

MicroRNA-542-3p represses OTUB1 expression to inhibit migration and invasion of esophageal cancer cells

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Received August 20, 2018; Accepted April 24, 2019

DOI: 10.3892/mmr.2019.10836

Abstract. Dysregulation of microRNAs (miRNAs) is involved in the pathogenesis of esophageal cancer. miRNA (miR)-542-3p is a tumor suppressor in multiple types of cancer. However, whether and how miR-542-3p contributes to the progression of esophageal cancer remains unknown, and this is the aim of the present study. In the current study, decreased expression of miR-542-3p was detected in tumor tissues compared with normal tissues from patients with esophageal cancer, and miR-542-3p expression was negatively correlated with mRNA expression levels of ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1 (*OTUB1*) in tumor tissues from patients with esophageal cancer. In KYSE150 human esophageal squamous cell carcinoma cells, overexpression of miR-542-3p significantly decreased *OTUB1* at mRNA and protein levels, whereas downregulation of miR-542-3p significantly increased *OTUB1* expression. Using a dual-luciferase assay, *OTUB1* was validated to be a target gene of miR-542-3p in KYSE150 cells. Functionally, miR-542-3p significantly inhibited the migration and invasion of KYSE150 cells by repression of *OTUB1* expression. These results demonstrated that miR-542-3p may promote the metastasis of esophageal cancer cells, and indicated that miR-542-3p may be a treatment target for esophageal cancer.

Introduction

Esophageal cancer is the sixth most commonly diagnosed cancer and the fifth leading cause of cancer motility globally (1). It was estimated that esophageal cancer accounted for >4% of cancer-related mortality in the USA in 2017 (2). In East Asia, the risk of incidence for esophageal cancer is almost 4-fold higher compared with North America (1). The two major types of esophageal cancer are esophageal squamous-cell

carcinoma and esophageal adenocarcinoma (3). Owing to its aggressive nature and difficulty to diagnose, the overall 5-year survival rate of patients with esophageal cancer is ~20% (4,5). Therefore, there is an urgent necessity to discover the molecular mechanisms that lead to the metastasis of esophageal cancer.

MicroRNAs (miRNAs) are a class of small, non-coding, single-stranded RNAs that are ubiquitously expressed in eukaryotic cells (6). Through directly binding to the 3' untranslated region (UTR) of target gene mRNA, miRNAs induce degradation of mRNA or inhibit mRNA translation, resulting in downregulation of target gene expression (7). Previous studies have revealed that mRNA-miRNA regulatory networks are crucial for normal biological processes, including cell differentiation, migration and apoptosis (8-10). Dysregulation of miRNA expression contributes to a number of human diseases such as cancer (11). A microarray study has demonstrated that the expression levels of several miRNAs are promising biomarkers for esophageal cancer (12). miRNA (miR)-542-3p downregulation has been reported in several cancer types (13,14); however, the potential role and molecular mechanism of miR-542-3p in esophageal cancer remains to be determined.

Ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1 (*OTUB1*) is a hydrolase that is able to specifically remove ubiquitin from proteins to prevent protein degradation (15). By upregulating the expression of oncogenes, *OTUB1* facilitates tumor progression in several types of cancers (15-17). In esophageal cancer, *OTUB1* stabilizes Snail protein to promote the metastasis of cancer cells (18); however, it is unknown how *OTUB1* is regulated in esophageal cancer.

In the present study, miR-542-3p and *OTUB1* mRNA expression levels were examined in normal and tumor tissues from patients with esophageal cancer. A negative correlation was observed between miR-542-3p and *OTUB1* mRNA expression in tumor tissues. Western blotting and reverse transcription-quantitative PCR (RT-qPCR) data indicated that *OTUB1* was negatively regulated by miR-542-3p in esophageal cancer cells. A dual-luciferase assay validated *OTUB1* as a target gene for miR-542-3p. Furthermore, function assays demonstrated that miR-542-3p inhibited the migration and invasion of KYSE150 human esophageal squamous cell carcinoma cells through repression of *OTUB1* expression. The data from the present study suggested a potential tumor suppressor role for miR-542-3p in esophageal cancer.

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Key words: microRNA-542-3p, ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1, esophageal cancer

Materials and methods

Tissue collection. Tumor tissues and matched normal tissues were collected from 40 patients (mean age 56.33±7.21, male:female = 25:15) with esophageal squamous cell carcinoma or esophageal adenocarcinoma in Sheyang People's Hospital (Sheyang, China) between June 2015 and July 2017. Written consent was provided by all participants before the experiments and all procedures were approved by the Ethics Committee of Sheyang People's Hospital (IRB no. SYPH1506). Tissues were stored in -80°C upon collection.

