Acta Biochim Biophys Sin, 2016, 48(9), 810–819
doi: 10.1093/abbs/gmw073
Advance Access Publication Date: 25 August 2016
Original Article

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MiR-630 inhibits invasion and metastasis in esophageal squamous cell carcinoma

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Received 22 March 2016; Accepted 18 April 2016

Abstract

Esophageal squamous cell carcinoma (ESCC) is among the most aggressive malignancies and has a high incidence in China. MicroRNAs (miRNAs) are small endogenous RNAs that regulate multiple tumorigenic processes, including proliferation, invasion, metastasis and prognosis. Using miRNA expression profiling analysis, we found that miR-630 was markedly down-regulated in three ESCC tissue samples compared with that in paired normal esophageal tissues. Differential miR-630 expression was subsequently confirmed using quantitative real-time PCR. To determine whether miR-630 down-regulation could be considered as a diagnostic indicator and adverse prognostic factor, we investigated the association between miR-630 and clinicopathological characteristics in patients with ESCC. It was found that decreased miR-630 expression was associated with poor overall survival in these patients. In addition, we also explored the biological function of miR-630 by targeting Slug and investigated the correlation between miR-630 expression and epithelial–mesenchymal transition (EMT) progression *in vivo* and *in vitro*. Ectopic miR-630 expression could inhibit proliferation, invasion and metastasis, whereas miR-630 knockdown induced proliferation, invasion, metastasis and EMT traits. Overall, our study supports a role for miR-630 as a critical novel modulator in ESCC.

Key words: miR-630, esophageal squamous cell carcinoma, suppressor, epithelial–mesenchymal transition, invasion and metastasis

Introduction

Esophageal cancer (EC) is one of the most common types of digestive tract cancer and approximately 90% of EC cases in China involve esophageal squamous cell carcinoma (ESCC), which is characterized by poor prognosis and a high mortality rate [1–4]. Poor prognosis is partially due to the fact that most patients with ESCC are diagnosed at an advanced stage [5].

MicroRNAs (miRNAs) are small, non-coding, endogenous RNAs (ncRNA) that regulate the expression of their target genes post-trancriptionally by binding to the 3′-untranslated regions (3′-UTRs) of target mRNAs [6]. Recently, several studies have identified the aberrant expression of various miRNAs in human tumor tissues compared with normal tissues using microarray and RNA sequencing analyses [7–9]. Some miRNAs act as oncogenes, whereas others act as tumor suppressors. Both types of miRNAs play critical roles in various aspects of carcinogenesis and cancer progression, including cell proliferation, migration and metastasis [10]. Oncogenic miRNAs, such as miR-21, miR-31 and miR-92a, are usually found to be overexpressed in ESCC [11–13]. In contrast, miR-143, miR-145 and miR-133a/b are frequently down-regulated in

ESCC and are often considered as tumor suppressors [14–16]. However, the underlying molecular mechanisms and the detailed role of miR-630 in ESCC carcinogenesis remain unclear.

The objective of this study is to identify the biological function of miR-630 as well as the correlation between miR-630 expression and EMT progression in ESCC. From various miRNAs, we validated the down-regulation of miR-630 in ESCC via microarray analysis. We also determined a prognostic role for miR-630 in ESCC using cancer and adjacent normal specimens from 44 patients by real-time PCR analysis. Furthermore, the inhibitory roles of miR-630 against proliferation, migration, metastasis and epithelial–mesenchymal transition (EMT) in ESCC were clarified *in vivo* and *in vitro*. These results indicate that miR-630 may serve as a diagnostic and prognostic biomarker of ESCC, and as a potential therapeutic target for the treatment of human ESCC as well.

Material and Methods

Patients and specimens

Forty-four carcinogenic tissues and matched adjacent normal tissues were selected from patients with ESCC who underwent esophagectomy at Jinling Hospital of Nanjing from April 2006 to April 2011. All patients were pathologically diagnosed with ESCC and were not subject to preoperative chemotherapy and/or radiotherapy. Tissue specimens were obtained with the consent of all patients. Tissues were stored in liquid nitrogen until RNA was extracted. ESCC diagnosis was defined according to the tumor node metastasis stage classification and World Health Organisation (WHO) criteria. This study was approved by the ethics committee of Jiangsu Province Medical Association.

Cell cultures and transfection

The ESCC cell lines eca109, EC9706, TE1, Kyse-30 and Kyse-70 and the normal esophageal epithelial cell line HEEC were purchased from Fudan University Shanghai Cell Bank and were cultured at 37° C in a humidified incubator 5% CO_2. Cells were incubated in RPMI 1640 (Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin. Cell transfection was performed using RNA mate (Genepharma, Shanghai, China) according to the manufacturer's protocol. MiR-630 mimics (miR-630), miR mimics negative control (miR-NC), miR-630 inhibitor (anti-miR-630) and anti-miR negative control (anti-miR-NC) were synthesized by the Genechem Company (Shanghai, China).

RNA extraction and quantitative real time (RT)-PCR

Total RNA was extracted from tissues using TRIzol® reagent (TaKaRa, Dalian, China) according to the manufacturer's instruction. MiR-630 expression was measured via relative fluorescence collection assay, using SYBR Green fluorescent dye and a STEP ONE RT-PCR apparatus (Applied Biosystems, Foster, USA). U6 expression was measured as an internal reference.

