SOX17-mediated MALAT1-miR-199a-HIF1α axis confers sensitivity in esophageal squamous cell carcinoma cells to radiotherapy

Yifei Yun1,2, Yutong Zhang1,2, Qiqi Xu1, Yao Ou1, Xifa Zhou1✉ and Zhonghua Lu1✉

© The Author(s) 2022

Radiotherapy is a main modality of esophageal squamous cell carcinoma (ESCC) treatment, while radioresistance largely limits the effect of this therapy. Evidence exists reporting that SOX17 may sensitize ESCC cells to irradiation, but the downstream mechanism remains poorly understood. Therefore, we attempt to explore the molecular basis of SOX17 effect on radioresistance in ESCC. The SOX17 expression was measured in ESCC tissues and cells, followed by evaluation of its relationship with patient survival. The fractionated irradiation-induced irradiation-resistant cell line KYSE150R was subjected to gain- and loss-of function studies to explore the effect of SOX17 and downstream effectors MALAT1, miR-199a, and HIF1α on the malignant phenotypes of ESCC. The interaction among these factors was explained using ChIP, dual lucerase reporter, RNA pull-down and RIP assays. Further, the in vivo effect of SOX17 on ESCC irradiation tolerance was assessed in nude mice. SOX17 was underexpressed in ESCC tissues and cells, which was negatively correlated with the prognosis of patients with ESCC. Besides, SOX17 inhibited irradiation tolerance of ESCC cells by suppressing MALAT1 transcription. Notably, MALAT1 acted as miR-199a sponge and thereby enhanced HIF1α expression. Moreover, SOX17 reduced the irradiation tolerance of ESCC cells by reducing HIF1α expression via the MALAT1-miR-199a axis, and attenuated tumor formation in nude mice. Our results indicate that SOX17 can impede the radioresistance of ESCC cells through the MALAT1-miR-199a-HIF1α axis, in support of further research for ESCC radiotherapy.

Cell Death Discovery (2022)8:270; https://doi.org/10.1038/s41420-022-01012-6

INTRODUCTION

Esophageal cancer is a common aggressive malignancy globally, which is listed as one of the four most fatal cancers in China, while esophageal squamous cell carcinoma (ESCC) is the major form of esophageal cancer around the world [1–3]. In the past few years, significant progress has achieved in the prevention and treatment of many cancers, but the overall 5-year survival rate of patients with ESCC is still below 20%, with the incidence of ESCC rapidly increasing worldwide [1]. Radiotherapy and surgical resection are the most important therapies for ESCC; however, the recurrence rates remain at high level by employing these managements [4]. The unsatisfactory outcomes may be caused by tumor radioresistance [5]. Therefore, an urgent need exists to explore the molecular mechanism of ESCC radioresistance and develop strategies to decrease the irradiation tolerance of ESCC cells.

SRY-box 17 (SOX17) transcription factor, an important member of high mobility group superfamily, is involved in various physiological processes [6]. Notably, emerging studies have provided evidence reporting the involvement of SOX17 in the pathogenesis of ESCC [7, 8]. For example, in cells and animal models, SOX17 poorly expresses in ESCC cells and tumor tissues; at the same time, overexpression of SOX17 could sensitize ESCC irradiation-resistant cells to irradiation, cisplatin and concurrent chemoradiation therapy treatment [9]. However, the effect of SOX17 on radioresistance and its related mechanisms still need further study.

Interesting, SOX17 could inhibit MALAT1 expression at the transcription level and subsequently affect the migration and invasion of ESCC cells by binding to the SRY element of the MALAT1 promoter region [10]. In addition, miRNAs (miRs) have become promising targets and tools for novel therapeutic approaches of cancers [11]. Thus, by bioinformatics analysis, we predict that several miRs, in the downstream of MALAT1, are related to ESCC in this study. Notably, among them, it has been demonstrated that miR-199a lowly expresses in esophagus cancer [12]. Following this, we further predicted the downstream regulatory factors of miR-199a, and found that hypoxia-inducible factor-1α (HIF1α), a putative target gene of miR-199a, significantly overexpresses in ESCC. Besides that, multiple studies have implied that inhibiting HIF1α could be a potential approach for ESCC treatment [13, 14], indicating that MALAT1 may upregulate the expression of HIF1α by targeting miR-199a and thereby mediate radioresistance.

Therefore, we hypothesized that SOX17 may affect the ESCC radioresistance by regulating MALAT1 expression and further modulating MALAT1-miR-199a-HIF1α axis, which may provide valuable strategies and targets for clinical management of ESCC.
**RESULTS**

**SOX17 is poorly expressed in ESCC and this low expression indicates poor prognosis of ESCC patients**

First, we aimed to investigate the effect of SOX17 on the radiosensitivity of ESCC and its relatively downstream mechanism. As shown in Fig. 1A, B, the expression of SOX17 was lower in ESCC tissues than adjacent normal tissues. The overall survival (OS) of the patients with lower SOX17 expression was shorter (Fig. 1C), suggesting the association of low expression of SOX17 with poor prognosis of ESCC patients. In addition, the results of qRT-PCR and Western blot demonstrated that versus HET-1A cells, the expression of SOX17 was reduced in ESCC cell lines (KYSE70, KYSE170, KYSE150, and KYSE510), with KYSE150 cells presenting the lowest SOX17 expression (Fig. 1D, E) and thus selected for the experiments related to SOX17. The above results illustrated that SOX17 was under-expressed in ESCC tissues and cells, and this downregulation linked to the poor prognosis of ESCC patients.

