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Original Article

miR-542-3p inhibits colorectal cancer cell proliferation, migration and invasion by targeting OTUB1

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Abstract: Although miR-542-3p has been found to be aberrantly downregulated in variety of human tumors, little is known about its role in colorectal cancer (CRC). This study was designed to assess the prognostic value of miR-542-3p in CRC by examining the expression profile of miR-542-3p in patients with CRC and investigate the possible molecular mechanism underlying the function of miR-542-3p. Our results showed that low levels of miR-542-3p were significantly associated with advanced tumor stage and lymph node metastasis and miR-542-3p can serve as an independent prognostic marker for CRC. Furthermore, ectopic induced expression of miR-542-3p significantly suppressed cell proliferation, induced apoptosis, inhibited migration and invasion in vitro and in vivo. Mechanistically, we identified OTUB1 as a direct and functional target for miR-542-3p, at least partly responsible for the anti-tumor effect of miR-542-3p in CRC. Our study demonstrates the importance of miR-524-3p/OTUB1 signaling in CRC development and suggests that targeting this signaling may highlight a new therapeutic approach for treatment of CRC.

Keywords: Colorectal cancer, miR-542-3p, OTUB1

Introduction

Colorectal cancer (CRC) ranks the third most common human malignancies, accounting for one million new diagnoses and over 693,900 deaths worldwide each year [1]. In China, the incidence of CRC has been increasing and now is the fourth most common cancer [2, 3]. With recent advances of comprehensive CRC treatment, patients who are diagnosed early with only localized disease can be cured. However, in most CRC patients, patients present with metastatic disease by the time it is diagnosed, resulting in a low five-year survival rate [4]. Therefore, identifying the factors involved in CRC tumorigenesis and progression is imperative in order to identify novel potential targets for improving the clinical outcome of patients with metastatic CRC.

As a posttranslational modification mechanism, ubiquitination can regulate the protein function by interfering its degradation, modifying its activity, adjusting its function, changing its subcellular location and altering protein-protein interactions [5]. Interestingly, ubiquitination is reversible process which can be is a dynamic process and can be reversed by biological processes catalysed by ubiquitin hydrolases or deubiquitinating enzymes called DUBs. Deubiquitinating enzymes (DUBs) confer to a number of proteases that can promote the cleavage of monoubiquitin or polyubiquitin from target proteins. The involvement of a number of DUBs, including USP46, USP22, UCHL1, and USP9X, in the proliferation, metastasis, and drug resistance of CRC has been documented [6-9]. Among these DUBs, OTUB1 (OTU domain-containing ubiquitin aldehyde-binding proteins 1; also called Otubain 1), which is a member of ovarian tumor domain protease (OTU) subfamily of DUBs, has attracted the attention of researchers due to its ability to interfere ubiquitination and regulate the stability and funcyion of protein through a non-canonical mechanism in addition to conventional catalytic process. In the context of CRC, preclinical and clinical evi-

dence has showed that OTUB1 promotes CRC metastasis by facilitating epithelial-to-mesenchymal transition (EMT) and acts as a potential distant metastasis marker and prognostic factor in CRC, making OTUB1 an appealing therapeutic target for CRC.

Lately, a family of endogenous non-coding mRNA molecules with a length of 18-25 nucleotides called microRNAs has attracted a lot of attention due to their roles in tumorigenicity and tumor progression [10]. MiRNAs function either as oncogene or tumor suppressor by regulating the expression of target protein at the post-transcriptional level by binding the 3'-UTR portion of mRNAs through translational repression or degradation [11]. By modulating different target genes, miRNA have been found to play a role in a variety of cellular activities such as cell apoptosis, cell proliferation, invasion, migration and stem cell differentiation [12-14]. MiR-542-3p, located in Xq26.3, has been found down-regulated in a variety of human malignancies, including esophageal squamous cell carcinoma [15], astrocytoma [16], and bladder cancer [17]. However, the role f miR-542-3p in CRC is largely unclear. In this study, we examined the expression profile of miR-542-3p in patients with CRC to assess the prognostic value of miR-542-3p in CRC. Moreover, we investigate the possible molecular mechanism underlying the function of miR-542-3p is that OTUB1 is a novel target gene of miR-542-3p.

Materials and methods

Clinical tissue samples

The clinical study protocol was reviewed and approved by Medical Ethics Committee of The Affiliated Cancer Hospital of Zhengzhou University and consent forms were signed by all patients or guardians. Between January, 2011 and March, 2016, 69 CRC samples along with matched normal tissues from CRC patients were collected in the Affiliated Cancer Hospital of Zhengzhou University. Patients who have undergone chemotherapy or radiotherapy before the surgery or patients with familial adenomatous polyposis CRC were excluded from the study. The collected tissue was snap frozen and kept in liquid (-70°C) until analysis. All samples were blindly examined by two senior pathologists for diagnosis and histological classification. The expression level of miR-340 in tissue was determined by quantitative RT-PCR. The expression of OTUB1 was analysed by automated capillary western blot (WES) as previously described [18].

Cell lines and cultures

Human CRC cells LOVO, HT29, SW620, SW480, HCT116 were purchased from American Type Culture Collection (ATCC). Normal human colon cell NCM460 cells were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were maintained in DMEM medium (Invitrogen, Carlsbad, CA) containing 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA) and 10% fetal bovine serum(FBS; Invitrogen, Carlsbad, CA). Cells were cultured in a humidified incubator at 37°C with 5% CO_2.

Quantitative real-time PCR (qRT-PCR)

TRIzol Reagent (Life Technology, Carlsbad, CA) was used to isolate total RNA from cultured CRC cells. The PrimeScriptTM RT Master Mix (Takara, Dalian, China) was used to synthesize First strand cDNA. Then the expression level of OTUB1 mRNA was determined by RT-PCR using Power SYBR Green PCR Master Mix (Carlsbad, CA) with GAPDH was used as an internal control. The forward and reversed primer sequences synthesized by Sangon (Shanghai, China) based on published sequence [19]. To assess the miR-542-3p in cells and tissues, miRVANA Kit (Ambion, Carlsbad, MA) was utilized to isolate miRNA. Then miR-542-3p level was measured by RT-PCR using the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA) with U6 as an internal control. The $2^{-\Delta\Delta Ct}$ method was used to analyse the relative expression of target genes [20].