Cell culture. Human esophageal squamous cell carcinoma cell line KYSE150 was purchased from American Type Culture Collection and used within 6 months. Cells were maintained in DMEM (Sigma-Aldrich, Merck KGaA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C.

Overexpression and downregulation of miR-542-3p. miR-542-3p mimic (5'-UGUGACAGAUUGAUAAACUGAAA-3'), miR-negative control (NC) mimic (5'-AAUUCUCCGAACGUGUCACTT-3'), miR-542-3p inhibitor (5'-UUUCAGUUAUCAAUCUGUCACA-3') and miR-NC inhibitor (5'-GUGACACGUUCGGAGAAUUTT-3') were synthesized by and purchased from Shanghai GenePharma Co., Ltd. For overexpression or downregulation of miR-542-3p, miR-542-3p mimic (50 nM) or miR-542-3p inhibitor (50 nM) was mixed with Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) in serum-free DMEM medium for 15 min. The mixtures were added into each well in 6-well plates and incubated for 48 h before the cells (2×10⁵) were harvested for subsequent experiments.

RNA extraction and RT-qPCR. Total RNA from cell lines (1×10⁴) or tissue samples (50-100 mg) was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For mRNA quantification, RNA was reverse-transcribed into first-stranded cDNA using PrimeScript RT Master Mix (Takara Bio, Inc.). RT-qPCR was conducted using a SYBR-Green qPCR Master Mix kit (Takara Bio, Inc.) on an ABI PRISM 7900HT Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the followed thermocycling parameters: Initial denaturation at 95°C for 2 min, 40 cycles of 95°C for 15 sec and 64°C for 30 sec. For miRNA quantification, RNA was reverse-transcribed with a Mir-X miRNA First Strand Synthesis kit (Takara Bio, Inc.) followed by qPCR with Mir-X™ miRNA qRT-PCR SYBR kit (Takara Bio, Inc.). Relative expression levels of miRNA and mRNA were calculated using the 2^{-ΔΔC_q} method (19). GAPDH and U6 served as internal controls for mRNA and miRNA, respectively. The primer sequences were as listed: miR-542-3p forward, 5'-TGTGACAGATTGATAACT-3' and stem-loop RT primer, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGA TACGACCTGCGGTTTCAGT-3'; OTUB1 forward, 5'-TCG GTCCTATACAAGGAGTATGC-3' and reverse, 5'-GGTCTT GCGGATGTACGAGT-3'; U6 forward, 5'-GTGCTCGCTTCG GCAGCACAT-3' and reverse, 5'-AATATGGAACGCTC ACGAAT-3'; GAPDH forward, 5'-GGAGCGAGATCCCTC CAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTTCAT GG-3'. Expression level of miR-542-3p was normalized to U6. Expression level of OTUB1 was normalized to GAPDH.

Protein lysate preparation and western blotting. The antibodies used were as follows: OTUB1 (cat. no. A302-917A; 1:1,000; Bethyl Laboratories, Inc.); GAPDH (cat. no. G8795; 1:5,000; Sigma-Aldrich; Thermo Fisher Scientific, Inc.); Snail (cat. no. sc-393172; 1:1,000; Santa Cruz Biotechnology, Inc., CA, USA); horseradish peroxidase-conjugated secondary antibodies against rabbit (cat. no. 7074, 1:10,000; Cell Signaling Technology, Danvers, MA, USA) and mouse (cat. no. 7076, 1:10,000; Cell Signaling Technology). Protein lysates from cells (1×10⁴) were prepared using RIPA lysis buffer (Roche Diagnostics GmbH) followed by determination of protein concentration by the BCA method. Then, protein samples (20 μg) were separated on an 10% SDS-PAGE gel and transferred to a PVDF membrane. The PVDF membrane was blocked by 5% bovine serum albumin (BSA, Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 2 h. Following incubation with the primary antibody at 4°C overnight and a secondary antibody at room temperature for 2 h, the bands were visualized using ECL Western Blot Substrate (Pierce; Thermo Fisher Scientific, Inc.). Densitometric analysis was performed by ImageJ software (version 1.8.0; National Institutes of Health). GAPDH was used to normalize expression data.

