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miR-145 Regulates the sensitivity of esophageal squamous cell carcinoma cells to 5-FU via targeting REV3L

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ABSTRACT

Aberrant expression of miR-145 was associated with chemotherapy in multitype cancers. However, the underlying role and molecular mechanism of miR-145 in the sensitivity of esophageal squamous cell carcinoma (ESCC) to 5-FU remained largely unknown. Cell viability was determined by Cell Counting Kit-8 (CCK-8) assay. Gene expression levels were detected by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). Protein expression levels were evaluated by Western blot. TargetScan was used for the prediction of binding sites for miRNA in mRNAs. The interaction between mRNA 3′ UTR and miRNA was verified by dual luciferase reporter assay. The results showed that miR-145 was downregulated in ESCC tumor tissues and cells, while REV3L was upregulated in ESCC tumor tissues. Overexpression of miR-145 decreased REV3L mRNA and protein level in ESCC cell line KYSE150, while decreased miR-145 increased REV3L mRNA and protein level in esophageal epithelium cell line (HEEC). In addition, the luciferase activity of ESCC cells was decreased after the treatment of miR-145 mimic and mRNA 3'UTR-WT. Overexpressed miR-145 significantly inhibited cell viability and elevated cell apoptosis rate upon 5-FU treatment. Additionally, transfection of miR-145 mimic further altered expression of key genes involved in cell apoptosis (Bcl-2, Bax, Caspase3) in ESCC cells treated with 5-FU. miR-145 might be a therapeutic target for the treatment of ESCC.

1. Introduction

Globally, esophageal cancer (EC) ranks the 8th most common cancer and the 6th most common cause of cancer-related death [1]. Meanwhile, EC was one of the most lethal malignancies in China and other Asian countries with the poor 5-year survival rate after curative surgery [2,3]. Esophageal squamous cell carcinoma (ESCC), which accounts for 90% of EC, [3,4], is a serious health problem in China, leading to 375,000 deaths in 2015 [5]. 5-Fluorouracil (5-FU) is a chemotherapy agent which induces cell death mainly through the inhibition of thymidylate synthase and misincorporation into newly synthesized DNA and RNA during cell cycle progression [6]. 5-FU is a commonly used drug alone or in combination with other chemotherapy agents for the treatment of ESCC [7]. However, intrinsic or acquired chemotherapy resistance frequently occur after the chemotherapy treatment of cytotoxic drugs, led to chemotherapy failure and eventually patient death [8]. It was urgent to further investigate molecular mechanism of drug resistance to improve the prognosis of ESCC patients.

MicroRNAs (miRNAs) are a class of endogenous and conserved noncoding 20–22 nt small RNAs which regulates gene expression at posttranscriptional level by binding to 3'-UTR of target mRNAs, resulting in mRNA degradation or translation inhibition [9]. miRNAs may act as an oncogene or a tumor suppressor, through regulating cell differentiation, cell proliferation and cell apoptosis [10,11]. miR-145 is a well-characterized tumor suppressor in various cancer types [12–14]. For EC, miR-145 is firstly reported to be downregulated in ESCC [15]. Recently, miR-145 is reported to inhibit cancer cell migration, invasion and epithelial-mesenchymal transition as well as cell proliferation in ESCC [16,17]. In human colon cancer cells, miR-145 inhibits 5-FU resistance [18]. The role of miR-145 in the sensitivity of ESCC to 5-FU has not been previously reported.

2. Materials and methods

2.1. Tissues

ESCC tissues and normal esophageal tissues were collected from 25 patients with ESCC between May 2015 and Nov, 2016 in Jingjiang Peoples' Hospital. Histopathologic sections from patients diagnosed with ESCC. The grades were based on "WHO Classification Tumors of the Digestive System" in 2010. All patients hadn't received radiotherapy or chemotherapy prior to the present study. All patients had

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provided informed consent. The current study was approved by Ethical Committee of Jingjiang Peoples' Hospital.

2.2. Cell culture

HEEC, TE-8, KYSE150, and TE-1 cells were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin (Sigma) in an incubator at 37 °C and 5% CO_2. KYSE150 cells were cultured in RPMI-1640 /F12 medium supplemented with 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin (Sigma) in an incubator at 37 °C and 5% CO_2.

