Suppression of Elk1 inhibits thyroid cancer progression by mediating PTEN expression

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Abstract. ETS-domain containing protein (Elk1) is reported to be a member of the ETS oncogene family, and promotes tumorigenesis in cancer such as bladder, prostate and ovarian. Nevertheless, the role of Elk1 in thyroid cancer progression remains unclear. In the present study, we aimed to investigate the role and underlying molecular mechanism of Elk1 in thyroid cancer. The results indicated that Elk1 was significantly upregulated in thyroid cancer tissues and cells. We found that loss of Elk1 function obviously induced the expression of early growth response-1 (Egr-1) and PTEN, promoted apoptosis and constrained the proliferation of thyroid cancer cells. Furthermore, Egr-1 inhibition obviously abrogated the induction of PTEN induced by Elk1 reduction. Moreover, Egr-1 suppression prevented the promotion of apoptosis and the inhibition of cell proliferation caused by Elk1 reduction. In conclusion, Elk1 inhibition induced thyroid cancer cell apoptosis and restrained their proliferation by regulating Egr-1/PTEN, indicating a potential role for Elk1 in thyroid cancer treatment.

Introduction

Thyroid cancer (TC) is the most common endocrine malignancy, accounting for approximately 3% of all malignant tumors (1,2). Moreover, it has a higher incidence in women, and is the most common cancer of the head and neck (3,4). In recent years, the incidence of TC has significantly increased. A better understanding of the underlying mechanism of TC would provide novel insights for the treatment of TC.

ETS-domain containing protein (Elk1), a transcription factor belonging to the ETS oncogene family, regulates the oncogene c-fos by phosphorylation through activation of the PKC/ERK pathways (5-8). Studies have reported that Elk1 has roles in cell proliferation, the cell cycle, apoptosis and tumorigenesis (9,10). It has been demonstrated that Elk1 expression is upregulated, and promotes cell viability in bladder cancer (10). Elk1 has also been shown to be induced, and to play a crucial role in hormone-resistant or metastatic prostate cancers (11) and is reported to play an important role in breast cancer and ovarian cancer (7,12-14). However, the molecular mechanism of Elk1 in TC remains unknown. In our study, we investigated the role of Elk1 in cell proliferation and apoptosis in TC. A previous study has shown that in SH-SY5Y neuroblastomas Elk1 represses the expression of Egr-1, which is implicated in different cellular processes containing cell proliferation, differentiation and apoptosis (15).

Early growth response-1 (Egr-1), also called Zif268, NGF1-A, and Krox24, is a transcription factor containing a zinc-finger DNA binding domain, and is known as an important immediate-early gene (IEG) (16-21). Egr-1 promotes quiescent cells to enter the proliferative phase, regulating cell growth and differentiation (20,22,23). Egr-1 is found in eukaryotic genomes, and is highly conserved evolutionarily (24). Many factors can activate Egr-1, and activated Egr-1 regulates target gene transcription by interacting with the binding sites of target genes. The biological function of Egr-1 is realized by upregulating or downregulating target gene expression. Egr-1 is considered to be a class II tumor suppressor gene (25). It has been demonstrated that the expression of Egr-1 is decreased in breast cancer, non-small cell lung cancer (NSCLC) and glioma (26,27). Nevertheless, other studies have demonstrated that Egr-1 expression is increased in prostate cancer, lymphoma and Wilms' tumor, among others (28,29). It has been reported that Egr-1 could directly regulate the transcription of the phosphatase and tensin homolog deleted on chromosome ten (PTEN) (30).

