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Long Non-Coding RNA Taurine Upregulated Gene 1 (TUG1) Downregulation Constrains Cell Proliferation and Invasion through Regulating Cell Division Cycle 42 (CDC42) Expression Via MiR-498 in Esophageal Squamous Cell Carcinoma Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BC 1 **Zhifeng Wang***
AD 2 **Jingmei Liu***
DE 1 **Rong Wang**
CD 3 **Qinqin Wang**
BC 1 **Rong Liang**
EF 4 **Jinliang Tang**

1 Department of Digestive Endoscopy, Shanxi Provincial People's Hospital, Taiyuan, Shanxi, P.R. China
2 Department of Gastroenterology, Shanxi Cancer Hospital, Taiyuan, Shanxi, P.R. China
3 Department of Normal Surgical, Shanxi Provincial People's Hospital, Taiyuan, Shanxi, P.R. China
4 Department of Gastroenterology, Jincheng People's Hospital, Jincheng, Shanxi, P.R. China

* Zhifeng Wang and Jingmei Liu contribute to this work equally as co-first authors

Corresponding Author: Jinliang Tang, e-mail: vxfgdk@163.com

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Background: Esophageal squamous cell carcinoma (ESCC) is a malignant tumor of the gastrointestinal tract. Taurine upregulated gene 1 (TUG1), a long non-coding (lnc) RNA, also known as LIN00080 or TI-227H, was connected with the tumorigenesis of various diseases. Hence, we plumed the role and molecular mechanism of TUG1 in the progression of ESCC.


Material/Methods: Expression patterns of TUG1, microRNA-498 (miR-498), and cell division cycle 42 (CDC42) mRNA were assessed using quantitative real time polymerase chain reaction (qRT-PCR). The expression level of CDC42 protein was evaluated via western blot analysis. Cell proliferation and invasion were determined with Cell Counting Kit-8 (CCK-8) assay or Transwell assay. The relationship between miR-498 and TUG1 or CDC42 was predicted by on-line bioinformatics database LncBase Predicted v.2 or microT-CDS and confirmed through dual-luciferase reporter system or RNA immunoprecipitation assay (RIP).

Results: TUG1 and CDC42 were upregulated while miR-498 was strikingly decreased in ESCC tissues and cells ($P < 0.0001$). Besides, TUG1 suppression blocked the proliferation and invasion of ESCC cells ($P < 0.001$). Importantly, TUG1 decrease restrained CDC42 expression via binding to miR-498 in ESCC cells. Also, the suppressive impacts of TUG1 silencing on the proliferation and invasion of ESCC cells were mitigated by miR-498 reduction. Meanwhile, the repression of proliferation and invasion induced by miR-498 elevation was weakened by CDC42 overexpression.

Conclusions: Inhibition of TUG1 hampered cell proliferation and invasion by downregulating CDC42 via upregulating miR-498 in ESCC cells. Thus, TUG1 might be an underlying therapeutic target for ESCC.

MeSH Keywords: **Carcinoma, Pancreatic Ductal • Esophageal Diseases • Esophageal Neoplasms**

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Background

Esophageal cancer (ESCA), with a 5-year survival rate approximately 20% to 30%, is deemed to be one of the most aggressive gastrointestinal malignancies all over the world and the sixth leading cause of cancer death [1–3]. Results from the Gepia databases showed that TUG1 was upregulated in a range of tumors, including ESCA, implying that augmented TUG1 expression was implicated in the development of ESCA (Supplementary Figure 1). And OncoPrint databases showed that elevated TUG1 expression possessed a worse overall survival rate, implying that TUG1 overexpression was related to the poor prognosis of ESCA (Supplementary Figure 1). ESCA principally includes esophageal adenocarcinoma and esophageal squamous cell carcinoma (ESCC), and ESCC accounts for about 90% of ESCA cases [4]. Up to now, although patients with ESCC can receive comprehensive treatment through surgery, biotherapy, chemotherapy, and radiotherapy, the mortality rate of ESCC is still relatively high, chiefly due to local invasion and distant metastasis [5,6]. Hence, it is important to consider the molecular mechanism of the progression of ESCC for improving therapies and the survival rate of ESCC patients.

Long non-coding RNAs (lncRNAs) are a kind of non-coding RNA that plays a role in the abnormal regulation of multifarious cancers [7–9]. lncRNAs, as competitive endogenous RNAs, participated in the regulation of miRNA target through binding to microRNAs (miRNAs) in diverse cancers, including ESCC [10,11]. For example, lncRNA PVT1 promoted cell migration and invasion and repressed cell apoptosis in ESCC through FSCN1 via miR-145 [12]. Furthermore, lncRNA CYTOR accelerated the tumorigenesis of ESCC via miR-153-3p/FYN axis [13]. Taurine upregulated gene 1 (TUG1), a long non-coding RNA named for its upregulation with the addition of taurine, and is also known as LINC00080 or TI-227H, has attracted much attention as a potential oncogene [14]. Substantial evidence confirmed that the normal expression of TUG1 was connected with the occurrence of diverse cancers such as nasopharyngeal carcinoma [15], prostate cancer [16], and ovarian cancer [17]. Thus far, the precise pathogenesis of TUG1 in ESCC remains to be further elucidation.

MiRNAs, another type of non-coding RNA (approximately 19–25 nucleotides), are concerned with a variety of cellular biological processes, such as cell proliferation, invasion differentiation, metabolism metastasis, and apoptosis. For instance, miR-216a-5p [18] and miR-488-3p [19] impeded the progression of ESCC via suppressing cell proliferation and inducing cell apoptosis. Recently, enhanced research indicated that miR-498 participated in the occurrence and progression of lung cancer [20,21], ovarian cancer [22], breast cancer [23], gastric cancer [24], as well as other cancers. Islam et al. reported that abnormal expression of miR-498 might affect the

clinicopathological parameters of ESCC and management for ESCC patients [25]. However, it is unclear whether miR-498 can be modulated by TUG1 in ESCC.

Cell division cycle 42 (CDC42), which is connected with cell division, invasion, migration and invadopodia formation, is a member of the Rho GTPase family [26]. CDC42 was revealed to be involved in the development of diverse cancers. It was reported that the CDC42/PAK1 pathway was activated by lncRNA H19 via miR-15b to accelerate the invasion, proliferation, and migration of hepatocellular cancer [27]. Also, lncRNA MALAT1 mediated cell invasion and migration via the miR-1/CDC42 axis in breast cancer cells [28]. Previous research stated that CDC42 was concerned with the tumorigenesis and progression of ESCC [29–31]. For instance, miR-195 could impede cell invasion and proliferation in ESCC cells by reducing CDC42 expression [30]. Currently, whether the development of ESCC is regulated by the TUG1/miR-498/CDC42 network has not been reported.

Hence, we evaluated the expression of TUG1 in ESCC tissues and cells. And the biological function of TUG1 and the potential regulation network of TUG1/miR-498/CDC42 in ESCC cells were investigated.

Material and Methods

ESCC patient specimens

There were 27 paired ESCC tissues and adjoining normal esophageal tissues harvested from Shanxi Provincial People's Hospital. The inclusion criteria were exhibited as follows: ESCC, resection, no other malignancies and no radiotherapy or chemotherapy before surgery. All participants in the study signed informed consents. Each ESCC case was determined by experienced pathologists. All tissue samples were frozen stored at -80°C . Moreover, the Ethics Committee of Shanxi Provincial People's Hospital approved this study.