Western blotting analysis

Proteins were isolated from cultured cell using lysis buffer (Beyotime, Shanghai, China). Extracted proteins were then subjected to SDS-PAGE for separation before transferred onto a PVDF membrane (Millipore, Billerica, MA) for protein probing. Proteins were detected with specific primary antibodies. Goat anti-rabbit IgG-HRP used as the second antibodies were purchased from Beyotime(Shanghai, China). Protein blots were visualized with enhanced chemiluminescent substrate (Thermo Fisher

Scientific, Waltham, MA) and analyzed using BandScan software (Glyko, Novato, CA).

Luciferase reporter assay

The luciferase reporter assay was performed as previously described [21]. Briefly, OTUB1 3'UTR region including miR-542-3p binding sites were amplified by PCR from HCT116 cells and inserted into a pMIR-REPORT vector (Applied Biosystems., Carsbad, CA). HCT116 cells were co-transfected with luciferase reporter vector and miR-542-3p mimic (or negative control, NC) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). 48 hours post transfection, the luciferase activity was measured using a luciferase assay kit (Promega., Madison, WI).

Transfection of miR-542-3p mimic

The lentiviral constructs of miR-542-3p mimics, anti-miR-542-3p (miR-542-3p inhibitor) and negative control were synthesized by Genepharma (Shanghai, China). HCT116 and LOVO cells were infected with the constructed lentivirus to induce ectopic expression of miR-542-3p while SW480 and SW620 cells were transfected with miR-542-3p inhibitor to suppress the constitutive expression of miR-542-3p.

Re-expression of OTUB1 in CRC cells

The expression of OTUB1 was restored with a vector constructed as previously described with an empty vector as control [19]. The construct was verified by sequencing. HCT116 and LOVO cells were transfected with vectors using Lipofectamine 3000 (Invitrogen, Carlsbad, CA).

Cell proliferation assay

Cell Counting Kit-8 (Beyotime, Shanghai, China) was used to assess the cell proliferation. Briefly, a total of 1×10^5 cells were plated in culture plates. Following an incubation of indicated time, the viable cells were examined by measuring absorbance at 450 nm (Tecan Group Ltd, Männedorf, Switzerland).

Flow cytometry

Following treatment, CRC cells were harvested and stained with Annexin V-PE and propidium iodide using an Apoptosis kit (BD Pharmingen, Franklin Lakes, NJ) according to the manufacturer's instructions. Then the apoptotic percentage of treated cells was determined by a flow cytometer (Beckman Coulter Inc., Miami, FL).

Wound scratch assay

Cells were seeded into 6-well plates and cultured to 90% confluence. The confluent cell monolayer was wounded using a sterile 200 μL pipette tip. The suspended cells were washed using normal growth medium. The scratch wound was captured after 24 hours using a microscope in three fields of view at 100× magnification. The cellular migration was determined by the ratios of decreased open area after 24 hours relative to the open area at 0 hours. Three independent assays were performed.

Transwell invasion assay

24-well Transwells coated with Matrigel (8-μm pore size; BD Biosciences, San Jose, CA) were used for cell invasion assays. Equal numbers (1×10^5) of non-transfected cells as well as cells stably transfected were plated on separate wells. Cells were cultured overnight in serum-free medium before trypsinization and re-suspended at a density of 2×10^5 cells/ml in DMEM containing 1% FBS. The cells were loaded to the upper chamber, with MEM containing 10% FBS as chemoattractant in the lower chamber. The medium containing 1% FBS in the lower chamber was used as a control. The Matrigel and the cells remaining in the upper chamber were removed by cotton swabs following 24-hour incubation. The cells in the lower surface of the membrane were stained with hematoxylin after the cells were fixed with formaldehyde solution. The cells in at least five random microscopic fields (×200) were counted and photographed.

In vivo xenograft model

The protocol of animal experiments were reviewed and approved by The Affiliated Cancer Hospital of Zhengzhou University. For tumor growth assay, BALB/c nude mice of four-week-old were used for the CRC xenograft models (n=6 per group). 1×10^7 LOVO cells transfected with control vector or miR-542-3p mimic construct were suspended in 100 μl medium was injected subcutaneously into the lower left flank regions of mice model. The tumor volume