PTEN is also recognized as a tumor suppressor (31,32), and it was observed that PTEN is inactivated or inhibited in multiple types of cancer including thyroid carcinoma (33). Research over the past few year has shown the mechanism by which loss of PTEN function contributes to tumor development (34). It has been reported that PTEN inhibition induces cell survival and cisplatin resistance in human ovarian cancer,

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Abbrevations: Elk1, ETS-domain containing protein; Egr-1, early growth response-1; TC, thyroid cancer; DMEM, Dulbecco's modified Eagle's nedium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; BrdU, bromodeoxyuridine; Annexin V FITC/PI, Annexin V fluorescein isothiocyanate conjugate/propidium iodide; RT-qPCR, real-time quantitative polymerase chain reaction; TBS, Tris-buffered saline

Key words: Elk1, thyroid cancer, Egr-1, PTEN

and promotes the risk of breast and endometrial cancers and leukemia (35,36). Furthermore, it has been demonstrated that PTEN suppression causes thyroid cancer development, progression and invasion, authenticating PTEN as a crucial tumor suppressor in thyroid carcinogenesis (37). Moreover, transient ectopic expression of PTEN promotes cell cycle arrest and cell death in thyroid cancer cell lines (38).

In the present study, we investigated the molecular mechanism underlying Elk1 action in thyroid cancer progression *in vitro*. We found that Elk1 expression was upregulated in thyroid cancer cell lines and tissues. Loss of Elk1 function significantly inhibited the proliferation and induced apoptosis in thyroid cancer cell lines. Furthermore, the results also demonstrated that Elk1 inhibition induced PTEN expression by upregulating Egr-1. Therefore, this study proposes the potential role of Elk1 in preventing thyroid cancer, providing a potential novel target for treatment.

Materials and methods

Cell lines and tissues. Human thyroid cancer cell lines FTC-133 and TPC-1 were purchased from the Protection Agency Culture Collections (HPACC, Salisbury, Wiltshire, UK). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) containing 10% fetal bovine serum (FBS, HyClone; GE Healthcare Life Sciences, Logan, UT, USA). The normal thyroid cell line FRTL-5 was obtained and cultured according to a previously described protocol (39). The cell lines were incubated at 37˚C with a 5% CO_2 atmosphere. Additionally, 10 pairs (sample collection: From February 2017 to October 2017) of tumor tissue samples and matched adjacent normal tissues were obtained from Xinxiang Central Hospital along with written informed consent of patients (4 males and 6 females; 39-50 years old), and were immediately stored in liquefied ammonia. The study was approved by the Xinxiang Central Hospital Ethics Board.

Cell transfection. The cells (FTC-133 and TPC-1) were separately seeded in 12-well plates and incubated in a humid atmosphere with 5% CO_2 at 37˚C until 80% fusion was achieved. The transfection procedure was performed according to the manufacturer's instructions. Elk1 siRNA (5'-AACCACCCGCCACTCTTCCT-3'), Egr-1 siRNA (5'-GTAGGTTGCTGTCGTCAGGGTAAAT-3'), and non-specific siRNA were separately diluted in FBS-free DMEM medium (200 μl) with 6 μl TurboFect (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the mixtures were added in the well. The cells were then cultured under conditions of 5% CO_2 at 37˚C for 24 h.

Cell growth and viability. Cell growth and viability were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and the assay was performed in accordance with standard protocols. The cells ($1x0^5$ cells/well) were cultured in 96-well plates with DMEM containing 10% FBS. The medium in each well was then replaced by 18 μl MTT (5 g/l) diluted in phosphate-buffered saline (PBS) followed by incubation at 37˚C for 5 h. Subsequently, a total of 150 μl dimethyl sulfoxide was added per well, in order to dissolve the crystals. Finally, the result was read using a microplate reader (Thermo Fisher Scientific) at 490 nm. The analysis was repeated three times.

Bromodeoxyuridine (BrdU) assay. The BrdU cell proliferation assay kit (Cell Signaling Technology, Danvers, MA, USA) was used to detect cell proliferation based on the manufacturer's protocol. Briefly, the cells were plated in 96-well plates and incubated with BrdU solution (10 μl per well) for 1.5 h. A total of 150 μl denaturing solution was added per well to replace the medium followed by culturing for 30 min, and cells were incubated with anti-BrdU conjugated with peroxidase. After addition of the substrate and incubation for 20 min, the optical density at 450 nm was determined at room temperature using a SpectroFluor Plus multiwell plate reader (Tecan, Research Triangle Park, NC, USA). The experiment was repeated three times.

