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Original Article

microRNA-155 acts as an oncogene by targeting the tumor protein 53-induced nuclear protein 1 in esophageal squamous cell carcinoma

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Abstract: MicroRNA-155 (miR-155) is overexpressed in many human cancers; however, the function of miR-155 is largely unknown in esophageal squamous cell carcinoma (ESCC). In the present study, we found that miR-155 is dramatically increased in ESCC tissues compared with the paired adjacent normal tissues, which suggested that miR-155 acts as an oncogene in ESCC. We predicted that *tumor protein p53-induced nuclear protein 1* (*TP53INP1*) is a candidate target gene of miR-155 given that miR-155 expression decreased mRNA and protein levels of *TP53INP1* as determined by RT-PCR and Western blot analysis. In addition, miR-155 and *TP53INP1* showed a negative relation in ESCC tissues. Dual luciferase-based reporter assay indicated direct regulation of *TP53INP1* by miR-155. Furthermore, we demonstrated that RNA interference of *TP53INP1* increased the proliferation and colonies formation of EC-1 cells. Up-regulation of *TP53INP1* abrogated miR-155 induced growth in EC-1 cells and mutation of *TP53INP1* in 3'-UTR restored the effects when co-transfected with miR-155. We also indicated that overexpression of miR-155 significantly promoted the proliferation of EC-1 cells *in vitro* and the development of tumors in nude mice. Taken together, our study reveals that miR-155 acts as an oncogene by targeting *TP53INP1* in ESCC.

Keywords: ESCC, *TP53INP1*, miR-155

Introduction

Esophageal squamous cell carcinoma (ESCC) is the major histologic subtype of esophageal cancer. ESCC is about 2% of total human malignant tumors and is the sixth leading cause of death from cancer [1]. Its morbidity shows a significant geographic difference and appears to be high in china. ESCC occurs in familial aggregates. Cohort studies in high-risk areas like Shanxi, Shandong province of China reported that 25%-50% ESCC patients had the family history of esophageal cancer [2]. It is well-known that Nitrite chronic stimulation, inflammation and trauma, genetic factor and microelement in food are risk factors of esophageal cancer. Statistics reveal that mutation of tumor suppressor gene p53 is associated with the development of ESCC in the cohort [3]. The most frequent symptom of ESCC is progressive dysphagia. Generally, patients apply to a physician at a stage when they cannot swallow solid foods anymore, which mean an advanced stage for ESCC, and in this stage patients lose their chances of curable surgical treatment. Therefore, developing effective early diagnosis method is particularly important for detecting and treating ESCC.

MicroRNAs (miRNAs) are a group of small non-coding RNAs with 18-25 nucleotides in length that negatively regulate gene expression by imprecisely binding to complementary sequence in the 3'-UTR of their target mRNAs [4]. MiRNAs play an important role in biological and pathologic processed including cell differentiation, proliferation, apoptosis and metabolism. They can function as oncogenes or tumor suppressors [5]. Aberrant miRNA expression may contribute to many types of human disease and they have been associated with every aspect of tumorigenesis. A recent study has shown that differential expression of miRNA was correlated with esophageal carcinoma survival [6].

In this study, we discovered that the expression of miR-155 in ESCC tissues was higher than that of adjacent tissues, which was consistent with Ran Liu [7]. Furthermore, *TP53INP1* (*tumor protein 53-induced nuclear protein 1*) is a target gene of miR-155. Meanwhile, *TP53INP1* is a proapoptotic stress-induced p53 target gene [8]. Regulation of miR-155 expression affected the expression of *TP53INP1* in EC-1 cell lines. Finally, we validated that *TP53INP1* is a direct target of miR-155 in the context of human ESCC.

Materials and methods

Tissue specimens and cell lines

30 pairs of fresh frozen ESCC and their adjacent non-tumor tissue specimens were obtained from surgical specimens from Anyang Tumor Hospital (Anyang, Henan, China) with approval of the Ethics Committee of Anyang Tumor Hospital. All samples used in this study were approved by the committee for ethical review of research. The whole procedure of consent was approved and documented by the Ethics Committee of Anyang Tumor Hospital. The ESCC cell lines EC-1 (provided by professor Kui-sheng Chen, Department of Pathology. The University of Zhengzhou, Henan, China) were stored in our own laboratory. Cells were maintained in 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hy-Clone, Logan, UT, USA) and cultivated at 37°C in 5% CO_2.