Cell culture and transfection

KYSE30 and TE-1 cells were procured from the Cell Bank of the Chinese Academy of Medical Science. The normal esophageal epithelial cells Het-1A were procured from American Tissue Culture Collection (Manassas, VA, USA). RPMI 1640 (Gibco, Waltham, MA, USA) replenished with fetal bovine serum (FBS, 10%) (Hyclone, Logan, UT, USA) and penicillin/streptomycin (1%, Invitrogen, Carlsbad, CA, USA) were employed to culture the aforementioned cells. The cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C .

Small interference RNA (siRNA), negative control (si-NC), and siRNA targeting TUG1 (si-TUG1) were obtained from Genepharma (Shanghai, China). The pcDNA-CDC42 overexpression vector (CDC42) was acquired by inserting the full sequence of CDC42 into pcDNA (Genepharma). The miRNA mimic or inhibitor targeting miR-498 (miR-498 or in-miR-498) and their negative control (miR-NC or in-miR-NC) were obtained from RIBOBIO (Guangzhou, China). Also, Lipofectamine 2000 reagent from Invitrogen was utilized for the transfection of the oligonucleotides or plasmids into ESCC cells based on the instructions of the manufacturer. The sequence of si-TUG1 was displayed as the following: si-TUG1#1 (3'-GGGAUUAUAGCCAGAGAACAUAUCUA-5'), si-TUG1#2 (3'-CAGCUGUUACCAUUAACUUCUUA-5') and si-TUG1#3 (3'-AUCGAGUGCCGAGAAAAGU-5').

Quantitative real time polymerase chain reaction (qRT-PCR)

TRIzol reagent from Invitrogen was utilized for the harvest of total RNA of ESCC tissues and cells. Also, GoScript Reverse Transcription System from Promega (Madison, WI, USA) or All-in-One™ miRNA First strand cDNA Synthesis Kit (GeneCopoeia, Rockville, MD, USA) was applied to reverse transcription RNA into cDNA of TUG1, CDC42, and miR-498. Then qRT-PCR was conducted for the assessment of the levels of TUG1, miR-498, and CDC42 by SYBR® Premix DimerEraser Kit (TaKaRa, Dalian, China). The special primers for TUG1, CDC42, and GAPDH were as follows:

TUG1, 5'-GACAGAGGCGACAGGAACGACG-3'(F) and 5'-CACCATGCAACATCGAACCG-3'(R);
CDC42, 5'-CCATCGGAATATGTACCGACTG-3'(F) and 5'-CTCAGCGGTCGTAATCTGTCA-3'(R);
miR-498, 5'-TTTCAAGCCAGGGGCGGTTTTTC-3'(F) and 5'-GCTTCAAGCTCTGGAGGTGCTTTTC-3'(R),
GAPDH (glyceraldehyde 3-phosphate dehydrogenase), 5'-CGGAGTCAACGGATTGGTCTGAT-3'(F) and 5'-AGCCTTCTCCATG GTGGTGAAGAC-3'(R),
U6 snRNA, 5'-GACAGAGGCGACAGGAACGACG-3'(F) and 5'-CACCATGCAACATCGAACCG-3'(R).

The expression of TUG1, CDC42, and miR-498 was computed by the $2^{-\Delta\Delta CT}$ method with normalization to U6 snRNA or GAPDH, respectively.

Western blot

The total protein of ESCC tissues and cells was isolated via RIPA buffer (Beyotime, Beijing, China). The isolated total protein (50 µg) was parted through using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis, 15%). After that, the parted proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland) by electrophoresis. Following this, the PVDF membranes were blocked in phosphate buffered saline (PBS) with 5%

non-fat milk. After blocking for 2 hours, the PVDF membranes were incubated with primary antibodies anti-CDC42 (11A11, 1: 1000) and anti-GAPDH (D16H11, 1: 1000), respectively. The PVDF membranes were incubated with the second antibody goat anti-rabbit IgG (Thermo Scientific, Rockford, IL, USA). An enhanced chemiluminescence system (Pierce, Rockford, IL, USA) and ImageJ software (NIH, Bethesda, MD, USA) was employed to assess the protein bands. The quantitative indicators of protein were based on the gray value of the protein band. Primary antibodies utilized in the research were bought from Cell Signaling Technology (Danvers, MA, USA).

Cell Counting Kit-8 (CCK-8) assay

CCK-8 reagent (Beyotime) was applied to assess the proliferation capacity of transfected ESCC cells. In brief, transfected ESCC cells (3×10^3) were seeded in 96-well plates and maintained for 24 hours, 48 hours, or 72 hours. The cells were irradiated after adhering to the wall. Afterward, CCK-8 reagent was added to each well at the appointed time. After that, the Microplate Absorbance Reader (Thermo Fisher Scientific) was employed for the evaluation of the color reaction at 450 nm.

Transwell invasion assay

The invasion capacity of transfected ESCC cells was assessed by Transwell plates (8 µm pore size, Millipore) with Matrigel (BD Bioscience, Mountain View, CA, USA). Briefly, the RPMI 1640 medium with free FBS in the upper chamber was replenished with control or treated cells (2×10^4). Meanwhile, the lower well of the Transwell chamber was replenished with the RPMI 1640 medium with 10% FBS. Following this, crystal violet (0.1%) was applied to stain the invaded cells under the bottom of the Transwell membrane. In the end, the invaded cells were measured by an inverted microscope.

Dual-luciferase reporter assay

The online bioinformatics database LncBase Predicted v.2 or microT-CDS was employed to predict the binding sites between miR-498 and TUG1 or CDC42. The luciferase reporter vectors of wild-type TUG1 (TUG1 WT) and CDC42 3'-UTR-WT (CDC42 3'-UTR-WT), as well as mutant TUG1 (TUG1 MUT) and CDC42 3'-UTR-MUT (CDC42 3'-UTR-MUT), were established utilizing the pGL3 control luciferase reporter vector (Promega). Then, the luciferase reporter vectors and miR-498 or miR-NC were co-transfected into KYSE30 and TE-1 cells using Lipofectamine 2000 reagent. After transfection for 48 hours, the luciferase activities of luciferase reporter vectors were evaluated with the dual-luciferase reporter system (Promega).

