Fentanyl inhibits proliferation and invasion via enhancing miR-302b expression in esophageal squamous cell carcinoma

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Abstract. Fentanyl is one of the most commonly used intravenous anesthetic agents during cancer resection surgery, but the effect of fentanyl on esophageal squamous cell carcinoma (ESCC) remains unclear. The aim of the present study was to investigate the involvement of microRNA 302b (miR-302b) in the anti-proliferation and anti-invasion effects of fentanyl in ESCC. In the present study, the effects of fentanyl on cell proliferation, apoptosis and invasion were detected using MTT assays, flow cytometry and Transwell assays in ESCC Eca109 and TE1 cell lines. Subsequently, expression of miR-302b was determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR and western blot analysis were performed in order to evaluate the expression of ErbB4, a target of miR-302b. Furthermore, anti-miR were used to inhibit miR-302b in fentanyl-treated ESCC cells in order to evaluate the role of miR-302b in the effect of fentanyl on malignant behaviors. Fentanyl inhibited the proliferation of Eca109 and TE1 cells in a dose- and time-dependent manner. Following exposure to fentanyl for 48 h, Eca109 and TE1 cells exhibited increased apoptosis and decreased invasion. Furthermore, fentanyl upregulated miR-302b expression, but downregulated ErbB4 expression. Finally, loss of miR-302b using the anti-miR technique reversed the effect of fentanyl on cell proliferation, apoptosis and invasion in the two ESCC cell lines. Taken together, the results of the present study indicated that fentanyl inhibits the proliferation and invasion of ESCC cells through upregulation of miR-302b.

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Introduction

Esophageal carcinoma (EC) remains one of the leading causes of cancer-associated mortality (1), with a 5-year survival rate of <20% (2). EC usually occurs as either adenocarcinoma or squamous cell carcinoma (ESCC), the latter of which is more dominant in East Asia and accounts for 95% of all Chinese EC cases (3). Given this, there is an urgent requirement for research to prevent and treat this disease.

Fentanyl, a strongly anesthetic analgesic drug, is an agonist for the μ-opioid receptor and is widely used in surgery, including tumor radical resection (4). Furthermore, it is considered to be an effective analgesic for breakthrough cancer pain in patients with terminal cancer (5). Recently, an increasing number of studies reported that fentanyl is able to inhibit cancer progression, including proliferation, cell cycle, apoptosis, invasion and chemotherapy sensitivity (6-10). In brief, fentanyl may serve a potential therapeutic role in cancer treatment. However, the effect of fentanyl on ESCC and the mechanism underpinning this remain unknown.

MicroRNAs (miRNAs) represent a class of small non-coding RNAs that regulate gene expression at the post-transcriptional level. One study demonstrated that miRNAs were aberrantly regulated in different oncogenic pathways and/or various types of cancer, indicating that certain miRNAs may function as oncogenes or tumor suppressor genes (11). Previous studies revealed the expression profiles of different miRNAs and identified certain specific miRNAs with biological functions and significance in ESCC (12-14). miR-302b, which was downregulated in ESCC, inhibited cell proliferation, induced apoptosis and reversed invasion in ESCC (13). In addition, it was believed that miR-302b inhibited the malignant behaviors of ESCC by directly targeting ErbB4, a molecular therapeutic target for ESCC (13).

Since fentanyl is able to change miRNA expression profiles in human cancer cells (8), we hypothesized that fentanyl may inhibit the proliferation and invasion of ESCC cells through the miR-302b/ErbB4 pathway. Therefore, in the present study, the effects of fentanyl on the proliferation, apoptosis and invasion of ESCC Eca109 and TE1 cells were investigated. Furthermore, the regulatory effect of fentanyl on the expression of miR-302b and its target, ErbB4, was also examined in order to elucidate the exact mechanism of the antitumor effect of fentanyl in ESCC.

Materials and methods

Cell culture and reagents. The ESCC Eca109 and TE1 cell lines were obtained from the Shanghai Institute for Biological Sciences (http://www.cellbank.org.cn/), Chinese Academy of Sciences (Beijing, China). Cells were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), supplemented with 10% fetal bovine serum and 100 U/ml penicillin and streptomycin, at 37˚C in a humidified atmosphere with 5% CO_2. Fentanyl was purchased from Sigma-Aldrich; Merck KGaA, was dissolved in dimethyl sulfoxide (DMSO) and was added into the culture medium at various concentrations (0, 0.5, 5, 50 and 500 ng/ml) for *in vitro* assays.

Cell proliferation assay. Cells were seeded at a density of $5x10^3$ cells/well in 96-well plates at a final volume of 180 μl in incubation, at 37˚C in a 5% CO_2 atmosphere. Following various incubation times (24, 48 and 72 h), 20 μl of 5 mg/ml solution of MTT (Sigma-Aldrich; Merck KGaA) in 1xphosphate-buffered saline (PBS) was added to each well. The plates were subsequently incubated for 4 h at 37˚C, prior to the reaction being solubilized in 100% DMSO (20 μl/well) and agitated at 37˚C for 15 min. The absorbance of each well was measured on a multi-detection microplate reader (BMG Labtech GmbH, Ortenburg, Germany) at a wavelength of 570 nm.

