Downregulation of miR-106b-3p increases sensitivity to cisplatin in esophageal cancer cells by targeting TGM3

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Abstract. Esophageal cancer (EC) is one of the most malignant and lethal digestive-related tumors worldwide. However, acquired drug resistance is a major obstacle concerning anticancer chemotherapy. An increasing number of studies have reported that microRNAs (miRNAs/miRs) are implicated in regulating the sensitivity of drug resistance in esophageal squamous cell carcinoma (ESCC). The aim of the present study was to investigate the role of miR-106b-3p in the sensitivity of cisplatin for ESCC. Initially, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to analyze miR-106b-3p and protein-glutamine γ-glutamyltransferase E (TGM3) expression levels in ESCC and non-tumor adjacent tissues. By using bioinformatics software TargetScan, TGM3 was predicted to be a potential downstream target of miR-106-3p. Following verification that TGM3 was a downstream target of miR-106b-3p by the dual-luciferase reporter assay, the effects of miR-106b-3p transfection on KYSE30 cell viability and apoptosis following treatment with cisplatin were confirmed using Cell Counting Kit-8 and flow cytometry assays, respectively. The results revealed that miR-106b-3p levels were upregulated, whereas TMG3 levels were downregulated in ESCC tissues. Dual-luciferase reporter assays confirmed that miR-106b-3p negatively regulated TGM3 expression by binding to its 3'UTR sequence. It was also shown that inhibition of miR-106b-3p could enhance the anti-proliferative effects, while promoting the apoptotic effects of cisplatin in the KYSE30 cell line by targeting TGM3. In conclusion, the present study demonstrated that downregulation of miR-106b-3p may increase the sensitivity of KYSE30 cell to cisplatin by targeting TGM3.

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Introduction

Esophageal cancer (EC) is one of the most aggressive and lethal types of malignant tumor, and is considered the sixth leading cause of cancer-related mortality, and the eighth most common cancer worldwide (1). Esophagectomy resection remains the major treatment strategy for patients with EC that are at the early stage of the disease (2). However, in order to improve prognosis, ~half of these patients required chemotherapy due to systemic or local recurrence (3). During chemotherapy, drug resistance is a major obstacle. Cisplatin was discovered in 1845 and is a chemotherapy medication widely used to treat various types of cancer, including EC (4). Tumor resistance and low sensitivity to cisplatin frequently occurs in EC, resulting in ineffective treatment and poor prognosis. Therefore, it is imperative to identify suitable biomarkers that could be used to overcome the potential resistance of patients with EC in chemotherapy.

MicroRNAs (miRNAs/miRs) constitute a class of endogenous, small non-coding RNAs that are involved in regulating the expression of target mRNAs (5). Previously, miRNAs were reported to be associated with the response of chemotherapy in a number of cancer types (6-8). For instance, miR-106b-3p was reported to act as a potent tumor promoter participating in tumor progression, development and sensitivity to chemotherapeutic drugs (9-11). In EC cells, it was also reported that miR-106b could promote cell proliferation, migration and invasion by targeting Smad7 (12). Furthermore, it was also demonstrated in another study that miR-106b could regulate the chemosensitivity of lung cancer cells to cisplatin (13). However, the exact mechanism of the contribution of miR-106b-3p in regulating cisplatin sensitivity to EC remains unknown.

Indeed, miRNAs regulate cellular processes or cancer development by modulating hundreds of target genes and signaling pathways (14,15). Recently, a number of studies have reported the promising target genes of miR-106-3p concerning cellular networks (16,17). By using the TargetScan online tool, thousands of potential targets of miR-106-3p were verified, including protein-glutamine γ-glutamyltransferase E (TGM3). As commonly known, TGM3 is a tumor suppressor gene in various cancer types, such as human neck and head cancer and colorectal cancer (18,19). However, the relationship between miR-106-3p and TGM3 in regulating the development of EC remains unknown. Hence, in the present study, the effects of

miR-106b-3p on the sensitivity of KYSE30 cells to cisplatin were investigated by targeting TGM3, along with the potential molecular mechanism of action.

Materials and methods

Specimens. A total of 30 pairs of esophageal squamous cell carcinoma (ESCC) and non-tumor adjacent tissues (>5 cm away from tumor tissues) were obtained from Baoji Central Hospital (Shaanxi, China) between June 2015 and November 2017. All patients were diagnosed with ESCC by a pathological evaluation, and they had not received biotherapy, chemotherapy or any other treatment prior to the initiation of the study. Ethical approval was granted by the Ethics Committee of the Baoji Central Hospital and written informed consent was received from each patient in accordance with the institutional guidelines.