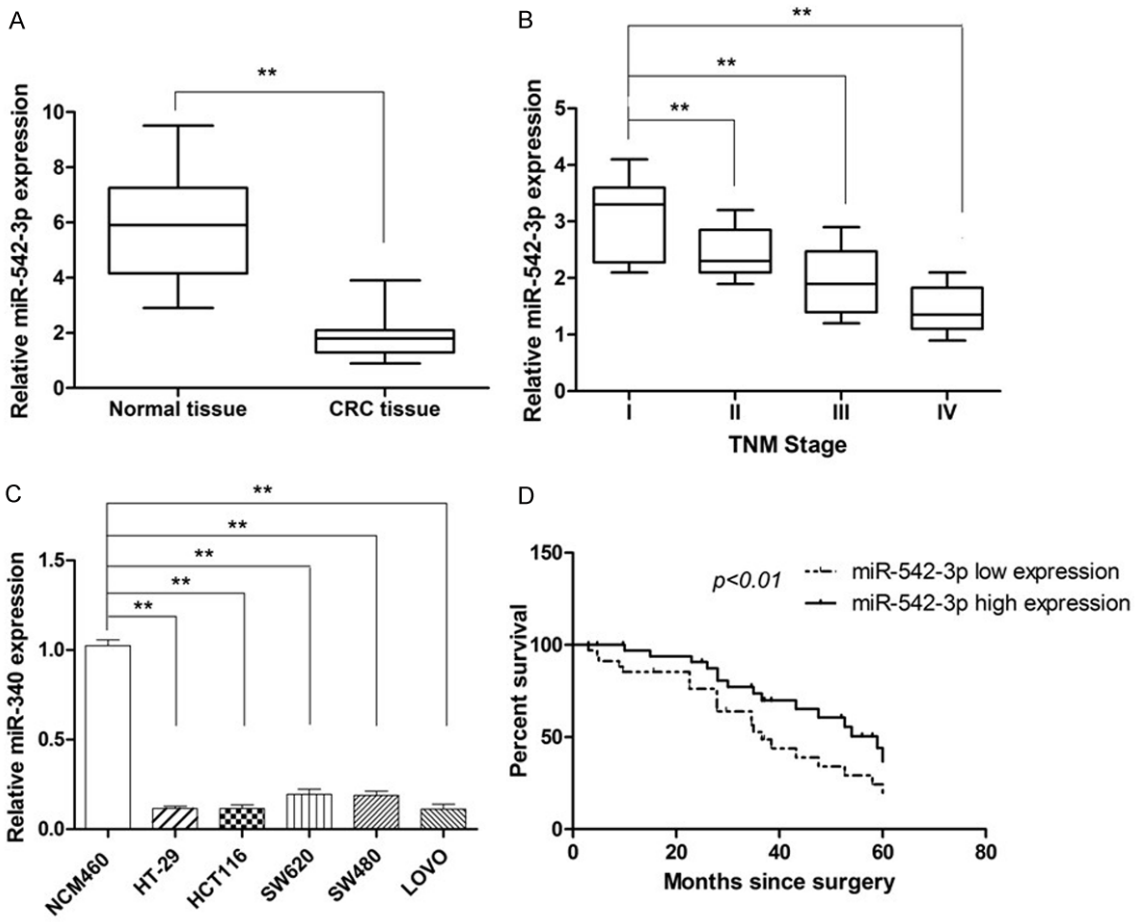

Figure 1. MiR-542-3p was downregulated in CRC tissues samples and cell lines, and associated with overall survival of CRC patients. A. MiR-542-3p was aberrantly low expressed in CRC tissue compared with normal tissue. B. Expression of miR-542-3p in CRC tissues correlated with TNM stage. C. MiR-542-3p was downregulated in human CRC cell lines compared with NCM cells. D. Low expression of miR-542-3p was associated with shorter overall survival of CRC patients. **P<0.01.

was measured every 3 days and the mice were sacrificed after 30 days. Immunohistochemistry assay was performed to detect the expression of cleaved caspase-3 and Ki-67.

For the tumor metastasis assays, BALB/C-nu/nu nude mice of six-week-old were used and 1×10^6 LOVO cells transfected with control vector or miR-542-3p mimic construct were injected into the lateral tail vein of mice model (n=3 per group). The mice were sacrificed 6 weeks later and the metastasis modules in lung were counted.

Statistical analysis

Values were presented as the mean ± SD. The comparison of miR-542-3p levels in tumor and normal tissue were performed using student's t test. Statistical comparisons between cell lines were performed by one-way ANOVA followed by Dunnett's t-test. The overall survival of patients was assessed by Kaplan-Meier survival analysis. GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) was used to analyze experimental data and a *P* value less than 0.05 was considered to be statistically significant.

Results

MiR-542-3p is downregulated in CRC tissues and cell lines

First, the level of miR-542-3p in CRC tissues and CRC cell lines were determined. As shown in **Figure 1A**, the expression level of miR-542-3p was significantly lower in CRC tissues relative to corresponding non-tumor tissues. Then, we examined the level of miR-542-3p in the tissue samples from patients of different TNM

Table 1. Association between miR-542-3p with clinicopathological characteristics of CRC patients

Variables	miR-542-3p expression		*P* value
	High (33)	Low (36)	
Age			0.4693
≤50	13	18	
>50	20	18	
Sex			0.2172
Male	23	19	
Female	10	17	
Tumor size			0.1484
≤5 cm	21	16	
>5 cm	12	20	
Histological grade			0.8095
Well, moderate	14	17	
Poor	19	19	
TNM stage			0.0030**
I and II	18	7	
III and IV	15	29	
Site			0.2172
Colon	23	19	
Rectum	10	17	
Lymph node metastasis			0.0079**
Absence	20	10	
Presence	13	26	

**P<0.01.

stage. Our findings revealed that miR-542-3p was decreased with advanced stage of CRC (**Figure 1B**). The level of miR-542-3p in CRC cell lines were also measured, which showed that miR-542-3p expression was suppressed in CRC cells compared with normal colon cell NCM460 (**Figure 1C**). Taken together, our results showed that the expression of miR-542-3p was suppressed in CRC.

MiR-542-3p expression correlates with clinicopathological parameters and predicts prognosis

To establish the association between level of miR-542-3p and clinicopathological features of CRC patients, CRC tissues expressing miR-542-3p at levels less than the median expression level were allocated to the low group while and those samples with expression above the median value were allocated to the high expression group. As listed in **Table 1**, the statistic analysis of 91 CRC cases showed that low levels of miR-542-3p were significantly associated with advanced tumor stage and lymph node metastasis. In contrast, no significant correlation was found between miR-542-3p levels with other clinicopathological features, such as histological grade, tumor location and tumor size. To further evaluate the prognostic significance of miR-542-3p, a Kaplan-Meier survival curve was plotted. As show in **Figure 1D**, our results showed that decrease in miR-542-3p expression significantly correlated with shorter median survival time (36.6 months for low miR-542-3p group vs. 59 months for high miR-542-3p group). Furthermore, both univariable and multivariable analysis were performed to assess the prognostic value of miR-542-3p. As shown in **Table 2**, the overall survival of patients significantly correlated with miR-542-3p expression, absence of lymph node metastasis and TNM stage and miR-542-3p can be considered as an independent prognostic marker for overall survival of patients with CRC.

