Mechanism of RET gene mediated EGFR signaling pathway on epithelial-mesenchymal transition, proliferation and apoptosis of papillary thyroid carcinoma cells

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Abstract. – OBJECTIVE: To explore the mechanism of RET gene mediated EGFR signaling pathway on the epithelial-mesenchymal transition (EMT), proliferation and apoptosis of papillary thyroid carcinoma (PTC) cells.

PATIENTS AND METHODS: PTC TPC-1 cells and human normal thyroid follicular epithelial cells Nthy-ori 3-1 were collected to identify the expression of RET in PTC. Seven groups were divided according to different transfection protocols, including blank group, negative control group, si-RET group, oe-RET group, AG-490 group, NSC 228155 group, and si-RET + NSC 228155 group. After transfection, qRT-PCR was used to identify whether the transfection was successful or not. qRT-PCR and Western blot were performed to detect the mRNA and protein expressions of RET, EGFR signaling pathway related genes, and EMT related genes. Cell migration, invasion, proliferation and apoptosis abilities were further detected by CCK8, cell scratch, transwell and flow cytometry assays, respectively.

RESULTS: RET gene was highly expressed in PTC cells ($p<0.05$). Compared with blank group, oe-RET group and NSC 228155 group had activated EGFR signaling pathway manifesting in the increased expression of EGFR, p-Src, p-FAK, accelerated EMT showing in the increased expression of N-cadherin and Vimentin expression, but decreased E-cadherin expression, increased cell migration, invasion and proliferation, while decreased apoptosis (all $p<0.05$); si-RET group and AG-490 group had inhibited activation of EGFR signaling pathway, suppressed EMT, decreased cell migration, invasion and proliferation, while increased apoptosis (all $p<0.05$); while no evident difference was found in si-RET + NSC 228155 group (all $p>0.05$). Meanwhile, compared with si-RET group, si-RET + NSC 228155 group showed activated EGFR signaling pathway, accelerated EMT, increased abilities of cell migration, invasion and proliferation, while decreased apoptosis (all $p<0.05$).

CONCLUSIONS: RET gene is highly expressed in PTC acting as an oncogene. Silencing RET gene expression may inhibit the invasion and promote the apoptosis of PTC cells by inhibiting the activation of EGFR signaling pathway and mediating the process of EMT. It suggests that RET may offer the possibility of a promising therapeutic target for the treatment of PTC on the basis of the explored mechanism.

Key Words:

RET gene, EGFR signaling pathway, Papillary thyroid carcinoma, Epithelial-mesenchymal transition, Proliferation, Apoptosis.

Introduction

Cancer is a serious threat to human health. Thyroid cancer (TC) is the most common head and neck cancer[1]. TC is the fastest-growing malignant cancer worldwide in recent decades, and there is a similar trend in China[2]. Papillary thyroid carcinoma (PTC) is the most common pathological type in TC[3]. Surgery, chemotherapy, radiotherapy and other therapeutic approaches have achieved evident clinical therapeutic effects. However, recurrence and metastasis are still the main causes of death of PTC patients, showing high cervical lymph node metastasis and local recurrence rate[4,5]. PTC is one of the malignant tumors with

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features of high malignancy, high mortality, great difficulty in diagnosis and poor prognosis. Therefore, it is of great clinical value to further reveal the changes of biological behaviors of PTC cells and related driving mechanism for effectively controlling the recurrence and metastasis of PTC patients and prolonging the total survival period.

Epidermal growth factor receptor (EGFR) is one of the ErbB family members of type I receptor, whose ligands include EGF, TGF-α, etc.[6]. When EGFR binds to the ligand, it activates the tyrosine kinase activity in the receptor, resulting in the phosphorylation of its tyrosine kinase residues, activation of multiple downstream signaling pathways, and regulation of cell proliferation and differentiation[7]. EGFR has been detected to be highly expressed in PTC, which has been discovered to be related to the increase of gene copy number and the high expression of its ligands[8,9]. In addition, RET gene plays an important role in the screening and diagnosis of medullary TC[10]. Besides, KIF5B-RET fusion gene was associated with EGFR mutation in lung adenocarcinoma[11]. It is thus speculated in this study that RET might be involved in the development of PTC by regulating the activation of EGFR pathway. Meanwhile, epithelial-mesenchymal transition (EMT) refers to the transformation from epithelium to stromal cells, which gives cells the ability to transfer and invade[12]. The progression of EMT involves in embryo development, inflammation and wound healing, as well as tumor progression[13]; besides, it plays an important role in the occurrence and metastasis of cancer[14-16]. EMT can be characterized by the decrease of E-cadherin as epithelial phenotype, the increase of N-cadherin and vimentin as interstitial phenotypes[17].

