

Original Article

Regulation of β -catenin-mediated esophageal cancer growth and invasion by miR-214

Yunhua Xu, Shun Lu

Shanghai Lung Cancer Center, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai 200030, China

Received August 12, 2015; Accepted October 12, 2015; Epub November 15, 2015; Published November 30, 2015

Abstract: The malignancy of esophageal cancer (EC) is largely due to its fast growth and invasion, in which WNT/ β -catenin signaling plays a critical role. Hence, suppression of β -catenin signal transduction in EC cells may inhibit cancer growth and metastases. Among all microRNAs (miRNAs), miR-214 has been shown as a tumor suppressor in many cancers, but has yet studied in EC. Here we found that EC specimens had significant higher levels of β -catenin, and significantly lower levels of miR-214, compared to paired non-EC tissue. The levels of β -catenin and miR-214 were inversely correlated in EC specimens. Bioinformatics analyses showed that miR-214 bound to 3'-UTR of β -catenin mRNA in EC cells to inhibit its translation. Overexpression of miR-214 decreased β -catenin protein, while depletion of miR-214 increased β -catenin protein in EC cells, without altering β -catenin mRNA levels. Overexpression of miR-214 in EC cells inhibited cell growth and invasion, while depletion of miR-214 in EC lines increased cell growth and invasion. Taken together, our data demonstrate a previously unappreciated role for miR-214 in suppression of β -catenin-mediated EC cell growth and invasion, and highlight miR-214 as a potent suppressor of EC.

Keywords: Esophageal cancer (EC), β -catenin, miR-214, bioinformatics analyses, miRNAs

Introduction

Recent investigations have revealed that esophageal cancer (EC) is the eighth most common cancer worldwide and the sixth most common cause of cancer death. Recently, the incidence of EC has dramatically increased [1-3]. EC has two major types, adenocarcinoma and squamous cell carcinoma [1-3]. Both have invasiveness manners. Indeed, the early invasiveness and metastases of EC cells account for the high lethality of the cancer [4-7]. Thus, understanding of the mechanisms underlying the growth and invasion of EC are extremely important for its therapy.

WNT signaling pathway has been shown to play a critical role in the carcinogenesis of EC [8-11]. Several WNT pathway components may be overexpressed in EC, including the key factor of WNT signaling, β -catenin [8-11]. Moreover, aberrant activation of WNT/ β -catenin appears to be a predictor for poor prognosis [11-13]. On the other hand, suppression of WNT signaling inhibited EC cell proliferation and growth, and reduced cell motility and invasion [8, 14]. These

data suggest that β -catenin may be a critical target protein for effective EC therapy.

MicroRNA (miRNA) is a class of 18-23-nucleotide non-coding small RNAs that regulate gene expression at translational level, through their base-pairing to the 3'-untranslated region (3'-UTR) of target mRNAs [15, 16]. It has been acknowledged that miRNAs regulate many biological events, and specifically play a critical role during carcinogenesis of various cancers, including EC [17-20]. Among all miRNAs, miR-214 has been extensively studied in many cancers, and its role was mainly found to be a tumor suppressor [21-27]. However, whether miR-214 may be similarly involved in the growth and metastases of EC has not been reported.

Here we found that EC specimens had significant higher levels of β -catenin, and significantly lower levels of miR-214, compared to paired non-EC tissue. The levels of β -catenin and miR-214 were inversely correlated in EC specimens. Bioinformatics analyses showed that miR-214 bound to 3'-UTR of β -catenin mRNA in EC cells to inhibit its translation. Overexpression of miR-

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214 decreased β -catenin protein, while depletion of miR-214 increased β -catenin protein in EC cells, without altering β -catenin mRNA levels. Overexpression of miR-214 in EC cells inhibited cell growth and invasion, while depletion of miR-214 in EC lines increased cell growth and invasion. Taken together, our data demonstrate a previously unappreciated role for miR-214 in suppression of β -catenin-mediated EC cell growth and invasion, and highlight miR-214 as a potent suppressor of EC.

Materials and methods

Patient specimen

Resected EC specimens from 30 EC patients were obtained together with the matched tumor-adjacent non-EC tissue (NT) from 2010 to 2014 at the Shanghai Lung Cancer Center of Shanghai Chest Hospital. All patients provided signed, informed consent for their tissues to be used for scientific research. Ethical approval for the current study was obtained from the Shanghai Lung Cancer Center of Shanghai Chest Hospital. All diagnoses were based on pathological and/or cytological evidence. The histological features of the specimens were evaluated by senior pathologists according to the World Health Organization classification criteria. Tissues were obtained prior to chemotherapy and radiotherapy and were immediately frozen and stored at -70°C prior to mRNA and protein analyses.