Caspase-3 activity detection. The caspase-3 activity assay was performed as per the manufacturer's instructions using the caspase-3 activity assay kit (Beyotime Institute of Biotechnology, Nantong, China). Briefly, cells were lysed on ice for 15 min, and 10-μl cell lysate per sample in 90 μl reaction buffer [1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM Nad and 10% glycerol] containing 12 μl caspase-3 substrate (Ac-DEVD-pNA) (2 mM) were added into 96-well microtitre plates. Lysates were incubated at 37˚C for 2 h. The results were measured with an ELISA reader (Tecan) at an absorbance of 405 nm.

Annexin V fluorescein isothiocyanate conjugate and propidium iodide (Annexin V FITC/PI). Apoptosis was measured using the BD Pharmingen™ Annexin V FITC/PI apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) following the standard protocol. In brief, cells were precooled in cold PBS and suspended in binding buffer. Then, Annexin V FITC solution (10 μl) was added followed by incubation for 23 min. Afterwards, 10 μl PI was added and the reaction was allowed to proceed for 7 min. Cellular apoptosis was measured using a FACS analyzer (Thermo Fisher Scientific, Inc.).

Real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using TRIzol (Thermo Fisher Scientific, Inc.). Total RNA extraction from tissues was performed according to a previously published method (40). RNA (5 μg) was then synthesized into cDNA using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RT-qPCR was performed in 20 μl reaction volumes containing 10 μl Applied Biosystems® SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Inc.). The genes were normalized to GAPDH. The primers used were as follows: Elk1 sense primer, 5'-CCTTGCGGTACTACTATGAC-3' and antisense primer, 5'-GGCTGCGGCTGCAGAGACTGG-3'; Egr-1 sense primer, 5'-TTTGCCAGGAGCGATGAAC-3' and antisense primer, 5'-CCGAAGAGGCCACAACACTT-3'; GAPDH sense primer, 5'-CGTCTTCACCACCATGGAGA-3' and antisense primer, 5'-CGGCCATCACGCCACAGTTT-3'. The protocol: 94˚C for 30 sec; 35 cycles of 95˚C for 30 sec, 58˚C (Elk1) or 60˚C (Egr-1) for 30 sec and 72˚C for 30 sec; 72˚C for 10 min. The relative gene expression levels were estimated using the $2^{-\Delta\Delta Ct}$ method.

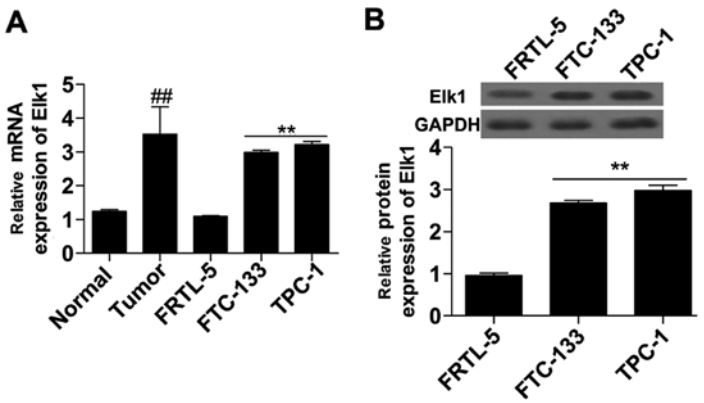

Figure 1. Elk1 expression in TC tissues and cell lines. (A) Relative mRNA expression of Elk1 was measured using RT-qPCR in TC tissues and cell lines. (B) Relative protein expression of Elk1 was measured using western blot analysis in TC cell lines. Normal, matched adjacent normal tissues. Tumor, TC tissues. FRTL-5, normal thyroid cell line. FTC-133 and TPC-1, thyroid cancer cell lines. GAPDH was used as the internal control. **P<0.01 vs. the FRTL-5 group. $^{\#\#}$P<0.01 vs. the Normal group. TC, thyroid cancer.

