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Knockdown of Long Noncoding RNA POU5F1B Promotes Radiosensitivity in Esophageal Carcinoma

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Funds Collection G

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Background: POU5F1B, serving as a carcinogen, participates in radiosensitivity of several tumors. However, in esophageal cancer, its potential mechanism and function in regulating radiosensitivity remain unclear.

Material/Methods: The expression level of POU5F1B was detected in plasma of esophageal tumor patients and cancer cell lines. The effect of POU5F1B knockdown on cell proliferation and colony formation was determined using CCK-8 assay and colony formation assay. Cell apoptosis rate was detected by flow cytometry.

Results: POU5F1B expression level declined after radiotherapy in the plasma of esophageal cancer patients (p=0.025). Compared with HEEPIC, the level of POU5F1B was upregulated in ECA109 (p<0.01), ECA9706 (p<0.01), KYSE410 (p<0.01), and KYSE510 (p=0.036). The silencing of POU5F1B played a role in inhibiting colony formation. After radiotherapy, the apoptosis rates in the ECA109 with 4Gy si-POU5F1B group and 4Gy si-NC group were 39.1±0.1% and 35.3±0.1%, respectively (p=0.0193). The rate was 21.00±0.1 and 29.1±0.1% (p<0.0072) in the si-NC group and si-POU5F1B group, respectively. For proliferation rate, 4Gy si-POU5F1B ECA109 performed better than 4Gy si-NC.

Conclusions: Radiotherapy contributed to the decline in the expression level of POU5F1B in plasma, which was upregulated in ECA109, ECA9706, KYSE410, and KYSE510, but not in HEEPIC. The knockdown of POU5F1B increased the radiosensitivity of esophageal cancer cell lines.

MeSH Keywords: Esophageal Neoplasms • Octamer Transcription Factor-3 • Radiation-Sensitizing Agents • RNA, Long Noncoding

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Background

Esophageal cancer ranks seventh in incidence and sixth in mortality among all cancers. Generally, patients diagnosed with esophageal cancer undergo radical radiation therapy or palliative radiotherapy, depending on their clinical stage and tolerance. However, the existence of radio resistance results in poor outcome of radiotherapy. Recent studies showed that the expression of lncRNA in several cancers is associated with patient characteristics, prognosis, and tumorigenesis [1,2]. lncRNA has been found to participate in the formation of chromosomes and the regulation of the encoded gene expression in transcription or post-transcription. Several lncRNAs are closely associated with cancer diagnosis [3], such as PTENP1, which inhibits cell proliferation by AKT and MAPK pathways in breast cancer [4]. The overexpression of lncRNA SchAP1 in prostate cancer is correlated with metastasis [5].

lncRNA also promotes the growth of cells and inhibits apoptosis in hepatocellular cancer cells [6]. Long noncoding RNA (lncRNA) POU5F1B (Putative POU domain, Class 5, transcription factor 1 b, also known as OCT4 – PG1, OTF3C, OTF3P1, and POU5F1P1), is located on chromosome 8 q24, near the MYC gene. POU5F1B is a progressive pseudogene highly homologous to OCT4. Among 11 subtypes of OCT4, isoform6-10 has different mRNA but encodes the same protein. For isoform1-5 and isoform11 encoding different proteins, isoform1 is the most common and main transcription subtype, also known as oct4a. The oct4 gene contains homologous and specific domains at the C terminal and N terminal, respectively, which are connected by the volatile region. The homologous region of POU with octamer domain is called the oct4 structure. The conserved binding domain has transcriptional activation, and the homologous domain is the regulatory site of phosphorylation. The Oct4 molecule is involved in activating the corresponding target gene by binding to the homologous domain.

Material and Methods

Patient samples

A total of 11 ESCC patients who had not undergone any therapy at Taixing People's Hospital were involved in this study. Blood was collected before and after patients underwent radiotherapy. Esophageal cancer was diagnosed by pathologists. All experimental procedures were performed following approved guidelines. This study was approved by the Ethics Committee of Taixing People's Hospital. Informed consent was signed by all patients.

