MicroRNA-544 inhibits esophageal squamous cell carcinoma cell proliferation and enhances sensitivity to cisplatin by repressing E2F transcription factor 5

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Abstract. Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies worldwide. MicroRNA (miRNA)-544 is an important cancer-associated RNA that is downregulated in multiple types of cancer. However, the role of miR-544 in ESCC progression remains unknown. In the present study, miR-544 expression level was determined via RT-qPCR in 30 pairs of ESCC and adjacent normal tissues and in a panel of ESCC cell lines. Cell proliferation and cell apoptosis were assessed by MTT and flow cytometry assays. Luciferase reporter assay and western blot analysis were conducted to verify E2F transcription factor 5 (E2F5), an oncogene in ESCC, as a novel target gene of miR-544. The results illustrated that miR-544 is frequently downregulated in ESCC tissues and cell lines. Overexpression of miR-544 in ESCC cells resulted in decreased cell proliferation and increased cell apoptosis. Thus, E2F5 was identified as a target of miR-544, and its expression was negatively correlated with miR-544 expression in clinical ESCC tissues. More importantly, overexpression of miR-544 led to increased sensitivity of ESCC cells to cisplatin, an anticancer drug. Overall, these findings indicate that miR-544 serves as a tumor suppressor by targeting E2F5; thus, miR-544 may be a therapeutic target for the treatment of ESCC.

Introduction

Human esophageal cancer is a malignancy that is associated with a high mortality rate worldwide; ~>90% of all cases of this disease present as esophageal squamous cell carcinoma (ESCC) (1,2). Despite advancements in therapeutic techniques, including chemotherapeutic, radiotherapeutic and surgical treatments, the prognosis of ESCC remains poor; its 5-year survival rate is only 10-15% (3). Therefore, further research on the molecular mechanisms underlying the development and progression of ESCC and the development of effective treatments for ESCC is required. Cisplatin is a first-line chemotherapeutic drug that is commonly used to treat several types of cancer, including ESCC (4). However, cancer cells appear to be chemoresistant to cisplatin during long-term treatment (4). Thus, the inhibition of cancer chemoresistance to cisplatin may improve cisplatin chemotherapy in various types of cancer.

MicroRNAs (miRNAs/miRs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level; they function in various processes, such as cell proliferation, apoptosis and differentiation (5). miRNAs control gene expression by degrading their target mRNAs or inhibiting their translation into functional proteins (5). Accumulated evidence has illustrated that miRNAs play diverse roles in the regulation of cancer initiation and progression of ESCC (6,7). Certain studies have demonstrated that miR-544 serves as an oncogene in osteosarcoma, liver cancer, gastric cancer and colorectal cancer (8-11). However, miR-544 acts as a tumor suppressor and inhibits tumor progression in breast cancer and glioma (12,13); this contrast in function may be tissue-specific. To date, the function and mechanism of miR-544 in the progression of ESCC have not been well illustrated.

The present study for the first time determined whether miR-544 was deregulated in ESCC tissues and cells. Furthermore, the effects of miR-544 overexpression on ESCC cellular proliferation and chemoresistance to cisplatin were determined. In addition, the correlation between miR-544 and E2F5 expression in ESCC was demonstrated.

Materials and methods

Tissue specimens. A total of 30 tumor tissues and paired adjacent normal tissues were obtained from 30 patients with ESCC (22 males and 8 females; age range, 49-77 years) who underwent surgery at the Department of Oncology at Linyi Central Hospital, China between June 2016 and June 2017. The normal

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paired tissues were obtained from the distal resection margins (>2 cm away from the tumor tissue). The histological grade and clinical stage of the tumors were defined based on the seventh edition of the American Joint Committee on Cancer system for esophageal cancer (14). The histological differentiation of the tumors were graded as follows: Well, n=8; moderate, n=13; and poor, n=9 Tumor-Node-Metastasis stage was also assigned: I, n=5; II, n=18; III, n=7. All specimens were stored at -80˚C until analysis. No patients received radiotherapy or chemotherapy prior to surgery. The present study was approved by the Research Ethics Committee of Linyi Central Hospital. Written informed consent was obtained from all patients prior to enrollment.

Cell culture and cell transfection. The EC9706, KYSE450 and TE-1 human ESCC cell lines and the human immortalized normal esophageal epithelial cell line HET-1A were purchased from the Type Culture Collection of the Chinese Academy of Sciences. The cells were incubated in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator containing 5% CO_2. The target sequences of miR-544 mimic were as follows: 5'-GTCGTA TCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ACGAACTTT-3'. The target sequences of NC were as follows: 5'-CAGUACUUUUGUGUAGUACAA-3'. The transfection efficiency was confirmed by RT-qPCR.