Cell line and transfection. The KYSE30 human esophageal cancer cell line (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) and the human squamous epithelial cell line Het-1A (BeNa Culture Collection; Beijing Beina Chunglian Biotechnology Research Institute) were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in the presence of 5% CO_2 and penicillin-streptomycin (Sigma-Aldrich; Merck KGaA). miR-106b-3p mimics, inhibitor and scramble (NC) were purchased from Shanghai GenePharma Co., Ltd. KYSE30 cells were seeded into 6-well plates at an initial density of $2x10^5$ cells/well. Cells were transfected with 40 nM NC or miR-106b-3p inhibitor or miR-106b-3p mimics sequences using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. After 24 h transfection, subsequent experimentation was conducted and the transfection efficiency of miR-106b-3p was assessed. The sequences were as follows: miR-106b-3p mimics, 5'-TAAAGT GCTGACAGTGCAGAT-3'; miR-106b-3p inhibitor, 5'-AUC UGCACUGUCAGCACUUUA-3'; and NC, 5'-CAGUACUUU UGUGUAGUACAA-3'.

Small interfering (si)RNA, siRNA-1 TGM3 and siRNA-2 TMG3, were designed and purchased from Shanghai GenePharma Co., Ltd. KYSE30 cells were seeded into a 6-well plate at a density of $2x10^5$ cells/well. Subsequently, 40 nM siRNA-1 TGM3 and siRNA-2 TMG3 sequences were transfected into cells with Lipofectamine 2000 reagent, according to the manufacturer's instructions. siRNA-1 TGM3 sequence, 5'-TATGAATTCTGTACGGGAGGCCACCAG CGC-3'; siRNA-2 TGM3 sequence, 5'-TATGAATTCTGT ACGGGAGGCCACCAGCGC-3'.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues and cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.). Subsequently, cDNA was synthesized using the miScript Reverse Transcription kit at room temperature for 10 min, and qPCR was performed using the miScript SYBR-Green PCR kit (both purchased from Qiagen, Inc.). The U6 small nuclear RNA was used for normalization. Determination of the relative levels of *TGM3* was performed using the TaqMan™ PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), while glyceraldehyde phosphate dehydrogenase (GAPDH) functioned as an internal control. The relative expression levels were calculated according to the $2^{-\Delta\Delta Cq}$ method (20).

Cell proliferation assay. The transfected cells were seeded into a 96-well plate at a density of $1x10^4$ cells/well and cultivated for 24 h at 37˚C in the presence of 5% CO_2. As performed previously, cells were treated with 4 μmol/l cisplatin for an additional 24 h (21). Cell viability was detected using Cell Counting Kit-8 reagent (CCK-8; Dojindo Molecular Technologies, Inc.), according to the manufacturer's instructions, and the absorbance was measured at 490 nm with a microplate reader.

Flow cytometry assay. The transfected cells were seeded into a 96-well plate at a density of $1x10^5$ cells/well. Medium containing 4 μmol/l cisplatin was added for 48 h at 37˚C, in the presence of 5% CO_2. Following trypsinization and washing with PBS (Thermo Fisher Scientific, Inc.), the cells were double stained with FITC Annexin V and propidium iodide (PI) for 10 min in the dark at room temperature. Finally, the apoptotic rate at the early and late period was determined via a FACScan flow cytometer and analyzed with CellQuest software version 5.2.1 (both from BD Biosciences).

Western blot analysis. The transfected cells were isolated and protein was extracted using RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.) containing Protease Inhibitor Cocktail (Bimake). The concentrations of proteins were measured using BCA reagent (Solarbio Life Sciences) and 20 μg/lane proteins were separated by 10% SDS-PAGE (Thermo Fisher Scientific, Inc.) and transferred to PVDF membranes (Beijing Solarbio Science & Technology Co., Ltd.). The membranes were blocked with 5% non-fat milk for 50 min at room temperature and the primary antibodies were added to the membranes at 37˚C overnight. The primary antibodies were as follows: Rabbit anti-TGM3 (cat. no. NBP1-86950; Bio-Techne), rabbit anti-Bcl-2 (cat. no. IMG-5685; Bio-Techne), rabbit anti-Bax (cat. no. AF820; Bio-Techne), rabbit anti-caspase 3 (cat. no. AF835; Bio-Techne) and rabbit anti-GAPDH (cat. no. IMG-5143A; Bio-Techne). Subsequently, the HRP-conjugated secondary antibody IgG H&L (1:1,000; cat. no. ab7090; Abcam) was added and cultured for another 2 h at room temperature. Finally, the signals were detected using the electrochemiluminescence assay (BD Pharmingen; BD Biosciences). GAPDH was used as the endogenous reference.

