

microRNA-144 inhibits cell proliferation and invasion by directly targeting TIGAR in esophageal carcinoma

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Received September 1, 2018; Accepted October 18, 2019

DOI: 10.3892/ol.2020.11420

Abstract. microRNAs (miRNAs) have been identified to play vital roles in the development and progression of numerous different types of human malignancy, including esophageal squamous cell carcinoma (ESCC). In the present study, the biological function of microRNA-144 (miR-144) was investigated, as well as its underlying molecular mechanism in ESCC. The results revealed that miR-144 expression was significantly decreased, whereas the expression of TP53-inducible glycolysis and apoptosis regulator (TIGAR) was significantly increased in human ESCC tissues when compared with adjacent non-tumor tissues. An increase in TIGAR was significantly associated with tumor size and Tumor-Node-Metastasis staging in patients. Functional analysis revealed that the overexpression of miR-144 using lentivirus particles significantly inhibited cell proliferation and tumor colony formation, and induced cell apoptosis in EC9706 and EC109 cells. The autophagy activity was also enhanced by miR-144 activity. In addition, overexpression of miR-144 significantly inhibited tumor growth *in vivo*. In the present study, TIGAR was confirmed to be the downstream target of miR-144 in ESCC. siRNA-mediated downregulation of TIGAR inversely regulated the inhibition effect of miR-144 on ESCC cells. To conclude, the present study demonstrated that miR-144 inhibits proliferation and invasion in esophageal cancer by directly targeting TIGAR.

Introduction

Esophageal cancer (EC) is one of the most malignant types of tumor in humans (1-4). As the eighth most common type of cancer worldwide, the yearly estimated number of new

EC cases in the USA alone is >16,000, with an estimated >150,000 cases of EC-associated mortality each year (1-4). Histologically, as the two major subtypes of EC, esophageal adenocarcinoma and esophageal squamous carcinoma (ESCC) represent ~90% of all EC cases (1,5-7). Among them, ESCC is the most dominant subtype in developing countries, including China (8,9). Despite advances in the early diagnosis, comprehensive therapy and surgical techniques, the prognosis for patients with ESCC remains far from satisfactory, with an overall 5-year survival rate of <25% (10-12). Therefore, it is essential that the molecular mechanisms underlying the neoplastic progression of ESCC be elucidated, and that novel molecular markers associated with ESCC are identified, in order to facilitate the development of an effective diagnostic and therapeutic strategy for ESCC.

Due to the vital regulatory effects in cell proliferation, apoptosis and differentiation, microRNAs (miRNAs) have been widely studied and proven to be associated with carcinogenesis and tumor progression, and even the response to therapy (2,3,13,14). As a large class of conserved 20-22 nucleotides long small non-coding single stranded RNAs, miRNAs negatively regulate gene expression through targeting the 3'untranslated region (3'UTR) of mRNAs. The aberrant expression of several miRNAs has been identified in ESCC (5,15,16). Chen *et al* (6) reported that miR-26b regulates the proliferation, migration and cell cycle transition of cancer cells by suppressing tumor necrosis factor receptor associated factor 5 in ESCC. Fan *et al* (17) revealed that miR-125b inhibits cell proliferation and induces cell apoptosis in ESCC by targeting Bcl2 modifying factor. In another clinical study by Dong *et al* (16), miR-216 a/b was demonstrated to be inversely correlated with lymph node metastasis and the Tumor-Node-Metastasis (TNM) stage (18) of ESCC, which indicated that plasma miRNA-216a/b may serve as potential biomarkers for the diagnosis of ESCC, and that dysregulation of miR-216a/b may be involved in the progression of ESCC. Collectively, these data indicate that miRNAs are important regulators of ESCC and have been recognized as biomarkers for the diagnosis of ESCC.

miR-144 has been demonstrated to act as a tumor suppressor in multiple types of human malignancy, such as non-small cell lung, gastric, papillary thyroid, bladder and colorectal cancers (19-22). In chronic and acute myeloid leukemia, the expression of miR-144 was also demonstrated

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Key words: microRNA-144, esophageal carcinoma, TP53-inducible glycolysis and apoptosis regulator

to be downregulated (19,20). Furthermore, using principal component regression analysis, Gao *et al* (21) reported that the miR-144/451 cluster may play an important role in the progression of ESCC and may also be considered as a biomarker for the detection of ESCC at an early stage of disease. In another study, Wu *et al* (22) demonstrated that miRNA-144 was highly expressed in the saliva of patients with EC and can be used as a genetic marker for the early diagnosis of these patients. However, the detailed role of miR-144 and the molecular mechanism underlying ESCC remains unknown and requires elucidation. In the present study, the role of miR-144 in ESCC and the underlying molecular mechanism both *in vitro* and *in vivo* are reported.

Materials and methods

Patients and sample collection. The present study was approved by the Medical Ethics Committee of the Affiliated Hospital of Taishan Medical University (Tai'an, China). Written informed consent was obtained from all patients included within the present study. In total, 80 patients with ESCC were recruited in the present study between January 2017 and November 2018. The inclusion criteria of the present study were as follows: Patients who underwent ESCC surgical resection without prior chemotherapy or radiotherapy. The exclusion criteria were as follows: Patients without confirmed diagnosis of ESCC or patients with ESCC who had previously received chemotherapy, radiotherapy or biological medication (monoclonal antibodies). ESCC tissues and corresponding normal tissues (<5 cm away from the ESCC tissues) were collected during surgery and immediately stored in liquid nitrogen. Patient diagnoses was validated blindly by pathologists from the Department of Pathology, Affiliated Hospital of Taishan Medical University (Tai'an, China).

Cell lines and cell culture. The ESCC cell line EC109 and EC9706 were purchased from Changsha Yingrun Biotechnology Co., Ltd. The cells were grown in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% streptomycin and 1% penicillin. Cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C and the medium was replaced every 3 days.