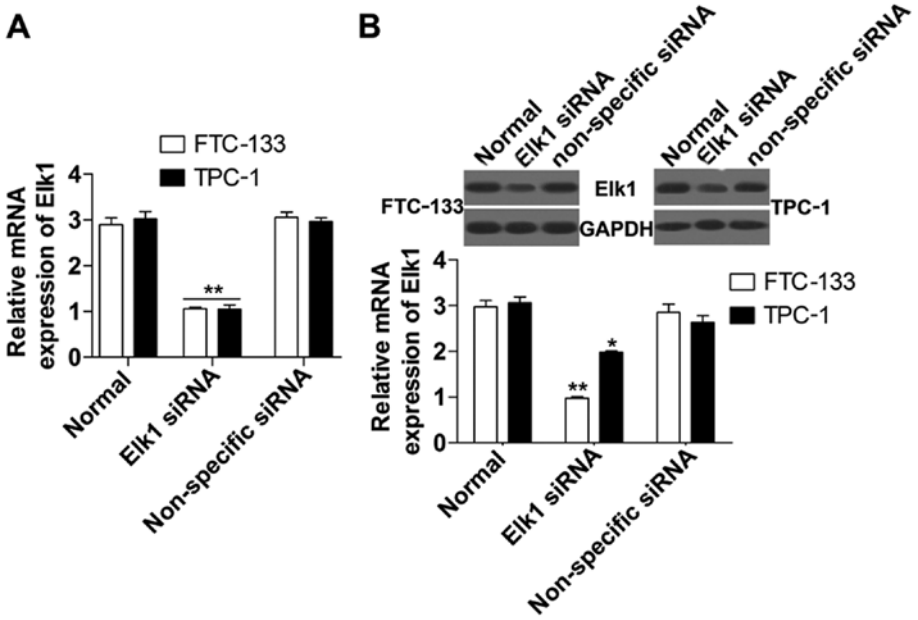

Figure 2. Inhibition of the expression of Elk1 in TC cells. (A) Relative mRNA expression of Elk1 was measured using RT-qPCR in transfected cell lines. (B) Relative protein expression of Elk1 was measured using western blotting in transfected cell lines. Normal, FTC-133 and TPC-1 cells without transfection. Elk1 siRNA, FTC-133 and TPC-1 cells transfected with Elk1 siRNA. Non-specific siRNA, FTC-133 and TPC-1 transfected with non-specific siRNA. GAPDH was used as the internal control. **P<0.01 and *P<0.05 vs. the non-specific siRNA group. TC, thyroid cancer.

Immunoblotting analysis. Proteins were extracted from the cells treated with the lysate (Beyotime Institute of Biotechnology), and quantified using the BCA kit (Beyotime). A total of 25 μg of protein was separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane using a semi-dry blotting apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes containing the proteins were incubated in Tris-buffered saline (TBS) containing 2% non-fat dry milk at room temperature for 2 h followed by washing with TBS. The nitrocellulose membranes were then incubated overnight at 4˚C with the primary antibodies against Elk1 (1:500; cat. no. ab131465), Egr1 (1:500; cat. no. ab182624), PTEN (1:800; cat. no. ab31392) and GAPDH (1:1,000; cat. no. ab37168; all from Abcam Inc., Cambridge, MA, USA), and then incubated with a horseradish peroxidase conjugated secondary antibody (1:1,000; cat. no. ab205718; Abcam) for 1 h at room temperature. Finally, proteins were visualized using Pierce enhanced chemiluminescence (Thermo Fisher Scientific, Inc.) in a Bio-Rad ChemiDoc apparatus.

Statistical analysis. Data are expressed as mean ± standard deviation (SD). Statistical significance was determined by Student's t-test for two groups or by one-way ANOVA for multiple groups. A P-value of <0.05 was considered statistically significant.

