ACCSCIENCE PUBLISHING

Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

Alkylation repair homolog 3-regulated esophageal squamous cell carcinoma associated long non-coding RNA 1 is required for maintaining the stemness of esophageal cancer

Yuanbo Cui[1], Yanan Lou[2], Pengju Lv[1], and Wei Cao[1]*

[1]Translational Medicine Center, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou 450007, China

[2]Department of Breast Surgery, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou, 450007, China

Abstract

N[1]-methyladenosine (m[1]A) RNA modification represents one of the essential post-transcriptional modifications in gene expression regulation. Long non-coding RNAs (lncRNAs) are involved in the development of malignant tumors, including esophageal cancer (ESCA). However, whether m[1]A can regulate that lncRNA in cancer cells remains unclear. ESCA cell lines TE1 and KYSE70 were used for functional experiments. The mRNA and protein levels were detected by quantitative reverse transcription polymerase chain reaction and Western blot, respectively. Colony formation and tumor sphere formation assays were used for evaluating ESCA stemness. The m[1]A modification on esophageal squamous cell carcinoma associated long non-coding RNA 1 (ESCCAL-1) transcript was examined by methylated RNA immunoprecipitation. In this study, we report that RNA m[1]A demethylase alkylation repair homolog 3 (ALKBH3)-mediated ESCCAL-1 is implicated in maintaining stem cell-like properties of ESCA. Clinically, ESCCAL-1 was up-regulated in ESCA and positively correlated with tumor stage. In addition, patients with higher ESCCAL-1 expression in tumors had shorter median survival. Functionally, the knockdown of ESCCAL-1 attenuated the stemness of ESCA cells as indicated by decreased sphere formation and colony formation capacities, while overexpression of ESCCAL-1 elicits the opposite biological effects. Moreover, ESCCAL-1 manipulation positively regulated both mRNA and protein levels of KLF4 and CD44, two stemness-related markers. Mechanistically, ALKBH3 upregulated ESCCAL-1 expression in an m[1]A demethylation-dependent manner. Notably, the downregulation of ALKBH3 mimicked the effects of ESCCAL-1 deficiency on ESCA stemness, and this phenomenon is significantly reversed by the enforced expression of ESCCAL-1. Our results revealed the role of m[1]A-mediated ESCCAL-1 in ESCA self-renewal, which expands the understanding of lncRNA post-transcriptional modification in cancer development.

Keywords: ALKBH3; *N*[1]-methyladenosine; ESCCAL-1; Stemness; Esophageal cancer

***Corresponding author:**
Wei Cao
(caowei7@zzu.edu.cn)

Citation: Cui Y, Lou Y, Lv P, *et al.*, 2023, Alkylation repair homolog 3-regulated esophageal squamous cell carcinoma associated long non-coding RNA 1 is required for maintaining the stemness of esophageal cancer. *Gene Protein Dis,* 2(1):305.
https://doi.org/10.36922/gpd.305

Received: December 28, 2022
Accepted: February 21, 2023
Published Online: March 13, 2023

1. Introduction

Esophageal cancer (ESCA), a common digestive system malignancy, is the sixth leading cause of cancer-related death worldwide[1]. Esophageal squamous cell carcinoma (ESCC) is the primary pathological type of ESCA in Asia, while esophageal adenocarcinoma (EAC) is more common in Western countries[2,3]. Although researchers have discovered in recent years that genetic mutations alter susceptibility to ESCA and that epigenetic changes contribute to the development of ESCA[4-6], the detailed mechanisms that drive the tumorigenesis of ESCA are still not well understood. Therefore, uncovering the molecular mechanism of ESCA is expected to contribute to the development of new diagnostic and therapeutic strategies.

Long non-coding RNAs (lncRNAs), non-coding transcripts longer than 200 nucleotides, widely mediate tumor development and influence disease prognosis[7]. We previously used transcriptome sequencing technology to detect differentially expressed lncRNAs in ten pairs of ESCA and adjacent normal tissues and identified esophageal squamous cell carcinoma associated long non-coding RNA 1 (ESCCAL-1) as an upregulated molecule closely related to ESCA[8]. It was further found that ESCCAL-1 can promote the proliferation, metastasis, cycle progression, and apoptosis resistance of ESCA cells[9,10], suggesting that ESCCAL-1 may be a critical oncogenic lncRNA in ESCA occurrence. However, the molecules responsible for the uncontrolled expression of ESCCAL-1 in ESCA and their biological roles still need to be fully understood.

N^1-methyladenosine (m^1A) methylation is one of the eukaryotic cell's most common RNA modifications. Deregulated methylase regulates RNA stability, splicing, translation, and other processes by affecting the m^1A modification of transcripts[11,12]. RNA m^1A modification controls intracellular gene expression profile at the post-transcriptional level and participates in the regulation of tumor initiation and development[13-15]. However, the function of m^1A modification in ESCA and its regulation of lncRNA expression remains unclear.

In this study, we found that high expression of ESCCAL-1 was closely related to the progression and prognosis of ESCA. The absence of ESCCAL-1 inhibits the stem-like properties of ESCA cells, and the forced expression of ESCCAL-1 promotes the self-renewal ability of ESCA. Alkylation repair homolog 3 (ALKBH3), an RNA demethylase, erases the m^1A modification of ESCCAL-1 and causes the upregulation of the latter expression in ESCA. ALKBH3/ESCCAL-1 axis is involved in the stemness maintenance of ESCA, providing a new therapeutic target for this disease.

2. Materials and methods

2.1. Cancer databases

UALCAN is a comprehensive cancer database containing multiple omics data (http://ualcan.path.uab.edu/index.html). We used this database to verify the expression of ESCCAL-1 in ESCA and its relationship with various clinical indicators of patients. Kaplan–Meier Plotter (KMP, http://kmplot.com/analysis/index.php), an online server designed to provide users with clinical data on pan-cancer, was used to analyze the relationship of patient survival between ESCCAL-1 and ESCA. GEPIA (http://gepia.cancer-pku.cn/about.html) is an online interactive website based on RNA-seq data, which is used to analyze the expression of ESCCAL-1 and ALKBH3 as well as their correlation in ESCA.