Dual-luciferase reporter assay. The position 107-114 of 3'UTR of *OTUB1* (5'...UUCACCCCCUUCUCCUGUCA CA...) mRNA containing the putative target site of miR-542-3p (3'AAAGUCAAUAGUAGACAGUGU) was determined by TargetScan version 7.1 (20) and amplified from the cDNA of KYSE150 cells and ligated into the pGL3-basic vector (Promega Corporation). pGL3-*OTUB1*-3'UTR-mutant (Mut, 5'...UUCACCCCCUUCUCCUGGAACA...) was created by introducing two site mutations into miR-542-3p potential target sites using QuickChangeSite-Directed Mutagenesis kits (Agilent Technologies, Inc.). pGL3-*OTUB1*-3'UTR-WT (200 ng) or pGL3-*OTUB1*-3'UTR-Mut (200 ng) was co-transfected with *Renilla* plasmid into KYSE150 cells using Lipofectamine® 3000, followed by transfection of miR-NC mimic (10 nM) or miR-542-3p mimic (10 nM) for 48 h at 37°C. The Dual-Luciferase Reporter Assay System (Promega Corporation) was used to measure the relative luciferase activity of each well. The firefly luciferase expression was normalized to *Renilla*.

OTUB overexpression plasmid construction and transfection. Full-length *OTUB1* cDNA was amplified from KYSE150 cells and ligated to a pcDNA3.1 vector (https://www.70dir.com/se/report_www_youbio_cn.html, YouBio) with the restriction sites of *KpnI* and *XhoI*. For overexpression of *OTUB1*, pcDNA3.1-*OTUB1* was mixed with Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) in serum-free DMEM medium for 15 min. The mixtures were then added into each well in 6-well plates (1×10⁶) and incubated for 48 h at 37°C before the cells were harvested for subsequent experiments.

Cell migration assay. A wound healing assay was used to examine the migratory ability of transfected KYSE150 cells. Briefly, 1×10⁶ cells were seeded in each well of 6-well plates. Following transfection, as aforementioned, the cells were cultured in 37°C to 90-100% confluence. A wound area was made in the center of each well using a 10 μl pipette tip. The culture medium of the cells was replaced with serum-free

