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Original Article

The resistance of esophageal cancer cells to paclitaxel can be reduced by the knockdown of long noncoding RNA DDX11-AS1 through TAF1/TOP2A inhibition

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Abstract: Esophageal cancer (EC) is one of the most common malignancies in the world. The currently used chemotherapeutic drug for the treatment of EC is paclitaxel (PTX), the efficacy of which is affected by the development of drug resistance. The present study aims to define the role of the long noncoding RNA (lncRNA) DDX11-AS1 in the progression of EC with the involvement of PTX-resistant EC cells. First, EC and adjacent normal tissue samples were collected from 82 patients with EC, after which the expression levels of DDX11-AS1, TOP2A and TAF1 were determined. The results showed that DDX11-AS1, TOP2A and TAF1 were highly expressed in EC tissues, and there was a positive correlation between the expression levels of DDX11-AS1 and TOP2A. A PTX-resistant EC cell line was constructed. Next, we evaluated the effects of DDX11-AS1 and TOP2A on the resistance of EC cells to PTX, and the regulatory relationships between DDX11-AS1, TOP2A and TAF1 were investigated. DDX11-AS1 could promote TOP2A transcription via TAF1, and the knockdown of TOP2A or DDX11-AS1 could increase the sensitivity of EC cells to PTX. The effect of DDX11-AS1 on the growth of PTX-inhibited tumors was confirmed using a tumor formation assay in nude mice. It was verified that knocking down DDX11-AS1 reduced the expression level of TOP2A and inhibited tumor growth. In conclusion, our findings suggest that DDX11-AS1 knockdown results in reduced resistance of EC cells to PTX by inhibiting TOP2A transcription via TAF1. Therefore, DDX11-AS1 knockdown could be a promising therapeutic strategy for EC.

Keywords: DDX11-AS1, esophageal cancer, paclitaxel, TOP2A, TAF1, resistance

Introduction

Esophageal cancer (EC) is the eighth most common cancer worldwide and the sixth leading cause of cancer-related mortality [1]. The incidence of EC in China has been reported to account for 50% of cases worldwide, with 223,306 new EC cases reported in China in 2012 [2]. The incidence and mortality of EC are increased as a result of obesity, smoking tobacco, and nutritional deficiencies [2, 3]. Surgery remains one of the most traditional therapeutic approaches in the management of EC at the early stage of the disease, with perioperative chemotherapy or chemo-radiotherapy performed for patients with locally advanced disease [4-6]. Recently, the anti-tumor agent paclitaxel (PTX) has been identified as an effective drug for EC treatment; however, the development of resistance to PTX greatly influences its clinical effect [7]. Therefore, understanding the underlying molecular mechanisms of EC progression is essential to come up with new and improved therapeutic approaches for EC and to suppress PTX resistance.

Long noncoding RNAs (lncRNAs) reportedly play crucial roles in both tumor development and progression [8]. The association between lncRNAs and chemosensitivity to PTX has been

revealed in EC [9]. LncRNA DDX11 antisense RNA 1 (DDX11-AS1), also known as cohesion regulator noncoding RNA (CONCR), is known to play a critical role in multiple carcinomas, including lung adenocarcinoma, colon adenocarcinoma, breast carcinoma, kidney carcinoma, and bladder carcinoma [10, 11]. DDX11-AS1 has been confirmed to be activated by MYC while being negatively mediated by p53 [10]. Moreover, N-MYC binds to topoisomerase alpha 2 (TOP2A) to form a subunit of cohesion, and p53 is phosphorylated by TATA-box binding protein-associated factor 1 (TAF1) [12, 13]. TOP2A is a marker of proliferation and chemotherapy resistance in different cancers, such as adrenocortical carcinoma and breast carcinoma [14, 15]. TAF1 is reported to be involved extensively in the activation of gene transcription in various cell types in eukaryotes [16]. TAF1 and TAF1L together participate in tumorigenesis of gastric and colorectal carcinomas through somatic mutations and mutational intratumoral heterogeneity [17]. These findings have led to the hypothesis that DDX11-AS1 could be involved in EC via TOP2A and TAF1. Therefore, in the present study, we investigated the relationship between DDX11-AS1, TOP2A, and TAF1 in EC, and the effect on resistant-PTX EC cells, to broaden our understanding of the tumorigenesis of EC with the aim of providing a promising therapeutic strategy for the treatment of EC.

Materials and methods

Ethics statement

Each participant signed a written informed consent prior to the experiments, and all aspects of the study complied with the guidelines provided by the Declaration of Helsinki. This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the guidelines established by the Ethics Committee of Cancer Hospital of Shantou University Medical College.

Subjects and EC tissue collection

The EC tissues and adjacent normal tissues (over 5-10 cm away from the tumor tissues) were obtained from 82 EC patients who had undergone consecutive surgical resection in the Cancer Hospital of Shantou University Medical College from May 2017 to October 2018. Part of the tissues was used for mRNA extraction, while the remaining portion was considered pathological tissues and fixed in neutral buffered formalin, embedded in paraffin, and sectioned routinely. All EC patients had not received any chemotherapy or radiotherapy prior to the surgery. The World Health Organization Classification of Tumors of the Digestive System was applied for histological diagnosis and classification [18].

Patient follow-up

The patients continued their follow-up once a month by an outpatient visit or a phone call after surgery, and the corresponding information was obtained using a questionnaire. The follow-up began following diagnosis and hospitalization and ended if the patients died or at the last follow-up. The follow-up time was 7-24 months, with the last follow-up dated on May 31, 2019.

Immunohistochemistry

The sections were deparaffinized conventionally, hydrated via gradient ethanol, and repaired with the use of the high-pressure cooker antigen repairing method. Next, endogenous peroxidase activity was eliminated in each section with the addition of 50 μL 3% H_2O_2. Subsequently, each section was incubated with primary antibody rabbit anti-TOP2A antibody (ab52934, Abcam Inc., Cambridge, MA, USA) or rabbit anti-TAF1 antibody (#12781, 1:100, Cell Signaling Technologies [CST], Beverly, MA, USA) at 4°C overnight. In the negative control (NC), the primary antibody was substituted with normal rabbit serum. Each section was incubated with 50 μL polymer reinforcing agent at 37°C for 20 min and 50 μL enzyme-labeled rabbit antipolymer at 37°C for 30 min. Afterwards, each section was developed with 100 μL of diaminobenzidine (DAB) for 3-10 min, followed by microscopic observation. The cells with a brown color were regarded as positive cells. The sections were then counterstained, dehydrated by gradient ethanol, sealed with neutral resin and photographed under the microscope.

The EC cells expressing TOP2A and TAF1 appeared to have brown and yellow granules. The staining intensity was estimated using the

score points from the reaction intensity level as well as the percentage of positive cells. The percentage of positive cells was scored as follows: 0 points: ≤ 10%; 2 points: 11%-51%; 3 points: 51%-81%; 4 points: ≥ 81%. The staining intensity was scored as follows: 1 point: weak intensity; 2 points: moderate intensity; 3 points: strong intensity. The score for the percentage of positive cells and staining intensity were summed. Finally, the cells were categorized into negative expression (-) (≤ 10% positive cells stained regardless of intensity), weak expression (+) (3 points), positive expression (++, 4-5 points), and strong positive expression (+++, 6-7 points) groups.

Cell culture

The HEK-293T cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), and the EC109 and KYSE150 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The 293T and EC109 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (31800022, Gibco, Grand Island, NY, USA) containing 1.5 g/L $NaHCO_3$, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. The KYSE150 cells were cultured in RPMI-1640 (12430-054, Invitrogen Inc., Carlsbad, CA, USA) containing 10% FBS and 1% penicillin-streptomycin. The culture medium was changed every 1 or 2 days (d).