Full-length E2F5 from the human cDNA library was inserted into pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.) to generate the pcDNA3.1/E2F5 overexpression vector. The pcDNA3.1 empty vector was the used as a NC. The KYSE450 and TE-1 cells were transfected with miR-NC (50 nM) or miR-544 mimic (50 nM) and/or pcDNA3.1/ E2F5 vector (100 nM) or pcDNA3.1 (100 nM) using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Transfection efficiency was confirmed by using western blot analysis. Subsequent experiments were performed 24-48 h after transfection.

Western blot analysis. Total protein was extracted from ESCC and HET-1A cells and tissues using lysis buffer (Beyotime Institute of Biotechnology), and protein concentrations were measured with a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). A total of 30 μl protein extract was separated via SDS-PAGE (10% gel) and transferred onto a PVDF membrane. The membranes were blocked with 5% non-fat milk for 60 min at room temperature, followed by incubation with primary antibodies against E2F5 (1:500; cat. no. ab44996; Abcam), cleaved caspase-3 (1:500; cat. no. ab2302; Abcam), cleaved poly (ADP-ribose) polymerase (1:500; cleaved-PARP, cat. no. AF1567; Beyotime Institute of Biotechnology) and β-actin (1:500; cat. no. ab8227; Abcam) overnight at 4˚C, then treated with a Horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:1,000; cat. no. ab150077; Abcam) for 2 h at room temperature. The protein bands were visualized using an enhanced chemiluminescence system (Beyotime Institute of Biotechnology). β-actin was acted as a loading control for normalization.

Total mRNA extraction and reverse transcription-quantitative (RT-q)PCR. According to the protocol of mRNA extraction, the cells were dissolved in TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) to extract total mRNA. Total RNA was reverse transcribed with oligodT primers using the miRcute Plus miRNA First-strand cDNA Synthesis kit (Tiangen Biotech Co., Ltd.) at 37˚C for 60 min and 85˚C for 1 min. qPCR was performed using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.). The PCR thermocycling conditions were: 94˚C for 2 min followed by 30 cycles of 94˚C for 30 sec, 61˚C for 30 sec, and 72˚C for 30 sec. The relative expression levels of miR-544 and E2F5 were calculated as the inverse log of ΔΔCq and normalized to the reference (15). The sequence of the primers used for amplification were: E2F5 forward, 5'-CGGCTAGCTTCTGGATTTCAACTTTTC TTC-3'; E2F5 reverse, 5'-GCGTCGACGGAAAGTGGAATG TCAGAAGTC-3'; miR-544 forward, 5'-GCCCGATTCTGC ATTTTTAGC-3'; miR-544 reverse 5'-CGGGCTAAGACG TAAAAACG-3'; β-actin forward 5'-GATCATTGCTCCTCC TGAGC-3'; β-actin reverse, 5'-ACTCCTGCTTGCTGATCC AC-3'; U6 forward, 5'-TGCGGGTGCTCGCTTCGCAGC-3'; and U6 reverse 5'-CCAGTGCAGGGTCCGAGGT-3'.

MTT assay. In total, ~1x10^3 KYSE450 and TE-1 cells/well were seeded onto a 96-well plate and incubated overnight. Subsequently, 20 μl MTT solution was added per well, and incubated for 4 h. The liquid was removed from the plate, and 150 μl DMSO was added per well. All plates were read at 490 nm.

Colony-formation assay. The proliferation of cells was analyzed using a plate colony-formation assay. Briefly, KYSE450 and TE-1 cells transfected with miR-544 mimic and E2F5 plasmid (300 cells/well) were added to each well of the 6-well plates and cultured for 10 days in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). The cells were washed with phosphate-buffered saline (PBS) twice and fixed using 0.1% crystal violet for 20 min at room temperature. Images of the colonies were captured and the number of colonies was counted under a light microscope (Olympus Corporation) (magnification, x100).

Flow cytometry analysis of apoptosis. KYSE450 and TE-1 cells transfected with miR-544 mimic and E2F5 plasmids were treated with or without 15 μM cisplatin for 24 h at 37˚C, followed by determination of the apoptotic rate. Apoptotic rates were determined using an annexin V and propidium iodide Apoptosis Detection kit (cat. no. C1062M; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. The apoptotic rate was determined using a FACSAria flow cytometer and analyzed by the FASCDiva version 4.1 software (BD Biosciences).

Target prediction. The TargetScanHuman database and TargetScanHuman Release version 7.1 software (http://www.targetScan.org) were used to predict the potential target genes of miR-544. TargetScan target gene prediction software identified the 617-624 site at the 3'-end of the 3'-UTR of E2F5 mRNA as a possible site of action of miR-544.