Western blot analysis

Total protein was extracted from cells and tumor tissues using RIPA lysis buffer. Extracted proteins were mixed with loading buffer, subject to 10% SDS-PAGE and transferred to PVDF membranes, which were subsequently blocked in a 5% solution of non-fat milk for 2 h. The membranes were subsequently incubated with antibodies specific to E-cadherin, N-cadherin, vimentin, β-catenin and Slug (Abcam, Cambridge, UK) for 16 h. GAPDH expression was used as an internal reference.

MTT assay

Cell proliferation was analyzed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at a density of 1×10^3 cells per well of a 96-well plate and incubated for 1–3 days. Then, 20 μl of 0.5 mg/ml MTT (Sigma, St Louis, USA) was added to each well, followed by 4 h of incubation. Then, 200 μl of DMSO (Sigma) were added to each well and the absorbance at 560 nm was measured with a microplate reader (Thermo, Waltham, USA). Three independent experiments were performed.

Colony formation inhibition assay

Clonogenic cells were seeded at a density of 500 cells per well in six-well plates and incubated for 12 days. Cells were subsequently stained with 0.5% crystal violet. The clone formation rate was determined. Independent experiments were conducted in triplicate.

In vitro transwell assay

Cell invasion was analyzed using Matrigel-coated transwell chamber according to the manufacturer's instructions (Corning Co, Corning, USA). A total of 5000 cells were added to the upper chamber and cultured in serum-free RPMI 1640. Conditioned medium was placed into the lower chamber as chemoattractants. The cells that penetrated to the bottom of the membrane from upper surface were fixed in 4% paraformaldehyde and stained with crystal violet after 24 h. Five random fields per well were selected and counted under the microscope (Thermo), and the number of invading cells were recorded. Each experiment was conducted in triplicate.

Immunofluorescence assay

Cells were allowed to grow on cover slips for 24 h, fixed in cold acetone for 20 min, and incubated with antibodies against E-cadherin, N-cadherin, vimentin or β-catenin overnight, followed by incubation with goat anti-rabbit FITC conjugated secondary antibody (Cell Signaling Technology, St Louis, USA) for 1 h. After extensive washing, fluorescent staining was observed under a fluorescence microscope (Olympus, Tokyo, Japan). 4′,6-diamidino-2-phenylindole (DAPI) staining was used to visualize cell nuclei.

Luciferase reporter gene assay

A Slug 3′-UTR luciferase reporter gene plasmid was constructed, and the fragment containing putative binding sites for miR-630 was amplified. Plasmids named pSlug-WT and pSlug-Mut were generated via subcloning downstream of the luciferase vector. Luciferase reporter experiments were performed in 96-well plates using a Dual-Luciferase Reporter Assay kit (Dual-Glo Luciferase Assay System; Promega, Madison, USA) and SpectraMax M5 instrument software (Molecular Devices, Sunnyvale, USA) to analyze the results. Cells were co-transfected with 100 ng of pSlug-miR-630-UTR-WT or pSlug-miR-630-UTR-Mut in the presence of 50 nM Lipofectamine 2000 (Life Technologies, Carlsbad, USA). After 48 h, cells were assayed using a Dual-Luciferase Reporter Assay kit (Promega) according to the manufacturer's instructions.

In vivo tumor metastasis assay

Twelve 5-week-old athymic female nude mice (Jinling Hospital, Nanjing, China) were inoculated subcutaneously with 1×10^7 ESCC cells (EC9706) that had been stably transfected with pcDNA/anti-miR-630 or pcDNA/anti-miR-NC. Mice were randomized into two groups of six mice. This process was repeated once a week. After 6 weeks, the mice were sacrificed and the subcutaneous tumors were harvested and measured. qPCR and western blotting tests were performed using groups of mice ($n = 6$/group) treated as above. The experiment was conducted under standard conditions according to the guidelines of the Jiangsu Province Experimental Animal Care and Use Committee.

Statistical analysis

All statistical analyses were conducted using SPSS 17.0 statistical software and the experimental data were presented as the mean ± SD. Categorical variables were compared using Fisher's exact test. The Pearson χ^2 test was used to evaluate the relationship between miR-630 expression and clinical features. Kaplan–Meier method was used to compare the overall survival curves between high-miR-630 and low-miR-630 expression groups via the log-rank test. Cox proportional hazards regression model was generated to identify factors associated with overall survival through a multivariate survival analysis of ESCC. The results of *in vitro* invasion assays and luciferase reporter assays were evaluated using Student's *t*-test. *P*-value < 0.05 was considered statistically significant.

Results

Down-regulated miR-630 expression in ESCC tissues and cells

To identify the trigger point of ESCC tumorigenesis, the miRNA profiles of ESCC and corresponding controls were compared in the gene chip assay. The expressions of more than 50 miRNAs were dysregulated in ESCC tissues compared with normal squamous epithelial tissues. Eleven of these miRNA exhibited dramatically (greater than 100-folds change) aberrant expression. Six of these 11 miRNAs (miR-4419a, miR-4667-5p, miR-6131, miR-1183, miR-5703 and miR-4306) were up-regulated, whereas the other five miRNAs (miR-630, miR-143-3p, miR-133b, miR-143-5p and miR-29c-3p) were down-regulated. MiR-630 was the most dramatically affected one according to the gene chip assay (**Fig. 1A**).