MiR-542-3p inhibits CRC cell growth and induces apoptosis in vitro and in vivo

To investigate the role of miR-542-3p in the tumor growth of CRC, miR-542-3p mimic and miR-542-3p inhibitor were used to change the expression of miR-542-3p in CRC cells. As shown in **Figure 2A**, the expression of miR-542-3p was significantly increased by miR-542-3p mimic whereas decreased by miR-542-3p inhibitor in CRC cell lines. Then proliferation and apoptosis assay as performed to explore the role of miR-542-3p in these cellular activities. As presented in **Figure 2B**, the proliferation of both LOVO and HCT116 cells were significant suppressed by miR-542-3p overexpression. The flow cytometric analysis showed that miR-542-3p significantly induced apoptosis in both LOVO and HCT116 cell lines (**Figure 2C**). Moreover the anti-proliferative and apoptosis-inducing effect of miR-542-3p was supported by the results that miR-542-3p significantly repressed the level of p-Akt and Bcl-2 while significantly elevated the level of Bax and cleaves caspase-3 (**Figure 2D**). In line with the in vitro results, in vivo experiments also showed that LOVO cells transfected with miR-542-3p correlated with significantly suppressed tumor growth along with lower expression of Ki-67 and higher level of activated caspase-3 (**Figure**

Table 2. The prognostic value of miR-542-3p

Variable	Univariable analysis		Multivariable analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender (Male or Female)	1.123 (0.621-2.965)	0.452	-	-
Age (≤50/>50)	1.321 (0.891-3.158)	0.865	-	-
Histological grade (Well, moderate/Poor)	1.369 (0.658-3.697)	0.322	-	-
Tumor size (≤5 cm/>5 cm)	1.545 (1.118-3.852)	0.218	-	-
Lymph node metastasis (Absence/Presence)	5.698 (2.682-10.945)	P<0.001	4.568 (2.987-9.245)	0.021*
TNM stage (I/II/III/IV)	3.568 (1.441-9.672)	0.003**	3.201 (2.129-7.652)	0.008**
miR-542-3p expression (high/low)	2.364 (1.115-6.324)	0.009**	1.852 (1.019-3.326)	0.012*

*P<0.05, **P<0.01.

2E). In contrast, in two CRC cells lines SW480 and SW620, which have relatively high endogenous level of miR-542-3p, miR-542-3p knockdown resulted in significantly enhancement in cell proliferation and decrease in apoptotic cell population (**Figure 2F** and **2G**). Collectively, these data suggested that miR-542-3p functions as tumor suppressor and ectopic overexpression of miR-542-3p suppresses tumor growth.

MiR-542-3p suppresses CRC cell invasion in vitro and metastasis in vivo

Given that miR-542-3p expression is associated with metastasis of CRC, we examined the effect of miR-542-3p on cell invasion and tumor metastasis. As shown in **Figure 3A**, wound scratch assay clearly demonstrated that miR-542-3p mimic transfection significantly suppresses the migration of CRC cells. The invasive capacity was determined by Transwell assay, which showed that miR-542-3p markedly suppressed the invasion of both tested cell lines (**Figure 3B**). The suppression of migration and invasion was also associated with downregulation of metastasis makers such as MMP-2 and MMP-9 (**Figure 3C**). In addition, the expression levels of EMT markers E-cadherin and N-cadherin were also modulated by miR-542-3p (**Figure 3C**). Then an in vivo metastasis model was established by injecting LOVO cells with stable ectopic overexpression of miR-542-3p into nude mice through the tail vein. The LOVO cells transfected with empty vector was injected as control. The metastasis of tumor was evaluated by examining the metastatic nodules in the lungs. As shown in **Figure 3D**, both nodule numbers and sized was remarkably decreased in the miR-542-3p groups when compared with the control groups. Conversely, the migration and invasiveness of SW480 and SW620 increased when endogenous miR-542-3p was knockdown with a miR-542-3p inhibitor (**Figure 3E** and **3F**). Taken together, our results indicated that miR-542-3p significantly suppresses CRC cell invasion in vitro and metastasis in vivo.

OTUB1 is a direct target of miR-542-3p in CRC cells

To elucidate the molecular mechanisms for the anti-cancer effect of miR-542-3p, candidate target genes of 542-3p was searched in bioinformatics database (MicroCosm and Target scan). Based on the results from bioinformatic algorithm, we postulated that miR-542-3p might exert anti-tumor effect by targeting OTUB1. The putative binding sites for miR-542-3p in the 3'-UTR of OTUB1 were shown in **Figure 4A**. To further investigate the correlation between the expression levels of miR-542-3p and OTUB1, 30 tissue specimens were randomly chosen for RT-PCR and simple western analysis. As shown in **Figure 4B** and **4C**, both the protein and mRNA levels of OTUB1 as significantly inversely associated with miR-542-3p levels. Moreover, the mRNA and protein levels of OTUB1 in CRC cells transfected with miR-542-3p mimic and miR-542-3p inhibitor were also examined to further demonstrate the regulatory role of miR-542-3p on OTUB1. As we expected, miR-542-3p overexpression led to a significant decrease in OTUB1 mRNA and protein expression compared with parental cell lines (**Figure 4D**). On the other hand, downregulation of miR-542-3p was associated with significantly higher expression of both OTUB1 mRNA and protein (**Figure 4E**). Taken together,