**SOX17 inhibits irradiation tolerance of ESCC cells**

After measurement of qRT-PCR and Western blot, the results showed that the KYSE150 cells exposed to X-ray irradiation of higher dose presented with higher expression of SOX17 (Fig. 2A, B, Fig. S1A). Besides, no significant decline in the expression of SOX17 was measured when the X-ray irradiation dose reached 4 Gy and above, so the dose of 4 Gy was chosen for single dose irradiation. When a single dose of X-ray (4 Gy) was employed to irradiate KYSE150 cells, the expression of SOX17, examined by qRT-PCR and Western blot, was proportional to the exposure time (Fig. 2C, D, Fig. S1B). In comparison with that in KYSE150 cell line, the expression of SOX17 mRNA and protein in irradiation-resistant cell line KYSE150R was significantly lower (Fig. 2E, F, Fig. S1C). The above results demonstrated that SOX17 expression in ESCC cells was related to X-ray irradiation dose and time, and SOX17 expression was lower in irradiation-resistant cells, which may participate in the process of X-ray radiotherapy.

In order to investigate the impact of SOX17 on irradiation tolerance of KYSE150R cell line, we overexpressed SOX17 in KYSE150R cells. Relative to oe-NC group, the expression of SOX17 in KYSE150R cells was obviously higher in the oe-SOX17 group (Fig. 2G, H, Fig. S1D). MTT assay, clonogenic assay and flow cytometry results exhibited that after exposure to X-ray irradiation of 4 Gy, KYSE150R cells in oe-NC group showed higher survival rate (Fig. 2I), higher proliferation ability (Fig. 2J), and lower apoptosis (Fig. 2K), versus KYSE150 cells. In comparison with KYSE150R cells in oe-NC group, KYSE150R cells in oe-SOX17 group displayed lower survival rate (Fig. 2G), lower proliferation ability (Fig. 2J), and higher apoptosis (Fig. 2K) after treatment with X-ray irradiation of 4 Gy. After exposure KYSE150R cells in oe-SOX17 group and oe-NC group to different doses of X-ray, the result of clonogenic assay showed that KYSE150R cells in the oe-SOX17 group exposed to irradiation of higher dose displayed with faster decrease of cell proliferation. (Fig. 2L, Fig. S2A), suggesting that KYSE150R cells had increased sensitivity and reduced tolerance to X-ray irradiation through overexpressing SOX17 in KYSE150R cells.

The above results indicated that high expression of SOX17 could reduce the irradiation tolerance of ESCC cells.

**SOX17 reduces irradiation tolerance of ESCC cells by transcriptional inhibition of MALAT1**

SOX17 could inhibit the transcription of MALAT1 by binding to SRY component in MALAT1 promoter region in the context of ESCC, and further affect the invasion and migration of ESCC cells [10]. Therefore, in the next studies, we investigated whether SOX17 could affect the irradiation effect of ESCC cells by suppressing the expression of MALAT1.

Based on bioinformatics tool GEPIA, we obtained 2636 significantly overexpressed genes by genetic difference analysis of ESCC samples in the TCGA database (Fig. 3A). After sorting the expression of differentially expressed genes (DEGs) related to ESCC, we found the differential expression of MALAT1 was the...
Fig. 2 High expression of SOX17 could decrease the irradiation tolerance of ESCC cells. After treatment with different doses of X-ray, the mRNA and protein expression of SOX17 in KYSE150 cells was measured by qRT-PCR (A) and Western blot (B). *p < 0.05 compared with 0 Gy group. After exposure to a single dose of X-ray, the expression of SOX17 mRNA and protein in KYSE150 cells was examined by qRT-PCR (C) and Western blot (D). The expression of SOX17 mRNA and protein was measured by qRT-PCR (E) and Western blot (F) in KYSE150 cells and its corresponding irradiation-resistant cell line KYSE150R. G-H, the mRNA and protein expression of SOX17 examined by qRT-PCR (G) and Western blot (H) in KYSE150R cells in response to oe-SOX17. I, Survival rates of KYSE150R cells in response to oe-SOX17 measured by MTT assay. J The proliferation ability of KYSE150R cells in response to oe-SOX17 examined by clonogenic assay. K Flow cytometry was employed to evaluate the apoptosis of KYSE150R cells in response to oe-SOX17. L Clonogenic assay was adopted to measure the proliferation ability of KYSE150R cells in response to oe-SOX17. *p < 0.05. Measurement data were expressed as mean ± standard deviation. Data between two groups were analyzed by unpaired t test. Comparisons between multiple groups were performed by one-way ANOVA, followed by Tukey’s post-hoc test. Two-way ANOVA with Bonferroni post-hoc test was employed to compare the data of groups at different time points. Cell experiments were repeated in triplicate.

The expression of SOX17 was higher in oe-SOX17 + oe-NC group than oe-NC group (Fig. 3I, J, Fig. S1E). Besides, in comparison with that in oe-NC group, MALAT1 expression was inhibited in oe-SOX17 + oe-NC group. Additionally, MALAT1 expression was elevated in the oe-SOX17 + oe-MALAT1 group versus the oe-SOX17 + oe-NC group (Fig. 3F). Versus oe-SOX17 + oe-NC group, the KYSE150R cells in oe-SOX17 + oe-MALAT1 group had higher survival rate (Fig. 3K), promoted cell proliferation (Fig. 3L), and lowered apoptosis (Fig. 3M).