To confirm the gene chip assay results, qPCR assay was employed to evaluate miR-630 expression in paired tumor and adjacent normal tissues from 44 patients with ESCC. As shown in **Fig. 1B**, the qPCR assay results revealed an average 50% reduction in miR-630 expression in tumor tissues compared with matched adjacent normal tissues, which confirms the gene chip assay results. Next, qPCR assay was performed to further detect miR-630 expression in five ESCC cell lines (eca109, EC9706, TE1, kyse-30 and kyse-70) and a normal esophageal epithelial cell line (HEEC). The results revealed a reduced miR-630 expression in ESCC cells compared with HEEC cells (**Fig. 1C**). These findings suggest that miR-630 may act as a specific tumor marker for the distinction of ESCC from normal esophageal squamous epithelium *in vivo* and *in vitro*.

Lower miR-630 expression correlates with poor prognosis in patients with ESCC

Kaplan–Meier method analysis (log-rank test) was performed to determine the association between miR-630 expression and overall survival in patients (**Fig. 1D**, $P = 0.000$). Notably, miR-630 expression was found to be correlated with tumor range, lymphatic metastasis, pathological stage and tumor location (**Table 1**, $P = 0.000$, 0.000 and 0.000, respectively). Proportional hazards method analysis revealed that low miR-630 expression ($P = 0.005$) was an adverse prognostic factor, in addition to tumor range and pathological stage (**Table 2**, $P = 0.034$ and 0.002). These results suggest that miR-630 plays a critical role in ESCC and may be considered as a specific biomarker of poor prognosis.

MiR-630 inhibits the proliferation and invasion of ESCC cells

Although miR-630 expression is correlated with tumor range, lymphatic metastasis, pathological stage and tumor location in ESCC and high miR-630 expression may yield aprognostic benefit, the roles of miR-630 in ESCC cell proliferation, invasion and metastasis remain to be elucidated.

Therefore, the effect of miR-630 on the proliferation, invasion and metastasis of the 3 most distinct ESCC cell lines (eca109, TE1, and EC9706) which had been transfected with miR-630, miR-NC, anti-miR-630 or anti-miR-NC was analyzed. Through an MTT assay, miR-630 up-regulation was found to inhibit the proliferation of ESCC cells compared with miR-NC. However, miR-630 could boost ESCC cell proliferation compared with the negative control (**Fig. 2A**). As shown in **Fig. 2B**, the clone formation rate of eca109, TE1, and EC9706 cells transfected with miR-630 was decreased by approximately 33.9%, 24.8% and 39.0% compared with controls, respectively. Nevertheless, the clone formation rate of eca109, TE1, EC9706 cell transfected with anti-miR-630 was increased by approximately 35.5%, 42.5% and 39.8% compared with controls, respectively. Furthermore, the effects of miR-630 were also explored in the *in vitro* transwell assay. As illustrated in **Fig. 2C**, miR-630 up-regulation reduced the number of invaded cells by approximately 50.0%, 43.2% and 41.8% in eca109, TE1, and EC9706 cells, respectively, compared with controls. In contrast, miR-630 down-regulation noticeably increased the numbers of invaded cells by approximately 2.07, 1.92 and 1.86 folds in eca109, TE1, EC9706 cells, respectively, compared with controls.

These data suggest that miR-630 up-regulation could significantly inhibit proliferation and invasion, whereas miR-630 down-regulation could induce proliferation and invasion in ESCC cells *in vitro*.

EMT regulation occurs with Slug-targeting via a miR-630 expression variant

EMT, which is associated with a shift to aggressive cell migration characteristics, is a well-known feature of many carcinomas. ESCC cell lines exhibited markedly different capacities for invasion and metastasis relative to miR-630 expression levels, suggesting that miR-630 might play a significant role in ESCC progression and that a relationship between miR-630 and EMT might be relevant. To investigate whether the loss of miR-630 was consistent with EMT, qPCR assay was performed to determine the expression levels of epithelial protein markers (E-cadherin and β-catenin) in ESCC cell lines