Apoptosis analysis. The cells were washed twice with cold 10 mM 1xPBS and were resuspended in 1xbinding buffer (BD Biosciences, San Jose, CA, USA). Cells were then washed twice with PBS and 400 μl 1x binding buffer was added followed by 5 μl Annexin V-fluorescein isothiocyanate (FITC) conjugate from the FITC Annexin V Apoptosis Detection kit (cat. no. 556547; BD Biosciences). The cells were then incubated in the dark for 15 min at 2-8˚C, then 5 μl PI was added and incubation was continued for 5 min. The samples were analyzed using a flow cytometer (FACSCalibur; BD Biosciences) and analyzed by the Cell Quest software (version 3.3; BD Biosciences).

Cell invasion assay. For the invasion assay, the membrane invasion culture system Transwell membranes with a diameter of 6.5 mm diameter and a pore size of 8 μm; Costar (Corning Incorporated, Corning, NY, USA) was used according to the manufacturer's protocol. Briefly, harvested cells ($1x10^5$), resuspended in 100 μl of serum-free RPMI-1640 medium, were added into the upper chamber. A total of 1,000 μl conditioned RPMI-1640 medium with 20% (v/v) fetal bovine serum was used as a chemoattractant and was placed into the lower chamber. After 48 h, the un-invaded cells on the upper surface of the membrane were removed with a cotton swab. The transformed cells that had invaded through the Matrigel matrix and stuck to the lower surface of the membrane were fixed with 4% paraformaldehyde for 1 h at room temperature and stained with 1% crystal purple for 15 min at room temperature. The invasive cells were then counted (in 5 high-power fields/chamber) using an inverted microscope (Olympus Corporation, Tokyo, Japan; magnification, x200). Each experiment was repeated in triplicate.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from Eca109 and TE1 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. RT-qPCR was performed using a Bio-Rad iQ5 Real-Time PCR Detection system to confirm the mRNA expression levels. A reverse transcription kit and SYBR-Green both from Takara Biotechnology Co., Ltd. (Dalian, China) were used. In brief, reverse transcription (RT) was performed in a 20 μl volume with 1 μg total RNA, by incubation at 16˚C for 30 min, 42˚C for 42 min and 85˚C for 5 min. A total of 1 μl of the RT product was used in each PCR. The PCR cycling began with template denaturing at 95˚C for 5 min, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 20 sec, 72˚C for 20 sec and 78˚C for 20 sec. Final PCR products were resolved by agarose gel electrophoresis and a single band of expected size indicated the specificity of the reaction. Relative quantification was performed using the $2^{-\Delta\Delta Cq}$ method normalized to GAPDH (15). Each PCR amplification was performed in triplicate to verify the results. The primers were as previously described (14).

Western blot analysis. Total proteins were extracted from cells using lysis buffer containing phenylmethyl sulfonylfluoride (both from Beyotime Institute of Biotechnology, Haimen, China) at 25˚C. The protein concentration was determined using BCA Protein Assay kit (Beyotime Institute of Biotechnology). For western blot analyses, 20 μg total protein was electrophoresed on a 10% SDS gel, transferred onto polyvinylidene difluoride membranes, blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 for 1 h at room temperature, and incubated with anti-ErbB4 (cat no. sc-283; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-β-actin (cat no. sc-7210; 1:200; Santa Cruz Biotechnology, Inc.) primary antibodies at 4˚C for 12 h. A corresponding bovine anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (cat no. sc-2370; 1:1,000; Santa Cruz Biotechnology, Inc.) was subsequently applied at room temperature for 2 h. Following chemiluminescence reactions with enhanced chemiluminescence detection reagents kits (GE Healthcare, Chicago, IL, USA), according to the manufacturer's protocol, the membranes were visualized by exposure to X-ray film in the dark. Densitometric analysis was performed using Scion Image software (Scion Corporation, Frederick, MD, USA).

Anti-miR design and transfection. miR-302b inhibitor (A) and miR Inhibit or Negative Control (NC) were purchased from AngRang Inc. (Xi'an, China). The sequence for miR-302b inhibitor was 5'-CTACTAAAACATGGAAGCACTTA-3'. Cells were seeded on to a 24-well plate at a concentration of $1x10^5$ cells/well. RNA oligonucleotides transfection (50 nM) was performed with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Fresh growth medium (RPMI-1640) was changed 6 h after transfection, and the cells were harvested for analysis 48 h after transfection.