2.2. Cell culture and transfection

Three ESCA cell lines, including TE1, KYSE70, EC1, and one immortalized esophageal epithelial cell line Het-1A, were cultured in an incubator containing 5% carbon dioxide at 37°C. All cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution. The lentivirus-based recombinant vectors were purchased from Shanghai GeneChem Company (China) for knockdown (sh-AL1#1, sh-AL1#2) or overexpression (OE-AL1) of ESCCAL-1. The vectors were transfected into ESCA cells with the transfection reagent HitransGA (GeneChem, China). ALKBH3 silencing siRNA was purchased from Shanghai GenePharma Company (China) and transfected into ESCA cells with the transfection agent INTERFERin (Polyplus, France).

2.3. Real-time quantitative reverse transcription polymerase chain reaction

Total RNAs were extracted from the cells using Trizol reagent (Invitrogen, USA). After concentration and purity measurements, 1 μg of RNAs in a 20-μL reaction system were reverse-transcribed into cDNA using a reverse transcription kit (Novoprotein, China). Finally, real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using the SYBRGREEN kit (Novoprotein, China) and amplification system (Applied Biosystems, USA). The housekeeping gene *GAPDH* was used as the internal reference, and the relative expression level of the target gene was calculated by method $2^{-\Delta\Delta Ct}$. The primers used are shown in Table S1.

2.4. Tumor sphere formation assay

The transfected cells were uniformly inoculated in low-adhesion six-well plates (CORNING, USA), each

containing 4 mL of sphere-forming medium and 10,000 cells. Dulbecco's modified eagle medium (DMEM)/F12 medium contained 20 ng/mL epidermal growth factor (EGF), 10 ng/mL basic fibroblast growth factor (bFGF), 5 μg/mL insulin, 0.4% bovine serum albumin (BSA), and 1× B27. The plates were placed in an incubator containing 5% carbon dioxide at 37°C for 7 – 12 days. Finally, the spheres were photographed and the number of spheres were analyzed.

2.5. Colony formation assay

The transfected cells were uniformly inoculated in 12-well plates with 1000 cells per well, and placed in an incubator containing 5% carbon dioxide at 37°C for 7 – 10 days. The cell clones were immobilized with paraformaldehyde and stained with crystal violet solution. Finally, cell colonies were photographed and the area of colonies in each well was analyzed.

2.6. Western blot analysis

Total proteins were extracted by RIPA lysis buffer (EpiZyme, China) containing protease inhibitors. After concentration and purity determination, the total proteins were denatured with loading buffer at 100°C for 10 min. Then, 10% polyacrylamide gel was prepared, and an equal amount of protein was added to each lane and electrophoresis was performed. Subsequently, the proteins on the gel were transferred to the polyvinylidene fluoride membrane. After blocking with skimmed milk and incubation with the primary antibody and secondary antibody, the ECL kit and the luminescence imaging analysis system were used to detect the target protein attached to the membrane. Primary antibodies include anti-GAPDH (1:5000, Bioworld, China), anti-CD44 (1:2000, Bioss, China), and anti-KLF4 (1:2000, Bioss, China).

2.7. Methylated RNA immunoprecipitation (MeRIP)

N^1-methyladenosine (m^1A) modification on the ESCCAL-1 transcript was detected by the m^1A MeRIP Kit (GenSeq, China). The experimental procedures were performed according to the user manual. In brief, the Trizol reagent was used to extract total RNAs from cells and the RNA concentration was adjusted to 1 μg/μL with enzyme-free water. The fragment buffer was used to process RNA transcripts. Subsequently, m^1A antibody or control antibody IgG, fragmented RNAs and immunoprecipitation (IP) buffer were added to the prepared immunomagnetic beads and incubated at 4°C for 1 h. Then, m^1A-labeled RNA samples obtained by immunoprecipitation were dissolved in enzyme-free water and detected by polymerase chain reaction (PCR). Specific primers of ESCCAL-1 transcript used for MeRIP-PCR are shown in Table S2.

2.8. Statistical analysis

Statistical analysis and illustration of experimental data were completed by SPSS 19.0 and GraphPad 9.0. Comparison of experimental data between the two groups was conducted by *t*-test, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. ESCCAL-1 is upregulated in ESCA and correlates with patient outcome

We previously identified the dysregulation of lncRNA ESCCAL-1 in ESCA using RNA-seq from ten pairs of tumor samples and adjacent normal samples[8] and verified its elevation in multiple ESCA cohorts[10]. In this study, we analyzed the expression of ESCCAL-1 in 11 normal samples, 80 EAC samples, and 81 ESCC samples by interrogating the cancer database UALCAN. ESCCAL-1 was significantly overexpressed in ESCA (Figure 1A). Moreover, the expression of ESCCAL-1 in ESCC was higher than in EAC (Figure 1A). Then, using this study cohort, we analyzed the relationship between ESCCAL-1 expression and race, sex, and tumor stage in ESCA patients. ESCCAL-1 was found to be more highly expressed in African Americans and Asians than that in Caucasians (Figure 1B). In addition, the expression of ESCCAL-1 in male ESCA was higher than in female ESCA (Figure 1C). To a certain extent, ESCCAL-1 expression increased with the progression of the ESCA tumor stage (Figure 1D). ESCCAL-1 was reported to be closely related to the outcome of ESCA patients in our previous study[8]. Here, we further analyzed the relationship between ESCCAL-1 expression and ESCA prognosis using the survival database KMP. It was found that the median survival time of patients with high ESCCAL-1 expression was significantly shorter than that of patients with low ESCCAL-1 expression in both EAC (16.5 months vs. 46.7 months, Figure 1E) and ESCC (25.4 months vs. 42.1 months, Figure 1F). These findings, in combination with our previous results, suggest that ESCCAL-1 is a bona fide risk factor for ESCA progression and survival.