2.3. Cell transfections

The miR-145 mimic and miR-negative control (NC) mimic were obtained from Qiagen Company (Hilden, Germany). HEEC, TE-8 and TE-1 cells were transfected with miRNA-145 mimic and miRNA-NC mimic were obtained from Qiagen Co mimics or miRNA-NC mimics by Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA, USA) and prepared for the following experiments.

2.4. 5-FU treatment

For 5-FU treatment (commonly used to treat esophageal cancer), cells were incubated with 35 mM 5-FU (Sigma) (resuspended in DMSO) for 72 h. Cells were then washed, trypsonized and counted.

3. real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)

miRNA was isolated by a miRNA Extraction Kit (Qiagen, Hilden, Germany). Reverse transcription was carried out by miRNA cDNA Synthesis Kit (Qiagen) in accordance with the manufacturer's instructions. Total RNA was isolated TRIzol® (Invitrogen, Thermo Fisher Scientific, Inc.). cDNA was prepared using the PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. PCR amplification was conducted by SYBR green Premix Ex Taq II (Qiagen). The procedure was performed by ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The expression level was calculated by $2^{-\Delta\Delta Ct}$. U6 acted as the control for miR-145. GAPDH acted as the control for genes. The sequences of the primers used were listed in Table 1.

3.1. Western blot

Cells were lysed by RIPA (Solarbio). Each protein sample (10 μl) was separated by 6%–15% SDS-PAGE. Then the protein were transferred onto PVDF membranes (0.45 μm, Millipore, USA). The PVDF membranes were first blocked by 5% non-fat milk at room temperature for 2 h prior to incubation with the following primary antibodies overnight at 4 °C: REV3L (ab159329, Abcam, USA), Bcl-2 (ab32124, Abcam, USA), Bax (ab32503, Abcam, USA), Caspase 3 (ab13847, Abcam, USA), and GAPDH (ab181602, Abcam, USA). On the next day, the PVDF membranes were incubated with the corresponding secondary antibody anti-human (ab6759, Abcam, USA) and anti-rabbit (ab97051,Abcam, USA) at room temperature for 2 h. At last, the PVDF membranes were visualized by ECL reagent. Protein was normalized with GAPDH was set as an internal control.

Table 1
Oligonucleotide primers used for Q-PCR.

Name	Primer sequence
miR-145U6	Forward 5'-CCTTGTCCTCACGGTCCAGT-3' Reverse 5′-AACCATGACCTCAAGAACAGTATTT-3′
	Forward 5'-CTCGCTTCGGCAGCACA-3' Reverse 5'-AACGCTTCACGAATTTGCGT-3'
REV3L	Forward 5′-TGATGTCTTCAGCTGGTAT-CATGA-3′ Reverse 5′-CCGCCCTTCAGGTTCACTT-3′
Bax	Forward 5'-CACCAGCTCTGAACAGATCATGA-3' Reverse 5'-TCAGCCCATCTTCTTCCAGATGT-3'
Caspase 3	Forward 5'-AACTGGACTGTGGCATTGAG-3' Reverse 5'-ACAAAGCGACTGGATGAACC-3'
Bcl-2	Forward 5'-CACCCCTGGCATCTTCTCCTT-3' Reverse 5'-AGCGTCTTCAGAGACAGCCAG-3′
GAPDH	Forward 5'-GGAGCGAGATCCCTCCAAAAT-3' Reverse 5'-GGCTGTTGTCATACTTCTCATGG-3'

3.2. Dual luciferase reporter assay

The wild-type (WT) sequence of the 3UTR of REV3L containing miR-145 binding sites and the mutant-type (MT) sequence of the 3UTR of REV3L lacking miR-145 binding sites were amplified by PCR and individually subcloned into the psiCHECK-2 vector (Promega Corp., Madison, WI, USA). Lipofectamine® 2000 was used to co-transfect cells with WT or MT miR-145 3UTR luciferase reporter gene plasmid, and miR-NC or miR-145 mimics, respectively. For the detection of dual luciferase activity, HEEC and KYSE150 cells were co-transfected with miR-145 mimic (20 mM) or miR-NC mimic (20 mM) and psiCHECK-2-REV3L-3'-UTR-WT (200 ng), psiCHECK-2-REV3L-3'-MUT (200 ng), and Renilla luciferase vector (10 ng) through the reagents of Lipofectamine 2000 (Invitrogen). At 48 h after transfection, the HEEC and KYSE150 cells were used for the detection of the Dual-Luciferase Reporter Assay (Promega, CA, USA). The luciferase activity in each group was examined by GloMax fluorescence reader (Promega). The pRL-CMV sea renal fluorescent acted as a control.