Cell lines and cell culture

Human esophageal cancer cell lines (ECA109, ECA9706, KYSE410, and KYSE510) and a normal esophageal epidermis cell line (HEEPIC) were purchased from ATCG and cultured in RPMI-1640 medium (10% FBS). The atmosphere of the culture chamber kept at 37°C and 5% CO_2. Pancreatic enzymes were used to digest cells at cell fusion of 85%. RPMI-1640 medium was from Gibco (USA), 0.1% crystal violet staining solution was from Solarbio (USA), and cell culture flasks and culture plates were acquired from Corning (USA). The inhibitor of POU5F1B (GAAGAGTTCCTAACACATTCA) was synthesized by GenePharma (China), Lipofectamine 2000 and Trizol were purchased from Invitrogen (USA), the RT-PCR kit was bought from Takara (Japan), and the penicillin-streptomycin, trypsin-EDTA solution, cell cycle kit, apoptosis assay kit, and annexin V-FITC apoptosis detection kit were from Beyotime Biotechnology (China).

Cell transfection

The plasmid was transfected into competance5α, cultured in LB culture medium, vibrated on a shaker, and coated to solid LB culture medium with kanamycin. Then, the coated culture plate was put into a 37°C incubator for 1 h and cultured overnight. One bacterial colony was placed into the LB liquid medium and vibrated for 8 h in 37°C. The plasmid was extracted according to instructions from TransGen Biotech. The plasmid carrying si-POU5F1B was transfected into ECA109 cells using Lipofectamine™ 2000, and then subcloned into 24 wells at 4×10^4 cells per well. When the fusion reached 80%, the RPMI-1640 medium was changed to RPMI 1640 with serum and penicillin-streptomycin. The target cells were screened using 800 ng/ml of G418, with the medium changed every 2–3 days for 21 days.

Cell radiotherapy

The cells (5×10^4) were transfected using a linear accelerator (Elekta) and exposed to various doses (0, 2, 4, 8 Gy) at 6 MV. After 24–96 h, other experiments were carried out.

PCR

lncRNA was extracted from plasma using miRNeasy Serum Plasma (Qiagen), then transcription was reversed to cDNA (Takara) fluorescence using SYBR green (Takara, R0036A). PCR analysis of POU5F1B was calculated by $2^{-\Delta\Delta}$ method, with GAPDH as control. Total RNA in cells was extracted using Trizol (Qiagen). The genetic sequences were: forward: 5'-GCCATACGGTCACAGAGCTT-3', reverse: 5'-CCCCCACATAACTCATACGG-3'; GAPDH forward: 5'-GGA GTCCACTGGCGTCTT-3', reverse: 5'-GAGTCCTTCCACGATACCAA-3'. Condition of POU5F1B RT-PCR: 94°C for 30 s; 57.5°C for 30 s

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for annealing; 72°C for 75 s for extension; and finally 72°C for 7 min for elongation, for 32 cycles.

CCK-8

A total of 2000 cells were planted in 1 of 96 cells and then irradiated. The OD value was read at 0, 24, 48, 72, and 96 h.

Colony formation assay

We planted 200, 500, 1000, and 5000 cells per cell in 6-well plates before and incubated them for 24 h with complete medium. Cells subjected to radiation (0, 2, 4, and 8 Gy) were incubated for 14 days at 37°C, washed twice with PBS, and then fixed for 15 min with methyl alcohol, and finally stained with crystal violet staining solution.

Flow cytometry

We planted 106 cells in 6-well plates irradiated at 4 Gy and collected after 24 h. The detection of apoptosis rate was performed by FACS (BD), Annexin V (FITC), and PI apoptosis detection kits (Beyotime). Each experiment was repeated 3 times.

Statistical analysis

The *t* test and ANOVA were used to evaluate statistical differences. SPSS 23.0 statistical software package (SPSS, Inc., Chicago, IL) was used for all statistical analyses. GraphPad prism 5.0 (USA) software was used and the results are shown as mean ±SD. The level of statistical significance was set at $P<0.05$.

Results

POU5F1B was downregulated in response to irradiation in EC plasma and upregulated in EC cell lines.

Expression of POU5F1B (with radiation or not): After radiation, the expression level of POU5F1B declined, as identified in the plasma of patients with esophageal cancer (p=0.025) (Figure 1). Its expression in the 5 esophageal cancer cell lines was higher than in HEEPIC, with the highest level in ECA109 cells (Figure 2).