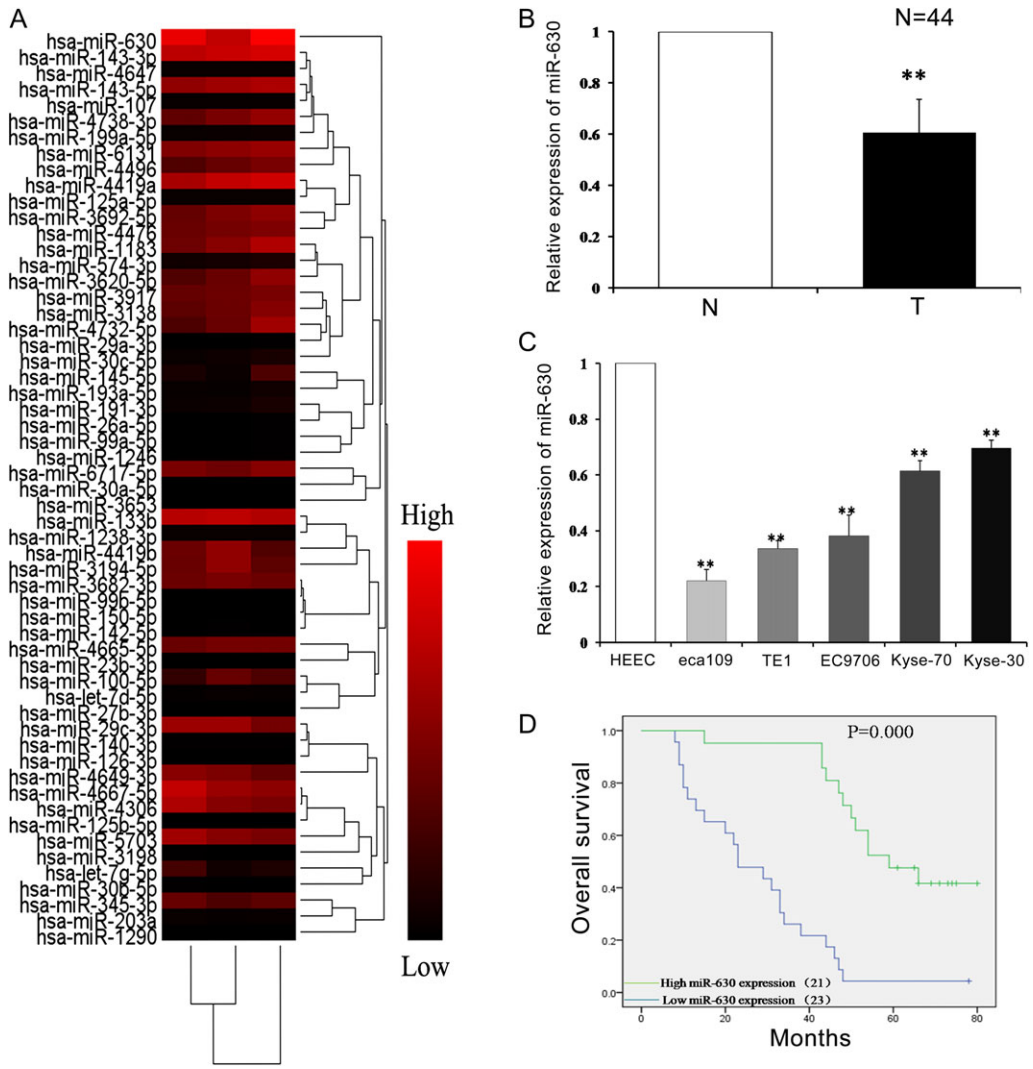

Figure 1. Low miR-630 expression in ESCC is inversely correlated with poor prognosis (A) Microarray chip analysis of miRNA expression in both ESCC tissues and normal squamous epithelial tissues. (B) Confirmation of miR-630 expression in ESCC tissues using qRT-PCR. (C) Confirmation of miR-630 expression in cell lines using qRT-PCR. (D) Univariate survival method (Kaplan–Meier method) of patients with ESCC indicating a strong correlation between miR-630 expression and overall survival ($P = 0.000$; log-rank test). U6 was used as an internal control. Each experiment was performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$.

transfected with anti-miR-630. They were found to be dramatically down-regulated compared with the levels in cells transfected with anti-miR-NC. An analysis of the expression of mesenchymal markers (N-cadherin and vimentin) in cells transfected with anti-miR-630 revealed obvious up-regulation of these two markers compared with the levels in cells transfected with anti-miR-NC (**Fig. 3A**). Furthermore, western blot assay was used to detect the protein expression levels of EMT-related markers. As shown in **Fig. 3B**, the E-cadherin and β-catenin protein expression levels were always lower in ESCC cell lines transfected with anti-miR-630 than in those transfected with anti-miR-NC. However, the N-cadherin and vimentin protein expression levels appeared to be higher in cells transfected with anti-miR-630 than in those transfected with anti-miR-NC. Moreover, immunofluorescence assay was conducted to allow a confocal microscopic analysis of the appearances of ESCC cell lines. As illustrated in **Fig. 3C**, ESCC cell lines transfected with anti-miR-630 exhibited a mesenchymal phenotype and lost the epithelial phenotype. In contrast, cells transfected with anti-miR-NC retained the epithelial phenotype.

These data suggest that the down-regulation of miR-630 makes ESCC cells to acquire a partly mesenchymal phenotype, lose the epithelial phenotype and exhibit enhanced capacities for migration and metastasis. Therefore, loss of miR-630 might affect the EMT of ESCC cells.

Although we have discovered the function of miR-630, the underlying molecular mechanism of this effect is not defined, particularly with regard to the EMT-related targets of miR-630. The TargetScan, miRanda and PicTar online tools were used to predict the downstream regulators and identify Slug as a potential target. Slug was subsequently tested *in vitro* using luciferase reporter assays, which demonstrated that miR-630 significantly inhibited luciferase activity with 3′-UTR plasmids (**Fig. 3D**). This finding suggests that miR-630 targets Slug directly in ESCC cells.