Luciferase reporter assay. TargetScan 7.2 software (targetscan.org) was used to predict the potential binding sequences of miR-106b-3p and TGM3 according to a previous study (22). Het-1A cells were cultured in a 24-well plate at a density of $1x10^4$ cells/well. Afterwards, the full length of TGM3 was amplified from the cDNA of Het-1A cells, and inserted into the pGL3-Basic vector (Promega Corporation) in order to construct wild-type (WT) TGM3. Subsequently, Het-1A cells were co-transfected with WT and mutant TGM3 along with NC and miR-106b-3p mimics sequences using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Inc.) at 37˚C, in the presence of 5% CO_2. Following 48 h of cell

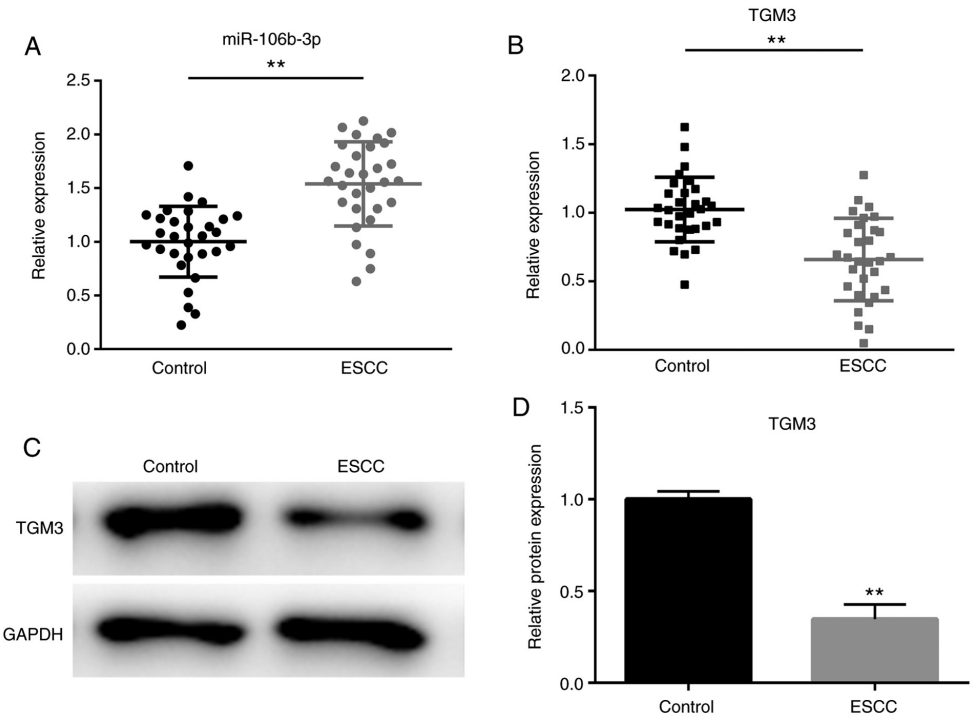

Figure 1. Relative expression levels of miR-106b-3p and TGM3 in ESCC and adjacent non-tumor tissues (n=30). (A) The mRNA expression levels of miR-106b-3p were determined by RT-qPCR in ESCC and adjacent non-tumor tissues. (B) The mRNA expression levels of TGM3 were determined by RT-qPCR in ESCC and adjacent non-tumor tissues. (C) The protein expression levels of TGM3 were determined by western blotting in ESCC and adjacent non-tumor tissues. (D) Semi-quantification of western blotting. **P<0.01 vs. control group. Control, non-tumor adjacent tissues; ESCC, esophageal squamous cell carcinoma; TGM3, protein-glutamine γ-glutamyltransferase E; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR.

culture after transfection, luciferase activity was determined using the dual-luciferase reporter gene assay kit (Promega Corporation) and normalized to *Renilla* luciferase enzyme activity, according to the manufacturer's protocol.