3.2. ESCCAL-1 is essential for maintaining the stemness of ESCA cells

We have previously shown that knockdown of ESCCAL-1 inhibits ESCA cell growth and metastasis, while overexpression of ESCCAL-1 promotes these malignant phenotypes of ESCA[9,10]. As stem cell-like property is an intrinsic characteristic of tumors and closely related to malignant proliferation and metastasis[16,17], we speculate that ESCCAL-1 might be involved in the stemness maintenance of ESCA. First, we detected the expression of ESCCAL-1 at the ESCA cell level by qRT-PCR and found

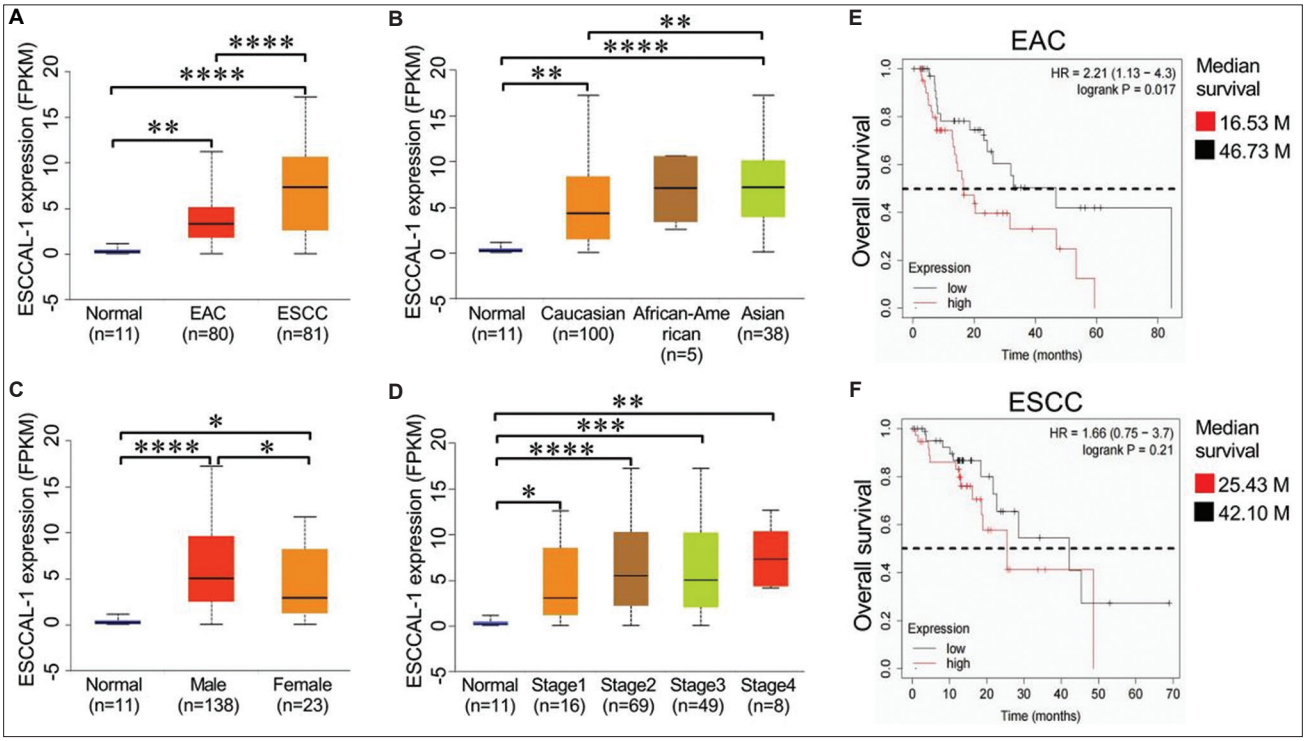

Figure 1. Expression of ESCCAL-1 in ESCA and its relationship with clinical parameters of patients. (A) UALCAN database was used to investigate the transcription levels of ESCCAL-1 in 11 esophageal epithelial samples, 80 EAC samples, and 81 ESCC samples. $^{**}P < 0.01$, $^{****}P < 0.0001$. (B) UALCAN was employed to analyze the relationship between ESCCAL-1 expression and patients' race with ESCA. $^{**}P < 0.01$, $^{****}P < 0.0001$. (C and D) UALCAN was applied to observe the relationship between ESCCAL-1 expression and gender (C) or tumor stage (D) in ESCA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (E and F) Kaplan-Meier Plotter database was utilized to analyze the relationship between the expression of ESCCAL-1 and the survival of EAC (E) or ESCC (F) patients.

ESCCAL-1: Esophageal squamous cell carcinoma associated long non-coding RNA 1; ESCA: Esophageal cancer; EAC: Esophageal adenocarcinoma.

that the expression of ESCCAL-1 in the three ESCA cell lines (TE1, KYSE70, EC1) was significantly higher than that of the normal esophageal epithelial cell line Het-1A (Figure 2A). We then used recombinant lentiviral vectors to knockdown (sh-AL1#1, sh-AL1#2; Figure 2B) and overexpress (OE-AL1; Figure 2C) ESCCAL-1 in ESCA cells to perform loss-of-function and gain-of-function experiments. Subsequently, both tumor sphere formation and colony formation assays were used to evaluate the stemness of ESCA cells. Results from tumor sphere formation experiments showed that the knockdown of ESCCAL-1 reduced the sphere-forming ability of TE1 cells by about 60% (Figure 2D and E). In comparison, the overexpression of ESCCAL-1 promoted the sphere-forming capacity of KYSE70 cells by about 40% (Figure 2D and F). Moreover, colony formation experiments confirmed that silencing ESCCAL-1 significantly limited the cloning ability of TE1 cells *in vitro* by more than 60%, while upregulation of ESCCAL-1 enhanced the colony formation of KYSE70 cells by more than two folds (Figure 2G–I). These data indicate that ESCCAL-1 participates in maintaining ESCA stemness.

3.3. ESCCAL-1 regulates stemness-related markers expression

Considering that ESCCAL-1 plays a key role in ESCA stemness, we wondered whether ESCCAL-1 might regulate the expression of stemness-related genes. We used qRT-PCR to detect the changes in mRNA levels of a set of markers associated with stemness[17] (including SOX2, CD44, ALDH1A1, Nanog, ZEB1, KLF4, Myc) in ESCA cells after ESCCAL-1 overexpression or knockdown. We found that overexpression of ESCCAL-1 led to significant upregulation of the transcription levels of these stemness-related genes (Figure 3A), among which CD44 and ALDH1A1 showed the greatest changes. In contrast, the knockdown of ESCCAL-1 resulted in decreased mRNA levels of some stemness markers (Figure 3B). We then cross-compared the results of Figure 3A and B, and found that two stemness markers, CD44 and KLF4, were uniformly positively regulated by ESCCAL-1 (Figure 3C). Subsequent Western blot analysis further confirmed that upregulation of ESCCAL-1 increased the protein levels of CD44 and KLF4 in ESCA cells, while downregulation of ESCCAL-1 decreased the levels of both proteins

