperMix (TransGen Biotech, Beijing, China) and quantified by the CFX96 Real-Time System (Bio-Rad, California, USA). The relative expression of genes was normalized to ACTB (β-actin), and the fold changes of the target gene expression in relative to the control were calculated by the formula 2^(-ΔΔCt). Primers were listed as follow: β-actin: CTCCA TCTGGGCCTCGCTGT, GCTGTCCACCTTCCACGGT TCC; CPSF6: GGCGAAGAGTTCAACCAGGA, ATCTCGGTCTTTCTGGGGCAT; circCPSF6: GAGTCTGAGCTCATAAATTCGCCGT, GTCTTTCTGCGCCATCT CCATTAT.

**Protein extraction and Western blot analysis**

Cells were harvested and lysed for 10–20 min in cold Pierce IP Lysis Buffer (Thermo Scientific, New York, USA) which was supplemented with protease and phosphatase inhibitor cocktail (NCM biotech, Suzhou, China), and then the supernatant was collected into a new clear 1.5 mL centrifuge tube after centrifuging for 15 min (12,000× g, 4 °C). The protein concentration was quantified by the BCA protein assay kit (Applygen, Beijing, China). Denatured total proteins (30 μg) per sample were loaded into 10% SDS-PAGE gels and separated, followed by transferring to polyvinylidene difluoride (PVDF) membranes. After blocking in Tris buffered saline with Tween-20 (TBST) containing 5% skim milk for 1 h at room temperature, membranes were incubated with primary antibodies at 4 °C overnight. Then, the membranes were washed with TBST three times for 10 min each and incubated with conjugated secondary antibodies for 1 h at room temperature, followed by washing with TBST three times for 10 min each. The targets were developed with Enhanced Chemiluminescent HRP substrate (NCM biotech, Suzhou, China) and imaged on ImageQuant LAS 4000 (GE, Massachusetts, USA). The targeting protein levels were normalized to β-actin using ImageJ for gray-scale statistics.

**Cell proliferation assay**

Firstly, cells were digested by 0.25% trypsin into individual cells and cleaned by PBS twice. After that, the cells were resuspended with fresh medium and counted with the TC10 Automated Cell Counter (Bio-Rad, California, USA). Then, cells were diluted at a density of 3,000 cells per 100 μL for seeding. Each 100 μL suspension was plated into a well of 96 E-Plate after background-testing. Every sample made 6 parallels. The cell index was tested every 15 min lasting for at least 96 h by the xCELLigence Real-Time Cell Analyzer MP System (ACEA, Agilent Technologies, California, USA).

**Colony formation assay**

A total of 1,000 single cells were plated into a well of a six-well plate and incubated at 37 °C in humidified air with 5% CO₂. Colonies would format for about 2 weeks after seeding. During fostering, the medium would be replaced every 3–5 d for up to colony formation. Each group made three parallels. Colonies were stained with 2% crystal violet and then imaged. Colony number was counted using particle analysis of ImageJ.

**In vivo xenograft assay**

Twelve five-week-old female BALB/c nude mice purchasing from Vitalriver (Beijing, China) were housed under specific-pathogen-free (SPF) conditions for a one-week acclimation period and randomly divided into 2 groups with 6 mice in each. Each nude mouse was subcutaneously injected in the left dorsal flank with a total of 3×10⁶ CPSF6-depleted KYSE450 cells or negative control KYSE450 cells, respectively. After 30 d, nude mice were sacrificed and the subcutaneous tumors were removed and weighted. The differences in tumor weight between “CPSF6-depleted KYSE450 cell injection” group and
“negative control KYSE450 cell injection” group were statistically analyzed. All animal cares and procedures were complied with the national policies for animal health and well-being, and the protocol was approved by Animal Care and Use Committee, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College.

**Cell cycle assay**

Firstly, the CPSF6-knockdown cells and negative control cells were respectively starved using serum-free medium for 24 h in order to synchronize most of the cells at G0/G1 stage. After that, cells were digested and re-cultured in complete medium for another 24 h. Then, adherent cells were rinsed twice with cold PBS. Eventually, cells were digested into single cells and fixed with 70% ethanol for 2 h at −20 °C prior to propidium iodide (PI) staining and flow cytometric analysis. Each group was conducted in triplets.

**Cell apoptosis assay**

The CPSF6-knockdown cells and negative control cells were respectively seeded into 60-mm dishes and cultured for 48 h so that cell confluence reached about 90%. Then, the cells were digested into individual cells, washed twice with pre-chilled PBS and resuspended with 1× binding buffer to achieve a density of 1.0×10^5 cells/mL. Annexin V-FITC (5 μL) were added into 195 μL of the resuspension, followed by adding 10 μL of PI and mixing thoroughly. Protecting from light, the resuspension was incubated for 30 min at room temperature with rotation. After that, the beads were washed twice with 500 μL protein-RNA binding buffer and then the mixture was incubated for 30 min at room temperature with agitation. Next, cell lysates with 1 μg total protein were diluted in protein-RNA binding buffer to a final volume of 1 mL. The 1 mL diluted lysates were mixed with previously prepared probe-bound beads and incubated for 1–2 h at room temperature with rotation. Finally, the beads were washed three times with wash buffer (20 mmol/L pH 7.4 Tris-HCl, 150 mmol/L NaCl, 2 mmol/L MgCl2, 5% glycerol, 0.1% Tween-20), and subsequently boiled in 50 μL 2× sodium dodecyl sulfate (SDS) loading buffer. Western blot assay was used for further analysis. GAPDH (#10494-1-Ig, Proteintech, Wuhan, China) was considered as a negative control.

**RNA-binding protein RIP assay**

For the native RIP assay, cell lysates (1 mg total protein, containing protease and phosphatase inhibitor cocktail and RNase inhibitor) were incubated with 2 μg of targeting antibodies for 4 h at 4 °C on a rotator, and then 25 μL pre-cleared Protein A/G PLUS agarose beads (Santa Cruz, California, USA) were added into the mixture and incubated for 30 min at room temperature with rotation. Next, the slurry was washed 3 times with wash buffer (20 mmol/L pH 7.4 Tris-HCl, 150 mmol/L NaCl, 0.1% Tween-20), and subsequently boiled in 50 μL 2× sodium dodecyl sulfate (SDS) loading buffer. The captured RNAs were extracted using TRIzol followed by RT-qPCR analysis.