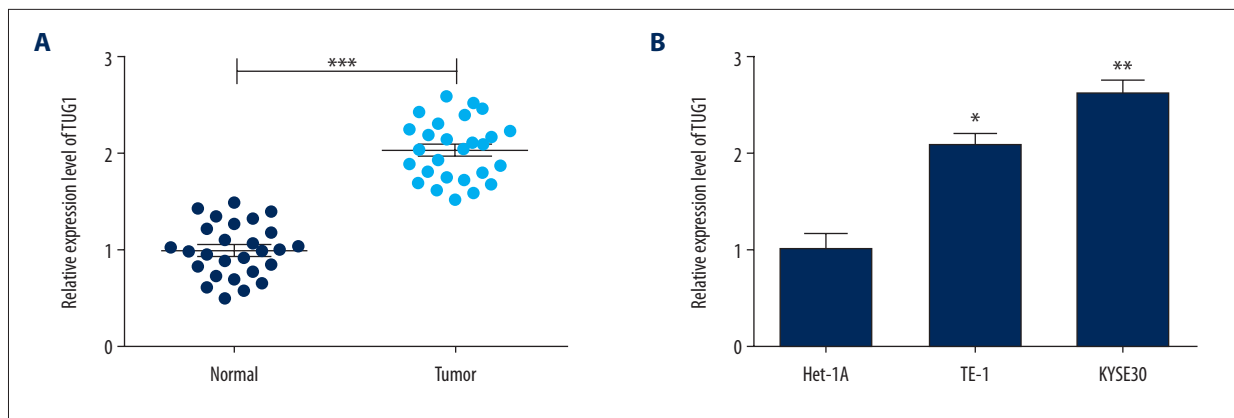


Figure 1. Expression levels of TUG1 in ESCC tissues and cells. **(A)** The expression of TUG1 in ESCC tissues and adjoining normal esophageal tissues were analyzed using qRT-PCR. *** $P < 0.001$ **(B)** qRT-PCR was applied to analyze TUG1 expression in ESCC and Het-1A cells. * $P < 0.05$ and ** $P < 0.01$. GAPDH was deemed as the endogenous for TUG1. Data are shown as a mean \pm SD from 3 independent experiments. Student's *t* test assessed the significance of the differences. qRT-PCR – quantitative real time polymerase chain reaction; TUG1 – taurine upregulated gene 1; ESCC – esophageal squamous cell carcinoma; qRT-PCR – quantitative real-time polymerase chain reaction; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; SD – standard deviation.

RNA immunoprecipitation (RIP) assay

EZ-Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) was employed to confirm the interaction between miR-498 and TUG1. In short, the miR-498 or miR-NC was transfected into KYSE30 and TE-1 cells. Then, transfected cells were lysed in RIP buffer containing magnetic beads conjugated that are coupled to Ago2 or IgG antibody (Millipore). After the separation of TUG1, the enrichment level of TUG1 was detected by qRT-PCR.

Statistical analysis

All the experiments were repeated more than 3 times. GraphPad Prism 6.00 (GraphPad, La Jolla, CA, USA) and SPSS 16.0 (SPSS, Chicago, IL, USA) were utilized for statistical analysis. Also, Student's *t*-test or one-way ANOVA was applied to evaluate the differences between 2 or among more groups. The difference was statistically significant when *P* value was less than 0.05. Data on repeated experiments were presented as mean \pm standard deviation (SD).

Results

TUG1 was augmented in ESCC tissues and cells

At the outset, we assessed the expression pattern of TUG1 in ESCC tissues and cells (KYSE30 and TE-1) via qRT-PCR to better understand the role of TUG1 in ESCC. Comparing to that in the adjoining normal esophageal tissues and Het-1A cells, TUG1 was conspicuously upregulated in ESCC tissues

and cells ($P < 0.0001$) (Figure 1A, 1B). Furthermore, high TUG1 expression was correlated with tumor size ($P = 0.032$), TNM stage ($P < 0.001$), and lymph node status ($P < 0.001$) (Table 1). Therefore, enhanced TUG1 expression might be related to the development of ESCC.

TUG1 silencing impeded cell proliferation and invasion in ESCC cells

In view of the aforementioned results, the impacts of TUG1 reduction on cell proliferation and invasion in ESCC cells were further investigated. First, the si-NC or 3 specific si-RNAs for TUG1 (si-TUG1#1, si-TUG1#2, si-TUG1#3) was transfected into ESCC cells for silencing of the expression of TUG1. The results of the qRT-PCR exhibited that the expression of TUG1 was drastically declined in KYSE30 and TE-1 cells transfected with 3 specific si-RNAs in comparison to the si-NC group, and the si-TUG1#3 was selected for subsequent studies (Figure 2A, 2B). Subsequently, CCK-8 assays manifested that TUG1 downregulation effectively impeded cell proliferation both in KYSE30 and TE-1 cells (Figure 2C, 2D). Next, Transwell assays demonstrated that TUG1 knockdown retarded the invasion of KYSE30 and TE-1 (Figure 2E). Therefore, these results revealed that inhibited TUG1 expression impeded the proliferation and invasion of ESCC cells.

TUG1 negatively regulated miR-498 expression in ESCC cells

Given the biological role of TUG1 in ESCC, we further explored its potential molecular mechanism. Online bioinformatics database LncBase Predicted v.2 was utilized to predict potential

Table 1. Analysis of the correlation between expression of TUG1 in esophageal squamous cell carcinoma and its clinicopathological parameters.

Variable	Patients, n	TUG1 expression		P-value
		Low	High	
Age, years	27	12	15	0.758
<60	10	4	6	
≥60	17	8	9	
Sex				0.325
Male	15	7	8	
Female	12	5	7	
Tumor size				0.032
<2	11	7	4	
≥2	16	5	11	
TNM stage				<0.001
<III stage	9	6	3	
≥III stage	18	6	12	
Lymph node status				<0.001
Yes	17	5	12	
No	10	7	3	

$P < 0.05$ was considered to be statistically significant. TUG1 – taurine upregulated gene 1.

miRNAs that might interact with TUG1. As shown, our analysis showed that miR-498 possessed possible binding sites for TUG1 (Figure 3A). Accordingly, the luciferase reporter vectors TUG1-MUT and TUG1-WT (carrying miR-498 binding sites) were constructed and co-transfected with miR-NC or miR-498 into KYSE30 and TE-1 cells. The results of dual-luciferase reporter assay showed that enhanced miR-498 expression prominently impeded the luciferase activity of TUG1-WT relative to the control group in KYSE30 and TE-1 cells (Figure 3B, 3C), whereas the luciferase activity of TUG1-MUT was no evident difference ($P < 0.001$). Then, we implemented the RIP assay to verify whether TUG1 can interact with miR-498. The results showed that TUG1 was apparently elevated in Ago2 immunoprecipitates in KYSE30 and TE-1 cells when compared with the control group, indicating TUG1 could directly couple with miR-498 by an Ago2-dependent manner ($P < 0.0001$) (Figure 3D). Moreover, qRT-PCR analysis implied that a conspicuous elevation of miR-498 was discovered in ESCC tissues ($P < 0.0001$) and KYSE30 and TE-1 cells ($P < 0.001$) (Figure 3E, 3F). Furthermore, miR-498 was dramatically upregulated in TUG1-inhibited KYSE30 and TE-1 cells (Figure 3G). Therefore, these findings demonstrated that miR-498 was negatively regulated by TUG1 in ESCC cells.

Inhibition of miR-498 reversed the impacts of TUG1 depletion on cell proliferation and invasion in ESCC cells

To further confirm whether TUG1 worked in ESCC via miR-498, we first assessed the expression of miR-498 in KYSE30 and TE-1 cells transfected with si-TUG1, si-TUG1+in-miR-498, si-NC, or si-TUG1+in-NC. QRT-PCR revealed that miR-498 was remarkably enhanced in TUG1-decreased KYSE30 and TE-1 cells than that in the si-NC group, while inhibition of miR-498 attenuated TUG1 knockdown-induced miR-498 expression (Figure 4A). Next, results from CCK-8 and Transwell assay manifested that transfection of si-TUG1 dramatically impeded the proliferation and invasion in KYSE30 and TE-1 cells, while this inhibition was attenuated by inhibiting of miR-498 (Figures 4B–4D). Conclusively, these results proved that inhibition of miR-498 could attenuate TUG1 downregulation-mediated the repression of proliferation and invasion of ESCC cells.