Results

Induction of Elk1 in thyroid cancer. To investigate the expression of Elk1 in thyroid cancer, we detected the expression of Elk1 in thyroid cancer tissues and thyroid cancer cells. The

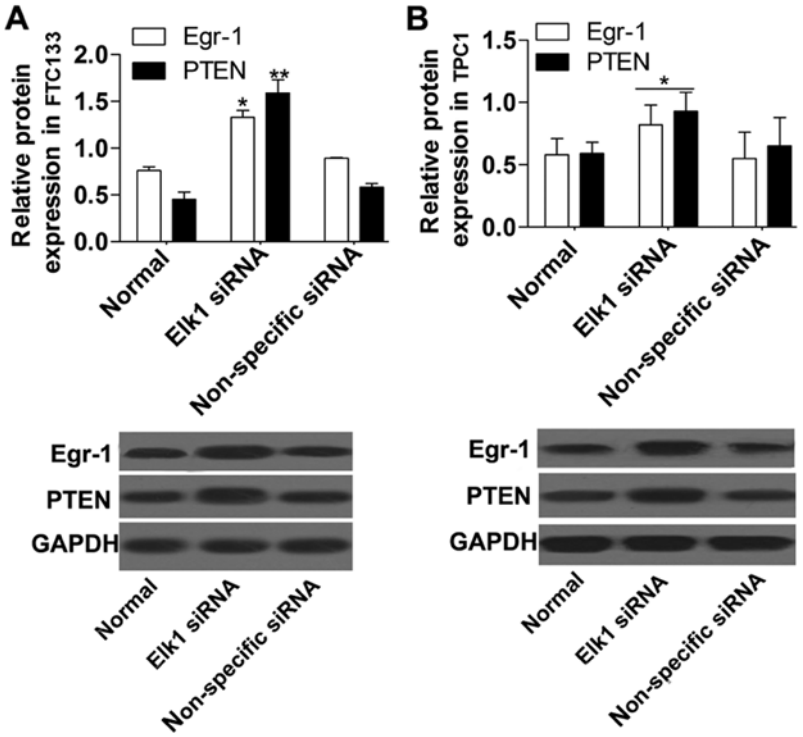

Figure 3. Expression of Egr-1 and PTEN after Elk1 inhibition. Relative protein expression of Egr-1 and PTEN in FTC-133 (A) and TPC-1 (B) cells was measured using western blotting. GAPDH was used as the internal control. **P<0.01 and *P<0.05 vs. the non-specific siRNA group.

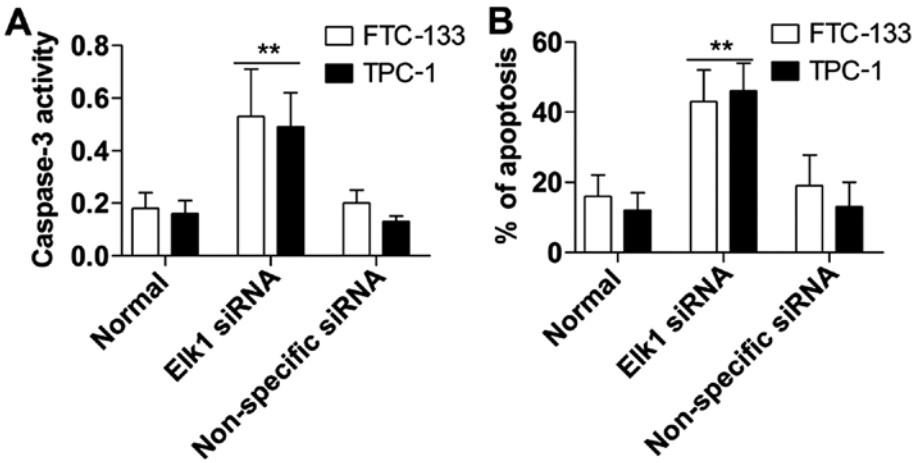

Figure 4. TC cell apoptosis after Elk1 inhibition. Apoptosis of TC cells was assessed by measuring the caspase-3 activity (A) and using the Annexin V FITC/PI assay (B) after Elk1 inhibition. **P<0.01 vs. the non-specific siRNA group. TC, thyroid cancer.

results showed that the mRNA (Fig. 1A) expression level of Elk1 in the thyroid cancer tissue was significantly higher than that in the normal tissue. Additionally, mRNA (Fig. 1A) and protein (Fig. 1B) were both significantly increased in the thyroid cancer cells (FTC-133 and TPC-1) compared with that in the normal thyroid cells (FRTL-5). Thus, Elk1 expression was upregulated in thyroid cancer.