When KYSE150R cells of the oe-SOX17 + oe-NC group and the oe-SOX17 + oe-MALAT1 group were exposed to X-ray irradiation at different doses, the decrease in the proliferation of KYSE150R cells in oe-SOX17 + oe-MALAT1 group was impeded with higher irradiation dose, as examined by clonogenic assay (Fig. 3N, Fig. S2B). This result illustrated that simultaneous overexpression of SOX17 and MALAT1 reversed the promoting effect of SOX17 overexpression on the radiosensitivity of ESCC cells.

most evident presenting with the lowest p value (Table S1), and MALAT1 was highly expressed in ESCC (Fig. 3B). Thus, MALAT1 was selected for further studies. Chipbase v2.0 website was adopted to analyze the co-expression between SOX17 and MALAT1 in ESCC (Fig. 3C). qRT-PCR results showed higher expression of MALAT1 in tumor tissues than that in adjacent normal tissues (Fig. 3D). In addition, the luciferase activity of MALAT1 promoter was significantly increased following SOX17 overexpression, as detected by luciferase reporter (Fig. 3H). The above results suggested that SOX17 could bind to the promoter region of MALAT1 to restrict the transcription of MALAT1 in ESCC cells.
Fig. 3  SOX17 suppressed the radioresistance of ESCC cells via transcriptional inhibition of MALAT1. A  Chromosome map showing location of 2636 DEGs which were significantly upregulated in ESCC. **B** A box plot of MALAT1 expression in normal samples and ESCC samples. Red box represents cancer samples, and gray box represents normal samples. **C** The co-expression relationship between SOX17 and MALAT1 in ESCC samples. D, MALAT1 expression in tumor tissues (ESCC-Tumor group) and adjacent normal tissues (ESCC-Adjacent normal group) from ESCC patients as measured by qRT-PCR. n = 95 in ESCC patients. **E** Correlation analysis of MALAT1 expression and SOX17 mRNA expression in tumor tissues of 95 ESCC patients analyzed by Pearson correlation analysis. **F** The expression of MALAT1 in KYSE150R cells in oe-NC group and oe-SOX17 group as examined by qRT-PCR. **G** MALAT1 binding to SOX17 in KYSE150R cells of oe-NC group and oe-SOX17 group measured by ChIP assay. **H** The luciferase activity of MALAT1 promoter in 293T cells of oe-NC group and oe-SOX17 group measured by dual luciferase reporter assay. **J** The expression of SOX17 and MALAT1 in KYSE150R cells in response to oe-SOX17 alone or combined with oe-MALAT1 examined by qRT-PCR. **K** The protein expression of SOX17 in KYSE150R cells in response to oe-SOX17 alone or combined with oe-MALAT1 measured by Western blot. **L** The survival rate of KYSE150R cells in response to oe-SOX17 alone or combined with oe-MALAT1 measured by MTT assay. **M** The proliferation ability of KYSE150R cells in response to oe-SOX17 alone or combined with oe-MALAT1 examined by clonogenic assay. **N** The apoptosis of KYSE150R cells in response to oe-SOX17 alone or combined with oe-MALAT1 measured by flow cytometry. **O** The proliferation ability of KYSE150R cells in response to oe-SOX17 alone or combined with oe-MALAT1 examined by clonogenic assay. *p < 0.05. Measurement data were expressed as mean ± standard deviation. Paired t test was employed to compare the difference between tumor tissues and adjacent normal tissues. Unpaired t test was adopted to analyze the other data between two groups. One-way ANOVA followed by Tukey’s post-hoc test was used for comparison between multiple groups. Two-way ANOVA with Bonferroni post-hoc test was employed to analyze the data between groups at different time points. Cell experiments were repeated in triplicate.

Overall, the above results indicated that SOX17 suppressed the irradiation tolerance of ESCC cells through transcriptional inhibition of MALAT1

MALAT1 competitively binds to miR-199a

In order to further explore the downstream regulatory mechanism of MALAT1 affecting ESCC cell radioresistance, we first predicted the downstream miRNAs of MALAT1 using starBase and LncBase databases. Besides, through the GeneCards database, we found 60 miRs related to ESCC, which were then intersected with the above predicted miRs, with 10 candidate miRs obtained (Fig. 4A). Among them, miR-199a was previously reported to be poorly expressed in ESCC [12], and thus we selected miR-199a for further study.

starBase website predicted the binding site of MALAT1 and miR-199a (Fig. 4B). qRT-PCR data revealed poor miR-199a expression in ESCC tissues versus normal adjacent tissues (Fig. 4C). A negative correlation was found between miR-199a expression and MALAT1 expression in ESCC tissues (Fig. 4D). The luciferase activity of MALAT1-WT in the miR-199a mimic group was decreased while no alteration was found in the MALAT1-MUT luciferase activity (Fig. 4E). In the RNA-pull down experiment, the biont-labeled miR-199a-WT probe could significantly pull down MALAT1, and the biont-labeled MALAT1-WT probe could markedly pull down miR-199a (Fig. 4F, G). These data confirmed that MALAT1 could bind to miR-199a.

MALAT1 was overexpressed in KYSE150R cells (Fig. 4H). Three si-MALAT1 sequences were applied to knock down MALAT1 gene in KYSE150R cells, and the expression of MALAT1 in KYSE150R cells was measured by qRT-PCR. As shown in Fig. 4I, the expression of MALAT1 was reduced after transfection of si-MALAT1, with si3-MALAT1 showing the superior efficiency and thus selected for the following experiments.

