SERPINE2 feedback regulates EGF/EGFR signaling in human papillary thyroid carcinoma cells

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Abstract. Thyroid cancer (TC) is the most prevalent malignant tumor in the endocrine system. Serpin peptidase inhibitor clade E member 2 (SERPINE2) is closely associated with tumor metastasis. The aim of the present study was to investigate whether SERPINE2 forms a feedback loop with epidermal growth factor (EGF)/EGF receptor (EGFR) that regulates cellular processes in human papillary thyroid carcinoma (TPC-1) cells. Reverse transcription-quantitative PCR and western blotting were utilized to analyze the expression of SERPINE2. Cell proliferation ability was detected with a cell proliferation and cytotoxicity assay kit (MTT) and by clone formation assay. The proliferation markers, including proliferating cell nuclear antigen and Ki-67, were also investigated to analyze the proliferative activity of TPC-1 cells. Besides, cell migration and invasion were analyzed by wound healing and Transwell assays, respectively, while cell apoptosis was analyzed by TUNEL staining. The results showed that SERPINE2 expression was increased in TPC cells, and SERPINE2 and EGF/EGFR regulated each other. Furthermore, SERPINE2 overexpression and silencing regulated TPC cell proliferation, migration, invasion and apoptosis. Besides, an EGFR inhibitor blocked the effects of SERPINE2 overexpression on the aforementioned biological processes. Therefore, the present study confirmed that SERPINE2 formed a positive feedback with EGF/EGFR to regulate the proliferation, invasion and migration of TPC cells, possibly providing novel insights into potential therapeutic targets of papillary TC.

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Introduction

Thyroid cancer (TC) is the most common malignant tumor of the endocrine system, and is one of the top ten cancer types threatening women's health. Serpin peptidase inhibitor clade E member 2 (SERPINE2), also named as protease nexin-1 (PN-1), is a single chain glycoprotein with a molecular weight of 45-50 kDa, which acts as a secreted serine protease inhibitor. It is overexpressed in various cancer types and is involved in tumor formation (1-3). SERPINE2 has been demonstrated to play vital roles in the progression of papillary TC (4). SERPINE2 expression is closely associated with the poor survival of patients with gastric cancer, and silencing SERPINE2 inhibits the migration and invasion of gastric cancer cells (2).

SERPINE2 enables primary tumor cells to form a vascular-like network in a variety of cancer tissues, including lung, brain, head and neck and breast cancer (5-8). Although increased expression of SERPINE2 has been demonstrated in papillary thyroid carcinoma tissues (4), its role in the pathogenesis of TC cells is still unknown. Epidermal growth factor receptor (EGFR), the product of proto-oncogene CerB1, is a transmembrane glycoprotein with tyrosine kinase activity that is commonly expressed in human epithelial cells. EGF and EGFR are highly expressed in TC (9), and EGF/EGFR is closely associated with the migration and invasiveness of TC cells (10).

Both EGF and its receptor partake in the pathogenesis of multiple carcinomas, thus constituting attractive targets for molecular therapy. Previous studies have demonstrated that EGF and EGFR are implicated in the invasion and migration of thyroid tumors, and that PN-1 can form a positive feedback with EGF/EGFR signaling to promote the biological activity of breast cancer cells (11-13). Therefore, the present study aimed to explore whether SERPINE2 could form positive feedback signals with EGF/EGFR, and participate in the proliferation, invasion and migration of papillary thyroid carcinoma.

Materials and methods

Clinical samples. Clinical specimens were collected from 30 patients diagnosed with papillary thyroid carcinoma at the Central Hospital of Wuhan (Wuhan, China) between July 2018 and October 2019. Informed consent was obtained from each patient, and the present study was approved by the

Human Ethics Committee of the Central Hospital of Wuhan. Cancerous and adjacent normal tissues were obtained after surgical resection, and the specimens were immediately stored at -80˚C. Adjacent normal tissue samples 2 cm away from the cancerous tissue were collected. TC tissue specimens were pathologically confirmed to be papillary TC.

Cell lines. Normal human thyroid epithelial cells (Nthy-ori 3-1), human anaplastic thyroid carcinoma cell lines (8505C, SW1736 and HTH83), and a human papillary thyroid carcinoma cell line (TPC-1; all purchased from American Type Culture Collection) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 g/ml). The medium was changed once every 24 h. After adherent cell growth, 0.25% trypsin was used for digestion and passaging. EGF (10 ng/ml; cat. no. SRP3027; Sigma-Aldrich; Merck KGaA) or AG1478 (10 μM; cat. no. T4182; Sigma-Aldrich; Merck KGaA) was used to treat the cells at 37˚C for 24 or 48 h.

Reverse transcription-quantitative PCR (RT-qPCR). In the first experiment, total RNA of Nthy-ori 3-1, HTH83, 8505C, SW1736 or TPC-1 cells was respectively extracted with TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. In the following experiments, TPC-1 cells were used. Following the experimental treatments, the total RNA of TPC-1 cells was extracted with TRIzol. Then, RNA was reverse transcribed into cDNA at 37˚C for 15 min and 95˚C for 5 min using PrimeScript™ RT Reagent Kit (Takara Bio, Inc.). LightCycler® 480 software (Roche Diagnostics) was employed to analyze cDNA in the LightCycler 480 RT-qPCR instrument (Roche Diagnostics) with SYBR® Premix Ex Taq™ (Takara Bio, Inc.).