CDC42 served as a target for miR-498 and was positively regulated by TUG1 in ESCC cells

According to the aforementioned results, we speculated that TUG1 might exert its competitive endogenous RNA (ceRNA) function by regulating the miR-498 target gene. To verify this speculation, we utilized microT-CDS to predict potential target

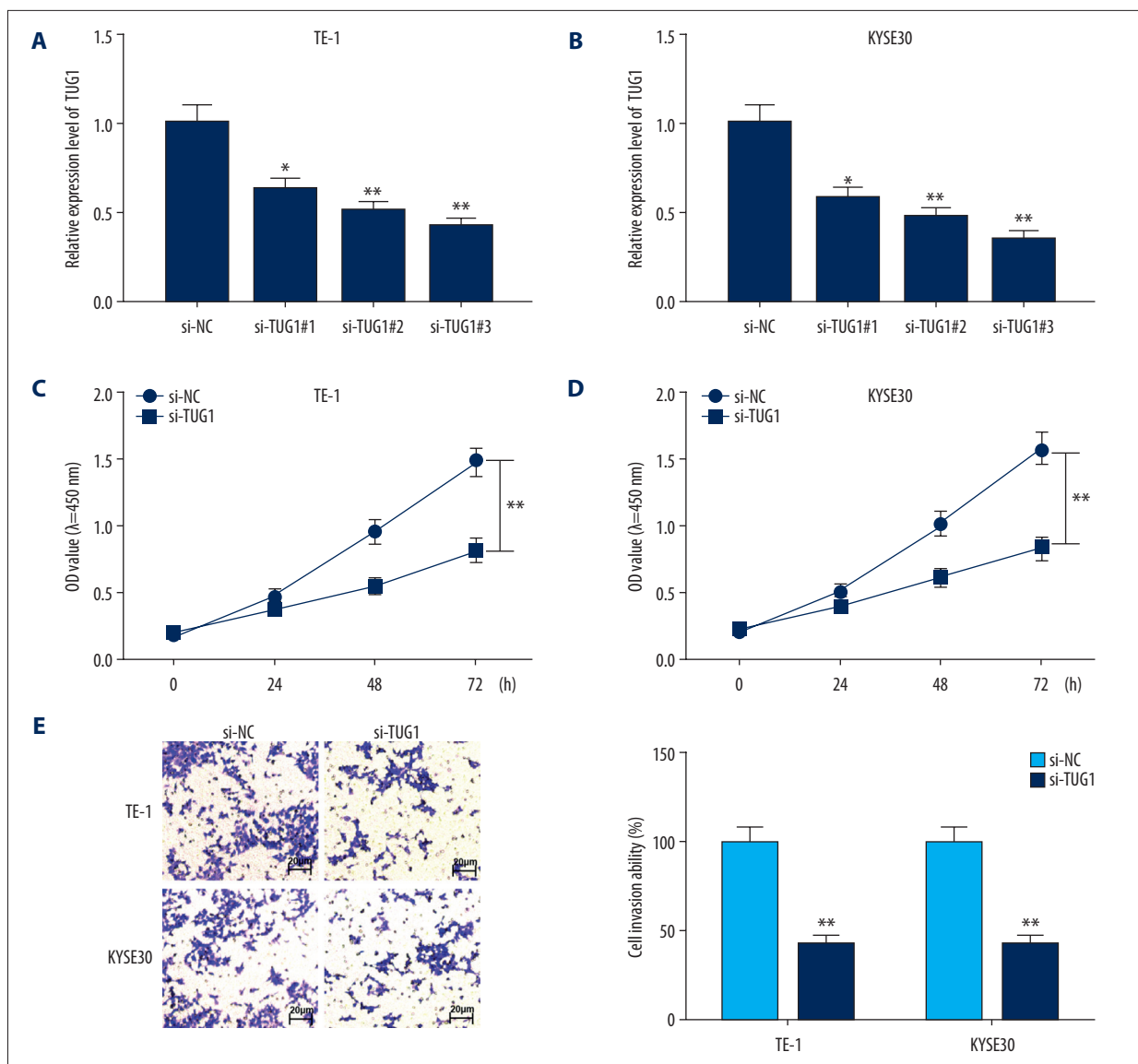


Figure 2. TUG1 silencing suppressed the proliferation and invasion of ESCC cells. (A, B) Knockdown efficiency of TUG1 was proved by qRT-PCR in TE-1 and KYSE30 cells. TE-1 and KYSE30 cells were transfected with 3 si-RNAs (si#TUG1-1, si#TUG2, si#TUG3) or si-NC. GAPDH was used as the endogenous for TUG1. * $P < 0.05$ and ** $P < 0.01$. (C, D) CCK-8 assay was employed to analyze the role of TUG1 knockdown on the proliferation of TE-1 and KYSE30 cells. ** $P < 0.01$. (E) Transwell assay was employed to analyze the role of TUG1 downregulation on the invasion of TE-1 and KYSE30 cells. ** $P < 0.01$. Data are shown as a mean \pm SD from 3 independent experiments. Student's *t*-test was employed to analyze the significance of the differences. TUG1 – taurine upregulated gene 1; ESCC – esophageal squamous cell carcinoma; qRT-PCR – quantitative real-time polymerase chain reaction; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; CCK-8 – Cell Counting Kit-8; SD – standard deviation.

genes that might be bind with miR-498. The results showed that CDC42 3'-UTR containing the complementary sequence for miR-498 (Figure 5A). The dual-luciferase reporter assay manifested that miR-498 augmentation remarkably blocked the luciferase activity of CDC42 3'-UTR-WT in KYSE30 and TE-1 cells in comparison to the control group (Figure 5B, 5C). Moreover, qRT-PCR implied that CDC42 mRNA was robustly suppressed in ESCC tissues ($P < 0.0001$) and cells ($P < 0.001$) (Figure 5D, 5E).

These data of western blot analysis exhibited that the levels of CDC42 protein was declined in miR-498-increased KYSE30 and TE-1 cells (Figure 5F). Meanwhile, TUG1 knockdown also constrained the CDC42 protein expression in KYSE30 and TE-1 cells (Figure 5G). Conclusively, these data collectively manifested that CDC42 was positively regulated by TUG1 and was negatively regulated by miR-498 in ESCC cells.