Quantitative real-time PCR analysis

Total RNA was extracted from isolated from tissues/cells by Trizol method (Invitrogen, Carlsbad, CA, USA). The first-strand of cDNA was synthesized with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). Quantitative real-time PCR (qRT-PCR) was performed as follows: 20 μl PCR mix was initial incubated at 95°C for 45 s, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The primers sequences are as follows: mir-155 RT: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTT-GAG ACCCCTAT-3'; mir-155 F: 5'-ACACTCCAGCTGGGTTAATGCTAATCGTGAT-3', R: 5'-TGGTGTCGTGGAGT CG-3'. U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'. *TP53INP1*F: 5'-CCA CGTACAATGACTCTTCT-3', R: 5'-TTCTTGGTTGGAGGAAGAAC-3'.

MTT assay

Two groups including the experimental group and the control group were involved in this study. Cells were dispensed in a 96-well plate with 1500 cells per well. Each group consisted of three wells. The cells were incubated for 24 h, 48 h, 72 h, 96 h and 120 h after transfection respectively. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μl of MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) for 4 h. When MTT incubation was completed, the supernatants were removed. Then, 150 μl of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was added to each well. After 15 min, the absorbance (OD) of each well was measured and the value was recorded using a microplate reader set at a wavelength of 490 nm. The experiments were performed in triplicate.

BrdU staining assay

For analysis of EC-1 proliferation, 5-bromo-2-deoxyuridine (BrdU) (10 μmol/L) was used to label the cells transfected with miRNA-155. Cells were then visualized with the fluorescence microscope.

Plate clone formation assay

200 cells were added to each well of a 6-well culture plate and incubated for 14 days, the cells were fixed by 70% ethanol and stained with 0.5% crystal violet. The number of colonies was analyzed using Image J software. These experiments were performed in triplicate.

Immunohistochemical staining (IHC)

We use IHC to study the expression and localization of the *TP53INP1* proteins on paraffin tissue sections (4 μm). The tissue sections were from ESCC and their adjacent non-tumor tissues. The antibody was bought from Abcam (Inc. Cambridge, MA). We can see the localization of the *TP53INP1* proteins, and the staining intensity was examined and classified into: absent and positive.

Tumorigenicity assay

A lentiviral based system of miR-155 was constructed and used to infect EC-1 cells. Cells (5×10^6) were suspended in 100 μl PBS and

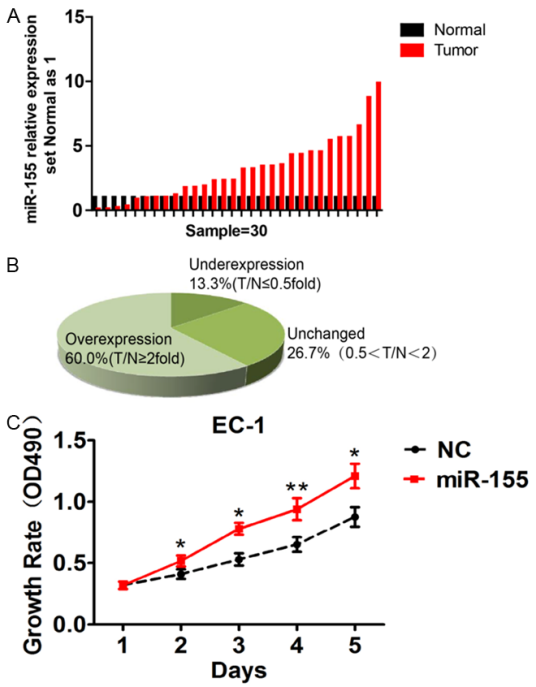

Figure 1. miR-155 was upregulated in ESCC tissues and promoted the growth of EC-1 cells. A: Quantitative real-time PCR analysis showed upregulation of miRNA-155 in ESCC tissues compared to paired adjacent normal tissues. B: 60% of ESCC samples showed twofold higher expression of miRNA-155. C: MTT assay showed that miR-155promoted proliferation in EC-1 cells. (*, P<0.05; **, P<0.01).

then injected into nude mouse (Bikai, Shanghai, China) at 5 to 6 weeks of age. According to the recommendations guidelines of the Animal Care and Use Committee of The Tenth People's Hospital of Shanghai, the studies were performed strictly with the Permit number: 2011-RES1. The protocol was approved by Science and Technology Commission of Shanghai Municipality (ID: SYXK 2007-0006). Each group consisted of 3 mice. Tumor growth was examined every week for 6 weeks. After 6 weeks, mice were killed and tumors were collected to measure the volume and weight.