The qPCR conditions included initial denaturation for 10 min at 95˚C and subsequently 40 cycles at 95˚C for 10 min and 60˚C for 20 sec. GAPDH mRNA was used as the internal reference. SERPINE2 forward, 5'-AATGAAACCAGGGATATGATTGAC-3' and reverse, 5'-TTGCAAGATATGAGAAACATGGAG-3'; GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'. The relative levels of SERPINE2 mRNA were calculated using $2^{-\Delta\Delta Cq}$ method (14).

Western blotting. TPC-1 cells were seeded into a 6-well plate ($1x10^6$/well). Following the experimental treatments, the culture medium was discarded and the cells were washed twice with pre-cooled PBS. The tissue or cells were lysed with lysis buffer (Beyotime Institute of Biotechnology) on ice for 30 min. The proteins were quantified using the BCA method (BCA Protein Assay kit; cat. no. ab102536; Abcam). The proteins (50 μg) were separated via 10% SDS-PAGE, then transferred to PVDF membranes and blocked with 5% skimmed milk powder at room temperature for 2 h. Subsequently, the following primary antibodies were added and incubated at 4˚C overnight: Anti-SERPINE2 (1:1,000; cat. no. ab154591; Abcam), anti-Ki67 (1:500; cat. no. sc-23900; Santa Cruz Biotechnology, Inc.), anti-proliferating cell nuclear antigen (1:500; PCNA; cat. no. sc-56; Santa Cruz Biotechnology, Inc.), anti-MMP2 (1:1,000; cat. no. ab92536; Abcam), anti-MMP9 (1:1,000; cat. no. ab76003; Abcam), anti-Bcl-2 (1:1,000; cat. no. ab32124; Abcam), anti-Bax (1:1,000; cat. no. ab32503; Abcam), anti-cleaved caspase-3 (1:500; cat. no. ab32042; Abcam), anti-caspase-3 (1:500; cat. no. ab13847; Abcam), anti-EGF (1:1,000; cat. no. ab184265; Abcam), anti-phosphorylated (p)-EGFR (1:1,000; cat. no. ab40815; Abcam) and anti-EGFR (1:1,000; cat. no. ab52894; Abcam). Next, the horseradish peroxidase-conjugated secondary antibody (10,000; cat. no. ab97040; Abcam) was applied and incubated at 37˚C for 1 h. The bands were visualized using Super ECL Detection Reagent kit (Shanghai Yeasen Biotech Co., Ltd.). The grey value of the protein bands was analyzed using ImageJ 1.46r software (National Institutes of Health).

Plasmid transfection. TPC-1 cells were seeded into a 24-well plate ($1x10^4$ cells/well), when cell growth reached 65-75%, the cells were transfected using TurboFect™ reagent (Thermo Fisher Scientific, Inc.) as per the manufacturer's protocols. Additionally, SERPINE2 overexpression plasmids or empty plasmids (1 μg), and SERPINE2 silencing plasmids or its negative control (1 μg scramble shRNA) were constructed by Shanghai GenePharma Co., Ltd., and were dissolved in 25 μl serum-free medium and thoroughly mixed. The mixture was then added to the culture medium, and after 6 h the culture medium was replaced, and the culture was incubated at 37˚C for 24 h for further experiments. Next, the cells were treated with AG1478 at 37˚C for 48 h.

MTT assay. TPC-1 cell concentration was adjusted to $3x10^4$ cells/ml and seeded into 96-well plates. A cell suspension of 200 μl was added to each well for culture at 37˚C with 5% CO_2 for 24, 48 and 72 h. Next, MTT reagent (10 μl; cat. no. C0009; Beyotime Institute of Biotechnology) was added to the cells and incubated for 4 h. A 150-μl volume of DMSO was added to each well to dissolve the crystals. After 10 min, the absorbance value was detected at 490 nm and the cell proliferation rate was calculated.

Colony formation assay. In total, 500 TPC-1 cells were seeded into a 6-well culture plate, and fixed with 4% paraformaldehyde at room temperature for 15 min and stained with crystal violet (Shanghai Yuanye Bio-Technology Co., Ltd.) at room temperature for 15 min. The number of clones containing >50 cells were counted under a light microscope.

Wound healing and Transwell Matrigel™ assays. TPC-1 cells were grown in 6-well plates ($6x10^4$ cells/ml). When the cells reached confluence, scratches were made with a 10-μl tip pipette perpendicular to the plate, and the width of each scratch was as close as possible. The cell culture medium was removed, and the plates were rinsed with PBS three times to remove cell fragments and serum-free medium was added. The experiment was repeated three times.

The invasion of TPC-1 cells was detected using Transwell chambers (EMD Millipore). Matrigel solution (50 μl) was used to pre-coat upper chamber at 37˚C for 5 h. TPC-1 cells ($2x10^6$ cells/ml) were subsequently seeded into the upper chamber of the Transwell invasion chamber (500 μl). DMEM