Statistical analysis. Data are expressed as the mean ± standard error of the mean from ≥3 separate experiments performed in

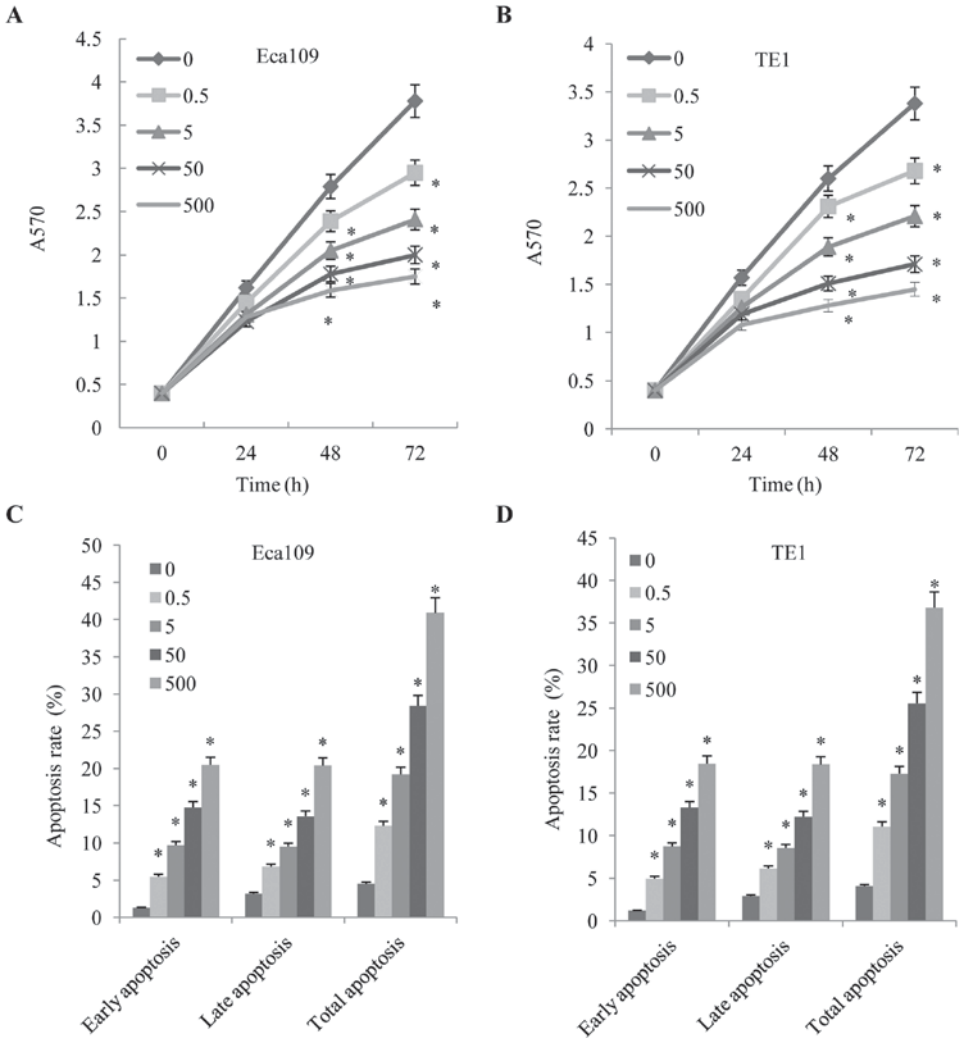

Figure 1. Effects of fentanyl stimulation on cell proliferation and apoptosis. Cells were incubated with increasing concentrations (0, 0.5, 5, 50 and 500 ng/ml) of fentanyl. Fentanyl inhibited the proliferation of (A) Eca109 or (B) TE1 cells in a time- and dose-dependent manner. Apoptosis analysis using flow cytometry demonstrated that fentanyl promoted the apoptosis of (C) Eca109 and (D) TE1 cells. All these results confirmed that fentanyl (at a concentration ≥5 ng/ml) significantly inhibited proliferation and induced apoptosis in the two cell lines. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. the group with the previous (lower) dose of fentanyl. A, absorbance.

triplicate. Differences among groups were assessed by a one-way analysis of variance (followed by Student-Newman-Keuls) using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of fentanyl on cell proliferation, apoptosis and invasion. The present study initially investigated the effects of fentanyl on cell proliferation, apoptosis and invasion. The Eca109 and TE1 cell lines were cultured in the presence of various concentrations (0.5, 5, 50 and 500 ng/ml) of fentanyl and the cell proliferation were measured using MTT assays. As demonstrated in Fig. 1A and B, the proliferation of the Eca109 and TE1 cells was inhibited by fentanyl in a dose- and time-dependent manner. Fentanyl significantly inhibited cell proliferation at 48 and 72 h. In order to further quantify cell death, Annexin V/PI analysis was performed. Following exposure to fentanyl for 48 h, Eca109 cells exhibited a decreasing rate of apoptosis (Fig. 1C and D). The cell invasion assay also revealed that fentanyl significantly stimulated invasion in a concentration-dependent manner (Fig. 2A-C). Concentrations of fentanyl >5 ng/ml exhibited a significant inhibitory effect on cell proliferation and metastasis. Therefore, a concentration of 5 ng/ml was selected for the subsequent experiments.