Suppression of Elk1 induces the expression of Egr-1 and PTEN. To detect the role of Elk1 in the regulation of Egr-1 and PTEN, we inhibited Elk1 expression using cell transfection in FTC-133 and TPC-1 cells. The results indicated that the loss-of-function experiment was successful with a significant reduction of Elk1 mRNA (Fig. 2A) and protein (Fig. 2B) expression in the Elk1 siRNA group compared with the non-specific siRNA group. Furthermore, the protein (Fig. 3) expression levels of Egr-1 and PTEN were obviously increased in the Elk1 siRNA group compared with the non-specific siRNA group.

Suppression of Elk1 promotes thyroid cancer cell apoptosis. Annexin V FITC/PI and caspase-3 activity detection were used to assess the role of Elk1 in the downregulation of thyroid cancer cell apoptosis. The results demonstrated that the caspase-3 activity in the Elk1 siRNA group was also obviously induced compared with that noted in the non-specific

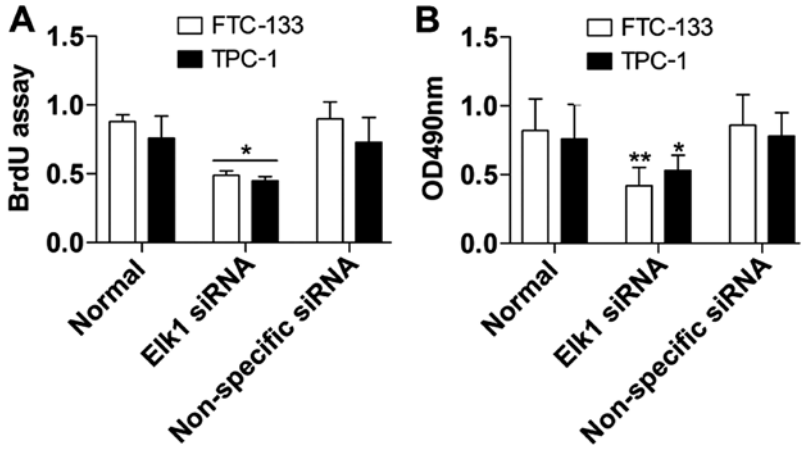

Figure 5. TC cell proliferation after Elk1 inhibition. Proliferation of TC cells was assessed by the BrdU assay (A) and MTT (B) after Elk1 inhibition. **P<0.01 and *P<0.05 vs. the non-specific siRNA group. TC, thyroid cancer.