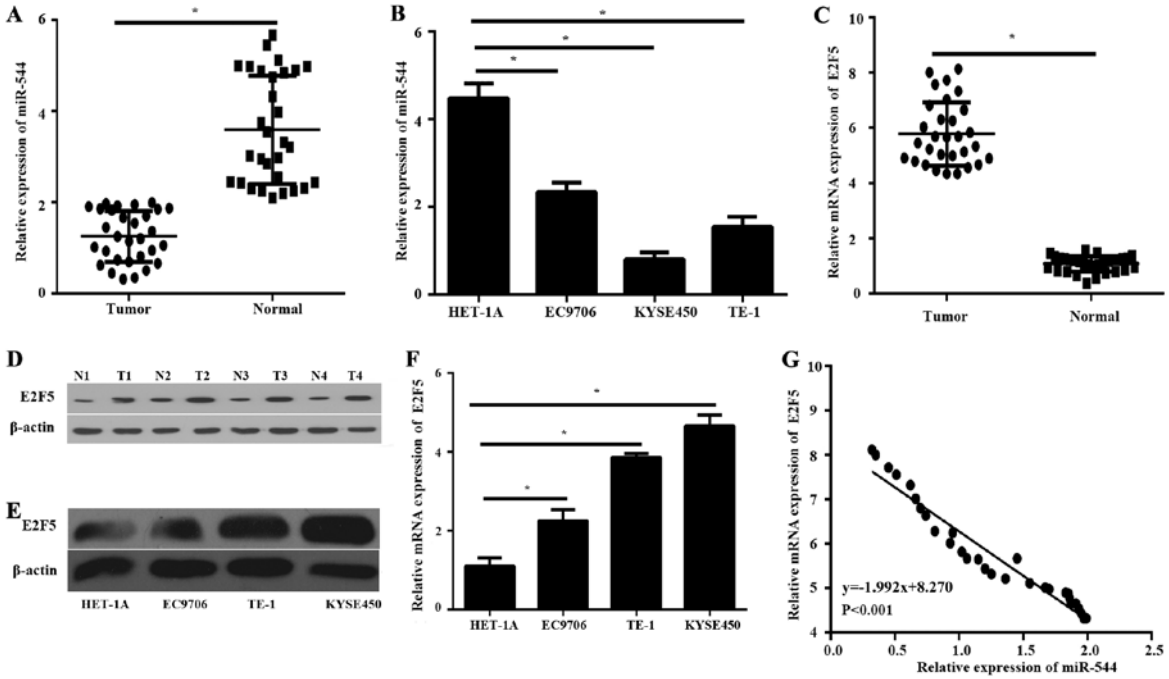

Figure 1. Expression of miR-544 and E2F5 in ESCC tumors and cell lines. (A) The expression of miR-544 in ESCC tissues and normal tissues was detected by RT-qPCR. (B) The expression of miR-544 in ESCC cells lines and normal esophageal epithelial HET-1A cells was detected by RT-qPCR. (C) The mRNA expression of E2F5 in ESCC tissues and normal tissues was detected by RT-qPCR. (D) Western blot analysis was performed to determine the expression of E2F5 in human ESCC and adjacent normal tissues. (E) The protein expression of E2F5 in ESCC cell lines and normal HET-1A cell line was detected by western blot analysis. (F) The mRNA expression of E2F5 in ESCC cells lines and HET-1A cell line was detected by RT-qPCR. (G) The correlation between miR-544 and E2F5 expression in ESCC tumor tissues. *P<0.05. miR, microRNA; E2F5, E2F transcription factor 5; ESCC, esophageal squamous cell carcinoma; RT-qPCR, reverse transcription-quantitative PCR.

Dual-luciferase assay. Wild-type (wt) and mutated (mut) putative miR-544-binding sites in E2F5 3'-untranslated region (UTR) were cloned into the downstream region of the luciferase gene in the pGL3-REPORT luciferase vector (Invitrogen; Thermo Fisher Scientific, Inc.). For the reporter assay, the cells were co-transfected with wt or mut pGL3-E2F5-3'-UTR vectors and miR-544 mimics using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 24 h, Luciferase activities were measured with a Dual Luciferase Reporter Assay kit (Promega Corporation), according to the manufacturer's protocol. Data were normalized against the activity of the *Renilla* luciferase activity.

Statistical analysis. All statistical analyses were performed using Prism GraphPad version 6.0 (GraphPad Software, Inc.). Data of more than two groups were analyzed using one-way analysis of variance with Tukey's post hoc test. Correlations between miR-544 and E2F5 mRNA levels were analyzed using Pearson's correlation analysis. The statistical analysis of two unpaired groups was evaluated using an unpaired Student's t-test. Statistical analysis of miR-544 and E2F5 expression between ESCC tissues and paired adjacent normal tissues was evaluated using a paired Student's t-test. P<0.05 was considered to indicate a statistically significant difference. The data were presented as the mean ± standard deviation.