EC cell line culture

Two EC cell lines (TE-1 and TE-2) have been established from a well or poorly differentiated human squamous cell carcinoma of EC, and were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and L-glutamine in a humidified chamber with 5% CO_2 at 37°C .

Modification of miR-214 levels in EC cell lines

MiR-214-modification-plasmids were prepared using routine methods. Briefly, the constructs for miR-214, or antisense (as)-miR-214 or control null were cloned into pcDNA3.1-EGFP to generate the corresponding plasmids. The se-

quences were: miR-214 sequence: 5'-ACAGCAGGCACAGACAGGCAGU-3', miR-214 antisense sequence (as-miR-214): 5'-ACUGCCUGUCUGUGCCUGCUGU-3', control null sequence: 5'-UUGUACUACACAAAAGUAAUG-3'. These plasmids of 2 μg were transfected into cultured EC cells using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen). The plasmids also contained a GFP reporter to allow determination of transfection efficiency, which was nearly 100% in the current study.

MTT assay

For assay of cell growth, cells were seeded at 5×10^3 per well into a 96 well-plate and subjected to a Cell Viability Kit (MTT, Roche, Indianapolis, IN, USA), according to the instruction of the manufacturer. The MTT assay is a colorimetric assay for assessing viable cell number, taking advantage that NADPH-dependent cellular oxidoreductase enzymes in viable cells reduce the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its insoluble formazan in purple readily being quantified by absorbance value (OD) at 570 nm in a microtiter plate reader (Promega, Fitchburg, WI, USA). Experiments were performed 5 times.

Quantitative PCR (RT-qPCR)

MiRNA and total RNA were extracted from patients' specimens or from the cultured cells with miRNeasy mini kit or RNeasy kit (Qiagen, Hilden, Germany), respectively. For cDNA synthesis, complementary DNA (cDNA) was randomly primed from 2 μg of total RNA using the Omniscript reverse transcription kit (Qiagen). RT-qPCR was subsequently performed in triplicate with a 1:4 dilution of cDNA using the Quantitect SyBr green PCR system (Qiagen) on a Rotorgene 6000 series PCR machine. All primers were purchased from Qiagen. Data were collected and analyzed using $2^{-\Delta\Delta\text{Ct}}$ method. Values of genes were first normalized against β -actin, and then compared to the experimental controls.

Western blot

For analysis of total protein, the protein was extracted from the patients' specimens or from the cultured cells, and homogenized in RIPA lysis buffer (1% NP40, 0.1% SDS, 100 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride, 0.5% sodium de-

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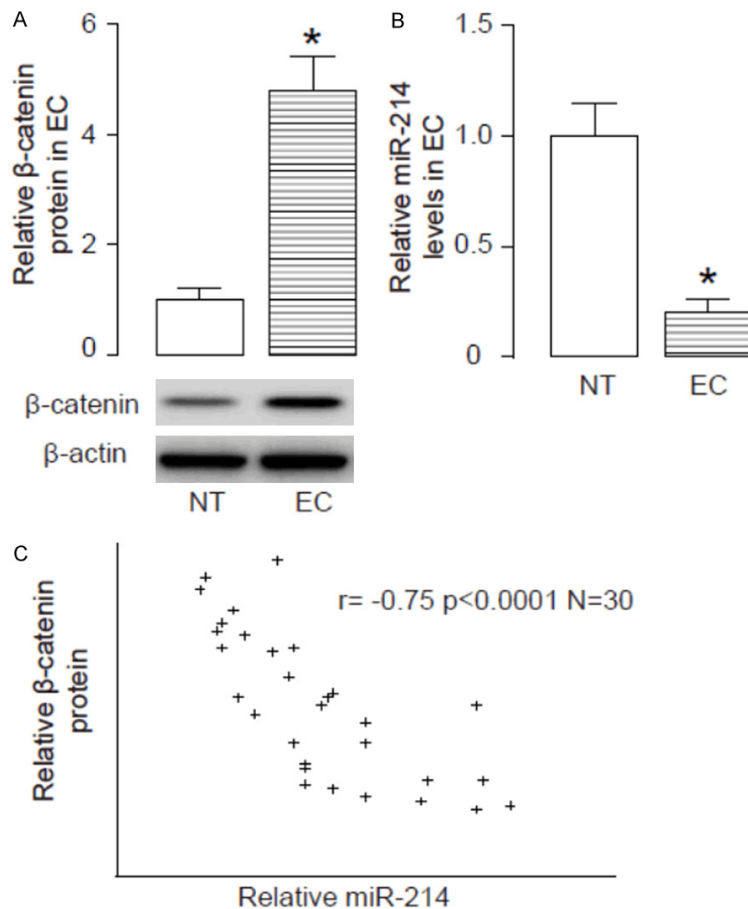


