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TNFAIP8 regulates cisplatin resistance through TAF-Iα and promotes malignant progression of esophageal cancer

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Abstract. – OBJECTIVE: Previous studies have demonstrated that TNFAIP8 is a cancer-promoting gene. However, the role of TNFAIP8 in esophageal cancer (ECa) has not been reported. The aim of this investigation was to investigate the expression of TNFAIP8 in ECa, and to further explore whether it could regulate cisplatin resistance to this cancer *via* modulating TAF-Iα expression and promote malignant progression of ECa.

PATIENTS AND METHODS: Quantitative Real Time-PCR (qRT-PCR) was performed to examine the expression of TNFAIP8 in 47 tumor tissue specimens and adjacent ones of ECa patients, and the interplay between TNFAIP8 expression and prognosis of patients with ECa was then analyzed. Further, qRT-PCR was applied to verify TNFAIP8 level in ECa cell lines. In addition, the TNFAIP8 knockdown model was constructed in ECa cisplatin-resistant cell lines including EC-109/DDP and OE19/DDP, and then, CCK8 and transwell assays were performed to analyze the impact of TNFAIP8 on the biological function of ECa cells; meanwhile, the Luciferase reporter gene assay and cell reverse experiments were finally conducted to explore its underlying mechanisms.

RESULTS: The qRT-PCR results revealed that the TNFAIP8 level in tumor tissue samples of ECa patients was remarkably higher than that in adjacent ones, and the difference was statistically significant. Similarly, the overall survival rate of patients with high expression of TNFAIP8 was lower when compared with patients with low expression of TNFAIP8. EC-109/DDP and OE19/DDP, the ECa cisplatin-resistant cell lines, were successfully constructed; subsequently, it was found that the proliferation, invasiveness, and metastasis ability of ECa cells in TNFAIP8 knockdown group was remarkably decreased compared with those in the sh-NC group. At the same time, the Western blot results illustrated that the expression of TAF-Iαwas remarkably elevated in the TNFAIP8 knockdown group. In addition, the Luciferase reporting assay and cell reverse experiments also demonstrated that there existed a mutual regulation effect between TNFAIP8 and TAF-Iα, which might together affect the malignant progression of ECa.

CONCLUSIONS: The expression of TNFAIP8 was found remarkably enhanced in ECa tissues and cell lines, which might be closely relevant to the poor prognosis of patients with ECa. Additionally, it was found that TNFAIP8 may regulate cisplatin resistance and promote malignant progression of ECa by modulating TAF-Iα expression.

Key Words:
TNFAIP8, TAF-Iα, ECa, Cisplatin resistance.

Introduction

Esophageal cancer (ECa) is one of the common malignant tumors in humans, and China is the country with the highest incidence and mortality of ECa[1,2]. The early symptoms of this disease are concealed, and there is no specific early diagnosis method. Lymph node metastasis can occur in the early stage, and about 50% of the patients are diagnosed in advanced tumor stage at the time of treatment, for the submucosa of the esophagus is rich in lymphatic vessels and there is no serosal membrane outside the muscular layer, which leads to a 5-year survival rate of only about 20%[3,4]. However, the specific pathogenesis of ECa still remains elusive. As we know, invasiveness and metastasis are the main causes of poor prognosis and death of patients with tumors. Therefore, it is of great significance to explore the mechanism of invasiveness and metastasis ability of ECa[5,6]. Among the treatments of ECa, radiotherapy and chemotherapy can effectively improve the local control rate of ECa and improve long-term survival rate. It is an important auxiliary method for clinical treatment of ECa. Currently, multidisciplinary comprehensive treatment based on surgery, radiotherapy, and chemotherapy is becoming more and more used[7,8]. However, radiotherapy and chemotherapy have serious toxic and side effects, which will also cause damage to the

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normal tissues of the body while killing the tumor cells, and the long-term survival rate of patients with ECa is not satisfactory[9,10]. Local recurrence and distant metastasis are the main factors leading to poor prognosis in patients with ECa. Finding more effective adjuvant treatment and therapeutic targets to inhibit local recurrence and distant metastasis is a key and difficult problem to be solved[11,12].

Recently, a new candidate oncogene, the tumor necrosis factor-1-induced protein 8, (TNFAIP8) has gradually entered the eyes of scholars, causing widespread concern[13,14]. The members of this family include TNFAIP8, TNFAIP8L1 (TIPEl), TIPE2, and TIPE3[14]. Although there is high homology between them, they have been shown[14] to have different biological behaviors in previous related experiments. Among them, TNFAIP8 is the first subtype found in this family, which is located on the 5q23.1 chromosome and is expressed in most malignant tumor tissues[15,16]. This gene is involved in the regulation of multiple signaling pathways, thus affecting many processes, such as apoptosis, and therefore plays an important role in the formation and development of tumors[13,16].