Luciferase reporter assays

TP53INP1-3'-UTR were constructed into psi-CHECK-2 report plasmid (Progma, Madison, WI, USA). Plasmids and miR-155 were co-transfected into EC-1 cells using Lipofectamine™ 2000 (Invitrogen, USA). The luciferase activity was measured 24 h post transfection using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Fold-activation values were measured relative to the levels of Renilla Luciferase activity in cells transfected with negative control and normalized by luciferase activities.

Western blot analysis

EC-1 cells were seeded into 6-well plates (1×10^5 cells per well) and transfected when cells outgrowth was confluent. Cells were collected and lysed 48 h post transfection. Equal amounts of proteins were separated by 10% gradient SDS-polyacrylamide gels. The proteins were transferred onto a NC membrane, blocked with 5% fat-free milk powder for 1 h. Blots were then incubated with rabbit monoclonal *TP53INP1* antibody (Abcam Inc. Cambridge, MA), followed by incubation with HRP-conjugated secondary antibody. β-actin was used as control to verify equal amounts of protein.

Statistical analysis

The SPSS 18.0 version (SPSS Inc. Chicago, IL, USA) was used for conducting the statistical analyses. Data was tested using Student's t-test, One-way ANOVA and Chi-square test. In all samples, P≤0.05 (*) and P≤0.01 (**) was considered to be statistically significant.

Results

MiR-155 is upregulated in ESCC tissues and promotes the proliferation of EC-1 cells

MiR-155 expression level between ESCC tissues and paired adjacent non-tumor tissues from 30 individual patients were measured using quantitative real-time PCR. miRNA-155 was markedly upregulated (>3 times) respectively in ESCC samples compared with normal samples (**Figure 1A**). Among all the samples, miR-155 was expressed more than two-fold higher in 60% of ESCC tissues (**Figure 1B**). To confirm the role of miR-155 in ESCC cells proliferation, miR-155 was over-expressed in EC-1 cell lines in vitro and then detected cell viability by MTT assay. MTT assay results indicated that cells over-expressed miR-155 showed stronger proliferation ability than control (**Figure 1C**).

TP53INP1 is the putative candidate target gene of miR-155

Putative miR-155 targets were predicted using target prediction programs TargetScan. Our