**Statistical analysis and graphics production**

IBM SPSS Statistics (Version 26.0; IBM Corp., New York, USA) and GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA) were used for statistical and graphics production. Survival analyses were performed according to the Kaplan-Meier method, and the statistical
differences were gathered by log-rank test. Wilcoxon rank-sum test and paired-sample t-test were both used for comparing the IHC staining strength of CPSF6 protein between ESCC tissues and adjacent normal tissues. The rest of statistical analyses were all gathered by Student’s t-test. P<0.05 (two-tailed) was considered significantly difference.

Results

CPSF6 is high expressed in ESCC tissues and ESCC cell lines

Previous studies have demonstrated that CPSF6 plays a pro-oncogenic role in several human cancers (13,18,19). Herein, we investigated the expression and functions of CPSF6 in ESCC. Firstly, through the Gene Expression Profiling Interactive Analysis 2 (GEPIA2) (20), we found that CPSF6 was universally up-regulated in tumor (statistically significant differences marked in red). Although GEPIA2 didn’t distinguish subtypes of esophageal cancer, a trend of high expression of CPSF6 in esophageal cancer was observed (Figure 1A). In order to know the expression of CPSF6 in ESCC, we collected ESCC tissues (n=29) and adjacent non-tumor tissues (n=30) for RT-qPCR assay. The results showed the mRNA level of CPSF6 in tumors was on average about 1.8-fold higher than that in non-tumor tissues (P<0.001) (Figure 1B). In addition, both mRNA and protein level of CPSF6 were prevalently up-regulated in ESCC cell lines (YES2, KYSE30, KYSE70, KYSE140, KYSE150, KYSE180, KYSE410, KYSE450, KYSE510 and COLO680N), compared with two immortalized esophageal epithelial cell lines (NE2 and NE3) (Figure 1C,D). These results suggest that CPSF6 may positively contribute to tumorigenesis and progression in ESCC.

High level of CPSF6 mRNA is associated with poor prognosis in ESCC

Next, we retrieved the mRNA expression level of CPSF6 and clinical characteristic information of ESCC patients from public databases. Consistent with above results, the mRNA expression level of CPSF6 in ESCC was significantly higher in tumor tissues than in normal tissues (TCGA: P<0.001; GSE53624: P<0.001) (Figure 2A,B). In addition, overall survival analysis of all ESCC cases showed that high expression of CPSF6 was associated with a worse patient survival (Figure 2C). Furthermore, we conducted survival analyses of stage I+II cases, stage III cases and lymph node metastasis cases of ESCC, individually. Not only in the early-stage patients (stage I+II), but also in the late-stage patients (stage III and lymph node metastasis), all analyses showed that the “CPSF6 low” groups had better median overall survival time than the “CPSF6 high” groups (Figure 2D,E,F). Together, these results indicate that down-regulation of CPSF6 mRNA may have a survival benefit for ESCC patients and CPSF6 may play pro-cancer roles in both early and advanced stages of ESCC.

High level of cytoplasmic CPSF6 protein is detrimental to ESCC patient prognosis

Besides, we examined the protein level of CPSF6 in clinical ESCC tissues (n=55) and adjacent normal tissues (n=49) by immunohistochemistry (IHC) assay (Figure 3A). As expected, CPSF6 protein was significantly up-regulated in ESCC tissues comparing with adjacent normal tissues through Wilcoxon rank-sum test (P=0.005) and paired-sample t-test (49 pairs, P<0.001), respectively (Figure 3B,C). Unexpectedly, we found that the expression level of total CPSF6 protein was not associated with the median overall survival time of ESCC patients (P=0.934) (Figure 3D). By looking closely at the IHC images, we observed that the staining strength of cytoplasm-located CPSF6 protein was likely higher in ESCC cases than in normal cases. Moreover, we also confirmed that cytoplasm-located CPSF6 was expressed indeed higher in ESCC cell lines than in immortalized esophageal epithelial cell lines, but there were no such differences in the nucleus generally (Figure 3E). Thus, we rejudged the expression level of CPSF6 protein in the cytoplasm and nucleus, respectively, according to the IHC staining indexes. Wilcoxon rank-sum tests showed that, in the cytoplasm, there was statistically significant difference between ESCC tissues and adjacent normal tissues (P=0.001) (Figure 3F), while no statistically significant difference in the nucleus (P=0.051) (Figure 3G). Similarly, through paired-sample t-test, a significantly statistic difference of CPSF6 protein expression between ESCC tissues and adjacent normal tissues was only discovered in the cytoplasm (P<0.001), not in the nucleus (P=0.760) (Figure 3H,I). Given that, extending survival analyses were done based on CPSF6 protein level in the cytoplasm and nucleus, individually. Notably, we observed that the patients with augmented cytoplasmic CPSF6 protein had shorter median overall survival time (15 months) than those with low cytoplasmic CPSF6 protein
Figure 1 CPSF6 is highly expressed in ESCC. (A) CPSF6 mRNA expression profile across all types of tumor and normal tissues in GEPIA 2. Each dot represents CPSF6 expression (TPM) of one sample. Statistically significant differences between tumor and normal samples are marked in red; (B) RT-qPCR analysis for relative CPSF6 mRNA expression of ESCC tumor tissues (n=29) and normal tissues (n=30) (P<0.001); (C) RT-qPCR analysis for relative CPSF6 mRNA expression in 2 immortalized esophageal epithelial cell lines and 10 ESCC cell lines; (D) Western blot assay for CPSF6 protein expression in 2 immortalized esophageal epithelial cell lines and 10 ESCC cell lines. Bar plot shows the relative CPSF6 protein normalized to β-actin. ESCC, esophageal squamous cell carcinoma; TPM, transcripts per million; RT-qPCR, quantitative real-time polymerase chain reaction.
Otherwise, expression level of CPSF6 protein in the nucleus was not associated with overall survival time of ESCC patients (P=0.554) (Figure 3K). Above results reveal that it could be the cytoplasmic CPSF6 protein, rather than the total CPSF6 protein or the nucleoplasmic CPSF6 protein, that serves as a potential indicator for poor patient prognosis.