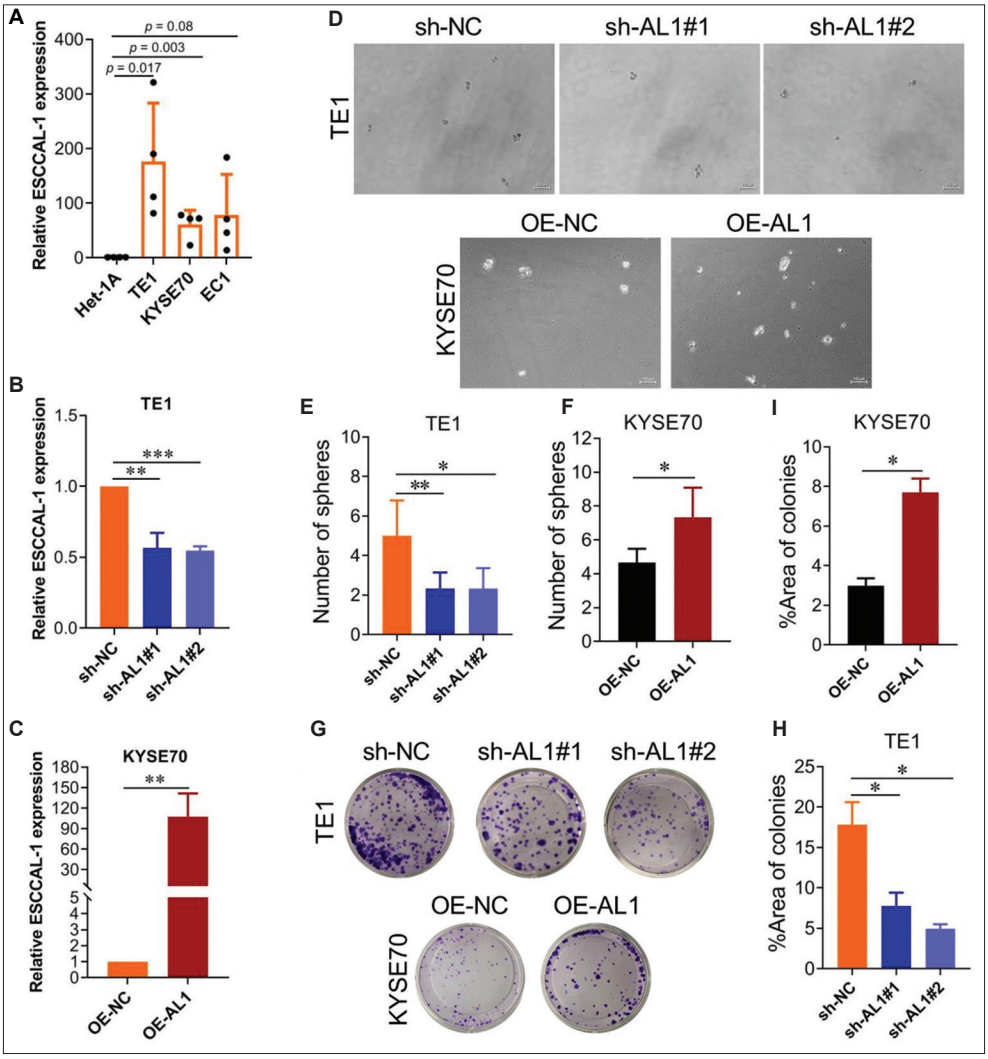

Figure 2. ESCCAL-1 is essential for maintaining ESCA stemness. (A) The transcription levels of ESCCAL-1 in Het-1A, TE1, KYSE70, and EC1 cells were detected by qRT-PCR, n = 4. (B and C) The knockdown (B) and overexpression (C) effects of ESCCAL-1 were tested by qRT-PCR, $n = 3$. $^{**}P < 0.01$, $^{***}P < 0.001$. (D and F) The effects of deletion or overexpression of ESCCAL-1 on the stemness of ESCA cells were evaluated by tumor sphere formation assay, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bar = 100 μm. In panel (D), TE1 cells were transfected with sh-AL1, and KYSE70 were transfected with OE-AL1. (G–I) A colony formation assay was used to observe the effects of knockdown ($n = 2$) or upregulation (n = 3) of ESCCAL-1 on ESCA growth. $^{*}P < 0.05$. In panel (G), TE1 cells were transfected with sh-AL1, and KYSE70 were transfected with OE-AL1.
ESCCAL-1: Esophageal squamous cell carcinoma associated long non-coding RNA 1; ESCA: Esophageal cancer; qRT-PCR: quantitative reverse transcription polymerase chain reaction.

(Figure 3D). These results suggest that ESCCAL-1 may be involved in the stemness maintenance of ESCA by regulating the expression of CD44 and KLF4.

3.4. RNA m^{1}A demethylase ALKBH3 mediates ESCCAL-1 expression in ESCA

Given that ESCCAL-1 is upregulated in ESCA, we intended to investigate the upstream mechanism that causes its dysregulation. As a novel RNA modification mode, ALKBH3-mediated m^{1}A modification has been reported recently to play a vital regulatory role in some tumor types, such as liver cancer and cervical cancer[13-15]. To explore whether m^{1}A may regulate ESCCAL-1, we first used the GEPIA database to analyze the expression of m^{1}A demethylase ALKBH3 in ESCA and its correlation with ESCCAL-1. Surprisingly, we found that ALKBH3 and ESCCAL-1 showed increased expression and significant positive correlation in 182 ESCA samples (Figure 4A and B), implying that ALKBH3 may be involved in the regulation of ESCCAL-1 and may play a role in ESCA.