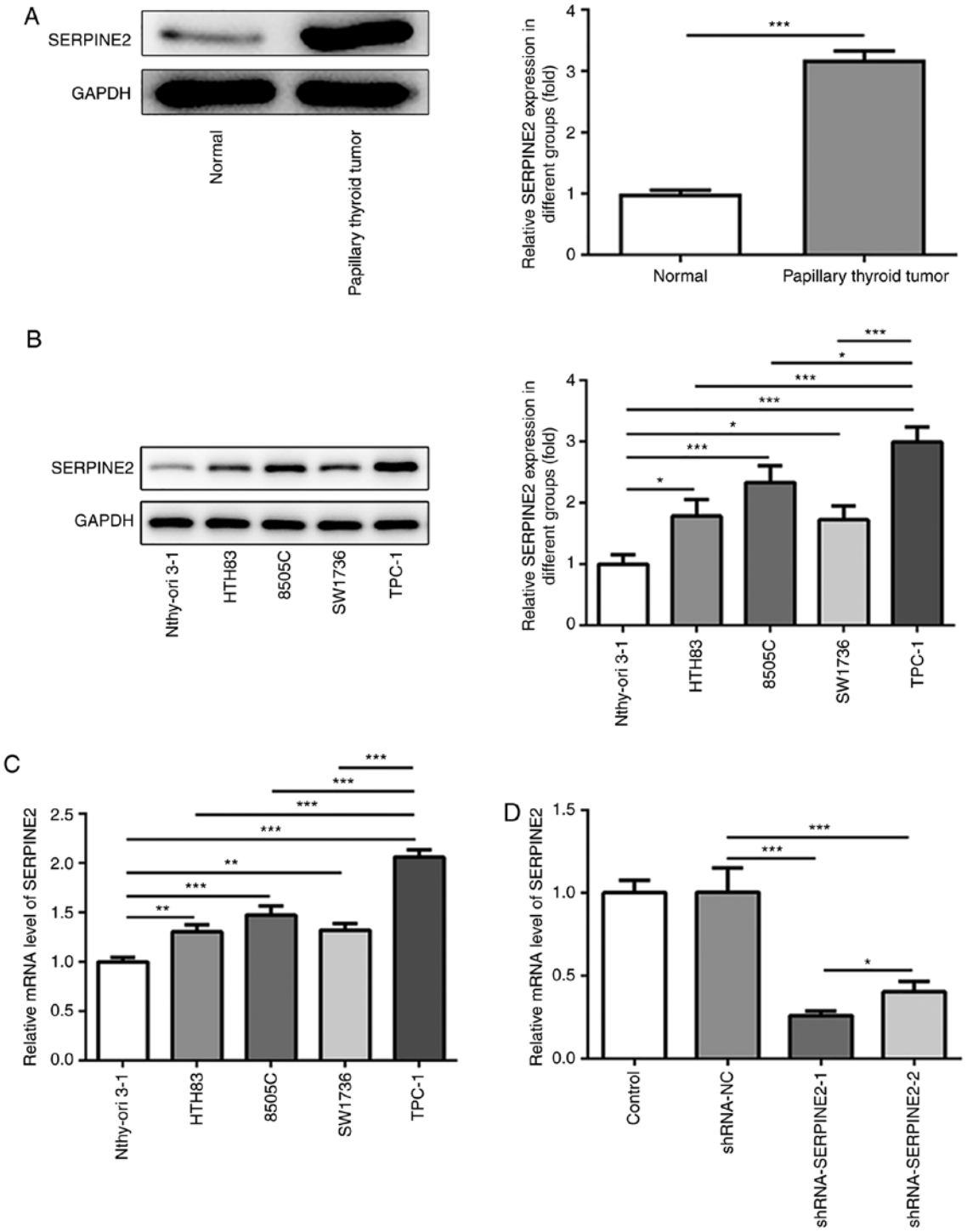

Figure 1. SERPINE2 expression is upregulated in papillary thyroid tumor tissue and cells. (A) Protein expression of SERPINE2 in thyroid cancer tissue and adjacent normal tissue were detected by western blotting. (B) Western blotting was performed to analyze the expression of SERPINE2 in Nthy-ori 3-1, HTH83, 8505C, SW1736 and TPC-1 cells. (C) RT-qPCR assay was employed to detect the mRNA expression of SERPINE2. (D) RT-qPCR was performed to measure the expression of SERPINE2 in TPC-1 cells following transfection with shRNA-SERPINE2-1 and shRNA-SERPINE2-2 plasmids. *P<0.05, **P<0.01, ***P<0.001. SERPINE2, serpin peptidase inhibitor clade E member 2; RT-qPCR, reverse transcription-quantitative PCR; shRNA, short hairpin RNA; NC, negative control.

medium containing 5% FBS (600 μl) was added to lower chamber. After cells were cultured for 24 h, the upper chamber was removed, and the non-invasive cells were removed from the substrate membrane. After fixing with 40% paraformaldehyde for 15 min at room temperature, cells were stained with 0.1% crystal violet for 30 min at room temperature, and then cells were washed with PBS three times before images were observed under a fluorescence microscope.

TUNEL staining. TPC-1 cell concentration was adjusted to 2x10^{6} cells/ml and then seeded cells into 6-well plates. After experimental treatment, cells were washed using PBS three times and then fixed with 4% paraformaldehyde for 1 h at room temperature.

Cell sections were prepared and soaked with xylene twice. Next, the sections were soaked and washed with a gradient of ethanol solutions. The TUNEL reaction mixture (50 μl) was