RNA extraction and reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from tissue samples and ESCC cell lines using a TRIzol[®] kit (Invitrogen; Thermo Fisher Scientific, Inc.) and a RNeasy Mini kit (Qiagen, Inc.). RNA was reverse transcribed into cDNA using an miScriptRT kit (Qiagen GmbH). The protocol for reverse-transcription was as follows: 37°C for 60 min followed by 85°C for 5 sec. qPCR was subsequently performed using a TaqMan miRNA PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) in an ABI 7500 Real-Time PCR system (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The following primer sequences were used for the qPCR: miR-144; Forward, 5'-TACAGTATAGATGAT-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'; TIGAR; Forward, 5'-ACTCAAGACTTCGGGAAGGA-3', and reverse, 5'-CACGCA TTTTCACCTGGTCC-3'; U6; Forward, 5'-CTCGCTTCG

GCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTGCGT-3'; β -actin; Forward, 5'-CTCCATCTGGCCTC GCTGT-3', and reverse, 5'-GCTGTACCTTCACCGTTC-3'. The following thermocycling conditions were used for the PCR: initial denaturation at 95°C for 30 sec; 40 cycles PCR extension of 95°C for 3 sec and 60°C for 30 sec. Small nuclear RNA U6 (U6) (Fig. 1A) and β -actin (Fig. 1B) were used as the internal reference genes for miR-144 and TIGAR PCR analysis, respectively. mRNA levels were calculated as fold-changes using the 2^{- $\Delta\Delta C_q$} method (23).

Cell transfection. The overexpression lentiviral particles (10⁸ TU/ml) of miR-144-3p were purchased from GenePharma, Inc. 293FT cells (American Type Culture Collection) were used as the interim cell line to generate the packaged lentivirus. The sequences of miR-144-3p were as follows: Forward, 5'-GCGCGAATTCGAGATCTTAACAGACCCTAGCTC-3' and reverse, 5'-GCGCGGATCCGTGCCCTGGCAGTCAGT AGG-3'. In detail, lentivirus particles were generated using co-transfection of pCDH-miR-144-3p or pCDH with psPAX2 and pMD2G vectors into 293FT cells. Cells were transfected with Lipofectamine[®] 3000 reagent (Invitrogen; Thermo fisher Scientific, Inc.). EC109 and EC9706 cells were transfected into three different groups: Blank group, miR-144 negative control (miR-NC with empty lentivirus packing vector) and miR-144 overexpression group, according to the manufacturer's protocol that supplied the miR-144-3p lentiviral particles (GenePharm, Inc.). Following transfection, the expression of miR-144 was determined via RT-qPCR, as aforementioned. Cells were collected for further experiments 48 h-post transfection. The targeting siRNA-TIGAR and control siRNA (20 μ M, respectively) were also purchased from GenePharma, Inc., and the sequences were as follows: siRNA-TIGAR forward, 5'-CCUACACCAUCAUCUAAUUTT-3' and reverse, 5'-AUU UACAUCAUCCUGUACCTT-3' and control siRNA forward, 5'-UUCUCCCAACGUCUACCUTT-3', and reverse, 5'-ACG UCACACGUUCGCAGAATT-3'.

Cell proliferation assay. The effect of miR-144 on the proliferation of ESCC cells was evaluated using a Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology). In detail, EC109 and EC9706 cells (2x10⁴ cells/well in 100 μ l medium) were seeded into 96-well plates. After 24 h of incubation at 37°C, the cells were transfected with miR-144 overexpression lentiviral particles, negative control lentiviral particles or blank medium for 48 h and incubated in a humidified atmosphere of 5% CO₂ at 37°C for 0, 24, 48, 72 and 96 h following transfection. Subsequently, 15 μ l of CCK-8 reagent was added to each well. After 4 h incubation at 37°C, the absorbance values at 450 nm were measured using a microplate reader (Bio-Rad Laboratories, Inc.). Each experiment was performed in triplicate.

Tumor colony formation assay. EC109 and EC9706 cells were transfected with miR-144 overexpression lentiviral particles, negative control lentiviral particles or blank medium, and trypsinized 24 h post-transfection. Each well had 2x10³ EC109 and EC9706 cells added to the 6-well plates and were incubated at 37°C for 7 days. The colonies were fixed with 4% methanol for 15 min at room temperature and stained with crystal violet for