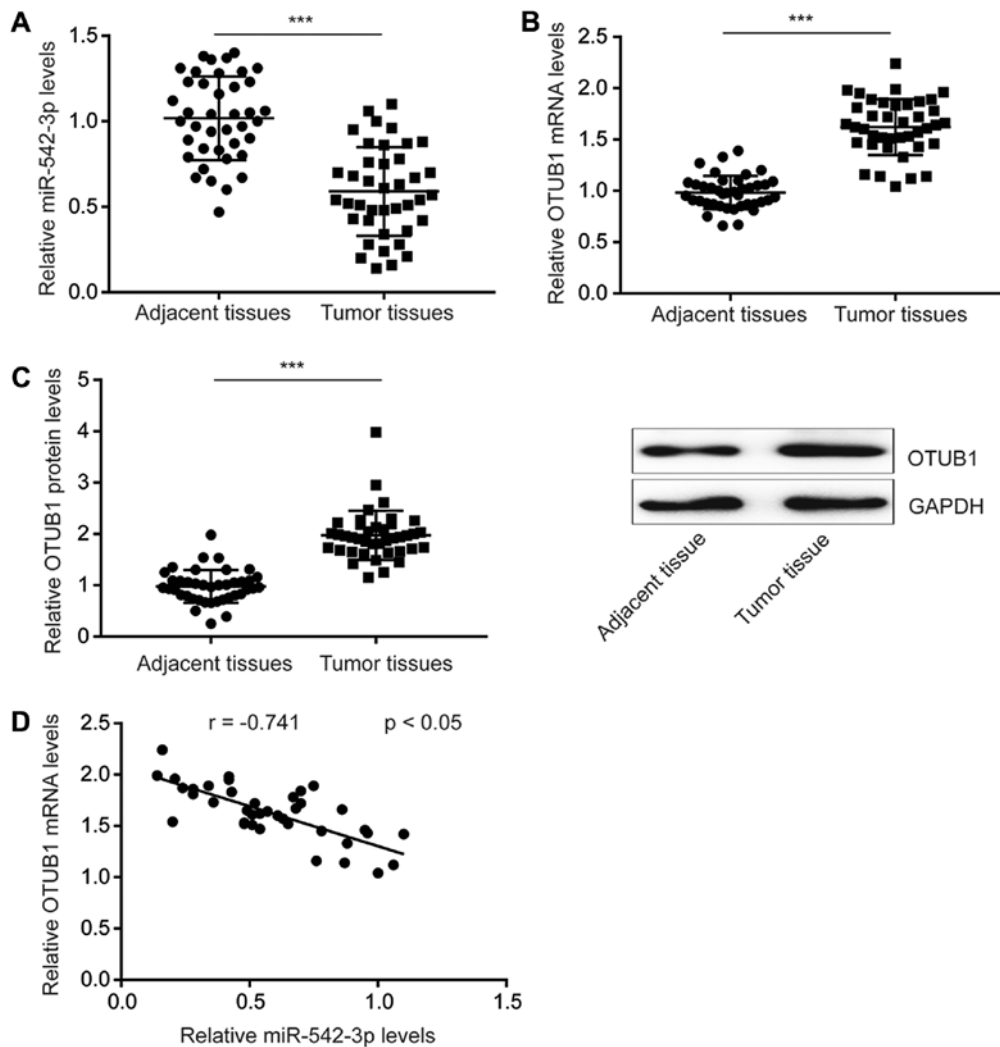


Figure 1. miR-542-3p expression is decreased in esophageal tumor tissues. (A) Compared with adjacent normal tissues, expression of miR-542-3p was decreased in tumor tissues from 40 patients with esophageal cancer. (B and C) Compared with adjacent normal tissues, expression of *OTUB1* (B) mRNA and (C) protein was increased in tumoral tissues from 40 patients with esophageal cancer. (D) Expression of miR-542-3p was negatively correlated with *OTUB1* mRNA expression in esophageal tumor tissues. *** $P < 0.0001$. miR-542-3p, microRNA-542-3p; *OTUB1*, ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1.

medium. Images were captured using an inverted microscope (Nikon Corporation) at 0 and 24 h to observe the cells (magnification, $\times 100$) that migrated into the wound area. The migrated areas were quantified using Image Pro Plus and normalized to the miR-NC mimic + pcDNA3.1 group.

Cell invasion assay. For the cell invasion assay, BD Matrigel Invasion Chambers (8- μ m pore; BD Biosciences) were used. In brief, KYSE150 cells (1×10^5) transfected with miR-NC mimic + pcDNA3.1, miR-542-3p mimic + pcDNA3.1 or miR-542-3p mimic + pcDNA3.1-OTUB1 were cultured in the upper chamber in serum-free DMEM at 37°C. Following 72 h of invasion, cells on the upper side of the filter were removed and cells that invaded to the underside of the membranes were fixed using 8% formaldehyde at room temperature for 15 min, followed by staining with crystal violet at room temperature for 30 min. The number of invaded cells ($\times 400$) was counted using a light microscope (Olympus Corporation).

Statistical analysis. Data were analyzed with GraphPad Prism 7 (GraphPad Software, Inc.) and presented as the

mean \pm standard error of the mean. For *in vitro* experiments, the statistical differences were evaluated using Student's t-test (two groups) or using ANOVA followed by Newman Keuls method (three groups). For *ex vivo* experiments in tumor tissues, the statistical analyses were performed using paired Student's t-test (two groups). Pearson's correlation analysis was performed to determine the correlation between miR-542-3p and *OTUB1* mRNA expression levels in esophageal tumor tissues. $P < 0.05$ was considered to indicate a statistically significant difference. All experiments were performed thrice.

Results

miR-542-3p is downregulated in esophageal tumor tissues. To investigate the role of miR-542-3p in esophageal cancer, RT-qPCR was performed to detect miR-542-3p expression levels in tumoral and adjacent normal tissues from 40 patients with esophageal cancer. The results demonstrated that the expression of miR-542-3p was significantly downregulated in tumor tissues compared with adjacent normal tissues (Fig. 1A).