**CPSF6 plays oncogenic roles in ESCC**

We subsequently retrieved the expression information of CPSF6-related genes (adjusted P<0.05 and |Pearson correlation coefficient| ≥ 0.5) from the GSE53624 dataset for GO biological process (BP) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. As the results, CPSF6-related genes were mostly enriched in cell cycle- and apoptosis-related pathways (Figure 4A,B). So, we speculated that CPSF6 may affect cell growth and death in ESCC. For exploring these biological roles of CPSF6, we firstly knocked down and overexpressed CPSF6 in ESCC cells. Obviously, depletion of CPSF6 caused a sharp decrease in CPSF6 expression while overexpression of CPSF6 led to a large increase of CPSF6 expression (Figure 5A,B). Functionally, depletion of CPSF6

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**Figure 2** High mRNA level of CPSF6 is associated with poor prognosis in ESCC. (A) Relative CPSF6 mRNA expression (Log2 FPKM) of ESCC tumor samples (n=11) and normal samples (n=81) from the TCGA (P<0.001); (B) Relative CPSF6 mRNA expression (Log2 FPKM) of ESCC tumor samples (n=119) and normal samples (n=119) from the GSE53624 dataset (P<0.001); (C-F) Kaplan-Meier survival analyses for the correlation between CPSF6 mRNA expression and overall survival with all cases (C), stage I+II cases (D), stage III cases (E), and lymph node metastasis cases (F) using the data of GSE53624 dataset. ESCC, esophageal squamous cell carcinoma; TCGA, The Cancer Genome Atlas.
Figure 3 High cytoplasmic protein level of CPSF6 is detrimental to ESCC patient prognosis. (A) IHC staining of CPSF6 protein in ESCC and adjacent normal tissues; (B) Distribution of samples with different grades of total CPSF6 protein in ESCC (n=55) and adjacent normal tissues (n=49) (P<0.001); (C) Comparing total expression of CPSF6 protein in ESCC and adjacent normal tissues by IHC index [intensity × positive rate (%)] (P<0.001); (D) Kaplan-Meier survival analysis of cases according to the level of total expression of CPSF6 protein [HR=1.027 (95% CI: 0.637−1.965), P=0.934]; (E) Western blot assay for detecting the subcellular locations of CPSF6 protein in ESCC cell lines; (F) Distribution of samples with different grades of cytoplasmic CPSF6 protein in ESCC and adjacent normal tissues (P<0.001); (G) Comparing cytoplasmic expression of CPSF6 protein in ESCC and adjacent normal tissues by IHC index (P=0.051); (H) Kaplan-Meier survival analysis of cases according to the level of cytoplasmic expression of CPSF6 protein (P<0.001); (I) Distribution of samples with different grades of nucleoplasmic CPSF6 protein in ESCC and adjacent normal tissues (P=0.760); (J) Comparing nucleoplasmic expression of CPSF6 protein in ESCC and adjacent normal tissues by IHC index [HR=1.820 (95% CI: 0.964−3.439), P=0.062]; (K) Kaplan-Meier survival analysis of cases according to the level of nucleoplasmic expression of CPSF6 protein [HR=0.821 (95% CI: 0.427–1.576), P=0.554].

ESCC, esophageal squamous cell carcinoma; IHC, immunohistochemistry; HR, hazard ratio; 95% CI, 95% confidence interval.
significantly attenuated cell proliferation and colony formation, while overexpression of CPSF6 significantly bolstered up these two malignant phenotypes in ESCC cells (Figure 5C, D). Otherwise, in vivo xenograft assay also demonstrated that the proliferation rate of subcutaneous tumor was significantly reduced after knocking down CPSF6 in ESCC cells (Figure 5E). Collectively, these observations indicate that CPSF6 plays an oncogenic role in ESCC.

Knockdown of CPSF6 induces cell-cycle arrest and apoptosis of ESCC

Recalling the results of signaling pathway analyses, we hypothesized that the pro-oncogenic effect of CPSF6 might be mediated by impacting cell cycle and apoptosis of ESCC cells. We thus conducted cell cycle and apoptosis assays with knockdown of CPSF6 in ESCC cell lines, respectively. As the results showed, the cell cycle was
Figure 5 CPSF6 plays oncogenic roles in vitro and in vivo. (A,B) RT-qPCR assay (A) and Western blot assay (B) for detecting the mRNA and protein level of CPSF6 in ESCC cell lines (KYSE410 and KYSE450) after knockdown or overexpression of CPSF6; (C) Proliferation curves of ESCC cell lines (KYSE410 and KYSE450) after knockdown or overexpression of CPSF6; (D) Colony formation assay of ESCC cell lines (KYSE410 and KYSE450) after knockdown or overexpression of CPSF6. The number of colonies is presented in bar plots; (E) Subcutaneous tumorigenesis assay was performed after knockdown of CPSF6 in KYSE450 cells. Tumor weight was measured on d 30 after subcutaneous injection. A box-plot is presented for tumor weight. ESCC, esophageal squamous cell carcinoma.
significantly arrested at G_0/G_1 phase in CPSF6-depleted cells (Figure 6A,B). Further experiments proved the resistance to apoptosis of ESCC cells was also significantly diminished after knockdown of CPSF6 (Figure 6C,D). In parallel, we found that the level of cleaved caspase 3 was dramatically increased in CPSF6-depleted cells (Figure 6E),

Figure 6 Knockdown of CPSF6 induces cell-cycle arrest and apoptosis of ESCC. (A,B) Cell cycle assay was performed after knockdown of CPSF6 in KYSE410 (A) and KYSE450 (B) cells, and the percentage of cells in each cell cycle phase is presented in bar plots; (C) Apoptosis assay was performed after knockdown of CPSF6 in ESCC cell lines (KYSE410 and KYSE45); (D) Bar plots are presented for the proportion of apoptotic cells; (E) Caspase 3 and cleaved caspase 3 were detected by Western blot after knockdown of CPSF6 in ESCC cell lines (KYSE410 and KYSE45). ESCC, esophageal squamous cell carcinoma.
which meant cell apoptosis was strongly activated after knockdown of CPSF6. Taken together, these results unveil that CPSF6 boosts the rate of cell division and the ability of cells to resist apoptosis, which may be the causes of promoting malignant phenotypes in ESCC.