Owing to an insufficient study of the role of RET gene expression and EGFR signaling pathway in PTC, our research group carried out a research to explore the mechanism of RET gene mediated EGFR signaling pathway on the EMT, proliferation and apoptosis of PTC.

Materials and Methods

Cell Culture

The frozen PTC TPC-1 cells purchased from Nanjing COBIOER Biotechnology Co., Ltd. (Jiangsu, China) were rapidly placed in a 37°C water bath and gently shaken to melt rapidly. All the contents of the cryopreservation tube were transferred into a 15 ml centrifuge tube, followed by the addition of 5 ml of the corresponding complete medium (DMEM high-glucose medium) slowly, with gentle shaking of the centrifuge tube with hand while dropping. After suspension by blowing gently, cells were centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. Another amount of 5 ml of the same complete medium was added again, followed by blowing repeatedly to suspend the cells, which was then transferred to T25 cell culture flask, and placed in the CO_2 incubator at 37°C. The next day, the growth of the cells was observed, and cell passage was performed on according to the degree of confluence and growth speed of the cells. Cells were subcultured when the confluence of cells reached about 80%. Cells in good growth and at the logarithmic stage of growth were selected for subsequent experiment. Meanwhile, following a full blowing and mixing of the cell suspension to be counted, cell counts were calculated according to the following formula: the number of living cells (cells/ml) = 3 × total cells - dead cells for cell count.

At the same time, normal human thyroid follicular epithelial cells Nthy-ori 3-1 (Shanghai GaiNing Biotechnology Co., Ltd., Shanghai, China) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS) at 37°C, and the culture fluid was changed once every two days. Cell passage was also conducted when the confluence of cells reached about 80%. The expression of RET in PTC was identified by testing in PTC TPC-1 cells and in Nthy-ori 3-1 cells.

Cell Transfection and Grouping

In view of the steps of transfection, it was performed strictly in accordance with the manual of Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). To be specific, taking the 6-well plate as an example, all the quantity and volume were calculated according to each well by referring to the transfection scale. On the day before transfection, cells were inoculated into the 6-well plate and 2 ml of antibiotic-free medium was added to achieve 50-70% of confluence. An amount of 125 µL Opti-MEM culture medium (Invitrogen, Carlsbad, CA, USA) was used to dilute and mix fully with 5 µL of Lipofectamine 3000 reagent (tube A). Then, another amount of 125 µL Opti-MEM medium was taken to dilute 2500 ng target plasmid or 2500 ng control plasmid, followed by the addition of 5 µL P3000 reagent (tube B). DNA diluted in tube B

was then added into tube A at the ratio of 1:1 ratio to mix well, followed by incubation at room temperature for 5 min. Corresponding transfection solution was added into each well, after which the tube was shaken by cross-shaped method, and incubated in a routine incubator at 37°C. According to the requirement of the experiment, 24-96 h after transfection, the experimental cells were divided into 7 groups: blank group (cells without any treatment), negative control group (transfection with empty vector), si-RET group (transfection with si-RET plasmid), oe-RET group (transfection with oe-RET plasmid), AG-490 group (specific EGFR inhibitor treatment), NSC 228155 group (specific EGFR activator treatment), and si-RET + NSC 228155 group (transfection with si-RET plasmid combined with specific EGFR activator treatment). After 48 h of transfection, the total RNA was extracted, and the cDNA was obtained by reverse transcription. SYBR Green real-time fluorescent quantitative PCR was used to detect the transfection outcome. Each sample was set with three parallel replicates.