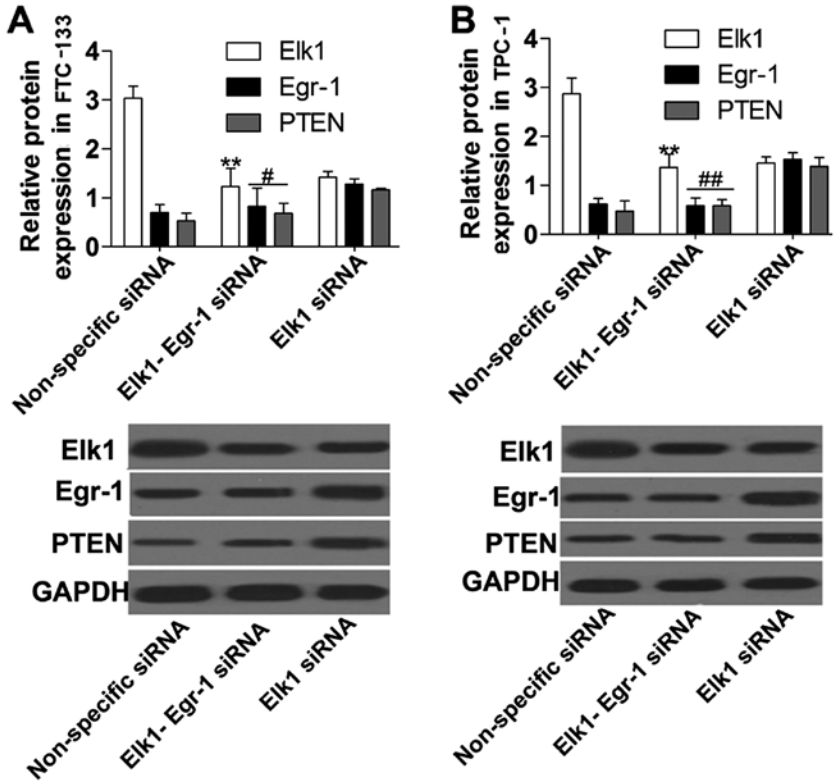

Figure 6. Protein expression after co-transfection. Relative protein expressions of Elk1, Egr-1 and PTEN in FTC-133 (A) and TPC-1 cells (B) were measured using western blotting. Elk1-Egr-1 siRNA, cells co-transfected with Elk1 siRNA and Egr-1 siRNA. GAPDH was used as the internal control. **P<0.01 vs. the non-specific siRNA group. $^{\#\#}$P<0.01 and $^{\#}$P<0.05 vs. the Elk1 siRNA group.

siRNA group (Fig. 4A). Furthermore, cellular apoptosis in the Elk1 siRNA group was markedly increased compared with the non-specific siRNA group as determined using the Annexin V FITC/PI assay (Fig. 4B).

Suppression of Elk1 constrains thyroid cancer cell proliferation. Cell proliferation was assessed using the BrdU and MTT assays to further detect the biological effect of Elk1 inhibition on thyroid cancer cells. In the BrdU assay, the results demonstrated that FTC-133 and TPC-1 cell proliferation (Fig. 5A) was markedly inhibited in the Elk1 siRNA group compared with the non-specific siRNA group. In the MTT assay, FTC-133 and TPC-1 growth and viability (Fig. 5B) were both constrained in the Elk1 siRNA group compared with the non-specific siRNA group, and the differences were significant.

Elk1 inhibition upregulates PTEN via increased Egr-1 expression. To explore the mechanism of Elk1 regulation of PTEN and Egr-1, we performed co-transfection of Elk1 siRNA and Egr-1 siRNA into FTC-133 and TPC-1 cells, and the results showed that the expression of Elk1 and Egr-1 protein (Fig. 6) was significantly suppressed in the Elk1-Egr-1 siRNA group

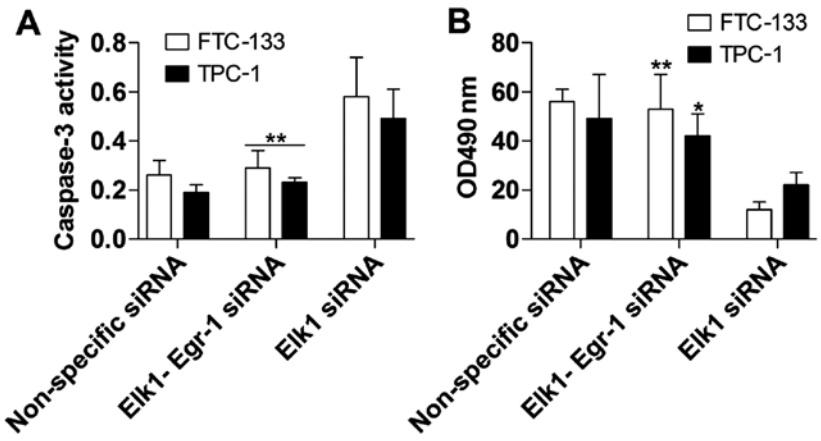

Figure 7. TC cell apoptosis and proliferation after co-transfection. Apoptosis (A) and proliferation (B) were measured using caspase-3 activity detection and the MTT assay, respectively. *P<0.05 and **P<0.01 vs. the Elk1 siRNA group. TC, thyroid cancer.

compared with the non-specific siRNA group and with the Elk1 siRNA group, respectively. Furthermore, PTEN protein expression (Fig. 6) was significantly downregulated in the Elk1-Egr-1 siRNA group compared with the Elk1 siRNA group. The promotion of apoptosis (Fig. 7A) and inhibition of cell proliferation (Fig. 7B) caused by the suppression of Elk1 were markedly overcome by Egr-1 inhibition.