Cisplatin is one of the basic drugs for chemotherapy of malignant tumors[17]. However, a large number of clinical studies have found that the clinical efficacy of platinum-based combination chemotherapy is remarkably reduced, mainly due to platinum-induced chemotherapy resistance[18,19]. Conventional chemotherapy drugs can cause different types of DNA damage in tumor cells, thereby activating DNA damage detection points and promoting DNA damage repair in tumor cells, allowing cells to escape apoptosis[17,20]. We aimed to silence the expression of TNFAIP8 in ECa cells by TNFAIP8 siRNA and to explore the effects of cisplatin on the chemosensitivity of ECa after TNFAIP8 gene silencing both *in vitro* and *in vivo*, hoping to lay a theoretical foundation for clinically enhancing the chemosensitivity of malignant tumors such as ECa.

Patients and Methods

Patients and ECa samples

Forty-seven pairs of tumor tissue samples and paracancerous ones of 43 ECa patients undergoing radical resection and routine HE staining were collected. All the samples were frozen and stored in a refrigerator at -80°C for subsequent RNA extraction. The study was approved by the Ethics Oversight Committee of Beijing Chao-Yang Hospital. All patients did not receive chemotherapy before surgery, the patients and their families were fully informed that the specimens would be used for scientific research, and they signed informed consent. This study was conducted in accordance with the Declaration of Helsinki. The pathological classification and staging criteria of ECa were performed according to the International Association of Cancer (UICC) ECa staging criteria.

Cell Lines and Reagents

Four human ECa cells (OE19, OE33, TE-1, and EC-109) and one human normal esophageal epithelial cell (HEEC) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) and fetal bovine serum (FBS; Gibco, Rockville, MD, USA) were purchased from Life Technologies (Waltham, MA, USA, USA). The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin in a 37°C incubator containing 5% CO_2.

Transfection

The control group (sh-NC) and TNFAIP8 (sh-TNFAIP8) containing the TNFAIP8 lentiviral sequence was purchased from Shanghai Jima Company. The cells were plated in 6-well plates and grown to a cell density of 70%; then, lentiviral transfection was performed according to the manufacturer's instructions. After 48 h, the cells were collected for qRT-PCR, Western Blot, and cell function experiments.

Cell Counting Kit (CCK-8) Assay

After 48 h of transfection, the cells were harvested and plated into 96-well plates at 2000 cells per well. The cells were cultured for 24 h, 48 h, 72 h, and 96 h respectively, and then added with CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent. After incubation for 2 hours, the OD value of each well was measured in the microplate reader at 490 nm absorption wavelength. In addition, for the drug sensitivity test, cisplatin (Solarbio, Beijing, China) was dissolved in physiological saline at 1 mg/ml and diluted by gradient (0, 1, 2, 4, 8, 16, 32, 64, 128 µm) for 48 hours. The ECa cell viability was then detected. After incubation, the OD value of each well at 450 nm absorption wavelength was measured in a microplate reader and the data were analyzed.

Transwell Cell Migration and Invasion Assay

After transfection for 48 h, the cells were trypsinized and resuspended in serum-free medium. After cell counting, the diluted cell density was adjusted to 2.0×10^5/ml, and the transwell chamber containing Matrigel and no Matrigel was placed in a 24-well plate. 200 µl of the cell suspension was added in the upper chamber of the chamber, and 500 µl of a medium containing 10% FBS was added to the lower chamber. After incubated in a 37°C incubator for 48 hours, the chamber was removed, fixed with 4% paraformaldehyde for 30 minutes, and stained with crystal violet for 15 minutes. Next, the cells were washed with phosphate-buffered saline (PBS), and the inner surface of the basement membrane of the chamber was carefully cleaned to remove the inner layer cells. The perforated cells stained in the outer layer of the basement membrane of the chamber were observed under the microscope, and 5 fields of view were randomly selected.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

After transfected cells were cultured for 48 h, RNA was extracted and reverse transcribed, and the relative expression levels of TNFAIP8 and TAF-Iα were determined according to the previous method. For TAF-Iα, the amount of expression relative to β-actin was calculated. Data analysis was performed using ABI Step One software and the relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were repeated 3 times. Primers used were shown as follows: TNFAIP8: Forward: 5'-ATAGACGACACAAGTAGTGAGGT-3'; Reverse: 5'-CCACGGTCATAGCAAGCTGAT-3'; TAF-Iα: Forward: 5'-AGCAAGAAGCGATTGAACACA-3'; Reverse: 5'-TGGTTGGCGGAGTTTGTTATATT-3'; GAPDH: Forward: 5'-GGAGCGAGATCCCTCCAAAAT-3'; Reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'.

Western Blot

The transfected cells were collected after 72 h of culture, and the proteins were extracted for quantitative detection. The proteins were extracted according to the protein extraction steps and the concentration was determined using bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The total protein was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and electroporated onto a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Skim milk was then used to block the non-specific sites in the membrane, which was then incubated with TNFAIP8 and TAF-Iα monoclonal antibody (1:500) at 4°C overnight, while the control was incubated with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:2000). In the next day, the membrane was washed with TBST for 3 times and then incubated with secondary antibody (1:1000) at room temperature for 1 h. After that, the protein expression was detected by enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA). All experiments were repeated 3 times.