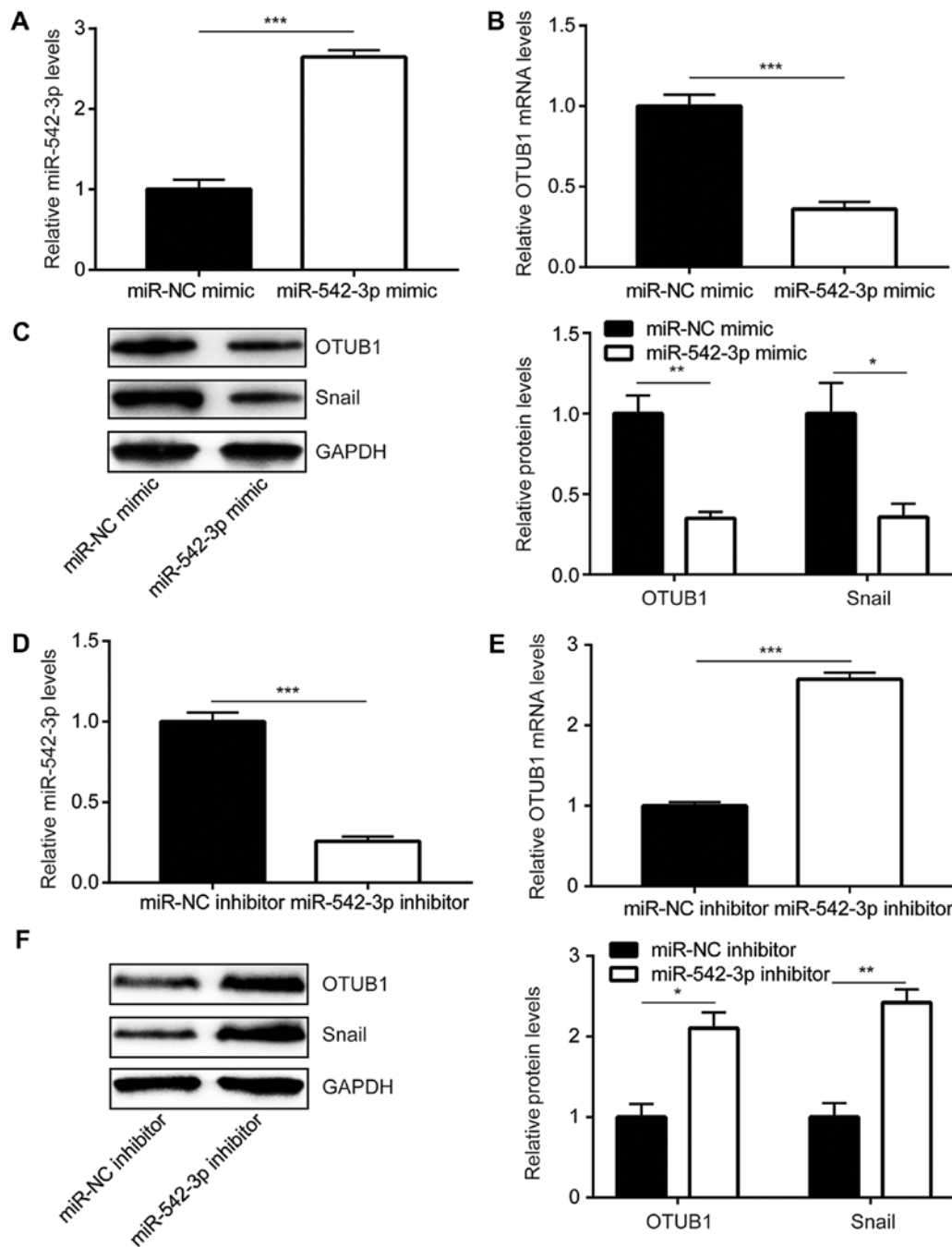


Figure 2. miR-542-3p represses *OTUB1* expression in esophageal cancer cells. (A) Transfection with miR-542-3p mimic successfully increased miR-542-3p expression in KYSE150 cells. (B and C) Overexpression of miR-542-3p decreased (B) *OTUB1* mRNA and (C) *OTUB1* and Snail protein expression in KYSE150 cells. (D) Transfection of miR-542-3p inhibitor successfully decreased miR-542-3p expression in KYSE150 cells. (E and F) Downregulation of miR-542-3p increased (E) *OTUB1* mRNA and (F) *OTUB1* and Snail protein expression in KYSE150 cells. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$. miR-542-3p, microRNA-542-3p; NC, negative control; *OTUB1*, ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1.

OTUB1 is a newly identified oncogene in esophageal cancer and has been reported to be regulated by miR-542-3p in colorectal cancer (13,18).

In the present study, *OTUB1* mRNA levels were significantly increased in tumor tissues compared with adjacent normal tissues (Fig. 1B). Western blotting revealed that the protein expression of *OTUB1* was also significantly increased in tumor tissues compared adjacent normal tissues (Fig. 1C). In addition, Pearson's correlation analysis indicated that expression of miR-542-3p was significantly negatively correlated with *OTUB1* mRNA expression levels in esophageal tumor tissues (Fig. 1D).

These results suggested that miR-542-3p may inhibit esophageal cancer progression and act as a tumor suppressor.

Expression of OTUB1 is repressed by miR-542-3p in esophageal cancer cells. To further study the regulatory association between *OTUB1* and miR-542-3p, miR-542-3p mimic was used to elevate miR-542-3p expression in KYSE150 cells. Compared with cells transfected with miR-NC mimic, transfection with miR-542-3p mimic significantly increased miR-542-3p expression in KYSE150 cells (Fig. 2A). Overexpression of miR-542-3p significantly decreased *OTUB1* mRNA levels and