qRT-PCR Detection of RET, E-cadherin, N-cadherin, Vimentin and EGFR

The total RNA was extracted from the treated cells in each group by EZ-press Cell to Ct Kit (Invitrogen, Carlsbad, CA, USA). Forty-eight h after the cells were transfected into the culture plate, the cell culture medium was discarded, and phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA) was used to wash cells gently twice to ensure that the culture medium and phosphate-buffered saline (PBS) were sucked clean. Cell Lysis Buffer (80 μL/100,000 cells) was added to lyse cells, followed by gent blowing and sucking for 5 times, and reaction at room temperature for 5 min. After the cell lysis was completed, it was blown and sucked 5 times again to ensure that the cell completely split and fell off the culture plate. The lysate was transferred into a 1.5 ml RNase free centrifuge tube and placed on ice for reverse transcription within 30 min. After the preparation of the reverse transcription reaction system, the cell lysate was added for reverse transcription reaction (at 42°C for 25 min), and then placed on ice. The reverse transcription product was diluted 5 times (20 μL reverse transcription product was added with 80 μL water) before being used as the template of PCR. After the PCR reaction system was prepared on ice, subsequent steps were continued, including reaction for 30 s at 95°C, 5 s at 95°C, and 30 s at 60°C (40 cycles). The primers of β-actin (used as the reference gene), RET, E-cadherin, N-cadherin, Vimentin and EGFR genes were designed and synthesized by Shanghai Sagon, with corresponding primers described in Table I. The multiple ratio relationship of the target genes in the experimental and control groups were realized by using the $2^{-\Delta\Delta Ct}$ method. The experiment was repeated three times, and three replicates were set each time.

Western Blot Detection of RET, E-cadherin, N-cadherin, Vimentin, EGFR, p-Src and p-FAK

Experimental cells were cultured to reach the confluence of 80-90% in good condition without evident dead cells. After 48 h of transfection, the cells in the 6-well plate were lysed with radioimmunoprecipitation assay (RIPA) lysate containing PMSF. The amount of lysate was about 200-300 μL per well. The cells were lysed on ice for 30 min, during which the cell culture dishes were shaken with oscillators at regular intervals to be fully lysed. The protein was collected by cell scraping, and the protein concentration was

Table I. Primer sequences of RET, E-cadherin, N-cadherin, Vimentin, EGFR, and β-actin for qRT-PCR.

Genes	Primer sequences
RET	F: 5'-CTGGCTAAGGTGTTCCCCTG-3' R: 5'-ACTGCTTTTCTCAAAGGGCA-3'
E-cadherin	F: 5'-CTCGGCCTGAAGTGACTGTAAC-3' R: 5'-CAGCAACGTGTTTCTGCATTTC-3'
N-cadherin	F: 5'-GTGCCATTAGCCAAGGGAATTCAGC-3' R: 5'-GCGTTCCTGTTCCACTCATAGGAGG-3'
Vimentin	F: 5'-GAAATGGCTCGTCACCTTCG-3' R: 5'-CCAGATTAGTTTCCCTCAGGTTCA-3'
β-actin	F: 5'-CATGTACGTTGCTATCCAGGC-3' R: 5'-CTCCTTAATGTCACGCACGAT-3'

measured by ultra-micro UV spectrophotometer (Shanghai Spectrum Instruments Co. Ltd., Shanghai, China; in order to ensure the accuracy, each sample was repeated for 2-3 times, and the average value was obtained), which was then stored at –20°C for subsequent process. After the separation glue and concentrated glue are prepared, the protein sample was loaded, followed by protein concentration at the positive and negative electrodes with a voltage of 60V, and then adjusted to a voltage of 120V after entering the separation gel. After the bromophenol blue was moved to the bottom of the gel, the protein was transferred to the polyvinylidene difluoride (PVDF) membrane, and 5% milk was placed on the shaking table at room temperature for 1 h. Appropriate amount of Tris-Buffered Saline and Tween-20 (TBST) solution was added to remove the milk on the membrane. In the next step, the diluted rabbit anti-human polyclonal antibodies of RET, E-cadherin, N-cadherin, Vimentin, EGFR, p-Src, p-FAK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were added for cell incubation in the shaking table at 4°C overnight. The membrane with TBST for 3 times in the next day, each time for 10 min. After that, after diluting the IgG of goat anti-rabbit polyclonal antibody IgG with 5% skimmed milk, it was incubated at room temperature and shaken in a shaking table for 1 h, followed by another washing with TBST (3 times, 15 min each). Then, after adding the developer of enhanced chemiluminescence (ECL), the membrane was placed into the darkroom for ECL chemiluminescence development. Finally, the membrane with photographic fixing process completed was washed with tap water and dried, followed by scanning and drawing.