3.3. Immunohistochemistry assay

Human ESCC tissues and normal tissues were fixed in paraformaldehyde immediately after resection and stained. The slides were incubated with the primary antibodies against REV3L (ab159329, Abcam, USA). The IHC score was calculated by multiplying the stain intensity.

3.4. Cell viability assay

To explore the influence of miR-145 in the cell viability, the cell viability was measured by CCK8 cell counting kit (Dojindo, Japan) based on the manufacture's protocol. Briefly, 1×10^4 TE-1 cells were seeded into 96-well plates overnight in complete DMEM medium at 37 °C. On the next day, 10 μl CCK8 solution was added into each well and maintained for 2 h. The absorbance of TE-1 cells at 450 nm was measured by microplate reader (Biorad, USA). At 24 h, 48 h and 72 h after miR-145 mimic or miR-NC mimic transfection, the cell number was analyzed using CCK8, respectively.

3.5. Flow cytometric assays

To detect the apoptotic rate, KYSE150 cells were seeded into 12-well plates (3×10^5/well) and cultured for 48 h at the temperature of 37 °C. KYSE150 cells were collected through digestion by 0.025% trypsin (Thermo Fisher Scientific). After washing with PBS, 5 μl fluorescein isothiocyanate-labeled Annexin V (FITC) and 5 μl PI was added into KYSE150 cells and incubated for 15 min in the dark at the temperature of 37 °C. Cell apoptosis was analyzed within 1 h by flow cytometry.

3.6. Statistical analysis

The SPSS 13.0 was applied in the statistical analyses. Data were

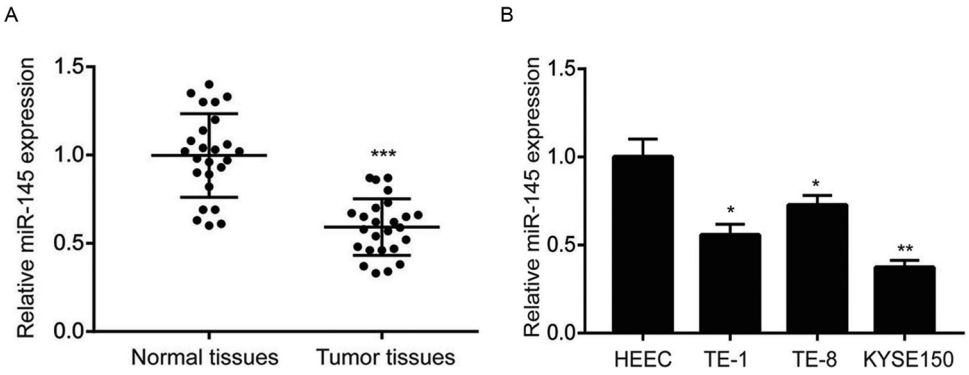

Fig. 1. miR-145 expression was downregulated in human ESCC tissues and cells.
A: The expression of miR-145 was decreased in ESCC tissues. B: The expression of miR-145 decreased in ESCC cells. Each experiment was performed in triplicate. *P < 0.05 vs. HEEC cells. **P < 0.01 vs. HEEC cells. ***P < 0.001 vs. Normal tissues.

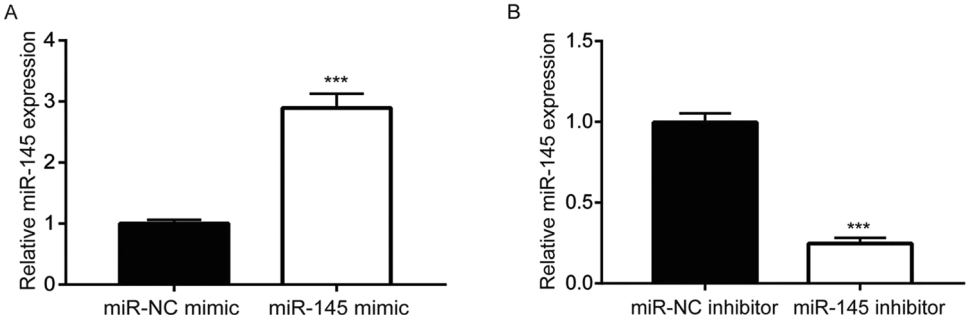

Fig. 2. The expression of miR-145.
A: The expression of miR-145 was upregulated after the transfection of miR-145 mimic. B: The expression of miR-145 was downregulated after the transfection of miR-145 inhibitor. Each experiment was performed in triplicate. ***P < 0.001 vs. miR-NC mimic or miR-NC inhibitor.