Loss of miR-630 induced tumor growth and EMT *in vivo*

Nude mice with transplanted EC9706 cell tumors were stably transfected with anti-miR-630 and anti-miR-NC. As shown in **Fig. 4A**,

Table 1. Correlation between miR-630 expression and clinical features ($n = 44$)

Variable	miR-630 expression		*P*-value
	low	high	
Age			
<60	11	8	0.557
≥60	12	13	
Gender			
Male	12	16	0.125
Female	11	5	
Pathological grading			
≤G2	10	13	0.246
G3	13	8	
Tumor range			
T1	1	14	0.000*
≥T2	22	7	
Lymph nodes			
negative	3	19	0.000*
positive	20	2	
Pathological stage			
<III	3	18	0.000*
≥III	20	3	
Tumor location			
Locussuperior	2	0	0.111
Locusmedilis	10	15	
Locusinferior	11	6	

Low/high by the sample median. Pearson χ^2 test. $P < 0.05$ was considered statistically significant.

Table 2. Multivariate analysis of prognostic parameters in patients with esophageal squamous cell carcinoma (ESCC) by Cox regression analysis

Variable	Category	HR	*P*-value
Age	<60	0.757	0.384
	≥60		
Gender	Male	1.824	0.177
	Female		
Pathological grading	<G2	0.863	0.353
	≥G2		
Tumor location	Locussuperior	0.082	0.775
	Locusmedilis		
	Locusinferior		
Tumor range	T1	4.490	0.034*
	T2		
	T3		
	T4		
Lymph node number	<7	0.260	0.610
	≥7		
Pathological stage	<III	9.797	0.002*
	≥III		
miR-630 expression	Low	7.964	0.005*
	High		

Proportional hazards method analysis showed a positive, independent prognostic importance of miR-630 expression ($P = 0.005$), in addition to the independent prognostic impact of tumor range ($P = 0.034$) and pathological stage ($P = 0.002$). $P < 0.05$ was considered statistically significant.

HR, hazard ratio.

tumors in the anti-miR-630 group had a larger average size than those in the anti-miR-NC group at 2 months after transplantation.

qPCR assay further demonstrated relatively higher Slug expression, reduced E-cadherin and β-catenin expression and strong N-cadherin and vimentin expression in the anti-miR-630 group compared with those in the anti-miR-NC group (**Fig. 4B,D**). Similarly, Western blot assay illustrated that the anti-miR-630 group exhibited relatively higher Slug expression, lower E-cadherin and β-catenin expression, and higher N-cadherin and vimentin expression, than the anti-miR-NC group (**Fig. 4C,E**). These results indicate that miR-630 may target Slug in part to influence EMT and thus manipulate ESCC tumorigenesis *in vivo*.

Discussion

miRNAs are small non-coding RNAs with approximately 22–24 nt that promote mRNA degradation or translation inhibition mainly at the post-transcriptional level in mammals. Increasing evidence clearly suggests that the expression a specific panel of miRNAs is dysregulated in an overwhelming majority of tumors, including breast cancer, non-small cell lung cancer, ovarian cancer, hepatocellular carcinoma, gastric carcinoma and pancreatic cancer [6,8,17–23]. Some miRNAs also strongly modulate the invasive and metastatic aspects of tumorigenesis [24–26]. During cancer initiation and progression, miRNAs can regulate diverse biological functions as either tumor oncogenes or suppressors. Therefore, the elucidation of abnormal miRNA expression might facilitate a deeper understanding of ESCC tumorigenesis.

In our previous studies, we identified the specific expression of miRNAs in ESCC via gene chip assay and revealed via qPCR assay that miR-630 was the most consistently expressed miRNA *in vivo* and *in vitro*. From the effects of miR-630 on tumor tissues and normal tissues from patients, we have concluded that miR-630 is down-regulated in tumor tissues and can induce the ESCC tumorigenesis.

Previous studies have revealed that miR-630 could induce cell death and inhibit proliferation in cisplatin-induced non-small cell lung cancer cells by targeting CDC7 kinase [27,28]. Similarly, Kuo *et al.* [29] demonstrated that miR-630 was a suppressor, whereas inhibition of miR-630 restored the motility and invasive ability of ANGPTL1-overexpressing lung cancer cells. Furthermore, Song *et al.* [30] reported that miR-630 targeted LMO3 to reduce lung cancer cell growth and metastasis. A recent study showed that E2F1-regulated DROSHA could be targeted by miR-630, which was considered as an independent prognostic factor among patients with renal cancer [31]. In human prostate cancer, miR-630 induction was found to cause growth arrest in PC-3 cells via the inhibition of cyclin G-associated kinase [32]. In addition, miR-630 suppressed tumor metastasis through the TGF-β signaling pathway in hepatocellular carcinoma [33]. miR-630 also induced apoptosis in colorectal cancer cells [34]. Moreover, miR-630 regulated responses to human epidermal growth factor receptor (HER)-targeting drugs and cancer cell progression in HER2-overexpressing breast cancer [35,36].

In accordance with previous reports, the transfection-enforced down-regulation of miR-630 appears to promote the proliferation and invasion of ESCC cells. However, miR-630 up-regulation inhibits both the proliferation and invasion of these cells. These losses or gains in invasive ability at different miR-630 expression levels suggest that miR-630 might act as a cancer suppressor gene to inhibit the proliferation, invasion and metastasis of ESCC cells.