miR-302b is involved in the effect of fentanyl on ESCC behaviors. It was previously revealed that miR-302b suppressed proliferation by inducing apoptosis and repressed the invasion of ESCC cells through targeting ErbB4 (13). The present study further investigated whether or not miR-302b is also involved in the effect of fentanyl on the biological behaviors of ESCC. An miR-302b inhibitor (anti-miR-302b) was used to block miR-302b expression in ESCC cells, with the results demonstrating that fentanyl increased the expression of miR-302b, and that anti-miR-302b reversed the upregulation of miR-302b (Fig. 3A and B). Subsequently, the effects of altered miR-302b expression on the anti-proliferation, pro-apoptosis and anti-invasion effects induced by fentanyl

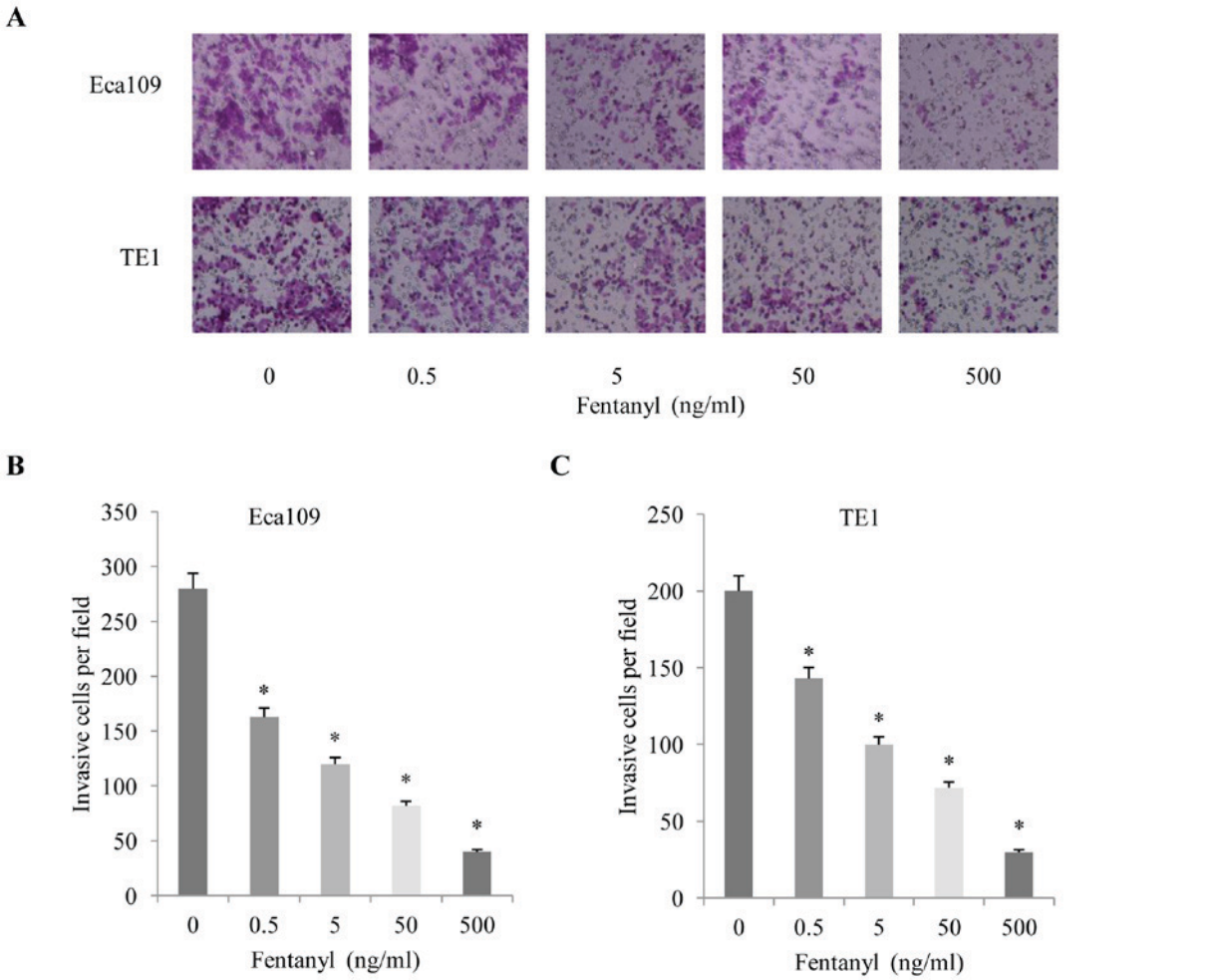

Figure 2. Effects of fentanyl stimulation on cell invasion. Cells were incubated with increasing concentrations (0, 0.5, 5, 50 and 500 ng/ml) of fentanyl. (A) Effect of fentanyl on cell invasion was detected using a Transwell assay. The cell invasion assay revealed that fentanyl significantly reversed the invasion of (B) Eca109 and (C) TE1 cells. All these results confirmed that fentanyl (at a concentration of ≥5 ng/ml) significantly reversed invasion. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. the group treated with the previous (lower) dose of fentanyl.

in ESCC were detected. It was revealed that downregulation of miR-302b reversed the anti-proliferation (Fig. 3C and D), pro-apoptosis (Fig. 3E and F) and anti-invasion (Fig. 4A-C) effects of fentanyl in the two ESSC cell lines.