**CPSF6 protein binds to and regulates its circular isoform circCPSF6 (hsa_circ_0000417)**

CPSF6 is an RNA-binding protein (RBP) which contains an N-terminal RNA recognition motif (21). For investigating the RNAs interacting with CPSF6 protein, we downloaded the genome-wide cross-linking immunoprecipitation sequencing (CLIP-seq) data of CPSF6 from the Encyclopedia of RNA Interactomes (ENCORI) database and the CLIP-seq data showed that CPSF6 protein can interact with different kinds of RNAs, including mRNA, circRNA, lncRNA and so on. Apart from a large number of mRNAs bound with CPSF6 protein, the number of CPSF6 protein-binding circRNA far exceeded that of other categories of RNA (Figure 7A). Accidentally, we noticed a circular isoform of CPSF6 bound to CPSF6 protein in CLIP-seq data. However, the circular isoform was not labeled with cirRNA ID in the CLIP-seq data. To identify the specific circular isoform of CPSF6, we queried our own RNA-seq data and determined that the major circular isoform of CPSF6 in ESCC was circCPSF6 (hsa_circ_0000417) (unpublished data). CircCPSF6 consists of exons 2-9 of CPSF6 gene and is formed by back-splicing. We confirmed that circCPSF6 was resistant to RNase R and had a conventional back-splicing junction site consistent with annotation in Human circRNA Database (circbank) (Figure 7B). The circRNA pulldown assay and RIP assay showed that these two molecules could interact with each other indeed (Figure 7 C,D). Furthermore, we demonstrated the subcellular distribution of circCPSF6 and found it mainly located in the cytoplasm (79.7%) in ESCC cells (Figure 7E). Reminded that a portion of CPSF6 protein was also detected in the cytoplasm, we wondered if there was any regulatory relationship between these two molecules. For this purpose, CPSF6 and circCPSF6 were both detected in CPSF6- or circCPSF6-depleted cells. As the results, circCPSF6 underwent significant downregulation in CPSF6-depleted cells (Figure 7F). In contrast, no significant changes of CPSF6 were seen in circCPSF6-depleted cells (Figure 7G). As with the impacts of CPSF6, depletion of circCPSF6 arrested cell cycle in G_0/G_1 phase and increased apoptosis of ESCC cells (Figure 7H, I). Above results encourage us that CPSF6 may act as an upstream regulator of circCPSF6 and may play the oncogenic roles, at least in part, through circCPSF6.

**Discussion**

CPSF6 protein, as a regulator of APA, was mainly detected in nucleoplasm and nuclear speckles, and only small amount of that was located in the cytoplasm (22,23). Recent studies have reported that CPSF6 is dysregulated in hepatocellular carcinoma and gastric cancer and functions predominantly in the nucleus at the level of APA regulation to promote tumorigenesis and development (13,18). However, there were no studies reporting the clinical values and functions of cytoplasmic CPSF6 protein. In the present study, we found that cytoplasmic CPSF6 was up-regulated in ESCC and associated with poor prognosis of ESCC patients, which indicates that cytoplasmic CPSF6 could be a potential biological marker for prognostic assessment in ESCC. The high expression of cytoplasmic CPSF6 protein in ESCC also suggests that CPSF6 may provide previously unknown functions to sustain tumor cell survival.

The development of tumor is an extremely complex and systemic process in which a large number of genes are hyperactive or super-silenced. Dysregulation of gene expression is a key feature of ESCC (5,6,9,24). It has been reported that aberrant genes of ESCC mostly cluster in cell cycle, apoptosis and DNA damage control pathways (25), which affect malignant phenotypes, including cell proliferation, clone formation, migration and invasion, of cancer in different ways. In order to study the potential biological roles of CPSF6 in ESCC, we firstly retrieved the CPSF6-related genes in GSE53624 dataset and performed GO and KEGG enrichment analyses. Notably, CPSF6-related genes are mainly participated in the pathways associated with cell division and apoptosis, such as cell cycle, DNA replication, DNA repair and p53 signaling. These results prompted us that CPSF6 might affect growth and death of ESCC cells. Indeed, we revealed that overexpression of CPSF6 promoted cell proliferation and colony-formation capacity, while knockdown of CPSF6 diminished these two malignant phenotypes, inhibited subcutaneous tumor growth rate, as well as raised cell cycle arrest and apoptosis of ESCC cells. Disrupting cell cycle and promoting apoptosis of tumor cells are the keys to radiotherapy and chemotherapy for tumor treatment. For instance, radiotherapy can trigger cell cycle arrest at G_2/M
Figure 7 CPSF6 protein binds to and regulates its circular isoform circCPSF6 (hsa_circ_0000417). (A) A bar plot is presented for CPSF6 protein-binding RNA of CLIP-seq data from the ENCORI database; (B) Identification of circCPSF6 through RNase R treatment and Sanger sequencing of junction site; (C,D) Interaction between CPSF6 protein and circCPSF6 was detected by RIP assay (C) and RNA-protein pulldown assay (D); (E) Subcellular distribution of circCPSF6 in ESCC presented by a bar plot; (F,G) RT-qPCR assay for detecting the RNA level of CPSF6 and circCPSF6 after depletion of CPSF6 (F) and circCPSF6 (G), respectively; (H) Cell cycle assay was performed after knockdown of circCPSF6, and each cell cycle phase is presented by a bar plot; (I) Apoptosis assay was performed after knockdown of circCPSF6, and a bar plot is presented for the proportion of apoptotic cells. CLIP-seq, cross-linking immunoprecipitation sequencing; ESCC, esophageal squamous cell carcinoma.
phase and promote cell apoptosis through inducing DNA damage (26,27). In addition, cisplatin and paclitaxel, the first-line chemotherapeutic agents for the clinical treatment of esophageal cancer, exert their effects by inducing G_{0}/G_{1} arrest and apoptosis of tumor cells (28,29). Considering the effects of CPSF6 to cell cycle and apoptosis, CPSF6 may be a potential therapeutic target to improve the outcomes of ESCC patients with radiotherapy and chemotherapy.