POU5F1B knockdown suppressed cell proliferation and improved radiosensitivity of EC cells

Compared with the control group, cells treated with radiation exhibited some transformation. The CCK-8 experiment revealed that the cells treated with 4Gy si-POU5F1B obtained a higher proliferation rate than cells treated with 4Gy si-nc (p=0.003) (Figure 3). Flow cytometry showed that the apoptosis rate in

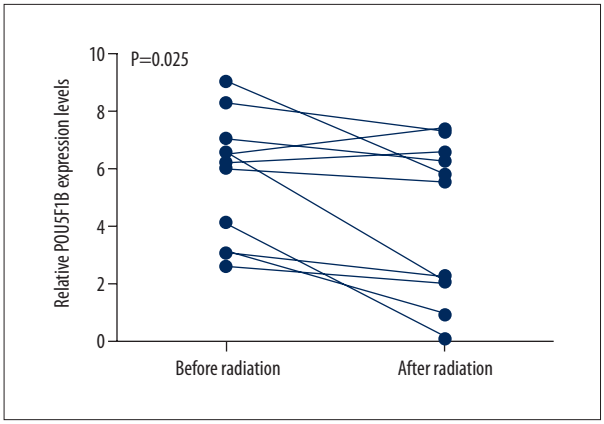

Figure 1. Expression alteration of POU5F1B in plasma of EC patients in response to irradiation.

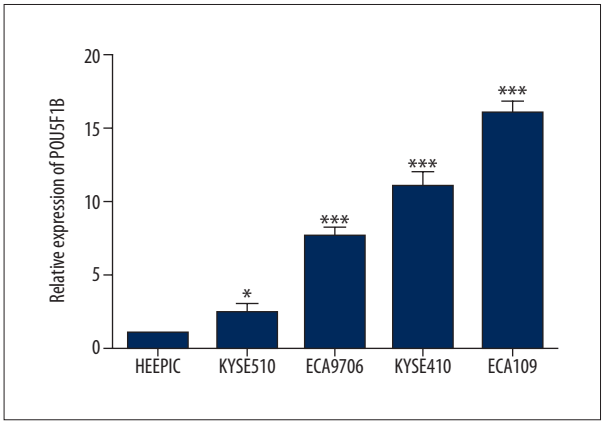

Figure 2. Expression alteration of POU5F1B in EC cells. qRT-PCR was performed to examine the expressions of POU5F1B in EC cell lines (ECA109, KYSE510, KYSE410, and ECA9706) and the primary normal human esophagus epithelial line HEEPIC.

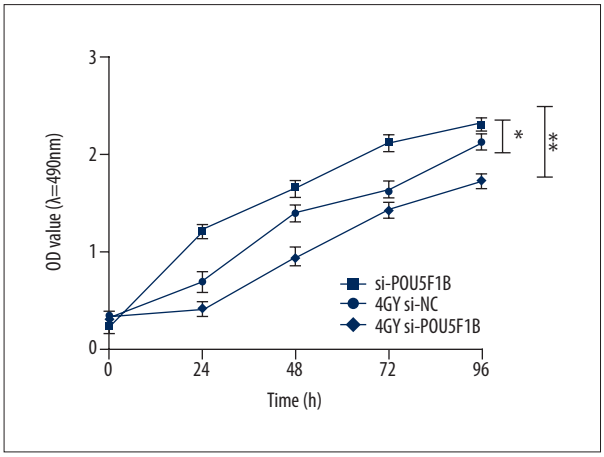

Figure 3. Effect of POU5F1B knockdown on cell proliferation and radiosensitivity of EC cells. CCK-8 assay was performed to determine cell proliferation at 24 h, 48 h,72, and 96 h in si-POU5F1B- or si-NC-transfected ECA109 cells.