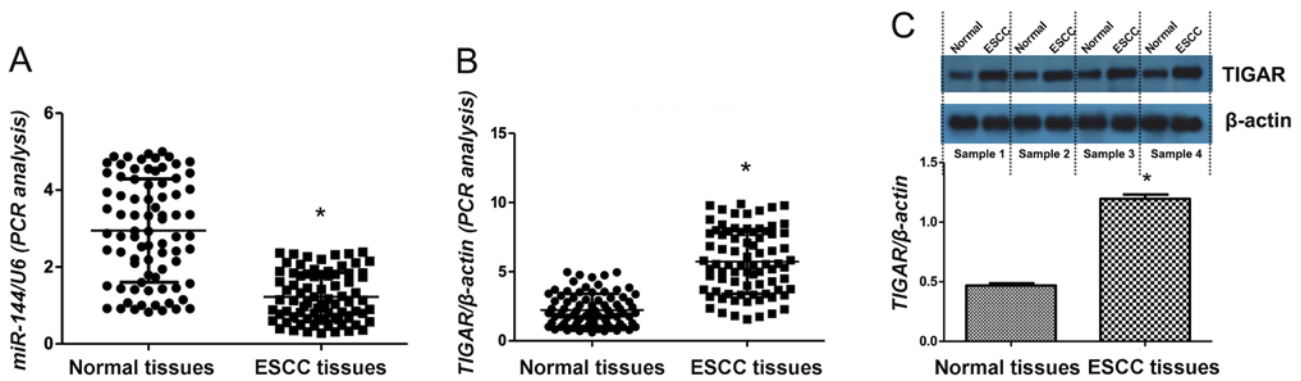


Figure 1. miR-144 is downregulated while TIGAR is upregulated in human ESCC. (A) PCR analysis revealed that the expression of miR-144 was significantly downregulated in ESCC tissues compared with the adjacent normal tissues. (B) PCR analysis of 80 samples revealed significantly higher mRNA expression levels of TIGAR in ESCC tissues compared with the adjacent normal tissues. (C) Western blot analysis of four randomly selected ESCC samples together with the adjacent noncancerous tissues confirmed that the protein expression of TIGAR in ESCC tissues was higher than the adjacent normal tissues. * $P < 0.05$ vs. control. miR, microRNA; TIGAR, TP53-inducible glycolysis and apoptosis regulator; ESCC, esophageal squamous cell carcinoma.

10 min at 37°C. Images were captured using a digital camera in order to assess the number of stained tumor cell colonies containing at least 50 cells. ImageJ software (version 1.47; National Institutes of Health) was used to quantify the results of tumor colony formation assay.

Bioinformatics. miRNA target gene predictions were taken from TargetScan (version 7.0) (24), which predicts the biological targets of miRNAs by searching for the presence of 8, 7 and 6 mer sites that match the seed region of each miRNA (25). The prediction of miRNA functions was determined using DIANA-miRPath (version 3.0; microrna.gr/miRPathv3) (26).

Cell apoptosis assay. In order to quantify the cell apoptosis rates, a flow Annexin V-FITC Apoptosis Detection kit (BD Biosciences) was applied according to the manufacturer's protocols. In brief, the transfected ESCC cells were collected and resuspended in 500 μ l Annexin V-binding buffer (BD Biosciences). Subsequently, 5 μ l of FITC-conjugated Annexin V and 5 μ l propidium iodide were added. Stained cell fluorescence was analyzed using the FACSCalibur flow cytometer (Beckman Coulter, Inc.) following incubation for 10 min at room temperature in the dark. The results of flow Annexin V-FITC Apoptosis Detection kit were analyzed using FlowJo software (version 9.6.2; FlowJo LLC). The caspase-3/-7 activity apoptosis assay was performed using a caspase-3/-7 Activity Apoptosis Assay kit (<https://www.aatbio.com>). ESCC cells were incubated with caspase-3/-7 assay solution (AAT Bioquest) for 1 h at room temperature. Fluorescence intensity was detected at Ex/Em=490/525 nm using a fluorescence microplate reader (Tecan Group, Ltd.).

Western blot analysis. The western blot analysis was performed as previously described (27,28). In brief, total protein was extracted using protein lysis buffer (Beyotime Institute of Biotechnology) and quantified using a bicinchoninic acid assay kit (Bio-Rad Laboratories, Inc.). A total of 30 μ g protein/lane was separated via SDS-PAGE on a 10% gel. The separated proteins were subsequently transferred onto a polyvinylidene difluoride membrane (EMD Millipore) and

blocked in 5% non-fat dried milk for 2 h at room temperature. The membrane were incubated with primary antibodies against TIGAR (1:1,000; cat. no. 14751) and β -actin (1:8,000; cat. no. 4970) (both from Cell Signaling Technology, Inc.) overnight at 4°C. Following the primary incubation, membranes were incubated with a horseradish peroxidase-conjugated donkey anti-goat IgG secondary antibody (1:1,000; cat. no. A0181; Beyotime Institute of Biotechnology) for 2 h at room temperature. Protein bands were visualized using enhanced chemiluminescence reagents (Pierce; Thermo Fisher Scientific, Inc.) and analyzed using Bio-Rad Quantity One software (version 4.4.0; Bio-Rad Technologies, Inc.).

Construction of 3'UTR reporter plasmid and luciferase assay. The 3'UTR of TIGAR, which contains a putative target region for miR-144, was amplified via PCR. The primer sequences were as follows: Forward, 5'-GTAGAGCTCTTAAGTATTTGTCCAAGTACAGTT-3', and reverse, 5'-GAACTC GAGAAATTCCAATAGTTGATTATACGTA-3'. The thermocycling conditions were as follows: Initial denaturation at 94°C for 3 min; 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 35 sec. Takara Taq™ DNA polymerase (Takara Bio, Inc.) was used for the PCR. The TIGAR 3'UTR mutant construct was generated via overlap extension PCR (72°C for 3 min). Fragments were inserted between the XbaI and XhoI sites in the pmirGLO (Promega Corporation). Co-transfection of the reporter vector (pmirGLO-wt-TIGAR or pmirGLO-mut-TIGAR) and miRNA (miR-144 mimics or scramble) was performed using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 24 h transfection, firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay kit (Promega Corporation), according to the manufacturer's protocol.

Tumor growth assay. All *in vivo* animal studies were conducted using a protocol approved by the Animal Care and Use Committee of the Affiliated Hospital of Taishan Medical University (Taian, China). A total of 15 male nude mice (5-weeks-old) weighing 25 \pm 5 g at the beginning of the study were purchased from the Laboratory Animal Centre of

Table I. Clinical profiles of the 80 patients with esophageal squamous cell carcinoma.