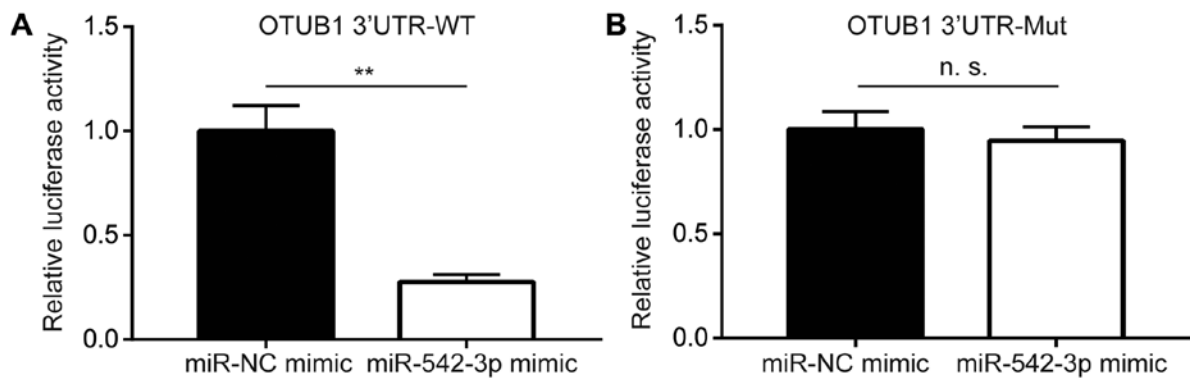


Figure 3. *OTUB1* is a target gene for miR-542-3p in esophageal cancer cells. (A) miR-542-3p mimic reduced luciferase activity of KYSE150 cells transfected with OTUB1 3'UTR-WT. (B) miR-542-3p mimic did not alter luciferase activity of KYSE150 cells co-transfected with OTUB1 3'UTR-Mut. **P<0.01. miR-542-3p, microRNA-542-3p; Mut, mutant; NC, negative control; n.s., not significant; *OTUB1*, ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1; UTR, untranslated region; WT, wild-type.