Construction of the PTX-resistant EC109 cell line

The EC109 cells at logarithmic phase were incubated with 0.625 μg/mL PTX (HY-B0015, MedChemExpress [MCE], NJ, USA) for 2 h. Next, the cells were detached using 0.25% trypsin, and centrifugation was carried out at 1000 rpm for 5 min at room temperature. Following removal of the supernatant, the EC109 cells were supplemented with culture medium and seeded in the culture flask. After 24 h, a large number of cells died and were removed, while the cells that were still alive continued to be cultured until the cell confluence reached 80%-90%, after which subculturing was conducted to the third passage. The above steps were repeated twice, that is, the EC109 cells were incubated with PTX 3 times (28 d each time). Afterwards, drug induction was prolonged to 4 h at an interval of 10 d. Drug induction was conducted in triplicate (18 d each time). Finally, the PTX-resistant EC109 cell line was obtained, which was R-EC109.

Cell counting kit (CCK)-8

PTX-resistant and parental EC109 cells in logarithmic growth phase were seeded into 96-well plates at 7×10^3 cells per well with 100 μL culture medium. After 24 h, the medium in the administration group was replaced with culture medium containing 100 μL PTX at different concentrations (0 μg/mL, 0.0125 μg/mL, 0.025 μg/mL, 0.05 μg/mL, 0.1 μg/mL, 0.5 μg/mL, 1 μg/mL, 10 μg/mL) with 6 parallel wells per concentration gradient. The medium in the control group was replaced with 100 μL routine culture medium. After 48 h, the cells in each well were supplemented with 10 μL CCK-8 (C0038, Beyotime Institute of Biotechnology, Shanghai, China), cultured for 2 h, and shaken for 10 min, following removal of the supernatant. The optical density (OD) value was measured at a wavelength of 450 nm using the enzyme-linked immunosorbent assay (ELISA) meter. The half inhibitory concentration (IC_{50}) and resistance index (RI) of PTX-resistant and parental EC109 cells were calculated using the following formula: $RI = IC_{50(\text{PTX-resistant EC109 cells})} / IC_{50(\text{parental EC109 cells})}$.

Construction of the cell lines

The Amp^+ shuttle plasmid pLKO.1, which was used for knockdown of β-catenin, DDX11-AS1 or TOP2A, and the Amp^+ shuttle plasmid pBABE applied for overexpression of DDX11-AS1 or TOP2A (both containing the puromycin resistance gene), as well as the packaging plasmids pVSVG, pREV and pMDL, were all purchased from Cyagen Biosciences Inc. (Suzhou, Jiangsu, China). The plasmids were then co-transfected into the HEK-293T cells using Turbofect transfection reagent (R0531, Thermo Fisher Scientific, Rockford, IL, USA). After 12 h, the culture medium was changed, and the supernatant was collected after 24 h and 48 h, respectively. The culture medium was filtered using a 0.22-μm filter, and the viruses were obtained. The viral titer was determined using the QuickTiter™ Lentivirus Titer Kit (VPK-107, Cell Biolabs, Gilroy, CA, USA), using 1×10^6 TU/Ml viruses to infect EC cells. Puromycin (2 μg/mL) was used to screen the EC cell lines with stable knockdown of β-catenin, DDX11-AS1, or TOP2A, and

Table 1. siRNA sequences of β-catenin, DDX11-AS1, and TOP2A

	Primer sequence
β-catenin	F: 5'-GGAAAUAGAUAGAAAUAAAGG-3'
	R: 5'-UUUAUUUCUAUCUAUUUCCUA-3'
DDX11-AS1	F: 5'-CTGTGTAGCTCTAGAGAAA-3'
	R: 5'-GGCCTTAAGTTTAGAGCAA-3'
TOP2A	F: 5'-GAUUGAUUAUGACAAAGUAUA-3'
	R: 5'-UACUUUGUCAUAAUCAAUCAG-3'

Note: F, forward; R, reverse; DDX11-AS1, DDX11 antisense RNA 1; TOP2A, topoisomerase alpha 2A.

overexpression of DDX11-AS1 or TOP2A. Primer sequences of β-catenin, DDX11-AS1, or TOP2A are shown in **Table 1**.

RNA isolation and quantification

The total RNA of cells and tissues was extracted with the concentration measured in accordance with the instructions supplied with the TRIzol kit (15596-018, Solabio Life Sciences Co., Ltd, Beijing, China). The primers, as shown in **Table 2**, were synthesized by Takara (Dalian, Liaoning, China). Reverse transcription was conducted according to the cDNA reverse transcription kit (K1622, Yaanda, Beijing, China). The cDNA was diluted to 50 ng/μL for subsequent fluorescence quantitative polymerase chain reaction (PCR). The quantity of cDNA was determined using the fluorescence quantitative PCR instrument (DaAn Gene Co., Ltd. of Sun Yat-sen University, Guangzhou, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference, and the relative transcription level of the target gene was calculated by the relative quantitative method ($2^{-\Delta\Delta CT}$ method) [19]. Each experiment was conducted 3 times independently.

Fluorescence in situ hybridization (FISH)

The FISH assay was conducted according to the instructions provided with the Ribo™ lncRNA FISH Probe Mix (RiboBio Co., Ltd., Guangzhou, Guangdong, China). The tissue on the slide was fixed in 1 mL 4% paraformaldehyde at room temperature for 10 min and treated with protease K (2 μg/mL), glycine and ethylphthalate reagents, followed by incubation with 250 μL prehybridization solution at 42°C for 1 h. Next, the prehybridization solution was removed, and the tissue was incubated with 250 μL hybridized liquid containing the probe (300 ng/mL) at 42°C overnight. The slide was then incubated with 4',6-diamidino-2-phenylindole (DAPI) (1:800) diluted in phosphate-buffered saline/Tween (PBST) for 5 min to stain the nucleus. The slide was sealed with anti-fluorescence quencher, five different visual fields were observed under the fluorescence microscope (Olympus Optical Co., Ltd, Tokyo, Japan), and images were obtained.

Dual luciferase reporter gene assay

The promoter and full gene sequences of TOP2A were retrieved from the National Center of Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/gene). The promoter region of TOP2A was cloned into the pmirGLO Luciferase vector (Promega, WI, USA) to construct the pmirGLO-TOP2A prom wild-type (wt) vector, i.e., pTOP2A-prom-wt vector. sh-TAF1 (5'-GCCTAGGTGGTTCACCTTTCC-3') was purchased from Shanghai GenePharma Co. Ltd. (Shanghai, China). The cells were transfected with TOP2A prom wt + NC vectors, TOP2A prom wt + sh-TAF1 + NC vectors, TOP2A prom wt + pLV-EGFP-N-DDX11-AS1 vectors, TOP2A prom wt + TAF1, TOP2A prom wt + pLV-EGFP-N-DDX11-AS1 + TAF1 and TOP2A prom wt + sh-TAF1 + pLV-EGFP-N-DDX11-AS1, according to the instructions supplied with the Lipofectamine 2000. All groups were transfected with luciferase expression vector pRL-TK (TaKaRa, Dalian, Liaoning, China) as the internal reference. The transfected cells were cultured for 24 h, after which the luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The experiment was conducted in triplicate.

Chromatin immunoprecipitation (ChIP)

After fixation with 4% formaldehyde (the final concentration of formaldehyde was 1%), the cells were collected and broken by ultrasound, followed by the addition of rabbit anti-human TAF1 antibody (#12781, 1:100, CST, Beverly, MA, USA) for binding to the TAF1-TOP2A promoter complex. Next, the TAF1 antibody-TAF1-TOP2A complex was enriched by combining it with the Protein A Agarose/SaLmon Sperm DNA. The precipitated complex was then washed, with removal of nonspecific binding proteins or nucleic acids, and the enriched TAF1-

Table 2. Primer sequences of DDX11-AS1 and GAPDH for RT-qPCR

Primers	Forward	Reverse
DDX11-AS1	CAGCAACCTTTCTGGGAAGC	ACAAGAGCTGAGCTTGTCTTT
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

Note: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

TOP2A promoter complex was eluted and collected. Subsequently, the complex was decrosslinked, and precipitated TOP2A promoter fragments were purified for PCR analysis.