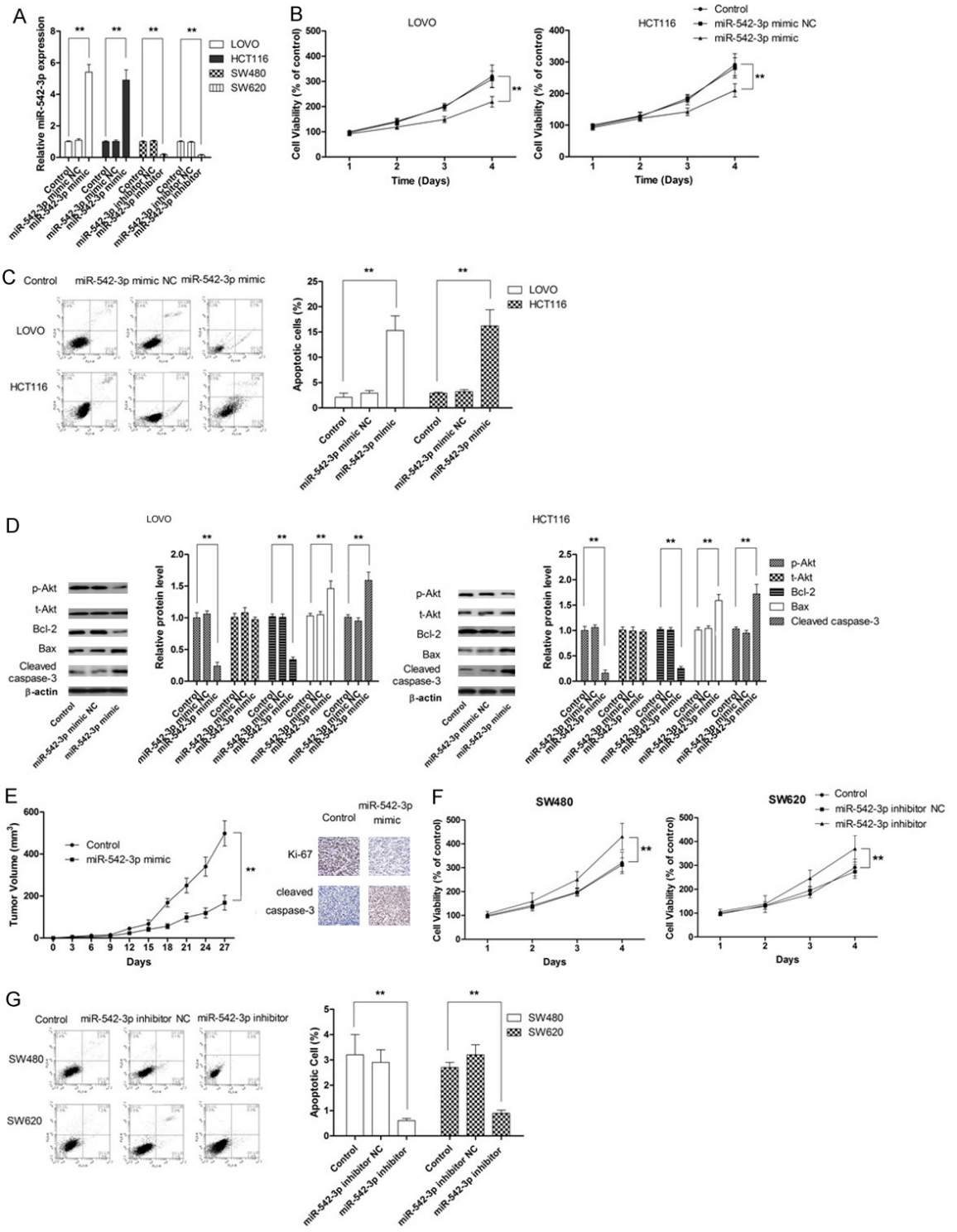

Figure 2. MiR-542-3p suppressed cell growth and induced apoptosis in vitro and in vivo. A. Expression of miR-542-3p was successful manipulated by miR-542-3p mimic and inhibitor. B. Ectopic overexpression of miR-542-3p inhibited CRC cell proliferation. C. Ectopic overexpression of miR-542-3p induced apoptosis in CRC cells. D. Ectopic overexpression of miR-542-3p modulated the expression of apoptotic and cell proliferation markers in CRC cells. E. MiR-542-3p suppressed the tumor growth in vivo along with decrease in Ki-67 expression and increase in cleaved caspase-3 in tumor tissue. F. Knockdown of miR-542-3p promoted cell proliferation in CRC cells. G. Knockdown of miR-542-3p protected CRC cells against apoptosis. **P<0.01.