Results

Expression of miR-544 and E2F5 in ESCC tumors and cell lines. RT-qPCR was performed in order to detect the expression levels of miR-544 and E2F5 in 30 ESCC tissues and corresponding normal tissues. The expression level of miR-544 in ESCC tissues was significantly decreased compared with the normal tissues (P<0.05; Fig. 1A). Furthermore, the expression level of miR-544 in ESCC cell lines was also significantly decreased compared with that in normal esophageal epithelial HET-1A cells (P<0.05; Fig. 1B). However, the mRNA level of E2F5 expression was significantly higher in ESCC tissues compared with normal tissues (P<0.05; Fig. 1C). The western blot analysis further demonstrated that the protein expression of E2F5 was upregulated in ESCC tissues compared with normal tissues (Fig. 1D). In addition, the protein and mRNA level of E2F5 expression was significantly higher in ESCC cell lines compared with that in HET-1A cells (P<0.05; Fig. 1E and F). Furthermore, Pearson's correlation analysis demonstrated a significant negative correlation between the expression of miR-544 and E2F5 mRNA expression in ESCC tissues (P<0.001; Fig. 1G). These findings suggest that miR-544 may play a critical role in the progression of ESCC and have internal correlation with E2F5 in ESCC.

miR-544 overexpression inhibits cell proliferation of ESCC cells. KYSE450 and TE-1 cells expressed lower levels of miR-544 compared with EC9706 cell lines, and were therefore selected for the further study. KYSE450 and TE-1 cells were transfected with miR-NC or miR-544 mimic. RT-qPCR demonstrated that the expression of miR-544 in the mimic-transfected cells was significantly higher compared with the miR-NC group (P<0.05; Fig. 2A). Overexpression of miR-544 resulted in decreased cell proliferation of KYSE450