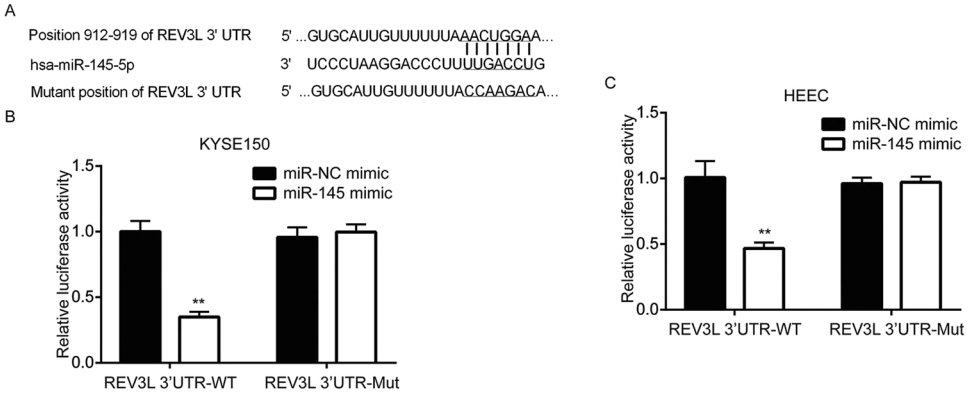

Fig. 3. REV3L was a target gene of miR-145.
A: REV3L mRNA 3'UTR contained putative miR-145 binding site. B: The luciferase activity of KYSE150 cells was decreased after the transfection of miR-145 mimics and REV3L 3'UTR-WT. C: The luciferase activity of HEEC cells was decreased after the transfection of miR-145 mimics and REV3L 3'UTR-WT. Each experiment was performed in triplicate. **P < 0.01 vs. miR-NC mimic.

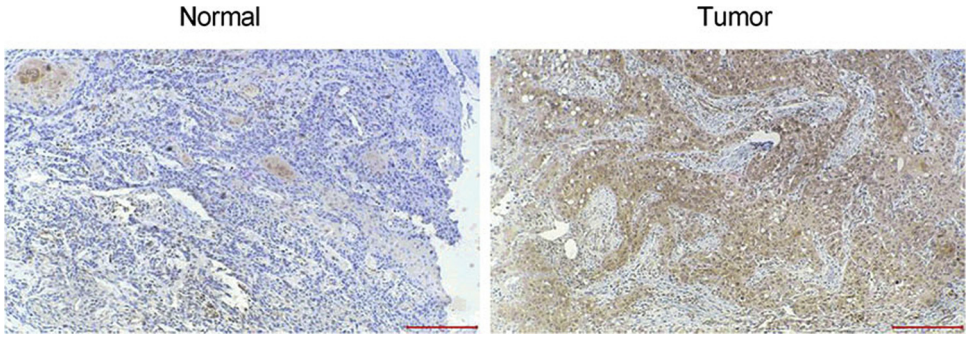

Fig. 4. The expression of REV3L.
REV3L was downregulated in ESCC tissues. Each experiment was performed in triplicate.

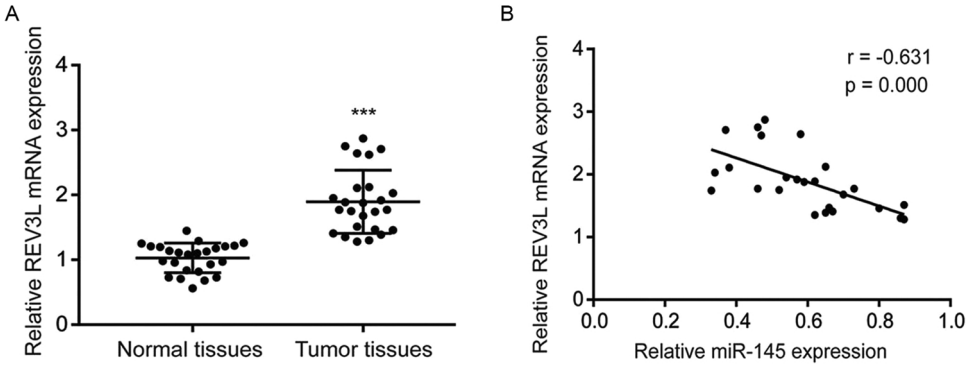

Fig. 5. REV3L was negatively correlated with miR-145.
A: The expression of REV3L decreased in ESCC tissues. B: The expression of REV3L was negatively corrected with miR-145 in ESCC tissues. Each experiment was performed in triplicate. **P < 0.01 vs. Normal tissues.