Cell Scratch Assay for the Detection of TPC-1 Cell Migration Ability

Cell scratch test is a simple method to measure cell migration and motility. To be specific in this experiment, prior to the test, on the monolayer of adherent cells cultured *in vitro*, the central area of cell growth was scratched with a micro-pipette head. After removal of the central part of the cell, cells were continued to be cultured to the time set in the experiment (e.g., 48 h). After taking out the cell culture plate, whether the peripheral cells migrated to the central scratch area can be observed to observe the cell migration ability. The migration ability of cells in each group can be determined by the reduction of scratch area between different groups. In this experiment, marker pen was used to draw 5 parallel horizontal lines on the back of the 6-well plate with ruler, and 5×10^5 cells were added into each well, so that it can be paved overnight. The next day, a sterilized ruler and a sterile pipette head were used to make a scratch perpendicular to the horizontal line on the back. Pay attention to the pipette head to be vertical. The cells were washed 3-4 times with PBS and added into serum-free medium, followed by incubation in a 5%CO_2 incubator at 37°C. Sampling was collected at 0 h and 48 h, followed by photography. Using Image J software to analyze the scratch area, the area of scratch reduction was obtained to calculate the migration index = the area of scratch reduction in the experimental group/ that in the control group.

Transwell Assay for the Detection of TPC-1 Cell Proliferation Ability

In the first step of transwell chamber preparation, ECM gel (Sigma-Aldrich, St. Louis, MO, USA) was added to the inner wall of the chamber, before which the ECM gel was taken out of the 20°C refrigerator and put it on the ice for melting. The box of pipette head was placed in the -20°C refrigerator for pre-cooling. Then, the ECM gel and serum-free medium were mixed on ice at a ratio of 1:9, followed by the addition of 40 μL per well to the inner surface of the chamber. Pay attention to remove bubbles, and the mixture was incubated in the incubator for 5 h to make the gel coagulate. Half an hour before the experiment, 70 μL serum-free medium was added to make the gel melt, and the supernatant was removed for standby. The cells were starved for 2 h before preparing cell suspension. After serum starvation, cells were digested with 0.25% trypsin, centrifuged, washed, and then the cell density was adjusted to $1\sim6\times10^5$ cells/mL with serum-free medium. An amount of 200 μL cell suspension (4×10^4 cells/well) and added to the upper chamber of transwell, with 500 μL cells/well complete medium containing 10% serum added to the lower chamber. Pay attention to that there was no bubble between the lower culture medium and the chamber, so as not to affect the chemotaxis of the culture medium. The plates were incubated in incubators for 24 h, followed medium removal in each well and cleaning of the lower surface of the chamber with PBS. The cotton swab was used to gently and repeatedly wipe off the untransferred cells, followed by direct transfer to the lower chamber in 600 μL 4% formalin solution (or methanol) for 60 min of fixation. When fixing, pay attention that

there were no bubbles on the lower surface of the film, otherwise, it was not easy to fall off completely. The chambers were taken out and dried at room temperature. After nuclear staining with hematoxylin for 8 min, samples were washed with water to remove the non-specific binding hematoxylin. Then, eosin was used to stain the cytoplasm for 5 min, followed by rinsing with tap water to remove the non-specific binding eosin. The cells on the lateral side of the chamber were observed with inverted microscopes to take the average value.

CCK-8 Assay for the Detection of TPC-1 Cell Proliferation Ability

According to the CCK-8 reagent (BOSTER Biological Technology, Wuhan, China) instruction, the cells were cultured until the confluence reached 80%. The cells in the logarithmic growth stage of each transfection group were inoculated with 5×10^6 cells per well in different 96-well plates, and the cell density was adjusted to about 1×10^4 cells/well. At the same time, the blank cell group and the zero adjustment group were set up with three replicates. The 96-well plate was cultured in the 5% CO_2 cell incubator at 37 °C for 24 h, 48 h, 72 h and 96 h, respectively. An amount of 10 μL CCK-8 solution was added to the corresponding well 2 h before the end of culture at the corresponding time point and continued to culture for 2 h in the incubator. The absorbance at 450 nm was measured by Microplate Reader, and the cell proliferation curve was drawn. Each experiment needed to be repeated three times.