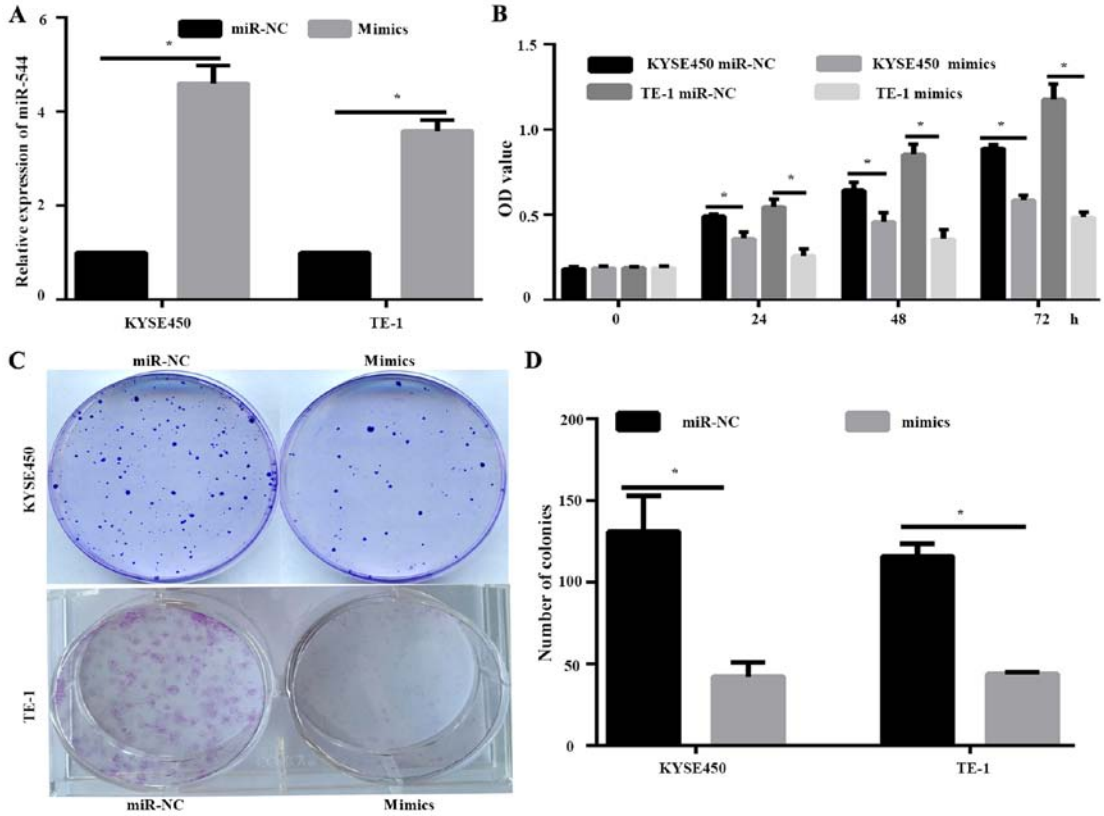

Figure 2. Overexpression of miR-544 inhibited cell proliferation of esophageal squamous cell carcinoma cells. (A and B) The expression level of miR-544 in KYSE450 and TE-1 cells transfected with miR-544 mimic or miR-NC was detected by RT-qPCR. U6 acted as the internal control. (C) MMT assay was used to detect the proliferation of KYSE450 and TE-1 cells. (D) Colony-formation assay was used to analyze the proliferative capability of KYSE450 and TE-1 cells. *P<0.05. miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control.

and TE-1 cells, as determined by MTT assay (P<0.05; Fig. 2B). Furthermore, the inhibition of proliferation by miR-544 mimic was further demonstrated by colony-formation assay (P<0.05; Fig. 2C).

E2F5 is a direct target gene of miR-544. To further explore the molecular mechanism of miR-544 in ESCC progression, computational algorithms were applied (using TargetScan) to predict potential target genes of miR-544 Dual-luciferase assays were performed to confirm this prediction. Two luciferase reporter recombinant vectors that contained either wt 3'UTR of E2F5 gene (wtE2F5) or E2F5 with a mut miR-544 binding site (mutE2F5) were constructed (Fig. 3A). KYSE450 and TE-1 cells were co-transfected with wtE2F5 and miR-544 mimics. The results of luciferase assay demonstrated that miR-544 mimics decreased the luciferase activity of wtE2F5 compared with the miR-NC (P<0.05; Fig. 3B). No significant difference in the luciferase activity of mutE2F5 was identified (Fig. 3B). Furthermore, RT-qPCR and western blot analysis showed decreased expression of E2F5 with miR-544 overexpression at both the mRNA (P<0.05; Fig. 3C) and protein levels (Fig. 3D) in KYSE450 and TE-1 cells.

E2F5 is a functional target of miR-544 in ESCC cells. The potential of E2F5 as a functional target of miR-544 was investigated. The KYSE450 and TE-1 cells were co-transfected with miR-544 mimics and E2F5 plasmid (Fig. 4). Western blot analysis demonstrated that the expression of E2F5 was significantly increased in KYSE450 and TE-1 cells that were transfected with E2F5 plasmid groups compared with the empty plasmid groups (Fig. 4A and B). In addition, E2F5 expression was increased in KYSE450 and TE-1 cells transfected with miR-544 mimics and E2F5 plasmid compared with that in cells transfected with miR-544 mimics and empty plasmid (Fig. 4A and B). Furthermore, MMT (P<0.05; Fig. 4C and D) and colony-formation assays (P<0.05; Fig. 4E) indicated that overexpression of E2F5 rescued the inhibitory effect of miR-544 mimics on the proliferation of KYSE450 and TE-1 cells. These data suggest E2F5 acts as a functional target of miR-544 in ESCC cells.

miR-544 increases the chemosensitivity of ESCC cells to cisplatin. The present study investigated the effect of miR-544 on the sensitivity of KYSE450 and TE-1 cells to cisplatin. Flow cytometry was used to analyze the apoptotic rate of KYSE450 and TE-1 cells transfected with miR-544 mimics and E2F5 plasmid that were treated with or without 15 μM cisplatin. The results of flow cytometry demonstrated that the miR-544 mimics significantly increased the apoptotic rate of KYSE450 and TE-1 cells compared with the miR-NC-transfected cells, which was further enhanced by cisplatin (P<0.05; Fig. 5A and B). Furthermore, the MTT assay demonstrated that miR-544 mimics and cisplatin groups significantly decreased cell proliferation compared with the cisplatin groups in both KYSE450 and TE-1 cells