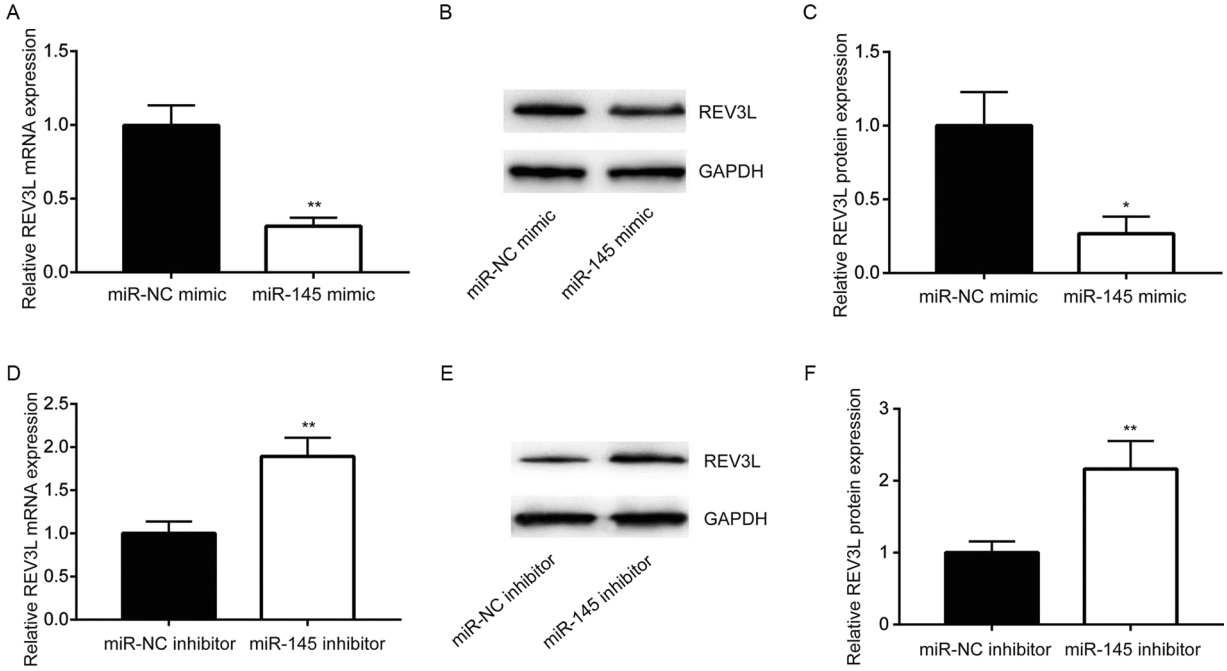

Fig. 6. miR-145 regulated the expression of REV3L.
A: Overexpressed miR-145 downregulated the mRNA level of REV3L. B-C: Overexpressed miR-145 downregulated the protein level of REV3L. D: Decreased miR-145 upregulated the mRNA level of REV3L. E-F: Decreased miR-145 upregulated the protein level of REV3L. Each experiment was performed in triplicate. **P < 0.01 vs. miR-NC mimic or miR-NC inhibitor.

presented as mean ± SD. Data between two groups were compared with the Student's t-test, while data among 3 groups were compared by one-way analysis of variance followed by Newman-Keuls analysis. The relation between miR-145 and REV3L by Spearman's correlation analysis. The difference was statistically significant when $p < 0.05$.

4. Results

4.1. miR-145 expression was downregulated in human ESCC tissues and cells

To investigate the expression of miR-145 in ESCC, RT-PCR was applied to compare the levels of miR-145 in ESCC tissues and matched normal tissues from 25 patients. As showed in Fig. 1A, in comparison with normal tissues, expression of miR-145 was significantly decreased in ESCC tissues. We next analyzed expression of miR-145 in esophageal epithelium cell line HEEC and ESCC cell lines TE-1, TE-8, KYSE150. MiR-145 was remarkably downregulated in ESCC cell lines compared with HEEC (Fig. 1B).