RNA-binding protein immunoprecipitation (RIP)

The lysate was extracted using radio-immunoprecipitation assay lysis buffer. The magnetic beads for immunoprecipitation were incubated with rabbit anti-human TAF1 antibody (CST, USA) to construct the magnetic bead-TAF1 antibody complex. The magnetic bead-TAF1 antibody complex was mixed with the lysate, and the magnetic bead-TAF1 antibody-TAF1-DDX11-AS1 complex was obtained by RIP. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was conducted for quantification of the purified RNA in the complex.

Immunoprecipitation (IP) assay

The cell lysate was extracted using precooled pyrolysis buffer (IP pyrolysis solution + phenylmethyl sulfonylfluoride [PMSF] + cocktail + phosphatase inhibitor). The protein concentration was determined and adjusted to 1 μg/μL. Protein A agarose beads (100 μL) were added to 1 mL total protein solution as well as the protein A/G agarose working buffer (excluding nonspecific binding proteins). The compounds were continuously mixed at 4°C for 1 h, after which centrifugation was carried out at 3000 rpm for 5 min at 4°C, and the supernatant was transferred to a new centrifuge tube and placed on ice. The supernatant (1 mL) was incubated with 5 μL primary antibody TOP2A (ab52934, 1:50, Abcam Inc., Cambridge, MA, USA) at 4°C for 2 h. Next, 20 μL Protein A/G agarose was added and incubated overnight or with rotation at 4°C for 1 h. The immunoprecipitation was obtained by centrifugation at 3000 rpm for 5 min at 4°C. The immunoprecipitate was then resuspended in sample buffer (30 μL) at two times the volume of the beads. The immunoprecipitation was denaturized at 95°C for 5 min and analyzed by Western blot analysis.

Western blot analysis

Western blot analysis was performed as previously described [20]. The total proteins in tissues and cells were extracted using radio-immunoprecipitation assay lysis buffer containing PMSF (R0010, Solarbio Science & Technology Co., Ltd, Beijing, China). The extracted protein was then separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was then transferred onto a polyvinylidene fluoride (PVDF) membrane by the wet transfer method and was then blocked with 5% evaporated skimmed milk at room temperature for 1 h. Afterwards, the membrane was incubated with the diluted primary rabbit antibodies TOP2A (ab52934, 1:10000), sex determining region Y (SRY)-box 2 (Sox2) (ab92494, 1:1000), organic cation/carnitine transporter 4 (Oct4) (ab109183, 1:1000), TAF1 (#12781, 1:100), β-catenin (ab32572, 1:1000), histone H3 (ab18521, 1:1000) and mouse antibody β-actin (ab8226, 1:100). The primary antibodies were all purchased from Abcam (Cambridge, MA, USA). The membrane was then incubated with the horseradish peroxidase (HRP)-labeled goat anti-mouse (ab205719, 1:2000) or goat anti-rabbit (ab205718, 1:2000) secondary immunoglobulin G (IgG) antibodies.

Nuclear and cytoplasmic protein extraction

The extraction and isolation of nuclear and cytoplasmic protein were performed using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology Co. Ltd., Shanghai, China). The detailed procedure has been described previously [21]. Western blot analysis was performed to detect the expression of β-catenin (ab32572, 1:1000, Rabbit, Abcam Inc., Cambridge, MA, USA) in the nucleus and cytoplasm.

Immunofluorescence

The R-EC109 and EC109 cells were plated on the slides and permeated with 2% Triton X-100 for 15 min and hydrochloric acid (HCl) for 20 min, and blocked with 2% bovine serum albumin (BSA) for 45 min. Next, the cells were incubated with rabbit anti-β-catenin antibody (ab16051, 1:300, Abcam, USA) and mouse anti-β-tubulin antibody (HC101-2, 1:5000, TransGen Biotech Co., Ltd., Beijing, China) at 4°C

overnight. Subsequently, the cells were incubated with goat anti-rabbit IgG H&L (ab150080, 1:400, Abcam, USA) and goat anti-mouse IgG H&L (ab150113, 1:400) fluorescent secondary antibodies at room temperature for 2 h. The cells were then stained with DAPI (2 μg/mL) and sealed. Nuclear expression of β-catenin was detected by fluorescence microscopy, and ImageJ software was applied for quantification of the fluorescence intensity.

Tumor formation in nude mice

Thirty BALB/c nude mice (four to seven weeks old, 18~24 g) were purchased from Lingchang Biology Co., Ltd. (Shanghai, China). The nude mice were housed and maintained in a specific pathogen-free (SPF) environment in the Animal Laboratory Center of Shantou University Medical College at a comfortable temperature, with sterilized feed and drinking water, an alternating day and night cycle of 12 h, and adaptive feeding for 7 d. The R-EC109 cells with or without knockdown of DDX11-AS1 were prepared in the cell suspension at 5×10^6 cells/mL, and 0.2 mL of the cell suspension was subcutaneously inoculated into the left side of BALB/c nude mice. Following inoculation, all nude mice were kept in the SPF-grade animal room and fed in the laminar flow hood, and tumor growth was observed every 3 d and recorded. After 6 d, PTX (100 μL, 5 mg/kg, PTX dissolved into PBS) or an equivalent amount of PBS was injected intraperitoneally every 3 d, after which the tumor volume and weight were measured [22, 23].

Statistical analysis

All data were processed using SPSS 21.0 statistical software (IBM Corp. Armonk, NY, USA). A normal distribution and homogeneity of variance were tested. Measurement data conforming to a normal distribution are expressed as the mean ± standard deviation. If the data did not conform to a normal distribution or homogeneity of variance, quantile spacing was applied. A paired *t*-test was used in EC tissues and adjacent normal tissues, and a nonpaired *t*-test was used for comparisons between the remaining two groups. A nonparametric Wilcoxon signed-rank test was used for data with a skewed distribution. The survival rate between the two groups was compared using the Kaplan-Meier test. One-way analysis of variance was applied for poc-test data, the Kruskal-Wallis H test was used for data with a skewed distribution, and repeated measurement one-way analysis of variance was conducted for comparisons of data at different time points. $P < 0.05$ was considered statistically significant.

Results

DDX11-AS1, TOP2A, and TAF1 were upregulated in EC tissues and DDX11-AS1 and TOP2A positively interacted

The EC tissues and EC adjacent normal tissues were collected to detect the expression of DDX11-AS1 in EC patients by conducting RT-qPCR, and the expression of TOP2A and TAF1 was determined in EC patients using immunohistochemistry. The results demonstrated high expression in DDX11-AS1 (**Figure 1A**, $P < 0.05$), TOP2A (**Figure 1C**, $P < 0.05$) and TAF1 (**Figure 1D**, $P < 0.05$) in EC tissues. The results from the correlation analysis of the interaction between DDX11-AS1 and TOP2A revealed a positive correlation between DDX11-AS1 and TOP2A expression (**Figure 1B**, $P < 0.05$), suggesting that the high expression of DDX11-AS1 might promote the expression of TOP2A and that the high expression of TOP2A is likely to be an important factor in improving the resistance of EC patients to PTX. Therefore, effective inhibition of DDX11-AS1 and TOP2A expression could potentially reduce the resistance of EC patients to PTX, enhancing the treatment efficacy of PTX resistance in EC.