Figure 1. MiR-214 and β -catenin levels inversely correlate in EC specimens. (A, B) Compared with the paired non-tumor tissue (NT), EC expressed significantly higher levels of β -catenin by Western blot (A), and significantly lower levels of miR-214 by RT-qPCR (B). (C) A correlation test in the resected EC specimens from 30 patients showing a strong inverse correlation between miR-214 and β -catenin ($r = -0.75$; $p < 0.0001$). * $p < 0.05$. $N = 30$.

oxycholate, in PBS) on ice. The supernatants were collected after centrifugation at $12000 \times g$ at 4°C for 20 min. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China), and whole lysates were mixed with $4 \times \text{SDS}$ loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Protein samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies for Western Blot are anti- β -

catenin and β -actin (all from Cell Signaling, San Jose, CA, USA). β -actin was used as protein loading controls. Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Images shown in the figures were representative from 5 individuals. Densitometry of Western blots was quantified with NIH ImageJ software (Bethesda, MA, USA). The protein levels were first normalized to β -actin, and then normalized to experimental controls.

MicroRNA target prediction and luciferase-reporter activity assay

MiRNAs targets were predicted as has been described before, using the algorithms TargetScan (<https://www.targetscan.org>) [28]. Luciferase-reporters were successfully constructed using molecular cloning technology. Target sequence was inserted into pGL3-Basic vector (Promega) to obtain pGL3- β -catenin-3'-UTR containing the miR-214 binding sequence (β -catenin-3'-UTR sequence). MiR-214-modified

EC cells were seeded in 24-well plates for 24 hours, after which they were transfected with $1 \mu\text{g}$ of Luciferase-reporter plasmids per well using PEI Transfection Reagent. Then luciferase activities were measured using the dual-luciferase reporter gene assay kit (Promega), according to the manufacturer's instructions.

Transwell cell migration assay

The transwell cell migration assay was performed using a Fluorometric Cell Migration Assay kit with polycarbonate membrane inserts ($5\text{-}\mu\text{m}$ pore size; Cell Biolabs, San Diego, CA, USA). Cells were serum-starved overnight in DMEM prior to initiation of the experiment. Cells were then incubated at 37°C for 24 hours to allow cell migration through the membrane. Migratory cells were detached from the under-