4.2. The expression of miR-145

miR-145 mimic was transfected into cells. Transfection of miR-145 mimic elevated miR-145 levels in cells in comparison with cells transfected with miR-NC mimic (Fig. 2A). Moreover, transfection of miR-145 inhibitor reduced miR-145 (Fig. 2B).

4.3. REV3L was a target gene of miR-145

To explore how miR-145 regulated REV3L expression, we performed bioinformatic analysis of miR-145 and REV3L mRNA sequences on miRDB software. Sequence alignment showed that REV3L mRNA 3'UTR contained putative miR-145 binding site (Fig. 3A). In dual luciferase reporter assay, miR-145 mimic significantly reduced relative luciferase activity of cells transfected with luciferase activity plasmid containing REV3L 3'UTR-WT (Fig. 3B). Consistently, in HEEC cells, miR-145 elevation also suppressed relative luciferase activity in cells transfected with REV3L 3'UTR-WT (Fig. 3C). Thus, miR-145 could directly regulate REV3L expression in both esophageal epithelium cells

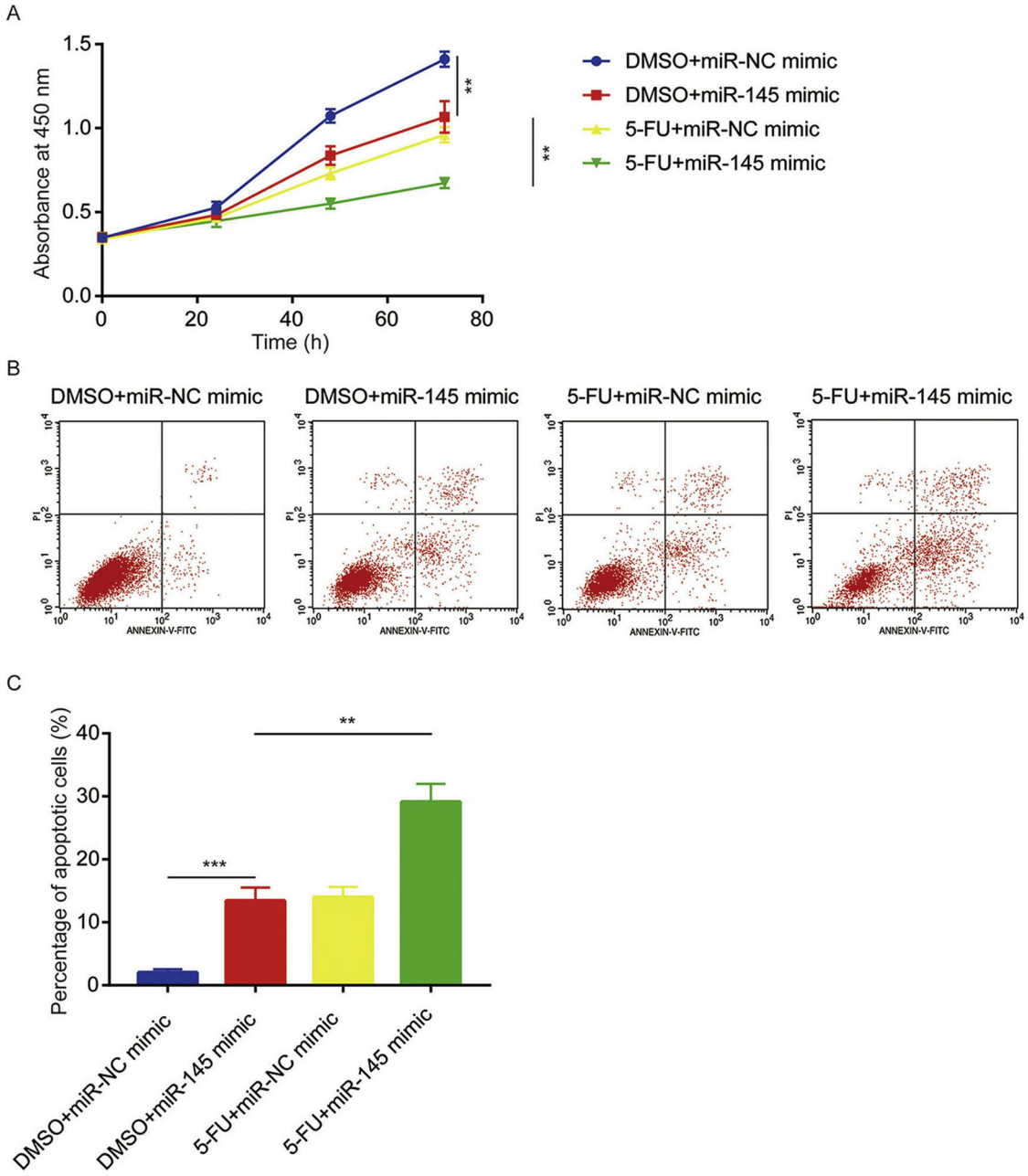

Fig. 7. Elevation of miR-145 enhanced 5-FU induced cell viability inhibition and cell apoptosis in ESCC cells.
A: miR-145 mimic + 5-FU was more potent in inhibiting the cell viability of ESCC cells. B-C: miR-145 mimic + 5-FU was more potent promoting the apoptosis of ESCC cells. Each experiment was performed in triplicate. **P < 0.01 vs. miR-145 mimic.

and ESCC cells.

4.4. The expression of REV3L