By RIP detection, MALAT1 and miR-199a were significantly enriched in Ago2 in KYSE150R cells with oe-MALAT1 while sh-MALAT1 reduced the enrichment (Fig. 4J, K). In KYSE150R cells, the expression of miR-199a was reduced in the presence of MALAT1 overexpression while it was increased after MALAT1 silencing (Fig. 4L).

Taken together, MALAT1 could competitively bind to miR-199a in ESCC cells.

MALAT1 upregulates HIF1α expression by competitively binding to miR-199a

HMDD website was employed to analyze the network diagram of target genes mediated by miR-199a (Fig. 5A). From the intersection of miR-199a target genes predicted by HMDD, PicTar, TargetScan and starBase databases, we obtained three candidate genes SIRT1, GSK3B and HIF1α (Fig. 5B). Among them, the expression of HIF1α in ESCC increased more significantly (Fig. 5C). The binding site of miR-199a and HIF1α was obtained using starBase website (Fig. 5D). The expression of HIF1α was higher in
ESCC tissues than that in adjacent normal tissues (Fig. 5E, F, Fig. S1F). Pearson correlation analysis revealed an adverse correlation between miR-199a and HIF1α in ESCC tissues (Fig. 5G).

The luciferase activity of HIF1α-WT in the miR-199a mimic group was repressed while that of HIF1α-MUT was almost unchanged (Fig. 5H). KYSE150R cells were treated with miR-199a mimic and miR-199a inhibitor (Fig. S1I). The mRNA and protein expression of HIF1α decreased evidently after miR-199a overexpression in KYSE150R cells, and it increased markedly after miR-199a overexpression in oe-NC group, a halted decrease was observed in the oe-SOX17 α group (Fig. 6C), while the mRNA and protein expression of HIF1α was decreased. Versus oe-SOX17 α group (Fig. 6D, E), and the apoptosis was obviously lower (Fig. 6F).

Collectively, MALAT1 could upregulate the expression of HIF1α by binding to miR-199a in ESCC cells.

SOX17 suppresses HIF1α through the MALAT1-miR-199a axis and thus reduces the irradiation tolerance of ESCC cells

Next, we aimed to examine whether SOX17 affects the radiosensitivity of ESCC cells by regulating the MALAT1/miR-199a/HIF1α axis. qRT-PCR and Western blot results showed that the mRNA and protein expression of SOX17 in the oe-SOX17 + oe-NC group was promoted, while the expression of MALAT1 was obviously lowered (Fig. 6A, B, Fig. S1I). Meanwhile, miR-199a expression was overexpressed in the oe-SOX17 + oe-NC group (Fig. 6C), while the mRNA and protein expression of HIF1α was decreased. Versus oe-SOX17 + oe-NC group, we found no difference in the expression of SOX17, MALAT1 and miR-199a in the oe-SOX17 + oe-HIF1α group, while HIF1α expression markedly restored.

Compared with oe-SOX17 + oe-NC group, the survival rate and proliferation of KYSE150R cells were enhanced after 4 Gy X-ray exposure in the oe-SOX17 + oe-HIF1α group (Fig. 6D, E), and the apoptosis was obviously lower (Fig. 6F). In comparison with oe-SOX17 + oe-NC group, a halted decrease was observed in the
SOX17 and miR-199a was downregulated and that of HIF1α expression via transcriptional repression of MALAT1 and upregulation of miR-199a, thus delaying the irradiation tolerance of ESCC cells in vivo.

**DISCUSSION**

Radiotherapy is an effective approach for ESCC treatment [15] but radiation tolerance often occurs, limiting the clinical application of radiotherapy in a large extent [16]. Interestingly, accumulating evidence shows that SOX17, expressed at low levels in many different types of cancers [17, 18], is associated with the radiotherapeutic sensitivity of ESCC [9]. Moreover, SOX17 could affect the invasion and migration of ESCC cells by inhibiting the expression of MALAT1 [10]. In addition, related evidence also shows that miRs are crucial regulators to radiosensitivity [19], which may have roles to play in the radiosensitivity of ESCC cells by mediating downstream target genes. However, few studies have focused on the influence of SOX17 on ESCC radiosensitivity,
Fig. 6  SOX17 inhibited HIF1α expression by modulating the MALAT1-miR-199a axis and thus reduced the resistance of ESCC cells to irradiation. A The expression of SOX17, HIF1α and MALAT1 in KYSE150R cells in response to oe-SOX17 alone or combined with oe-HIF1α measured by qRT-PCR. B The protein expressions of SOX17 and HIF1α in KYSE150R cells in response to oe-SOX17 alone or combined with oe-HIF1α examined by Western blot. C The expression of miR-199a in KYSE150R cells in response to oe-SOX17 alone or combined with oe-HIF1α measured by qRT-PCR. D The survival rate of KYSE150R cells in response to oe-SOX17 alone or combined with oe-HIF1α examined by MTT assay. E The proliferation ability of KYSE150R cells in response to oe-SOX17 alone or combined with oe-HIF1α assessed by clonogenic assay. F The apoptosis of KYSE150R cells in response to oe-SOX17 alone or combined with oe-HIF1α evaluated by flow cytometry. G The proliferation ability of KYSE150R cells in response to oe-SOX17 alone or combined with oe-HIF1α measured by clonogenic assay. *p < 0.05, ns, not significant. Measurement data were presented as mean ± standard deviation. One-way ANOVA with Tukey’s post-hoc test was employed to analyze data of multiple groups. Two-way ANOVA followed by Bonferroni post-hoc test was adopted to compare the data among multiple groups at different time points. Cell experiments were repeated for three times.

and the mechanisms still remain poorly understood. Therefore, in this investigation, we illustrated that SOX17 had an impact on the ESCC radioresistance by suppressing MALAT1 expression and further regulating the expression of its downstream miR-199a and HIF1α.