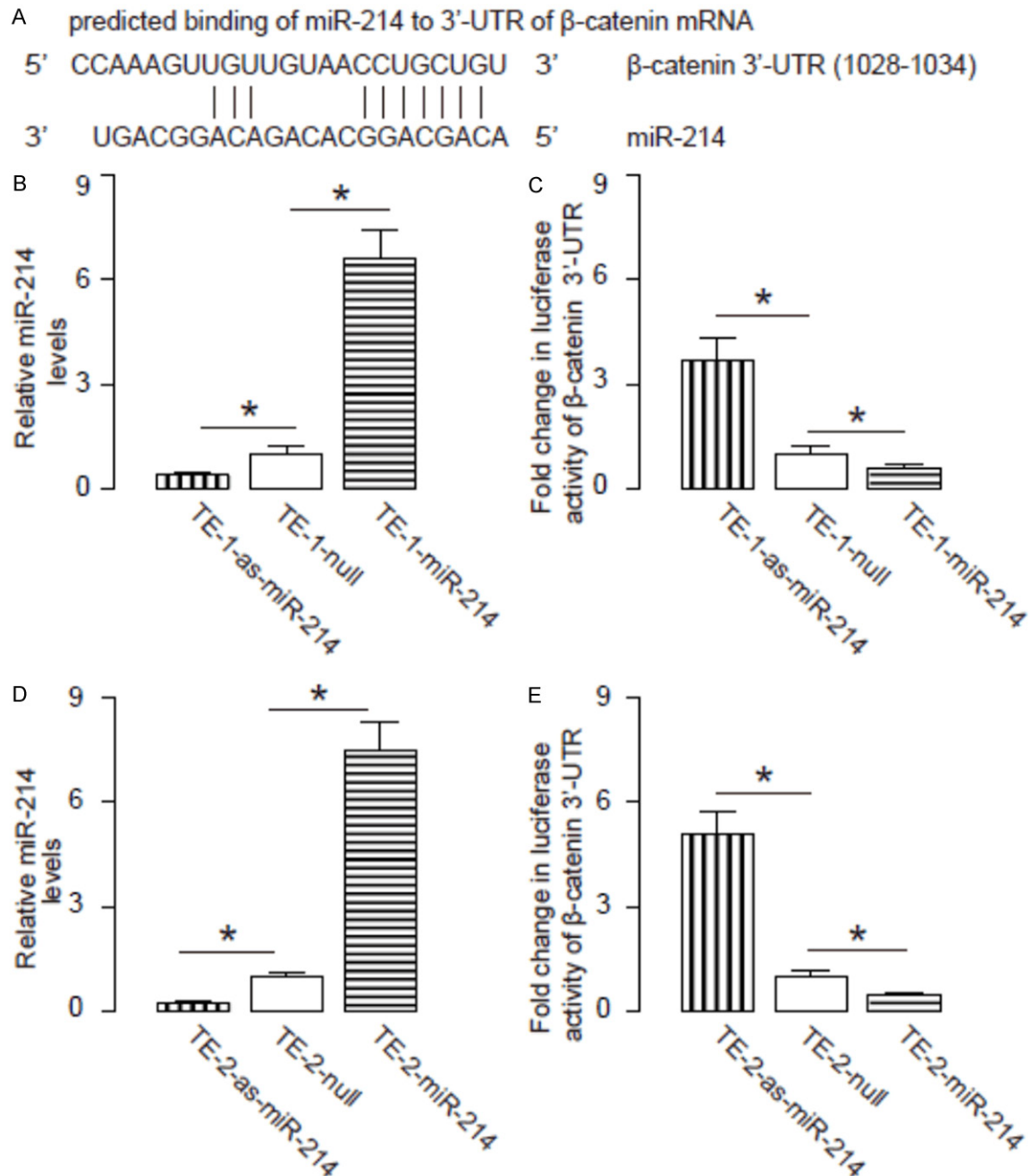


Figure 2. MiR-214 targets 3'-UTR of β -catenin mRNA to inhibit its expression in EC cells. A. Bioinformatics analysis of β -catenin targeting miRNAs, showing that miR-214 binds to 3'-UTR of β -catenin mRNA at 1028th-1034th base site. B. TE-1 cells were transfected with plasmids carrying either miR-214, or as-miR-214, or null as a control. The modification of miR-214 levels in TE-1 cells was confirmed by RT-qPCR. C. The miR-214-modified TE-1 cells were transfected with 1 μ g of β -catenin-3'-UTR Luciferase-reporter plasmid and the luciferase activities were examined. D. TE-2 cells were transfected with plasmids carrying either miR-214, or as-miR-214, or null as a control. The modification of miR-214 levels in TE-2 cells was confirmed by RT-qPCR. E. The miR-214-modified TE-2 cells were transfected with 1 μ g of β -catenin-3'-UTR Luciferase-reporter plasmid and the luciferase activities were examined. *p < 0.05. N = 5.

side of the membrane and subsequently lysed and detected by CyQuant GR dye (Invitrogen). Fluorescence measurement was performed in a FluoStar Optima fluorescence plate reader with a 485/520 nm filter set.

Statistics

All statistical analyses were carried out using the SPSS 18.0 statistical software package. All values in cell and animal studies are depicted