Immunohistochemistry was applied to examine the expression level of REV3L ESCC tissues. As showed in Fig. 4, the expression of REV3L was significantly increased in ESCC tissues. We examined the expression of REV3L with RT-qPCR. The results showed that REV3L was downregulated in ESCC tissues (Fig. 5A). Moreover, we also found that the expression of REV3L was negatively correlated with miR-145 (Fig. 5B).

4.5. miR-145 regulated the expression of REV3L

Cells were transfected with miR-145 mimic. As we expected, miR-145 overexpression reduced REV3L mRNA levels (Fig. 6A). Western blot showed that REV3L protein expression was decreased upon miR-145 mimic transfection (Fig. 6B and C). Relatively higher expression of miR-145 was found in normal esophageal epithelium cell line HEEC cells. We sought to investigate whether reduction of miR-145 could promote REV3L expression in HEEC. Indeed, transfection of miR-145 inhibitor reduced miR-145 levels and elevated REV3L mRNA levels in cells (Fig. 6D). Additionally, REV3L protein levels were also increased after miR-145 inhibitor transfection (Fig. 6E and F). These data indicated that miR-145 negatively regulated REV3L mRNA and protein expression.

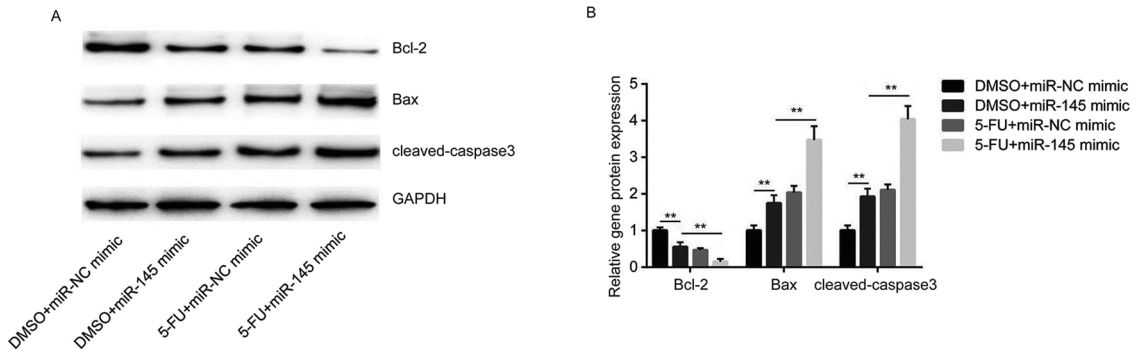

Fig. 8. Overexpression of miR-145 altered expression of apoptotic proteins upon 5-FU treatment in ESCC cells.
A-B: miR-145 mimic + 5-FUwas more efficacious in downregulating the expression of Bcl-2 and upregulating ax and cleaved-caspase3. Each experiment was performed in triplicate. **P < 0.01 vs. miR-145 mimic.