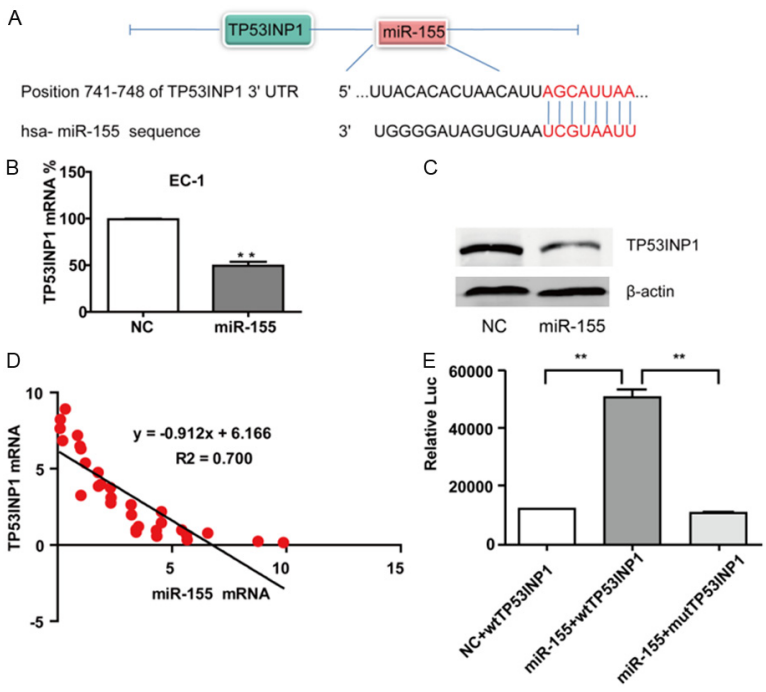

Figure 2. *TP53INP1* is a direct target of miR-155. (A) putative miR-155 binding sequence in the 3'-UTR of *TP53INP1* mRNA. (B) quantitative real-time PCR and (C) western blot were used to monitor the expression level of *TP53INP1* after transfection with miR-155. β-actin was used as an internal control (**, P<0.01). (D) Inverse Correlation between miR-155 and *TP53INP1* mRNA Levels. (E) dual luciferase reporter assay showed that wt*TP53INP1* (wild-type *TP53INP1* 3'-UTR) co-transfected with miR-155 was significantly increased (**, P<0.01).

analysis revealed that *TP53INP1* was a potential target of miR-155. The 3'-UTR of *TP53INP1* contained a binding site for the seed region of miR-155 (**Figure 2A**).

The effect of miR-155 on the expression of *TP53INP1* was further examined by quantitative real-time PCR and western blot. We found that over-expression miR-155 caused a significant decrease in mRNA (**Figure 2B**) and protein level (**Figure 2C**). Moreover, we tested mRNA level of *TP53INP1* and miR-155 in 30 ESCC tissues using quantitative real-time PCR, and the data showed obvious negative relation between expression of *TP53INP1* and miR-155 (r=-0.7, P=0.001) (**Figure 2D**).

To validate whether TP53INP1 is a direct target of miR-155, a human TP53INP1

3'-UTR was cloned into the reporter vector and the dual luciferase reporter assay was performed. The relative luciferase activity of the reporter that contains wild-type 3'-UTR was significantly increased when miR-155 was co-transfected (**Figure 2E**). In contrast, the activity of mutant 3'-UTR was unaffected by simultaneous transfection of miR-155 compared with NC. The results indicated that miR-155 may suppress *TP53INP1* expression through binding the 3'-UTR of *TP53INP1*.

The low expression of TP53INP1 is associated with tumor differentiation, stage and size

Next, we examined the expression of *TP53INP1* in ESCC tumor and normal tissue sections with IHC. Compared to the normal tissue, it's obvious that the positive staining intensity of ESCC is much stronger (**Figure 3**). Moreover, we found that the positive staining of *TP53INP1* was localized within the nucleus.

After that, we investigated the relevance of *TP53INP1* expression and clinical pathological features. Data analysis showed that *TP53INP1* mRNA low expression correlates with tumor grade (P=0.024), tumor differentiation (P=0.029) and tumor size (P=0.012) in ESCC (**Table 1**). Moreover, the *TP53INP1* expression in advanced TNM stage ESCC (III)is lower than in early TNM stage tumor (I, type IIA and IIB) (**Table 1**). However, *TP53INP1* mRNA is not related to the patients' age and gender.

Targeting TP53INP1 is a mechanism of miR-155 on oncomiR in EC-1 cells

To investigate whether miR-155 exerts its oncogenic function by targeting *TP53INP1*, we examined whether RNA interference (RNAi) knockdown of *TP53INP1* expression could recapitulate the oncogenic effects of miR-155 in EC-1 cells. We found that siRNA knockdown of *TP53INP1* in EC-1 cells significantly promoted cell proliferation (**Figure 4A**, **4B**) and anchorage-independent growth (**Figure 4C**). These results indicate that a reduction of *TP53INP1* expression can mimic miR-155 in promoting EC-1 cells to proliferate. We then performed