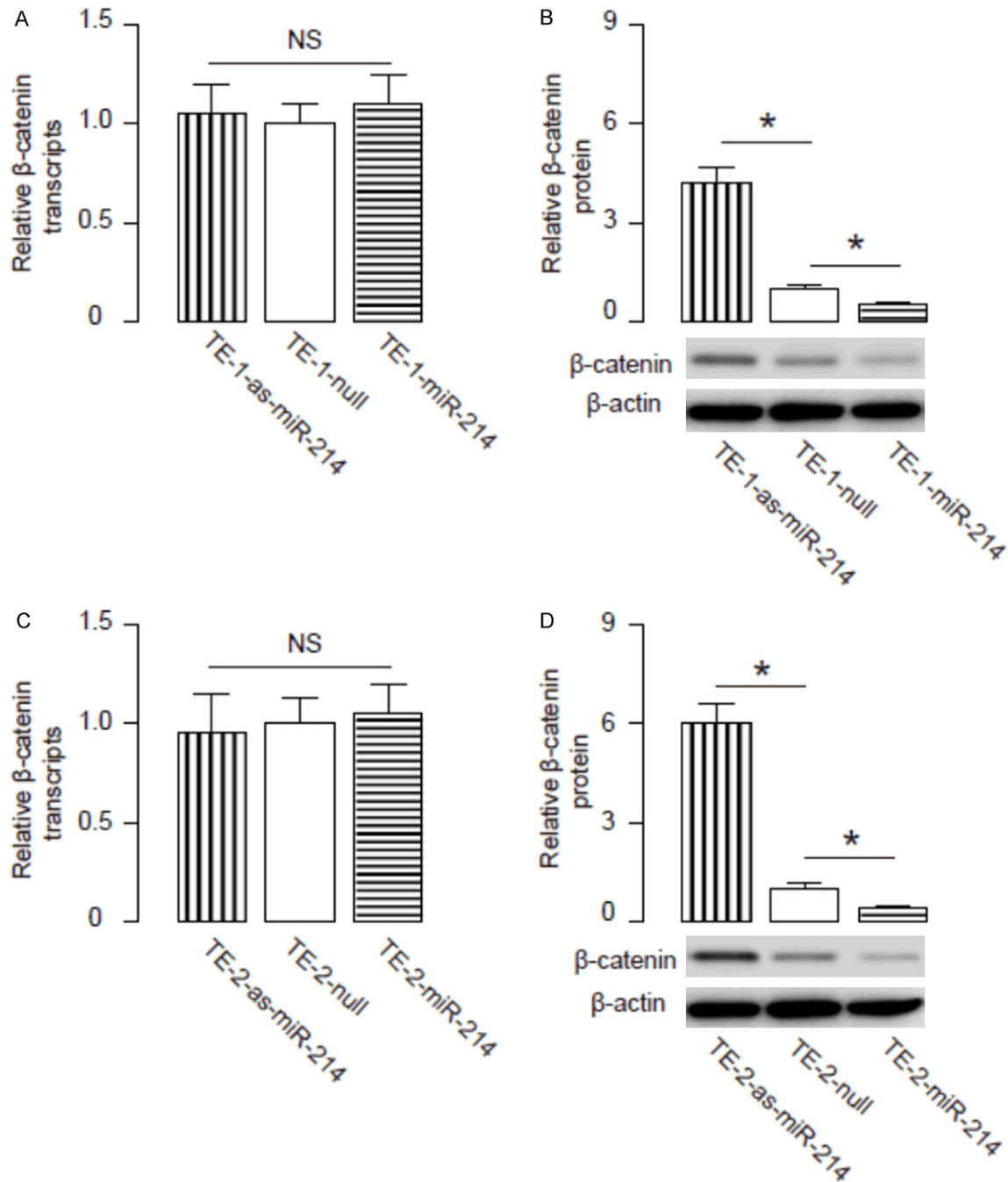


Figure 3. MiR-214 suppresses β -catenin protein, without affecting β -catenin mRNA in EC cells. A. The mRNA levels of β -catenin in miR-214-modified TE-1 cells by RT-qPCR. B. The protein levels of β -catenin in miR-214-modified TE-1 cells by Western blot. C. The mRNA levels of β -catenin in miR-214-modified TE-2 cells by RT-qPCR. D. The protein levels of β -catenin in miR-214-modified TE-2 cells by Western blot. * $p < 0.05$. NS: non-significant. N = 5.

as mean \pm standard deviation and are considered significant if $p < 0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher's Exact Test for comparison of two groups. Bivariate correlations were calculated by Spearman's rank correlation coefficients.

