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miR-146a-5p mediates epithelial–mesenchymal transition of oesophageal squamous cell carcinoma via targeting *Notch2*

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Background: Our previous study found that dysregulated *microRNA-146a-5p* (*miR-146a-5p*) is involved in oesophageal squamous cell cancer (ESCC) proliferation. This article aimed to evaluate its detailed mechanisms in ESCC epithelial–mesenchymal transition (EMT) progression.

Methods: Invasion assay, qRT-PCR and western blotting were used to validate the roles of *miR-146a-5p* and *Notch2* in EMT progression. miRNA target gene prediction databases and dual-luciferase reporter assay were used to validate the target gene.

Results: *miR-146a-5p* inhibitor led to increase of invaded ESCC cells, while *miR-146a-5p* mimics inhibited invasion ability of ESCC cells. Protein level of E-cadherin decreased, whereas those of Snail and Vimentin increased in the anti-*miR-146a-5p* group, which demonstrated that *miR-146a-5p* inhibits EMT progression of ESCC cells. miRNA target gene prediction databases indicated the potential of *Notch2* as a direct target gene of *miR-146a-5p* and dual-luciferase reporter assay validated it. Importantly, shRNA-*Notch2* restrained EMT and partially abrogated the inhibiting effects of *miR-146a-5p* on EMT progression of ESCC cells.

Conclusions: *miR-146a-5p* functions as a tumour-suppressive miRNA targeting *Notch2* and inhibits the EMT progression of ESCC.

Oesophageal cancer is one of the lethal cancers worldwide (Siegel *et al*, 2015). Oesophageal squamous cell cancer (ESCC) accounts for the majority of oesophageal cancer cases worldwide and is predominant in Middle East and central and eastern Asia (Chen *et al*, 2016). Although the medical technology has improved a lot in recent years, the prognoses of ESCC patients remain to be poor, ranging from 15% to 25%. Epithelial–mesenchymal transition (EMT) is an evolutionarily conserved development process during which epithelial cells lose polarity and develop a mesenchymal phenotype. EMT progression triggers the dissociation of carcinoma cells from primary carcinomas, which subsequently migrate and disseminate to distant sites (Nieto *et al*, 2016). This progression can be triggered by many signaling pathways, including Notch (Chen *et al*, 2010), transforming growth factor-β (Chen *et al*, 2016), epidermal growth factor (Liu *et al*, 2015), fibroblast growth factor (Du *et al*, 2015) and PLC-γ (Ji *et al*, 2015) pathways.

MicroRNAs (miRNAs) are small non-coding regulatory RNA molecules. They could bind to and cleave their target mRNAs or

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inhibit their translation into proteins. In this way, miRNAs inhibit the expression of specific target genes (Pichler and Calin, 2015). Accumulating evidence has shown that miRNAs can participate in tumourigenesis, progression and metastasis either as oncogenes or tumour suppressors and have potential as diagnostic, prognostic and therapeutics factors in cancer medicine (Sita-Lumsden *et al*, 2013; Krzeszinski *et al*, 2014; Wang *et al*, 2016a).

MiRNA-146a-5p has been widely explored in various cancers. Decreased expression level of *miR-146a-5p* is found in pancreatic cancer cells (Li *et al*, 2010), lung (Chen *et al*, 2013) and gastric carcinomas (Yao *et al*, 2013). On the other hand, it is upregulated in melanoma (Forloni *et al*, 2014), squamous cell carcinoma of the cervix (Wang *et al*, 2008) and thyroid carcinoma (Sun *et al*, 2015a). In our previous article, we found that *miR-146a-5p* level was significantly decreased in ESCC tissue and serum and it could act as a promising biomarker for the prognosis and diagnosis of ESCC (Wang *et al*, 2016a). In this study, we further investigated the mechanisms of *miR-146a-5p* in EMT progression of ESCC.

MATERIALS AND METHODS

Culture of ESCC cell lines and antibodies. Human ESCC cell lines (Eca109 and EC9706) were provided by Dr. Jiandong Zhang, Qianfoshan Hospital affiliated to Shandong University of China. Both cell lines were cultured in RPMI 1640 (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U ml^{-1} penicillin G and streptomycin in a 37 °C incubator with humidified atmosphere and 5% CO_2.

Anti-Notch2 rabbit polyclonal antibody (ab8926), anti-Snail rabbit monoclonal antibody (ab167609), anti-Vimentin rabbit monoclonal antibody (ab7752), anti-E-cadherin rabbit polyclonal antibody (ab15148) and anti-β-actin mouse monoclonal antibody (ab6276) were purchased from Abcam company (Cambridge, MA, USA).