CPSF6, as a canonical RBP (21,23,30,31), plays oncogenic roles in human cancers (12,13,18). A few studies have reported that RBPs could dynamically regulate circRNA biogenesis, functions and metabolism (32-36). CircRNA, a novel identified type of RNA, has been reported to exhibit aberrant expression in multiform types of human cancers (37-39). RBP-circRNA interactions may play important roles in tumorigenesis and progression. Apart from the CLIP-seq data described above, no studies have reported that CPSF6 could bind with circRNA. Interestingly, we explored that CPSF6 protein was able to bind with its circular isoform circCPSF6 (hsa_circ_0000417) and perturbed circCPSF6 expression in ESCC cells. In particular, we found that circCPSF6 exhibited similar functions to CPSF6 protein in accelerating cell cycle and repressing apoptosis in ESCC cells, implying that CPSF6 might exert its tumor-promoting effects through circCPSF6. In further studies, we will investigate the roles of circCPSF6 in tumorigenesis and progression of ESCC and attempt to reveal the regulatory manner of CPSF6 protein to circCPSF6. Considering the fact of CPSF6 as a m6A reader (40), is it possible for CPSF6 to act as a broad-spectrum circRNA-binding protein that regulates circRNA metabolism in trafficking and decay? It is also an attractive issue that deserves investigating in the future.

**Conclusions**

Our investigations show that CPSF6 is significantly up-regulated in ESCC and high level of cytoplasmic CPSF6 is detrimental to ESCC patient prognosis. Biologically, CPSF6 may function as an oncoprotein in ESCC, at least in part, through regulating circCPSF6 expression.

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**Footnote**

*Conflicts of Interest*: These authors have no conflicts of interests to declare.

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**Table S1** CPSF6 protein expression data and relevant clinical information of ESCC microarray (HEsoS105Su01)

| Sample code | Surgery time | Follow-up time (month) | Survival period (cut-off 2014/3) | Gender | Age (year) | Distant metastasis | Primary tumor site | Pathological grade | Pathological classification | CPSF6 expression | AJCC cancer staging (7 edition) | Intensity rate (cytoplasm) (%) | Positivity rate (cytoplasm) (%) | Intensity rate (nucleus) (%) | Positivity rate (nucleus) (%) | Tissue type |
|-------------|--------------|------------------------|----------------------------------|--------|------------|-------------------|-------------------|-------------------|--------------------------|-----------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------|
| D08A1704    | 2008/10/25   | 14                     | Female                           | 62     | No         | Yes               | Squamous cell carcinoma | II                | Esophagus                | Ulcerated          | T3 N1 M0                      | 1                           | 100                         | 2                           | 90                          | Cancer         |
| D08A1706    | 2009/11/10   | 15                     | Male                             | 51     | No         | Yes               | Squamous cell carcinoma | II                | Esophagus                | Ulcerated          | T3 N1 M0                      | 1                           | 100                         | 2                           | 100                         | Adjacent        |
| D08A1707    | 2008/8/19    | 31                     | Male                             | 65     | No         | Yes               | Squamous cell carcinoma | II                | Esophagus                | Bulge             | T3 N0 M0                      | 2                           | 90                          | 2                           | 45                          | Cancer          |
| D08A1712    | 2006/12/31   | 9                      | Male                             | 54     | No         | Yes               | Squamous cell carcinoma | III               | Esophagus                | Bulge             | T3 N2 M0                      | 2                           | 100                         | 2                           | 85                          | Cancer          |
| D08A1715    | 2008/3/13    | 67                     | Male                             | 51     | No         | Yes               | Squamous cell carcinoma | II                | Esophagus                | Bulge             | T2 N0 M0                      | 2                           | 90                          | 2                           | 75                          | Cancer          |
| D08A1728    | 2010/4/3     | 95                     | Female                           | 37     | No         | Yes               | Squamous cell carcinoma | II                | Esophagus                | Bulge             | T3 N0 M0                      | 2                           | 100                         | 2                           | 100                         | Adjacent        |
| D08A1729    | 2009/4/21    | 15                     | Female                           | 67     | No         | Yes               | Squamous cell carcinoma | III               | Esophagus                | Ulcerated          | T3 N2 M0                      | 2                           | 100                         | 2                           | 100                         | Cancer          |
| D08A1737    | 2006/6/16    | 93                     | Female                           | 62     | No         | Yes               | Basal cell-like squamous cell carcinoma | II                | Esophagus                | Negative          | T2 M0                         | 1                           | 10                          | 3                           | 45                          | Cancer          |
| D08A1739    | 2006/6/29    | 59                     | Male                             | 58     | No         | Yes               | Squamous cell carcinoma | II                | Middle and upper esophagus | Bulge             | T3 N1 M0                      | 1                           | 60                          | 2                           | 95                          | Cancer          |
| D08A1742    | 2005/7/16    | 8                      | Male                             | 57     | No         | Yes               | Squamous cell carcinoma | II-II              | Middle and lower esophagus | Ulcerated          | T2 N1 M1                      | 4                           | 100                         | 2                           | 100                         | Adjacent        |
| D08A1747    | 2008/11/27   | 4                      | Male                             | 73     | No         | Yes               | Squamous cell carcinoma | II                | Esophagus                | Bulge             | T2 N0 M0                      | 2                           | 75                          | 2                           | 95                          | Cancer          |
| D08A1749    | 2007/1/23    | 8                      | Male                             | 48     | No         | Yes               | Squamous cell carcinoma | II                | Esophagus                | Ulcerated          | T3 N1 M0                      | 3A                          | 100                         | 3                           | 95                          | Cancer          |
| Sample code | Surgery time | Survival status | Follow-up time | Gender | Age (year) | Distant metastasis | Pathological Classification | Pathological grade | Surgical margin | AJCC cancer staging (7 edition) | Number of lymph nodes | Positive lymph nodes | AJCC grade Intensity (cytoplasm) (%) | Positivity rate (cytoplasm) (%) | AJCC grade Intensity (nucleus) (%) | Positivity rate (nucleus) (%) | Tissue type |
|-------------|--------------|-----------------|----------------|--------|------------|-------------------|---------------------------|-----------------|----------------|---------------------------------|----------------------|-------------------|-------------------------------|-------------------------|----------------------|-------------------------|-------------|
| D08A1750    | 2007/2/28    | Death           | 2007/12/9      | Male   | 59         | No                | Squamous cell carcinoma | II               | Middle and lower esophagus | Negative          | 3                  | T3 N2 M0                     | 3B                    | 1                    | 100                        | 2                       | 90                   | Adjacent  |
| D08A1751    | 2007/3/6     | Death           | 2008/1/27      | Female | 73         | No                | Squamous cell carcinoma | III              | Middle and upper esophagus | Ulcerated Squamous epithelial heterogeneity, carcinoma | 10                | 0                  | T3 N0 M0                     | 2                      | 1                    | 50                        | 2                       | 85                   | Adjacent  |
| D08A1754    | 2007/3/29    | Death           | 2008/6/7       | Female | 58         | No                | Squamous cell carcinoma | II               | Middle and lower esophagus | Negative          | 10                | T3 N2 M0                     | 3B                    | 1                    | 100                        | 2                       | 100                  | Cancer     |
| D08A1758    | 2007/5/28    | Death           | 2008/1/9       | Male   | 70         | No                | Squamous cell carcinoma | II               | Middle esophagus            | Ulcerated         | 1                 | T3 N0 M0                     | 2                      | 1                    | 90                        | 2                       | 95                   | Cancer     |
| D08A1760    | 2007/8/28    | Death           | 2011/1/1/19    | Female | 57         | No                | Squamous cell carcinoma | II               | Esophagus                  | Negative          | 11                | T2 N0 M0                     | 2                      | 1                    | 95                        | 2                       | 95                   | Cancer     |
| D08A1761    | 2007/9/6     | Death           | 2008/12/6      | Male   | 78         | No                | Squamous cell carcinoma | II/III           | Esophagus                  | Ulcerated         | 3                 | T3 N0 M0                     | 2                      | 1                    | 100                        | 2                       | 80                   | Cancer     |
| D08A1763    | 2007/9/24    | Death           | 2008/4/1       | Female | 55         | Yes               | Supraduodenal lymph nodes | I               | Esophagus                  | Ulcerated         | 22                | T3 N2 M1                     | 4                      | 1                    | 100                        | 2                       | 95                   | Cancer     |
| D08A1764    | 2007/10/16   | Death           | 2008/9/20      | Male   | 57         | No                | Squamous cell carcinoma | II               | Esophagus                  | Narrowing         | 6                 | T3 N1 M0                     | 3A                     | 2                    | 100                        | 2                       | 100                  | Cancer     |
| D08A1772    | 2007/11/8    | Death           | 2010/2/20      | Male   | 64         | No                | Squamous cell carcinoma | II               | Esophagus                  | Ulcerated         | 31                | T3 N1 M0                     | 3A                     | 1                    | 100                        | 1                       | 95                   | Cancer     |