Statistical analysis. SPSS version 22.0 (IBM Corp.) was used to analyze the data. All data are presented as the mean ± standard deviation. The differences between groups were analyzed using the Student's t-test or one-way ANOVA followed by Newman-Keuls post hoc test. Pearson's correlation analysis was performed to measure the correlation between miR-106b-3p and TGM3 expression levels. P<0.05 was considered to indicate a statistically significant difference.

Results

Dysregulation of miR-106b-3p and TGM3 in ESCC tissues. As shown in Fig. 1A, the expression levels of miR-106b-p were significantly increased in the ESCC tissues compared with the non-tumor adjacent tissues (P<0.01); whereas the mRNA and protein expression levels of TGM3 were significantly down-regulated in ESCC tissues compared with those in non-tumor adjacent tissues (P<0.01; Fig. 1B-D).

TGM3 is a downstream target of miR-106b-3p. As shown in Fig. 2A, TGM3 expression was negatively correlated with miR-106-3p (r=-0.2726, P=-0.0323). Furthermore, the sequences of TGM3 3'-untranslated region (UTR) contained the putative miR-106b-3p binding sites as predicted by TargetScan (Fig. 2B). Following transfection with WT and

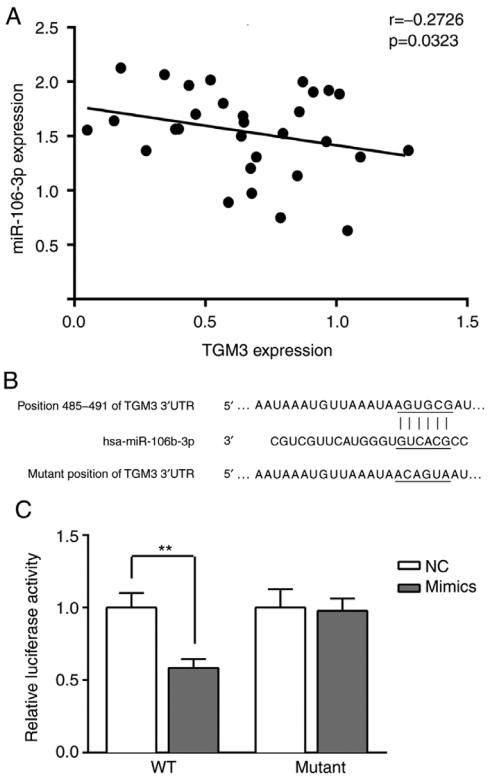

Figure 2. TGM3 is a putative downstream target of miR-106b-3p. (A) Correlation analysis between miR-106b-3p and TGM3. (B) Predicted binding sites of miR-106b-3p with the 3'-UTR sequence of TGM3. (C) Luciferase activity was determined following transfection. **P<0.05 vs. NC. NC, scrambled control; WT, wild-type; TGM3, protein-glutamine γ-glutamyltransferase E; miR, microRNA; UTR, untranslated region.