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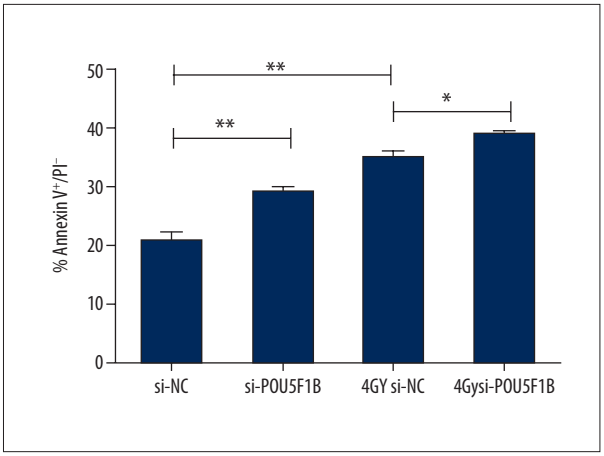

Figure 4. The effect of POU5F1B deficiency on apoptotic rate was detected in EC cells at 24 h postradiation by flow cytometry via doublestaining of Annexin-V-FITC and PI.

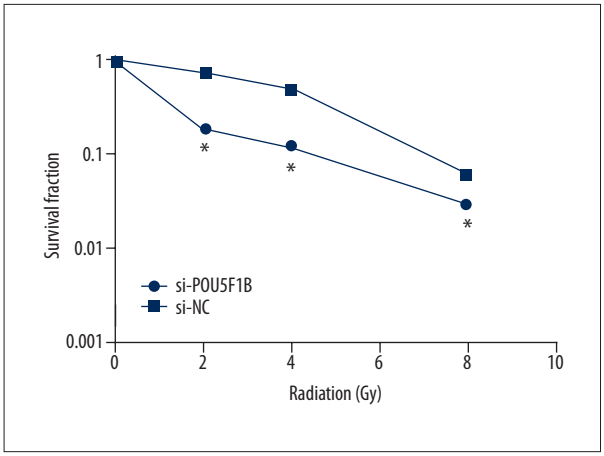

Figure 5. The effect of POU5F1B deficiency on apoptotic rate was detected in EC cells at 24 h postradiation by flow cytometry via doublestaining of Annexin-V-FITC and PI.

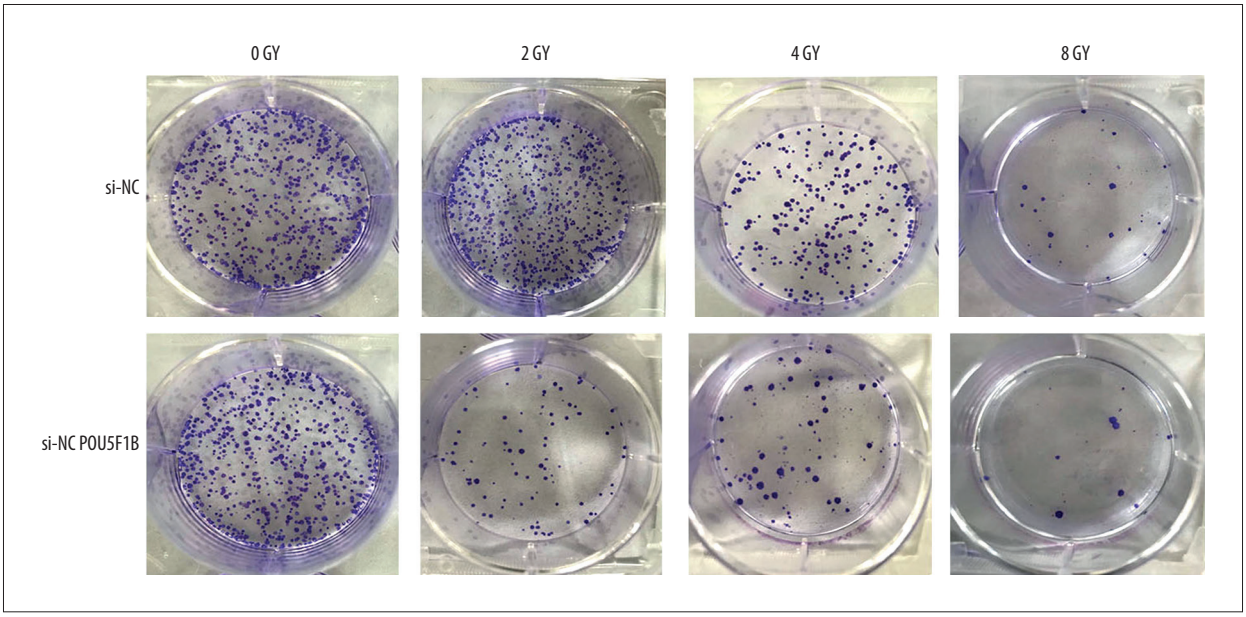

Figure 6. The clonogenic survival curves were compared in EC cells transfected with si-POU5F1B or si-NC with the indicated single doses of irradiation (0, 2, 4, or 8 Gy) treatment.