Table S1 (continued)
| Sample code | Surgery time | Follow-up time | Gender | Age (year) | Distant metastasis | Primary site | AJCC cancer staging (7th edition) | Surgical margin | Pathological pattern | Pathological grade | Tumor site | Pathological classification | Primary tumor | Number of lymph nodes | Positive lymph nodes | AJCC cancer staging (7th edition) | Positivity rate (cytoplasm) (%) | Positivity rate (nucleus) (%) | Intensity (cytoplasm) (%) | Intensity (nucleus) (%) | AJCC cancer staging (7th edition) | Positivity rate (cytoplasm) (%) | Positivity rate (nucleus) (%) | Intensity (cytoplasm) (%) | Intensity (nucleus) (%) | Tissue type | AJCC cancer staging (7th edition) | Positivity rate (cytoplasm) (%) | Positivity rate (nucleus) (%) | Intensity (cytoplasm) (%) | Intensity (nucleus) (%) | Tissue type |
|-------------|--------------|----------------|--------|------------|-------------------|-------------|----------------------------------|---------------|---------------------|----------------|-------------|-----------------------------|----------------|--------------------------|--------------------|---------------------------------|---------------------------|---------------------------|-----------------------------|---------------------------|----------------|--------------------------------|---------------------------|---------------------------|-----------------------------|---------------------------|----------------|
| D08A1774    | 2007/11/28   | 2015/7/7       | Female | 70         | No                | Esophagus    | T2 N1 M0                          | Negative      | II                  | Mysomatous      | Esophagus   | Squamous cell carcinoma       | Ulcerated       | 12                        | 1                  | 2                              | 100                       | 2                          | 100                        | 2                          | 100                      | Cancer                  | 1                              | 25                        | 3                          | 95                        | Adjacent             |
| D08A1785    | 2009/3/10    | 2009/3/10      | Male   | 53         | No                | Esophagus    | T2 N0 M0                          | Negative      | II                  | Bulge          | Esophagus   | Squamous cell carcinoma       | Nudged          | 4                         | 0                  | 2                              | 100                       | 3                          | 100                        | 3                          | 100                      | Cancer                  | 1                              | 30                        | 3                          | 75                        | Adjacent             |
| D08A1789    | 2008/8/17    | 2008/8/17      | Female | 52         | Yes               | Esophagus    | T3 N1 M1                          | Negative      | II                  | Ulcerated      | Esophagus   | Squamous cell carcinoma       | Narrowing       | 9                         | 2                  | 4                              | 70                        | 2                          | 90                         | 1                          | 90                       | Cancer                  | 1                              | 30                        | 3                          | 75                        | Adjacent             |
| D08A1793    | 2009/1/15    | 2014/3         | Male   | 57         | No                | Esophagus    | T3 N0 M0                          | Negative      | II                  | Narrowing      | Esophagus   | Squamous cell carcinoma       | Tumor site      | 17                        | 0                  | 2                              | 100                       | 3                          | 95                         | 2                          | 100                      | Cancer                  | 1                              | 35                        | 2                          | 85                        | Adjacent             |
| D08A1803    | 2008/3/24    | 2012/1/2       | Male   | 54         | No                | Esophagus    | T1a N0 M0                          | Negative      | II                  | Ulcerated      | Esophagus   | Squamous cell carcinoma       | Infiltrative     | 12                        | 0                  | Tia a N0 M0                   | 3A                        | 3                          | 100                        | 2                          | 100                      | Cancer                  | 1                              | 30                        | 3                          | 90                        | Adjacent             |
| D08A1809    | 2008/5/8     | 2013/6/21      | Female | 54         | No                | Esophagus    | T3 N0 M0                          | Negative      | II                  | Bulge          | Middle and upper esophagus | Ulcerated       | 8                         | 0                  | T3 N0 M0                      | 2                          | 2                          | 100                        | 2                          | 100                      | Cancer                  | 1                              | 35                        | 3                          | 85                        | Adjacent             |
| D08A1810    | 2008/5/19    | 2014/3         | Male   | 57         | No                | Esophagus    | T3 M0                            | Negative      | I                   | Infiltrating ulcer | Esophagus   | Squamous cell carcinoma       | Tumor site      | 30                        | 0                  | T2 N0 M0                      | 2                          | 1                          | 95                         | 3                          | 100                      | Cancer                  | 1                              | 40                        | 3                          | 95                        | Adjacent             |
| D08A1814    | 2008/9/11    | 2009/9/11      | Male   | 53         | No                | Esophagus    | T2 N0 M0                          | Negative      | I-II                | Ulcerated      | Esophagus   | Squamous cell carcinoma       | Ulcerated       | 30                        | 0                  | T2 N0 M0                      | 2                          | 1                          | 95                         | 3                          | 100                      | Cancer                  | 1                              | 40                        | 3                          | 95                        | Adjacent             |