Quantitative real-time PCR (qRT-PCR)

For Notch2. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Complementary DNA (cDNA) was generated using the qPCR-RT Kit (Toyobo, Osaka, Japan). Real-time PCR was carried out on the Bio-Rad Single Color Real-Time PCR system (Bio-Rad, Hercules, CA, USA) using gene-specific primers: for *Notch2*, sense primer: 5′-GGGACCCTGTCATAC CCTCT-3′, and anti-sense primer: 5′-GAGCCATGCTTACGCT TTCG-3′; and for β-actin, sense primer: 5′-CAAAGGCCAACAGA GAGAAGAT-3′ and anti-sense primer: 5′-TGAGACACACCAT CACCAGAAT-3′ (Sangon Biotech, Shanghai, China). PCR reaction conditions were set as follows: 95 °C for 1 min and 40 cycles of 95 °C for 15 s, 58 °C for 15 s and 72 °C for 45 s. *Notch2* expression was calculated according to the mathematical model $R = 2^{-\Delta\Delta Cq}$, where $\Delta Cq = Cq_{Notch2} - Cq_{\beta\text{-actin}}$, and $\Delta\Delta Cq = \Delta Cq_{test} - \Delta Cq_{control}$.

For miR-146a-5p. Total miRNA was extracted from cells using the miRNeasy Mini Kit (QIAGEN, Duesseldorf, Nordrhein-Westfalen, German) in accordance with the manufacturer's protocol. cDNA synthesis was performed using an All-in-One TM miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD, USA). The RT reaction system (25 μl) comprised 1 μl of 2.5 U μl^{-1} Poly A Polymerase, 1 μl of RTase Mix, 5 μl of 5 × PAP/RT buffer, 2 μg of total RNA templates and RNase/DNase-free ddH_2O. The reaction conditions were carried out as follows: 37 °C for 60 min and 85 °C for 5 min. The reaction system (20 μl) for qPCR comprised 10 μl of 2 × qPCR Mix, 2 μl of First-strand cDNA (diluted 1:5), 2 μl of Universal Adaptor PCR Primer, 2 μl of All-in-OneTM miRNA qPCR Primer (*miR-146a-5p*: HmiRQP0196; U6: HmiRQP9001, GeneCopoeia) and 4 μl of ddH_2O. Amplification was performed in a Bio-Rad Single Color Real-Time PCR system (Bio-Rad, Hercules, CA, USA) under the following reaction conditions: 95 °C for 10 min and 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 10 s. *RNU6* was used as an endogenous control for normalisation of the data. Relative quantification of *miR-146a-5p* expression was calculated with $2^{-\Delta\Delta Cq}$ method. All RT-PCRs were performed in triplicate, and the data are presented as the mean ± s.d.

Protein extraction and western blotting. RIPA buffer was used for total protein extraction. Prepared protein samples were separated by 10% SDS polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. The membrane was blocked with 5% skimmed milk in TBST and incubated with the appropriate antibody (anti-Notch2, 1 : 500; anti-Snail, 1 : 1000; anti-Vimentin, 1 : 1000; anti-E-cadherin, 1 : 500). Then horseradish peroxidase-conjugated secondary antibodies (1 : 5000) were added. Bands were subsequently visualised using a chemiluminescence detection system (EMD Millipore, Billerica, MA, USA) and the density was determined using ImageJ software (US National Institutes of Health, Bethesda, MD, USA). β-Actin (1 : 5000) was used as a loading control.

Transfection. The EC 9706 and Eca 109 cells were transfected with specific *Notch2* shRNA (sequence: CGGTGTACCATTGA-CATTG; Genechem, Shanghai, China) for 72 h using Lipofectamine reagent in serum-free 1640 medium according to the manufacturer's instruction. The multiplicity of infection was 20 for Eca 109 and 40 for EC 9706 cells. Untreated cells were used as a negative control. The efficacy of transfection was tested by qRT-PCR and western blotting.

For the manual alteration of *miR-146a-5p* expression, miRNA mimics (sense: UGAGAACUGAAUUCCAUGGGUU; antisense: CCCAUGGAAUUCAGUUCUCAUU) and miRNA inhibitor (AACCCAUGGAAUUCAGUUCUCA) and miRNA-NC (sense: UUCUCCGAACGUGUCACGUdTdT; antisense: ACGUGACAC-GUUCGGAGAAdTdT) (Genechem) were transfected into EC 9706 and Eca 109 cells using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions.

