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ORIGINAL RESEARCH

LncRNA NEAT1 contributes to paclitaxel resistance of ovarian cancer cells by regulating ZEB1 expression via miR-194

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Jihong An*
Weiling Lv*
Yongzhou Zhang

Department of Clinical Pharmacy, Huaihe Hospital of Henan University, Kaifeng, People's Republic of China

*These authors contributed equally to this work

Background: Chemoresistance is one of the major obstacles for cancer therapy in the clinic. Nuclear paraspeckle assembly transcript 1 (NEAT1) has been reported as an oncogene in most malignancies such as lung cancer, esophageal cancer, and gastric cancer. This study is designed to investigate the function of NEAT1 in paclitaxel (PTX) resistance of ovarian cancer and its potential molecular mechanism.

Patients and methods: The expressions of NEAT1 and miR-194 in ovarian cancer tissues and cells were estimated by quantitative real-time polymerase chain reaction (qRT-PCR). MTT, flow cytometry, and Western blot assays were used to assess the effect of NEAT1 on PTX resistance in PTX-resistant ovarian cancer cells. Luciferase reporter assay was applied to examine the association between NEAT1, zinc finger E-box-binding homeobox 1 (ZEB1) and miR-194. Xenograft tumor model was established to confirm the biological role of NEAT1 in PTX resistance of ovarian cancer in vivo.

Results: NEAT1 was upregulated, and miR-194 was downregulated in PTX-resistant ovarian cancer tissues and cells. Functionally, NEAT1 knockdown enhanced cell sensitivity to PTX via promoting PTX-induced apoptosis in vitro. NEAT1 was identified as a molecular sponge of miR-194 to upregulate ZEB1 expression. Mechanistically, NEAT1-knockdown-induced PTX sensitivity was mediated by miR-194/ZEB1 axis. Moreover, NEAT1 knockdown improved PTX sensitivity of ovarian cancer in vivo.

Conclusion: NEAT1 contributed to PTX resistance of ovarian cancer cells at least partly through upregulating ZEB1 expression by sponging miR-194, elucidating a novel regulatory pathway of chemoresistance in PTX-resistant ovarian cancer cells and providing a possible long noncoding RNA (lncRNA)-targeted therapy for ovarian cancer.

Keywords: lncRNA NEAT1, miR-194, ZEB1, PTX, ceRNAs

Introduction

Ovarian cancer is one of the most prevalent and deadly types of gynecological malignancy, with ~225,000 new diagnosed cases and an estimated 140,000 deaths worldwide.[1,2] Due to untypical early symptoms and unreliable screening approaches, the majority of ovarian cancer patients are diagnosed at an advanced stage and present a poor response to currently available therapeutic interventions, with a 5-year survival rate of 15%–30%.[3,4] Most ovarian cancer patients receive successful chemotherapy initially; however, tumor recurrence frequently occurs following the development of multidrug resistance (MDR).[5] Resistance to paclitaxel (PTX), a frontline chemotherapeutic agent with long-term clinical applications in the therapy of ovarian carcinoma, continues to be one of the primary causes of treatment failure.[6] Therefore, it is

Correspondence: Jihong An
Department of Clinical Pharmacy, Huaihe Hospital of Henan University, No 115, Ximen Street, Gulou District, Kaifeng 475000, People's Republic of China
Tel +86 371 2390 6849
Email anjihongjh@126.com

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imperative to identify an effective therapeutic target that can sensitize ovarian cancer to PTX and elucidate the molecular mechanism of drug resistance in ovarian cancer.

Long noncoding RNAs (lncRNAs) are defined as a class of transcripts of >200 nucleotides in length and possess the capability of regulating gene expression at the transcriptional, posttranscriptional, and epigenetic level. It is becoming increasingly apparent that dysregulated lncRNAs are implicated in carcinogenesis and progression of numerous cancers, acting as either oncogenes or tumor suppressors.[7] Moreover, abnormality of lncRNA levels has been shown to be associated with the development of chemoresistance in various tumors, including ovarian cancer.[8,9] For example, enrichment of lncRNA ROR promoted proliferation, migration, and chemoresistance in nasopharyngeal carcinoma.[10] Upregulation of lncRNA HOTTIP induced cellular resistance to cisplatin in osteosarcoma through activating the Wnt/β-catenin pathway.[11] LncRNA-TUG1 mediated cell growth and drug resistance of small cell lung cancer by regulating LIMK2b via EZH2.[12]

Nuclear paraspeckle assembly transcript 1 (NEAT1), transcribed from the familial tumor syndrome multiple endocrine neoplasia type 1 locus on chromosome 11, has been identified as a nuclear-restricted lncRNA with the following two isoforms: 3.7 kb NEAT1-1 and 23 kb NEAT1-2.[13] NEAT1 has been documented as an oncogene to facilitate tumorigenesis in different types of solid tumors.[14] Of note, NEAT1 was reported to be upregulated in ovarian cancer and contributed to the carcinogenesis of ovarian cancer.[15,16] However, the involvement of NEAT1 in PTX resistance of ovarian cancer has been poorly defined.

In recent years, the interaction between lncRNAs and miRNAs has drawn great attention. LncRNAs could function as miRNA sponges or decoys to downregulate the expressions and activities of miRNAs, eventually leading to the derepression of miRNA targets at posttranscriptional level.[17] A previous study showed that miR-194 was downregulated in PTX-resistant ovarian cancer cell lines and forced expression of miR-194 attenuated PTX resistance in ovarian cancer cells.[18] Hence, this study was designed to explore whether the influence of NEAT1 on PTX resistance is mediated by miR-194 in ovarian cancer.

In this study, we demonstrated that NEAT1 was upregulated and miR-194 was downregulated in PTX-resistant ovarian cancer tissues and cells. Furthermore, functional and mechanism analyses revealed that NEAT1 contributed to PTX resistance through upregulating zinc finger E-box-binding homeobox 1 (ZEB1) expression by sponging miR-194. This study first established a NEAT1-miR-194-ZEB1 regulatory network in ovarian cancer, hinting a promising therapy strategy for ovarian cancer with PTX resistance.

Patients and methods

Patients and tissue samples

This study was approved by the Ethical Committee of Huaihe Hospital of Henan University, and written informed consent was provided by every participant prior to surgery. Thirty-two ovarian carcinoma specimens were collected from ovarian cancer patients receiving oophorectomies between 2013 and 2015 at Huaihe Hospital. All biopsy samples were histopathologically examined by two independent pathologists. Samples were promptly frozen in liquid nitrogen and maintained at −80°C until use. Patient's samples were divided into two groups based on the response to the first-line chemotherapy: 18 treatment-responsive patients and 14 treatment-resistant patients. According to the NCCN guidelines, intrinsically treatment-resistant tumors were regarded as those with persistent or recurrent disease within 6 months after the initiation of first-line taxol-platinum-based combination chemotherapy. Treatment-responsive tumors were classified as those with a complete response to chemotherapy and a platinum-free interval of >6 months.

Cell lines and culture

Human ovarian carcinoma cell lines SKOV3 and HeyA-8 were obtained from American Type Culture Collection (Manassas, VA, USA). The corresponding PTX-resistant ovarian cancer cells SKOV3/PTX and HeyA-8/PTX cells were established from the parental cell lines by stepwise exposure to escalating concentrations of PTX, as previously described.[19] Cells were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin) (Sigma-Aldrich Co., St Louis, MO, USA) in a 95% air/5% CO_2 atmosphere at 37°C. To maintain the PTX-resistant phenotype of SKOV3/PTX and HeyA-8/PTX cells, 5 nM PTX was additionally added into the culture medium.