Luciferase Gene Reporter System

According to the instructions, the ECa cell lines in the logarithmic growth phase were prepared according to the Luciferase system. The relative fluorescence values were then measured by a luminometer and compared. The detection principle is that if the transcription factor activates the target promoter, the Luciferase gene is expressed, and the amount of Luciferase expression is directly proportional to the intensity of the transcription factor. After the specific Luciferase substrate is added, Luciferase can react with the substrate to generate fluorescence. By measuring the intensity of the fluorescence, the activity of the Luciferase can be used to determine whether the transcription factor can interact with the target promoter fragment.

Statistical Analysis

Data analysis was performed using SPSS 22.0 (SPSS IBM, Armonk, NY, USA) statistical software. The measurement data were expressed as mean ± standard deviation (x±s). The mean difference of TNFAIP8 expression between groups was analyzed by paired sample test. The relationship between TNFAIP8 level and clinicopathological features of ECa was tested using χ^2-test. Survival analysis was performed with Kaplan-Meier method, and survival curves were drawn; $p < 0.05$ was considered statistically significant.

Results

TNFAIP8 Was Highly Expressed in ECa Tissues and Cell Lines

The expression of TNFAIP8 in tumor tissues and their adjacent tissues and ECa cell lines was

detected by qRT-PCR. The results indicated that TNFAIP8 level in tumor tissue samples of ECa patients was remarkably increased compared with adjacent ones, with the difference statistically significant (Figure 1A); similarly, in cell lines, TNFAIP8 expression was also remarkably higher in ECa cells compared to human normal esophageal epithelial cells (HEEC) (Figure 1B). In addition, in order to explore the interplay between the expression of TNFAIP8 and the prognosis of patients with ECa, relevant follow-up data were collected. Kaplan-Meier survival curves revealed that high expression of TNFAIP8 was closely relevant to the poor prognosis of ECa patients, and the lower TNFAIP8 level, the better the prognosis ($p<0.05$; Figure 1C), which suggested that TNFAIP8 might be a new biological indicator for predicting the prognosis of ECa.

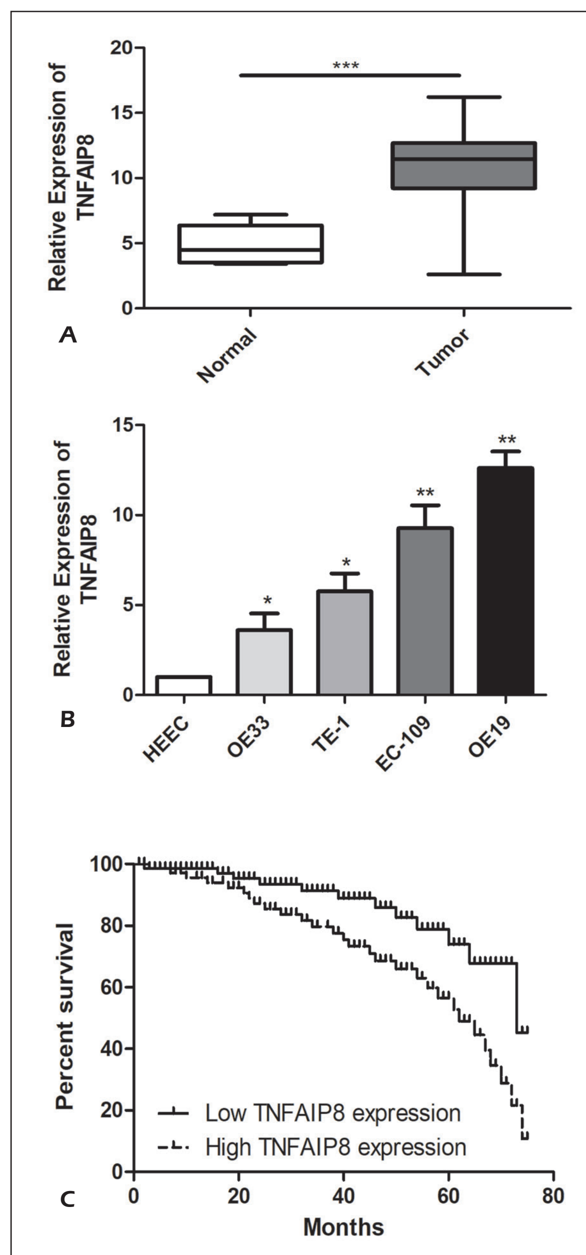

Figure 1. High expression of TNFAIP8 in esophageal cancer tissues and cell lines. **A**, qRT-PCR was used to detect the expression difference of TNFAIP8 in esophageal cancer tumor tissues and adjacent non-tumor tissues. **B**, qRT-PCR was used to detect the expression level of TNFAIP8 in esophageal cancer cell lines. **C**, Kaplan Meier survival curve of esophageal cancer patients based on TNFAIP8 expression was shown; the prognosis of patients with high expression was significantly worse than that of the low expression group. Data are mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

TNFAIP8 Contributed to Cisplatin Resistance in ECa

QRT-PCR results revealed that the expression of TNFAIP8 in patients with cisplatin resistance was significantly elevated when compared with the control ones (Figure 2A). In addition, Western Blotting also proved the above trend at the protein level (Figure 2B). Subsequently, ECa cisplatin-resistant cell lines including EC-109/DDP and OE19/DDP were established, and CCK-8 assay revealed that the knockdown of TNFAIP8 could remarkably weaken the ECa cell viability (Figures 2C, 2D), demonstrating that TNFAIP8 could contribute to cisplatin resistance in ECa.