Dual-luciferase reporter assay. EC 9706 and Eca 109 cells were transfected with luciferase vectors (a luciferase vector containing the wild-type target gene's 3′-UTR and a luciferase vector containing the mutant-type target gene's 3′-UTR) for *Notch2* (NCBI Reference Sequence: NM_024408.3; 3′UTR region: 7714–11474) together with *miR-146a-5p* mimics or inhibitor via Lipofectamine 2000 Reagent (Invitrogen). After 72 h, the luciferase activity was measured by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Data were presented as the ratios between the firefly and Renilla fluorescence activities.

Invasion assay. Invasion ability of ESCC cells was measured by the number of cells invading through matrigel-coated transwell inserts (Corning, Corning, New York, USA). Briefly, transwell inserts with 8-mm pores were coated with matrigel (matrigel/DMEM = 8 : 1; 60 μl per well; BD Bioscience, Franklin Lakes, NJ, USA). EC 9706 or Eca109 cells after transfection were seeded at a density of 1×10^5 per well in the upper chambers with 250 μl of 1640 medium supplemented without FBS. In all, 700 μl 1640 medium with 20% FBS were added to the 24-well plate. After 48 h of incubation, cells that had invaded to lower surface of the matrigel-coated membrane were fixed with methanol, stained with 0.1% crystal violet and counted in five randomly selected fields under a light microscope (Olympus BX51, Olympus, Tokyo, Japan).

Statistical analysis. The quantitative data are expressed as mean ± s.d. Comparison between two or more groups was subjected to a two-tailed Student's *t*-test or ANOVA when appropriate. Differences were considered significant for *P*-values

<0.05. All statistical analyses were performed with the SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA) and the GraphPad Prism 5 software (GraphPad Software Inc, San Diego, CA, USA).

RESULTS

***miR-146a-5p* inhibits EMT process.** Expression level of *miR-146a-5p* was downregulated in ESCC tissues compared with the adjacent normal tissues and it could predict overall survival (OS) and progression-free survival (PFS) of ESCC patients, which has been reported in our previous article (Wang *et al*, 2016a). The invasiveness of ESCC cells (EC 9706 and Eca 109) were examined after transfection. In the *miR-146a-5p* mimics group, *miR-146a-5p* was overexpressed (Figure 1A) and the invasiveness of ESCC decreased significantly (both $P<0.001$) (Figure 1B). On the contrary, knockdown of *miR-146a-5p* via miRNA-inhibitor transfection (Figure 1A) evidently promoted invasion of ESCC cells (both $P<0.001$) (Figure 1C). Expression level of E-cadherin was upregulated ($P=0.002$ for EC 9706, $P<0.001$ for Eca 109) while Snail ($P=0.004$ for EC 9706, $P<0.001$ for Eca 109) and Vimentin were downregulated ($P=0.003$ for EC 9706, $P<0.001$ for Eca 109) in the *miR-146a-5p* mimics group by western blotting. On the other hand, after inhibition of *miR-146a-5p*, the protein level of E-cadherin was reduced ($P=0.007$ for EC 9706, $P<0.001$ for Eca 109) while Snail ($P=0.005$ for EC 9706, $P=0.017$ for Eca 109) and Vimentin ($P<0.001$ for EC 9706 and Eca 109) were increased (Figure 1D and E).

***Notch2* is a direct target of *miR-146a-5p* in ESCC.** To investigate the target gene of *miR-146a-5p* in the EMT process, we first performed a miRNA target gene prediction with miRwalk, miRmap, miRanda, miRBase and Targetscan databases. We found that *Notch2* exhibits *miR-146a-5p*-binding sequences in its 3′-UTR regions (nucleotides 1884–1891, Figure 2A). Luciferase assays were performed to obtain direct evidence that *Notch2* is a target of *miR-146a-5p*. As expected, the luciferase activity was decreased with *miR-146a-5p* overexpression in the wt *Notch2*-3'-UTR group ($P=0.003$ for EC 9706 and $P<0.001$ for Eca 109) (Figure 2B), compared with the mu *Notch2*-3′-UTR group (both $P>0.05$), suggesting that *miR-146a-5p* reduced the luciferase activity of wt *Notch2*-3′-UTR but had no effect on mu *Notch2*-3′-UTR. Meanwhile, an increase in the luciferase activity of wt *Notch2*-3′-UTR was observed after *miR-146a-5p*-inhibitor transfection ($P=0.002$ for EC 9706 and $P<0.001$ for Eca 109) (Figure 2B). To confirm that *Notch2* acts as a *miR-146a-5p* target, we examined Nocth2 protein levels in *miR-146a-5p* mimics or inhibitor transfected ESCC cells. Notch2 expression level was decreased ($P=0.004$ for EC 9706 and $P=0.006$ for Eca 109) (Figures 2C and D) after *miR-146a-5p* mimics was transfected, compared with the control group. Reciprocally, the *miR-146a-5p* knockdown was accompanied by an increase in the Notch2 expression in EC 9706 and Eca 109 cells ($P=0.008$ for EC 9706 and $P=0.007$ for Eca 109) (Figure 2C and D).