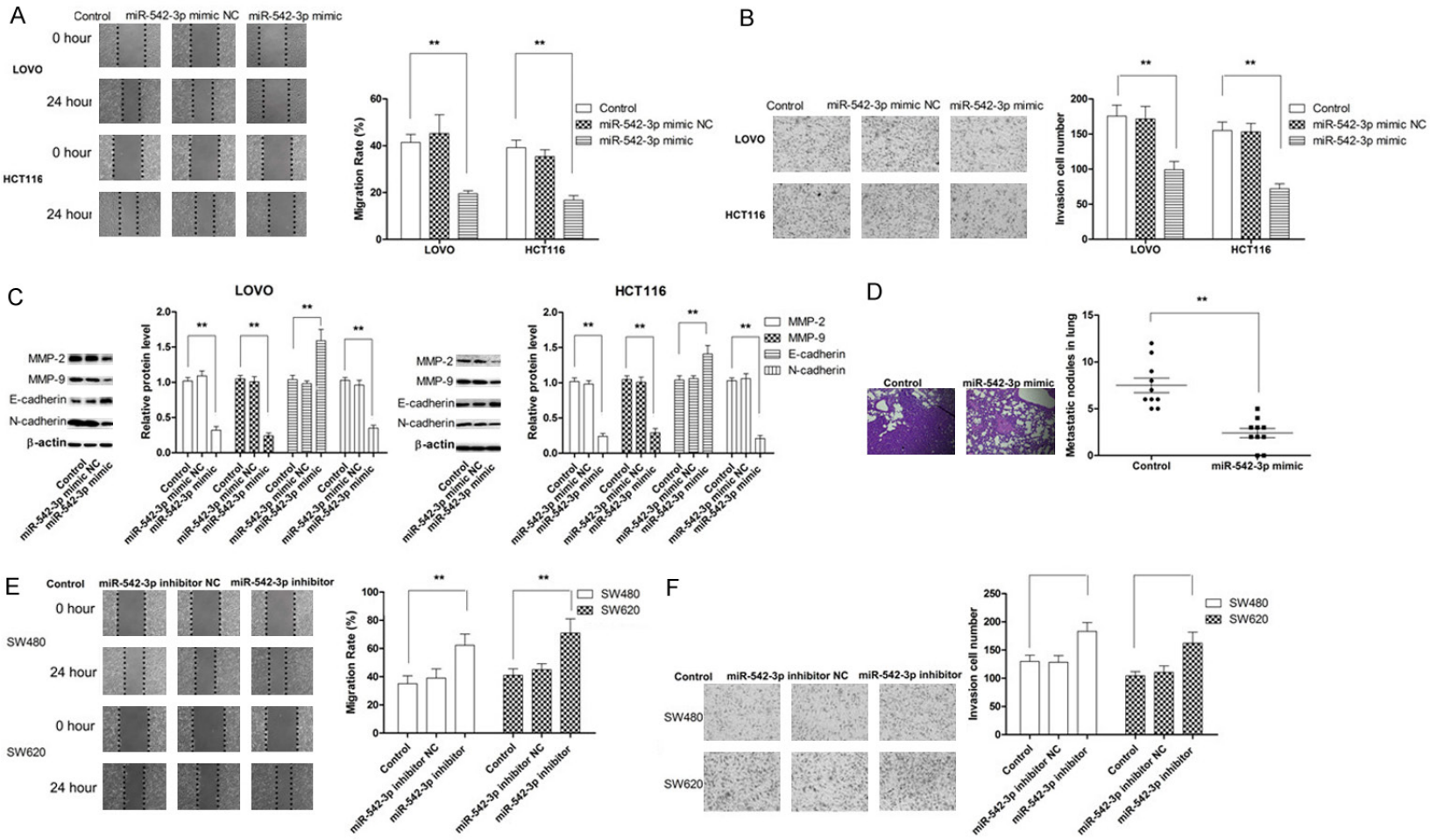

Figure 3. MiR-542-3p inhibited cell invasion and tumor metastasis. A. Ectopic overexpression of miR-542-3p suppressed the migration of CRC cells. B. Ectopic overexpression of miR-542-3p inhibited the invasiveness of CRC cells. C. Ectopic overexpression of miR-542-3p regulated the expression of marker molecules involved in invasion and EMT. D. MiR-542-3p associated with significantly less metastatic nodules in lungs of mice model. E. Knockdown of miR-542-3p promoted cell migration. F. Knockdown of miR-542-3p enhanced cell invasion. **P<0.01.

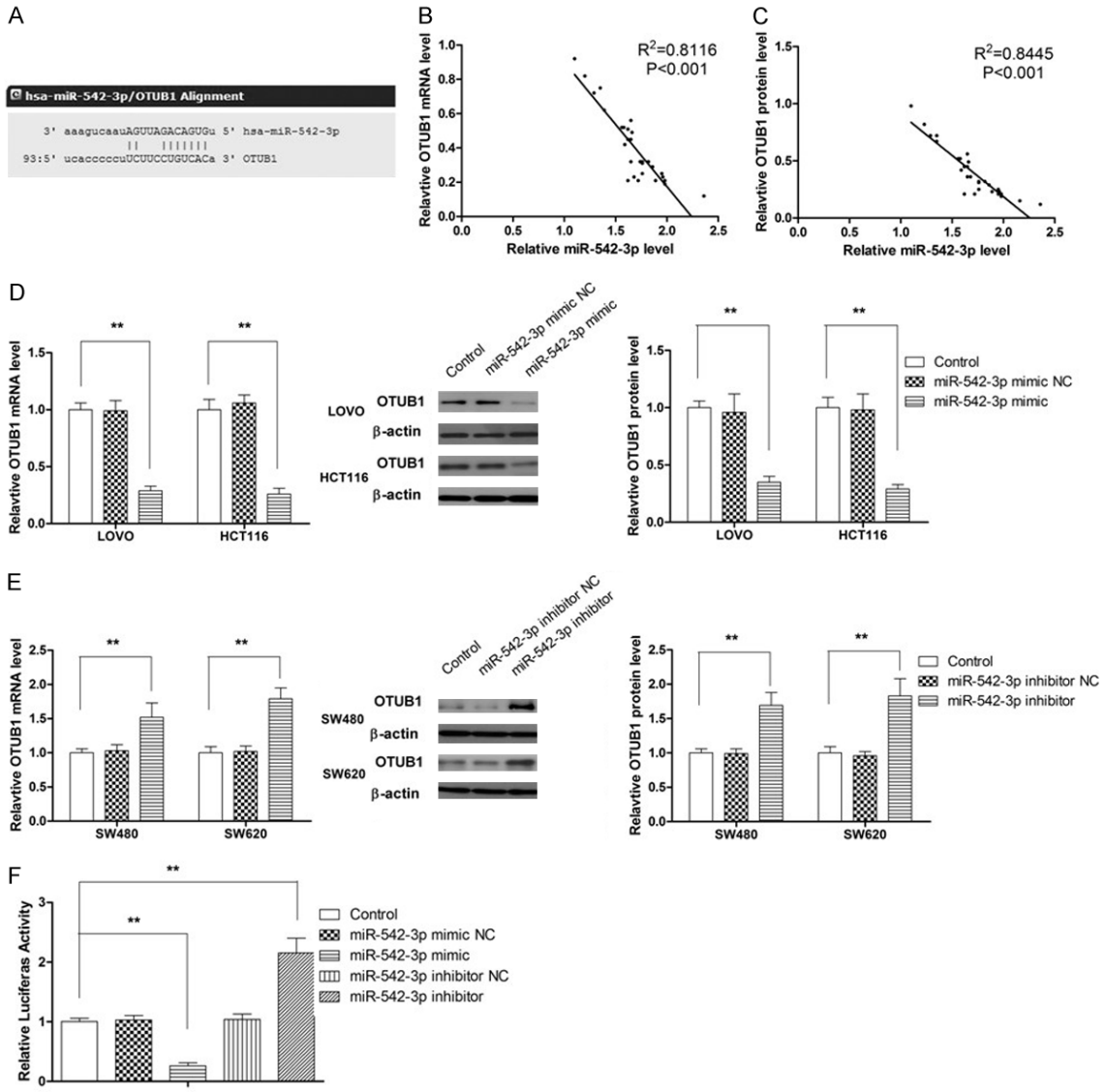

Figure 4. OTUB1 was a direct target of miR-542-3p. A. Schematic illustration of the hypothesized duplexes formed from interactions between the OTUB1 3'-UTR binding sites and miR-542-3p. B. Inverse correlation between miR-542-3p and OTUB1 mRNA level in CRC tissues. C. Inverse correlation between miR-542-3p and OTUB1 protein level in CRC tissues. D. MiR-542-3p mimic significantly elevates the expression of OTUB1 mRNA and protein in both LOVO and HCT116 cells. E. MiR-542-3p inhibitor significantly repressed the expression of OTUB1 mRNA and protein in both SW480 and SW620 cells. F. Luciferase activity is increased by miR-542-3p inhibitor and suppressed by miR-542-3p mimic. **P<0.01.

these data suggested that miR-542-3p might be involved in the degradation of mRNA of OTUB1 other than post-transcriptional regulation of protein expression of OTUB1. Next, luciferase assay was performed to confirm that OTUB1 was a direct target of miR-542-3p. As shown in **Figure 4F**, luciferase reporter activity was significantly increased in cells transfected with miR-542-3p mimic while decreased in cells transfected with miR-542-3p inhibitor, providing direct evidence that OTUB1 was directly targeted by miR-542-3p.