the 4Gy si-POU5F1B group, 4Gy si-NC group, si-NC group, and si-POU5F1B group was 39.1±0.1%, 35.3±0.1% (p=0.0193), 21.00±0.1, and 29.1%±0.1 (p<0.0072), respectively (Figures 4, 5). A difference was found in the clone formation experiment results in 0, 2, 4, 8 Gy, and the group with 8 Gy radiation had the lowest colony formation rate (p=0.015). The rate of 4 Gy was higher than that of the 0 Gy dose group (p=0.035) (Figures 6, 7).

Discussion

The evidence of esophageal cancer radiotherapy is extensive. Radiotherapy for patients can be divided into radical radiotherapy, radiotherapy before surgery or after surgery, and palliative radiotherapy. Radiotherapy before surgery improves the infection rate and reduces the lymph nodes transfer rate. Palliative radiotherapy helps to relieve difficulty in feeding and the pain caused by bone transfer or the pressure from swollen lymph nodes. Nevertheless, radio resistance occasionally causes poor clinical outcome of radiotherapy. In this context, the expression level of POU5F1B in the plasma of patients was detected before and after radiotherapy, so as to determine the relationship between the outcome and POU5F1B.

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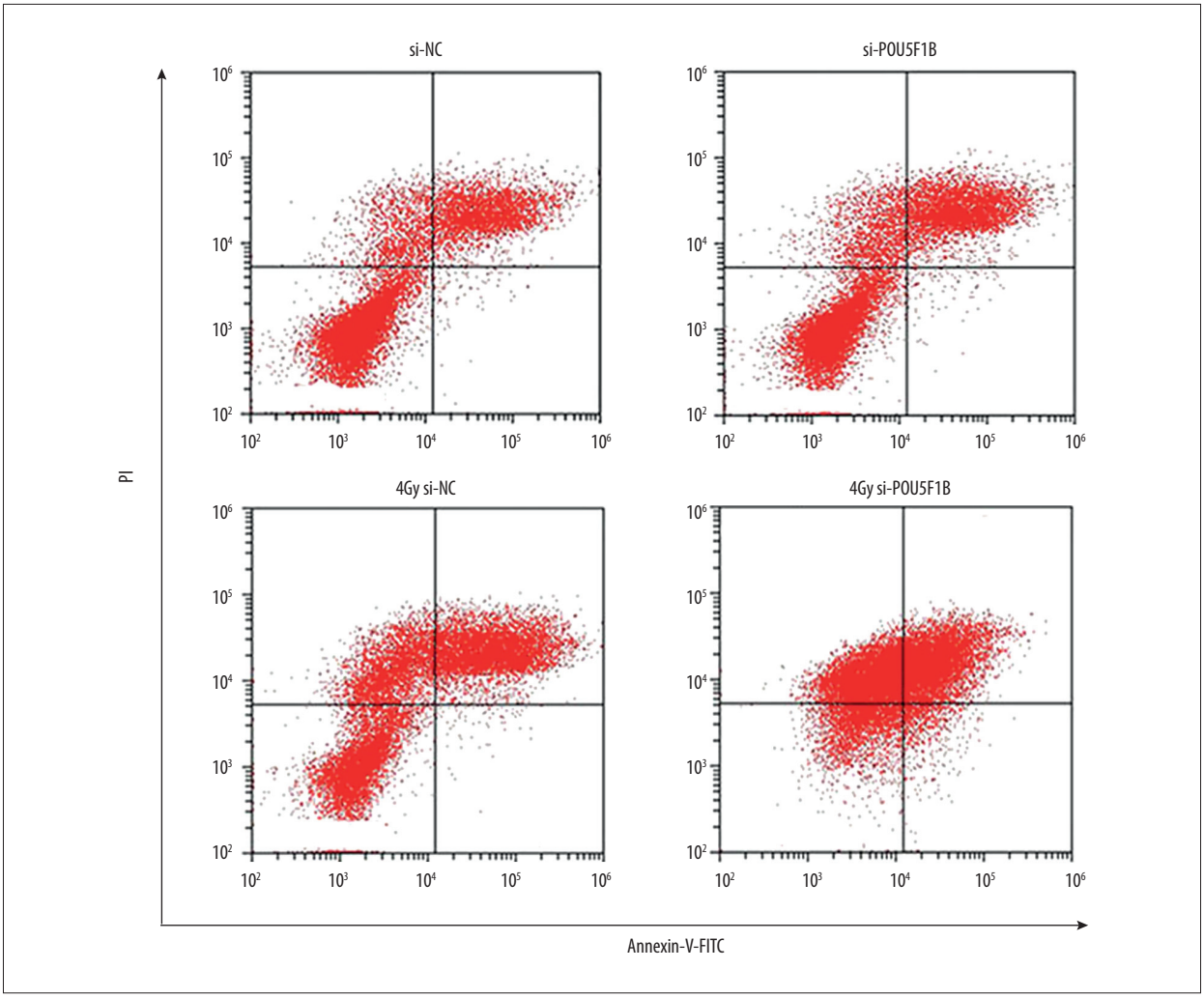