Clinicopathological feature	Number of cases	miR-144	P-value	TIGAR	P-value
Sex					
Male	44	1.23	0.847	5.68	0.764
Female	36	1.21		5.79	
Age					
≤60 years	43	1.18	0.496	5.87	0.509
>60 years	37	1.27		5.56	
Tumor size					
≤3 cm	56	1.22	0.745	4.59	<0.001
>3 cm	24	1.23		8.38	
Grading					
H	23	1.13	0.471	5.07	0.181
M	27	1.33		6.29	
L	30	1.20		5.73	
Metastasis					
Present	44	0.72	<0.001	5.84	0.575
Absent	36	1.83		5.59	
TNM staging					
I	21	1.90	<0.001	5.64	0.010
II	26	1.36		6.12	
III	21	0.73		6.25	
IV	12	0.58		4.10	

Mean values of miR-144 and TIGAR are presented in this table. miR, microRNA; TIGAR, TP53-inducible glycolysis and apoptosis regulator; H, high; M, moderate; L, low; TNM, Tumor-Node-Metastasis.

Affiliated Hospital of Taishan Medical University and divided into three groups (n=5 in each group) including blank group, miR-NC group and miR-144 group. All mice were kept at 25°C clean atmosphere with 50% relative humidity and a 12/12 h dark/light cycle. The mice had unlimited access to food and water. EC9706 with blank treated or stably transfected with miR-NC or miR-144 lentivirus (1.5×10^6 in 0.2 ml) were injected subcutaneously into the right dorsal flank of each mouse. Tumor size was calculated using the following formula: volume=[0.5x (length x width²)] at 1-, 2-, 3- and 4-weeks after inoculation of tumor cells. Mice were sacrificed using cervical dislocation at 4-weeks after inoculation of tumor cells.

Immunohistochemistry (IHC). The mouse tumor tissues were fixed in 4% paraformaldehyde for 48 h at room temperature and embedded in paraffin. Paraffin-embedded tissue samples were cut into 4-μm-thick sections. IHC analysis was subsequently carried out using standard techniques. In brief, deparaffinized sections were incubated with 3% H₂O₂ for 10 min at room temperature, followed by 0.1% trypsin (Beyotime Institute of Biotechnology) for 20 min at room temperature. Subsequently, sections were incubated with the primary antibody against Ki-67 (1:800; cat. no. 9449; Cell Signaling Technology, Inc.) overnight at 4°C, followed by incubation with a HRP-labeled goat anti-mouse IgG secondary antibody (1:50; cat. no. A0216; Beyotime Institute of Biotechnology) for 2 h at 37°C. IHC staining was visualized using a DAB horseradish peroxidase

color development kit (Beyotime Institute of Biotechnology) and images were captured using an Olympus Provis AX70 light microscope (magnification, x400). At least three sections from each specimen were analyzed blindly by two independent observers from Department of Pathology, Affiliated Hospital of Taishan Medical University (Tai'an, China). The percentage of Ki-67 positive cells was calculated after counting two hundred cells per region.

Statistical analysis. All experiments were repeated at least three times independently. Data are presented as the mean ± standard deviation. The results were statistically analyzed using an unpaired two-tailed Student's t-test or one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test using the SPSS software (version 23.0; IBM Corp.). For the data presented in Table I, an unpaired two-tailed Student's t-test was used to compare the results among two groups, while ANOVA followed by Bonferroni's post hoc test was applied for the comparison among >2 groups. P<0.05 was considered to indicate a statistically significant result.

Results

miR-144 is downregulated and TIGAR is upregulated in human ESCC specimens. The expression levels of miR-144 in clinical ESCC tissues and adjacent tissues were determined via RT-qPCR while the expression of TIGAR was evaluated