Our experimental results showed that SOX17 was under-expressed in ESCC cells and tissues, and SOX17 overexpression could reduce the irradiation tolerance of ESCC cells. SOX17 has been reported as an important transcription factor involved in the radiosensitization of ESCC cells [21]. Moreover, SOX17 inhibited HIF1α expression through the MALAT1-miR-199a axis to reduce the radioresistance of ESCC cells.

and provide potential therapeutic targets for better clinical outcomes [24, 25]. Through bioinformatics analysis, we screened several downstream regulators of MALAT1, which were also associated with ESCC. Among them, miR-199a is expressed at low levels in esophageal cancer [12]. Further, our experimental results showed that HIF1α inhibition could suppress tumor growth of ESCC and sensitize ESCC cells to therapeutic approaches [13, 14, 26]. For example, Dihydroartemisinin might sensitize ESCC cells to photodynamic therapy by inhibiting the HIF1α pathway [14]. Moreover, mitochondrial pyruvate carrier blocker UK5099 could activate HIF1α expression, and UK5099-treated ESCC cells showed obviously more resistant to irradiation, as well as higher invasive ability in comparison with the parental cells [27].

Similarly, in the current research, we also demonstrated that SOX17 could suppress the HIF1α expression through the MALAT1-miR-199a axis to reduce the radioresistance of ESCC.
cells. In addition, we further illustrated that in the model of nude mice, SOX17 overexpression could inhibit irradiation tolerance of ESCC by downregulating HIF1α.

In conclusion, our experimental results showed that SOX17 inhibited MALAT1 expression at the transcription level, and thus potentiated the irradiation sensitivity of ESCC cells. Mechanistically, MALAT1 upregulated HIF1α by sponging miR-199a. SOX17 could prevent the irradiation tolerance of ESCC through the MALAT1-miR-199a-HIF1α axis, which may provide potential targets against ESCC radioresistance.

MATERIALS AND METHODS

Ethics statement

The current study was performed in strict accordance with the Declaration of Helsinki. All experimental protocols have been approved by the Ethics Committee of Changzhou Tumor Hospital, Soochow University. All patients have signed informed consent. The procedures of animal assay were in compliance with Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Study subjects

From December 2014 to January 2017, 95 patients (54 males and 41 females, aged 40–70 years, a mean age of 58.57 ± 6.92 years), who were pathologically confirmed as ESCC after operation, were enrolled in this study. The part in the central of cancer tissue without bleeding around necrosis and the normal mucosa, 2 cm away from the distal esophagus, were collected as resected specimens. Patients received no radiotherapy or chemotherapy before surgery. The clinicopathological features of 95 patients with ESCC are shown in Table S2. All the subjects were followed up and their conditions and clinical outcomes post treatment were recorded in detail. The follow-up period was 3–30 months, from the end of surgery to July 2019. The relationship between gene expression and OS of patients was determined using the Kaplan-Meier method.

Bioinformatics analysis

DEGs in ESCC samples in TCGA database were analyzed using GEPIA database, with \( \log_{2}(F \text{C}) > 1 \) and \( \rho < 0.01 \) as the threshold. Differentially expressed lncRNAs were selected, and the co-expression relationships between SOX17 and DEGs in esophageal cancer were analyzed by Chipbase v2.0. Downstream miRs of lncRNAs were predicted using starBase and LncBase databases. Meanwhile, miRs related to esophageal cancer were retrieved from GeneCards database and then intersected with the downstream miRs of lncRNAs using jvenn tool. The expression of targets was calculated by 2^{-\Delta\Delta C_{T}}, normalized to U6 or GAPDH.

RNA extraction and qRT-PCR

Total RNA from tissues and cells was extracted using TRizol (Invitrogen, Carlsbad, CA), TaqMan MicroRNA Assay RT primer (4427975, Applied Biosystems) or PrimeScript RT Reagent Kit (RR047A, Takara, Japan) were employed to synthesize the cDNA from mRNA. qRT-PCR assay was conducted by Thermo’s TaqMan Multiplex Real-Time Solution (4461882) and ABI 7500 real-time PCR system, with primer sequences shown in Table S3. The expression of targets was calculated by 2^{-\Delta\Delta C_{T}}, normalized to U6 or GAPDH.

Western blot

Tissues and cells were lysed with RIPA buffer and the BCA kit was employed to determine protein concentration in the protein extract. The protein was separated and transferred onto membranes. The membranes were incubated with primary antibodies (rabbit anti-SOX17, 1:500, Abcam, Cambridge, UK; rabbit anti-HIF1α, 1:5000, Sigma). The membranes were
then incubated with horseradish peroxidase-labeled anti-rabbit IgG (1:1000, Santa Cruz Biotech, CA). Immunoblots were visualized and captured using Bio-Rad ChemiDoc™ imaging system. GAPDH (rat, 1:1000, Santa Cruz Biotech, CA) was used as an internal reference, and the protein band image was analyzed by ImageJ2x software.