Transient transfection

siRNA specially targeting NEAT1 (si-NEAT1), scrambled siRNA control (si-con), miR-194 mimic (miR-194), scrambled miRNA control (miR-con), miR-194 inhibitor (anti-miR-194), and inhibitor control (anti-miR-con) were purchased from RiboBio (Guangzhou, People's Republic of China). Ectopic expression of NEAT1 was achieved by introducing NEAT1 sequence into pcDNA3.1 vector (Thermo Fisher Scientific). SKOV3/PTX and HeyA-8/PTX cells were

seeded into six-well plates at a density of 5×10^4 cells/well and cultured overnight prior to transfection. Then, transient transfection with oligonucleotides or plasmids into SKOV3/PTX and HeyA-8/PTX cells was performed by Lipofectamine™ 2000 (Thermo Fisher Scientific). Cells were harvested 48 h posttransfection for subsequent analysis.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from ovarian cancer tissues and cells was extracted with TRIzol reagent (Thermo Fisher Scientific). The first strand of cDNA for NEAT1 and miR-194 was synthesized from 1 μg of RNA using the High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific) and MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific), respectively. qRT-PCR was carried out using SYBR® Premix DimerEraser Kit (TaKaRa, Dalian, People's Republic of China) on an Applied Biosystems 7500 Real-Time PCR System. The relative expressions of NEAT1 and miR-194 were calculated using $2^{-\Delta\Delta CT}$ method with normalization to GAPDH and U6 snRNA, respectively. The primers used were presented as below: NEAT1: 5′-CTTCCTCCCTTTAACTTATCCATTCAC-3′ (forward primer) and 5′-CTCTTCCTCCACCATTACCAACAATAC-3′ (reverse primer); miR-194: 5′-CAGGAGTTGTAAATCCGAGCCG-3′ (forward primer) and 5′-TTCATAGGTCAGAGCCCTGTGCA-3′ (reverse primer); GAPDH: 5′-TATGATGATATCAAGAGGGTAGT-3′ (forward primer) and 5′-TGTATCCAAACTCATTGTCATAC-3′ (reverse primer); U6: 5′-CTCGCTTCGGCAGCACA-3′ (forward primer) and 5′-AACGCTTCACGAATTTGCGT-3′(reverse primer).

Western blot

Protein samples from tissues or cells were subjected to 10% SDS-PAGE and transferred onto PVDF membranes. Following blocked in 5% skim milk for 2 h, the membranes were incubated overnight at 4°C with the primary antibodies against P-glycoprotein (P-gp) (1:1,000), glutathione *S*-transferase π (GST-π) (1:500), ZEB1 (1:2,000 dilution), and β-actin (1:5,000) from Santa Cruz Biotechnology Inc. (Dallas, TX, USA), followed by probed with horseradish peroxidase-conjugated goat anti-rabbit second antibody for 2 h at 1:5,000. The antigen–antibody complexes were visualized using chemiluminescence.

Drug resistance assay

PTX resistance was assessed by (4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. SKOV3/PTX and HeyA-8/PTX cells were incubated for 24 h prior to exposing to various doses of PTX for 48 h. Subsequently, 20 μL of 5 mg/mL MTT (Sigma-Aldrich) was added to each well for another 4 h, followed by the addition of 150 μL of DMSO to dissolve the generated formazan crystals. The absorbance at a wavelength of 570 nm was detected using a microplate reader. The concentration of PTX causing 50% inhibition of growth (IC_{50}) was determined by the relative survival curve.

Flow cytometry analysis of apoptosis

SKOV3/PTX and HeyA-8/PTX cells (3.5×10^5/well) were seeded into six-well plates and treated with PTX for 48 h. The ratio of apoptotic cells was detected using the Annexin V-FITC Apoptosis Detection Kit (Thermo Fisher Scientific) and analyzed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

Luciferase reporter assay

The NEAT1 full fragments or its mutant containing the putative miR-194-binding sites in NEAT1 were synthesized and cloned into downstream of firefly luciferase gene in pGL3 plasmids (Promega Corporation, Fitchburg, WI, USA), named as pGL3-NEAT1-wild type (Wt) and pGL3-NEAT1-mutant (Mut). Similarly, the 3′UTR of ZEB1 containing the predicted miR-194-binding sites or mutant sites was amplified by PCR and inserted into pGL3 plasmids, named as pGL3-ZEB1-3′UTR-Wt and pGL3-ZEB1-3′UTR-Mut. SKOV3/PTX and HeyA-8/PTX cells were maintained in 96-well plates and cotransfected with 400 ng of constructed luciferase reporter plasmids, 50 ng of renilla luciferase reporter vector (pRL-TK) and 50 nM of miR-194, miR-con, miR-194+ pcDNA-NEAT1 or miR-194+ vector using Lipofectamine™ 2000. Cells were harvested at 48 h after transfection, and luciferase activity was determined by using the Dual Luciferase Reporter Assay Kit (Promega Corporation). Renilla luciferase activities were used as the internal control for the normalization of firefly luciferase activity.

In vivo experiment

Lentiviral vector (Lenti-short hairpin [sh]-NEAT1) for stable NEAT1 overexpression was obtained from Genechem (Shanghai, People's Republic of China). The animal experiments were approved by the Animal Care and Use Committee of Huaihe Hospital and were performed in accordance with the institutional guide for the care and use of laboratory animals. Six-week-old male BALB/c athymic nude mice (n=5 per group) were purchased from Shanghai Experimental Animal Center (Shanghai, People's Republic of China). SKOV3/PTX cells (1×10^7) tansduced with sh-NEAT1 or

sh-con were injected subcutaneously into the flank of the nude mice. After 6 days of injection, mice were intraperitoneally administrated with phosphate buffered solution (PBS) or 3 mg/kg PTX (Sigma-Aldrich Co.) every 3 days for five cycles. The tumor size was measured every 3 days with a caliper, and tumor volume was calculated according to the formula: volume = length × width^2/2. All mice were sacrificed on day 21 after inoculation. The resected tumor masses were harvested for subsequent weight and qRT-PCR analysis.

Statistical analysis

All quantitative data were shown as mean ± standard deviation (SD) from three independent experiments. The comparisons between two or more groups were estimated by Student's *t*-test or one-way analysis of variance (ANOVA). $P<0.05$ was considered to be statistically significant.

Results

NEAT1 was upregulated and miR-194 was downregulated in PTX-resistant ovarian cancer tissues and cells

To examine the association of NEAT1 and miR-194 with PTX resistance in ovarian cancer, qRT-PCR was first performed to detect the expressions of NEAT1 and miR-194 in PTX-resistant and PTX-sensitive ovarian cancer tissues. As shown in Figure 1A and B, a significant increase in NEAT1 expression and a marked decrease in miR-194 expression were observed in treatment-resistant patients compared to that in treatment-responsive patients. Next, the expressions of NEAT1 and miR-194 in PTX-resistant ovarian cancer cells (SKOV3/PTX and HeyA-8/PTX) were further explored. Similarly, higher expression of NEAT1 (Figure 1C and D) and lower expression of miR-194 (Figure 1E and F)