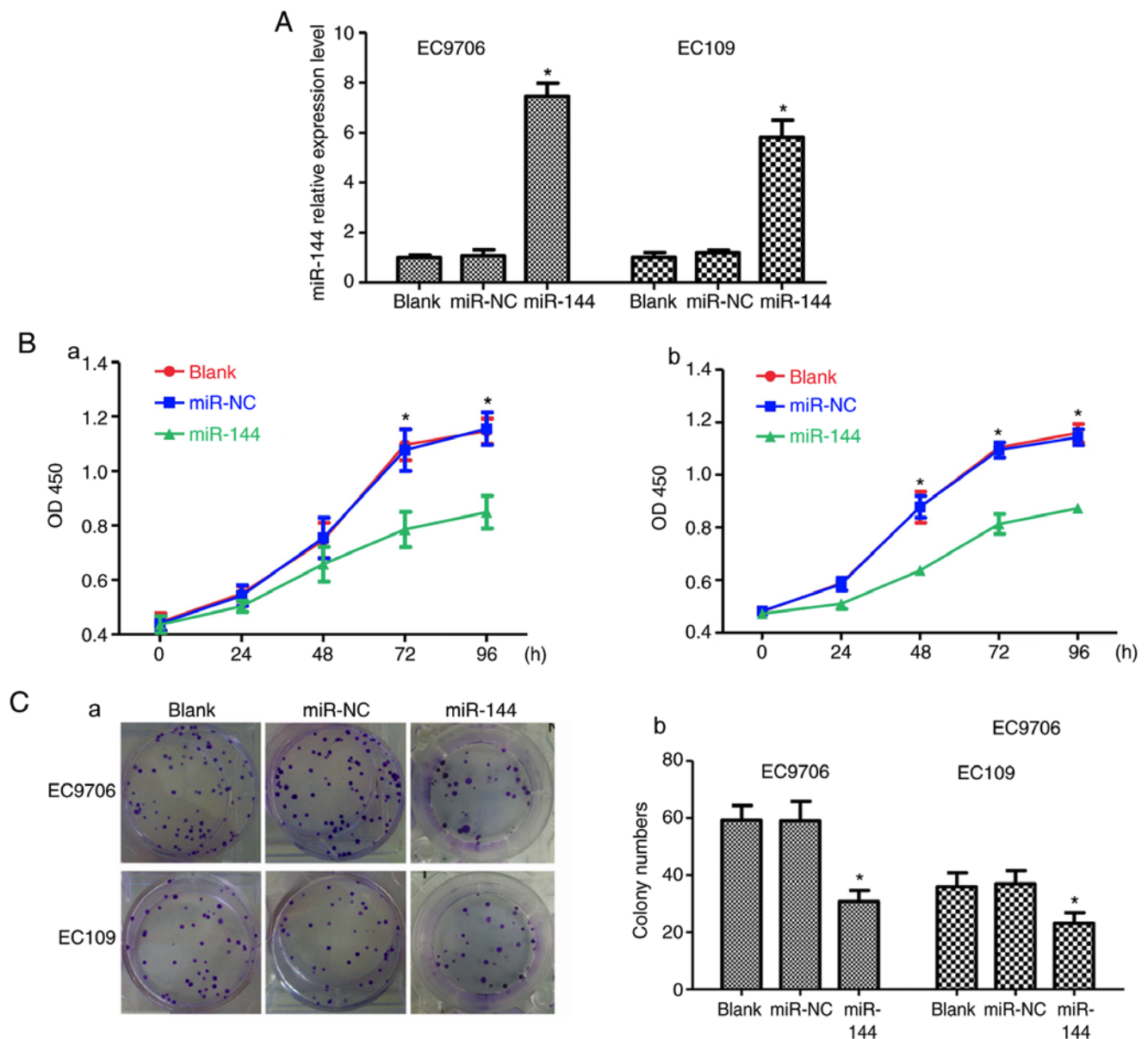


Figure 2. miR-144 suppresses cell proliferation and tumor formation and promotes cell apoptosis in EC9706 and EC109 cells. (A) miR-144 expression levels were upregulated in ESCC cells following lentiviral transfection. (B) (a-b) Overexpression of miR-144 significantly decreased cell proliferation in EC109 and EC9706 cells compared with the miR-NC group and the blank group, respectively. (C) (a-b) Overexpression of miR-144 significantly inhibited the tumor formation ability of EC109 and EC9706 cells. * $P < 0.05$ vs. control. miR, microRNA; ESCC, esophageal squamous cell carcinoma; NC, negative control.

via RT-qPCR and western blot analysis using TIGAR-specific antibodies. Compared with the adjacent non-cancer tissues, the expression of miR-144 was significantly downregulated in ESCC tissues (Fig. 1A). In addition, there were significantly higher mRNA and protein expression levels of TIGAR observed in ESCC tissues compared with the adjacent normal tissues ($P < 0.05$; Fig. 1B and C).

Expression levels of miR-144 and TIGAR are associated with ESCC progress. The baseline characteristic of all patients included in the present study are presented in Table I. There was no statistically significant association observed regarding the miR-144, TIGAR expression and sex, age and tumor grading. However, miR-144 expression was significantly associated with ESCC metastasis and TNM staging. In addition, TIGAR expression was significantly associated with ESCC

tumor size and TNM staging. These results indicated that the expression levels of miR-144 and TIGAR were closely associated with ESCC progress (Table I).

miR-144 suppresses the proliferation and tumor formation of ESCC cells. In order to investigate the effect of miR-144 on ESCC, human EC9706 and EC109 cells were transfected with miR-144 lentivirus, negative control lentivirus and the blank control. The successful transfection was confirmed by RT-qPCR (Fig. 2A). As presented in Fig. 2B-a and b, overexpressed miR-144 significantly decreased cell proliferation in EC109 and EC9706 cells, compared with the miR-NC group and the blank group. Furthermore, by conducting a tumor colony formation assay, it was revealed that miR-144 significantly inhibited the tumor formation ability of EC109 and EC9706 cells (Fig. 2C-a and b).