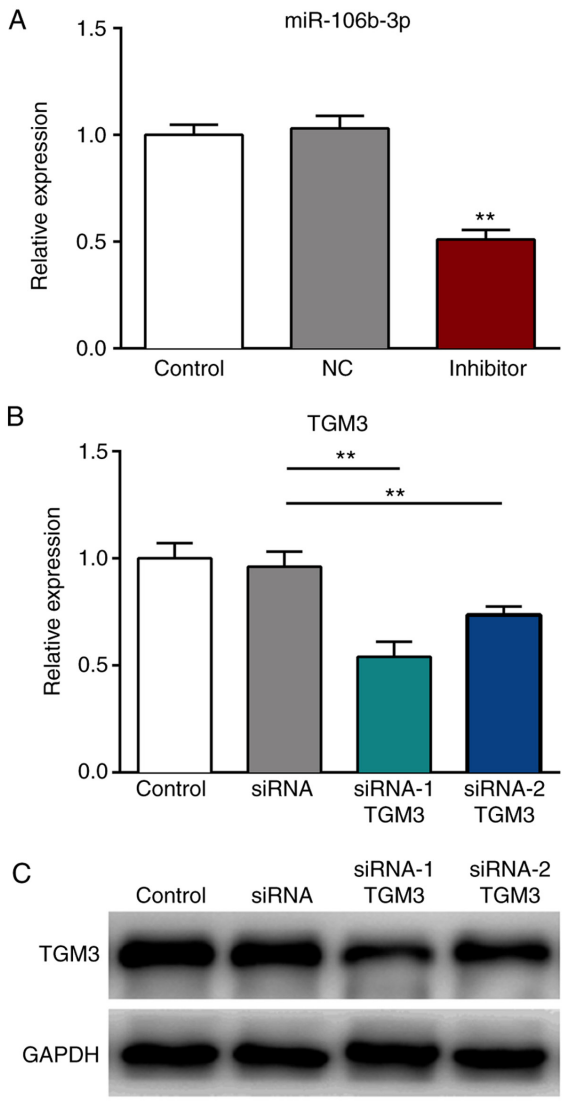

Figure 3. Transfection efficiency of miR-106b-3p and TGM3. (A) Relative expression levels of miR-106b-3p following transfection. (B) Relative mRNA expression levels of TGM3 following transfection. (C) The protein expression levels of TMG3 following transfection. **P<0.01 vs. NC or siRNA group. NC, scrambled control; TGM3, protein-glutamine γ-glutamyltransferase; miR, microRNA; siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

mutant TGM3 3'-UTR plasmids, luciferase activity was significantly decreased in the cells transfected with miR-106b-3p mimics (P<0.01; Fig. 2C).

Transfection efficiency. The expression levels of miR-106b-3p were significantly decreased following transfection with the miR-106b-3p inhibitor, compared with those transfected with the NC (P<0.01; Fig. 3A). Furthermore, the mRNA and protein expression levels of TMG3 were significantly reduced following transfection with siRNA-1 TMG3 or siRNA-2 TMG3 sequences, compared with those noted in the siRNA group (P<0.01; Fig. 3B and C).

Downregulation of miR-106b-3p suppresses ESCC cell viability following treatment with cisplatin. Following treatment with cisplatin, cell viability was significantly suppressed by transfection with the miR-106b-3p inhibitor (Fig. 4).

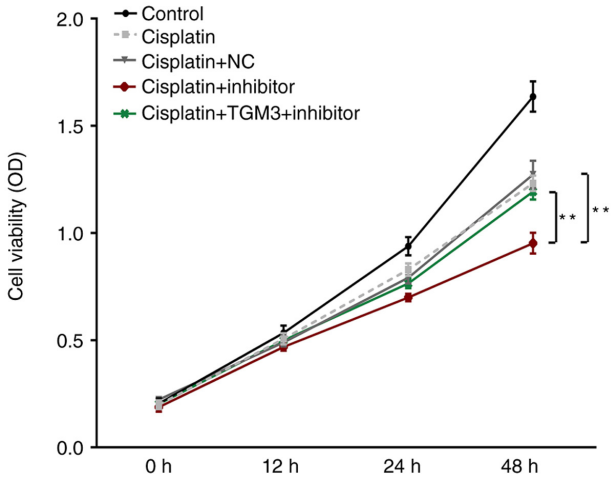

Figure 4. Downregulation of microRNA-106b-3p significantly decreases esophageal squamous cell carcinoma cell viability following cisplatin treatment. **P<0.01 vs. cisplatin + inhibitor group. NC, scrambled control; TGM3, protein-glutamine γ-glutamyltransferase.

However, this suppressive effect could be reversed by co-transfection with TMG3 siRNA (P<0.01).

Downregulation of miR-106b-3p promotes ESCC cell apoptosis following treatment with cisplatin. The induction rate of apoptosis was significantly increased by transfection with the miR-106b-3p inhibitor. However, this effect was reversed by co-transfection with TMG3 siRNA (P<0.01; Fig. 5A-F).

Effects of miR-106b-3p on the expression levels of apoptosis-related proteins. Following treatment of the cells with cisplatin, the expression levels of Bcl-2 were decreased, whereas the expression levels of TGM3, Bax and caspase-3 were increased following transfection with the miR-106b-3p inhibitor. This variation could be reversed by co-transfection with TMG3 siRNA (Fig. 6).