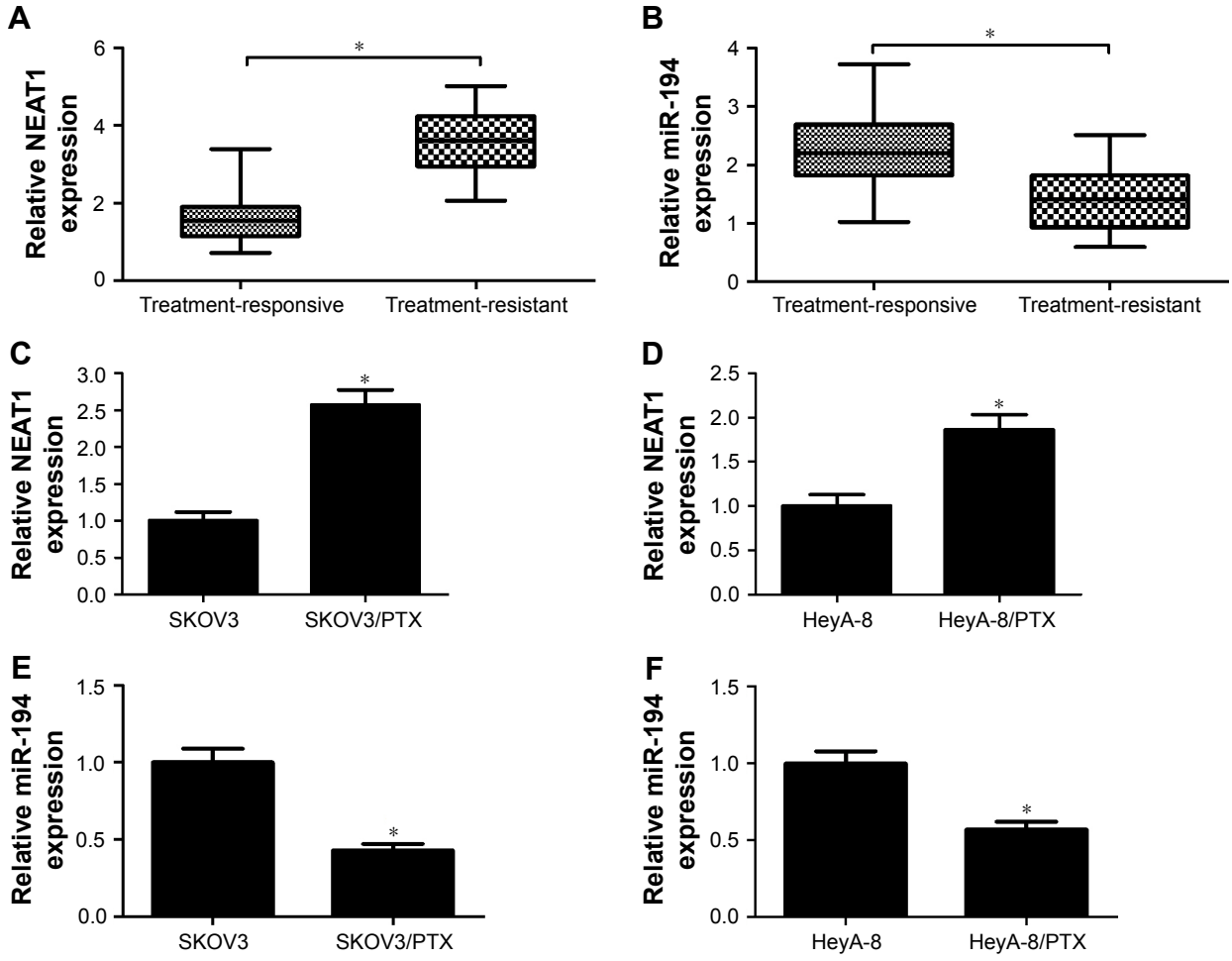

Figure 1 Expressions of NEAT1 and miR-194 in PTX-resistant ovarian cancer tissues and cells.
Notes: qRT-PCR analysis of NEAT1 (**A**) and miR-194 (**B**) expressions in 18 treatment-responsive patients and 14 treatment-resistant patients. qRT-PCR analysis of NEAT1 (**C** and **D**) and miR-194 (**E** and **F**) expressions in parental ovarian cancer cells (SKOV3 and HeyA-8) and PTX-resistant ovarian cancer cells (SKOV3/PTX and HeyA-8/PTX). *$P<0.05$.
Abbreviations: NEAT1, nuclear paraspeckle assembly transcript 1; PTX, paclitaxel; qRT-PCR, quantitative real-time polymerase chain reaction.

were discovered in PTX-resistant ovarian cancer cells in comparison with their parental cells SKOV3 and HeyA-8. These results demonstrated that dysregulation of NEAT1 and miR-194 may be associated with PTX resistance in ovarian cancer.

NEAT1 knockdown enhanced PTX sensitivity in PTX-resistant ovarian cancer cells

Based on the above results, the effects of NEAT1 knockdown on PTX resistance in ovarian cancer cells were further explored. First, cells were incubated with different concentrations of PTX for 48 h and, then, IC_{50} value was detected by MTT assay. As expected, IC_{50} value of PTX in SKOV3/PTX (Figure 2A) and HeyA-8/PTX (Figure 2B) cells was significantly higher than that in SKOV3 and HeyA-8 cells, confirming the production of PTX resistance in SKOV3/PTX and HeyA-8/PTX cells. Next, the role of NEAT1 in the acquisition of PTX resistance was evaluated by using loss-of-function experiments. qRT-PCR analysis confirmed the knockdown efficiency of NEAT1, demonstrated by the downregulation of NEAT1 expression in si-NEAT1-transfected SKOV3/PTX (Figure 2C) and HeyA-8/PTX (Figure 2D) cells. Since P-gp and GST-π, as MDR-associated marker proteins, were reported to play an important role in drug resistance of ovarian cancer,[20] we investigated the effect of NEAT1 knockdown on the protein levels of P-gp and GST-π by Western blot. The results exhibited that the protein levels of P-gp and GST-π were remarkably reduced in response to transfection of si-NEAT1 in SKOV3/PTX (Figure 2E) and HeyA-8/PTX (Figure 2F) cells. In addition, IC_{50} determination showed that NEAT1 knockdown significantly decreased PTX resistance in SKOV3/PTX (Figure 2G) and HeyA-8/PTX (Figure 2H) cells. To observe whether NEAT1-mediated alteration of PTX resistance was related to apoptosis, SKOV3/PTX and

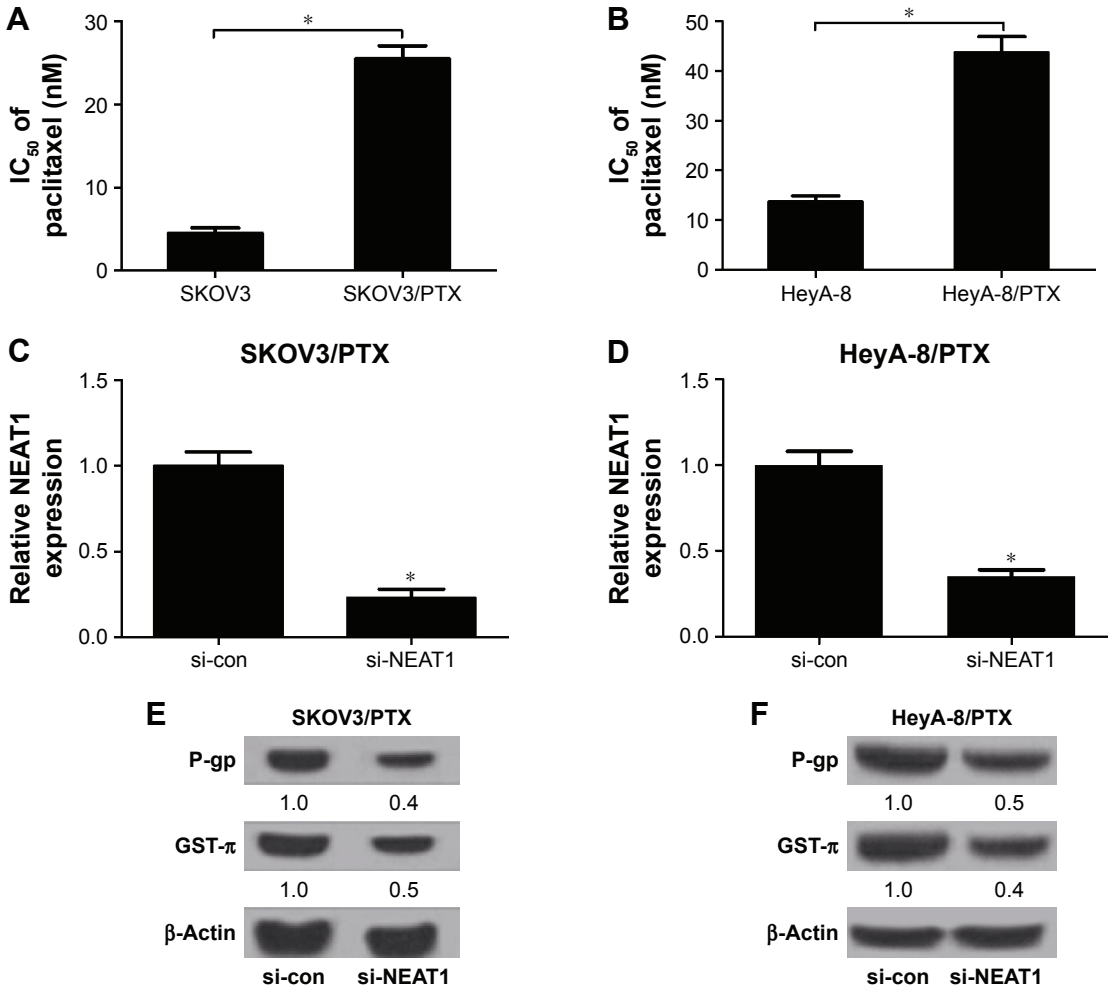

Figure 2 (*Continued*)