Knockdown of TNFAIP8 Inhibited Cell Proliferation and Metastasis

To explore the influence of TNFAIP8 on the proliferation of ECa cells, the TNFAIP8 silencing model was first constructed in the ECa cisplatin-resistant cell lines including EC-109/DDP and OE19/DDP (Figure 3A), and CCK-8, and transwell invasion and migration assays were performed to examine the cell proliferation and metastasis in the sh-NC group and TNFAIP8 knockdown group. As shown in Figure 3B, the rate of proliferation of ECa cells after knockdown of TNFAIP8 was remarkably reduced. At the same time, the transwell experiment also revealed a significant reduction in the number of transmembrane ECa cells in the transwell chamber after the knockdown of TNFAIP8 compared to sh-NC group (Figure 3C). These results indicated that the knockdown of TNFAIP8 inhibited cell proliferation and metastasis in ECa.

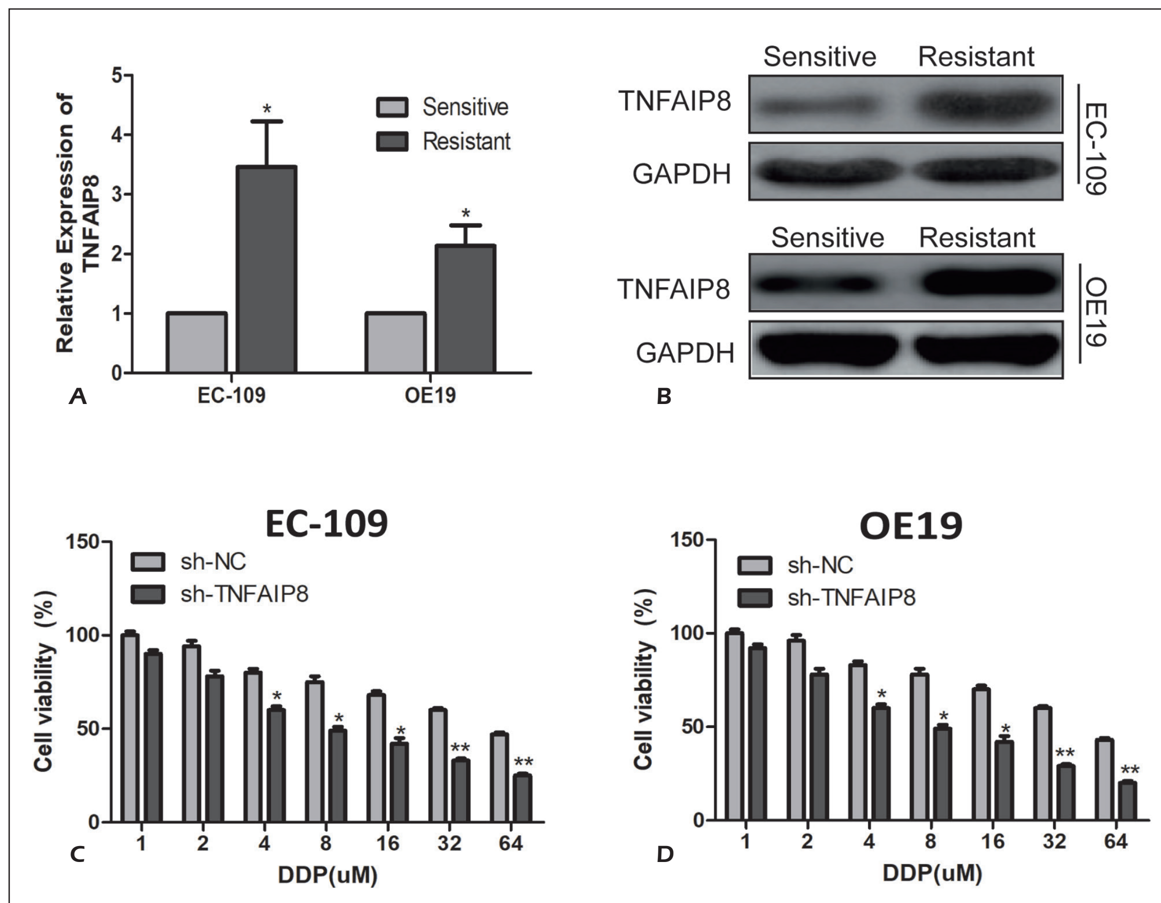

Figure 2. TNFAIP8 increases cisplatin resistance in esophageal cancer. **A**, qRT-PCR evaluated the expression of TNFAIP8 in cisplatin-resistant patients. **B**, Western Blotting was used to detect the expression of TNFAIP8 in cisplatin-resistant patients. **C**, CCK-8 assay revealed the effect of knockdown of TNFAIP8 on the viability of EC-109 esophageal cancer cell lines. **D**, CCK-8 assay detected the effect of knockdown of TNFAIP8 on the viability of OE19 esophageal cancer cell lines. Data are mean ± SD, *p<0.05.