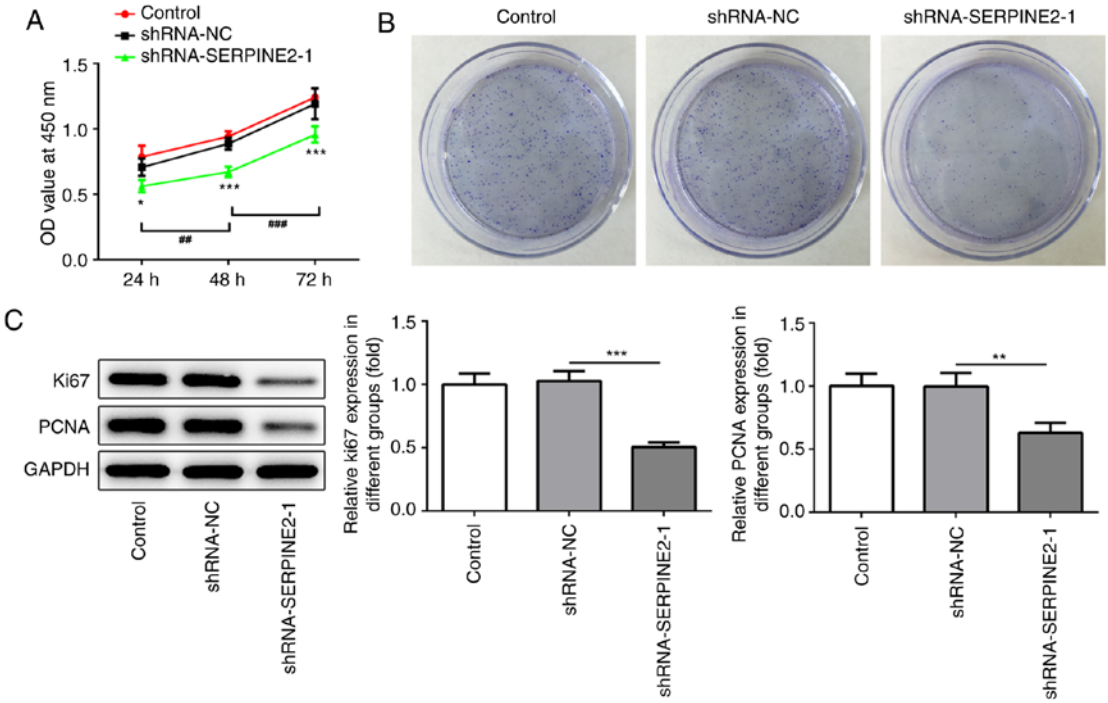

Figure 2. SERPINE2 silencing suppresses the proliferation of TPC-1 cells and the expression of Ki67 and PCNA. (A) MTT assay was performed to analyze cell proliferation. *P<0.05, ***P<0.001 vs. shRNA-NC; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. 48 h. (B) SERPINE2 silencing notably reduced clone formation. (C) SERPINE2 silencing significantly decreased Ki67 and PCNA expression, as detected by western blotting. **P<0.01, ***P<0.001. SERPINE2, serpin peptidase inhibitor clade E member 2; shRNA, short hairpin RNA; NC, negative control; PCNA, proliferating cell nuclear antigen.

prepared and added to the sections for incubation in dark for 1 h at 37˚C. The nucleus was stained with DAPI in the dark for 5 min. The excess DAPI was washed with PBS four times. The slides were sealed with anti-fluorescence quenching solution and observed under a fluorescence microscope. A total of six fields of view was randomly selected.

Statistical analysis. GraphPad Prism 8.0 software (GraphPad Software, Inc.) was used for data analysis. Data are expressed as the mean ± standard deviation. One-way ANOVA was used for comparison between multiple groups, followed by Tukey's test. All experiments were repeated at least three times. P<0.05 was considered to indicate a statistically significant difference.

Results

SERPINE2 levels are significantly increased in TC cells. To investigate the role of SERPINE2 in TC, TC and adjacent normal tissues were collected to detect the protein expression of SERPINE2 via western blotting. Increased protein expression of SERPINE2 was observed in TC tissue compared with those in the normal group (Fig. 1A). Next, the expression of SERPINE2 in Nthy-ori 3-1, HTH83, 8505C, SW1736 and TPC-1 cell lines was evaluated by western blotting and RT-qPCR (Fig. 1B and C). The results demonstrated that, compared with that in normal thyroid epithelial cells, there was a significant increase in the expression of SERPINE2 in HTH83, 8505C, SW1736 and TPC-1 cells, suggesting the possible oncogenic role of SERPINE2 in TC. Moreover, SERPINE2 showed the highest expression in TPC-1 cells. Therefore, TPC-1 cells were used in subsequent experiments (Fig. 1B and C). To investigate the loss of function of SERPINE2 in TPC-1 cells, SERPINE2 expression was silenced using shRNA. The knockdown effect of shRNA-SERPINE2-1 on SERPINE2 expression was stronger than that of shRNA-SERPINE2-2 (Fig. 1D). Thus, shRNA-SERPINE2-1 was employed to silence SERPINE2 expression in subsequent experiments.

SERPINE2 silencing significantly decreases cell proliferation and clone formation. The functions of SERPINE2 on TPC-1 cell proliferation and clone formation were analyzed through silencing SERPINE2 expression (Fig. 2A and B). The MTT assay revealed that the silencing of endogenous SERPINE2 suppressed the proliferation and clone formation of TPC-1 cells. In addition, western blotting revealed that SERPINE2 knockdown significantly reduced the expression of the proliferation makers Ki67 and PCNA (Fig. 2C).