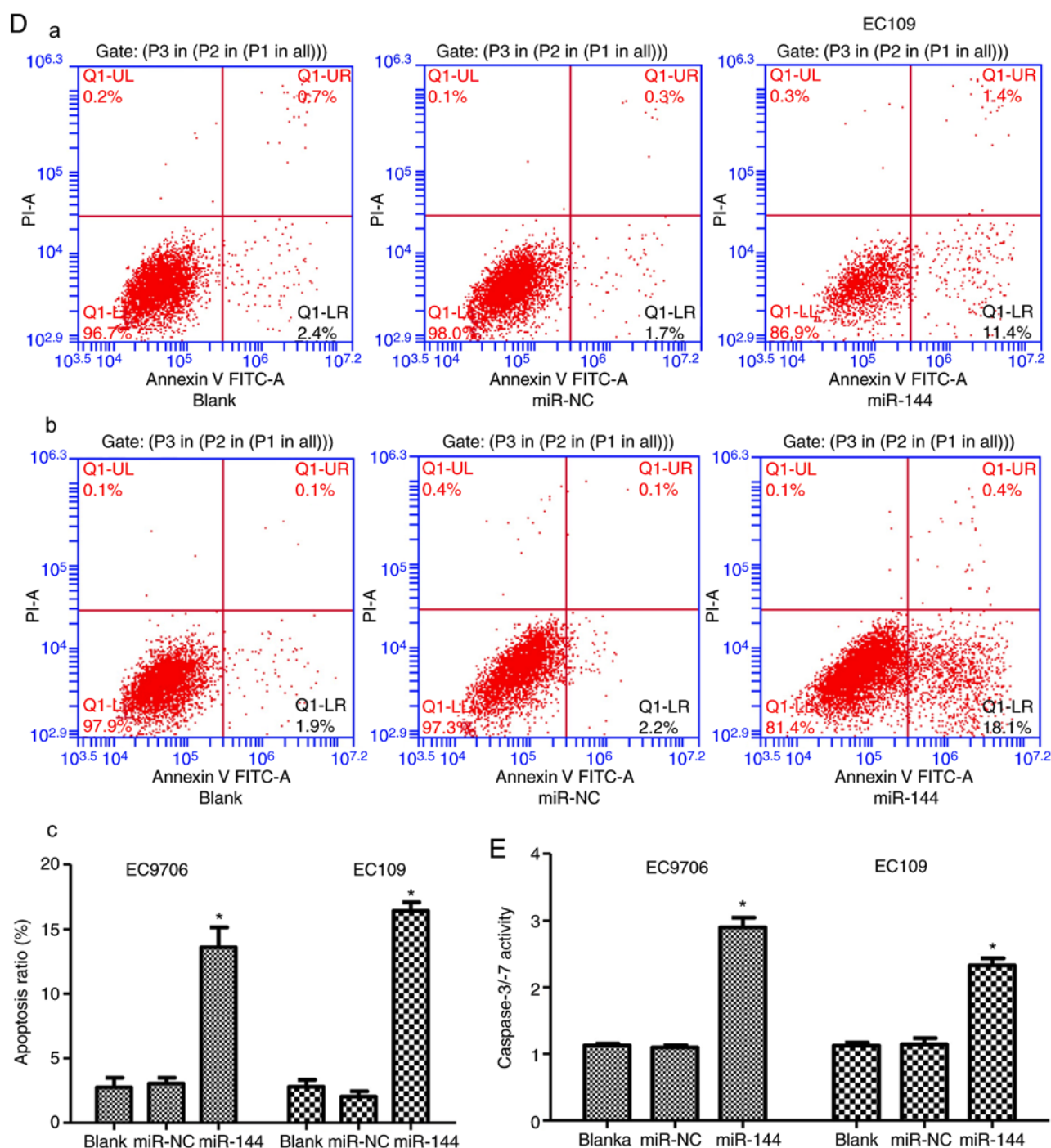


Figure 2. Continued. miR-144 suppresses cell proliferation and tumor formation and promotes cell apoptosis in EC9706 and EC109 cells. (D) (a-c) Flow cytometric analysis assay revealed that miR-144 promotes cell apoptosis in ESCC cells. (E) Caspase-3/-7 activity of miR-144 lentivirus transfected cells was almost 2-fold as much as the miR-NC group and the blank group. * $P < 0.05$ vs. control. miR, microRNA; ESCC, esophageal squamous cell carcinoma; NC, negative control.

miR-144 promotes cell apoptosis in ESCC cells. The results of the flow cytometry analysis revealed that the proportion of apoptotic cells (Q1UR + Q1LR) in the miR-144 group was significantly higher than that of the miR-NC group and the blank group for both EC109 and EC9706 cells, which revealed that miR-144 overexpression effectively increased the number of apoptotic cells (Fig. 2D-a and c). Caspase-3/-7 activity apoptosis assay also demonstrated that the caspase-3/-7 activity of miR-144 lentivirus transfected cells was almost 2-fold as much as the miR-NC group and the blank group (Fig. 2E).

miR-144 inhibits tumor growth in vivo. In order to further investigate the role of miR-144 on ESCC, an *in vitro* investigation was performed. EC9706 cells transfected with miR-144 overexpression lentivirus and the control cells were injected into nude mice. Compared with the control groups, miR-144 overexpression markedly inhibited tumor volume, which indicated that miR-144 could inhibit ESCC tumor growth *in vivo* (Fig. 3A and B). The largest tumor diameters observed were 1.1, 1.0 and 0.5 cm in the blank group, miR-NC group and miR-144 group, respectively. Furthermore, the results of

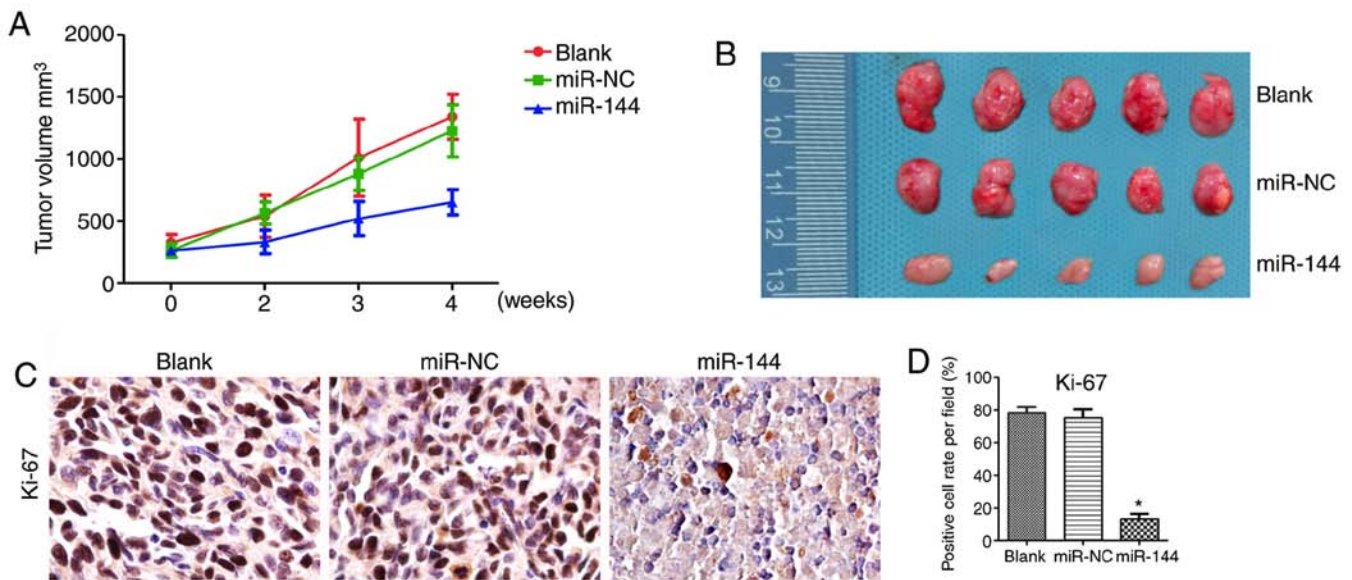


Figure 3. miR-144 inhibits tumor growth *in vivo*. (A) Overexpression of miR-144 significantly inhibited tumor volume compared with the control groups. (B) Images of the tumors from each group. (C) IHC staining data of Ki-67 on *in vivo* samples. (D) Quantification of IHC staining. * $P < 0.05$ vs. control. miR, microRNA; IHC, immunohistochemistry; NC, negative control.