TAF-Iα Was Lowly Expressed in ECa Tissues and Cell Lines

Bioinformatics studies have demonstrated that TNFAIP8 and TAF-Iα may have some association in ECa tissues. Subsequently, the targeting effect of TNFAIP8 on TAF-Iα was further verified, and TNFAIP8 and TAF-Iα were co-transfected into EC-109 and OE19 cell lines for Luciferase reporter gene experiments. The results indicated that TNFAIP8 can be targeted by TAF-Iα through this binding site (Figure 4A). ECa tissue verification illustrated that TAF-Iα level was remarkably decreased in tumor tissues of patients with ECa (Figure 4B). Therefore, we performed qRT-PCR assay to analyze the expression of TNFAIP8 and TAF-Iα in 43 cases of ECa tissue samples and adjacent ones, and a negative correlation between TNFAIP8 and TAF-Iα in ECa cell lines was observed (Figure 4C). Finally, in the ECa cisplatin-resistant cell lines, Western blotting result indicated that TAF-Iα level was remarkably upregulated after the knockdown of TNFAIP8 (Figure 4D), while TNFAIP8 expression was also found enhanced after the knockdown of TAF-Iα (Figure 4E).

TAF-Iα Modulated TNFAIP8 Expression in Human ECa Cells

Subsequently, we knocked down TAF-Iα in ECa cisplatin-resistant cell line that had silenced TNFAIP8 to figure out if there was some mutual regulation between them. qRT-PCR ex-