Cell culture and transfection
Human ESCC cell lines KYSE70, KYSE170, KYSE150, and KYSE150 were all purchased from American Type Culture Collection (Manassas, VA, USA), and the normal esophageal epithelial cell line HET-1A was obtained from Oulu Biotechnology (Guangzhou, Guangdong, China). They were all maintained in RPMI-1640 medium (Gibco, Waltham, MA) with 10% fetal bovine serum (FBS; Gibco), 100 μg/mL streptomycin and 100 μg/mL penicillin. Cells were cultured in a 3% CO2 incubator at 37 °C.

Human ESCC radiotherapy-resistant cell line KYSE150R was established by fractionated X-ray irradiation [28]. KYSE150 cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA) containing 10% FBS (HyClone, Logan) in a humidified incubator with 5% CO2 (v/v). After KYSE150 cells had grown to 50–60% density, they were treated with 2 Gy X-ray irradiation (2.5 Gy/min) using a Varian-6/100 linear accelerator (Varian Medical Systems, Inc, Palo Alto, CA). Subsequently, the KYSE150 cell medium was changed. As the cell density reached 30–35%, the medium was changed every 2 days. When many dead cells were observed, 15% FBS-containing RPMI-1640 medium was used for cell culture. When the irradiated cells reached 70–80% confluence, the above irradiation process was repeated until the total radiation dose reached 60 Gy. Finally, the obtained cells were named KYSE150R cells and continued to be cultured for ≥2 weeks before subsequent experiments.

Cells were trypsinized and plated into six-well plates (1 × 105 cells/well). After 24 h of routine culture, when cells reached 50% confluence, transient transfection was performed using Lipofectamine 2000 (Invitrogen). The experimental groups included oe-NC, oe-SOX17, oe-SOX17 = oe-NC, oe-SOX17 = oe-MALAT1, oe-MALAT1, si-NC, si-MALAT1 (forward: 5′-AUUGUAGACGCUAAUUCUCA-3′, reverse: 5′-AUUGUAGACGCUAAUUCUCA-3′), si-MALAT1 (forward: 5′-GCAAGAAGAGCAUCAAAGCU-3′, reverse: 5′-AUGUAUGUGCAUCUUCAUUCGUCC-3′), oe-MALAT1 (forward: 5′-GCAGAGGCAUUUCUUCAUUCGUCC-3′, reverse: 5′-AUGUAUGUGCAUCUUCAUUCGUCC-3′), oe-MALAT1 = oe-SOX17 (forward: 5′-CAUGAGGGAAACGAGTGGTTGGTAA-3′, reverse: 5′-TTACCAACCACTCGGTTCCTCTGTTG-3′)), mimic NC, miR-199a mimic, inhibitor NC, miR-199a inhibitor, oe-NC + mimic NC, oe-MALAT1 + mimic NC, oe-NC + miR-199a mimic, oe-MALAT1 + miR-199a mimic and oe-SOX17 + oe-HIF1α groups. Transfection plasmids, mimic and inhibitor were all purchased and synthesized in Sino Biotechnology Inc. (Beijing, China). After the transfected cells were cultured for 6 h, the medium was renewed. After incubation for 48 h, the cells were collected and used for subsequent experiments.

Cell viability assay
After ESCC cells in each group were exposed to X-ray irradiation, the cell culture plate was centrifuged for 5 min, and then the old medium in the well was replaced with MTT (1 mg/mL) dissolved the fresh medium without light exposure. After that, the culture plate was incubated for 3 h, and centrifuged for 6 min. MTT solution was discarded from the well and 100 μL DMSO was added. The absorbance was detected by a microplate reader at 450 nm.

Clonogenic assay for cell proliferation
ESCC cells were digested with 0.25% trypsin and pipetted to single cells (1 × 10^6 cells/mL). Cells (500 cells/well) were seeded into 24-well plates containing 1 mL of 37 °C preheated culture medium and incubated at 37 °C with 5% CO2 for 2–3 weeks, with medium renewed every 2–3 days. Next, the cells fixed by 5 mL of 4% parformaldehyde and incubated for 15 min. The cells were stained with GIEMSA (Invitrogen) for 10–30 min and air-dried. The colonies were photographed and counted with an inverted microscope (DMi8-M, Leica Co. Ltd, Solms, Germany), and colonies with more than 50 cells were considered as effective clones. Planting efficiency (% = (effective clone number/plated cell number) × 100%); Survival fraction (% = (Plant efficiency of radiation treatment group/Plant efficiency of control group) × 100%.

Flow cytometry
ESCC cells were seeded into six-well plates (2 × 10^6 cells/well) Experimental groups were classified into blank group, negative control group and transfection group. After transfection at 100 nmol/L and incubation for 72 h, the cells were trypsinized and centrifuged in a 15 mL centrifuge tube.

Afterwards, as per the instructions of AnnexinV-FITC Apoptosis Detection Kit I (BD Biosciences, San Jose, CA), the cells were resuspended in 500 μL binding buffer, and incubated with 5 μL FITC and 5 μL PI in the dark for 15 min. Cell apoptosis was measured by a FACS Calibur flow cytometer (BD Biosciences).