IHC staining revealed that miR-144 inhibited Ki-67 expression *in vivo* (Fig. 3C and D).

miR-144 inhibits ESCC by targeting TIGAR. In order to verify the target gene of miR-144 in human ESCC, the following experiments were performed. First, the predicted binding sites of miR-144 in the 3'-UTR of TIGAR mRNA were revealed according to the results from the Targetscan analysis. Dual luciferase reporter gene assays revealed that miR-144 effectively weakened the luciferase activity of TIGAR wild-type by ~50% ($P < 0.05$). However, miR-144 did not affect the luciferase activity of TIGAR with mutant type 3'UTR (Fig. 4A). PCR analysis confirmed the successful knockdown of TIGAR using the si-TIGAR (Fig. S1). Although the miR-144 group was demonstrated to exhibit a significant inhibiting effect on EC9706 cell proliferation, CCK-8 analysis revealed that the proliferation of EC9706 cells was also significantly inhibited when TIGAR was inhibited by si-TIGAR, when compared with the blank control ($P < 0.05$; Fig. 4B). Similar results were also observed when using the tumor colony formation assay and the flow cytometry analysis (Fig. 4C-E). Collectively, these results indicated that TIGAR was a direct target of miR-144.

Discussion

Despite advances having been made regarding the treatment of ESCC, the molecular mechanism underlying the development of ESCC remains largely unknown, and patient overall survival time in the disease remains far from satisfactory (2,6,29,30), which demonstrates an urgent need to identify novel molecular markers associated with ESCC and to facilitate the development of an effective diagnostic and therapeutic strategy for ESCC.

In the present study, miR-144 expression was observed to be downregulated in human ESCC tissues. Low miR-144

expression levels in tumors were significantly associated with metastasis and TNM staging in patients with ESCC, which revealed a possible association between miR-144 and ESCC tumorigenesis. The results of the present study demonstrated that the overexpression of miR-144 suppressed cell proliferation, and enhanced apoptosis in ESCC lines EC9706 and EC109. In addition, overexpression of miR-144 effectively inhibited tumor cell growth *in vivo*. Furthermore, it was revealed that miR-144 directly targeted TIGAR in ESCC cells. By decreasing the level of TIGAR using si-TIGAR, similar outcomes were observed to those with the overexpression of miR-144. These results indicated that miR-144 inhibits cell proliferation and invasion by directly targeting TIGAR in ESCC.

Various miRNAs have been reported to be aberrantly expressed in a number of different types of human cancer, including miR-144 (3,29,31). miR-144 has been widely researched and reported to be an important regulator of tumor development. However, the roles of miR-144 in human cancer are currently controversial (32-38), despite numerous reports having demonstrated that miR-144 is downregulated in a number of different types of human cancer, and that it may act as a tumor suppressor (19-22). There are studies demonstrating that miR-144 can promote cell proliferation of HeLa cells (39) and nasopharyngeal carcinoma cells (40). A recent study by Gao *et al* (21) demonstrated that the miR-144/451 cluster may serve an important role in the progression of ESCC and may be considered as a biomarker for the detection of ESCC at an early stage of disease. In their study, miR-144 was reported to be downregulated in ESCC tissues and cell lines, and overexpression of miR-144 was revealed to inhibit the proliferation of ESCC cells *in vitro* (21). In the present study, the gene expression profiles of miR-144 in human ESCC tissues were investigated using RT-qPCR. It was revealed that miR-144 was significantly downregulated in ESCC tissues when compared with the adjacent normal non-tumor tissues, which