***Notch2* promotes EMT process of ESCC.** Previously, we found that *Notch2* was upregulated in ESCC cancerous tissues compared with adjacent normal tissues, and it was significantly associated with the patients' OS and PFS. Besides, *Notch2* inhibition with shRNA decreased ESCC cell proliferation and survival ability (Wang *et al*, 2016b). The EC 9706 and Eca 109 cells were transfected with specific *Notch2* shRNA using Lipofectamine reagent. After transfection, mRNA expression level of *Notch2* was significantly decreased according to qRT-PCR results (both $P<0.001$, Figure 3A). We used invasion assay and western blotting to examine the effect of *Notch2* on EMT process. After transfection, invasion ability of EC 9706 and Eca 109 were decreased significantly (both $P<0.001$, Figure 3B and C). According to western blotting, the expression levels of Snail (both $P<0.001$) and Vimentin (both $P<0.001$) were decreased while

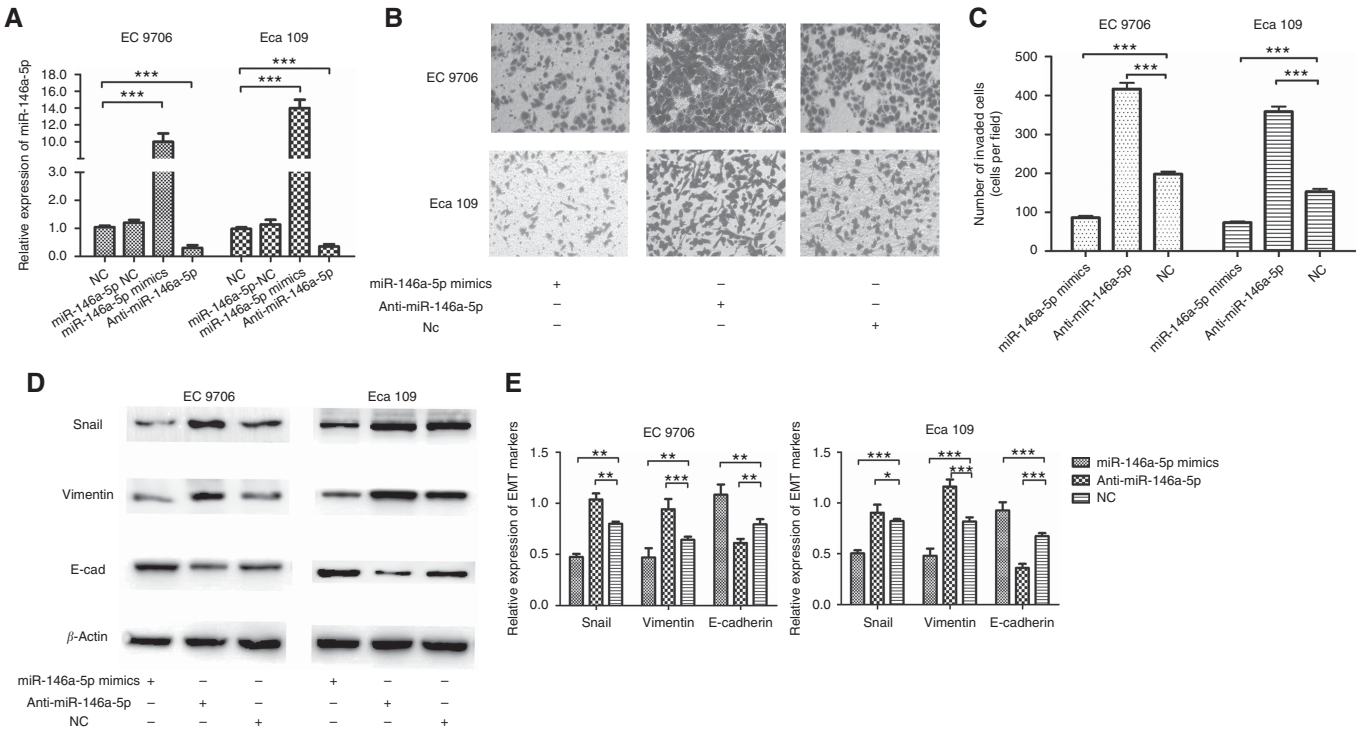

Figure 1. *miR-146a-5p* regulates EMT of ESCC. (**A**) The relative expression levels of *miR-146a-5p* after transfection. (**B**, **C**) The effects of *miR-146a-5p* mimics and inhibitor on invasion ability of ESCC. (**D**, **E**) *miR-146a-5p* mimics and inhibitor led to the changes of EMT markers' expression. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