RNA-pull down assay
Biotinylated probe MALAT1-WT and mutant control MALAT1-Mut were designed and synthesized by Ribó Biological Co., Ltd (Ribó, Guangzhou, China). ESCC cells were lysed in lysis buffer and respectively incubated with 3 μg MALAT1-WT or MALAT1-Mut for 2 h. The mixture was incubated with magnetic beads for 4 h to pull down the biotin-competent RNA complex. TRizol reagent was employed to extract the mRNA bound in the pull-down complex. The miR-199a levels in the pull-down samples were assessed by qRT-PCR. Biotin-labeled probe miR-199a-WT and mutant miR-199a-Mut were both designed and synthesized in Ribó Biological Co., Ltd (Ribó, Guangzhou, Guangdong China). The pull-down assay was carried out as described above. The expression of MALAT1 was detected by qRT-PCR.

ChIP assay
ESCC cells were fixed in 1% formaldehyde solution and incubated at 37 °C for 10 min, followed by addition of glycine solution. The cells were lysed in 200 μL SDS lysis buffer, sonicated to generate chromatin fragments and immunoprecipitated with antibodies to SOX17 (Rabbit, 2 μg for 25 μg of chromatin, Abcam, Shanghai, China), and IgG (Rabbit, Abcam, serving as NC). Precipitated protein-DNA complex was eluted and cross-linking was reversed. DNA fragments were purified and analyzed by qRT-PCR.

Luciferase activity assay
Reporter plasmids containing MALAT1-WT and MALAT1 mutated at the putative miR-199a binding site were inserted into Pmir-GLO Dual Luciferase miRNA Target Expression Vectors (Promega, Madison, WI). Similarly, reporter plasmids containing HIF1α-WT and HIF1α-MUT at the miR-199a binding site were inserted into the luciferase reporter vector. These reporter plasmids were co-transfected with mimic NC and miR-199a mimic into 293T cells. After 48 h of transfection, luciferase activity was measured by the luciferase assay kit (KB01-200, BioVision, Mountain View, CA) and the dual luciferase reporter assay system (Promega, Madison, WI), with Renilla luciferase an internal reference.

Reporter plasmids containing pGL3-luc, pGL3-MALAT1-WT-luc and pGL3-MALAT1-MUT-luc in the 3′ UTR of SOX17 were inserted into the pGL3 Luciferase Reporter Vectors (Promega) and then co-transfected with oe-NC and oe-SOX17 into 293T cells (Oulu Biotechnology). Activity of MALAT1 promoter was detected.

RIP assay
RIP assay was performed using Magna RIP Kit (Millipore, Billerica, MA). Cells were collected and lysed with 100 μL RIP lysis buffer with protease inhibitor and ribonuclease inhibitor. Next, the cell lysate was incubated with anti-Ago2 (1 μg, Rabbit, Abcam) and protein A/G-beads (30 μL). The samples were digested with proteinase K to isolate the immunoprecipitated RNA. The purified RNA was subjected to qRT-PCR.

Subcutaneous tumor growth experiments
Forty-eight 5-week-old SPF male BALB/c nude mice (18–22 g, Shanghai SLAC Laboratory Animal Co., Ltd, Shanghai, China) were used for tumorogenesis experiments. The mice were anesthetized with ether and routinely disinfected. KYSE150 cells (1 × 10^6 cells/200 μL) were injected into nude mice in the oe-NC group, oe-SOX17 = oe-NC group and oe-SOX17 + oe-HIF1α group were subcutaneously injected into the dorsal surface of the mouse right hind limb. Tumor volume was measured every 5 days. When the average tumor volume grew to 200 mm^3, the mice were irradiated with 4 Gy X-ray for 5 consecutive days. When the average tumor diameter of the mice in the oe-NC group without irradiation treatment reached 1.5 cm, the mice were euthanized by cervical dislocation.

Immunohistochemistry
The tumor tissue sections (5 μm) were subjected to antigen retrieval, blocked with 1% BSA for 1 h, and incubated with primary antibody (rabbit anti-HIF1α 1:1000, rabbit anti-α-SMA 1:100 Sigma) overnight at 4 °C and then with HRP-conjugated IgG (Boster, Wuhan, China) for 1 h. Further, the sections were exposed to DAB (Boster) for development. After counterstained with hematoxylin, the sections were dehydrated and subjected to microscopic examination after mounting.
REFERENCES