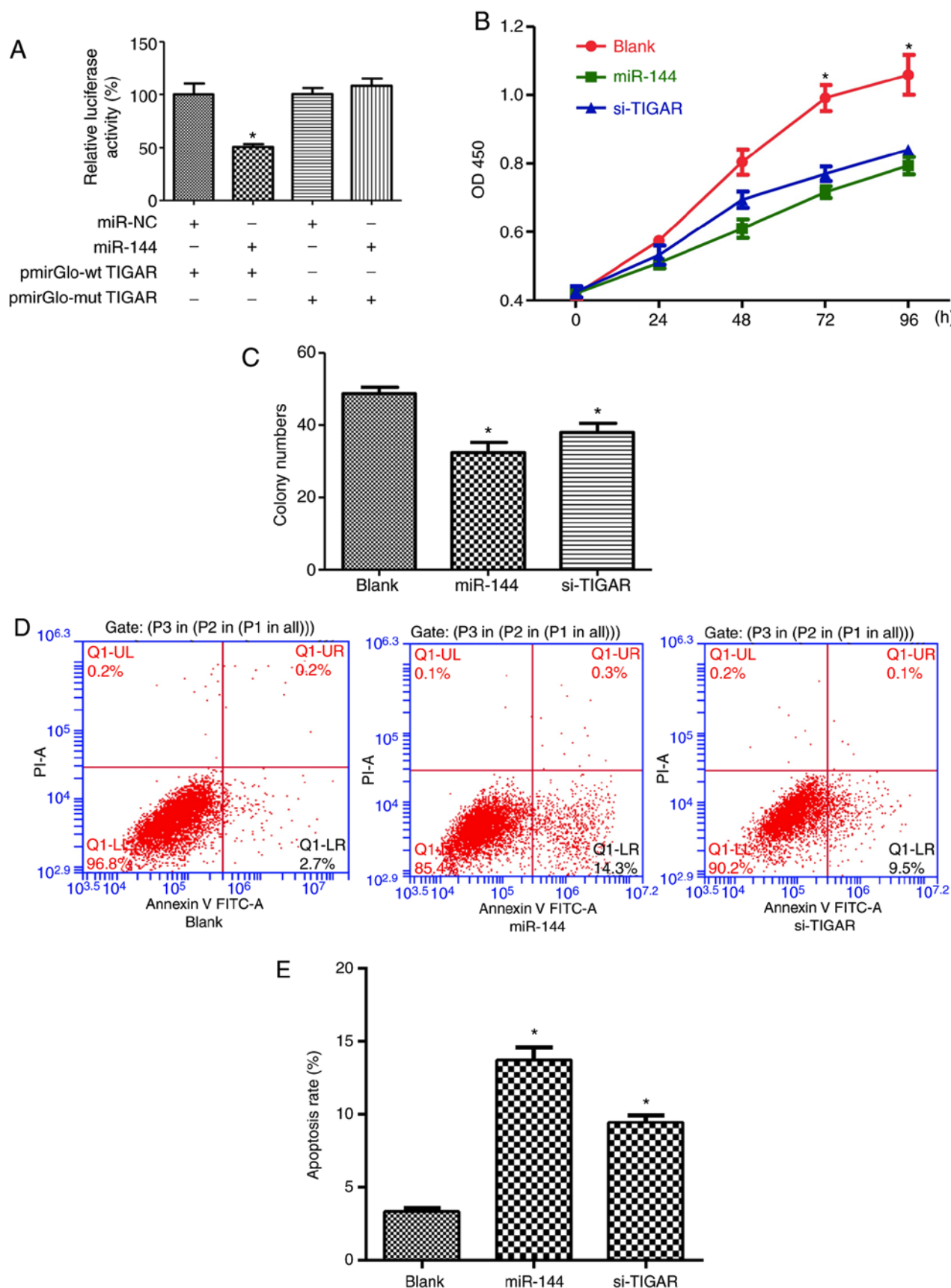


Figure 4. miR-144 inhibits ESCC by targeting TIGAR. (A) Dual luciferase reporter gene assays revealed that miR-144 effectively weakened the luciferase activity of TIGAR wild-type by ~50%, while miR-144 did not affect the luciferase activity of TIGAR with mutant type 3'UTR. (B) Cell Counting Kit-8 analysis revealed that the proliferation of EC9706 cells was significantly inhibited when TIGAR was inhibited by si-TIGAR, when compared with the blank control. (C) Tumor colony formation assay revealed that the proliferation of EC9706 cells was significantly inhibited when TIGAR was inhibited by si-TIGAR, when compared with the blank control. (D) Flow cytometric analysis revealed that the apoptosis rate of EC9706 cells was significantly increased when TIGAR was inhibited by si-TIGAR, when compared with the blank control. (E) Quantification of the flow cytometric analysis assay. * $P < 0.05$ vs. blank group. miR, microRNA; TIGAR, TP53-inducible glycolysis and apoptosis regulator; 3'UTR, 3'untranslated region; si, small interfering; NC, negative control.

was consistent with the results of previous studies (21,41). Furthermore, the *in vitro* results of miR-144 expression levels were confirmed by an *in vivo* evaluation.

It was also revealed in the present study that TIGAR was the direct downstream target gene of miR-144 in ESCC. Furthermore, it was demonstrated that siRNA-mediated TIGAR knockdown was able to obtain similar inhibitory outcomes as with the overexpression of miR-144. TIGAR is an inhibitor of glycolysis that is highly expressed in a number of different types of human carcinoma, and it is currently the only known phosphatase glycolytic modulator regulated by TP53 (42). Previous studies have demonstrated that TIGAR is an important factor in moderating stress levels, repairing DNA damage and inhibiting glycolysis. Silencing of TIGAR in glioblastoma cell lines was revealed to cause higher levels of Fru-2,6-P2 in these cells, increase glycolysis and lead to the accumulation of reactive oxygen species, which finally promoted cell death (43). In addition, the present study demonstrated that TIGAR expression was significantly upregulated in ESCC tissues and strongly connected with ESCC tumor size and TNM stage, which further confirmed the conclusion that the effects of miR-144 on ESCC are primarily based on the regulation of the TIGAR signaling pathway.

There were several limitations to the present study. First, the independent role of TIGAR in the development of ESCC was not investigated, and the expression levels of TIGAR in the animal model require confirmation in an *in vivo* model. Secondly, besides TIGAR, other miR-144 targets that have already been demonstrated to result in similar phenotypes (e.g., RUNX1, CEP55, etc.) should also be investigated in future studies, which may aid in the identification of, or a combination of, novel pathways responsible for the demonstrated phenotypes. In addition, although the association between TNM staging and miR-144, TIGAR expression were investigated in the present study, other parameters, such as lymph node metastasis, T stage and vascular invasion were not investigated. Additional groups, such as miR-144+ overexpression of TIGAR were also not investigated, which would be useful in confirming the results of the present study and should be the focus of future studies. Finally, due to the follow-up time, there was limited ability to analyze the association between miR-144, TIGAR and patient survival time. Thus, further studies are required to elucidate these queries.

Overall, the present study revealed that miR-144 inhibits cell proliferation and invasion by directly targeting TIGAR in esophageal carcinoma.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

BZ designed the study. YM acquired the data. QW and LT analyzed and interpreted the data. LL performed the statistical analyses. YM wrote the manuscript. BZ reviewed the manuscript for intellectual content.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of the Affiliated Hospital of Taishan Medical University. Written informed consent was provided by all patients that participated in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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