Levels of DDX11-AS1, TOP2A, and TAF1 were highly correlated with the prognosis of EC patients

Follow-up data for 82 patients were recorded and collected. The patients were assigned as high expression patients and low expression patients, with the median of DDX11-AS1, TOP2A, and TAF1 expression as the cut-off point. As shown by the Kaplan-Meier test, the survival rate of patients with a high DDX11-AS1 expression was lower than that of patients with low DDX11-AS1 expression (**Figure 2A**); in comparison to patients with low TOP2A expression, patients with high TOP2A expression presented a reduced survival rate (**Figure 2B**); patients with high TAF-1 expression had a decreased survival rate versus patients with low TAF-1 expression (**Figure 2C**). These results indicate

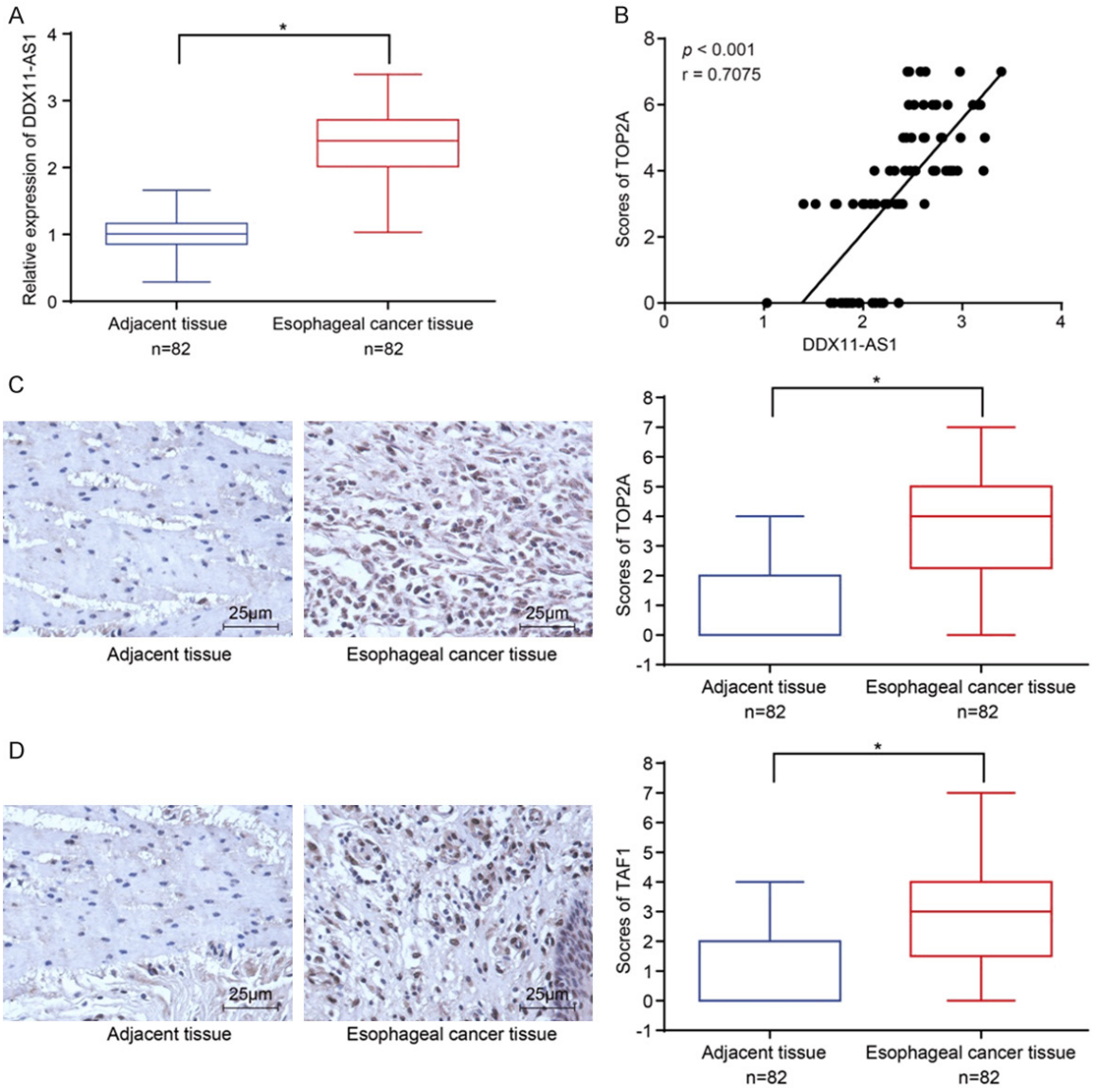

Figure 1. EC tissues present high expression levels of DDX11-AS1, TOP2A, and TAF1, DDX11-AS1 is positively associated with TOP2A. A. The expression of DDX11-AS1 in EC tissues and adjacent normal tissues detected by RT-qPCR. B. Correlation analysis between DDX11-AS1 and TOP2A. C. Expression of TOP2A in EC tissues and adjacent normal tissues determined by immunohistochemistry (400 ×). D. Expression of TAF1 in EC tissues and adjacent normal tissues measured using immunohistochemistry (400 ×). *$P < 0.05$. The data are measurement data and expressed as the mean ± standard deviation. Data between two groups were compared using the paired *t*-test. N = 82. EC, Esophageal cancer; TOP2A, topoisomerase alpha 2; TAF1, TATA-box binding protein-associated factor 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

that the expression of DDX11-AS1, TOP2A, and TAF1 is closely associated with the prognosis of clinical EC patients.

DDX11-AS1 knockdown decreased EC cell resistance to PTX through inhibition of TOP2A

Following the verification that DDX11-AS1 could promote the transcription of TOP2A, the effect of DDX11-AS1 on PTX resistance was further explored in EC cells. The changes in cell sensitivity to PTX were detected through the knockdown of DDX11-AS1 in R-EC109 cells and the overexpression of DDX11-AS1 in EC109 and KYSE150 cells. The results showed that the sensitivity of R-EC109 cells to PTX was significantly increased following the knockdown of DDX11-AS1, while the sensitivity of EC109 and KYSE150 cells to PTX was notably decreased after DDX11-AS1 overexpression (**Figure 3A**,

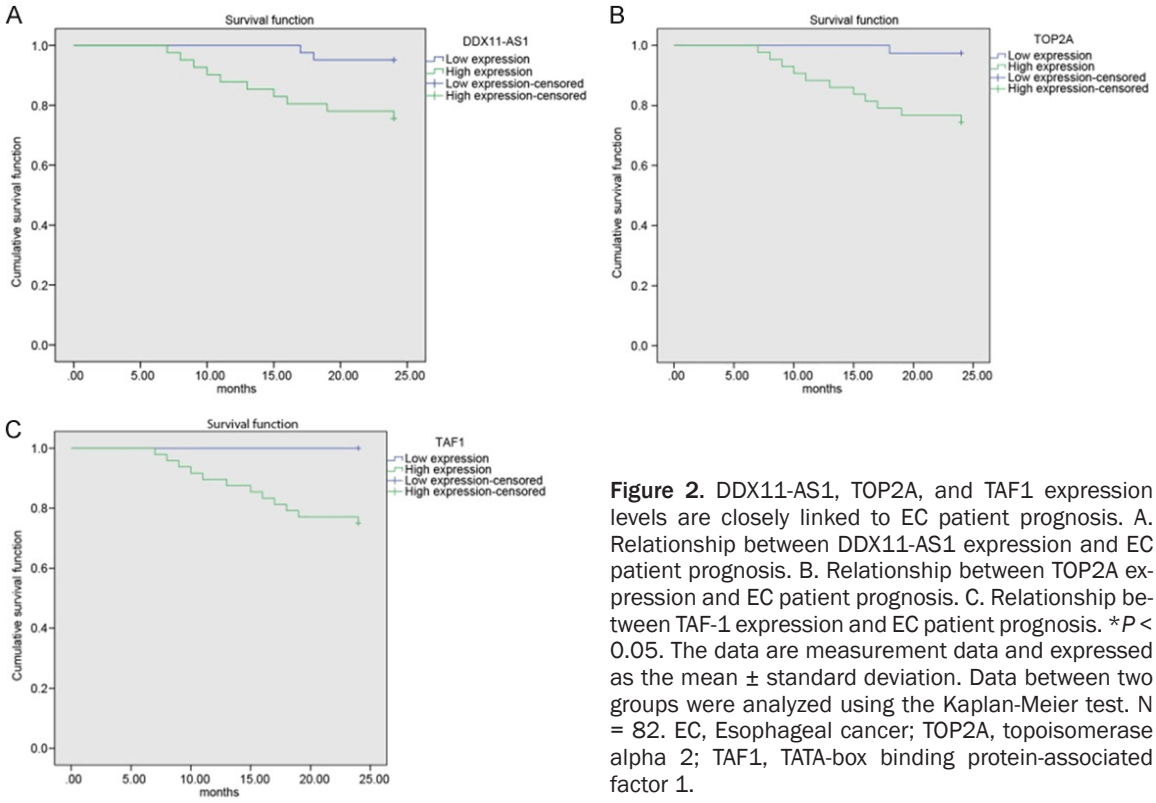

Figure 2. DDX11-AS1, TOP2A, and TAF1 expression levels are closely linked to EC patient prognosis. A. Relationship between DDX11-AS1 expression and EC patient prognosis. B. Relationship between TOP2A expression and EC patient prognosis. C. Relationship between TAF-1 expression and EC patient prognosis. *P < 0.05. The data are measurement data and expressed as the mean ± standard deviation. Data between two groups were analyzed using the Kaplan-Meier test. N = 82. EC, Esophageal cancer; TOP2A, topoisomerase alpha 2; TAF1, TATA-box binding protein-associated factor 1.