Data from qRT-PCR showed that the expression level of ALKBH3 in three ESCA cell lines (TE1, KYSE70,

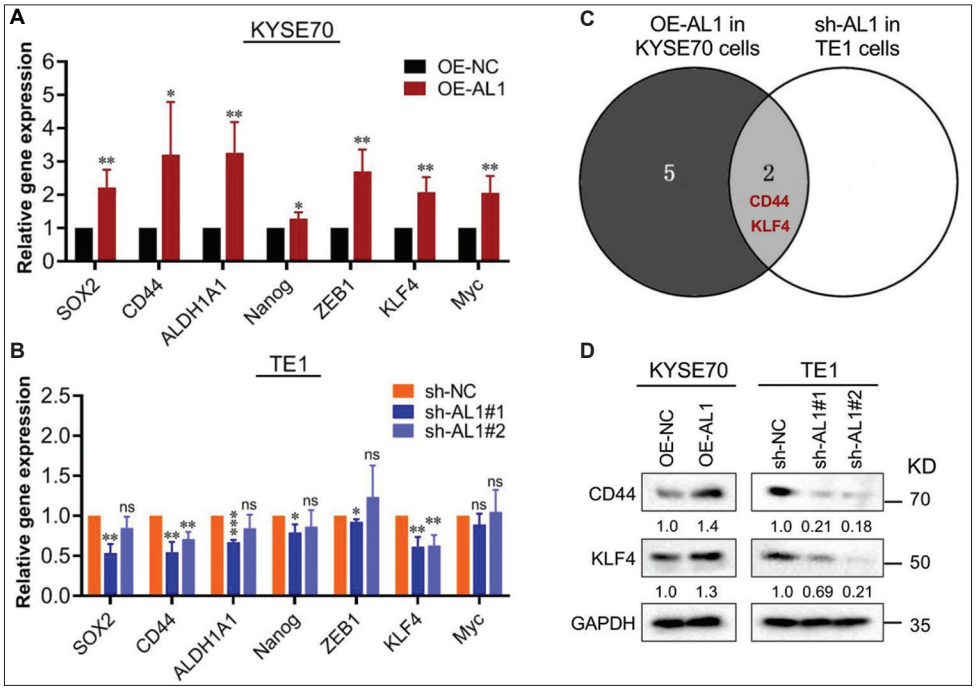

Figure 3. Effects of ESCCAL-1 manipulation on stemness-related gene expression in ESCA cells. (A and B) The effects of ESCCAL-1 overexpression (A, $n = 4$) or knockdown (B, $n = 3$) on mRNA levels of various stemness-associated markers, including SOX2, CD44, ALDH1A1, Nanog, ZEB1, KLF4, and Myc. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to OE-NC or sh-NC. (C) The intersection of results from panels (A) and (B) revealed that the expression of CD44 and KLF4 was consistent with that as a result of ESCCAL-1 manipulation. (D) The protein levels of CD44 and KLF4 in ESCA cells after ESCCAL-1 knockdown were detected by Western blot, $n = 3$.
ESCCAL-1: Esophageal squamous cell carcinoma associated long non-coding RNA 1; ESCA: Esophageal cancer.

EC1) was significantly higher than that of normal esophageal epithelial cell line Het-1A (Figure 4C). Moreover, silencing ALKBH3 expression with siRNA can significantly downregulate the level of ESCCAL-1 in ESCA cells (Figure 4D and E). This suggests that ESCCAL-1 is regulated by RNA m^{1}A demethylase ALKBH3. Next, to determine whether ESCCAL-1 harbors m^{1}A modification, we divided the transcript into six regions (R1 to R6), designed six pairs of specific primers (Figure 4F), then conducted an m^{1}A MeRIP assay. We found significant m^{1}A enrichment in the R4 region of ESCCAL-1 transcripts (Figure 4G). Then, ALKBH3 in ESCA cells was knocked down, and the MeRIP experiment was performed. The results showed that downregulating ALKBH3 significantly increased the m^{1}A modification level of ESCCAL-1 transcripts (Figure 4H). These results suggest that ESCCAL-1 is regulated by ALKBH3 in an m^{1}A-dependent manner.

3.5. ALKBH3/ESCCAL-1 axis maintains ESCA stemness

ALKBH3 regulates biological phenotypes of cancer cells by affecting gene expression[13,14]. Since ALKBH3 regulates the expression level of ESCCAL-1 in ESCA, and ESCCAL-1 is vital for the stemness maintenance of ESCA, we speculated that the ALKBH3/ESCCAL-1 axis might participate in ESCA self-renewal. To this end, we performed functional rescue assays. The results showed that silencing ALKBH3 reduced the protein levels of CD44 and KLF4 (Figure 5A) and significantly hindered the clonogenesis and tumor sphere formation ability of ESCA cells (Figure 5B and C), mimicking the biological effects of ESCCAL-1 deletion on ESCA. Notably, overexpression of ESCCAL-1 significantly rescued, at least in part, the effects of ALKBH3 knockdown on the stemness of ESCA cells (Figure 5A–C). These data suggest that the ALKBH3/ESCCAL-1 axis contributes to the stemness maintenance of ESCA (Figure 5D).

4. Discussion

LncRNA is involved in the progression of ESCA and other malignant tumors and can be modified after transcription[18,19]. RNA m^{1}A modification is essential in regulating gene expression in eukaryotic cells[20,21]. However, whether m^{1}A can deregulate lncRNAs in tumor cells remains unclear. Here, we report that RNA m^{1}A demethylase ALKBH3-mediated ESCCAL-1 is required for the stemness maintenance of ESCA.