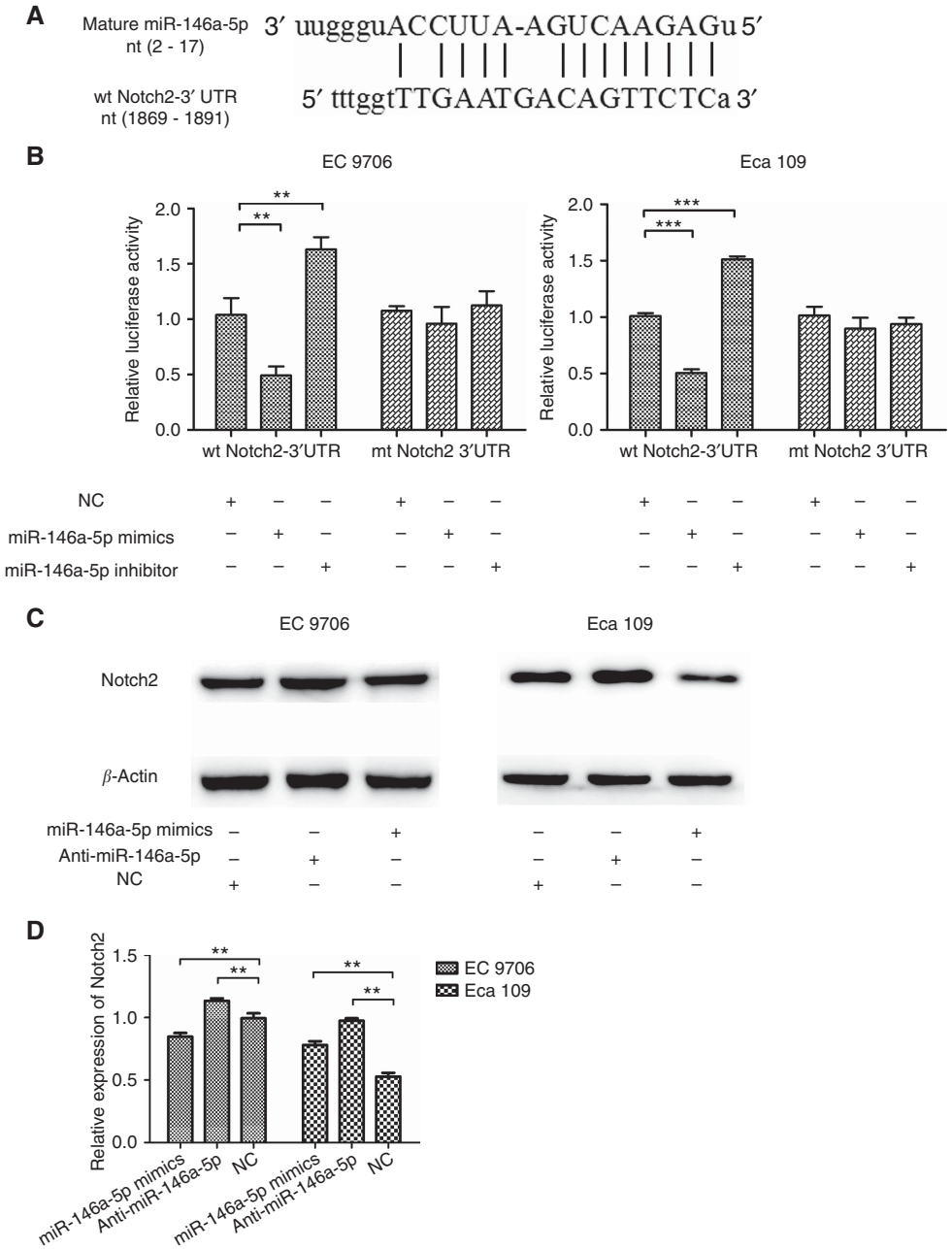

Figure 2. The prediction and validation of *miR-146a-5p* target gene. (**A**) *Notch2* exhibits *miR-146a-5p*-binding sequences in its 3′-UTR regions. (**B**) Luciferase assays validated that *Notch2* is a target of *miR-146a-5p*. (**C**, **D**) Further evidence of *miR-146a-5p* targeting *Notch2* by western blotting. $^{**}P<0.01$; $^{***}P<0.001$.