SERPINE2 knockdown suppresses the metastatic potential of TPC-1 cells and promotes apoptosis. To investigate whether MBZ could inhibit the metastatic potential of TC cells, TPC-1 cells transfected with shRNA-SERPINE2-1 were used. The migration and invasion of TPC-1 cells were significantly suppressed when SERPINE2 was silenced (Fig. 3A). Furthermore, MMP2 and MMP9 expression levels were also significantly reduced by knockdown of SERPINE2 (Fig. 3B). Next, the present study explored whether SERPINE2 knockdown regulated cell apoptosis. The results of TUNEL staining showed that cell apoptosis was notably increased (Fig. 3C). In addition, the expression of the anti-apoptotic protein Bcl-2 was significantly

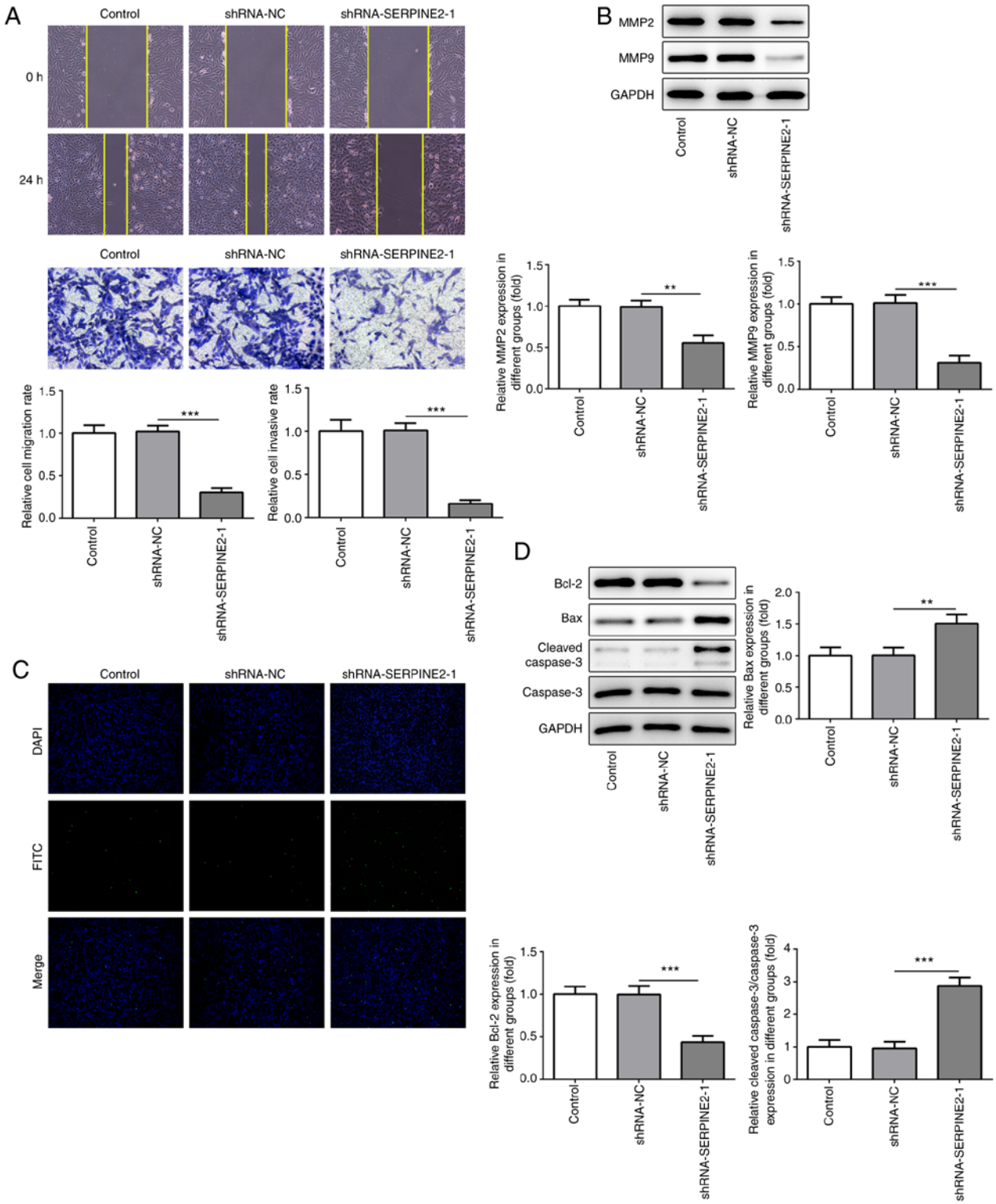

Figure 3. SERPINE2 silencing suppresses the migration and invasion while promoting apoptosis in TPC-1 cells. (A) SERPINE2 silencing significantly reduced TPC-1 cell migration and invasion, as determined through wound healing and Transwell assays. (B) MMP2 and MMP9 expression levels were detected by western blotting. (C) TUNEL staining was performed to analyze cell apoptosis. (D) The expression levels of apoptosis-related proteins were examined via western blotting. **P<0.01, ***P<0.001. SERPINE2, serpin peptidase inhibitor clade E member 2; shRNA, short hairpin RNA; NC, negative control.

decreased, whereas that of the pro-apoptotic proteins Bax and cleaved caspase-3 was significantly increased (Fig. 3D).