Table S1 (continued)
| Sample code | Surgery time | Survival status | Follow-up time | Gender | Age (year) | Distant metastasis | Tumor site | Pathological classification | Pathological grade | Surgical margin | Number of lymph nodes | Positive lymph nodes | AJCC cancer staging (7 edition) | Intensity (cytoplasm) (%) | Positivity rate (cytoplasm) (%) | Intensity (nucleus) (%) | Positivity rate (nucleus) (%) | Tissue type |
|------------|--------------|-----------------|----------------|--------|------------|-------------------|------------|---------------------------|-------------------|----------------|----------------------|---------------------|--------------------------|------------------------|-----------------------------|------------------------|--------------------------|----------------|
| D08A1819   | 2008/7/4     | Death           | 2008/3/9       | Male   | 58         | No                | Esophagus | II                        | Ectopic           | Negative      | 30                    | 2                   | T2 N1 M0                  | 2B                     | 1                           | 100                   | 3                        | 100                  | Cancer       |
| D08A1820   | 2008/7/1     | Death           | 2008/6/1       | Male   | 62         | No                | Esophagus | II-II                     | Ectopic           | Bulge         | Negative             | 14                  | T3 N1 M0                  | 3A                     | 2                           | 100                   | 3                        | 100                  | Cancer       |
| D08A1821   | 2008/7/8     | Death           | 2008/7/8       | Male   | 72         | No                | Esophagus | II                        | Pelvic            | Negative      | 23                    | 0                   | T3 N0 M0                  | 2                     | 1                           | 95                    | 3                        | 100                  | Cancer       |
| D08A1822   | 2008/7/14    | Death           | 2008/7/12      | Male   | 59         | No                | Esophagus | II                        | Lower esophagus   | Infiltrating ulcer | Negative          | 24                  | T3 N2 M0                  | 3B                    | 1                           | 85                    | 3                        | 100                  | Cancer       |
| D08A1823   | 2008/6/4     | Death           | 2008/5/16      | Male   | 59         | No                | Esophagus | I-II                      | Middle and lower esophagus | Negative          | 22                  | 16             | T3 N0 M0                  | 3C                    | 1                           | 95                    | 3                        | 100                  | Cancer       |
| D08A1824   | 2008/6/6     | Death           | 2008/4/1       | Male   | 59         | No                | Esophagus | II                        | Lower esophagus   | Ulcerated      | Negative             | 19                  | T3 N0 M0                  | 3C                    | 1                           | 100                   | 2                        | 100                  | Cancer       |
| D08A1825   | 2008/11      | Survival        | 2014/3         | Female | 59         | No                | Esophagus | II                        | Middle esophagus  | Ulcerated      | Negative             | 32                  | T3 N0 M0                  | 2                     | 1                           | 90                    | 3                        | 100                  | Cancer       |
| D08A1843   | 2009/12/5    | Survival        | 2014/3         | Female | 56         | No                | Esophagus | I-II                      | Middle and lower esophagus | Ulcerated Squamous epithelial hyperplasia on the upper cut edge | 21                  | 0             | T3 N0 M0                  | 2                     | 100                   | 3                     | 100                   | 2                        | 95                  | Cancer       |
| D08A1861   | 2009/5/8     | Survival        | 2014/3         | Male   | 53         | No                | Esophagus | II                        | Middle esophagus  | Ulcerated      | Negative             | 22                  | T3 N0 M0                  | 2                     | 1                           | 35                    | 2                        | 95                   | Cancer       |