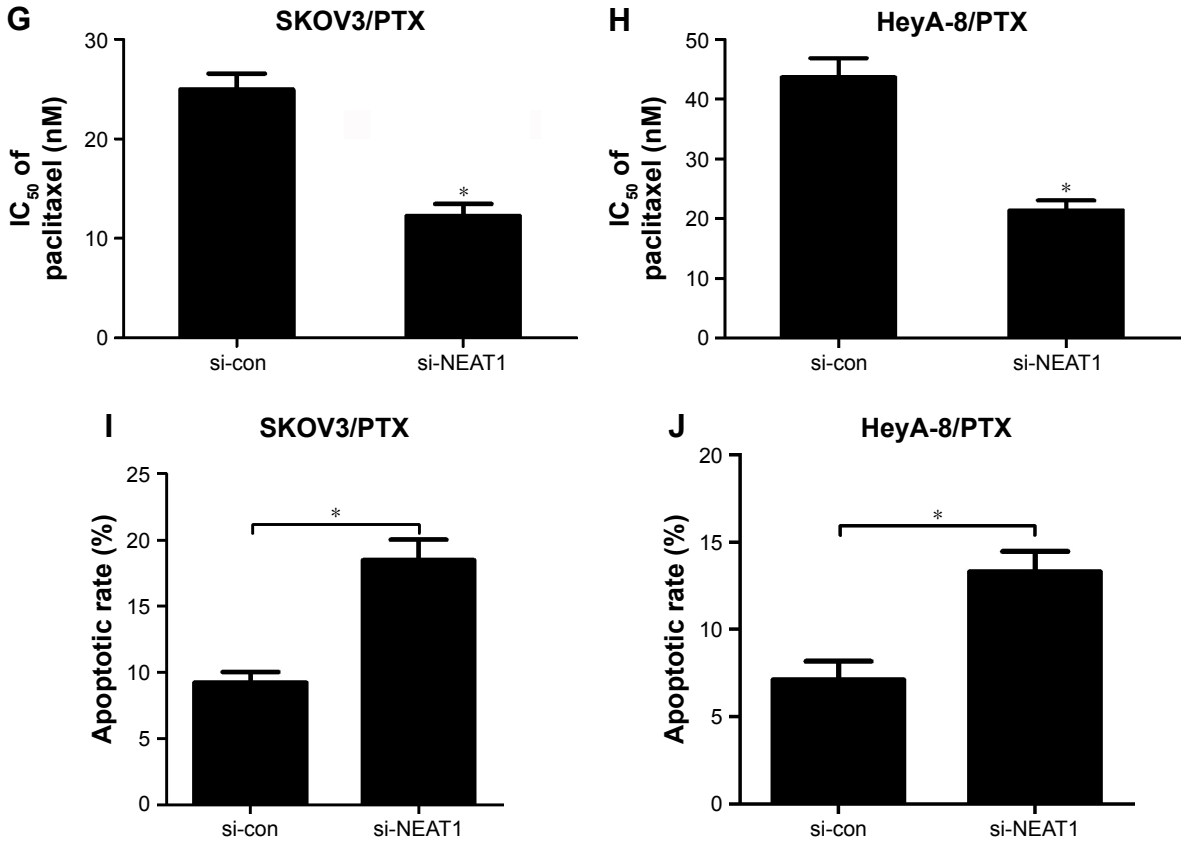

Figure 2 NEAT1 knockdown-sensitized SKOV3/PTX and HeyA-8/PTX cells to PTX.

Notes: IC_{50} value of PTX in SKOV3 and SKOV3/PTX cells (**A**) as well as in HeyA-8 and HeyA-8/PTX cells (**B**). Expression of NEAT1 in si-NEAT1- or si-con-transfected SKOV3/PTX (**C**) and HeyA-8/PTX (**D**) cells was estimated by qRT-PCR. Protein levels of P-gp and GST-π were determined by Western blot in SKOV3/PTX (**E**) and HeyA-8/PTX (**F**) cells after introduction with si-NEAT1 or si-con. PTX resistance of si-NEAT1- or si-con-treated SKOV3/PTX (**G**) and HeyA-8/PTX (**H**) cells was assessed using IC_{50} value of PTX by MTT assay. Apoptotic rate was measured by flow cytometry after SKOV3/PTX (**I**) and HeyA-8/PTX (**J**) cells transfected with si-NEAT1 or si-con were treated with PTX for 48 h. *$P<0.05$.

Abbreviations: GST-π, glutathione *S*-transferase π; IC_{50}, the concentration of PTX causing 50% inhibition of growth; MTT, (4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NEAT1, nuclear paraspeckle assembly transcript 1; P-gp, P-glycoprotein; PTX, paclitaxel; qRT-PCR, quantitative real-time polymerase chain reaction; si-con, siRNA control.

HeyA-8/PTX cells transfected with si-con or si-NEAT1 were treated with PTX for 48 h. Flow cytometry analysis revealed that PTX-induced apoptosis was obviously enhanced after introduction with si-NEAT1 in SKOV3/PTX (Figure 2I) and HeyA-8/PTX (Figure 2J) cells. Taken together, these data suggested that NEAT1 knockdown sensitized SKOV3/PTX and HeyA-8/PTX cells to PTX.

NEAT1 negatively regulated the expression of miR-194

IncRNAs have been reported as competing endogenous RNAs (ceRNAs) that share miRNAs recognition sites with mRNAs or compete for miRNAs binding, thus resulting in changes of target mRNAs expression.[21] Through web-based tool Starbase 2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php), miR-194 was predicted to have complementary bases pairing with NEAT1 (Figure 3A). Accordingly, luciferase reporter vectors (pGL3-NEAT1-Wt or pGL3-NEAT1-Mut) containing the wild-type or mutated miR-194-binding sites in NEAT1 were established and co-transfected with miR-194 mimic or miR-con into SKOV3/PTX and HeyA-8/PTX cells. The results showed that ectopic expression of miR-194 dramatically suppressed the luciferase activity of pGL3-NEAT1-Wt reporter but not that of pGL3-NEAT1-Mut reporter in SKOV3/PTX (Figure 3B) and HeyA-8/PTX (Figure 3C) cells. To further evaluate the regulatory impact between NEAT1 and miR-194, SKOV3/PTX and HeyA-8/PTX cells were transfected with pcDNA-NEAT1, si-NEAT1, or matched controls. It was observed that miR-194 expression was significantly downregulated in pcDNA-NEAT1-transfected SKOV3/PTX and HeyA-8/PTX cells and was exceptionally upregulated in si-NEAT1-transfected SKOV3/PTX and HeyA-8/PTX cells (Figure 3D and E). All these data indicated that NEAT1 suppressed the expression of miR-194.