SERPINE2 forms a positive feedback loop with EGF/EGFR in TPC-1 cells. According to a previous study, there is a feedback association between SERPINE2 and EGF/EGFR (12). The expression levels of EGF and p-EGFR were significantly reduced by SERPINE2 knockdown (Fig. 4), which suggested that SERPINE2 regulated EGF/EGFR signaling. Next, EGF (100 ng/ml) or the specific tyrosine kinase inhibitor AG1478 (10 μM) were added to stimulate the cells for 24 h or 48 h. EGF significantly increased the expression of SERPINE2 in

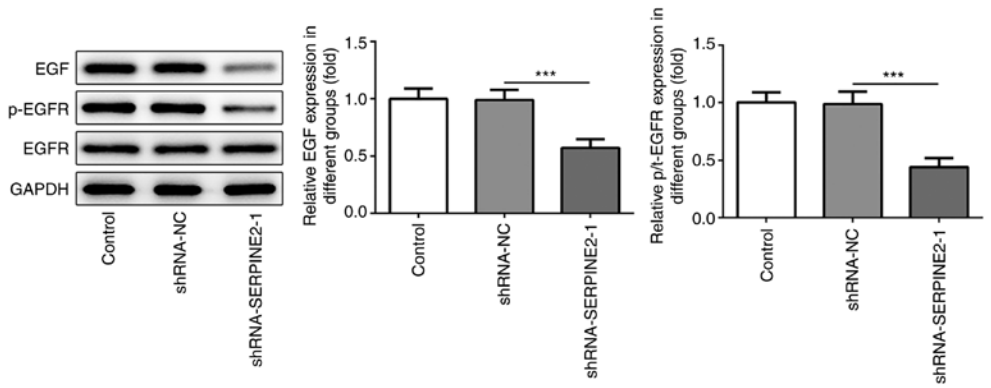

Figure 4. SERPINE2 knockdown significantly decreases EGF and p-EGFR expression. ***P<0.001. SERPINE2, serpin peptidase inhibitor clade E member 2; shRNA, short hairpin RNA; NC, negative control; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; p-, phosphorylated.

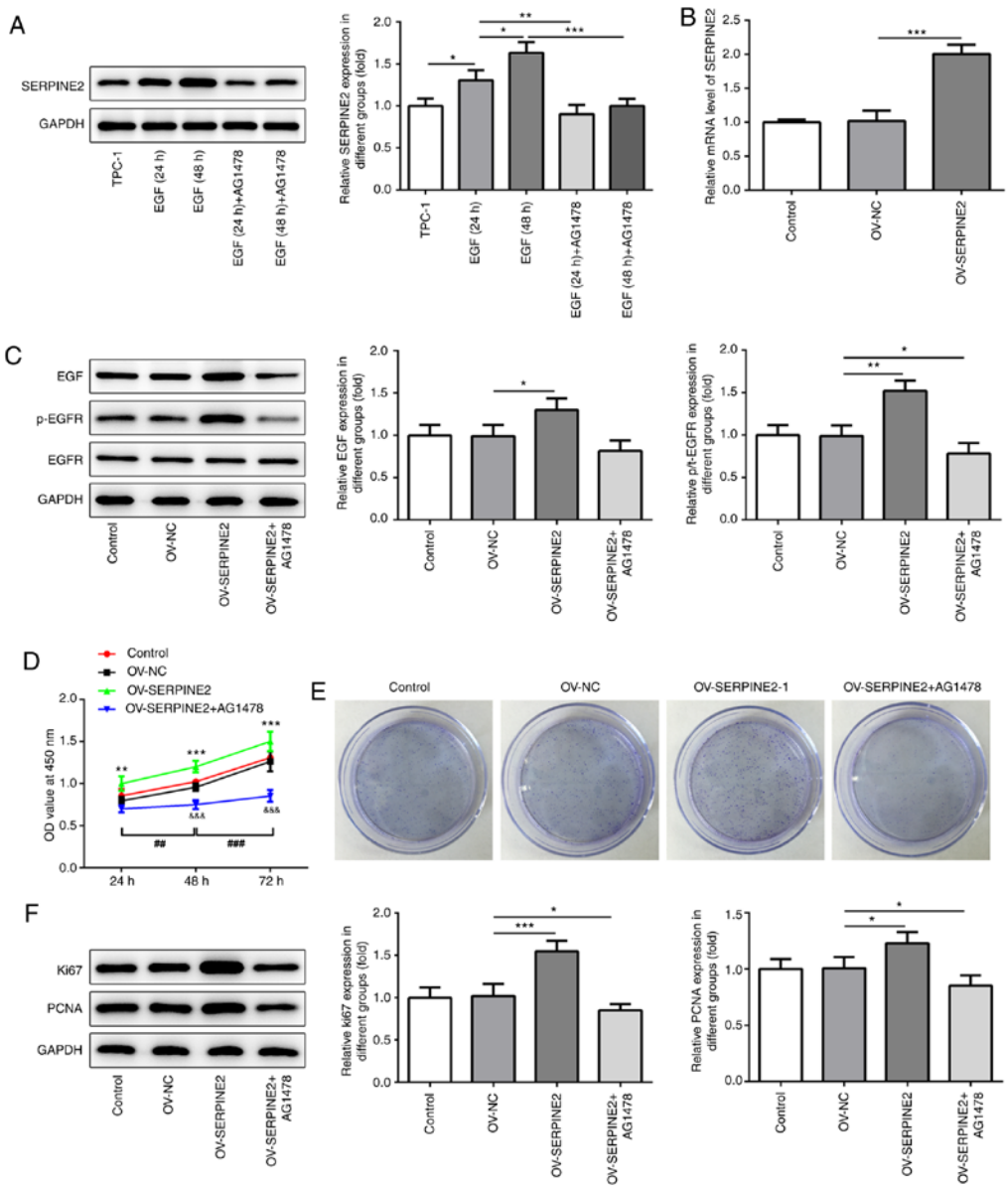

Figure 5. AG1478 inhibits the effects of SERPINE2 overexpression. (A) Western blotting and (B) reverse transcription-quantitative PCR demonstrated that EGF/EGFR signaling regulated SERPINE2 expression, as indicated by the addition of EGF and the EGFR inhibitor AG1478. (C) AG1478 counteracted the effects of SERPINE2 overexpression on inducing the expression of EGF and p-EGFR. AG1478 blocked the effects of AERPINE2 overexpression on the proliferation of TPC-1 cells, which was analyzed using (D) MTT assay. **P<0.01, ***P<0.001 vs. OV-NC; &&&P<0.001 vs. OV-SERPINE2; ##P<0.01, ###P<0.001 vs. 48 h. (E) Clone formation assays. (F) SERPINE2 overexpression regulated the expression of Ki67 and PCNA via EGF/EGFR signaling. *P<0.05, **P<0.01, ***P<0.001. SERPINE2, serpin peptidase inhibitor clade E member 2; OV, overexpression plasmid; NC, negative control; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; p-, phosphorylated; PCNA, proliferating cell nuclear antigen.