Fentanyl upregulated the expression of miR-302b in ESSC cells. The present study analyzed the effects of fentanyl on the expression levels of miR-302b. As demonstrated in Fig. 5, following treatment with fentanyl for 48 h, the expression level of miR-302b increased significantly in the two ESCC cells in a dose-(Fig. 5A and B) and time-(Fig. 5C and D) dependent manner, according to the RT-qPCR results.

Fentanyl downregulated the expression of ErbB4, but this effect was reversed by anti-miR-302b transfection in ESSC cells. ERBB4 is one of the down-stream targets of miR-302b (14); therefore, we hypothesized that fentanyl modified the behaviors of ESCC cells through suppressing ErbB4. As demonstrated in Fig. 6, fentanyl decreased the expression of ErbB4 at the transcriptional (Fig. 6A) and translational levels (Fig. 6B) in a dose-dependent manner. However, the suppressive effect of fentanyl on ErbB4 expression was subsequently reversed by anti-miR-302b transfection (Fig. 6A and B), demonstrating the effects of miR-302b on the ability of fentanyl to inhibit the activation of ErbB4.

Discussion

Cancer is a major public health issue in the majority of countries, including China. Cancer is often treated by chemotherapy, immunotherapy, radiation and surgery. Anesthesia serves an important role in surgery, ensuring the safety and comfort of patients during procedures (16). However, numerous anesthetic agents are used without knowledge of their effects on cancer. Recently, it has been suggested that certain anesthetic drugs may modify malignant biological behaviors, including proliferation, angiogenesis and apoptosis in certain cancer cells (17). Nevertheless, the possible role of anesthetic drugs in cancer development and progression remains unclear.

Fentanyl is widely used in clinic as an anesthetic, particularly in the treatment of different types of cancer, including ESCC (18). Previous studies have reported the potential antitumor effects of fentanyl, but there are limited reports regarding its role in ESCC (6-10). The results of the present study suggested that the mechanisms of the anti-proliferation and anti-invasion effects of fentanyl were associated with