3B). DDX11-AS1 expression was downregulated in R-EC109 cells and overexpressed in EC109 and KYSE150 cells, and the expression levels of TOP2A, nuclear β-catenin, Sox2 and Oct4 were determined. Based on the results, knockdown of DDX11-AS1 in R-EC109 cells could significantly reduce the expression levels of TOP2A, nuclear β-catenin, Sox2 and Oct4 (**Figure 3C**). Overexpression of DDX11-AS1 in EC109 and KYSE150 cells led to evidently increased contents of nuclear β-catenin and expression of Sox2 and Oct4 (**Figure 3D**). Furthermore, to explore the effects of DDX11-AS1 and TOP2A on PTX resistance *in vivo*, EC109 cells were subcutaneously injected into nude mice following overexpression of DDX11-AS1 or knockdown of TOP2A in EC109 cells, and the mice were treated with PTX. The weights and sizes of the tumors in mice were regularly measured. It was found that overexpression of DDX11-AS1 significantly decreased the sensitivity of EC109 to PTX, while knockdown of TOP2A inhibited this phenomenon (**Figure 3E**, **3F**, $P < 0.05$). PTX did not significantly affect the body weight of nude mice at this dosage (**Figure 3G**, $P > 0.05$). Western blot analysis was conducted to determine the expression of TOP2A, nuclear β-catenin, Sox2 and Oct4 in each tumor mass. The results revealed that overexpression of DDX11-AS1 increased the expression of TOP2A, nuclear β-catenin, Sox2 and Oct4, while knockdown of TOP2A presented the opposite trend (**Figure 3H**). The above results suggested that DDX11-AS1 could upregulate the expression of TOP2A, nuclear β-catenin, Sox2 and Oct4, strengthen the resistance of EC cells to PTX and aggravate the development of EC.

DDX11-AS1 knockdown inhibited TOP2A transcription through TAF1

LncRNAs can change the expression of multiple genes and play an important role in the occurrence and development of cancers [24-26]. The underlying mechanism regarding the effects of lncRNAs on the sensitivity of EC cells to TPX was determined by retrieving lncRNAs that affected TOP2A transcription from the LncMAP database, and lncRNA DDX11-AS1 was predicted to promote TOP2A transcription through the transcription factor TAF1 (**Figure 4A**). The dual luciferase reporter gene assay was conducted to detect the effects of TAF1 and DDX11-AS1 on the activity of the TOP2A promoter. The results revealed that both TAF1

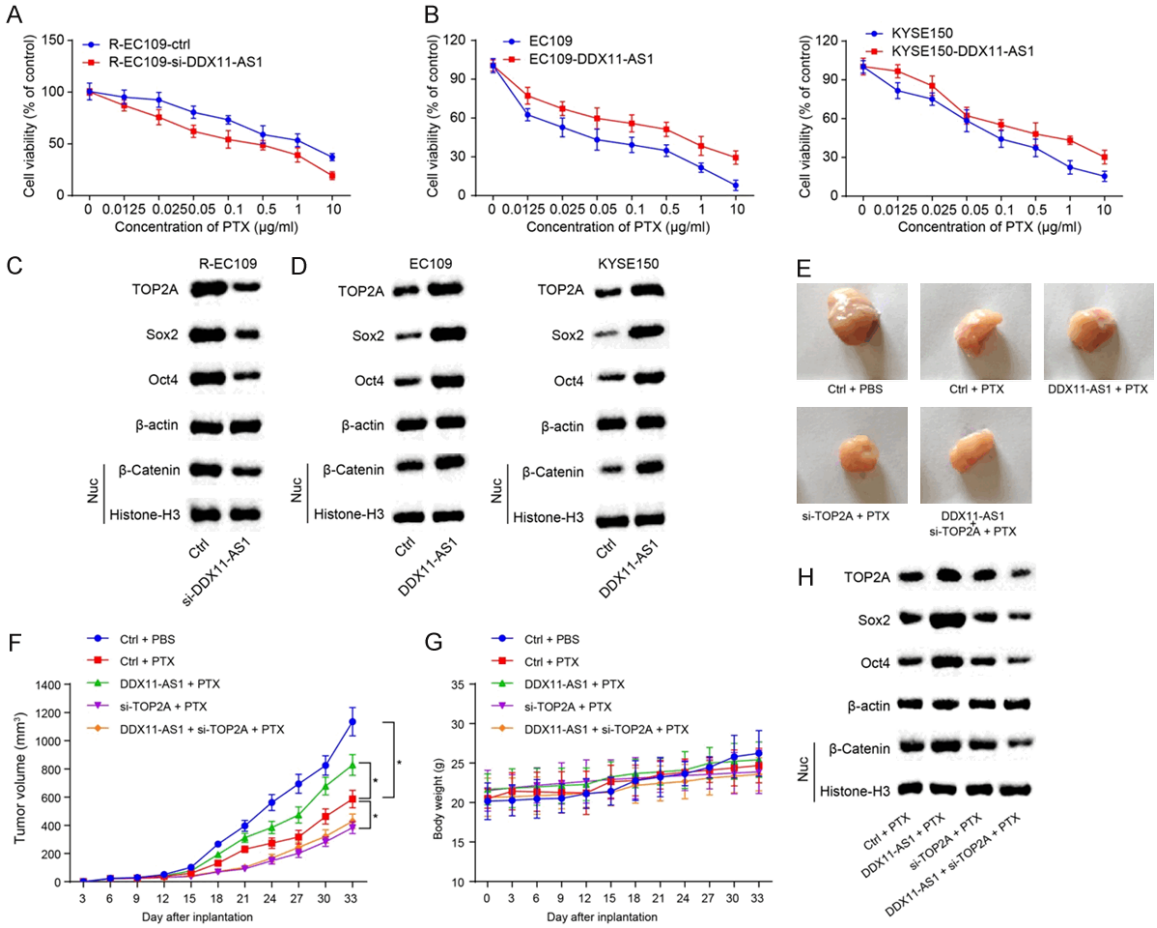

Figure 3. DDX11-AS1 knockdown inhibits the growth of EC and decreases the resistance of EC cells to PTX via inhibition of TOP2A. A. EC cell sensitivity to PTX in R-EC109 cells after knocking down DDX11-AS1, as detected by the CCK8 assay. B. EC cell sensitivity to PTX in EC109 and KYSE150 cells after overexpressing DDX11-AS1, as detected by the CCK8 assay. C. Expression of TOP2A, nuclear β-catenin, Sox2 and Oct4 in R-EC109 cells after knocking down DDX11-AS1, as determined by Western blot analysis. D. Expression of TOP2A, nuclear β-catenin, Sox2 and Oct4 in EC109 and KYSE150 cells after overexpressing DDX11-AS1, as determined by Western blot analysis. E, F. Effects of PTX on tumor growth in nude mice after overexpression of DDX11-AS1 and/or knockdown of TOP2A. G. The weight of tumors in nude mice after overexpression of DDX11-AS1 and/or knockdown of TOP2A. H. Expression of TOP2A, nuclear β-catenin, Sox2 and Oct4 after overexpression of DDX11-AS1 and knockdown of TOP2A in nude mice, as determined by Western blot analysis. *, $P < 0.05$. The above results are measurement data and expressed as the mean ± standard deviation. The non-paired *t*-test was used to analyze data between two groups. Cell viability and tumor volumes at different time points were analyzed by repeated measurements of variance. The experiment was repeated three times. N = 6. EC, Esophageal cancer; PTX, paclitaxel; TOP2A, topoisomerase alpha 2; TAF1, TATA-box binding protein-associated factor 1; Sox2, sex determining region Y (SRY)-box 2; Oct4, organic cation/carnitine transporter4; CCK8, cell counting kit 8.