1. Lagergren J, Smyth E, Cunningham D, Lagergren P. Oesophageal cancer. Lancet. 2017;390:2383–96.
2. Pennathur A, Gibson MK, Jobe BA, Luketich JD. Oesophageal carcinoma. Lancet. 2015;381:400–12.
3. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, et al. Cancer statistics in China. 2015. CA Cancer J Clin. 2016;66:115–32.
4. van Rossum PSN, Mohammad NH, VleugAAP, van Hillegersberg R. Treatment for unresectable or metastatic oesophageal cancer: current evidence and trends. Nat Rev Gastroenterol Hepatol. 2018;15:235–49.
5. Cohen DJ, Leichman L. Controversies in the treatment of local and locally advanced gastric and esophageal cancers. J Clin Oncol. 2015;33:1754–9.
6. Tan DS, Holzner M, Weng M, Srivastava Y, Jauch R. SOX17 in cellular reprogramming and cancer. Semin Cancer Biol. 2020;57:65–73.
7. Ma K, Cao B, Guo M. The detective, prognostic, and predictive value of DNA methylation in human esophageal squamous cell carcinoma. Clin Epigenet. 2016;8:43.
8. Chang WL, Lai WW, Kuo IY, Lin CY, Lu PJ, Sheu BS, et al. A six-CpG panel with DNA methylation biomarkers predicting treatment response of chemoradiation in esophageal squamous cell carcinoma. J Gastroenterol. 2017;52:705–14.
9. Kuo IY, Huang YL, Lin CY, Lin CH, Chang WL, Lai WW, et al. SOX17 overexpression sensitizes chemoradiation response in esophageal cancer by transcriptional down-regulation of DNA repair and damage response genes. J Biomed Sci. 2019;26:20.
10. Kuo IY, Wu CC, Chang JM, Huang YL, Lin CH, Yan JJ, et al. Low SOX17 expression is a prognostic factor and drives transcriptional dysregulation and esophageal cancer progression. Int J Cancer. 2014;135:563–73.
11. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov. 2017;16:203–22.
12. Pavlov K, Kluiver J, Meijer C, Boersma-van Ek W, Kruyt FAE, Karrenbeld A, et al. Circulating miRNAs in patients with Barrett’s esophagus, high-grade dysplasia and esophageal adenocarcinoma. J Gastrointest Oncol. 2018;9:1150–6.
13. Zhu Y, Zang Y, Zhao F, Li Z, Zhang J, Fang L, et al. Inhibition of HIF-1alpha by PX-478 suppresses tumor growth of esophageal squamous cell cancer in vitro and in vivo. Am J Cancer Res. 2017;11:1998–212.
14. Li Y, Sui H, Jiang C, Li S, Han Y, Huang P, et al. Dhidryoaromatinisin increases the sensitivity of photodynamic therapy via NF-kappaB/HIF-1alpha/VEGF pathway in esophageal cancer cell in vitro and in vivo. Cell Physiol Biochem. 2018;48:2035–45.
15. Mariette C, Robb WB, Piessen G, Adenis A. Neoadjuvant chemoradiation in oesophageal cancer. Lancet Oncol. 2015;16:1008–9.
16. Dai T, Shah MA. Chemoradiation in oesophageal cancer. Best Pract Res Clin Gastroenterol. 2015;29:193–209.
17. Fu DY, Wang ZM, Li C, Wang BL, Shen ZZ, Huang W, et al. Sox17, the canonical Wnt antagonist, is epigenetically inactivated by promoter methylation in human breast cancer. Breast Cancer Res Treat. 2010;119:601–12.
18. Du YC, Oshima H, Oguma K, Kitamura T, Ihadani H, Fujimura T, et al. Induction and down-regulation of Sox17 and its possible roles during the course of gastrointestinal tumorigenesis. Gastronterology. 2009;137:1346–57.
19. Chaudhry MA. Radiation-induced microRNA: discovery, functional analysis, and cancer radiotherapy. J Cell Biochem. 2014;121:436–49.
20. Zhang W, Gloeckner SC, Guo M, Machida EO, Wang DH, Easwaran H, et al. Epigenetic inactivation of the canonical Wnt antagonist SRY-box containing gene 17 in colorectal cancer. Cancer Res. 2008;68:2764–72.
21. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med. 2012;4:143–59.
22. Rupaimoole R, Calin GA, Lopez-Berestein G, Sood AK. miRNA deregulation in cancer cells and the tumor microenvironment. Cancer Discov. 2016;6:235–46.
23. Li Z, Rana TM. Therapeutic targeting of microRNAs: current status and future challenges. Nat Rev Drug Discov. 2014;13:622–38.
24. Liu A, Zhu J, Wu G, Cao L, Tan Z, Zhang S, et al. Antagonizing miR-455-3p inhibits chemoresistance and aggressiveness in esophageal squamous cell carcinoma. Mol Cancer. 2017;16:106.
25. Hong L, Han Y, Zhang H, Li M, Gong T, Sun L, et al. The prognostic and chemotherapeutic value of miR-296 in esophageal squamous cell carcinoma. Ann Surg. 2010;251:1056–63.
26. Natsuiizaka M, Naganuma S, Kagawa S, Ohashi S, Ahmadi A, Subramanian H, et al. Hypoxia induces IGFBP3 in esophageal squamous cancer cells through HIF-1alpha-mediated miRNA transcription and continuous protein synthesis. FASEB J. 2012;26:2620–30.
27. Li Y, Li X, Kan Q, Zhang M, Li X, Xu R, et al. Mitochondrial pyruvate carrier function is negatively linked to Warburg phenotype in vitro and malignant features in esophageal squamous cell carcinomas. Oncotarget. 2017;8:10589–73.
28. Luo W, Liu W, Yao J, Zhu W, Zhang H, Sheng Q, et al. Downregulation of H19 decreases the radioresistance in esophageal squamous cell carcinoma cells. Oncotarget. 2019;12:4779–88.

ACKNOWLEDGEMENTS

This study was supported by Changzhou Social Development Tech Program, China (Grant No. CE20195028) and Changzhou Sci & Tech Program, China (Grant No. CJ20190034).

AUTHOR CONTRIBUTIONS

YJ: Conceptualization, Writing—original draft. YZ: Data curation, Formal analysis. XQ: Investigation; Writing—review & editing. YO: Software, Visualization. XZ: Supervision, Validation. ZL: Methodology, Project administration.

COMPETING INTEREStS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The current study was performed in strict accordance with the Declaration of Helsinki. All experimental protocols have been approved by the Ethics Committee of Changzhou Tumor Hospital, Soochow University. All patients have signed informed consent. The procedures of animal assay were in compliance with Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41420-022-01012-6.

Correspondence and requests for materials should be addressed to Xifa Zhou or Zhonghua Lu.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022