E-cadherin was increased ($P<0.001$ for EC 9706 and $P=0.002$ for Eca 109) (Figure 3D and E) in the anti-*Notch2* group.

***miR-146a-5p* regulates EMT process in ESCC cells via targeting *Notch2*.** To investigate whether *miR-146a-5p* targets *Notch2* to inhibit EMT process, we set four groups including *miR-146a-5p*-NC together with *Notch2*-NC, anti-*miR-146a-5p* together with anti-*Notch2*, anti-*miR-146a-5p* together with *Notch2*-NC and *miR-146a-5p*-NC together with anti-*Notch2*. As shown in Figure 4A and B, *miR-146a-5p* inhibitor increased ESCC cells' invasive ability, whereas shRNA-*Notch2* could abolish this change (all $P<0.001$). Besides, *miR-146a-5p* inhibitor led to changes of EMT-related markers' expression (increase of Snail and Vimentin and decrease of E-cadherin) in the EC 9706 and Eca 109 cell lines, whereas co-transfection with shRNA-*Notch2* partially abolished these changes at the protein level (all $P<0.05$, Figure 4C and D). These findings suggest that *miR-146a-5p* regulates EMT progression by suppressing *Notch2* expression.

DISCUSSION

Accumulating studies have demonstrated that miRNAs regulate the expression of oncogene or tumour suppressor (Png *et al*, 2012; Krzeszinski *et al*, 2014; Cheng *et al*, 2015), which suggest a new mechanism involved in the initiation and development of ESCC. Aberrant expression of *miR-146a-5p* in other human cancers (Sun *et al*, 2014; Shi *et al*, 2015; Sun *et al*, 2015b; Cui *et al*, 2016; Lerner *et al*, 2016) promoted us to determine its expression status in ESCC. In previous article, we found that *miR-146a-5p* was