and DDX11-AS1 could enhance the transcription of luciferase through the TOP2A promoter (**Figure 4B**, $P < 0.05$). Detection of DDX11-AS1 by the FISH assay revealed that DDX11-AS1 was mainly located in R-EC109 nuclei (**Figure 4C**). To investigate whether DDX11-AS1 could affect the expression of TOP2A, CHIP assay was used to detect the interaction between the TOP2A promoter and transcription factor TAF1 in R-EC109 cells, and the results revealed an interaction between the TOP2A promoter and TAF1 (**Figure 4D**, $P < 0.05$). Next, the RIP assay was performed to detect the interaction between TAF1 and DDX11-AS1 in R-EC109 cells. The results demonstrated an interaction between TAF1 and DDX11-AS1 (**Figure 4E**, $P < 0.05$). Finally, the effects of overexpression/knockdown of DDX11-AS1 on the expression of TOP2A were analyzed. It was found that overexpression of DDX11-AS1 resulted in a significant increase in the expression of TOP2A, while knockdown of DDX11-AS1 led to an evident

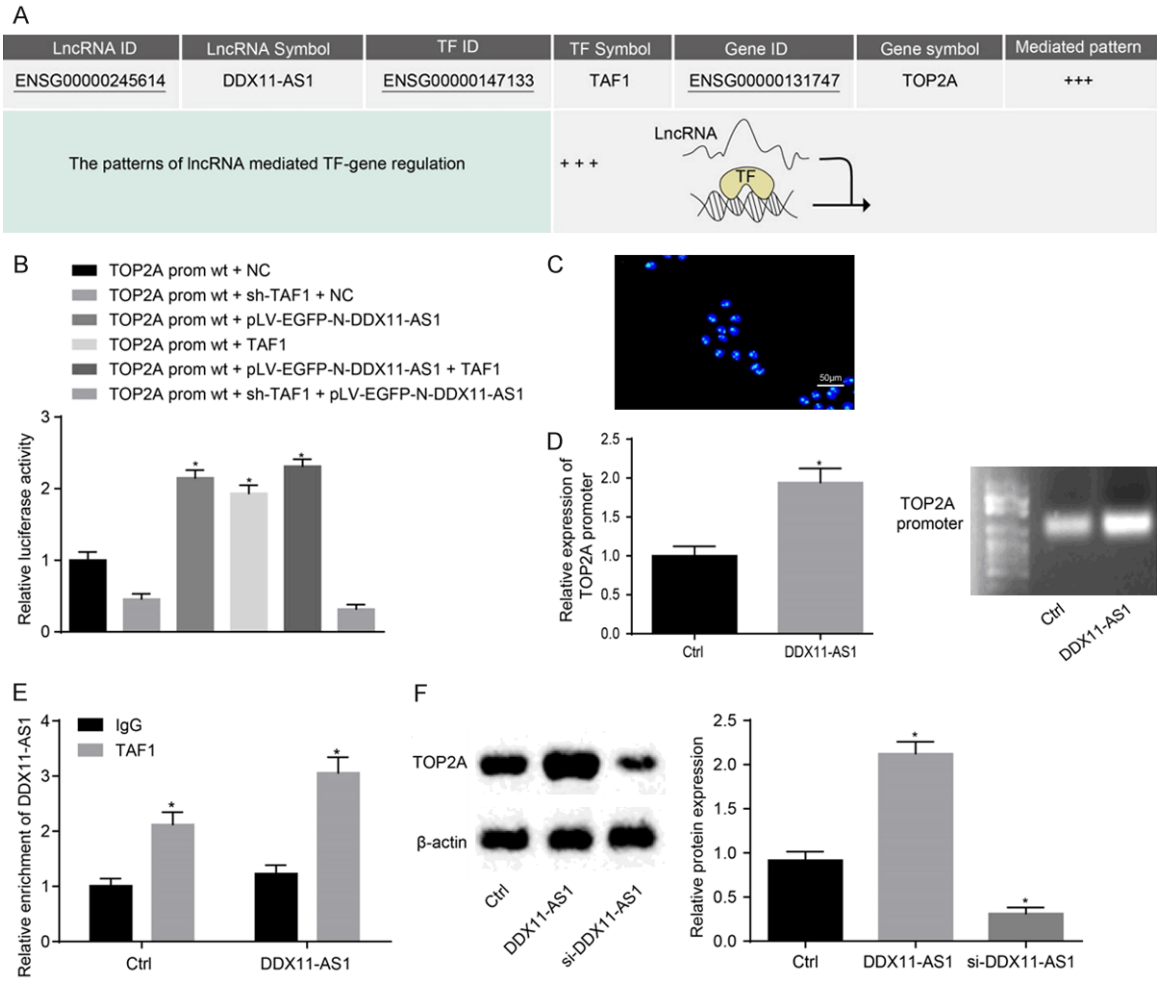

Figure 4. DDX11-AS1 binds to TAF1 to promote the transcription of TOP2A. A. lncRNAs that affected TOP2A transcription predicted in the LncMAP database. B. Fluorescence intensity changes induced by the TOP2A promoter after alteration, as detected by the dual luciferase reporter gene assay. C. Localization of DDX11-AS1 in R-EC109 cell detected by FISH (200 ×). D. The interaction between TAF1 and TOP2A promoter in R-EC109, as verified by the ChIP assay. E. The interaction between TAF1 and DDX11-AS1, as confirmed by the RIP assay. F. The expression of TOP2A after knockdown/overexpression of DDX11-AS1, as detected by Western blot analysis. *, $P < 0.05$. The above results are all measurement data and expressed as the mean ± standard deviation. The non-paired *t*-test was used to analyze data between two groups. Differences between groups were compared with one-way analysis of variance. The experiment was repeated three times. EC, Esophageal cancer; PTX, paclitaxel; TOP2A, topoisomerase alpha 2; TAF1, TATA-box binding protein-associated factor 1; CHIP, chromatin immunoprecipitation; RIP, RNA-binding protein immunoprecipitation; FISH, fluorescence in situ hybridization.

decrease in its expression (**Figure 4F**, $P < 0.05$). These results suggested that DDX11-AS1 could enhance the transcription of TOP2A by upregulating TAF1, thus increasing the expression of TOP2A.

TOP2A was highly expressed in PTX-resistant EC cells and promoted the resistance of EC cells to PTX

The PTX-resistant EC line R-EC109 was induced by treating EC109 cells with PTX using the high-dose intermittent induction and time-increasing method (**Figure 5A**). The IC_{50} of EC109 parental cells was measured by the CCK8 method and found to be 0.068 μg/mL, while the IC_{50} of R-EC109 was 2.963 μg/mL. The RI value was 43.57. This result indicated that R-EC109, a highly PTX-resistant cell line, was successfully constructed.