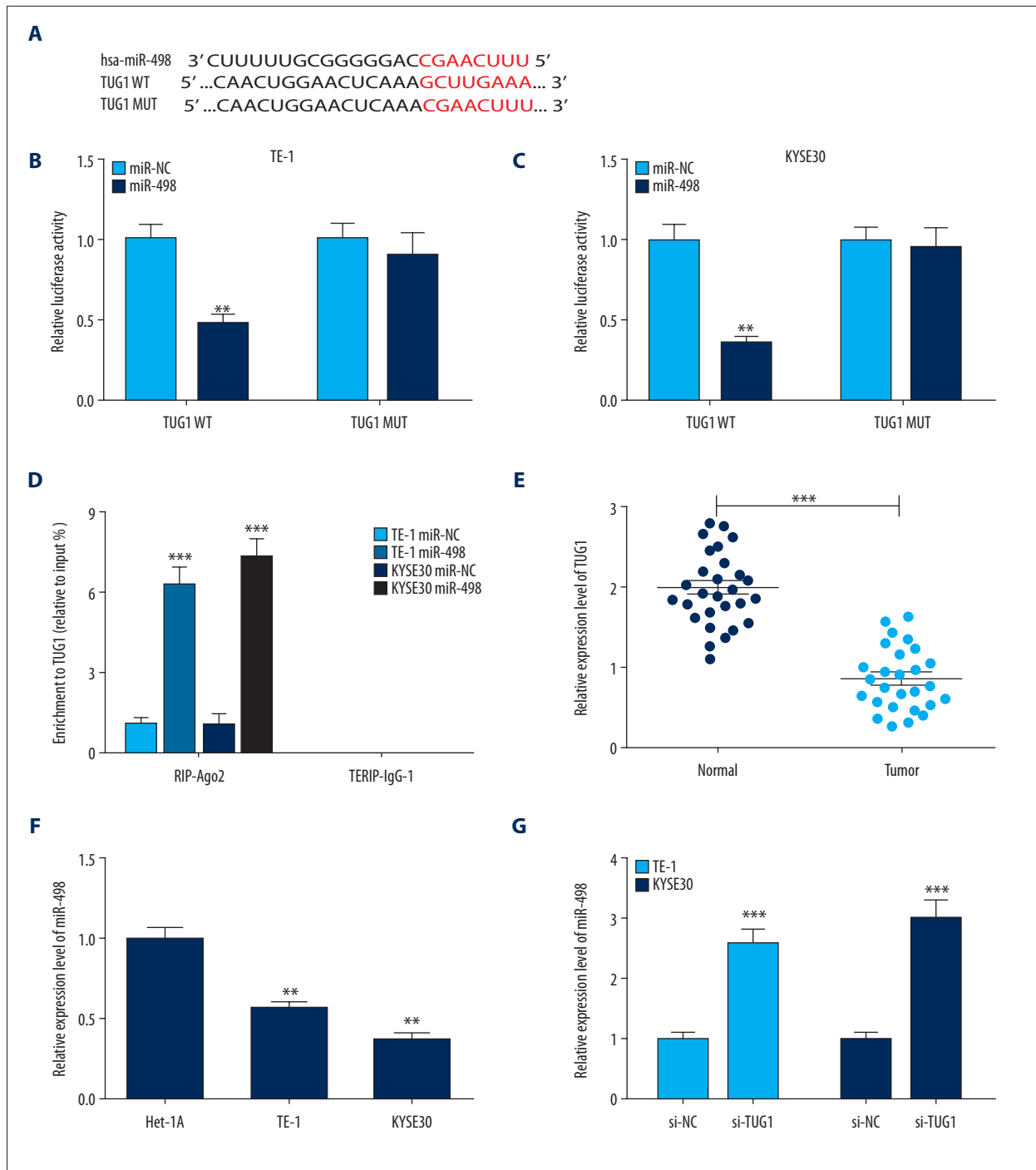


Figure 3. TUG1 interacted with miR-498 in ESCC cells. **(A)** The binding sites between miR-498 and TUG1 were predicted by LncBase Predicted v.2. **(B, C)** The luciferase activity of TUG1-MUT or TUG1-WT in TE-1 and KYSE30 cells transfected with miR-498 or TUG1-WT was assessed with dual-luciferase reporter assay. ** $P < 0.01$. **(D)** The interaction between miR-498 and TUG1 in TE-1 and KYSE30 cells was assessed through RIP assay. *** $P < 0.001$. **(E, F)** qRT-PCR analysis of miR-498 expression levels in ESCC tissues and cells. *** $P < 0.001$ and ** $P < 0.01$. **(G)** The effect of TUG1 knockdown on the expression of miR-498 in TE-1 and KYSE30 cells was analyzed via qRT-PCR. *** $P < 0.001$. U6 snRNA was used as the endogenous for miR-498. Data are shown as a mean \pm SD from 3 independent experiments. Student's *t*-test assessed the significance of the differences. TUG1 – taurine upregulated gene 1; ESCC – esophageal squamous cell carcinoma; MUT – mutant; WT – wild type; RIP – RNA immunoprecipitation; qRT-PCR – quantitative real time polymerase chain reaction; SD – standard deviation.

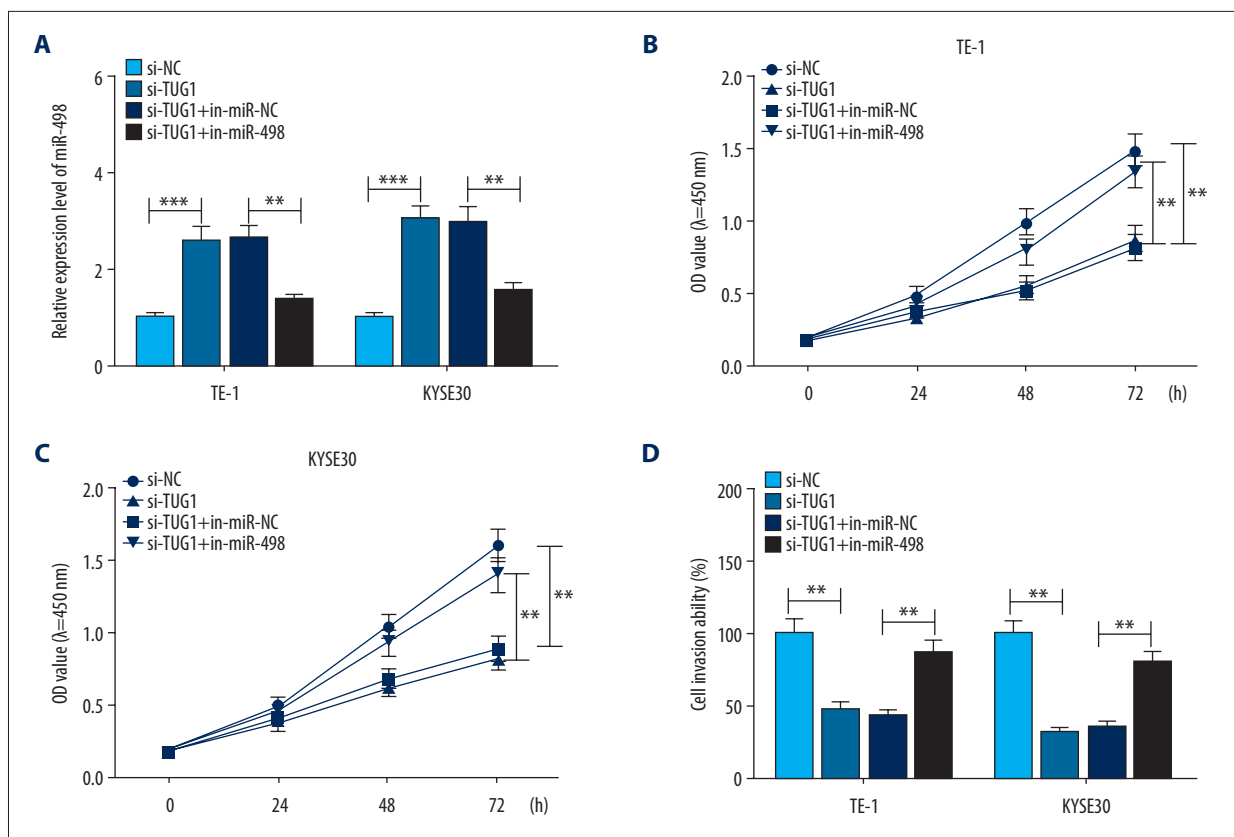


Figure 4. Inhibition of miR-498 attenuated the effects of TUG1 knockdown on ESCC cells. (A) qRT-PCR was executed for the detection of the expression level of miR-498 in both TE-1 and KYSE30 cells. *** $P < 0.001$ and ** $P < 0.01$. (A–D) TE-1 and KYSE30 cells were transfected with si-NC, si-TUG1, si-TUG1+in-miR-498, or si-TUG1+in-miR-NC, respectively. (B–D) CCK-8 or Transwell invasion assays analysis of the proliferation and invasion capacities of TE-1 and KYSE30 cells. ** $P < 0.01$. U6 snRNA was used as the endogenous for miR-498. Data are shown as a mean \pm SD from 3 independent experiments. Student's *t*-test assessed the significance of the differences. TUG1 – taurine upregulated gene 1; ESCC – esophageal squamous cell carcinoma; qRT-PCR – quantitative real time polymerase chain reaction; CCK-8 – Cell Counting Kit-8; SD – standard deviation.