Discussion

TGM3 is a member of the Ca2+-dependent enzyme family and is hypothesized to be involved in the formation of the cornified cell envelope and shape determination (23,24). Previously, TGM3 was reported to be involved in human head and neck cancer development (25,26). A number of previous studies revealed that dysregulation of TGM3 was associated with tumorigenesis and development of a variety of human cancer types, including basal cell carcinoma, laryngeal carcinoma, oral squamous cell carcinoma and ESCC (27-29). It was also found that TGM3 expression was significantly reduced in ESCC tissues (30). In addition, it was reported by another study that TGM3 could suppress tumor growth via the NF-κB signaling pathway in EC (31). More importantly, TGM3 was identified as a potential prognostic indicator in ESCC, suggesting that TGM3 may be a novel target in ESCC treatment (32). In the present study, TGM3 was downregulated in EC tissues, which was in accordance with the results reported in previous studies. However, the underlying molecular mechanisms of TMG3 in ESCC cisplatin sensitivity still remains unclear.

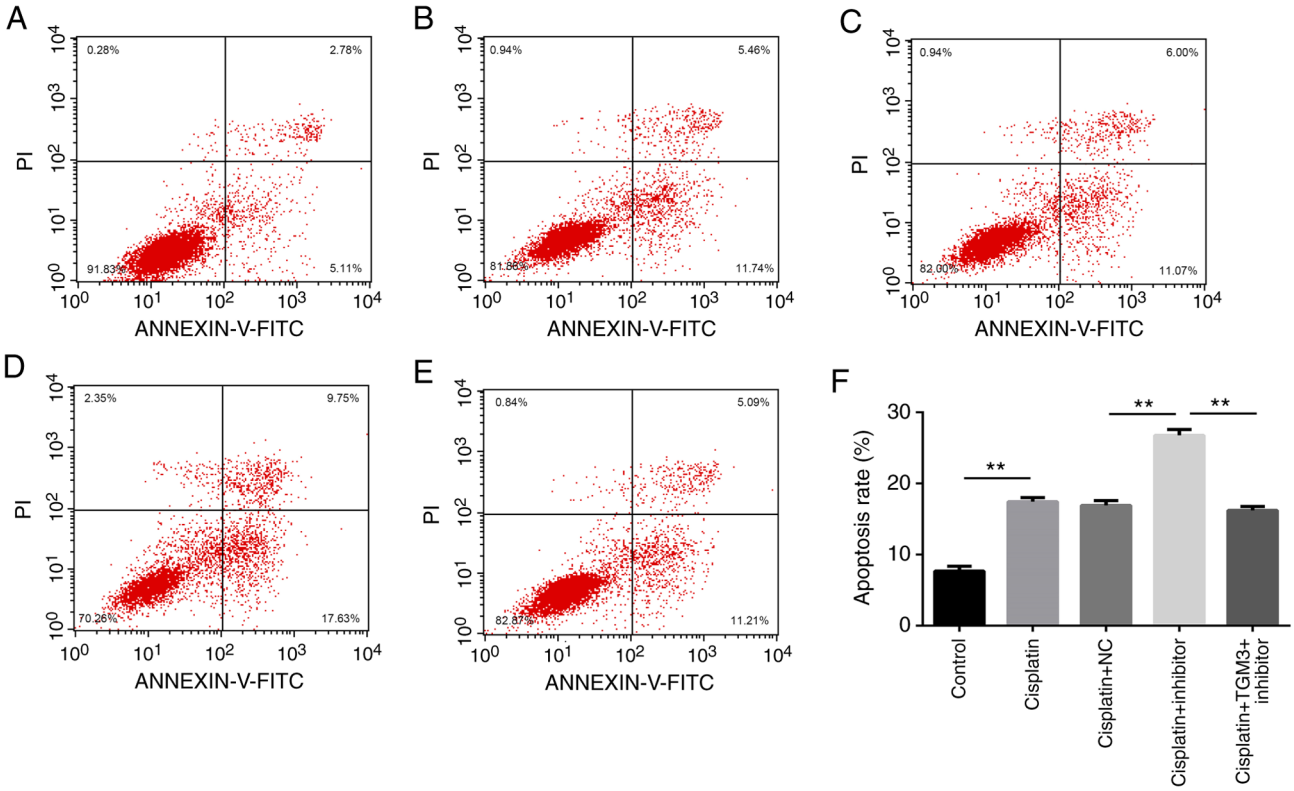

Figure 5. Downregulation of microRNA-106b-3p significantly increases esophageal squamous cell carcinoma cell apoptotic rate following treatment with cisplatin. (A) Control group; (B) cisplatin group; (C) cisplatin + NC group; (D) cisplatin + inhibitor group; and (E) cisplatin + TGM3 + inhibitor group. (F) Corresponding quantification of flow cytometry plots. **P<0.01 vs. control or cisplatin + inhibitor. NC, scrambled control; TGM3, protein-glutamine γ-glutamyltransferase E.