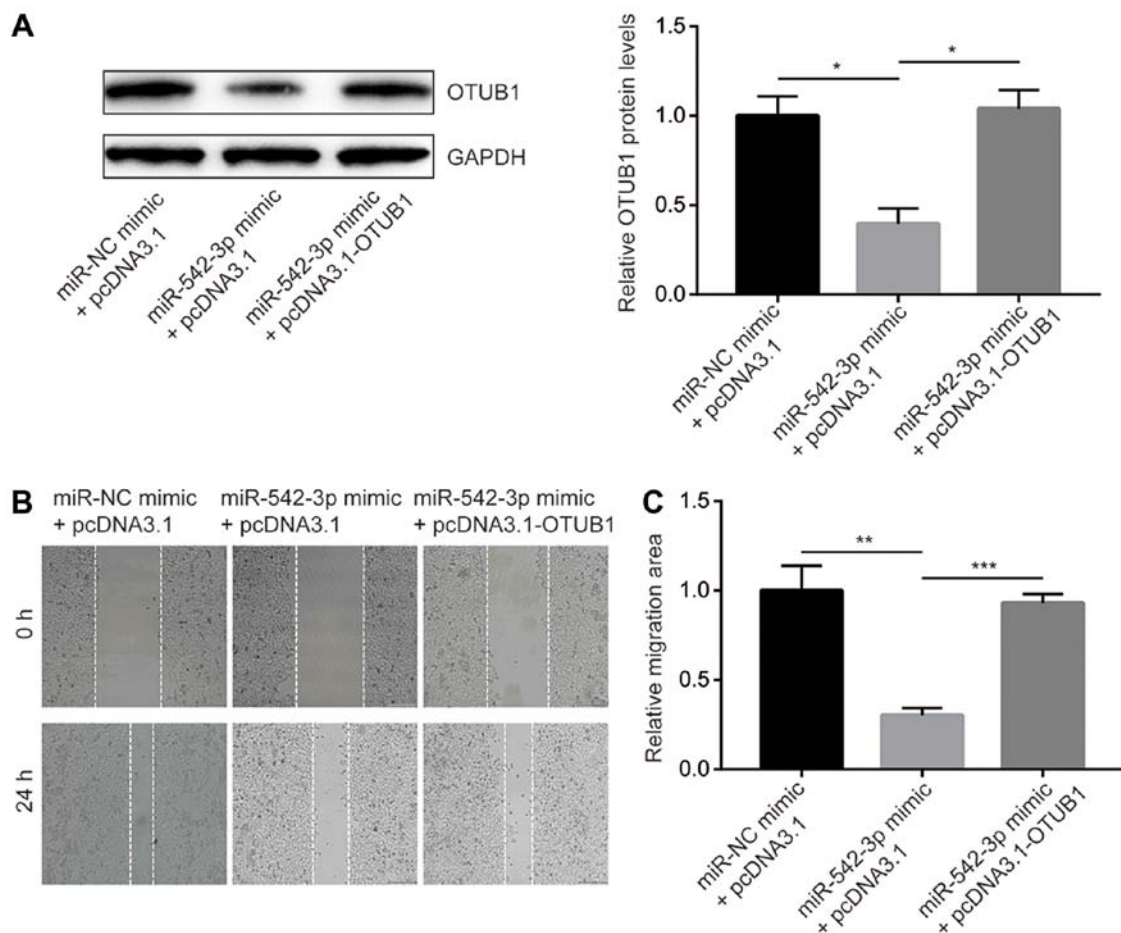


Figure 4. miR-542-3p inhibits migration of KYSE150 cells through the regulation of OTUB1. (A) Overexpression of miR-542-3p reduced OTUB1 protein expression in KYSE150 cells, which was reversed by co-transfection of recombinant *OTUB1*. (B) Overexpression of miR-542-3p inhibited the migratory ability of KYSE150 cells, which was reversed by co-transfection of recombinant *OTUB1*. (C) The migrated areas in B were quantified using Image Pro Plus and normalized to the miR-NC mimic + pcDNA3.1 group. *P<0.05, **P<0.01 and ***P<0.0001. miR-542-3p, microRNA-542-3p; NC, negative control; *OTUB1*, ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1.

OTUB1 protein levels in cells (Fig. 2B and C). Conversely, transfection with miR-542-3p inhibitor significantly decreased miR-542-3p expression in KYSE150 cells and led to an increased expression of *OTUB1* at mRNA and protein levels (Fig. 2D-F). These data demonstrated that miR-542-3p may negatively regulate *OTUB1* in esophageal cancer cells.

OTUB1 is a target gene of miR-542-3p in esophageal cancer cells. To confirm whether miR-542-3p directly regulated *OTUB1* expression, dual-luciferase reporter assay was performed in KYSE150 cells. Transfection of miR-542-3p mimic significantly reduced luciferase activity of OTUB1 3'UTR-WT compared with cells transfected with miR-NC

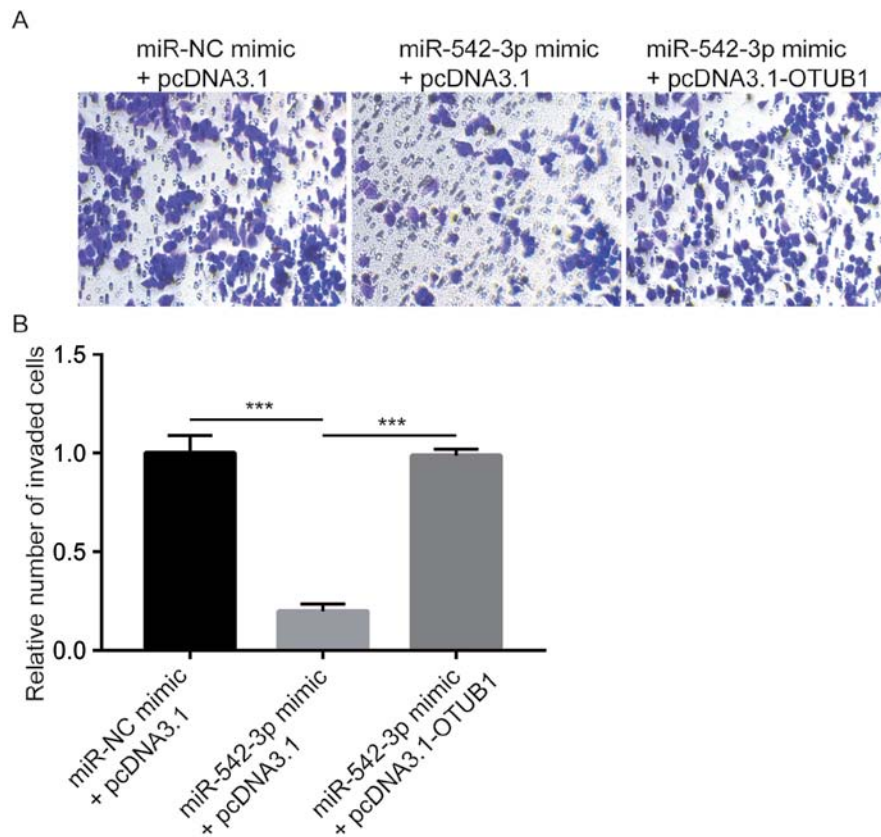


Figure 5. miR-542-3p inhibits invasion of KYSE150 cells. (A) Overexpression of miR-542-3p inhibited the invasive ability of KYSE150 cells, which was reversed by co-transfection with recombinant *OTUB1*. (B) Quantification of relative invaded cell number in A. *** $P < 0.0001$. miR-542-3p, microRNA-542-3p; *OTUB1*, ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1.

mimic (Fig. 3A), whereas overexpression of miR-542-3p did not alter the luciferase activity of *OTUB1* 3'UTR-Mut (Fig. 3B). These data suggested that miR-542-3p may bind directly to the putative binding site in 3'UTR of *OTUB1* to repress its expression.

miR-542-3p inhibits esophageal cancer cell migration and invasion through regulation of OTUB1. To study the biological function of miR-542-3p in esophageal cancer cells, wound healing and cell invasion assays were performed to detect cell migratory and invasive ability of cells transfected with the miR-542-3p mimic either with or without overexpression of *OTUB1*.