CDC42 overexpression overturned miR-498 upregulation-mediated inhibition of proliferation and invasion of ESCC cells

Knowing that miR-498 targeted CDC42 in ESCC cells, we transfected miR-NC, miR-498, miR-498+pcDNA, or miR-498+CDC42 into KYSE30 and TE-1 cells to further verify whether miR-498 played its role in ESCC via CDC42. The result of western blot presented that augmented miR-498 expression impeded CDC42 protein expression in KYSE30 and TE-1 cells, whereas this suppression was recovered by CDC42 overexpression (Figure 6A). In addition, the result of CCK-8 and Transwell assay presented that reinforced miR-498 expression blocked the proliferation and invasion of ESCC cells, whereas these impacts were recovered by CDC42 overexpression (Figure 6B–6D). In summary, miR-498 played its function in ESCC cells through CDC42.

Knockdown of TUG1 downregulated CDC42 expression through binding to miR-498 in ESCC cells

Based on all the results presented, we further explored whether TUG1 regulated CDC42 expression via miR-498. Subsequently, we measured the expression levels of CDC42 protein and mRNA in KYSE30 and TE-1 cells transfected with si-NC, si-TUG1, si-TUG1+in-miR-NC, or si-TUG1+in-miR-498. We observed that reduced TUG1 expression markedly decreased the levels of CDC42 mRNA and protein in KYSE30 and TE-1 cells, whereas inhibition of miR-498 recovered CDC42 mRNA and protein expression levels (Figure 7A, 7B). Conclusively, these data showed that knockdown of TUG1 modulated CDC42 expression by sponging miR-498.

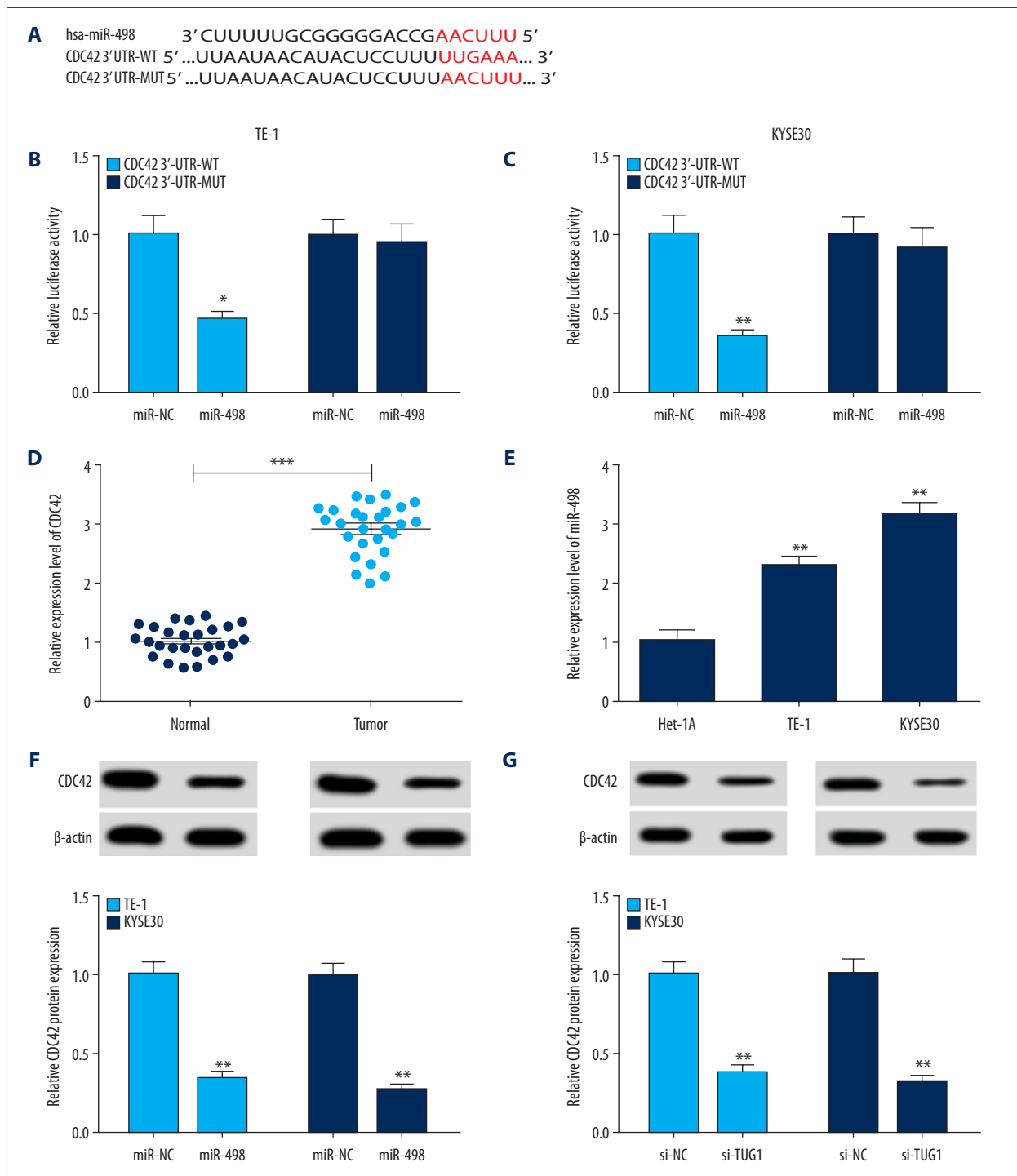


Figure 5. MiR-498 targeted CDC42 in ESCC cells. **(A)** The predicted binding sites of CDC42 in miR-498 by microT-CDS. **(B, C)** The luciferase activity of CDC42 3'URT-MUT or CDC42 3'URT-WT was estimated in TE-1 and KYSE30 cells transfected with miR-498 or miR-NC using dual-luciferase reporter assay. * $P < 0.05$ and ** $P < 0.01$. **(D, E)** qRT-PCR was utilized for the detection of the level of CDC42 mRNA in ESCC tissues and cells. *** $P < 0.001$ and ** $P < 0.01$. GAPDH was used as the endogenous for CDC42. **(E)** Western blot analysis of the role of miR-498 on CDC42 protein expression. ** $P < 0.01$. **(F)** Western blot analysis of the effect of knockdown of TUG1 on CDC42 protein expression. ** $P < 0.01$. Data are shown as a mean \pm SD from 3 independent experiments. Student's *t*-test assessed the significance of the differences. CDC42 – cell division cycle 42; ESCC – esophageal squamous cell carcinoma; MUT – mutant; WT – wild type; qRT-PCR – quantitative real time polymerase chain reaction; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; SD – standard deviation; TUG1 – taurine upregulated gene 1.