Figure 7. The clonogenic survival curves were compared in EC cells transfected with si-POU5F1B or si-NC with the indicated single doses of irradiation (0, 2, 4, or 8 Gy) treatment.

Recent research has mainly focused on the role of lncRNA in tumorigenesis.

lncRNA has many advantages due to rich subtypes and functional modes over other noncoding RNAs. The number of lncRNAs is also larger than that of encoded gene. Previous studies showed that the expression of several lncRNA in various cancers is abnormal and is upregulated to a certain extent [7]. At present, the studies of lncRNA focus more on its function in the early diagnosis and prognosis of cancer.

lncRNAs act not only as biomarkers for the diagnosis of cancer, but also as the target of tumor therapy [8]. In breast cancer, suppressing the expression of HOTAIR is reported to inhibit the Transwell invasion ability of breast cancer cells. Knocking down MEG3 plays a role in promoting the expression of angiogenesis-relevant genes [9]. Silencing H19 in gynecologic malignant cancers helps to enhance the ability of cells to take up glucose [10]. Researchers have reported a close correlation between the expression of MALAT1 in hepatocellular carcinoma before and after surgery and the recurrence of hepatocellular carcinoma [11]. The participation lncRNAs in regulation of genes, chromosomes, and histones is also confirmed.

According to GenBank, lncRNA POU5F1B is homologous with OCT4 located on chromosome 6, which is highly expressed in many digestive tract tumors like esophageal cancer and is associated with invasion and metastasis of various cancers such as pancreatic cancer and esophageal cancer. Previous studies have found the upregulation of POU5F1B in liver, cervical, and stomach cancers. OCT4 plays an important role in maintaining the pluripotency of embryonic stem cells and self-renewal of cells. In prostate cancer, inhibition of lncRNAROR expression can lead to increased expression of mir-145, thus reducing the expression of OCT4 and inhibiting cell proliferation. The 5-year survival rate of esophageal cancer patients is only 10–25%.

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However, the functional mechanism of POU5F1B in esophageal carcinoma remains unclear. It has been found that the lncRNA MALAT1 affects radiosensitivity of esophageal cancer by regulating CKS1 and also regulates tumor radiosensitivity. In esophageal cancer, the expression of lncRNA LOC285194 is related to the radiochemotherapy resistance and prognosis of esophageal cancer patients. The present study investigated the relationship between lncRNA POU5F1B expression and radiotherapy sensitivity in the esophageal cancer cell line ECA109.

Conclusions

To the best of our knowledge, this is the first study to investigate the functions of POU5F1B in regulating the radiosensitivity of esophageal cancer cell lines. Our results demonstrated that radiotherapy downregulated the expression of POU5F1B in plasma, and the knockdown of POU5F1B enhanced radiosensitivity, partly through inhibiting proliferation and inducing apoptosis. These findings may provide a new therapy target for esophageal cancer.

Conflict of interest

None.

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