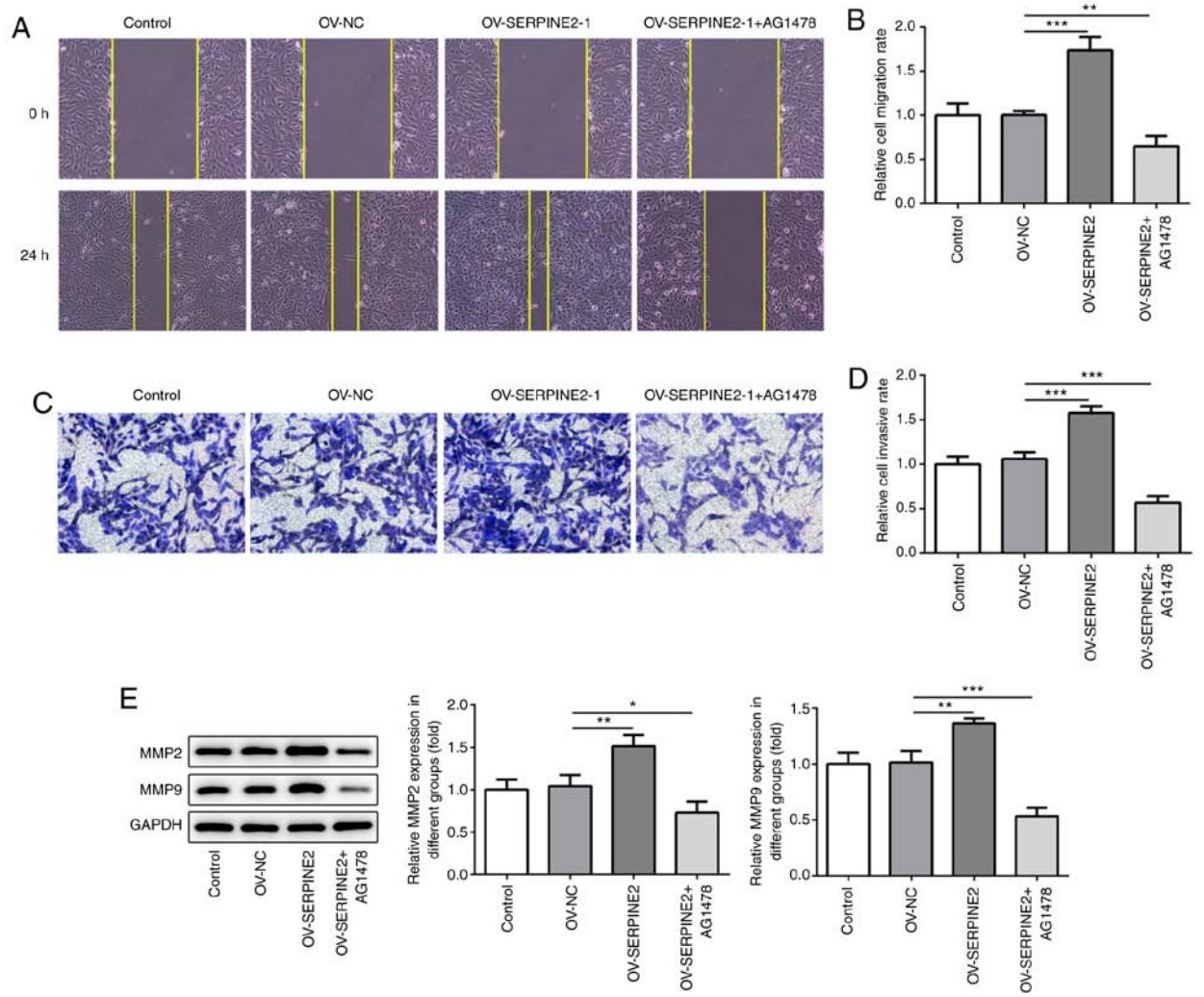

Figure 6. AG1478 inhibits the migration and invasion of TPC-1 cells. (A and B) Wound healing and (C and D) Transwell assays were performed to analyze the migratory and invasive abilities of TPC-1 cells. (E) MMP2 and MMP9 expression levels were determined following the overexpression of SERPINE2 and inhibition of EGFR using AG1478. *P<0.05, **P<0.01, ***P<0.001. SERPINE2, serpin peptidase inhibitor clade E member 2; OV, overexpression plasmid; NC, negative control.