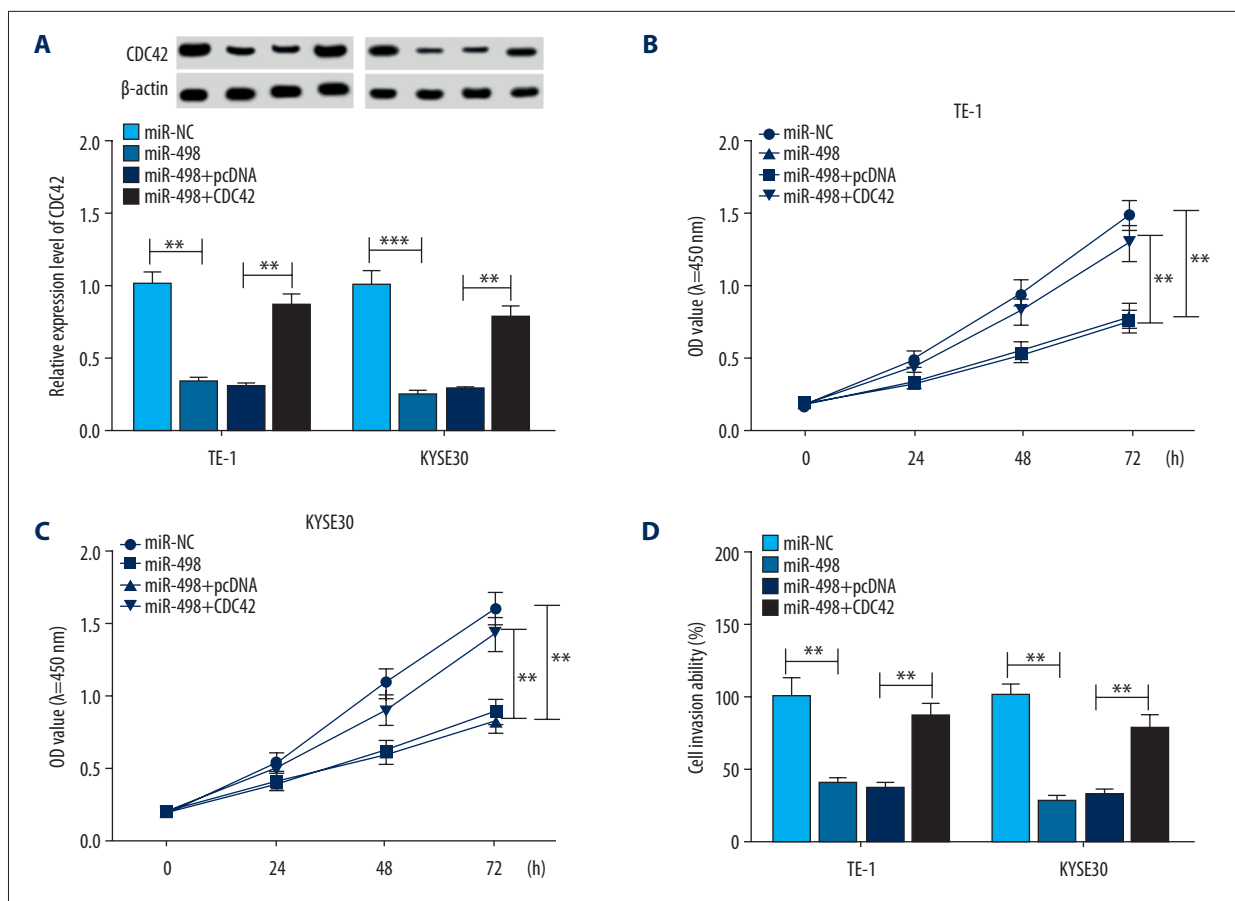


Figure 6. CDC42 overexpression recovered the effect of miR-498 on ESCC cells. (A) Western blot analysis of CDC42 protein expression level in TE-1 and KYSE30 cells. $** P < 0.01$. (A–D) The miR-NC, miR-498, miR-498+pcDNA, or miR-498+CDC42 TE-1 was transfected into KYSE30 cells, respectively. (B–D) CCK-8 or Transwell assays were carried out for the assessment of proliferation and invasion of TE-1 and KYSE30 cells. $** P < 0.01$. Data are shown as a mean \pm SD from 3 independent experiments. Student's *t*-test assessed the significance of the differences. CDC42 – cell division cycle 42; ESCC – esophageal squamous cell carcinoma; CCK-8 – Cell Counting Kit-8; SD – standard deviation.

Discussion

There is evidence that lncRNA TUG1 is abnormally expressed in ESCC, but its biological role and potential molecular mechanism in ESCC remain unclear [32,33]. Hence, the molecular mechanisms of TUG1 in ESCC need to be fully explored in order to develop an effective ESCC treatment regimen. As a consequence, we probed the role of TUG1 and the regulatory network of the TUG1/miR-498/CDC42 axis in ESCC cells.

Previous research has claimed that TUG1 was upregulated in ESCC tissues [32,33]. Jiang et al. stated that TUG1 was prominently augmented in ESCC tissues, and TUG1 upregulation was connected with chemotherapy resistance and poor prognosis of ESCC [32]. Xu et al. found that TUG1 was enhanced in cisplatin-resistance tissues and cells of ESCC and the poor prognosis of ESCC patients was associated with the upregulation of TUG1 [34]. Another report pointed out that reduced TUG1

expression restrained cell cycle, migration, and proliferation in ESCC cells [33]. The results of this study showed that a prominent reinforcement of TUG1 was discovered in ESCC tissues and cells. Also, TUG1 downregulation repressed cell proliferation and invasion in ESCC cells. Our results were consistent with the aforementioned studies, indicating that TUG1 exerted a carcinogenic role in ESCC.

Additional studies have pointed out that TUG1 could act as a sponge for multiple miRNAs and regulate the level of miRNA targets [35]. For instance, TUG1 accelerated the progression of prostate cancer through acting as a sponge for miR-26a [16]. In the present study, we uncovered that miR-498 served as a target for TUG1. Also, miR-498 was downregulated in ESCC tissues and cells. Besides, miR-498 inhibition attenuated the prohibitive impacts of TUG1 downregulation on proliferation and invasion of ESCC cells. Furthermore, increased studies had shown that miR-498 frequently decreased in other