To further explore the underlying association between miR-630 and metastasis, the EMT was analyzed. Slug which acts as a master regulator is known to alter the expression of some genes and thus

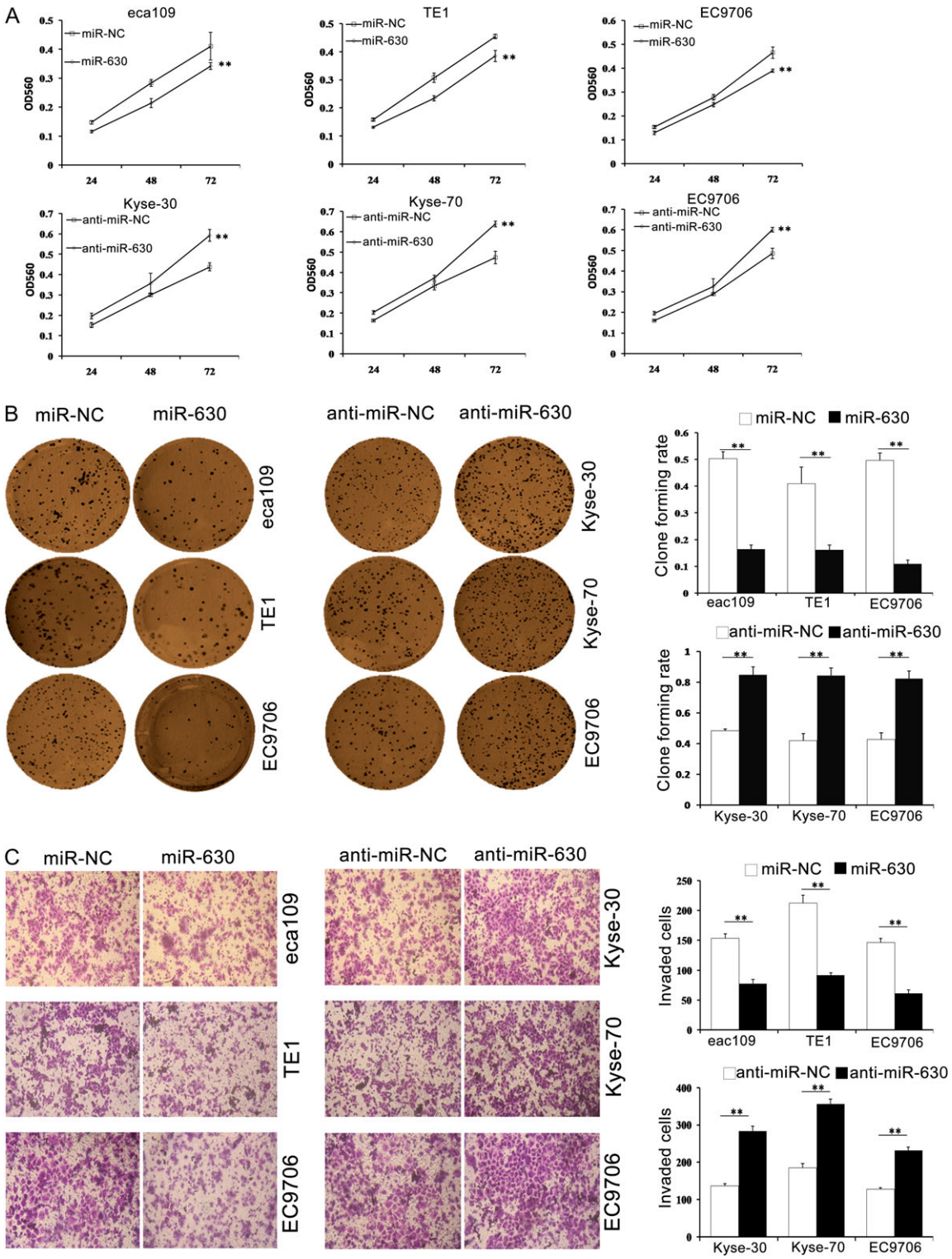

Figure 2. MiR-630 inhibits the proliferation and invasion of ESCC cells (A) MTT assay of cell proliferation and (B) colony formation assay of ESCC cell lines transfected with miR-NC, miR-630, anti-miR-NC and anti-miR-630. Cells were cultivated for 14 days and stained to visualize colonies, clone formation rates were calculated. (C) Transwell invasion assay of ESCC cell lines transfected with miR-NC, miR-630, anti-miR-NC and anti-miR-630. Cells in five random fields of view were observed at 100× magnification, invading cells were counted. Each experiment was performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$.

contribute to physiological changes that result in EMT [37,38]. We confirmed that Slug is a downstream target of miR-630 through a luciferase gene reporter assay. EMT is a major embryological mechanism by which cells lose epithelial markers (E-cadherin and β-catenin) but acquire mesenchymal markers (N-cadherin and vimentin) [39,40]. Notably, affected cells detach from the primary tumor and subsequently migrate to new sites. Studies have shown that miR-200 family members and miR-205 target ZEB1 and ZEB2 to regulate