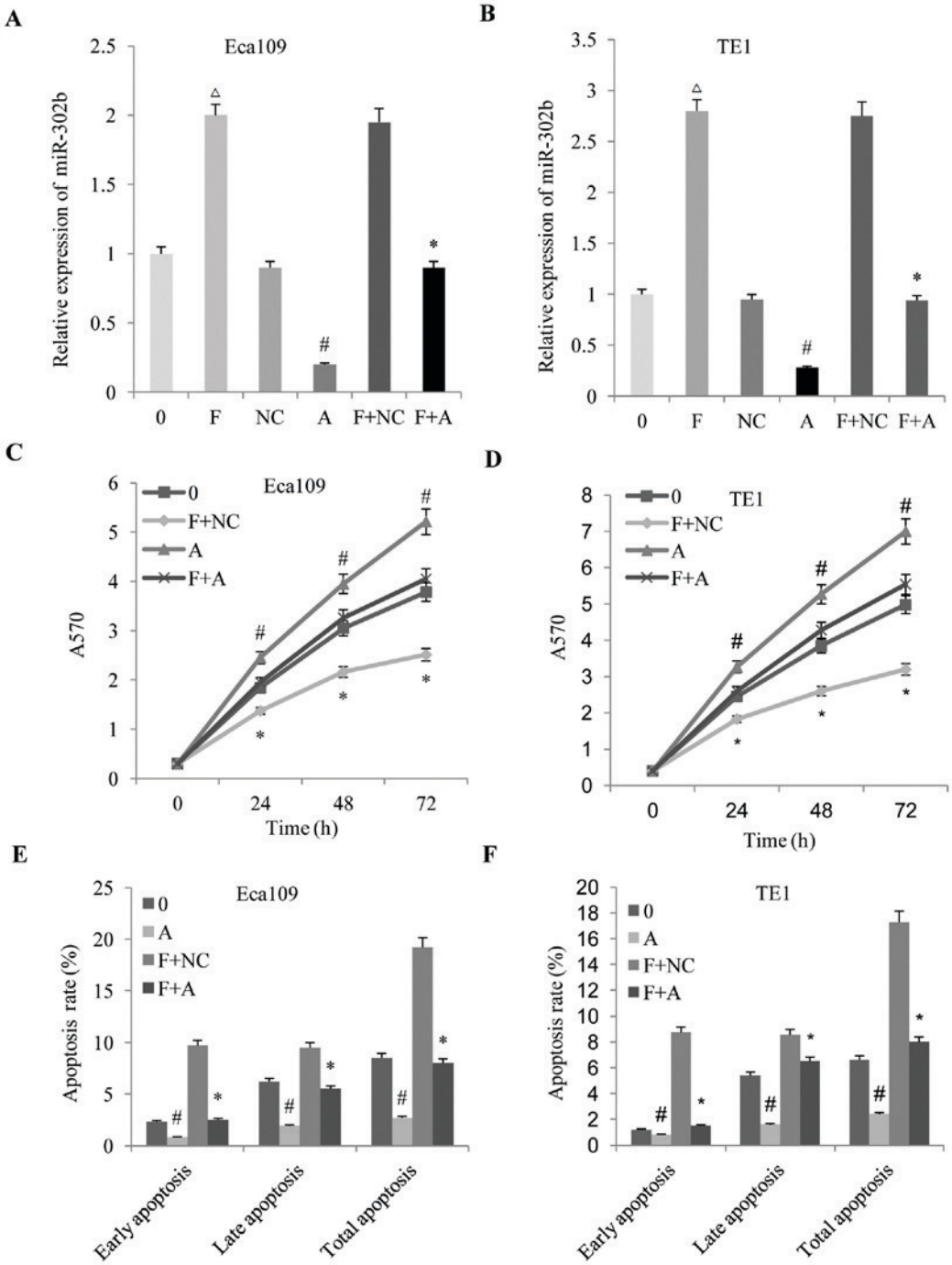

Figure 3. Effects of downregulation of miR-302b on the cell proliferation inhibition induced by fentanyl. Expression of miR-302b in (A) Eca109 or (B) TE1 cells treated withanti-miR-302b and/or fentanyl (5 ng/ml; detected by quantitative polymerase chain reaction. MTT was used to assess the effects of downregulation of miR-302b on the proliferation (induced by 5 ng/ml fentanyl) of (C) Eca109 and (D) TE1 cells, and flow cytometry was used to assess the effects of downregulation of miR-302b on the apoptosis (induced by 5 ng/ml fentanyl) of (E) Eca109 and (F) TE1 cells. Downregulation of miR-302b reversed the anti-proliferation and pro-apoptosis effects induced by fentanyl in Eca109 and TE1 cells. Data are presented as the mean ± standard deviation of three independent experiments. $^{\Delta}$P<0.05 vs. 0; $^{\#}$P<0.05 vs. 0 or NC; *P<0.05 vs. F+NC. miR, microRNA; 0, cells without fentanyl exposure; F, cells treated with fentanyl (5 ng/ml); NC, negative control of anti-miR-302b; A, cells treated with anti-miR-302b.

miR-302b expression in ESCC Eca109 and TE1 cell lines. Accompanied by the malignant biological behaviors changes, the expression of miR-302b was elevated by fentanyl treatment. Furthermore, ErbB4 was targeted and inhibited by increased miR-302b expression. Notably, the application of anti-miR-302b impaired the anti-proliferation and anti-invasion effects of fentanyl. These results supported our hypothesis that fentanyl inhibited the proliferation and invasion of ESCC cells by stimulating the expression of miR-302b which, in turn, downregulated the expression of ErbB4.

miR-302b is a member of the miR-302 cluster, which regulates the regulatory circuitry controlling ES cell 'stemness' (19). Previously, it was revealed that miR-302b acted as a tumor suppressor by post-transcriptionally regulating different types of oncogenes. miR-302b was able to inhibit proliferation (20,21), induce apoptosis (22,23) and