OTUB1 mediates the anti-cancer effect of miR-542-3p

A recent study by Zhou et al has reported that OTUB1 is frequently upregulated in CRC and associated with poor prognosis [19], and our beforementioned results indicates that miR-542-3p negatively modulates the expression of OTUB1 at the mRNA and protein levels by directly binding to its 3'-UTR. Thus, we hypothesized that the OTUB1 is involved in miR-542-3p mediated CRC cell proliferation, migration

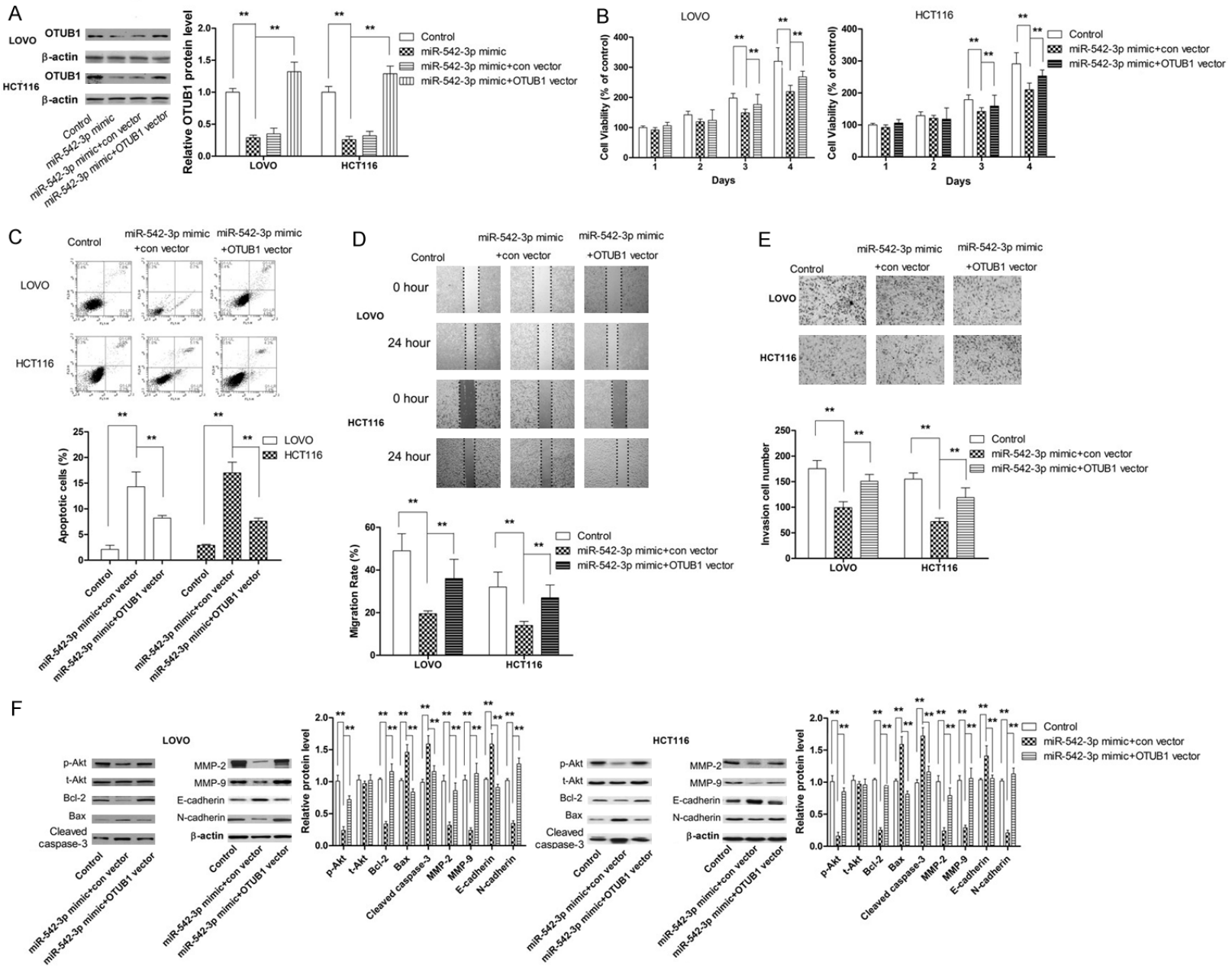
A
LOVO
OTUB1
β-actin
HCT116
OTUB1
β-actin
Control
miR-542-3p mimic
miR-542-3p mimic+con vector
miR-542-3p mimic+OTUB1 vector
Relative OTUB1 protein level
LOVO
HCT116
B
LOVO
HCT116
Cell Viability (% of control)
Days
Control
miR-542-3p mimic+con vector
miR-542-3p mimic+OTUB1 vector
C
Control
miR-542-3p mimic +con vector
miR-542-3p mimic +OTUB1 vector
LOVO
HCT116
Apoptotic cells (%)
D
Control
miR-542-3p mimic +con vector
miR-542-3p mimic +OTUB1 vector
0 hour
24 hour
LOVO
HCT116
Migration Rate (%)
E
Control
miR-542-3p mimic +con vector
miR-542-3p mimic +OTUB1 vector
LOVO
HCT116
Invasion cell number
F
LOVO
HCT116
p-Akt
t-Akt
Bcl-2
Bax
Cleaved caspase-3
MMP-2
MMP-9
E-cadherin
N-cadherin
β-actin
Relative protein level

Figure 5. Restoration of OTUB1 reversed the anti-tumor effect of miR-542-3p in CRC cells. A. OTUB1 vector successfully introduces ectopic overexpression of OTUB1. B. OTUB1 vector attenuated the anti-proliferative effect of miR-542-3p in LOVO and HCT116 cells. C. OTUB1 overexpression protected against miR-542-3p-induced apoptosis in CRC cells. D. OTUB1 overexpression reversed miR-542-3p-induced suppression in cell migration. E. OTUB1 overexpression reversed miR-542-3p-induced inhibition in cell invasion. F. OTUB1 overexpression reversed miR-542-3p-induced changes in expression of molecular markers for proliferation, apoptosis, invasion and EMT. **P<0.01.