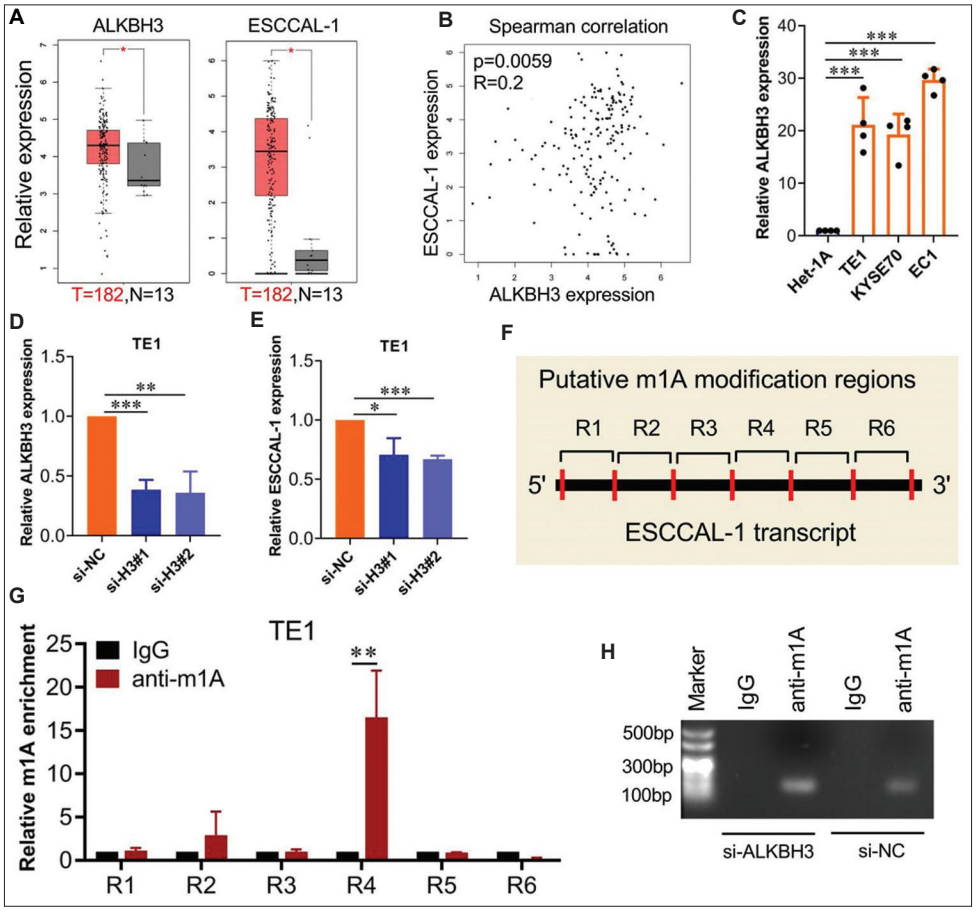

Figure 4. ESCCAL-1 is regulated by ALKBH3 in an m^{1}A-dependent manner. (A) The expression levels of ALKBH3 and ESCCAL-1 in 13 normal tissues and 182 ESCA tissues were analyzed by the GEPIA database. *$P < 0.05$. (B) The GEPIA database was used to investigate the expression correlation between ALKBH3 and ESCCAL-1 in 182 ESCA samples. (C) The mRNA levels of ALKBH3 in Het-1A, TE1, KYSE70, and EC1 cells were tested by qRT-PCR, $n = 4$. ***$P < 0.001$. (D and E) The effect of ALKBH3 silencing on the expression of ALKBH3 and ESCCAL-1 in ESCA cells was evaluated by qRT-PCR, $n = 3$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (F) The putative presence of m^{1}A-modified regions on ESCCAL-1 transcripts. (G) MeRIP-PCR assay was used to detect m^{1}A modification on ESCCAL-1 transcripts, $n = 3$. **$P < 0.01$. (H) MeRIP-PCR combined with agarose gel electrophoresis was used to evaluate the effect of ALKBH3 knockdown on m^{1}A modification of ESCCAL-1 transcript, $n = 2$.
ESCCAL-1: Esophageal squamous cell carcinoma associated long non-coding RNA 1; ESCA: Esophageal cancer; m^{1}A: N^1-methyladenosine.

Stem cell-like property refers to the self-renewing ability of malignant tumor cells to maintain continuous proliferation and metastasis[22,23]. The stemness nature of tumors allows them to adapt to extreme growth conditions and to combat multiple risk factors[22,23]. Therefore, reducing tumor stemness is critical in eliminating tumor therapeutic resistance[24,25]. In the previous studies, ESCCAL-1 has the carcinogenic effect of promoting ESCA proliferation and metastasis[9,10], two basic intrinsic properties of malignant tumors, and depend on a high degree of self-renewal ability. Therefore, ESCCAL-1 might be associated with the stemness of ESCA. Through a series of functional and molecular expression experiments, we demonstrated that the knockdown of ESCCAL-1 significantly weakened the stemness of ESCA cells and reduced the mRNA and protein levels of two stemness-related genes, *CD44* and *KLF4*. In contrast, overexpression of ESCCAL-1 caused the opposite biological effects. Although we have not clarified the exact mechanisms by which ESCCAL-1 is involved in the maintenance of ESCA stemness, our results preliminarily suggest that ESCCAL-1 may exert its function by regulating the expression of CD44, KLF4, and other stemness markers. This study provides new insights into targeting the ESCCAL-1/stemness axis for ESCA treatment.

In addition to the high expression of ESCCAL-1 in ESCA, we also found that ESCCAL-1 is closely related to tumor stage and patient survival, further supporting the view that ESCCAL-1 is a potential biomarker of ESCA. In addition, we noted that this lncRNA had a special relationship with the gender and race of ESCA patients. Its expression level in male tumor samples was higher than that in female tumor samples, and its expression level in