4.6. Elevation of miR-145 enhanced 5-FU induced cell viability inhibition and cell apoptosis in ESCC cells

REV3L was pivotal for DNA repair and aberrant expression of REV3L promoted cancer progression and chemotherapy resistance in many cancer types. Since miR-145 regulated REV3L in ESCC cells, we next sought to study whether miR-145 regulated 5-FU sensitivity in ESCC. In cell viability assay, transfection of miR-145 mimic or 5-FU treatment reduced cell viability of KYSE150 cells, while combination of miR-145 mimic transfection and 5-FU treatment was more potent in suppressing the cell viability of ESCC cells (Fig. 7A). Moreover, in comparison with single transfection of miR-145 mimic or 5-FU treatment, combination of miR-145 mimic and 5-FU promoted the apoptosis of KYSE150 (Fig. 7B-C). These data suggested that miR-145 elevation could sensitized ESCC cells towards 5-FU treatment.

4.7. Overexpression of miR-145 altered expression of apoptotic proteins upon 5-FU treatment in ESCC cells

As alteration of apoptotic proteins expression involved in cellular response to many chemotherapy agents, to detect whether it was associated with miR-145 contribution to 5-FU sensitivity in ESCC cells, we performed western blot to detect Bcl-2, Bax, cleaved-caspase3 in KYSE150 transfected with miR-145 mimic or treated with 5-FU or combination of miR-145 elevation and 5-FU treatment. Consistent with cell apoptosis assay result, single miR-145 elevation or 5-FU treatment slightly reduced Bcl-2 expression and increased Bax and cleaved-caspase3 expression, and combination of miR-145 mimic and 5-FU induced a significant reduction of Bcl-2 expression and strong elevation of Bax and cleaved-caspase3 expression (Fig. 8A-B).

5. Discussion

5-FU based chemotherapy is standard therapeutic approach for ESCC patients. However, development of 5-FU resistance remains a critical limitation to its clinical use [19]. The molecular mechanism of 5-FU resistance is quite complicated which involves altered expression of many genes [20]. Recent years, accumulating evidences suggest that aberrant expression of miRNAs contributes to gene expression alteration and chemotherapy resistance [21]. In current study, we found that miR-145 was elevated in ESCC tissues and cells and was associated with chemotherapy resistance via regulation of REV3L in ESCC cells.

Through analyzing miRNA expression profile in ESCC, miR-145 is discovered as one of most significantly downregulated miRNAs in ESCC tissues [15]. Subsequent study revealed that miR-145 represses ESCC cell proliferation and invasion [22,23]. In the present study, we found that miR-145 was downregulated in ESCC tissues. Further analysis validated REV3L as a target gene of miR-145. Moreover, overexpression of miR-145 enhanced 5-FU induced cell viability inhibition and elevated cell apoptosis rate in ESCC cells. Bcl-2/Bax ratio was critical mediator of cell apoptosis [24]. Moreover, caspase3 would be cleaved and activated pro-apoptotic activity [25]. Studies indicated that 5-FU treatment induced cell apoptosis via Bcl2/Bax and caspase3 [26,27]. In this study, overexpressed miR-145 was more efficient in regulating apoptotic proteins (Bcl2, Bax, caspase3) in cells treated with 5-FU. Taken together, our data revealed a chemotherapy sensitizer role of miR-145 in ESCC cells.

As the catalytic subunit of DNA polymerase ζ, REV3L functions as bypass of most lesion and is considered a cancer susceptibility candidate gene [28,29]. REV3L is overexpressed and mutated in several cancer types and proved to be mediator of chemotherapy resistance [30,31]. In consistent with a previous study [32], RT-PCR showed the expression of REV3L was increased in ESCC tissues. In addition, we found miR-145 expression was inversely associated with REV3L in ESCC tissues. Furthermore, overexpression of miR-145 decreased REV3L expression in KYSE150 cells and inhibition of miR-145 elevated REV3L expression in HEEC cells. Dual luciferase reporter assay confirmed REV3L as a target gene of miR-145. Previous studies showed that miR-29 and miR-340 could target REV3L [33,34]. Our data further extended the understanding miRNAs regulatory role in REV3L expression.

In conclusion, our results demonstrated that miR-145 was downregulated in ESCC tissues and contributed to chemotherapy resistance via regulation of REV3L. Therefore, miR-145 might be a promising biomarker and therapeutic target for ESCC.

Availability of materials and data

They are available on special requests.

Declaration of interest

None.

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