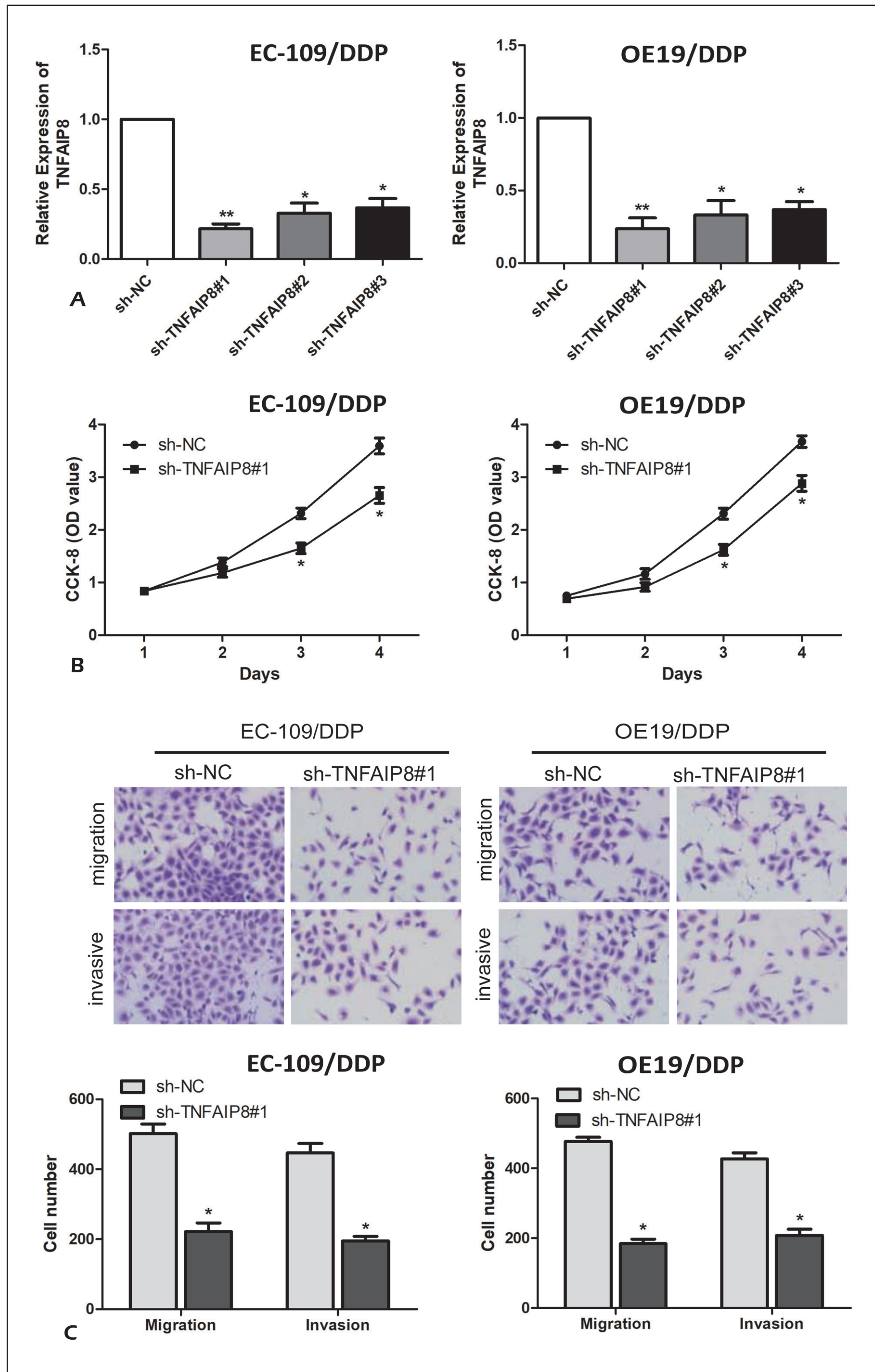

Figure 3. Inhibition of proliferation and metastasis of esophageal cancer cells after silencing TNFAIP8. **A**, qRT-PCR verified the interference efficiency after transfection of TNFAIP8 knockdown vector in esophageal cancer cisplatin-resistant EC-109/DDP and OE19/DDP cell lines. **B**, The CCK-8 assay detected the effect of transfection of TNFAIP8 knockdown vectors on the proliferation of esophageal cancer cells in EC-109/DDP and OE19/DDP cell lines. **C**, Transwell assay detected the effects of transfection of TNFAIP8 knockdown vectors on invasion and migration of esophageal cancer cells in EC-109/DDP and OE19/DDP cell lines (magnification: 40×). Data are mean ± SD, *$p<0.05$.