and invasion. To verify our hypothesis, a vector containing OTUB1 sequence was used to force ectopic OTUB1 expression in SW620 and HCT116 cells transfected with miR-542-3p mimic. As shown in **Figure 5A**, the expression of OTUB1 was successfully increased. Ectopic overexpression of OTUB1 significantly attenuated the inhibitory effect of miR-542-3p mimic on cell growth in both LOVO and HCT116 cells (**Figure 5B**). Meanwhile, the apoptosis-inducing effect of miR-542-3p in both cell lines was also reversed by OTUB1 vector (**Figure 5C**). Moreover, the restoration of OTUB1 significantly promoted CRC cell migration and invasion, which was suppressed by miR-542-3p (**Figure 5D** and **5E**). Correspondingly, the attenuated expression of Bcl-2, p-AKT, MMP-9, MMP-2, N-cadherin and the upregulated expression of E-cadherin, Bax and cleaved caspase-3 was abolished by the ectopic OTUBI expression (**Figure 5F**). Taken together, our results provided experimental evidence that OTUB1 directly mediates miR-542-3p-medicated anti-cancer effect.

Discussion

The involvement of miRNAs in the development of human malignancies has been evidenced in preclinical and clinical studies [22]. In the case of CRC, aberrant expression of miRNAs has been reported to implicated in tumor initiation, progression and metastasis [23], as miRNAs can regulate multiple signaling pathways that contribute to CRC, including inflammation, cell survival, cell death, EMT, invasion and metastasis, which renders miRNAs promising biomarkers for predicting prognosis. In fact, a number of miRNAs have been identified to be independent prognostic factor for CRC. For instance, miR-378 is found to aberrantly downregulated in CRC tissues compared with match normal tissue and lower expression of miR-378 predicts poor clinical outcome for CRC patients [24]. In contrast, upregulation of miR-183 is closely related to advanced clinical stage, lymph node and distant metastases, and poor prognosis of CRC [25]. In previous study, miR-542-3p was reported to significantly downregulated in colorectal cancer cells [26]. However, the association between clinical features and miR-542-3p has never been investigated. In this study, our findings showed that aberrantly low expression of miR-542-3p correlated with advanced TNM stage and lymph mode metastasis. CRC patients with low miR-542-3p expression had worse prognoses than did patients with a high miR-542-3p expression. Moreover, we used Cox regression analysis to evaluate some clinical and pathological factors, which showed low miR-542-3p expression is an independent indicator of poor patient prognosis. This result is in line with some previous studies showing miR-542-3p downregulation associated with advanced stage and poor clinical outcome of human cancers [15, 17].

To further investigate the role of miR-524-3p in cellular functions of CRC cells, the expression of miR-524-3p was manipulated using miR-524-3p mimic and inhibitor. Our results showed that ectopic miR-524-3p expression effectively suppresses CRC cell proliferation, induces apoptosis and inhibits migration and invasion. Meanwhile, in vivo studies with mice model also supported our in vitro findings, showing miR-524-3p is able to suppress tumor growth and inhibits metastasis of CRC. These results agrees with previous studies in neuroblastoma and gastric cancer, in which miR-524-3p was aberrantly low expressed, and forced overexpression of miR-524-3p suppressed cell proliferation and invasion [27, 28]. In addition to the cell growth and metastatic behavior, miR-524-3p has also been implicated in angiogenesis by directly targeting the key angiogenesis-promoting protein Angpt2 [29]. Therefore, therapy targeting miR-524-3p may be able to exert anticancer effect at different facets simultaneously. Since the role of miR-524-3p in the development of cancer was reported, a number of molecules have been identified as the direct downstream target gene of miR-524-3p. In non-small cell lung cancer, neuroblastoma and bladder cancer, miR-524-3p is found to exert anti-tumor effect by targeting survivin [17, 28, 30]. In breast carcinoma, angiopoietin-2 was found to

be the mediator for the anti-angiogenic effect of miR-524-3p [29]. In melanoma and gastric cancer, proto-oncogene serine/threonine protein kinase (PIM1) and astrocyte-elevated gene-1 (AEG1) were found to be responsible for the suppressing effect of miR-524-3p, respectively [27, 31]. In this study, OTUB1 was identified as a novel target gene for miR-524-3p, which mediated the inhibition on cell proliferation and invasion by miR-524-3p in CRC cell. Our results further support the concept that miRNAs could regulate the cellular activities of cancer cells by targeting different target genes.

DUBs are a family of enzymes playing crucial role in various cellular activities, thus are considered as the key molecular determinants of the aberrant cancer proteome [32]. The oncogenic role of OUTB1, a member of DUBs, has also been evidenced in prostate cancer [33], lung cancer [34], gastric cancer [33] and ovarian cancer [34, 35]. In the context of CRC, OTUB1 has been found to have higher expression in colon cancer than the matched normal tissues and correlate with tumor differentiation in clinical CRC specimen [36]. A later study also reported that OUTB1 positively regulates the metastasis of CRC and acts as a potential distant metastasis marker and prognostic factor in CRC [19]. In line with previous results, we found that both mRNA and protein expression of OTUB1 was significantly higher in the CRC tissues than that in the adjacent non-neoplastic tissues. Meanwhile, Pearson correlation analysis revealed a significant inverse correlation between the expression of miR-524-3p and OTUB1. Furthermore, introduction of ectopic expression of OTUB1 remarkably abolished miR-524-3p-mediated suppression on cell proliferation, migration and invasion as well as induction of apoptosis, suggesting that miR-524-3p regulates CRC proliferation and invasion, at least partly, by directly blocking OTUB1 expression.

In conclusion, the data in this study identified an important tumor-suppressive miRNA, miR-524-3p, that is associated with clinical features and clinical outcomes of CRC patients. MiR-524-3p plays important roles in the tumor growth and metastasis by suppressing colorectal cancer cell growth as well as invasion through directly targeting OTUB1. Hence, our study demonstrates the importance of miR-524-3p/OTUB1 signaling in CRC development and suggests that targeting this signaling may highlight a new therapeutic approach for treatment of CRC.

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Disclosure of conflict of interest

None.

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