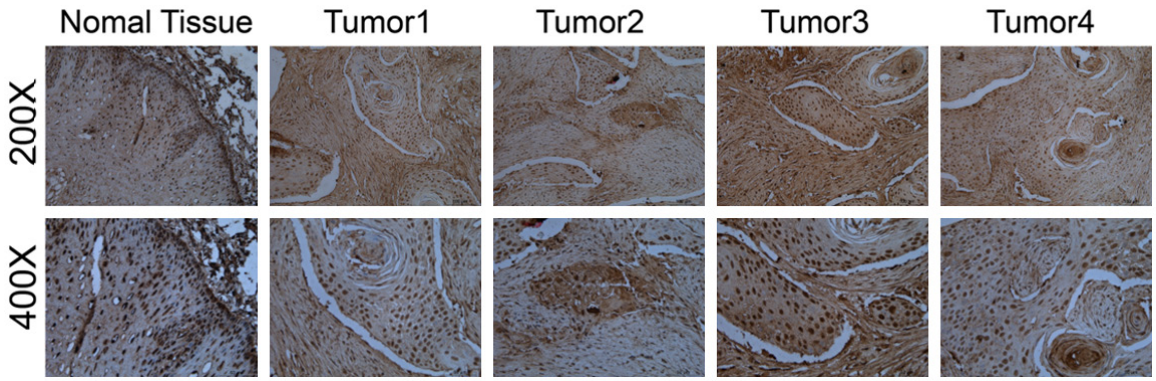

Figure 3. Representative IHC photos of *TP53INP1* expression in normal tissue (200× and 400×) and ESCC tumors. *TP53INP1* staining was mainly localized within the nucleus of cells in the form of yellow brown granules. It's obvious that the positive staining intensity of ESCC is much more stronger than that of normal tissue.

Table 1. The correlation of *TP53INP1* mRNA level with clinicopathologic features like age, gender, differentiation, tumor size and TNM stage

Characteristic	Total (n = 60)	TP53INP1 Expression		P
		low	high	
Age				
<60	24	16	8	1.000
≥60	36	24	12	
Gender				
Female	22	16	6	0.726
Male	38	26	12	
Differentiation				
well	12	7	5	0.029
Moderate	26	19	7	
Poor	22	21	1	
T Classification				
T1 T2	6	3	3	0.036
T3	35	20	15	
T4	19	17	2	
N Classification				
N0	34	18	16	0.112
N1	26	19	7	
TNM Stage				
I	4	2	2	0.024
II A	12	7	5	
II B	13	10	3	
III	31	29	2	
Tumor size(cm)				
<5	34	22	12	0.012
≥5	26	24	2	

rescue experiments to further validate that *TP53INP1* targeting is involved in miR-155-mediated oncogenesis in ESCC. MTT assays and BrdU staining assay were used to evaluate the proliferation level of EC-1 cells. Two expression vectors, wt*TP53INP1* and mut*TP53INP1* (whole *TP53INP1* sequence without 3'-UTR) were constructed. Overexpression of *TP53INP1* protein greatly suppressed the proliferation of EC-1 cells overexpressing miR-155 (**Figure 4D**, **4E**). This growth inhibition was significantly rescued when mut*TP53INP1* was co-transfection with miR-155. As expected, miR-155 acts as an oncomiR in EC-1 cells by down-regulating *TP53INP1* expression.

MiR-155 promotes cell proliferation in vitro and in vivo by targeting TP53INP1

The significant increased expression of miR-155 in ESCC tissues and EC-1 cells prompted us to explore the possible biological significance of miR-155 in tumorigenesis. BrdU staining assay found that 35.9% cells transfected with miR-155 were labeled by BrdU while control group was 20.3%, which verified that miR-155 could promote the proliferation ability of EC-1 cells (**Figure 5A**). The capacity of colony formation was evaluated on EC-1 cell lines transfected with miR-155. The data indicated that miR-155 significantly stimulated EC-1 cells to grow more and larger colonies on soft agar (**Figure 5B**).

To further confirm the biological function of miR-155, an in vivo model was used

MiR-155-transfected EC-1 cells were injected into nude mice and produced tumors with mean size of 611.3 ± 100.2 mm^3, mean weight of 456.0 ± 77.5 mg, whereas control group produced tumors in the size of 277.0 ± 77.0 mm^3 and weight of 184.0 ± 47.8 mg. The size and weight of the former showed significantly differ-