Results

MiR-214 and β -catenin levels inversely correlate in EC specimens

We examined the β -catenin levels in EC specimens by immunoblots and detected significant-

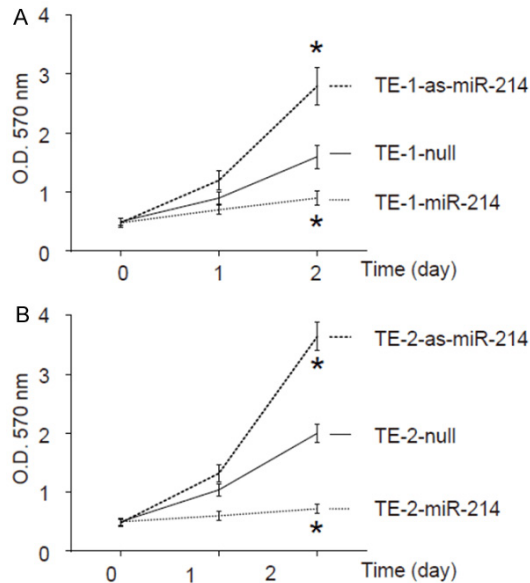


Figure 4. MiR-214 inhibits EC cell growth. (A, B) MTT assay for miR-214-modified TE-1 cells (A), and TE-2 cells (B). * $p < 0.05$. $N = 5$.

ly higher levels of β -catenin in EC specimens, compared to the paired adjacent non-tumor tissue (NT, **Figure 1A**). Moreover, we detected significantly lower levels of miR-214 in EC specimens, compared to NT (**Figure 1B**). To figure out whether these is a possible relationship between miR-214 and β -catenin levels, we performed a correlation test in the resected 30 EC specimens. A strong inverse correlation was detected between miR-214 and β -catenin (**Figure 1C**, $\gamma = -0.75$; $p < 0.0001$), suggesting the presence of a regulatory relationship between miR-214 and β -catenin in EC.

MiR-214 targets 3'-UTR of β -catenin mRNA to inhibit its expression in EC cells

We then performed bioinformatics analyses, and found that miR-214 bound to 3'-UTR of β -catenin mRNA at 1028th-1034th base site (**Figure 2A**). Then we used 2 EC lines, TE-1 and TE-2, to examine the biological relevance of the binding between miR-214 and 3'-UTR of β -catenin mRNA. We transfected TE-1 and TE-2 cells with plasmids carrying either miR-214, or as-miR-214, or null as a control. The modification of miR-214 levels in TE-1 cells was confirmed by RT-qPCR (**Figure 2B**). Then, these miR-214-modified TE-1 cells were transfected with 1 μ g of β -catenin-3'-UTR Luciferase-reporter plasmid. We found that the luciferase activities in TE-1-as-miR-214 cells were significantly higher

than the control, while the luciferase activities in TE-1-miR-214 cells were significantly lower than the control (**Figure 2C**). Similarly, the modification of miR-214 levels in TE-2 cells was confirmed by RT-qPCR (**Figure 2D**). Then, these cells were transfected with 1 μ g of β -catenin-3'-UTR Luciferase-reporter plasmid. We found that the luciferase activities in TE-2-as-miR-214 cells were significantly higher than the control, while the luciferase activities in TE-2-miR-214 cells were significantly lower than the control (**Figure 2E**). Together, these data demonstrate that miR-214 targets 3'-UTR of β -catenin mRNA to inhibit its translation in EC cells, and loss of miR-214 in EC may result in increases in β -catenin, and subsequently enhanced WNT signaling in EC cells.

MiR-214 suppresses β -catenin protein, without affecting β -catenin mRNA in EC cells

Moreover, we found that although the mRNA levels of β -catenin in TE-1 cells were not altered by miR-214 modification (**Figure 3A**), overexpression of miR-214 in TE-1 cells decreased β -catenin protein, while depletion of miR-214 in TE-1 cells increased β -catenin protein (**Figure 3B**). Similarly, although the mRNA levels of β -catenin in TE-2 cells were not altered by miR-214 modification (**Figure 3C**), overexpression of miR-214 in TE-2 cells decreased β -catenin protein, while depletion of miR-214 in TE-2 cells increased β -catenin protein (**Figure 3D**). Together, these data suggest that miR-214 suppresses β -catenin protein, but not β -catenin gene transcription in EC cells.

MiR-214 inhibits EC cell growth

In an MTT assay, we found that overexpression of miR-214 in TE-1 cells resulted in a significant decrease in cell growth, while depletion of miR-214 in TE-1 cells resulted in a significant increase in cell growth (**Figure 4A**). Similarly, overexpression of miR-214 in TE-2 cells resulted in a significant decrease in cell growth, while depletion of miR-214 in TE-2 cells resulted in a significant increase in cell growth (**Figure 4B**). Together, these data suggest that miR-214 inhibits EC cell growth.

