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...
miR-146a-5p mediates EMT via targeting Notch2

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% CO₂.

Anti-Notch2 rabbit polyclonal antibody (ab8926), anti-Snail rabbit monoclonal antibody (ab167609), anti-E-cadherin rabbit polyclonal 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) 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 the 2⁻ΔΔCq method. All RT-PCRs were performed in triplicate, and the data are presented as the mean ± s.d.

Transfection. The EC 9706 and Eca 109 cells were transfected with specific Notch2 shRNA (sequence: CGGTGTACCTGATT-CATTG; Genechem, Shanghai, China) for 72 h using Lipofectamine reagent in serum-free 1640 medium according to the manufacturer’s instructions. 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: UGAGAACUAUUCAGUGGU; antisense: CCAAUUGGAUCAUCCUAAUG) and miRNA inhibitor (AACCCAUUGGAUCAUCCUAA) and miRNA-NC (sense: UUCUCGAAGGUGAUCAUGGUAU; antisense: AGCUAGACAC-GUCGGAAGAxDdT) (Genechem) were then 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 × 10⁴ 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.
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 reduced (\( P = 0.002 \) 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 Notch2 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...
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 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
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

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.
The 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.

**ACKNOWLEDGEMENTS**

This work was supported by National Natural Science Foundation of China (no. 81572958) and Science and Technology Development Planning Project of Shandong Province (2014GSF118058).

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.
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