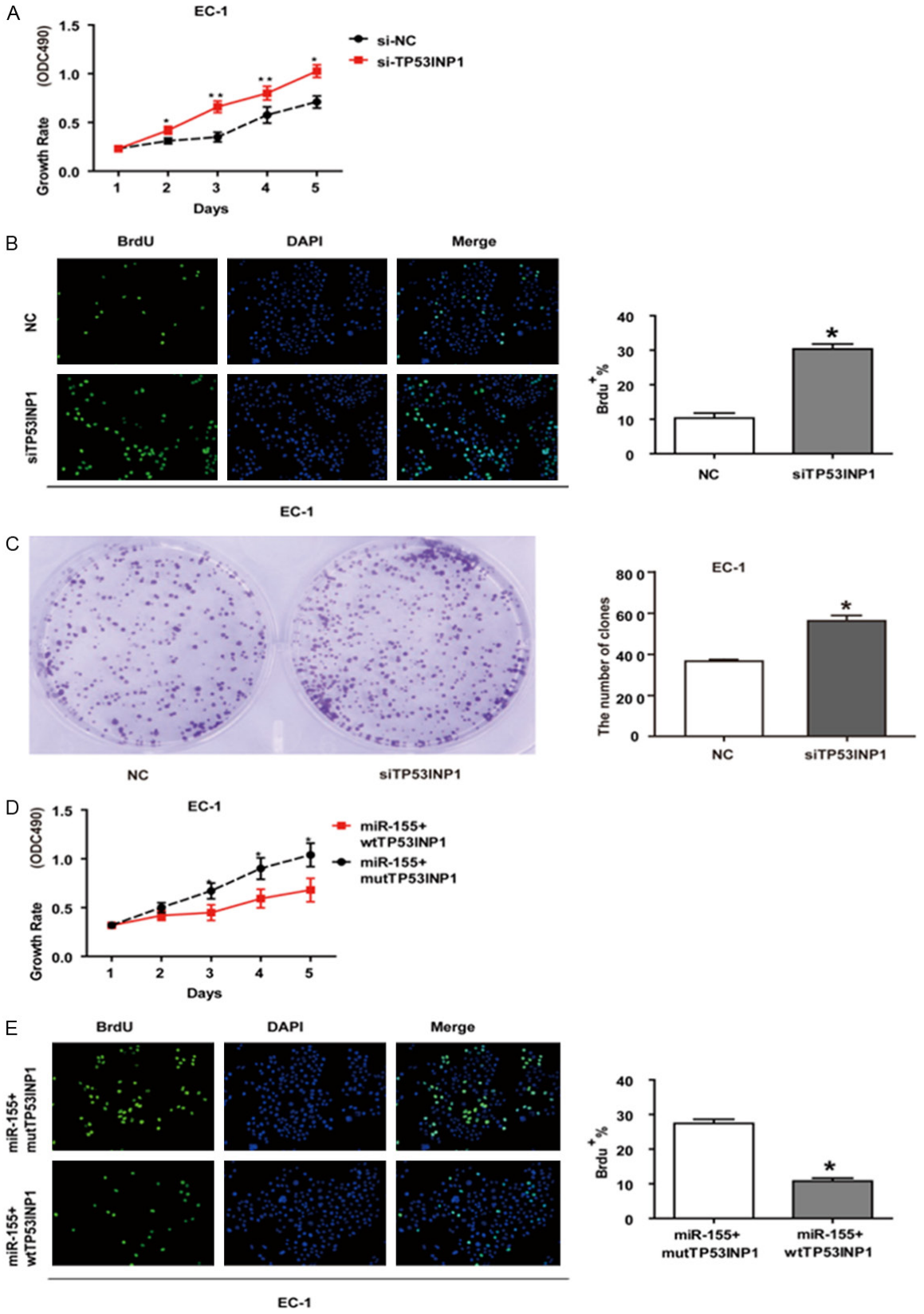

Figure 4. Targeting *TP53INP1* is involved in the oncogenic function of miR-155 in EC-1 cells. (A) MTT assay and (B) BrdU staining assay RNAi knockdown of *TP53INP1* in EC-1 cells significantly promoted cell proliferation. (C) RNAi

knockdown of *TP53IINP1* stimulated colonies growth in EC-1 cells. (D) MTT assay, (E) BrdU staining assay showed that *TP53INP1* overexprssion inhibits the oncogenic function of miR-155. (*, P<0.05; **, P<0.01).