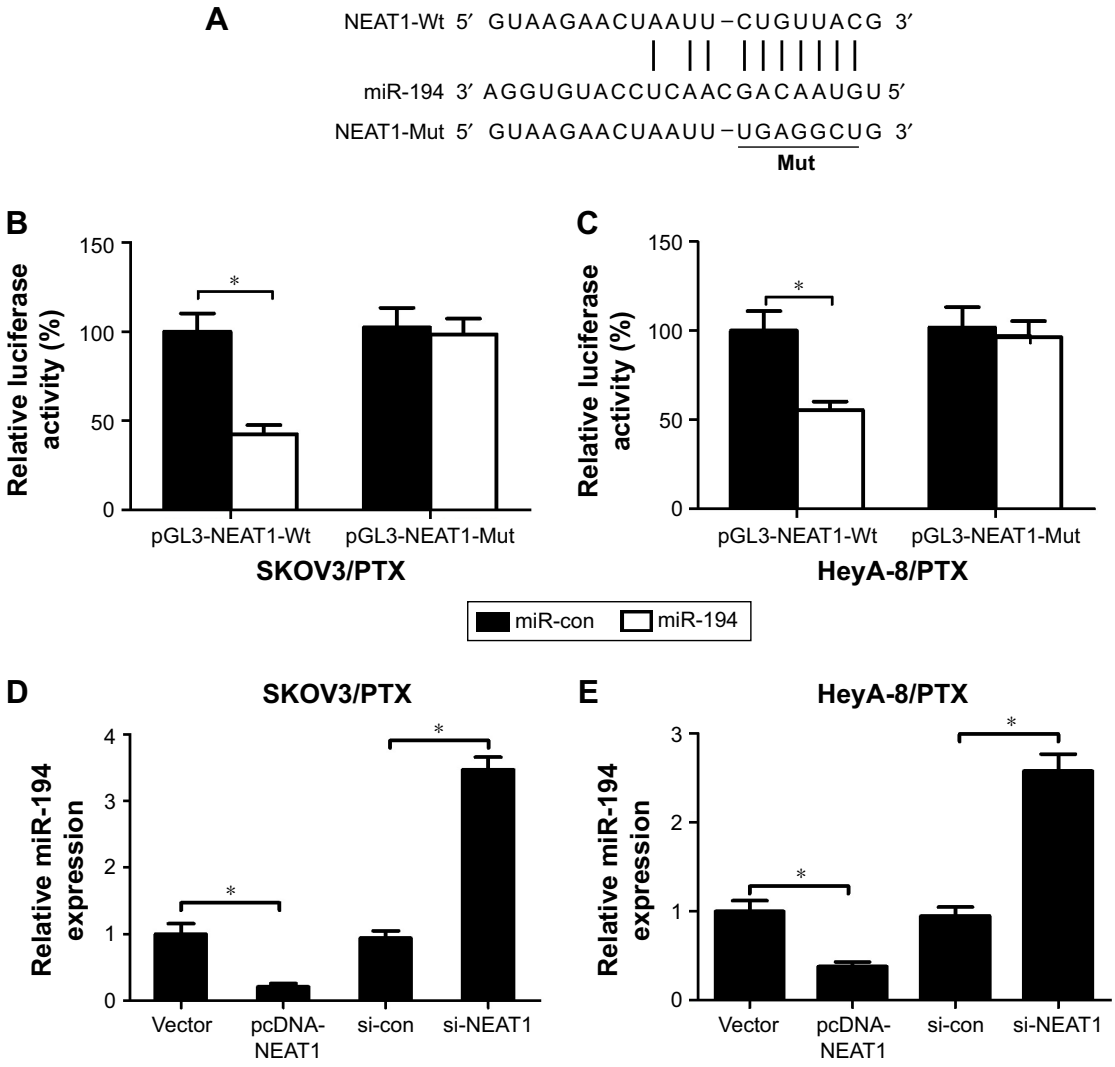

Figure 3 NEAT1 inhibited miR-194 expression.

Notes: (**A**) Graphical representation of predicted wild-type or mutated binding sequences in NEAT1. Luciferase reporter assay in SKOV3/PTX (**B**) and HeyA-8/PTX (**C**) cells after cotransfection with established luciferase reporter vectors (pGL3-NEAT1-Wt or pGL3-NEAT1-Mut) and miR-194 or miR-con. Expression of miR-194 was examined by qRT-PCR in SKOV3/PTX (**D**) and HeyA-8/PTX (**E**) cells treated with pcDNA-NEAT1, si-NEAT1, or respective controls. *$P<0.05$.

Abbreviations: miR-con, miRNA control; NEAT1, nuclear paraspeckle assembly transcript 1; PTX, paclitaxel; qRT-PCR, quantitative real-time polymerase chain reaction; si-con, siRNA control; Wt, wild type; Mut, mutant.

NEAT1 knockdown improved PTX sensitivity in PTX-resistant ovarian cancer cells by negatively regulating miR-194

In view of the regulatory role of NEAT1 in miR-194 expression in SKOV3/PTX and HeyA-8/PTX cells, whether the influence of NEAT1 in PTX resistance was correlative with miR-194 was further explored. SKOV3/PTX and HeyA-8/PTX cells were transfected with miR-194, si-NEAT1, si-NEAT1 + anti-miR-194, or matched controls. Western blot analysis demonstrated that miR-194 overexpression or NEAT1 knockdown led to an obvious reduction of P-gp and GST-π expressions in SKOV3/PTX (Figure 4A) and HeyA-8/PTX (Figure 4B) cells, while anti-miR-194 transfection significantly reversed si-NEAT1-mediated reduction of P-gp and GST-π expressions. Drug resistance assay showed that miR-194 upregulation or NEAT1 deficiency effectively enhanced PTX sensitivity in SKOV3/PTX (Figure 4C) and HeyA-8/PTX (Figure 4D) cells; however, anti-miR-194 introduction greatly abolished si-NEAT1-triggered increase in PTX sensitivity. Meanwhile, flow cytometry analysis revealed that forced expression of miR-194 or NEAT1 silence dramatically promoted PTX-induced apoptosis in SKOV3/PTX (Figure 4E) and HeyA-8/PTX (Figure 4F) cells, whereas anti-miR-194 treatment markedly abated the promotive effect of si-NEAT1 on PTX-induced apoptosis. These results illustrated that miR-194 downregulation partially

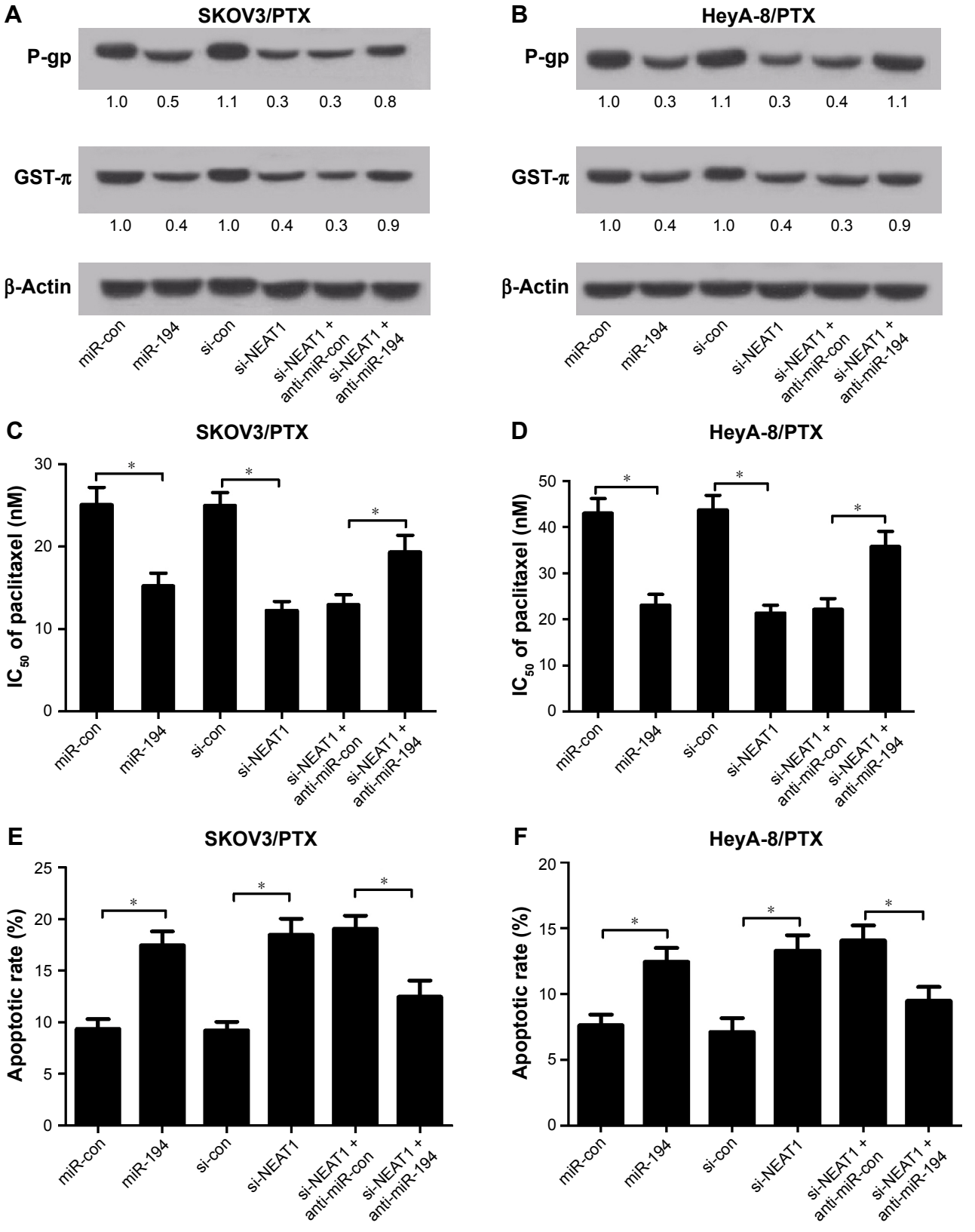

Figure 4 NEAT1 knockdown increased PTX sensitivity in PTX-resistant ovarian cancer cells by repressing miR-194.