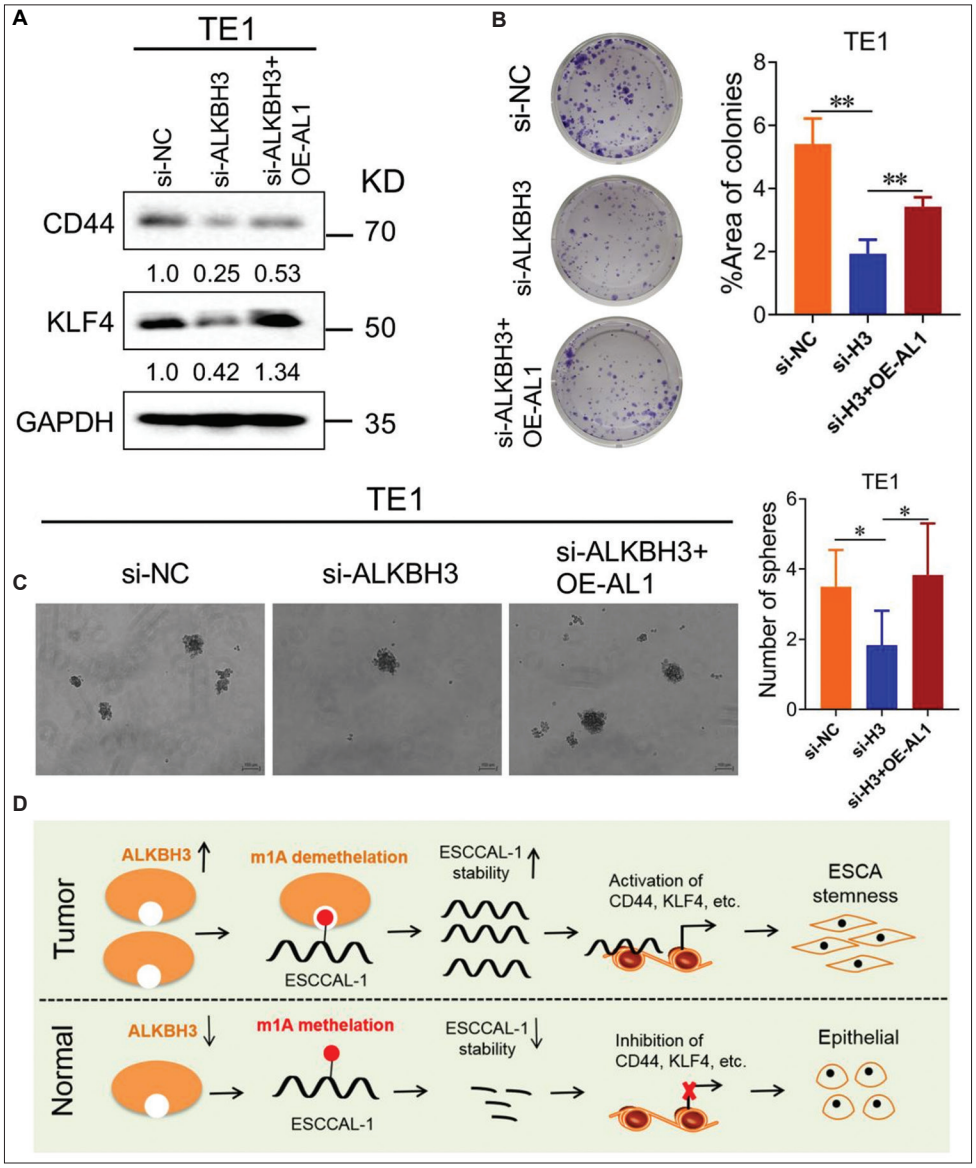

Figure 5. The role of ALKBH3/ESCCAL-1 axis in regulating ESCA stemness. (A) Western blot was applied to examine the protein levels of CD44 and KLF4 in ESCA cells following ALKBH3 silence alone or combined with ESCCAL-1 overexpression, $n = 3$. (B) A colony formation experiment was carried out to observe the role of the ALKBH3/ESCCAL-1 axis in ESCA growth, $n = 3$. $^{**}P < 0.01$. TE1 cells were transfected with si-ALKBH3 or si-ALKBH3 + OE-AL1. (C) Tumor sphere formation assay depicted that overexpression of ESCCAL-1 rescued the effects of ALKBH3 silence on ESCA stemness. $^{*}P < 0.05$. Scale bar = 100 μm. TE1 cells were transfected with si-ALKBH3 or si-ALKBH3 + OE-AL1. (D) A mechanistic model of ALKBH3-mediated ESCCAL-1 in ESCA stemness, $n = 3$.
ESCCAL-1: Esophageal squamous cell carcinoma associated long non-coding RNA 1; ESCA: Esophageal cancer.

Asian tumor samples was significantly higher than that in Caucasian patients. According to Global Cancer Statistics, the incidence of male ESCA patients is two to three folds higher than that of female patients, and the Asian region shows a higher incidence of ESCA compared with Western countries and regions[1]. Therefore, we speculate that ESCCAL-1 might be one of the key factors leading to the male ESCA and Asian ESCA. Androgen receptor signaling is widely considered a risk factor for male genitourinary tumors, such as prostate cancer[26,27]. Moreover, the activation of this signal can be regulated by upstream lncRNAs[26,27]. A recent study has found that the androgen receptor functions as an oncogenic promoter in ESCA[28]. Therefore, ESCCAL-1 might contribute to the difference in ESCA incidence between males and females by regulating the androgen receptor signaling. However, the relationship between ESCCAL-1 and the gender or ethnicity of ESCA patients needs to be further confirmed.

Recent studies have reported that the demethylase ALKBH3 can alter the post-transcriptional level of oncogenic mRNAs and tRNAs by erasing m^1A modification and thus participate in tumor progression[14,15,29,30]. However, whether lncRNAs can also be regulated by m^1A modification is unknown. This study revealed that ALKBH3 caused ESCCAL-1 overexpression in ESCA through m^1A demethylation modification. Significantly, ALKBH3 is involved in the stemness maintenance of ESCA in an ESCCAL-1-dependent manner. This is the first report on the biological role of lncRNA m^1A modification in ESCA, expanding our understanding on the role of lncRNA post-transcriptional modification in the development of ESCA.

5. Conclusions

This study further explored the expression of ESCCAL-1 in ESCA and its relationship with the clinical parameters of patients. High expression of ESCCAL-1 is necessary to maintain the stemness status of ESCA. ALKBH3 promoted the overexpression of ESCCAL-1 in an m^1A-dependent manner. These findings provide a new strategy for clinically targeting the ALKBH3/ESCCAL-1 axis against ESCA.

Acknowledgments

None.

Funding

This study was supported by the Medical Science and Technology Project of Henan Province (LHGJ20200765, SB201903032, SBGJ202003053) and the Key Project of Higher Education in Henan Province (20A310018).

Conflict of interest

The authors declare that there are no competing interests.

Author contributions

Conceptualization: Wei Cao
Formal analysis: Yuanbo Cui
Investigation: Yuanbo Cui, Yanan Lou, Pengju Lv
Writing – original draft: Yuanbo Cui
Writing – review & editing: Wei Cao

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The data are available from the corresponding author upon reasonable request.

References

1. Sung H, Ferlay J, Siegel RL, *et al*., 2021, Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*, 71(3): 209–249.

   https://doi.org/10.3322/caac.21660

2. Bray F, Ferlay J, Soerjomataram I, *et al*., 2018, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*, 68(6): 394–424.

   https://doi.org/10.3322/caac.21492

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

   https://doi.org/10.1038/nrdp.2017.48

4. Song Y, Li L, Ou Y, *et al*., 2014, Identification of genomic alterations in oesophageal squamous cell cancer. *Nature*, 509(7498): 91–95.

   https://doi.org/10.1038/nature13176

5. Cui Y, Chen H, Xi R, *et al*., 2020, Whole-genome sequencing of 508 patients identifies key molecular features associated with poor prognosis in esophageal squamous cell carcinoma. *Cell Res*, 30(10): 902–913.

   https://doi.org/10.1038/s41422-020-0333-6

6. Xi Y, Lin Y, Guo W, *et al*., 2022, Multi-omic characterization of genome-wide abnormal DNA methylation reveals diagnostic and prognostic markers for esophageal squamous-cell carcinoma. *Signal Transduct Target Ther*, 7(1): 53.

   https://doi.org/10.1038/s41392-022-00873-8

7. Slack FJ, Chinnaiyan AM, 2019, The role of non-coding RNAs in oncology. *Cell*, 179(5): 1033–1055.

   https://doi.org/10.1016/j.cell.2019.10.017

8. Cao W, Lee H, Wu W, *et al*., 2020, Multi-faceted epigenetic dysregulation of gene expression promotes esophageal squamous cell carcinoma. *Nat Commun*, 11(1): 3675.