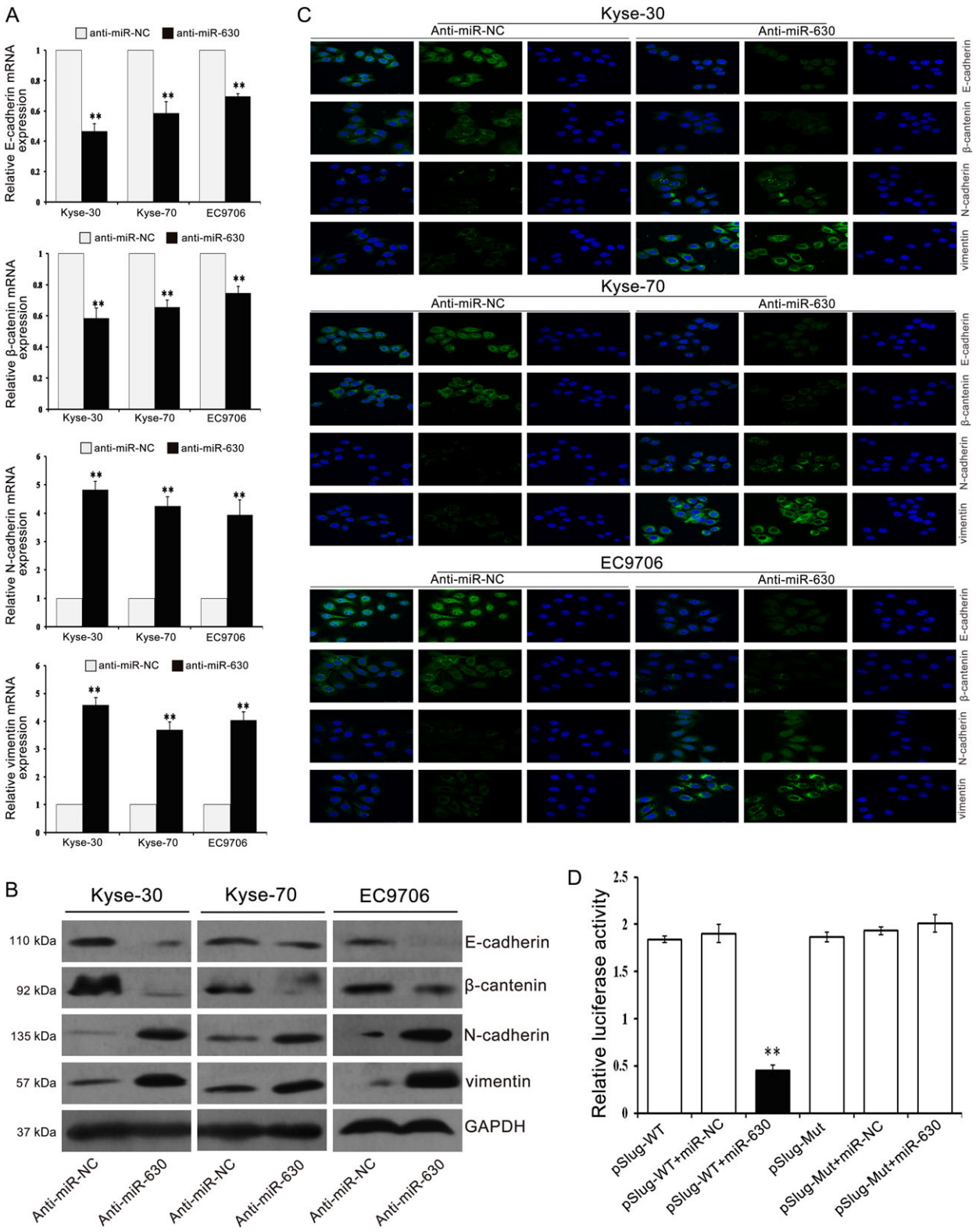

Figure 3. EMT regulation occurs with Slug-targeting via a miR-630 expression variant (A) Confirmation of the expression of epithelial markers (E-cadherin and β-catenin) and mesenchymal markers (N-cadherin and vimentin) in ESCC cell lines by qRT-PCR. (B) Confirmation of the expression of epithelial markers (E-cadherin and β-catenin) and mesenchymal markers (N-cadherin and vimentin) in ESCC cell lines by western blotting. (C) Down-regulation of miR-630 expression induces EMT characteristics in ESCC cell lines. DAPI was used to visualize nuclei. (D) Relative luciferase activity was analyzed after wild-type or mutant 3′-UTR reporter plasmids were co-transfected with miR-NC or miR-630 into EC9706 cells. GAPDH was used as an internal control. Each experiment was performed in triplicate. $*P < 0.05$, $**P < 0.01$.