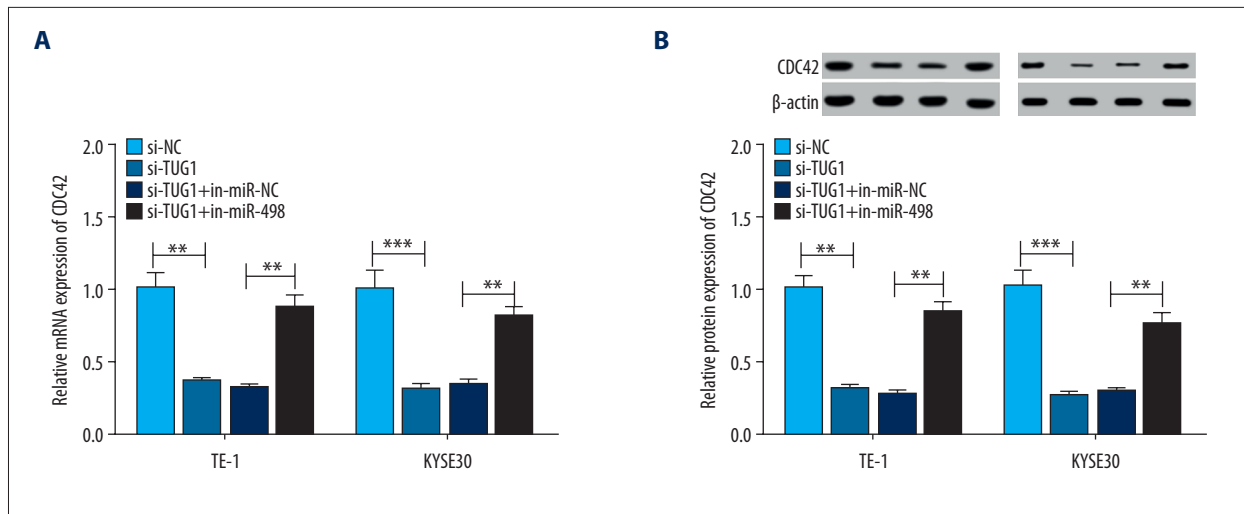


Figure 7. Knockdown of TUG1 downregulated CDC42 via miR-498 in ESCC cells. **(A)** qRT-PCR was executed for evaluation of the expression of CDC42 in TE-1 and KYSE30 cells. $** P < 0.01$. GAPDH was used as the endogenous for CDC42. **(A, B)** The si-NC, si-TUG, si-TUG1+in-miR-NC, or si-TUG1+in-miR-498 TE-1 was transfected into TE-1 and KYSE30 cells, respectively. **(B)** Western blot analysis was conducted for the assessment of CDC42 protein expression in TE-1 and KYSE30 cells. $** P < 0.01$. Data are shown as a mean \pm SD from 3 independent experiments. Student's *t*-test assessed the significance of the differences. TUG1 – taurine upregulated gene 1; CDC42 – cell division cycle 42; ESCC – esophageal squamous cell carcinoma; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR – quantitative real time polymerase chain reaction; SD – standard deviation.

cancer cells and exerted an anti-tumor effect, and our results were consistent with them [20,21,36,37]. One report uncovered that circFADS2 silencing curbed invasion and proliferation of lung cancer cells through upregulation miR-498 [20]. Besides, lncRNA UFC1 facilitated invasion, proliferation, and migration through modulating the miR-498/Lin28b axis [37]. Of note, Yang et al. indicated that miR-498 targeted CCPG1 to repress cell apoptosis and promote cell proliferation in retinoblastoma cells [38]. The different results might be due to the different microenvironments of miR-498 in different cancers, which leads to its different biological functions. These data indicated that TUG1 played its function via miR-498 in ESCC.

After confirming that TUG1 worked through miR-498 in ESCC, we further addressed whether TUG1 regulated the target of miR-498 through miR-498. We found that CDC42 acted as a target for miR-498. Also, CDC42 was upregulated in ESCC cells and was positively regulated by TUG1 in ESCC cells. Furthermore, CDC42 enhancement recovered the repressive impacts of miR-498 upregulation on cell proliferation and invasion in ESCC cells. Additionally, Sun et al. stated that CDC42 expression was boosted in ESCC cells and the miR-195/CDC42 axis was connected with the development of ESCC [30,31]. Sharma et al. indicated that miR107 targeted CDC42 to suppress

the migration, proliferation, and invasion of ESCC cells [29]. What's more, inhibition of miR-498 attenuated the prohibitive roles of TUG1 knockdown on CDC42 mRNA and protein expression, which was supported that TUG1 bound to miR-498 to regulate CDC42 expression in ESCC cells. Interestingly, miR-498 targeting CDC42 has never been reported in ESCC. Therefore, our data verified that TUG1 reduction impeded cell proliferation and invasion in ESCC via sponging miR-498 and repressing the expression of CDC42.

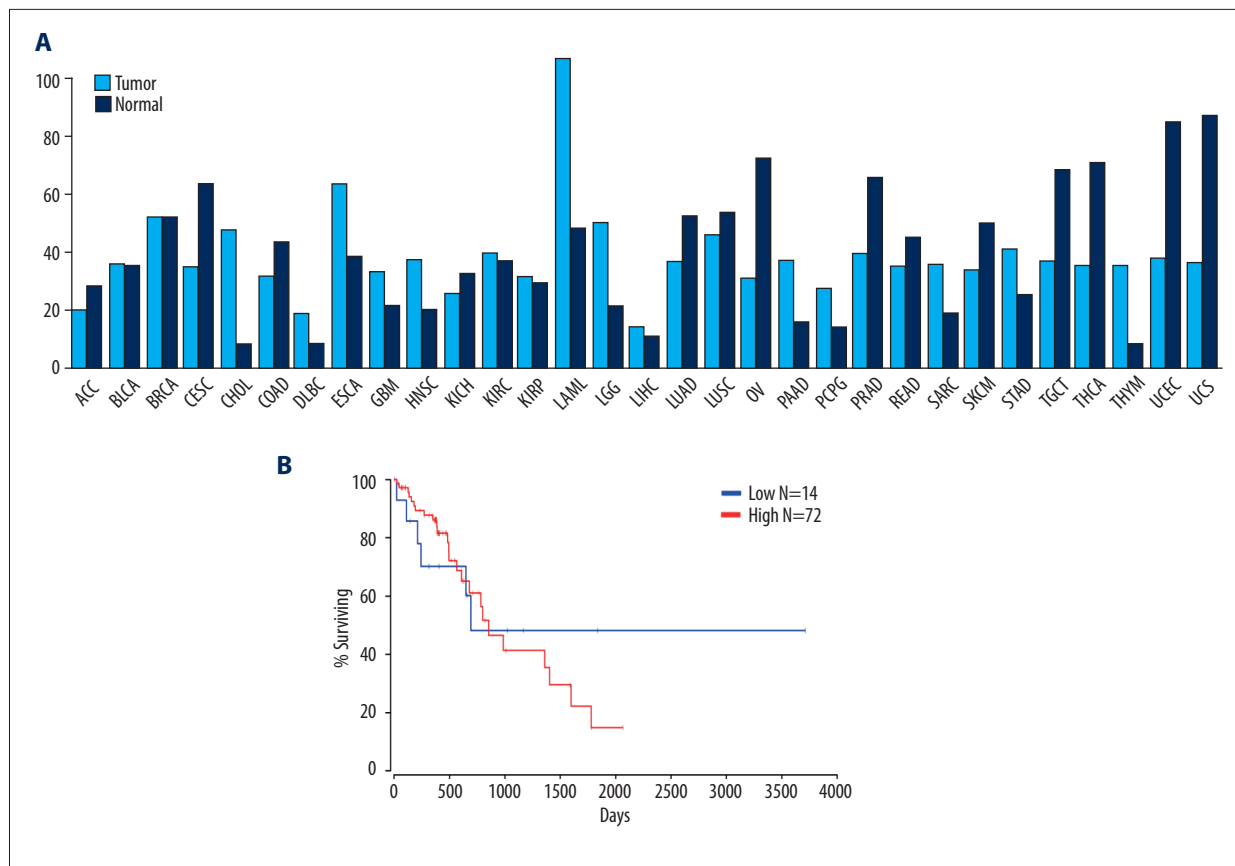
Conclusions

This study verified that TUG1 knockdown restrained the proliferation and invasion abilities of ESCC cells through downregulating CDC42 by sponging miR-498. Furthermore, this research helps us to better understand the molecular mechanism of ESCC development, and TUG1 might be an underlying target for the treatment of ESCC.

Conflicts of interest

None.

Supplementary Data



Supplementary Figure 1. (A) Gepia databases exhibited that TUG1 was upregulated in a range of tumors, including ESCA. (B) Oncolnc databases showed that high TUG1 expression in ESCA patients possessed a worse overall survival rate. TUG1 – taurine upregulated gene 1; ESCA – esophageal cancer.

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