   https://doi.org/10.1038/s41467-020-17227-z

9. Liu J, Mayekar MK, Wu W, *et al*., 2020, Long non-coding RNA ESCCAL-1 promotes esophageal squamous cell carcinoma by down regulating the negative regulator of APOBEC3G. *Cancer Lett*, 493: 217–227.

   https://doi.org/10.1016/j.canlet.2020.09.001

10. Cui Y, Yan M, Wu W, *et al*., 2022, ESCCAL-1 promotes cell-cycle progression by interacting with and stabilizing galectin-1 in esophageal squamous cell carcinoma. *NPJ Precis Oncol*, 6(1): 12.

    https://doi.org/10.1038/s41698-022-00255-x

11. Jin H, Huo C, Zhou T, *et al*., 2022, m^1A RNA modification in gene expression regulation. *Genes (Basel)*, 13(5): 910.

https://doi.org/10.3390/genes13050910

12. Chen Z, Qi M, Shen B, *et al.*, 2019, Transfer RNA demethylase ALKBH3 promotes cancer progression via induction of tRNA-derived small RNAs. *Nucleic Acids Res*, 47(5): 2533–2545.

https://doi.org/10.1093/nar/gky1250

13. Wang Y, Wang J, Li X, *et al.*, 2021, N1-methyladenosine methylation in tRNA drives liver tumourigenesis by regulating cholesterol metabolism. *Nat Commun*, 12(1): 6314.

https://doi.org/10.1038/s41467-021-26718-6

14. Wu Y, Chen Z, Xie G, *et al.*, 2022, RNA m^{1}A methylation regulates glycolysis of cancer cells through modulating ATP5D. *Proc Natl Acad Sci U S A*, 119(28): e2119038119.

https://doi.org/10.1073/pnas.2119038119

15. Lu Q, Wang H, Lei X, *et al.*, 2022, LncRNA ALKBH3-AS1 enhances ALKBH3 mRNA stability to promote hepatocellular carcinoma cell proliferation and invasion. *J Cell Mol Med*, 26(20): 5292–5302.

https://doi.org/10.1111/jcmm.17558

16. Nallasamy P, Nimmakayala RK, Parte S, *et al.*, 2022, Tumor microenvironment enriches the stemness features: The architectural event of therapy resistance and metastasis. *Mol Cancer*, 21(1): 225.

https://doi.org/10.1186/s12943-022-01682-x

17. Chaudhary A, Raza SS, Haque R, 2023, Transcriptional factors targeting in cancer stem cells for tumor modulation. *Semin Cancer Biol*, 88: 123–137.

https://doi.org/10.1016/j.semcancer.2022.12.010

18. Wilkinson E, Cui YH, He YY, 2022, Roles of RNA modifications in diverse cellular functions. *Front Cell Dev Biol*, 10: 828683.

https://doi.org/10.3389/fcell.2022.828683

19. Wang Z, He J, Bach DH, *et al.*, 2022, Induction of m6A methylation in adipocyte exosomal LncRNAs mediates myeloma drug resistance. *J Exp Clin Cancer Res*, 41(1): 4.

https://doi.org/10.1186/s13046-021-02209-w

20. Cui L, Ma R, Cai J, *et al.*, 2022, RNA modifications: Importance in immune cell biology and related diseases. *Signal Transduct Target Ther*, 7(1): 334.

https://doi.org/10.1038/s41392-022-01175-9

21. Li J, Zhang H, Wang H, 2022, N1-methyladenosine modification in cancer biology: Current status and future perspectives. *Comput Struct Biotechnol J*, 20: 6578––6585.

https://doi.org/10.1016/j.csbj.2022.11.045

22. Paul R, Dorsey JF, Fan Y, 2022, Cell plasticity, senescence, and quiescence in cancer stem cells: Biological and therapeutic implications. *Pharmacol Ther*, 231: 107985.

https://doi.org/10.1016/j.pharmthera.2021.107985

23. Du L, Cheng Q, Zheng H, *et al.*, 2022, Targeting stemness of cancer stem cells to fight colorectal cancers. *Semin Cancer Biol*, 82: 150–161.

https://doi.org/10.1016/j.semcancer.2021.02.012

24. Liu C, Zhang Y, Gao J, *et al.*, 2022, A highly potent small-molecule antagonist of exportin-1 selectively eliminates CD44+CD24-enriched breast cancer stem-like cells. *Drug Resist Updat*, 66: 100903.

https://doi.org/10.1016/j.drup.2022.100903

25. Ervin EH, French R, Chang CH, *et al.*, 2022, Inside the stemness engine: Mechanistic links between deregulated transcription factors and stemness in cancer. *Semin Cancer Biol*, 87: 48–83.

https://doi.org/10.1016/j.semcancer.2022.11.001

26. Taheri M, Khoshbakht T, Jamali E, *et al.*, 2021, Interaction between non-coding RNAs and androgen receptor with an especial focus on prostate cancer. *Cells*, 10(11): 3198.

https://doi.org/10.3390/cells10113198

27. Tortora F, Calin GA, Cimmino A, 2021, Effects of long non-coding RNAs on androgen signaling pathways in genitourinary malignancies. *Mol Cell Endocrinol*, 526: 111197.

https://doi.org/10.1016/j.mce.2021.111197

28. Huang F, Chen H, Zhu X, *et al.*, 2021, The oncogenomic function of androgen receptor in esophageal squamous cell carcinoma is directed by GATA3. *Cell Res*, 31(3): 362–365.

https://doi.org/10.1038/s41422-020-00428-y

29. Zhao Y, Zhao Q, Kaboli PJ, *et al.*, 2019, m^{1}A regulated genes modulate PI3K/AKT/mTOR and ErbB pathways in gastrointestinal cancer. *Transl Oncol*, 12(10): 1323–1333.

https://doi.org/10.1016/j.tranon.2019.06.007

30. Woo HH, Chambers SK, 2019, Human ALKBH3-induced m^{1}A demethylation increases the CSF-1 mRNA stability in breast and ovarian cancer cells. *Biochim Biophys Acta Gene Regul Mech*, 1862(1): 35–46.

https://doi.org/10.1016/j.bbagrm.2018.10.008