Discussion

Thyroid cancer (TC) has different histological and biological types, and the clinically significant human thyroid cancers are papillary and follicular carcinomas (41). Surgery-based treatment is the primary clinical treatment. However, specific targets for drugs to treat TC are still lacking, and the molecular mechanisms of TC remain unclear. Elk1 is reported to be a transcriptional factor that forms part of the ternary complex factor (TCF), and it can be phosphorylated by the MAPK cascade (42). Elk1 regulates different factors related to cell proliferation, differentiation and even tumorigenesis (43). Studies have demonstrated that Elk1 plays an important role in cancer progression. Kawahara *et al* found that Elk1 is induced in prostate cancer and promotes tumor development, whereas Elk1 inhibition suppresses tumor growth (44). Additionally, Elk1 is upregulated and promotes cell proliferation in bladder cancer and non-small cell lung cancer (NSCLC) (10). In this study, we found that Elk1 expression was significantly upregulated in TC tissues and cells. Moreover, the results showed that TC cell proliferation and apoptosis were constrained and promoted, respectively, after experimental downregulation of Elk1 by siRNA.

Egr-1 is an important immediate-early gene, and is also a tumor suppressor related to different cancers (45), as well as being implicated in cell proliferation and apoptosis (22). Additionally, studies have demonstrated that Egr-1 expression is down-regulated or absent in NSCLC and breast cancer, while it is upregulated in prostate cancer and lymphadenoma (26,28). Research has shown that Egr-1 can be regulated via Elk1, and Demir and Kurnaz demonstrated that Egr-1 could be repressed by Elk-1 expression in SH-SY5Y neuroblastomas (15). In our study, Elk1 inhibition markedly increased Egr-1 expression in the TC cell lines FTC-133 and TPC-1. Furthermore, it was previously indicated that Egr-1 positively regulates PTEN expression, and that loss of Egr-1 restrains the expression of PTEN. Thus, we investigated the role of the Elk1/Egr-1 pathway in TC.

PTEN is accepted as a tumor suppressor, and its suppression function is realized by inhibiting the activity of P13K (32). PTEN induction is reported to facilitate cell apoptosis in bladder cancer, lung squamous carcinoma and ovarian cancer (46). PTEN mutations are extremely common in melanoma cell lines, advanced prostate cancers, and endometrial carcinomas, and PTEN deficiency was found to accelerate the proliferation and invasion of a range of cancers such as gastric cancer, pancreatic cancer, prostate cancer, among other (47-49). Most importantly, many correlative data suggest that inhibition of PTEN leads to TC *in vivo*, and Guigon *et al* demonstrated that suppression of PTEN facilitates TC tumor development in a mouse model (50). In our study, PTEN expression was induced by Elk1 inhibition, decreasing TC cell proliferation and increasing apoptosis. Additionally, loss of Egr-1 significantly reversed the effect of Elk1 inhibition on PTEN expression, TC cell proliferation and apoptosis. Thus, the results showed that Elk1 inhibition could induce the expression of PTEN via upregulation of Egr-1.

In summary, this study revealed that Elk1 is induced in TC tissues and cell lines. The loss of Elk1 can markedly increase the expression of PTEN, promoting TC cell apoptosis and inhibiting proliferation. Furthermore, Elk1 suppression upregulates PTEN expression via increased Egr-1 expression, providing a novel target for the treatment of TC.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; YK and XG designed and prepared the experiments; YK performed the experiments; JY, YF, YC and YZ contributed to the reagents/materials/analysis tools; YK wrote the manuscript; XG modified and revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Xinxiang Central Hospital Ethics Board and written informed consent obtained from the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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