It has been reported that the high expression of TOP2A in patients undergoing chemotherapy may be one of the factors promoting drug resistance to chemotherapy [27]. To verify this hypothesis, we compared the difference in

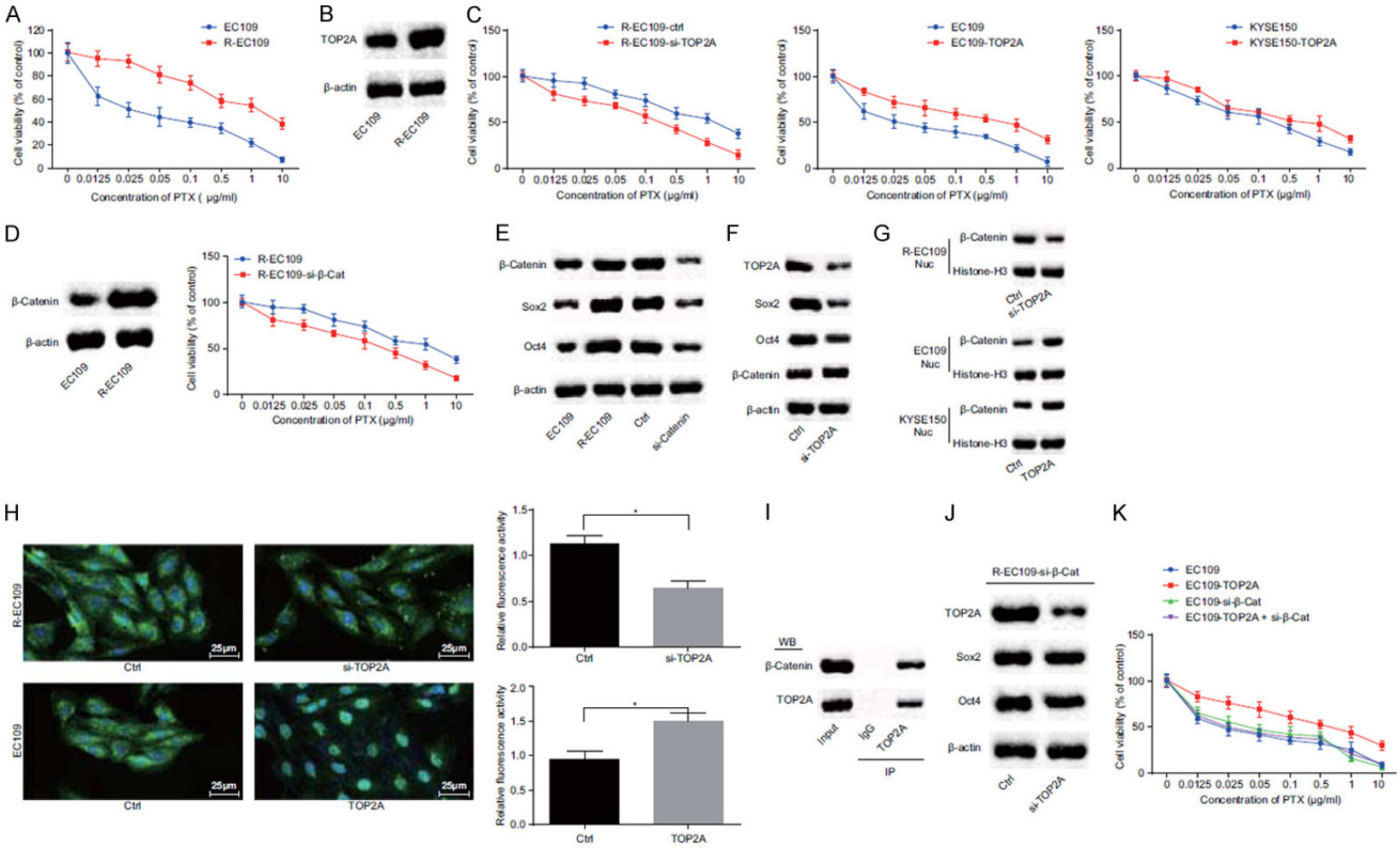

Figure 5. TOP2A knockdown suppresses EC cell resistance to PTX by inhibiting β-catenin translocation into nuclei. A. Sensitivity of parental and drug-resistant cells to PTX, as detected by the CCK8 method. B. Expression of TOP2A in parental and drug-resistant EC cells, as determined by Western blot analysis. C. Sensitivity to PTX, as detected by the CCK8 method after knockdown of TOP2A in the R-EC109 cell line and overexpression of TOP2A in EC109 and KYSE150 parental cell lines. D. Expression of β-catenin in EC PTX-resistant and parental EC cells, as determined by Western blot analysis, and the sensitivity of R-EC109 to PTX after knockdown of β-catenin in R-EC109 cells, as detected by the CCK8 assay. E. Expression of Sox2 and Oct4 in EC PTX-resistant and parental EC cells, and in R-EC109 cells, after knockdown of β-catenin, as detected by Western blot analysis. F. Expression of Sox2, Oct4 and β-catenin in R-EC109 cells after knockdown of TOP2A, as detected by Western blot analysis. G. Content of β-catenin in the nucleus of R-EC109, EC109 and KYSE150 cells after knockdown/overexpression of TOP2A, as detected by Western blot analysis. H. The effect of knockdown or overexpression of TOP2A on the localization of β-catenin in EC cells, as detected by the immunofluorescence assay (400 ×). I. Interaction between TOP2A and β-catenin in R-EC109 cells, as detected by the IP experiment. J. Expression of Sox2 and Oct4 in R-EC109 cells with

knockdown of both β-catenin and TOP2A. K. Sensitivity of EC109 cells to PTX after overexpression of TOP2A and knockdown of β-catenin in EC109 cells, as detected by the CCK8 assay. *, $P < 0.05$. The above data are all measurement data and expressed as the mean ± standard deviation. The non-paired *t*-test was used to analyze data between two groups. The cell viability at different time points was analyzed by repeated measurements of variance. The experiment was repeated three times. EC, Esophageal cancer; PTX, paclitaxel; TOP2A, topoisomerase alpha 2; TAF1, TATA-box binding protein-associated factor 1; Sox2, sex determining region Y (SRY)-box 2; Oct4, organic cation/carnitine transporter4; CCK8, cell counting kit 8; IP, immunoprecipitation.

TOP2A expression between resistant and parental strains and found that TOP2A was highly expressed in the R-EC109 cell line (**Figure 5B**). TOP2A was knocked down in R-EC109 and overexpressed in EC109 to investigate the effect of TOP2A on PTX resistance. The sensitivity of EC cells to PTX was detected using the CCK8 assay. KYSE150 is an EC cell line with low TOP2A expression and sensitivity to PTX, which was selected through the Oncomine database. The sensitivity of KYSE150 to PTX was detected following the overexpression of TOP2A. The results revealed that TOP2A knockdown in R-EC109 could significantly increase the sensitivity to PTX. However, the overexpression of TOP2A in EC109 and KYSE150 significantly reduced sensitivity to PTX (**Figure 5C**). The aforementioned findings suggested that TOP2A was expressed at a high level in the PTX-resistant EC cell line, and EC cells had increased resistance to PTX.

Knockdown of TOP2A decreased EC cell resistance to PTX by inhibiting β-catenin translocation into nuclei

β-catenin is an important oncogene that promotes cell proliferation and inhibits cell apoptosis, and it has been previously suggested that TOP2A may promote cell resistance by increasing the activity of β-catenin [28]. By comparing the expression of β-catenin in R-EC109 cells with that in EC109 cells, it was revealed that there was an upregulation in β-catenin in R-EC109 cells. After knockdown of β-catenin in R-EC109 cells, the sensitivity of R-EC109 cells to PTX was significantly improved (**Figure 5D**). In addition, the EC cell stemness-related proteins Sox2 and Oct4 that were regulated by β-catenin were also upregulated. Following the knockdown of β-catenin in R-EC109, the expression of Sox2 and Oct4 were significantly downregulated (**Figure 5E**). Therefore, we hypothesized that TOP2A increased the expression of Sox2 and Oct4 and promoted cell resistance by enhancing the activity of β-catenin. To determine whether TOP2A induced drug resistance by increasing the activity of β-catenin, TOP2A was knocked down in R-EC109 cells and overexpressed in EC109 and KYSE150 cells. The results showed that after knockdown of TOP2A in R-EC109 cells, the expression levels of Sox2 and Oct4 were significantly downregulated (**Figure 5F**).