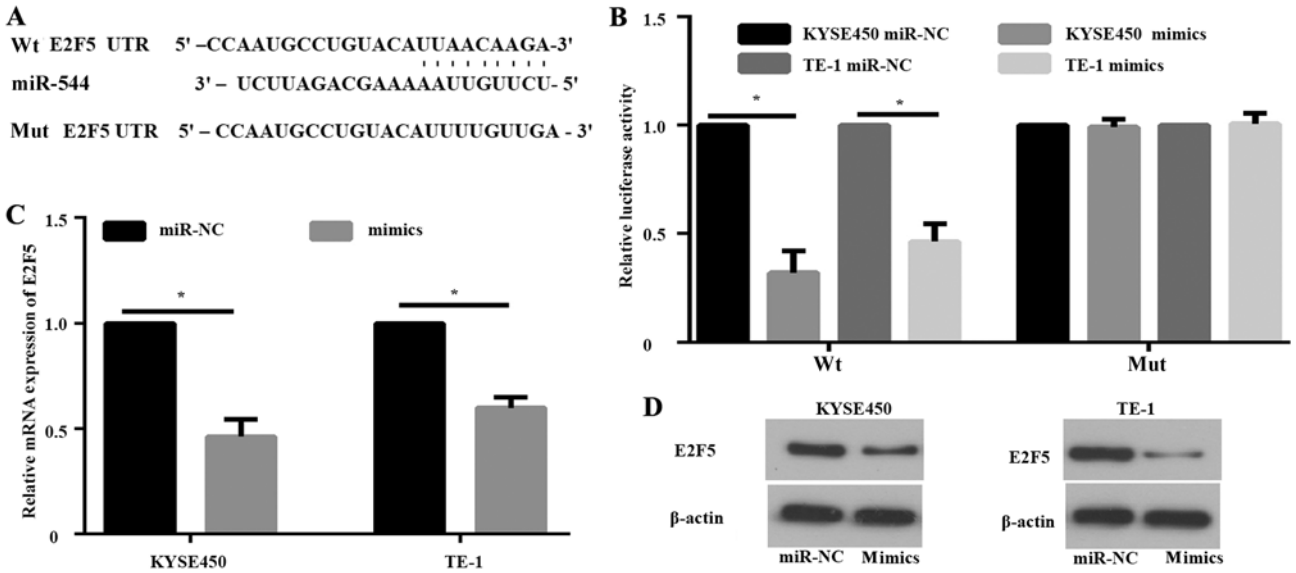

Figure 3. E2F5 is a direct target gene of miR-544. (A) Sequence alignment of miR-544 with 3'-UTR of wt and mut E2F5 predicted by TargetScan. (B) The luciferase activity of cells was examined via the dual-luciferase reporter assay. (C) The mRNA expression level of E2F5 in KYSE450 and TE-1 cells was analyzed by RT-qPCR. (D) The protein expression level of E2F5 in KYSE450 and TE-1 cells was analyzed by western blot analysis. β-actin acted as the internal control. *P<0.05. miR, microRNA; E2F5, E2F transcription factor 5; RT-qPCR, reverse transcription-quantitative PCR; wt, wild-type; mut, mutant; NC, negative control; UTR, untranslated region.