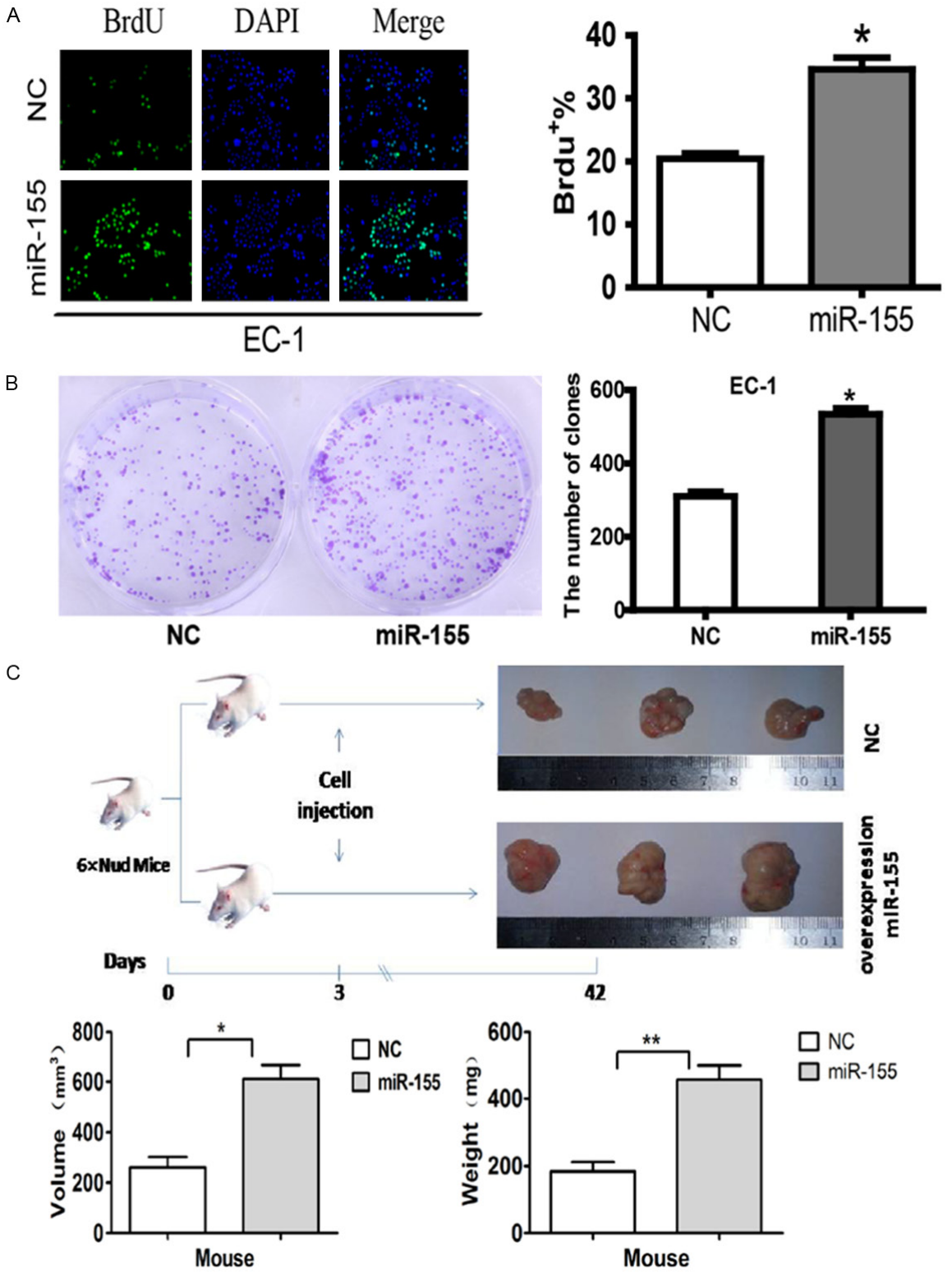

Figure 5. miR-155 promotes colony formation in vitro and tumorigenicity in vivo. A: BrdU staining assay showed that miR-155 promoted proliferation in EC-1 cells. B: miR-155 promotes colonies formation of EC-1 cells. C: miR-155 stimulates tumor growth of EC-1 cells in nude mouse. (*, P<0.05; **, P<0.01).

ence than the latter with P<0.05 and P<0.01 respectively (**Figure 5C**).