MiR-214 inhibits cell invasion in EC cells

Next, we evaluated the effects of miR-214 on EC cell invasiveness. We found that overexpression of miR-214 in TE-1 cells resulted in a sig-

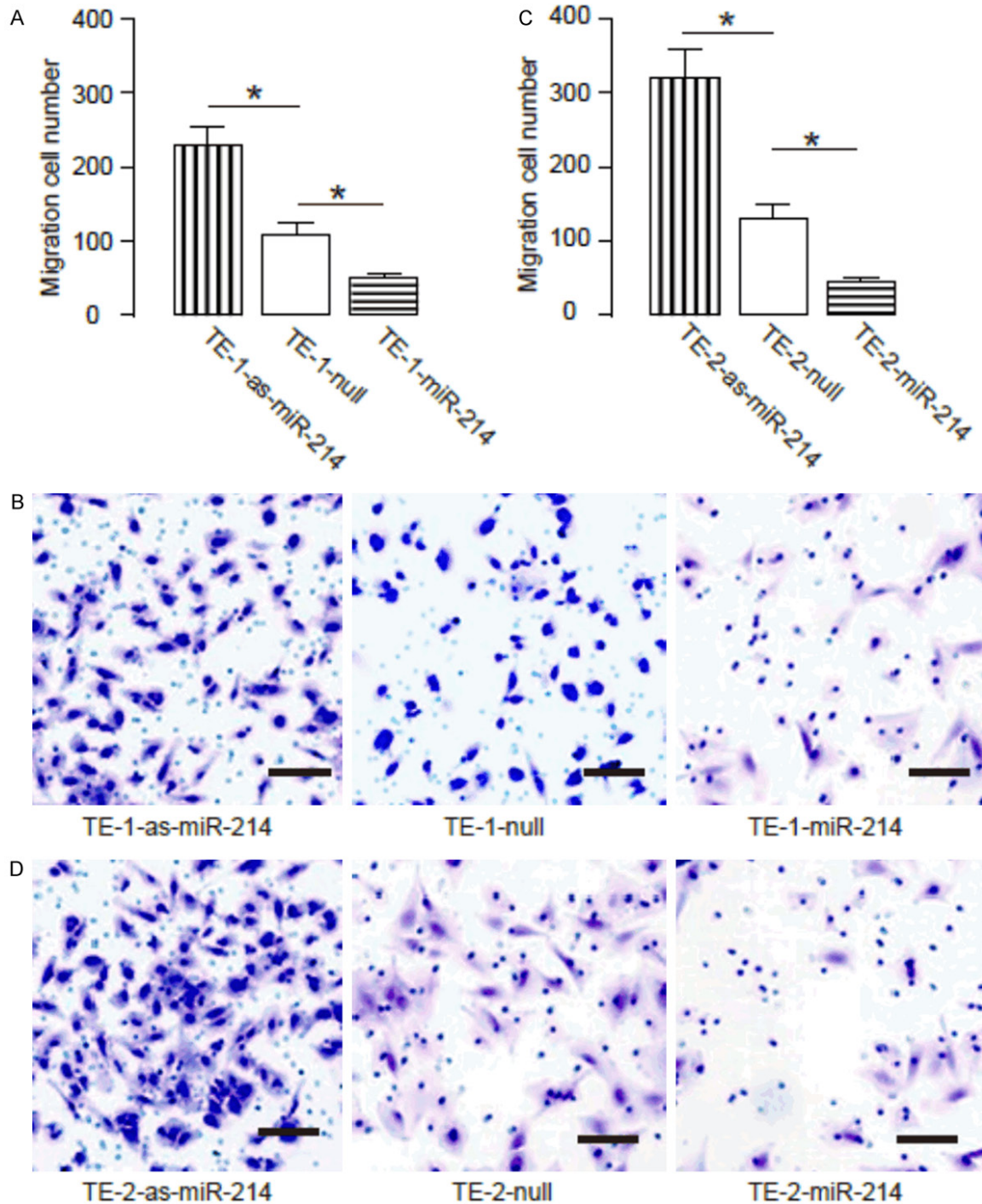


Figure 5. MiR-214 inhibits cell invasion in EC cells. (A, B) Analyses of migrated cells in a transwell cell migration assay of miR-214-modified TE-1 cells by quantification (A), and by representative image (B). (C, D) Analyses of migrated cells in a transwell cell migration assay of miR-214-modified TE-2 cells by quantification (C), and by representative image (D). * $p < 0.05$. $N = 5$. Scale bars are 20 μm .