Notes: SKOV3/PTX and HeyA-8/PTX cells were transfected with miR-194, si-NEAT1, si-NEAT1 + anti-miR-194, or matched controls. Protein levels of P-gp and GST-π in transfected SKOV3/PTX (**A**) and HeyA-8/PTX (**B**) cells were examined by Western blot. IC_{50} value of PTX was measured by MTT assay after the transfected SKOV3/PTX (**C**) and HeyA-8/PTX (**D**) cells were treated with various doses of PTX for 48 h. Flow cytometry analysis was performed to detect apoptosis after transfected. SKOV3/PTX (**E**) and HeyA-8/PTX (**F**) cells were exposed to PTX for 48 h. *$P<0.05$.

Abbreviations: GST-π, glutathione *S*-transferase π; IC_{50}, the concentration of PTX causing 50% inhibition of growth; MTT, (4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; miR-con, miRNA control; NEAT1, nuclear paraspeckle assembly transcript 1; P-gp, P-glycoprotein; PTX, paclitaxel; si-con, siRNA control.

overturned NEAT1-knockdown-induced PTX sensitivity in PTX-resistant ovarian cancer cells.

NEAT1 upregulated ZEB1 expression by sponging miR-194

TargetScan 6.2 (www.targetscan.org) and miRanDa (www.microrna.org) were used to predict the potential targets of miR-194. ZEB1-3′UTR was found to contain the complementary sequence for miR-194, as depicted in Figure 5A. To confirm the prediction, the 3′UTR sequence of ZEB1 and its mutated sequence were cloned into the luciferase reporter vectors, named as pGL3-ZEB1-3′UTR-Wt and pGL3-ZEB1-3′UTR-Mut, respectively. Luciferase reporter assay results showed that exogenetic expression of miR-194 significantly blocked luciferase activity of pGL3-ZEB1-3′UTR-Wt reporter, whereas NEAT1 overexpression

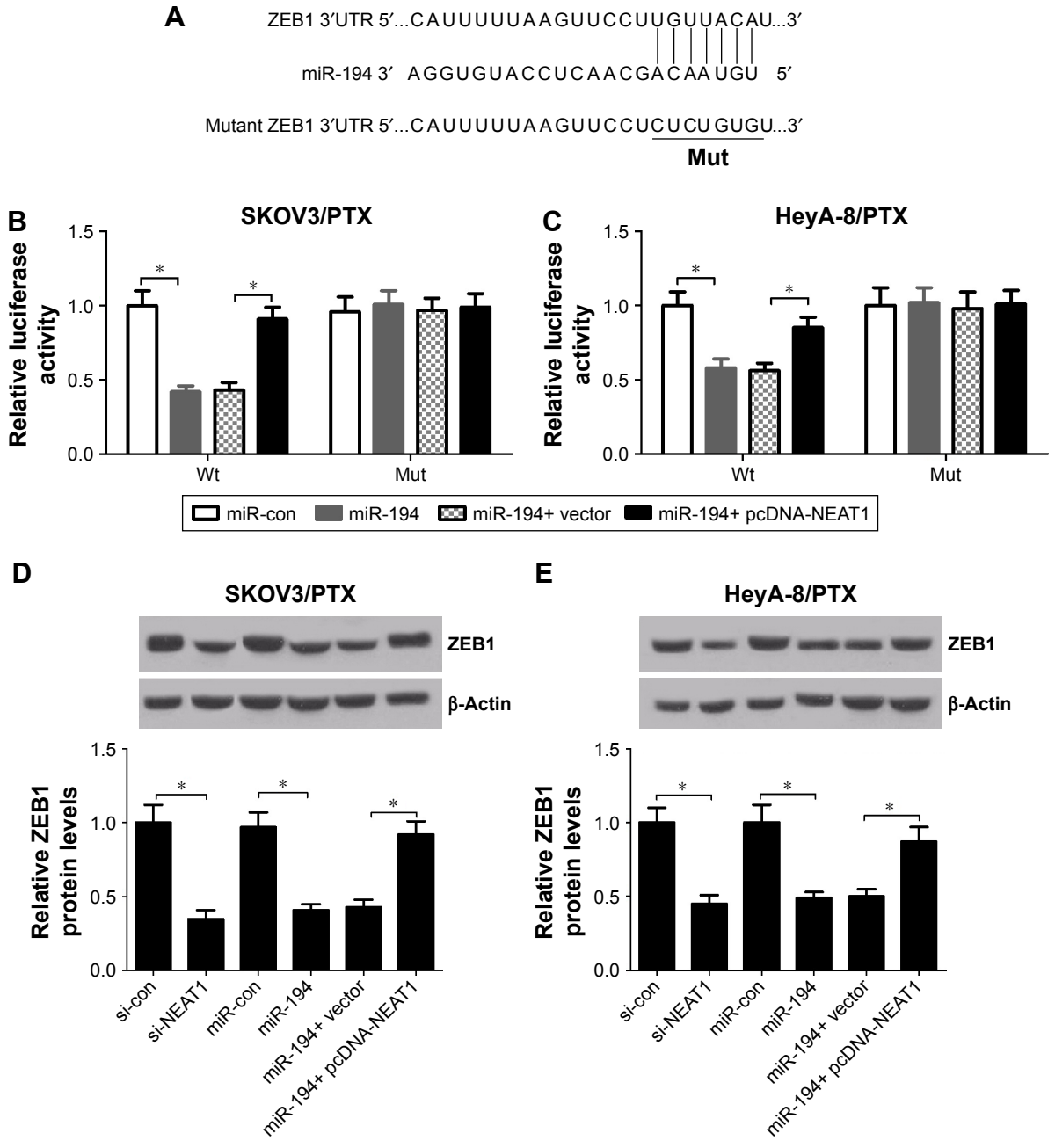

Figure 5 NEAT1 upregulated ZEB1 expression via sponging miR-194.

Notes: (**A**) Schematic representation of the predicted wild-type or mutant miR-194-binding sites in the 3′UTR sequences of ZEB1. The luciferase activity was detected by luciferase reporter assay in SKOV3/PTX (**B**) and HeyA-8/PTX (**C**) cells cotransfected with luciferase reporter plasmids (pGL3-ZEB1-3′UTR-Wt or pGL3-ZEB1-3′UTR-Mut) and miR-194, miR-194+ pcDNA-NEAT1, or corresponding control. Protein level of ZEB1 in SKOV3/PTX (**D**) and HeyA-8/PTX (**E**) cells introduced with si-NEAT1, miR-194, miR-194+ pcDNA-NEAT1, or matched controls was estimated by Western blot. *$P<0.05$.

Abbreviations: miR-con, miRNA control; NEAT1, nuclear paraspeckle assembly transcript 1; PTX, paclitaxel; si-con, siRNA control; ZEB1, zinc finger E-box-binding homeobox 1; Wt, wild type; Mut, mutant.

remarkably abrogated the inhibitory effect in SKOV3/PTX (Figure 5B) and HeyA-8/PTX (Figure 5C) cells. However, the luciferase activity in pGL3-ZEB1-3′UTR-Mut reporter was not affected by any treatment (Figure 5B and C). Subsequently, we assessed the protein level of ZEB1 in SKOV3/PTX and HeyA-8/PTX cells introduced with si-NEAT1, miR-194, miR-194+ pcDNA-NEAT1, or matched controls. As shown in Figure 5D and E, NEAT1 knockdown or miR-194 overexpression markedly suppressed the protein level of ZEB1, while revert of NEAT1 expression attenuated miR-194-induced decrease in ZEB1 expression. Taken together, these data demonstrated that NEAT1 positively regulated ZEB1 expression through miR-194.