Table S1 (continued)
| Sample code | Surgery time | Survival status | Follow-up time (month) | Gender | Age (year) | Apex | Distant metastasis | Primary foci | Pathological classification | Pathological grade | Tumor site | Pathological pattern | Surgical margin | Number of lymph nodes | Positive lymph nodes | AJCC cancer staging (7 edition) | Intensity (cytoplasm) (%) | Positivity rate (cytoplasm) (%) | Intensity (nucleus) (%) | Positivity rate (nucleus) (%) | Tissue type |
|-------------|--------------|-----------------|------------------------|--------|------------|------|---------------------|-------------|------------------------|-------------------|------------|---------------------|-----------------|----------------------|---------------------|-----------------------------|---------------------|----------------------|---------------------|----------------------|----------|
| D08A1862    | 2009/5/11    | Death           | 2/10/1/0               | Male   | 52         | Acute (7/1) | Yes            | Squamous cell carcinoma | II     | Middle esophagus | Ulcerated | Negative | 16          | 0          | T3 N0 M1 | 4        | 1               | 20                  | 1                    | 95                  | Cancer              | Adjacent |
| D08A1866    | 2009/6/23    | Death           | 2/13/2/11              | Female | 50         | No             | Yes            | Squamous cell carcinoma | II     | Middle and lower esophagus | Ulcerated | Negative | 35          | 1          | T3 N1 M0 | 3A       | 1               | 100                 | 2                    | 100                 | Cancer               | Adjacent |
| D08A1869    | 2009/7/2     | Survival        | 2014/3                 | Male   | 75         | No             | Yes            | Squamous cell carcinoma | II-III | Middle esophagus | Narrowing | Negative | 18          | 1          | T3 N1 M0 | 3A       | 1               | 45                  | 2                    | 100                 | Cancer               | Adjacent |
| D08A1872    | 2009/8/25    | Death           | 2/15/6/15              | Male   | 61         | No             | Yes            | Squamous cell carcinoma | III    | Esophagus          | Ulcerated | Moderate to severe squamous epithelial heterogeneity | 21          | 2          | T3 N1 M0 | 3A       | 1               | 45                  | 3                    | 100                 | Cancer               | Adjacent |
| D08A1874    | 2009/9/9     | Survival        | 2014/11 (Death)        | Male   | 53         | No             | Yes            | Squamous cell carcinoma | I-II   | Middle and lower esophagus | Ulcerated | Negative | 13          | 0          | T2 N0 M0 | 2        | 1               | 95                  | 3                    | 100                 | Cancer               | Adjacent |
| D08A1875    | 2009/9/7     | Survival        | 2014/3                 | Male   | 60         | No             | Yes            | Squamous cell carcinoma | I     | Middle and lower esophagus | Ulcerated | Negative | 24          | 0          | T3 N0 M0 | 2        | 1               | 80                  | 3                    | 100                 | Cancer               | Adjacent |
| D08A1878    | 2009/11/1    | Survival        | 2014/3                 | Male   | 67         | No             | Yes            | Squamous cell carcinoma | II    | Middle and lower esophagus | Bulge     | Negative | 27          | 0          | T3 N0 M0 | 2        | 1               | 75                  | 2                    | 90                  | Cancer               | Adjacent |
| D08A1882    | 2009/12/9    | Death           | 2/12/1/2               | Male   | 60         | No             | Yes            | Squamous cell carcinoma | III    | Esophagus          | Bulge     | Negative | 21          | 0          | T3 N0 M0 | 2        | 1               | 100                 | 3                    | 100                 | Cancer               | Adjacent |
| D08A1883    | 2009/12/23   | Survival        | 2014/3                 | Female | 63         | No             | Yes            | Squamous cell carcinoma | II-III | Esophagus          | Ulcerated | Negative | 34          | 0          | N0 M0   | 0        | 1               | 75                  | 2                    | 100                 | Cancer               | Adjacent |

Table S1 (continued)
Table S1 (continued)

| Sample code | Surgery time | Survival status | Follow-up time (months) | Remark | Gender | Age (year) | Distant metastasis | Primary foci | Pathological classification | Pathological grade | Tumor site | Pathological pattern | Surgical margin | Number of lymph nodes | Positive lymph nodes (cytoplasm) (%) | Intensity (cytoplasm) (%) | Positivity rate (cytoplasm) (%) | Intensity (nucleus) (%) | Positivity rate (nucleus) (%) | Tissue type |
|-------------|--------------|-----------------|-------------------------|--------|--------|------------|-------------------|-------------|---------------------------|-----------------|-----------|---------------------|----------------|------------------------|-------------------------------|---------------------------|--------------------------|----------------|--------------------------|--------|
| D08A1884    | 2009/12/30   | Survival       | 2014/3                 | 51     | Female | 57        | No                | Squamous cell carcinoma | I–II       | Esophagus                | Ulcerated       | 0         | N2 M0               | 1               | 95                     | 2                             | 100                       | Cancer               | Adjacent   |
| D08A1885    | 2010/3/8     | Survival       | 2014/3                 | 48     | Male   | 62        | No                | Squamous cell carcinoma | II         | Esophagus                | Ulcerated       | 21        | 0 N0 M0             | 1               | 100                   | 3                             | 100                       | Cancer               | Adjacent   |
| D08A1886    | 2010/3/18    | Death          | 2011/3/23              | 12     | Female | 69        | No                | Squamous cell carcinoma | II         | Esophagus                | Ulcerated       | 16        | 4 N2 M0             | 1               | 95                     | 2                             | 95                        | Cancer               | Adjacent   |
| D08A1887    | 2010/11/8    | Death          | 2011/11/2             | 9      | Male   | 70        | No                | Squamous cell carcinoma | II         | Esophagus                | Bulge           | 20        | 7 N3 M0             | 3C              | 100                   | 2                             | 95                        | Cancer               | Adjacent   |
| D08A1888    | 2010/1/13    | Death          | 2010/11/14            | 10     | Male   | 73        | No                | Squamous cell carcinoma | II         | Esophagus                | Ulcerated       | 0         | N0 M0               | 2               | 100                   | 3                             | 100                       | Cancer               | Adjacent   |
| D08A1890    | 2010/3/2     | Death          | 2011/2/28             | 22     | Male   | 48        | No                | Squamous cell carcinoma | II         | Esophagus                | Bulge           | 12        | 0 N0 M0             | 1               | 95                     | 3                             | 100                       | Cancer               | Adjacent   |
| D08A2234    | 2011/1/25    | Survival       | 2014/3                 | 38     | Female | 54        | No                | Squamous cell carcinoma, partial basal cell-like squamous cell carcinoma | III        | Esophagus                | Bulge           | 16        | 0 T1 N0 M0         | 1               | 100                   | 2                             | 100                       | Cancer               | Adjacent   |

ESCC, esophageal squamous cell carcinoma.