Transfection of miR-542-3p mimic reduced *OTUB1* protein expression whereas co-transfection of miR-542-3p mimic and recombinant *OTUB1* reversed the downregulation of *OTUB1* in KYSE150 cells (Fig. 4A). In the wound healing assays, overexpression of miR-542-3p significantly inhibited cell migration towards the wound areas, which indicated that the migration ability of cells was reduced (Fig. 4B and C). In addition, overexpression of *OTUB1* reversed migration inhibition induced by miR-542-3p mimic (Fig. 4B and C). Similarly, overexpression of miR-542-3p significantly inhibited the invasive ability of the cells, which was reversed by overexpression of *OTUB1* (Fig. 5). The results demonstrated that miR-542-3p may inhibit the migratory and invasive abilities of esophageal cancer cells through repression of *OTUB1*.

Discussion

miRNAs regulate a number of oncogenes and tumor suppressors in cells, which can lead to the initiation and progression of cancer (21,22). Several miRNAs have been identified as key regulators of esophageal cancer development and accurate predictors of clinical outcome for patients with esophageal cancer (23-25). Decreased expression of miR-542-3p has been observed in certain types of cancers, including hepatocellular carcinoma, osteosarcoma, colorectal cancer and melanoma (13,26-29). In the present study, miR-542-3p expression and function were investigated in esophageal cancer. RT-qPCR revealed that miR-542-3p expression was decreased in tumor tissues compared with adjacent normal tissues from patients with esophageal cancer. In KYSE150 cells, overexpression of miR-542-3p markedly reduced cell migratory and invasive abilities. Thus, consistent with its tumor suppressor role in other cancer types, miR-542-3p may also function as a tumor suppressor in esophageal cancer.

OTUB1 is a cysteine protease that removes ubiquitin from modified proteins to stabilize target proteins (30). *OTUB1* is involved in the regulation of several proteins that are pivotal for the progression of cancer, such as estrogen receptor, p53 and forkhead box M1 (17,30,31). Recently, *OTUB1* was demonstrated to promote the metastasis of esophageal cancer by stabilizing Snail (18). miR-542-3p has been demonstrated to bind to 3'UTR of *OTUB1* mRNA to downregulate *OTUB1* in colorectal cancer cells (13).

Consistent with the previous study, an increase in *OTUB1* mRNA expression was observed in tumor tissues compared with adjacent normal tissues from patients with esophageal cancer. Additionally, *OTUB1* expression levels were inversely correlated with miR-542-3p expression levels in tumor tissues, and in KYSE150 cells, overexpression of miR-542-3p decreased *OTUB1* expression.

Snail is a member of the *Snail* superfamily, which functions in cell survival and cell differentiation of cancer cells (32). Furthermore, the development and metastasis of cancer were blocked by *Snail* suppression (33). Recently, *Snail* silencing was found to inhibit cell migration of esophageal cells (34). Notably, in KYSE150 cells, overexpression of miR-542-3p decreased *Snail* expression.

Finally, cell migration and invasion inhibition induced by miR-542-3p overexpression was partially attenuated by co-transfection of recombinant *OTUB1* in KYSE150 cells. These results demonstrated that miR-542-3p may regulate *OTUB1* to inhibit cell metastasis of esophageal cancer, which were consistent with a previous study about the role of *OTUB1* in esophageal cancer (18). Future studies are needed to determine whether the expression of miR-542-3p may be used as a biomarker to predict distant metastasis and overall survival of patients with esophageal cancer.

The results of the present study indicated a potential tumor suppressor role for miR-542-3p in esophageal cancer. Overexpression of miR-542-3p inhibited migration and invasion of esophageal cancer cells. Therefore, upregulation of miR-542-3p may be a potential treatment approach for patients with esophageal cancer.

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 on reasonable request.

Authors' contributions

JuS and WY designed the study. JuS, JiS and YD acquired and interpreted the data. YD and JiS collected clinical samples. WY prepared the manuscript and supervised the study.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Sheyang People's Hospital (Sheyang, China). All patients signed written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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