NEAT1 contributed to PTX resistance of PTX-resistant ovarian cancer cells through elevating ZEB1 expression by sponging miR-194

To investigate the regulatory effect of NEAT1 and miR-194 on ZEB1 function, SKOV3/PTX and HeyA-8/PTX cells were transfected with si-ZEB1, si-ZEB1 + pcDNA-NEAT1, si-ZEB1 + anti-miR-194, or matched controls. The protein level of ZEB1 was apparently reduced in si-ZEB1-treated SKOV3/PTX (Figure 6A) and HeyA-8/PTX (Figure 6B) cells, while NEAT1 overexpression or miR-194 downregulation weakened this effect. IC_{50} determination showed that ZEB1 knockdown dramatically attenuated PTX resistance in SKOV3/PTX (Figure 6C) and HeyA-8/PTX (Figure 6D) cells, which was significantly relieved by reintroduction of pcDNA-NEAT1 or anti-miR-194. Furthermore, ZEB1 knockdown resulted in a marked promotion of PTX-induced apoptosis in SKOV3/PTX (Figure 6E) and HeyA-8/PTX (Figure 6F) cells, which was strikingly suppressed by NEAT1 up-regulation or miR-194 suppression, as demonstrated by flow cytometry analysis. These results indicated that NEAT1 overexpression or miR-194 downregulation reversed ZEB1-knockdown-induced sensitivity of PTX in ovarian cancer cells.

NEAT1 knockdown improved PTX sensitivity of ovarian cancer in vivo

To confirm the functional effect of NEAT1 on PTX resistance in vivo, a xenograft tumor mouse model was established. SKOV3/PTX cells transfected with sh-con or sh-NEAT1 were subcutaneously injected into nude mice. After 6 days, the mice were administrated intraperitoneally with PBS or 3 mg/kg PTX every 3 days. As presented in Figure 7A and B, PTX treatment or NEAT1 knockdown significantly blocked tumor growth (decreased tumor volume and weight), while combined sh-NEAT1 and PTX led to more obvious inhibition on tumor growth. qRT-PCR analysis showed that NEAT expression was prominently lower in tumor tissues from sh-NEAT1 group than sh-con group (Figure 7C). Thus, it is concluded that NEAT1 knockdown improved PTX sensitivity of ovarian cancer in vivo.

Discussion

Although PTX-based chemotherapy is particularly effective in most cancer cases, the development of chemoresistance is becoming a major impediment to the successful treatment of cancer patients.[22] Convincing evidence has indicated the essential roles of lncRNAs in drug resistance of diverse tumors.[23] In this study, we focused on the function and mechanism of NEAT1 in PTX resistance in ovarian cancer. The results showed that NEAT1 expression was upregulated in PTX-resistant ovarian cancer tissues and cells. Functional and mechanistic studies manifested that NEAT1 contributed to PTX resistance of ovarian cancer cells possibly through upregulating ZEB1 expression by sponging miR-194.

It is well documented that aberrant expression of NEAT1 plays a crucial role in the development of drug resistance in human cancers. For example, NEAT1 was upregulated in adriamycin-resistant gastric cancer cells and functioned as an oncogene to contribute to chemotherapy resistance of gastric cancer cells.[24] NEAT1 expression level was downregulated in leukemia patients and cells and overexpression of NEAT1-mitigated MDR induced by cytotoxic agent through inhibition of ATP-binding cassette G2 (ABCG2) in leukemia.[25] Another study reported that NEAT1 enhanced cisplatin sensitivity in non-small-cell lung cancer through functioning as a ceRNA to upregulate EGCG-induced cisplatin transporter CTR1 (copper transporter 1) by sponging miR-98-5p.[26] In the present study, it is demonstrated that NEAT1 was upregulated in PTX-resistant ovarian cancer tissues and cells. The efflux pump P-glycoprotein (P-gp), an important member of the ATP-binding cassette (ABC) transporters that facilitate the removal of anticancer drugs from cancer cells, is identified as an important mechanism of resistance to drug treatment.[27,28] GST-π, as one of the members of GST family, has been shown to affect drug metabolism and result in drug sequestration in intracellular vesicles.[29] Our results demonstrated that NEAT1 knockdown suppressed P-gp and GST-π levels in PTX-resistant ovarian cancer cells, suggesting that NEAT1 knockdown may attenuate drug resistance of ovarian cancer cells. Functional analysis further revealed that NEAT1 knockdown improved PTX sensitivity and

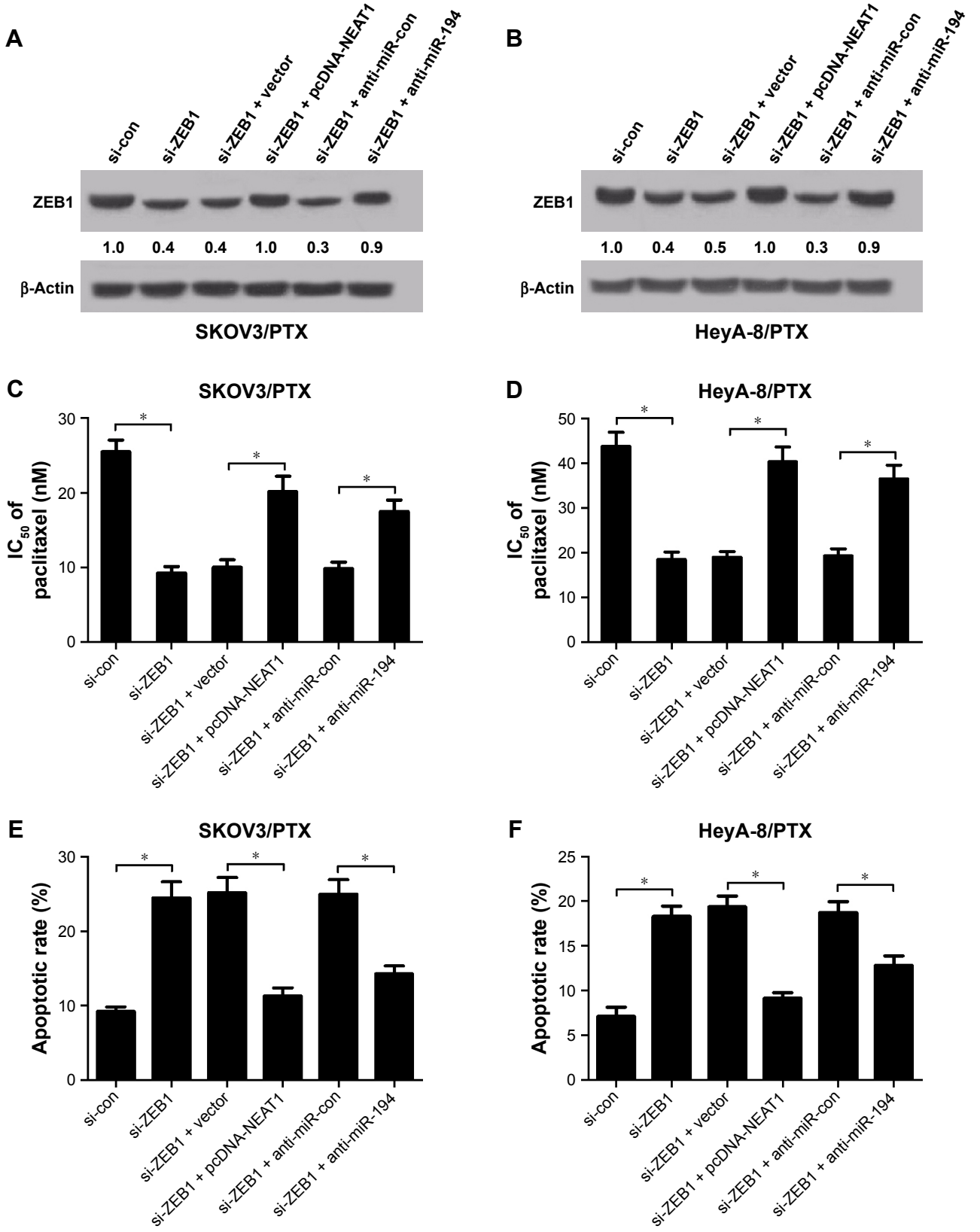

Figure 6 ZEB1-knockdown-induced sensitivity of PTX was suppressed by reintroduction of pcDNA-NEAT1 or anti-miR-194.