Discussion

MiRNAs are a class of small non-coding RNAs which inhibit gene expression by either blocking translation or inducing degradation of mRNA. MiR-155 is a classic immuno-related miRNA. In the immune system, miR-155 is unique due to its ability to shape the transcriptome of activated myeloid and lymphoid cells controlling multiple physiological processes ranging from inflammation to immunological memory [9]. Accumulating evidence shows that miR-155 is an oncogenic miRNA. Studies indicate frequent increase of miR-155 in various types of human malignancy, including breast cancer [10], squamous cell lung cancer [11], thyroid tumor [12] and pancreatic tumor [13]. The results demonstrate that miR-155 plays an important role in carcinogenesis.

In this study, we reveal the molecular mechanism for the first time that *TP53INP1* decreased as a direct target gene of miR-155 in ESCC. We show that miR-155 directly interacts with 3'-UTR of *TP53INP1* and blocks *TP53INP1* translation. Then we want to know how does miR-155 function on cells? Decreased *TP53INP1* expression has been found in 45 cases of breast carcinoma [14]. The *TP53INP1* expression level is inversely related to tumor size, positive lymph node metastasis, high histological grade and aberrant p53 expression. The study has verified that *TP53INP1* is a tumor suppressor gene in carcinogenesis. A previous study reports that *TP53INP1* expression is repressed by miR-155 in pancreatic ductal adenocarcinoma (PDAC) [13]. Therefore, we focus on the mechanism of *TP53INP1* in the p53 pathway. Our study shows that miR-155 acts as an oncogene in ESCC, its expression level is associated with the tumor malignance and overexpression of miR-155 promoted tumor development *in vivo*. These findings are consistent with previous reports.

MiR-155 has to modulate the expression of target transcript to realize its biological function. Therefore, we predicted the candidate target genes of miR-155 and *TP53INP1* gene turned out to be one of the candidates. Data above have confirmed that overexpression of miR-155 led to lower *TP53INP1* mRNA level and promoted the EC-1 cells proliferation. In addition, the expression of miR-155 and *TP53INP1* showed negative relation in ESCC tissues. Accumulating evidences show that *TP53INP1* are downregulated expression in many malignancy including breast cancer, lung cancer and pancreatic cancer [13-15], which is also in agreement with our data. Thus, our studies suggest that miR-155 interact along with *TP53INP1* in a regulatory pathway and play a crucial role in tumor development.

TP53INP1 is a proapoptotic stress-induced p53 target gene [8, 16]. *TP53INP1* acts as an anti-tumoral gene and allows regulation of cell cycle progression and apoptosis, dependently or independently from p53 in the p53-induced apoptotic pathway [17]. P53 pathway is a crucial tumor suppressor pathway, mutations of p53 gene increase with tumor progression in many tumors. P53 is a conserved nuclear transcription factor that regulates the transcription of numerous target genes. P53 protein can inhibit cell cycle, induce apoptosis, and hinder angiogenesis in response to stress or DNA damage. Here we show that *TP53INP1* inhibit the proliferation of EC-1 cells. Since *TP53INP1* is a direct target gene of miR-155, we propose that miR-155/*TP53INP1* might affect cell growth by p53 pathway.

Normal cell cycle is necessary for the cell growth and the disorder cell cycle control may induce unlimited cell proliferation [18, 19]. Numerous studies have shown that microRNAs can regulate the expression of protein-coding genes through imperfect base pairing with the 3'-UTR of target miRNAs. The abnormal expression of these proteins causes disordered cell cycle and cell proliferation. Oncogenic miRNAs may facilitate cell cycle entry and progression by targeting related proteins. Our studies reveal that miR-155 acts as an oncogene in ESCC and has important roles in the growth of EC-1 cells by directly targeting *TP53INP1*. *TP53INP1* is involved in p53 pathway. So we assume that miR-155 leads to the dysfunction of p53 pathway through decreasing expression of *TP53INP1*. The alteration of p53 pathway may disrupt the cell cycle control and drive the cell cycle to G1 progression and S-phase entry.

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Disclosure of conflict of interest

None.

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