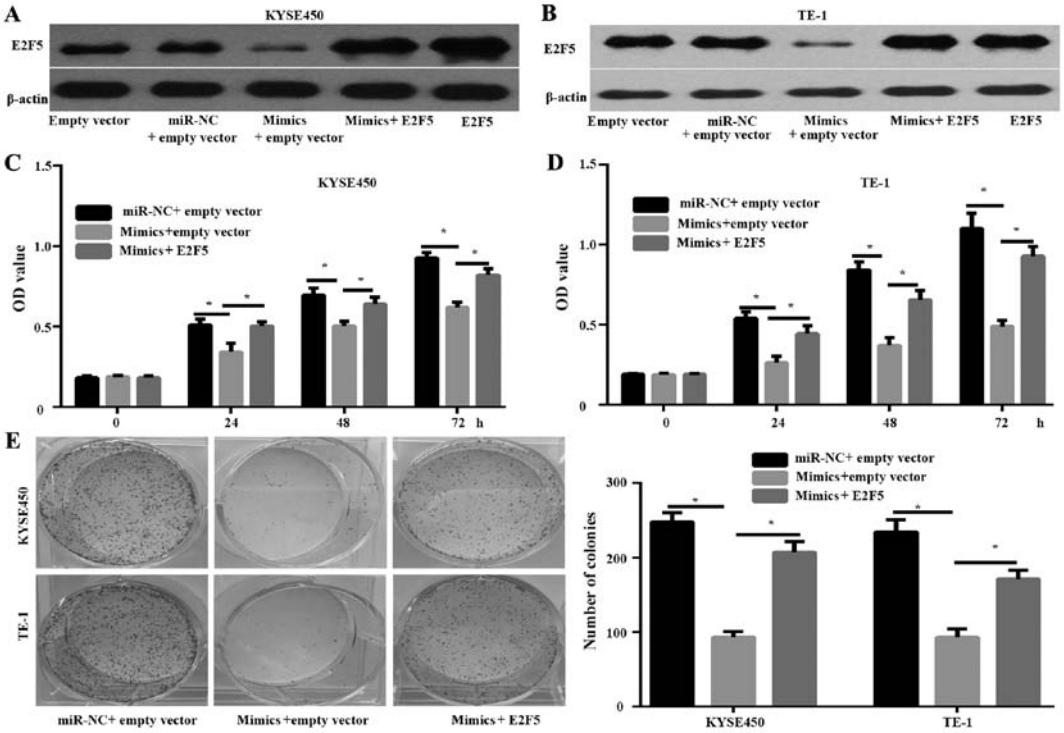

Figure 4. E2F5 is a functional target of miR-544 in esophageal squamous cell carcinoma cells. The KYSE450 and TE-1 cells were co-transfected with miR-544 mimic and E2F5 plasmid or empty vector. The protein expression of E2F5 in (A) KYSE450 and (B) TE-1 cells was detected by western blot analysis. β-actin acted as the internal control. Cell proliferation was detected by MTT assay with (C) KYSE450 and (D) TE-1 cells, and (E) colony-formation assays. *P<0.05. miR, microRNA; E2F5, E2F transcription factor 5; NC, negative control.

(P<0.05; Fig. 5C and D). In addition, the expression of apoptosis-associated proteins cleaved caspase-3 and cleaved PARP were detected. The expression of cleaved caspase-3 and cleaved PARP was significantly enhanced in the miR-544 mimics and cisplatin groups compared with the miR-544 mimics or cisplatin groups, respectively (Fig. 5E). These data demonstrated that miR-544 can enhance the chemosensitivity to cisplatin. Finally, the role of E2F5 in the enhanced chemosensitivity to cisplatin of miR-544 mimics was investigated. The protein expression of E2F5 in the cisplatin group was downregulated compared with the parental group, and the miR-544 mimics and cisplatin group had