nificant decrease in cell invasion, while depletion of miR-214 in TE-1 cells resulted in a significant increase in cell invasion, in a transwell cell migration assay, shown by quantifica-

tion (Figure 5A), and by representative images (Figure 5B). Similarly, overexpression of miR-214 in TE-2 cells resulted in a significant decrease in cell invasion, while depletion of miR-

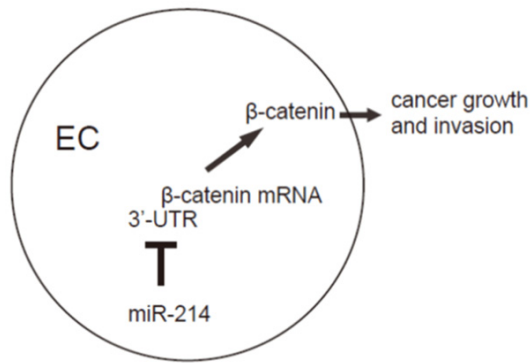


Figure 6. Schematic of the model. MiR-214 targets 3'-UTR of β -catenin mRNA to inhibit its translation in EC cells. Loss of miR-214 promotes EC cell growth and invasion through augmenting β -catenin.

214 in TE-2 cells resulted in a significant increase in cell invasion, in a transwell cell migration assay, shown by quantification (**Figure 5C**), and by representative images (**Figure 5D**). Together, these data suggest that miR-214 inhibits EC cell invasion. To summarize, our data demonstrate a previously unappreciated role of miR-214 in suppressing β -catenin-mediated EC cell growth and invasion (**Figure 6**).

Discussion

EC is a malignant cancer that affects millions of people worldwide, and has become one of the most common causes of cancer-associated death. The poor outcome of EC largely results from its fast growth and invasion, the molecular regulation of which is not clarified [8-14]. Here, we focused on the miRNA-regulated WNT signaling in the regulation of the growth and invasion of EC, paying special attention to the canonical pathway through β -catenin.

Generally, there are two ways to control protein regulation. First, protein levels are regulated by protein degradation via various protein modifications, e.g. phosphorylation, SUMOylation, acetylation and ubiquitination. Second, protein levels are regulated at the translation control point through miRNAs. In this study, we focused on miRNAs.

We compared the levels of β -catenin and some candidate miRNAs that target β -catenin in EC specimens, and we specifically found that miR-214 inversely correlated the levels of β -catenin

in EC tissue. Thus, we hypothesized that in EC, β -catenin may be regulated by miR-214 at post-transcriptional level. Based on bioinformatics analyses and promoter reporter assay, we found that the binding of miR-214 to β -catenin mRNA indeed suppressed the protein translation of β -catenin mRNA, which was further supported by the examination of β -catenin mRNA and protein levels in miR-214-modified EC cell lines.

The two cell lines that were used in the current study are well or poorly differentiated human squamous cell carcinoma of EC, respectively. We involved these two lines to make our study more representative. Here, we found similar data on two EC cell lines and got essentially same results, which ruled out a possibility of our findings to be cell-line dependent.

Since miR-214 overexpression resulted in suppression of EC cell growth and invasion, while miR-214 depletion increased EC growth and invasion, the significant reduction of miR-214 levels in EC specimens could have a significant clinical importance, in which it favors activation of WNT/ β -catenin signaling to directly contribute to the cancer growth and metastases.

Future approaches may be performed to improve our understanding of the regulation of miR-214 in EC and to determine how miR-214 is suppressed in EC. On the other hand, it is possible that miR-214 may have targets other than β -catenin that are also related to either cancer cell growth or cell invasion in EC cells. Elucidation of these targets of miR-214 other than β -catenin may improve the completeness of our understanding of the role of miR-214 in the carcinogenesis of EC.

In summary, our data demonstrate a previously unappreciated role of miR-214 in controlling of β -catenin-associated EC cell growth and invasion, and this regulation may be at least partially conducted through modulation of β -catenin protein translation.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shun Lu, Shanghai Lung Cancer Center, Shanghai Chest Hospital, Shanghai Jiao Tong University, 241 Huaihai West

Road, Shanghai 200030, China. Tel: +86212222-00000; Fax: +862162804970; E-mail: shun_lu14@163.com

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