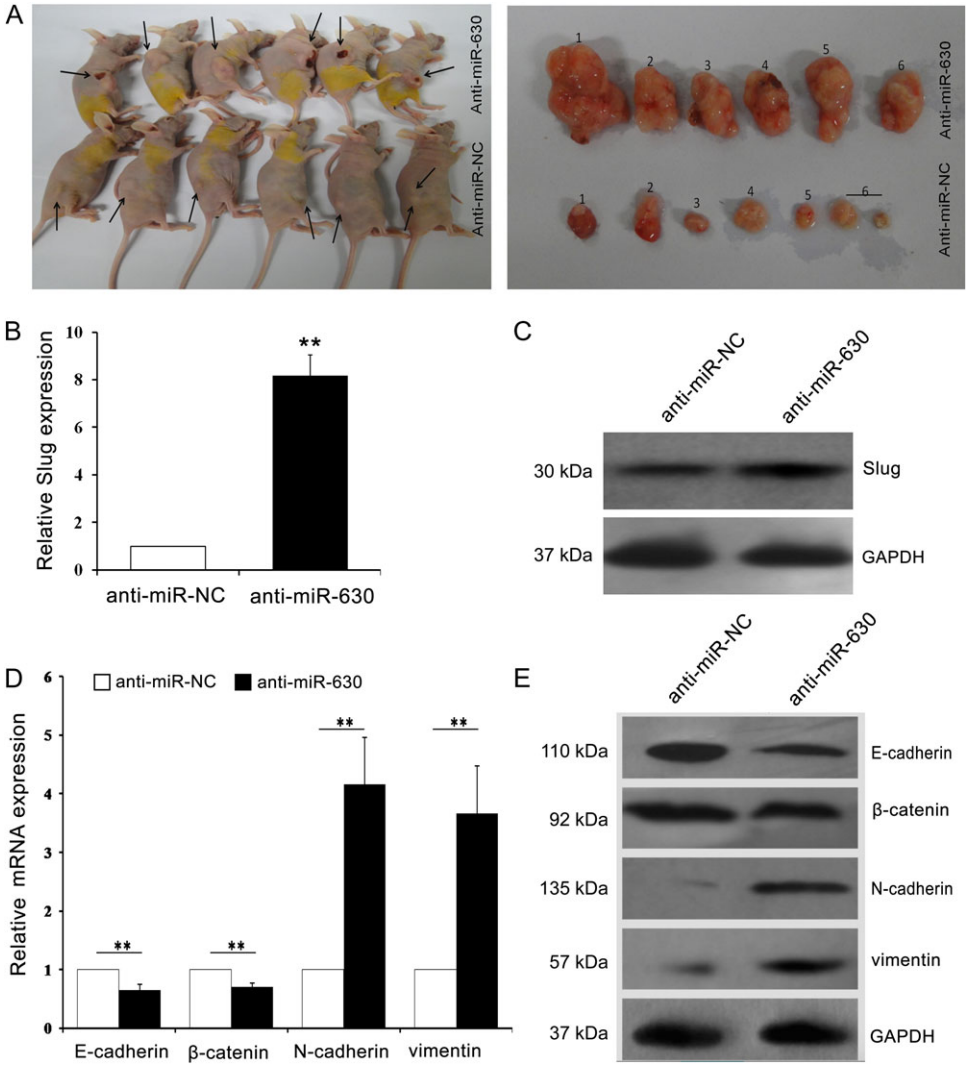

Figure 4. Loss of miR-630 induces tumor growth and EMT *in vivo* (A) Tumor nodules in nude mice treated with anti-miR-NC and anti-miR-630 (n = 6/group). (B) Confirmation of Slug expression in tumor nodules by qRT-PCR. (C) Confirmation of Slug expression with tumor nodules by western blotting. (D) Confirmation of epithelial markers (E-cadherin and β-catenin) and mesenchymal markers (N-cadherin and vimentin) expression in tumor nodules by qRT-PCR. (E) Confirmation of epithelial markers (E-cadherin and β-catenin) and mesenchymal markers (N-cadherin and vimentin) expression with tumor nodules by western blotting. GAPDH was used as an internal control. Each experiment was performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$.

EMT [41]. Furthermore, miR-10b was found to be up-regulated by the EMT transcription factor Twist [42]. Furthermore, miR-155 and miR-29a have been identified as regulators of TGF-β signaling pathways in breast cancer tissues [43,44]. It is important to identify the miRNAs involved in EMT, although these would not shed light on the regulation of gene expression in metastasis.

Our results illustrated that miR-630 reduced cell migration and metastasis. Slug was proven to be a target of miR-630 through luciferase assays. EMT marker expression was also evaluated to detect the transformation of ESCC cells with respect to miR-630 down-regulation. Using assays to evaluate the mRNA and protein expression of E-cadherin, β-catenin N-cadherin and vimentin, we found that miR-630, as expected, targeted Slug and induced an obvious EMT both *in vivo* and *in vitro*, further supporting the hypothesis that miR-630 might indeed be a *bona fide* tumor suppressor, whose loss is capable of inducing an EMT-related transformation in ESCC.

In summary, our study revealed that miR-630 expression was down-regulated in patients with ESCC. Accordingly, we have demonstrated that low miR-630 expression is correlated with poor patient survival, indicating a potential prognostic role of this miRNA. In addition, our results also emphasize the biological function of miR-630 in human ESCC and indicate that this tumor suppressor might play critical roles in invasion, metastasis and Slug targeting for EMT progression in ESCC both *in vivo* and *in vitro*. However, several issues are to be addressed in future studies. First and the foremost, the downstream signaling path of miR-630 and Slug must be further investigated with regard to ESCC development. In addition, the molecular mechanism by which miR-630 affects ESCC pathogenesis should be further elucidated. Last but not the least, the upstream targets of miR-630 in the promoter regions should be further explored.

Acknowledgements

We greatly appreciate the valuable help from the Department of Cardiothoracic Surgery of Jinling Hospital in sample collection.

Technical assistance from the Department of Medical Oncology of Jinling Hospital is also greatly appreciated.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (No. 81301914) and Six Big Talent of Jiangsu Province.

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