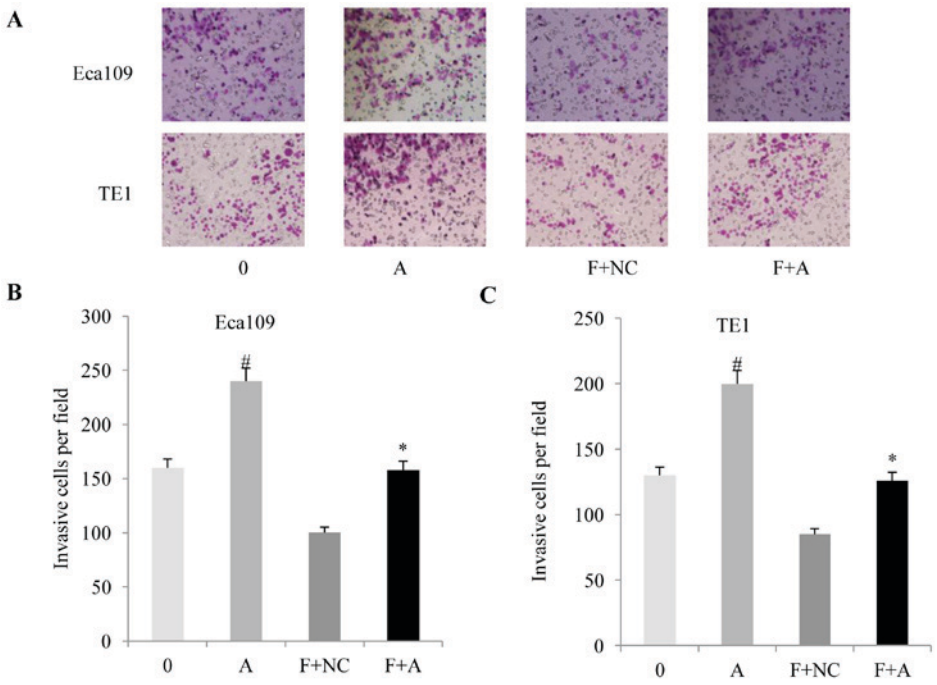

Figure 4. Effects of downregulation of miR-302b on the cell invasion inhibited by fentanyl. (A) Transwell assays were used to assess the effects of downregulation of miR-302b on the invasion inhibited by fentanyl (5 ng/ml). Downregulation of miR-302b reversed the anti-invasion induced by fentanyl in (B) Eca109 and (C) TE1 cells. Data are represented as the mean ± standard deviation of three independent experiments. #P<0.05 vs. 0; *P<0.05 vs. F+NC. miR, microRNA; 0, cells without fentanyl exposure; F, cells treated with fentanyl (5 ng/ml); NC, negative control of anti-miR-302b; A, cells treated with anti-miR-302b.

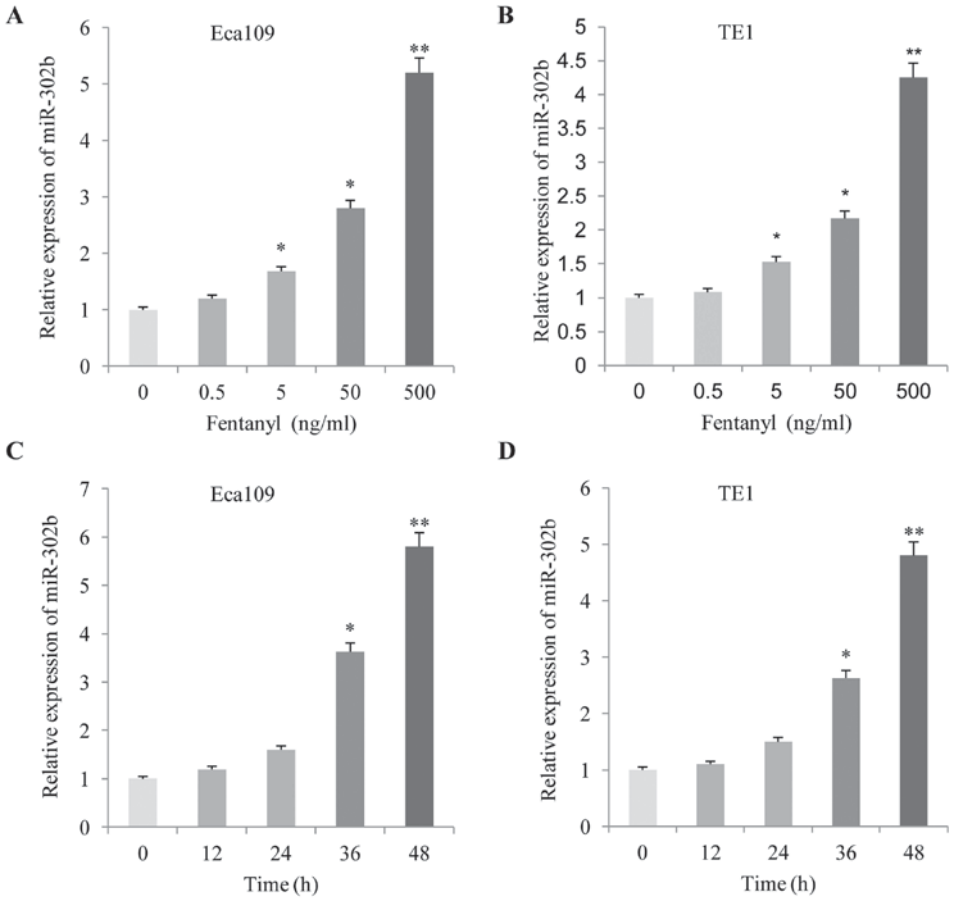

Figure 5. Effects of fentanyl on the expression of miR-302b in Eca109 and TE1 cells. (A) Eca109 and (B) TE1 cells were treated with different concentrations (0, 0.5, 5, 50 and 500 ng/ml) of fentanyl for 48 h. (C) Eca109 and (D) cells were treated with fentanyl (5 ng/ml) for different times (0, 12, 24, 36 and 48 h). Quantitative polymerase chain reaction was used to evaluate the expression of miR-302b. Fentanyl upregulated the expression of miR-302b in the two cell lines in a dose- and time-dependent manner. Representative results are from three independent experiments are demonstrated. *P<0.05 or **P<0.01 vs. the group treated with previous (lower) dose of fentanyl.