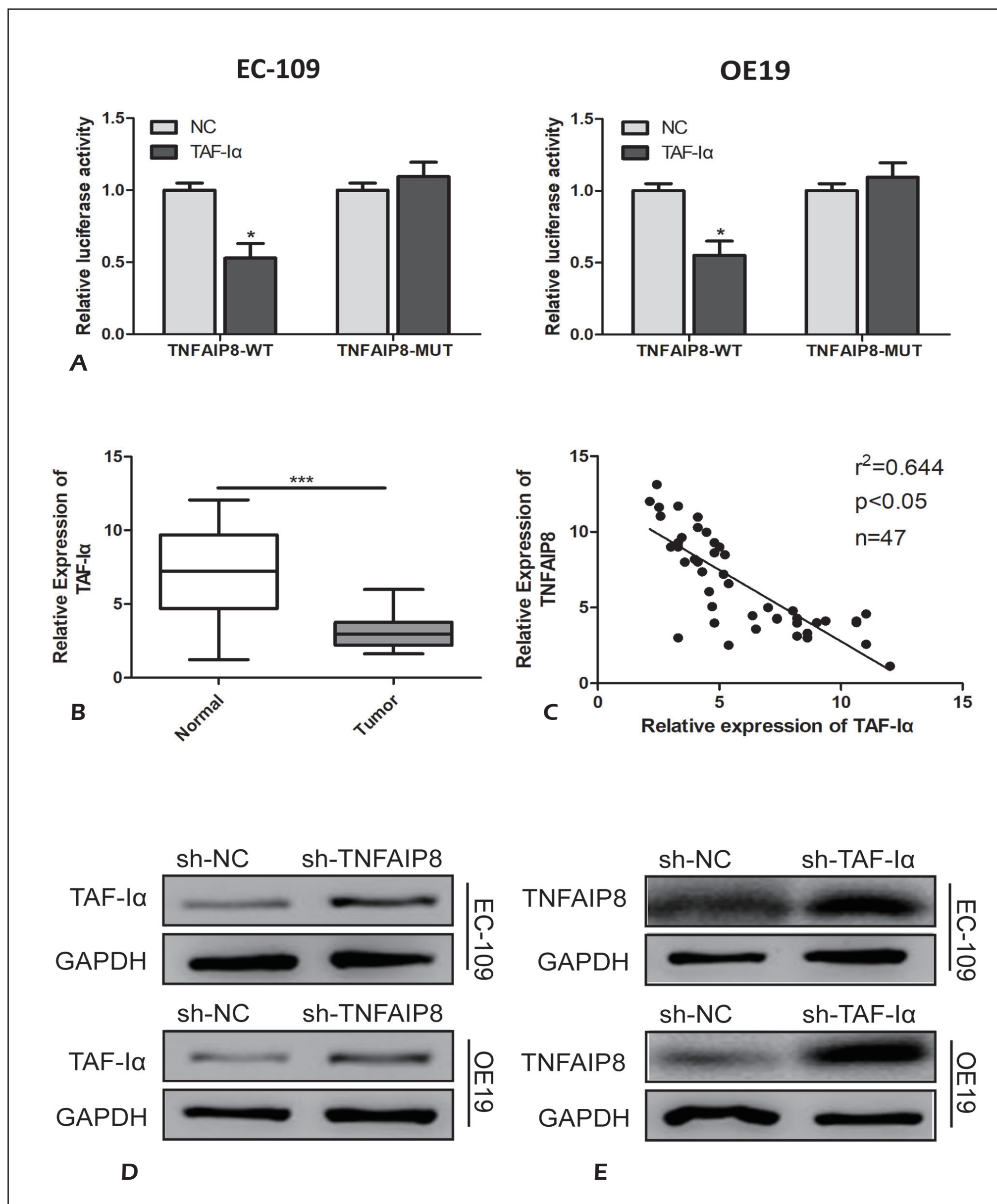

Figure 4. TNFAIP8 can be targeted to bind to TAF-Iα. **A**, Dual luciferase reporter assays demonstrated a direct targeting of TNFAIP8 to TAF-Iα. **B**, qRT-PCR was used to detect the difference in expression of TAF-Iα in tumor tissues and adjacent tissues of esophageal cancer. **C**, There was a significant negative correlation between the expression levels of TNFAIP8 and TAF-Iα in esophageal cancer tissues. **D**, qRT-PCR verified the expression level of TAF-Iα after interference with TNFAIP8 in EC-109/DDP and OE19/DDP cell lines. **E**, qRT-PCR verified the expression level of TNFAIP8 after interfering with TAF-Iα in EC-109/DDP and OE19/DDP cell lines. Data are mean ± SD, $*p<0.05$, $**p<0.01$, $***p<0.001$.

amined the transfection efficiency of TNFAIP8 after co-transfection of TNFAIP8 and TAF-Iα (Figure 5A). Subsequently, in the ECa cisplatin-resistant cell lines, silencing of TAF-Iα was found to be capable of reversing the influence of TNFAIP8 downregulation on the invasiveness and migratory ability of ECa cells using the transwell assay (Figure 5B), which revealed that TAF-Iα could modulate TNFAIP8 expression in ECa cells.

Discussion

Various DNA damage factors in the process of cell growth, such as the deletion, rearrangement,