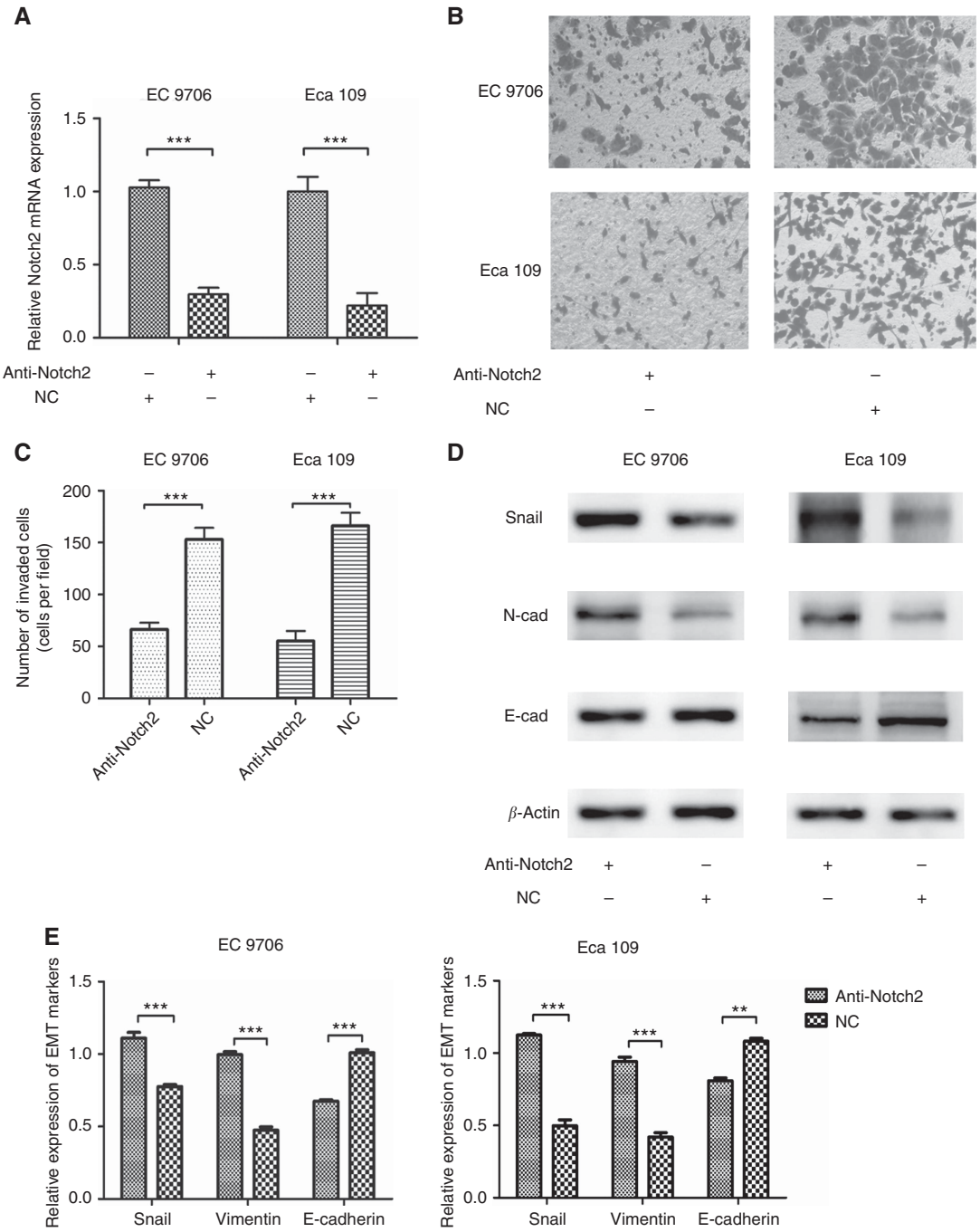

Figure 3. *Notch2* promotes EMT of ESCC. (**A**) shRNA transfection of *Notch2* could inhibit its expression. (**B**, **C**) shRNA-*Notch2* inhibited invasion ability of ESCC. (**D**, **E**) shRNA-*Notch2* regulated EMT markers of ESCC. $^{**}P<0.01$; $^{***}P<0.001$.

downregulated in ESCC cancerous tissues as well as serum, which indicated its potential antitumour function (Wang *et al*, 2016a). In this article, we demonstrated its role in EMT progression. It inhibits EMT progression of EC 9706 and Eca 109 cells depending on *Notch2*.

miR-146a-5p plays important roles in carcinogenesis and development of tumour. In this article, our gain- and loss-of-function experiments demonstrated that *miR-146a-5p* inhibitor led to the increase of invaded ESCC cells, whereas *miR-146a-5p* mimics inhibited the invasion ability. Meanwhile, the protein level of E-cadherin was reduced, whereas Snail and Vimentin were increased in the anti-*miR-146a-5p* group. These results indicate that *miR-146a-5p* inhibits EMT progression of ESCC cells. It is well established that miRNAs perform their function by regulating the expression of a target gene. Therefore, we decided to identify the functional target gene for *miR-146a-5p* that was involved in EMT regulation. MiRNA target gene prediction databases indicated the potential of *Notch2* as a direct target of *miR-146a-5p* in ESCC and the dual-luciferase reporter assay validated it. Accordingly, Notch signal pathway has been considered as a crucial regulator of EMT (Espinoza and Miele, 2013; Ishida *et al*, 2013; Yuan *et al*, 2014; Zoni *et al*, 2015). Furthermore, the overexpression level and oncogenic role of *Notch2* have been observed in numerous human cancer types, such as lung adenocarcinoma (Mimae *et al*, 2012), glioma (Yu *et al*, 2015), cervical cancer (Zhang *et al*, 2014), hepatoblastoma (Litten *et al*, 2011) and salivary adenoid cystic carcinoma (Qu *et al*, 2016). We have revealed that