Notes: SKOV3/PTX and HeyA-8/PTX cells were transfected with si-ZEB1, si-ZEB1 + pcDNA-NEAT1, si-ZEB1 + anti-miR-194, or matched controls. Protein level of ZEB1 was determined by Western blot in transfected SKOV3/PTX (**A**) and HeyA-8/PTX (**B**) cells. PTX resistance in transfected SKOV3/PTX (**C**) and HeyA-8/PTX (**D**) cells was assessed using IC_{50} value of PTX by MTT assay. Apoptotic rate in transfected SKOV3/PTX (**E**) and HeyA-8/PTX (**F**) cells with PTX treatment for 48 h was evaluated by flow cytometry analysis. *$P<0.05$.

Abbreviations: IC_{50}, the concentration of PTX causing 50% inhibition of growth; miR-con, miRNA control; NEAT1, nuclear paraspeckle assembly transcript 1; PTX, paclitaxel; si-con, siRNA control; ZEB1, zinc finger E-box-binding homeobox 1.

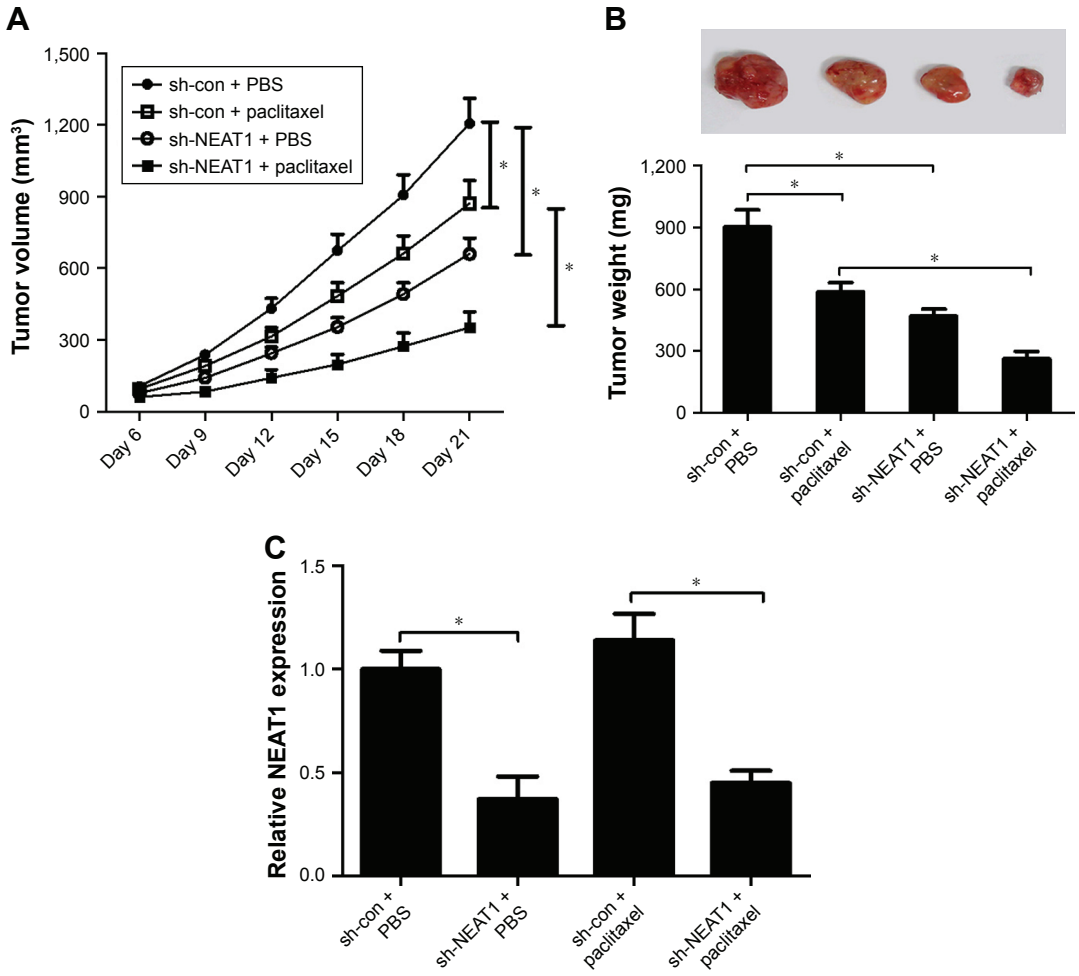

Figure 7 NEAT1 knockdown enhanced PTX sensitivity of ovarian cancer cells in vivo.
Notes: SKOV3/PTX cells introduced with sh-con or sh-NEAT1 were inoculated subcutaneously into the nude mice. The mice were given PBS or 3 mg/kg PTX (once every 3 days) via subcutaneous injection on day 6 after inoculation. (**A**) Growth curve of xenografted tumors. (**B**) Weights of resected tumor masses. (**C**) NEAT1 expression in xenografted tumors. *$P<0.05$.
Abbreviations: NEAT1, nuclear paraspeckle assembly transcript 1; PTX, paclitaxel; sh, short hairpin; PBS, phosphate buffered solution.

promoted PTX-induced apoptosis in PTX-resistant ovarian cancer cells in vitro. Moreover, chemoresistance of NEAT1 was validated on ovarian cancer xenografts in nude mice. These results demonstrated that NEAT1 conferred PTX resistance in PTX-resistant ovarian cancer cells in vitro and in vivo. In agreement with our data, NEAT1 was highly expressed in ovarian cancer tissues and cells and knockdown of NEAT1 elicited suppression on proliferation and invasion in ovarian cancer.[16]

Recently, a competing endogenous RNAs (ceRNAs) hypothesis proposed that lncRNAs may exert their biological function through acting as molecular sponge for miRNAs, thus in turn leading to derepression of miRNA targets.[30] In the present study, miR-194 was demonstrated to be down-regulated in PTX-resistant ovarian cancer tissues and cells. Interestingly, NEAT1 was first demonstrated to interact with miR-194 and negatively regulate its expression. Moreover, enforced expression of miR-194 attenuated PTX resistance in PTX-resistant ovarian cancer cells, consistent with a previous report.[18] Furthermore, inhibition of miR-194 reversed NEAT1-knockdown-mediated increase in PTX sensitivity. The inductive effect of miR-194 on drug sensitivity was also reported in non-small-cell lung cancer[31] and laryngeal squamous cell carcinoma.[32]

Additionally, our results demonstrated that ZEB1 was a direct target of miR-194 and NEAT1 derepressed ZEB1 expression by suppressing miR-194. Mechanistic analysis further revealed that miR-194 inhibition or NEAT1 overexpression abolished ZEB1-knockdown-mediated improvement of PTX sensitivity. ZEB1, as a epithelial–mesenchymal transition (EMT) inducing transcription factor, is involved in drug-resistant phenotype.[33] A previous document

demonstrated that increased expression of ZEB1 contributed to the development of drug resistance in ovarian cancer cells.[34] From all these data, it is concluded that NEAT1 acted as a ceRNA to positively regulate ZEB1 expression by sponging miR-194, thus contributing to PTX resistance of ovarian cancer cells. Moreover, it has been reported that TGF-β1 is involved in EMT and cell invasion and NEAT1 knockdown remarkably reduced the expression of TGF-β1 in ovarian cancer cells.[35] In the future research, we will focus on whether the regulatory effect of NEAT1/miR-194 on PTX resistance was mediated by TGF-β1 signaling pathway.

Conclusion

This study identified the involvement of NEAT1 in PTX resistance of ovarian cancer cells. NEAT1 enhanced PTX resistance in ovarian cancer cells by working as a ceRNA to sponge miR-194, thus reinforcing the protein level of ZEB1. Therefore, our study contributes to better understanding of the molecular mechanism of drug resistance in ovarian cancer, providing a promising lncRNA-targeted therapy for ovarian cancer.

Disclosure

The authors report no conflicts of interest in this work.

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