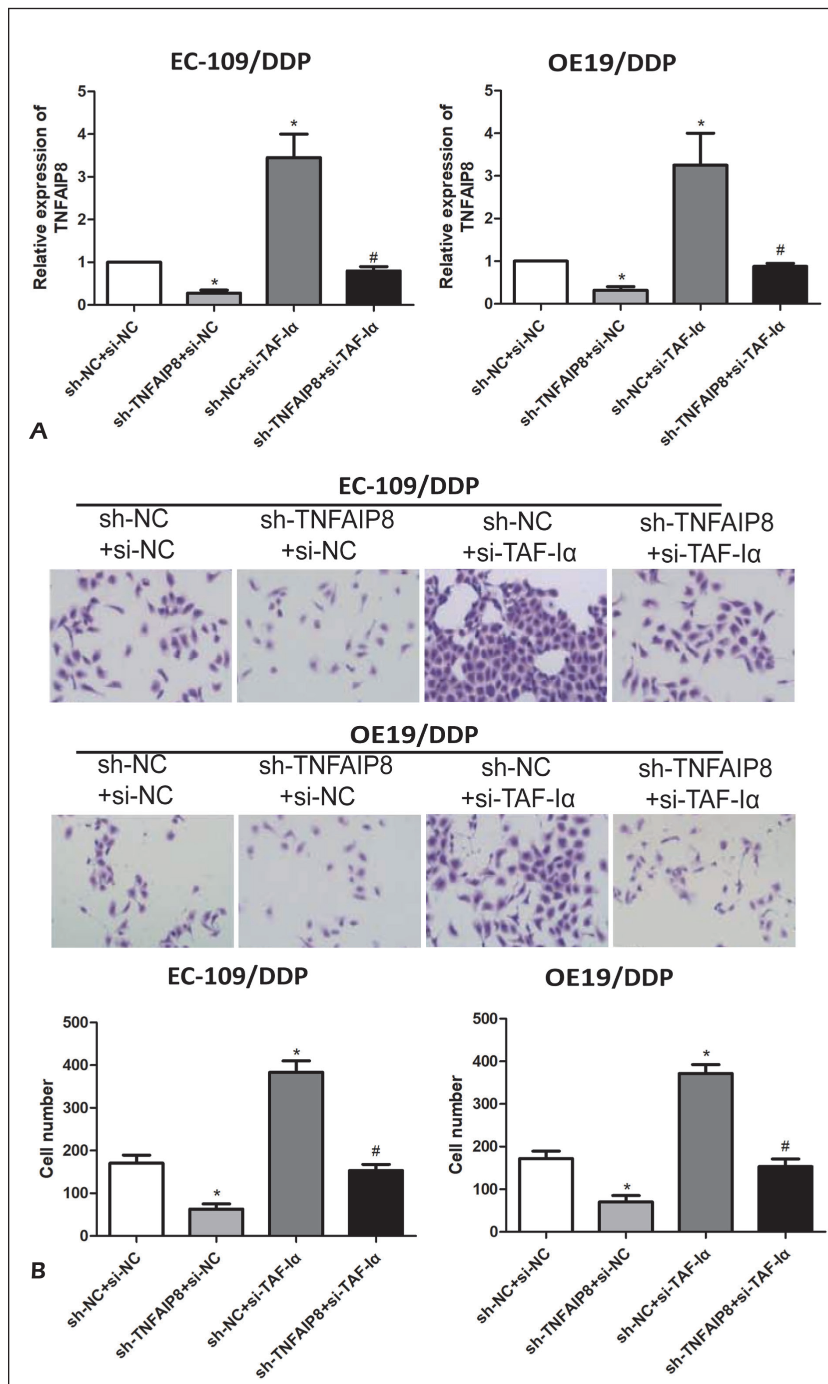

Figure 5. TNFAIP8 regulates the mechanism of action of TAF-Iα in esophageal cancer cells. **A**, TNFAIP8 expression levels in EBAIP8 and TAF-Iα co-transfected EC-109/DDP and OE19/DDP cell lines were detected by qRT-PCR. **B**, Transwell assay was used to detect the invasion and migration of TNFAIP8 and TAF-Iα after co-transfection of EC-109/DDP and OE19/DDP cell lines (magnification: 40×). Data are mean ± SD, *$p<0.05$.

and base modification of chromosomal DNA, and the gradual accumulation of such genomic abnormalities eventually lead to the occurrence of tumors[11,12]. That is to say, the mechanism of maintaining defects in the genome leads to chromosome aberrations, and the accumulation of such distortions can eventually lead to the occurrence of tumors, including cell cycle checkpoint pathways and DNA repair pathways[21,22]. Due to the concealment of ECa, most patients have been diagnosed at an advanced stage. However, the combination of radiotherapy and chemotherapy is not effective. Finding new treatments is the key to improve the overall survival of patients with ECa[9,10]. Proliferation, invasion, and migration are the most evident biological characteristics of ma-

lignant tumors, and they are also the root cause of malignant tumors. Therefore, how to inhibit the proliferation, invasion, and migration of malignant tumors is of great significance in controlling malignant tumor progression and improving survival rate[11,12]. Tumor proliferation and invasion and migration are a complex process involving multiple factors. The regulation of TNFAIP8 on tumor proliferation and invasion-related gene expression is a hot topic[13-15].

In recent years, a series of roles of TNFAIP8 in tumor formation have been observed. These results indicate that TNFAIP8 plays an important role in the development of tumors and is one of the most important components regulating apoptosis in different types of tumors. It is involved in the regulation of cell proliferation, affecting invasion, migration, apoptosis, and drug resistance in various tumors[16]. TNFAIP8 expression can be detected in most malignant tumor tissues such as breast cancer, ovarian epithelial cell carcinoma, and cervical cancer[13-16]. At present, there are few studies on the role of TNFAIP8 in ECa. Based on the previous reports, ECa cell lines EC-109 and OE19 were selected as the research subjects. TNFAIP8 was silenced to investigate the effects of TNFAIP8 on the biological characteristics of proliferation, invasion, and migration of EC-109 and OE19 cells, as well as its regulation of Survivin protein expression in cells. The expression of TNFAIP8 was firstly verified in 47 tumor tissues and adjacent tissues of ECa patients. The results showed that the TNFAIP8 expression was remarkably upregulated and inversely correlated with poor prognosis in patients with ECa. Therefore, TNFAIP8 may play a role in promoting cancer in ECa. However, the role of TNFAIP8 in the proliferation of ECa cells and its ability to affect invasion and migration has not been determined. Based on this, in order to further study the role of TNFAIP8 in tumor development, we constructed the ECa cisplatin-resistant cell line and found that the knockdown of TNFAIP8 can reduce the inhibition of cisplatin on ECa cell proliferation and inhibit cisplatin sensitivity.

TAF-Iα exhibits transcriptional activation activity but does not have this activity for naked DNA, thus it is shown that TAF-Iα relies on alteration of the adenoviral core (Adcore) structure to activate replication and transcription[23,24]. Further investigations have shown that TAF-Iα, through its interaction with histone H3/H4 *in vitro*, relies on the reorganization of chromatin structure to have transcriptional activation activity in *in vitro* recombinant chromatin[24,25].

Autoantibodies to TAF-Iα can be found in a variety of malignancies, while TAF-Iα is also a cytoplasmic protein that binds to mRNA encoding TNFAIP8. To demonstrate whether TNFAIP8 regulates cisplatin resistance by TAF-Iα and promotes the development of esophageal carcinoma, the expression of TAF-Iα after silencing TNFAIP8 was detected by Western blot, and both expressions were negatively correlated. The results showed that the expression level of TAF-Iα was remarkably upregulated after TNFAIP8 silencing, suggesting that TNFAIP8 may promote the malignant progression of ECa by regulating TAF-Iα. In this study, Luciferase reporter gene and cell recovery experiments revealed that there was a mutual regulation between TNFAIP8 and TAF-Iα. With the deepening of research, further understanding of the biological functions of TNFAIP8 and TAF-Iα genes and their role in the development of tumors will be more helpful for the diagnosis, treatment, and prognosis of tumors.

Conclusions

In summary, the expression of TNFAIP8 in ECa was found remarkably increased, which might be closely relevant to the poor prognosis of patients with ECa. Additionally, it was found that TNFAIP8 may regulate cisplatin resistance *via* modulating TAF-Iα and thus promote the malignant progression of ECa.

Conflict of Interests

The authors declare that they have no conflict of interest.

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