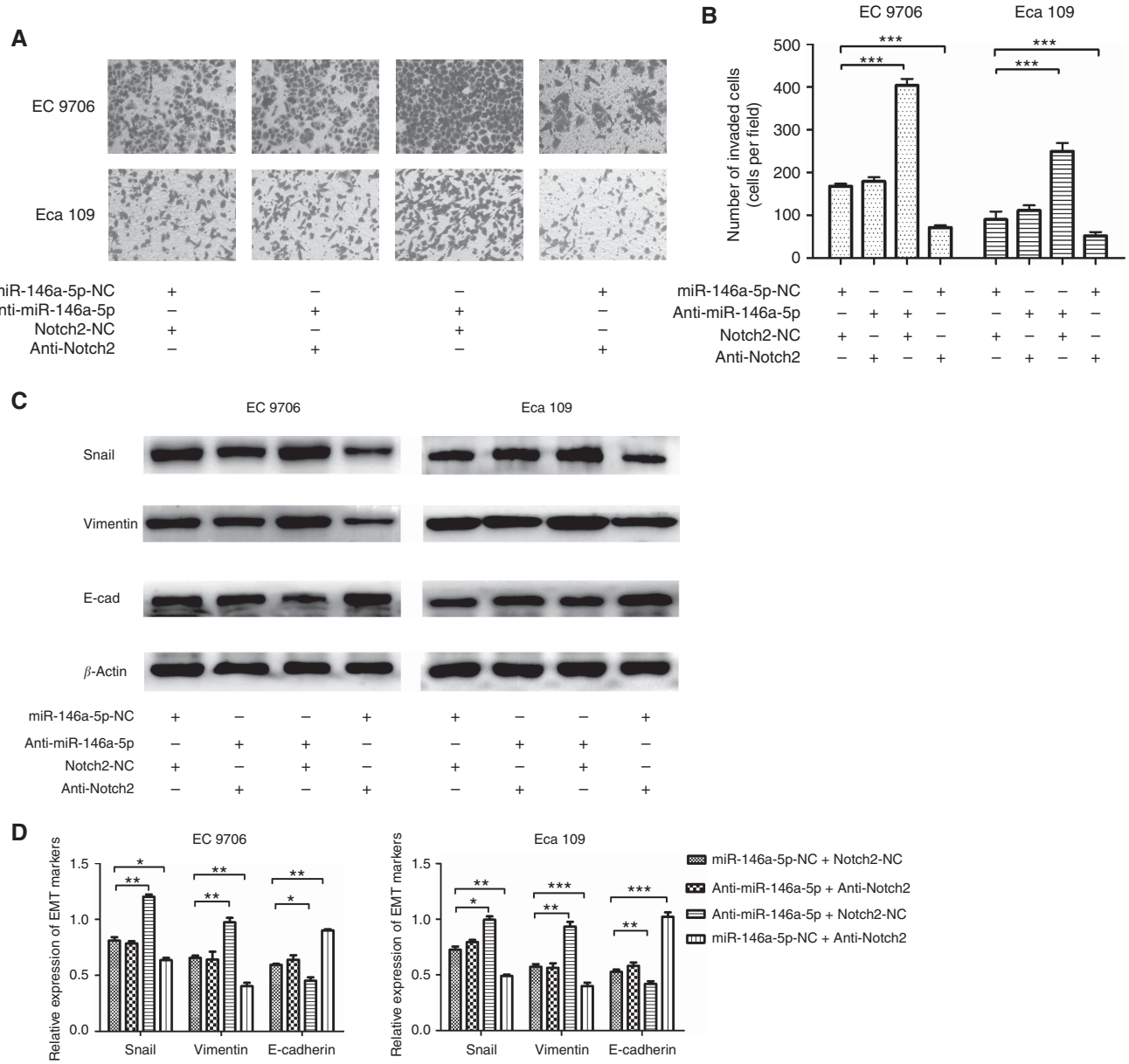

Figure 4. The *miR-146a-5p*-mediated EMT process depends on *Notch2*. (**A**, **B**) *miR-146a-5p* inhibitor caused an increase in ESCC cells' invasive ability, whereas shRNA-*Notch2* could abolish this change. (**C**, **D**) *miR-146a-5p* inhibitor led to increase of Snail and Vimentin and decrease of E-cadherin, whereas co-transfection with shRNA-*Notch2* partially abolished these changes. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

protein level of Notch2 was upregulated in ESCC cancerous cancer and it could promote the proliferation and survival ability of EC 9706 and Eca 109 (Wang *et al*, 2016b). Herein, it indicated that knockdown of *Notch2* decreased EMT progression and invasion ability of ESCC cells. Importantly, the inhibiting effect of *miR-146a-5p* on EMT progression and cell invasion were partially abrogated by *Notch2* knockdown in ESCC cells. Thus we propose that *miR-146a-5p* inhibits ESCC EMT by suppressing *Notch2*.

As is known to us, both *miR-146a-5p* and *miR-146a-3p* derive from *pre-miR-146a*. Their sequences and roles are different from each other (Gysler *et al*, 2016). *MiR-146a-5p* is wildly investigated, whereas researches on *miR-146a-3p* are not sufficient enough. To verify the specificity of the mimics and inhibitor used in our experiment, we blasted the sequences of our mimics and inhibitor via miRBASE database. Results indicated that the sequences match well with *miR-146a-5p* (both *E*-value <0.001), while similarity sequence test's *E*-values were both 5.1 for *miR-146a-3p*, which revealed that the mimics and inhibitor could unlikely change the level of *miR-146a-3p*.

In conclusion, we suggest that *miR-146a-5p* functions as a tumour-suppressive miRNA and inhibits the EMT progression of ESCC via suppressing *Notch2*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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