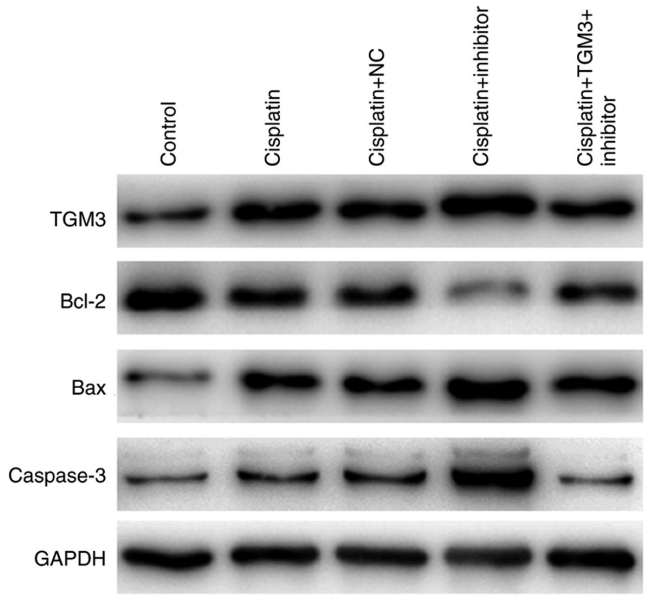

Figure 6. Protein expression levels of TGM3, Bcl-2, Bax and caspase-3 in the different groups. TGM3, protein-glutamine γ-glutamyltransferase E; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NC, scrambled control.

Acquired drug resistance and low sensitivity to chemotherapy have become major obstacles of successful cancer treatment. Growing evidence has highlighted the important roles of certain proteins in regulating sensitivity of cancer cells to chemotherapeutic agents. Accumulating evidence has reported that various miRNAs are involved in regulating cisplatin chemosensitivity in human cancer types, including ESCC. For instance, miR-218, miR-145, miR-338-5p and miR-125a-5p (33-36). Jiao *et al* (11) reported that miR-106b could regulate 5-fluorouracil resistance by targeting zinc finger and BTB domain-containing protein 7A in cholangiocarcinoma. Yu *et al* (13) proposed that miR-106b could enhance the sensitivity of A549/DDP cells to cisplatin by targeting polycystic kidney disease-2. Fang *et al* (37) revealed that miR-106b played a crucial role in causing gemcitabine resistance of pancreatic cancer as well. The present study further confirmed that inhibition of miR-106b-3p via transfection could increase chemosensitivity of KYSE30 cells to cisplatin *in vitro*, most likely by targeting TGM3. To the best of our knowledge, this is the first study to investigate the underlying mechanism of miR-106b-3p in regulating cisplatin sensitivity of EC cell lines via TGM3 targeting. Additional work must focus on the detailed molecular mechanisms by investigating how miR-106b-3p and TGM3 affect chemoresistance *in vivo*. Moreover, Bcl-2, Bax and Caspase-3 were chosen as the three typical apoptosis-related factors to verify the effects of TGM3 and miR-106b-3p on apoptosis. In the future, we will conduct immunohistochemical staining of apoptotic markers.

In conclusion, the present study validated that downregulation of miR-106b-3p may increase cisplatin sensitivity in KYSE30 cell lines by targeting TGM3. Therefore, miR-106b-3p may function as a promising sensitizer of cisplatin therapy in patients with EC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YoZ, YuZ, XL and YS drafted the manuscript. NW, MC and ZY contributed to the manuscript revision. YoZ, YuZ, XL and YS designed the study. XL, NW, MC and ZY performed the experiments. YoZ, XL, YS, NW, MC and ZY contributed to the data acquisition and supervision. YuZ, XL, MC and ZY contributed to data analysis and interpretation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was granted by the Ethics Committee of the Baoji Central Hospital (approval no. 2015010398) and written informed consent was received from each patient in accordance with the institutional guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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