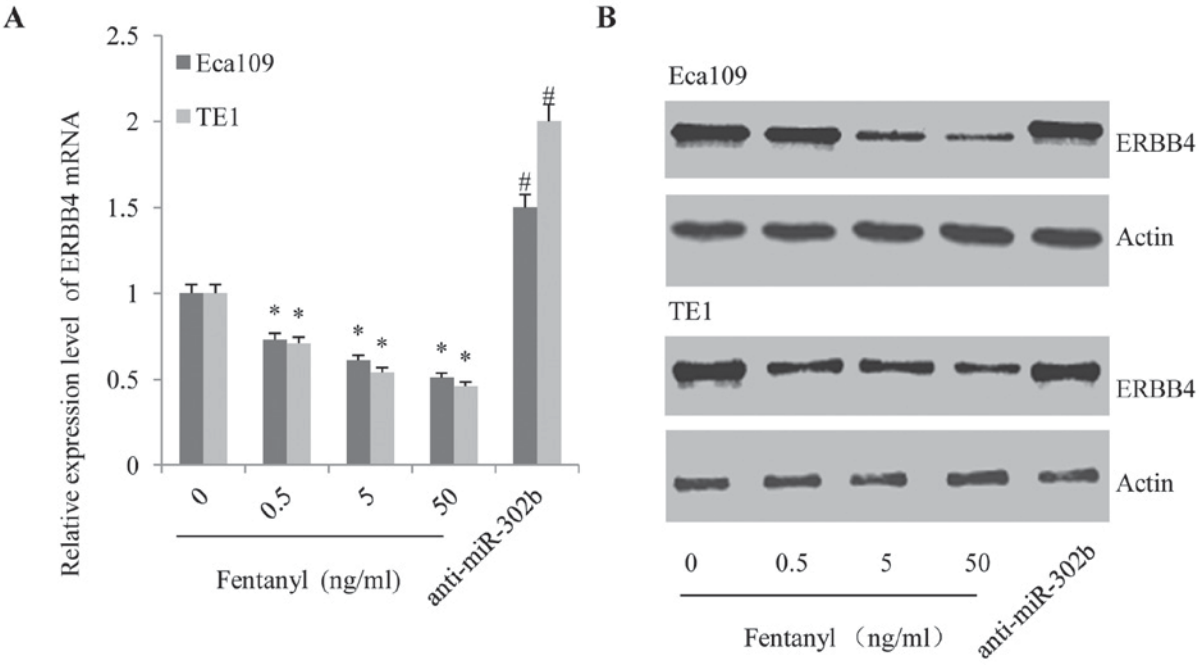

Figure 6. Effects of fentanyl and anti-miR-302b on the activation of ERBB4 in Eca109 and TE1 cells. The relative (A) mRNA and (B) protein expression of ERBB4 was detected by reverse transcription-quantitative polymerase chain reaction or western blot, respectively, in Eca109 and TE1 treated with increasing concentrations (0, 0.5, 5 and 50 ng/ml) of fentanyl or anti-miR-302b. The results demonstrated that fentanyl decreased ERBB4 expression while anti-miR-302b increased it. Each experiment was performed three times in triplicate. *P<0.05 vs. the group treated with the previous (lower) dose of fentanyl, #P<0.05 vs. control (cells without fentanyl exposure).

enhance chemotherapy sensitivity (24,25). A previous study revealed that miR-302b was a potential molecular marker of ESCC and that it acts as a tumor suppressor by targeting ErbB4 (13).

ErbB4, one of the potential targets in ESCC, is one of members of the ErbB/HER subfamily, which regulates cellular proliferation, differentiation and programmed cell death (26). Xu *et al* (27) revealed that extra-nuclear ErbB4 had negative effects on the progression of ESCC, while the nuclear translocation of ErbB4 exhibited a tumor-promoting property. Zhao *et al* (28) reported that ErbB4 served as a potential molecular target in the treatment of ESCC. Therefore, activation of ErbB4 may promote proliferation and invasion in ESCC. The results of the present study demonstrated that, accompanied by elevation of miR-302b in ESCC cells, fentanyl treatment also downregulated the expression of ErbB4, thereby inhibiting proliferation and invasion. Furthermore, fentanyl failed to downregulate the expression of ErbB4 in cells transfected with anti-miR-302b, which also impaired the inhibitory effects of fentanyl against the proliferation and invasion of these cells through miR-302b.

In summary, to the best of our knowledge, the present study is the first to identify the involvement of miR-302b in the anti-proliferation and anti-invasion effects of fentanyl in ESCC. Based on the results in the present study, it was concluded that fentanyl inhibited the proliferation and invasion of ESCC cells by elevating the expression of miR-302b and, in turn, suppressing the activation of ErbB4. However, the manner in which fentanyl treatment regulatesmiR-302b expression in ESCC cells remains unknown and requires further study.

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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

NW and ZZ conceived the study. NW and JL wrote and edited the main manuscript. JL and ZZ designed the experiments. NW, ZZ and JL performed the experiments and analyzed the data.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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