a time-dependent manner (Fig. 5A). The results showed that AG1478 significantly blocked the promoting effects of EGF on the expression of SERPINE2, indicating that EGF/EGFR signaling could also modulate SERPINE2 expression. Next, SERPINE2 was overexpressed through transfection with OV-SERPINE2 plasmids into TPC-1 cells (Fig. 5B). The EGFR inhibitor significantly reversed the effects of SERPINE2 overexpression on the upregulation of EGF and p-EGFR expression (Fig. 5C). The overexpression of SERPINE2 in TPC-1 cells significantly increased cell proliferation, as revealed by the MTT assay (Fig. 5D). However, this effect could be significantly abolished by the EGFR inhibitor AG1478. The same trend was observed in the clone formation assay (Fig. 5E). The aforementioned results implied that SERPINE2 regulated the proliferative ability of TPC-1 cells via EGF/EGFR signaling. As a cell proliferation marker, Ki67 plays a role in the occurrence and development of tumors, and is closely associated with the proliferation, infiltration, metastasis and prognosis of thyroid carcinoma (15-17). As revealed by western blotting, SERPINE2 overexpression enhanced the expression of Ki67 and PCNA, which was reversed by the addition of AG1478 (Fig. 5F).

SERPINE2 modulates the migratory and invasive abilities of TPC-1 cells via EGF/EGFR signaling. To investigate whether SERPINE2 regulated the migratory and invasive abilities of TPC-1 cells wound healing and Transwell assays were performed (Fig. 6A-D). SERPINE2 overexpression significantly increased the migratory and invasive abilities of TPC-1 cells after transfection for 72 h, which could be significantly abolished by inhibiting EGFR. Moreover, MMP2 and MMP9 expression was significantly increased by overexpression of SERPINE2 (Fig. 6E). Simultaneously, AG1478 significantly reversed the effect of SERPINE2 overexpression on MMP2 and MMP9 expression.

Discussion

To the best of our knowledge, the present study was the first to investigate the association between SERPINE2 and EGF/EGFR in TPC-1 cells. A potential feedback loop between SERPINE2 and EGF/EGFR was demonstrated. This study found that SERPINE2 exhibited higher protein expression in TPC-1 cells compared with Nthy-ori 3-1 or other TC cells, indicating that SERPINE2 had a potential

role in TPC-1 cells. It was further found that SERPINE2 could regulate TPC-1 cell proliferation, apoptosis, invasion and migration via EGF/EGFR signaling. Regarding the association between EGF and SERPINE2, a previous study demonstrated that EGF could upregulate PN-1 levels through EGFR/protein kinase C δ type/MEK/ERK (18). PN-1 upregulation could further activate EGF signaling by blocking serine protease HTRA1 (8). In the present study, EGF and p-EGFR levels were significantly decreased when TPC-1 cells were treated with AG1478 following SERPINE2 overexpression. AG1478 was previously used to block the phosphorylation of EGFR (19). Collectively, these results suggested that there is a positive feedback loop between EGF and SERPINE2.

The introduction of exogenous SERPINE2 significantly promoted the proliferation, migration and invasion of TPC-1 cells. In part, these results were similar to previously published data that suggested that SERPINE2 facilitates cell migration and invasion, but has no obvious effects on cell proliferation in gastric cancer cells (2). As observed in the present study, SERPINE2 regulated EGF/EGFR signaling to accelerate cell proliferation in TPC-1 cells. The activation of EGF/EGFR signaling is implicated in the proliferation of TC cells (20). Overall, the present study provided evidence for an underlying mechanism that links SERPINE2 with EGF/EGFR signaling, revealing a positive feedback loop between SERPINE2 and EGF/EGFR. In addition, SERPINE2 silencing notably activated TPC-1 cell apoptosis, and regulated Bcl-2, Bax and cleaved caspase-3 expression. A previous study demonstrated that SERPINE2 functions as an oncogene in endometrial cancer cells and regulates cell apoptosis (21).

Ki67 frequently exhibits increased expression in patients with anaplastic carcinoma and malignant nodule tumors (22,23). In the present study, it was observed that SERPINE2 knockdown reduced Ki67 and PCNA expression levels. Ki67 is a well-known cell proliferation marker in anaplastic thyroid carcinoma cells and is associated with the induction of mitotic arrest (24). PCNA, which is an auxiliary protein of DNA polymerase δ, plays an important role in the initiation of cell proliferation (25-27). It is a useful indicator of cell proliferation status, it is closely associated with the clinical stage of TC, and its expression is significantly higher in TC tissues from patients (28). Furthermore, the expression of PCNA is higher in thyroid nodules than in normal tissues (29). In the present study, the expression of Ki67 and PCNA was significantly reduced by the silencing of SERPINE2. Therefore, SERPINE2 knockdown significantly reduced the proliferative ability of TPC-1 cells. Besides, PN-1 has been revealed to regulate cell invasion and migration through MMP2/9, which degrades the extracellular matrix in C6 glioma cells (30). The present our study also identified a similar function of PN-1 in the regulation of MMP2/9 expression, and validated the involvement of EGF/EGFR in this process. Furthermore, this study also found that an EGFR inhibitor could block the effects of SERPINE2 overexpression on cell proliferation.

Taken together, the present study confirmed that SERPINE2 formed a positive feedback with EGF/EGFR to regulate the proliferation, invasion and migration of TPC-1 cells, which may provide potential new ideas for identifying novel therapeutic targets of papillary thyroid tumors.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HC, BH and XS conceived and designed the study, collected, analyzed and interpreted the data, and revised the manuscript. HC wrote the manuscript. All authors read and approved the final manuscript. HC and XS confirm the authenticity of the raw data.

Ethics approval and consent to participate

Informed consent was obtained from each patient, and the present study was approved by the Human Ethics Committee of the Central Hospital of Wuhan (Wuhan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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