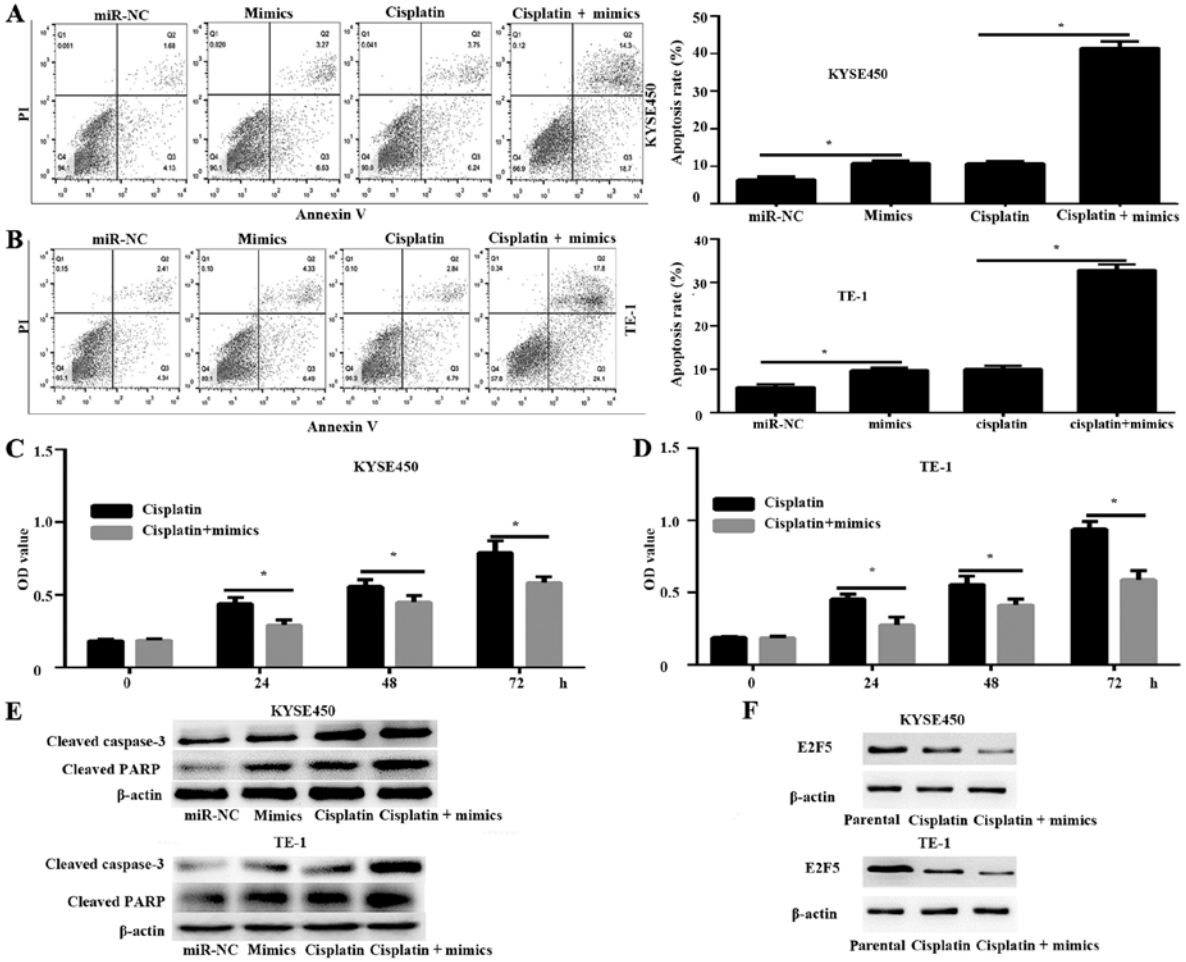

Figure 5. miR-544 increases the chemosensitivity of KYSE450 and TE-1 cells to cisplatin. Apoptosis rate was detected in (A) KYSE450 and (B) TE-1 cells transfected with miR-544 mimics and E2F5 plasmid, and treated with or without 15-μM cisplatin for 24 h. MMT assay was used to detect the proliferation of (C) KYSE450 and (D) TE-1 cells treated with cisplatin following no transfection or transfection with miR-544 mimics. β-actin acted as the internal control. (E) The protein expression of (E) cleaved caspase-3 and cleaved PARP and (F) E2F5 was detected by western blot analysis. β-actin acted as the internal control. *P<0.05. miR, microRNA; E2F5, E2F transcription factor 5; NC, negative control; PI, propidium iodide; OD, optical density; PARP, poly(ADP-ribose) polymerase.

decreased expression of E2F5 compared with the cisplatin group (Fig. 5F). Overall, these data demonstrate increased chemosensitivity of ESCC cells to cisplatin by miR-544 via E2F5.

Discussion

The rapid proliferative ability of ESCC is a critical factor contributing to its dismal prognosis (3); however, the molecular mechanisms involved remain incompletely understood. Thus, the identification of key genes that are dysregulated in ESCC tissues and the elucidation of the mechanisms leading to aberrant expression of genes promoting ESCC progression are essential to achieve successful management of ESCC. Previous studies have shown that miRNA expression is aberrant in ESCC, which suggests that miRNAs play an important role in ESCC progression (6,7). miR-544 was recently demonstrated to function either as a tumor suppressor or oncogene in different types of cancer (8-13,16). However, the expression, function and molecular mechanism of miR-544 in ESCC have not been well illustrated. Therefore, examining the effects of miR-544 in ESCC is important.

Previous studies have shown that miR-544 targets different genes in various cancer types. In osteosarcoma, miR-544 promotes cell proliferation by negatively regulating axin-2 (8). In hepatocellular carcinoma, it enhances immune escape through downregulation of natural cytotoxicity triggering receptor 1/NKp46 by targeting runt-related transcription factor 3 (9). miR-544 also promotes the cell proliferation and metastasis of colorectal cancer by directly targeting forkhead box O1 (10). In glioblastoma, miR-544 inhibits cell proliferation, invasion and migration and increases cell apoptosis by targeting Parkinsonism associated deglycase (13). Previous data have reported that E2F5 is overexpressed in various types of cancer, including breast cancer, epithelial ovarian cancer, ESCC, prostate cancer and hepatocellular carcinoma; it was also associated with cancer progression and prognosis (17-21). Certain miRNAs, such as miR-34a and miR-1179, could regulate the function of E2F5 (22,23). However, the mechanism of E2F5 in ESCC, as well as the correlation of miRNAs and E2F5 in ESCC, was not completely demonstrated. The present study demonstrates, for the first time, that miR-544 could negatively regulate E2F5 in ESCC by using a luciferase assay, western blot analysis and RT-qPCR. E2F5 overexpression

could rescue the inhibitory effect of miR-544 mimics in ESCC cells. These data demonstrate that E2F5 is a functional target gene of miR-544. Moreover, the expression levels of miR-544 and E2F5 were negatively correlated in ESCC tissues. The function of E2F5 in drug resistance is beyond the scope of the present study; however, it will be the focus of future studies.

In summary, miR-544 is downregulated in human ESCC and functions as a tumor suppressor by inducing cell proliferation and drug resistance. These functions are mediated by inhibiting the expression of its direct target gene, E2F5, an oncogene associated with tumorigenesis in certain types of cancer. Therefore, the miR-544/E2F5 axis may be considered as a potential therapeutic target in ESCC.

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Availability of data and materials

All data analyzed during the present study are included in this published article.

Authors' contributions

FS and KW conceived and designed the experiments. FS, CZ, DLM and KW performed all the experiments. CZ and KW wrote and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Linyi Central Hospital (Lin Yi, China). Written informed consent was obtained from all patients prior to enrolment.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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