Considering that β-catenin mainly takes effect after nucleation, TOP2A does not change the expression of total β-catenin but may change its localization. The content of β-catenin was determined in the nucleus. After knockdown of TOP2A in R-EC109 cells, there was an evident decrease in the content of β-catenin in the nucleus, while the contents of β-catenin in EC-109 and KYSE150 cells were markedly increased following overexpression of TOP2A (**Figure 5G**). Moreover, immunofluorescence to determine the localization of β-catenin revealed that its distribution in the nucleus decreased after knockdown of TOP2A in R-EC109 cells but increased in EC109 and KYSE150 cells after overexpression of TOP2A (**Figure 5H**). The interaction between TOP2A and β-catenin was verified in R-EC109 cells using the IP assay. The results revealed an interaction between TOP2A and β-catenin (**Figure 5I**, $P < 0.05$). After knockdown of both β-catenin and TOP2A in R-EC109, the downregulation of Sox2 and Oct4 that occurred as a result of TOP2A knockdown was fully promoted (**Figure 5J**), which suggested that the enhancement of resistance induced by TOP2A was related to β-catenin. To further explore whether the resistance induced by overexpression of TOP2A was related to β-catenin, TOP2A was overexpressed and β-catenin was downregulated in EC109 parental cells. It was found that knockdown of β-catenin could significantly inhibit the reduction of sensitivity to PTX induced by overexpression of TOP2A (**Figure 5K**). Therefore, we propose that TOP2A enhanced the resistance of EC cells to PTX by promoting the translocation of β-catenin into nuclei.

Discussion

Esophageal cancer is known to be associated with high mortality worldwide, partially due to

the late diagnosis of the disease, which is common due to the characteristics of EC, such as frequent local/distant metastasis and poor subjective symptoms [29]. The use of ionization radiation has been found to increase EC cell resistance to PTX, which makes EC treatment more complex [30]. LncRNAs have been found to be correlated with PTX resistance in different cancers, such as nasopharyngeal carcinoma and ovarian cancer [31, 32]. In the present study, we determined the interaction of DDX11-AS1, TAF1 and TOP2A in EC and their roles in PTX-resistant EC cells to investigate the mechanism underlying the development of drug resistance at the molecular level in human EC cells. Our results showed that DDX11-AS1 could promote TOP2A transcription through the transcription factor TAF1 and that TOP2A could promote β-catenin translocation into nuclei by interacting with β-catenin, resulting in the enhancement of EC cell resistance to PTX.

Our findings revealed that DDX11-AS1, TOP2A and TAF1 were upregulated in EC, along with increased expression of TOP2A in the EC109 cell line. Based on the global lncRNA-protein-coding gene network information, novel potential functional lncRNAs were identified to be highly expressed in esophageal squamous cell carcinoma (ESCC) [33]. EC cells have also been noted to exhibit high expression of lncRNAs, such as lncRNA-1, HNF1A antisense RNA 1, HOTAIR, and PCAT-1 [34]. A higher expression level of DDX11-AS1 is associated with poor survival and higher recurrence in HCC patients [35]. The expression of TOP2A has also been closely associated with the progression of disease in EC patients, and TOP2A is upregulated up to 55.2% in the tissues of EC patients [36]. In addition, the expression of TOP2A is correlated with the stages and clinical characteristics of EC patients [37]. TOP2A has been found to be upregulated in hepatocellular carcinoma based on several studies [38, 39]. A previous study found that TAF1 was upregulated in hepatitis C virus-positive hepatocellular carcinoma in patients with liver cirrhosis and involved in G1/S transition of the cell cycle [40]. Therefore, the aforementioned data highly support the conclusion that DDX11-AS1, TOP2A and TAF1 are negative prognostic factors in EC.

In addition, we found that knockdown of TOP2A resulted in a decrease in EC cell resistance to PTX by inhibiting β-catenin translocation into nuclei. Overexpression of TOP2A and microtubule-associated protein tau underexpression are associated with overexpressed HER2, which is correlated with a higher rate of pathologic complete response to preoperative PTX/FAC chemotherapy in breast cancer [41]. The Wnt/β-Catenin signaling pathway is known to participate in the development of metastasis and the progression of disease in EC cells [42]. In addition, β-catenin can be targeted by lncRNA MALAT1 to promote the development of malignancy in ESCC via Ezh2 [43]. DACT2 can arrest cells at G2/M to enhance the sensitivity of cells to PTX in nasopharyngeal carcinoma through direct inhibition of the β-catenin/Cdc25c signaling pathway [44]. The relationship between TOP2A and β-catenin has been verified in a previous study [28].

The most important finding of our study was that DDX11-AS1 knockdown resulted in the inhibition of TOP2A transcription through TAF1 and decreased the resistance of EC cells to PTX. TAF1 is a candidate gene in endometrial carcinomas, in which β-catenin is also found to participate in its progression [45], indicating a relationship between TAF1 and TOP2A. Furthermore, DDX11-AS1 is found to be activated by MYC, which binds to TOP2A, while it is negatively regulated by p53, which is phosphorylated by TAF1 [10, 12, 13], suggesting a potential interaction between DDX11-AS1 and TAF1/TOP2A. DDX11-AS1 is associated with gene expression involved in cell proliferation, differentiation and the cell cycle in hepatocellular carcinoma [35]. Additionally, the downregulation of DDX11-AS1 results in decreased cellular proliferation and increased apoptosis and arrests tumor cells at G0/G1 stage [10]. Therefore, inhibition of DDX11-AS1 might represent an effective therapeutic target in EC.

TOP2A was found to have a close correlation with drug resistance in esophageal cancer cells. In the current study, we provide evidence that DDX11-AS1, an upstream regulator of TOP2A, is closely related to PTX resistance in esophageal cancer. Therefore, TOP2A is likely to be associated with esophageal cancer PTX. A close relationship is observed between drug resistance and multidrug resistance. The next step in the process in our future studies would be to establish strict sputum standards, collect clinical samples from multiple centers, analyze the relationship between TOP2A and esopha-

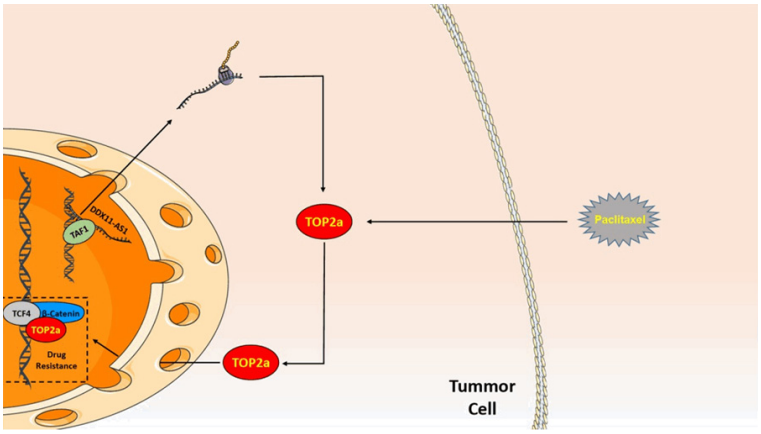

Figure 6. Mechanism of DDX11-AS1 in PTX-resistant EC cells. DDX11-AS1 promotes TOP2A transcription through the transcription factor TAF1, and TOP2A interacts with and promotes β-catenin translocation into the nucleus, leading to enhancement of EC cell resistance to PTX. EC, Esophageal cancer; PTX, paclitaxel; TOP2A, topoisomerase alpha 2; TAF1, TATA-box binding protein-associated factor 1.

geal cancer drug resistance, and confirm whether it can be used as a biomarker for esophageal cancer drug resistance. This target was validated by a prospective cohort study. Finally, we could design a relevant test kit for rapid clinical identification.

In conclusion, the present findings provide further insights regarding the major role of lncRNA DDX11-AS1 in the progression of EC by regulating the expression of TAF1/TOP2A, and the results demonstrate that DDX11-AS1 knockdown inhibits EC resistance to PTX (**Figure 6**); these results indicate that the inhibition of DDX11-AS1 may be a potential target for EC treatment. However, further experiments regarding the mechanism of TOP2A in β-catenin translocation into the nucleus are required to fully understand the specific mechanism underlying EC cell resistance to PTX.

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Disclosure of conflict of interest

None.

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