Flow Cytometry Assay for the Detection of TPC-1 Cell Apoptosis Ability

The cells at the logarithmic growth stage of each transfection group were inoculated into 6-well plates with 5×10^6 cells per well and cultured in 37°C and 5% CO_2 cell incubators. When the cells grew and fused to 90%, cells were washed with PBS three times, digested with trypsin without EDTA, and centrifuged for 10 min at 1,000r/min. The cells were washed with cold PBS once and centrifuged at 1,000r/min for 10 min. After cell washing, 300 μL of staining buffer was added for the re-suspension of cells. Then, a volume of 5 μL AnnexinV-FITC (Beyotime Biotechnology, Shanghai, China) was added to mix well, which was then incubated at room temperature in the dark for 20 min, followed by the addition of 5 μL propidium iodide (PI). The test shall be performed within 1 h.

Statistical Analysis

Using SPSS 21.0 (IBM, Armonk, NY, USA) for statistical analysis in the whole paper, each group should have at least 3 replicates, and each experiment should be repeated at least 3 times. GraphPad Prism 8 was used for drawing. The measurement data were expressed in mean ± standard deviation (SD). The comparison between groups was realized using Student's *t*-test. The comparison among multiple groups adopted one-way analysis of variance (ANOVA). The comparison between two groups used SNK-q test. $p<0.05$ was used as an inspection level of statistical significance.

Results

Cell Transfection Identification

Compared with Nthy-ori 3-1 cells, the expression of RET gene increased in TPC-1 cells (all $p<0.05$), suggesting that RET was highly expressed in PTC. Meanwhile, compared with blank group, there was no significant difference in the expression of RET gene in the negative control group, AG-490 group and NSC 228155 group ($p>0.05$). However, it was significantly higher in oe-RET group, but significantly lower in si-RET group and si-RET + NSC 228155 group (all $p<0.05$) (Figure 1). It suggested that the transfection was successful that was suitable for the following experiments.

mRNA and Protein Expression of RET gene, EGFR and its Downstream Signaling Pathway Factors, E-cadherin, N-cadherin and Vimentin Related to EMT

As shown in Figure 2, compared with blank group, the mRNA and protein expression levels of each gene in the negative control group did not change significantly (all $p>0.05$). The mRNA and protein expression of N-cadherin and Vimentin, EGFR and its downstream Src/FAK signaling pathway related indexes of p-Src and p-FAK increased markedly in oe-RET group and NSC 228155 group, while the expression of E-cadherin mRNA and protein decreased (all $p<0.05$). Meanwhile, the mRNA and protein expression of N-cadherin and Vimentin, EGFR, p-Src and p-FAK decreased remarkably in si-RET group and AG-490 group, while the expression of E-cadherin mRNA and protein increased (all $p<0.05$). In addition, compared with si-RET group, si-RET + NSC 228155 group had increased mRNA and

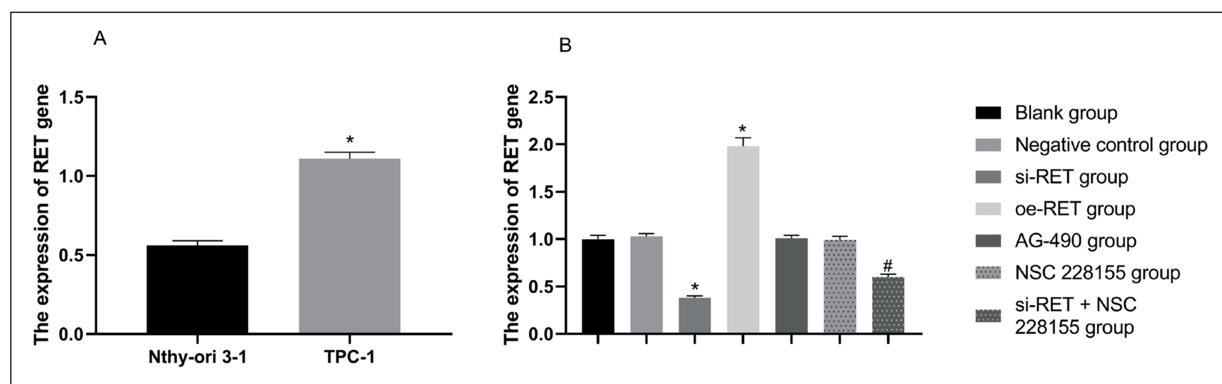

Figure 1. The expression of RET gene in different groups indicated that the transfection was successful. Note: **A**, Expression of RET gene in TPC-1 cells and in Nthy-ori 3-1 cells, *compared with Nthy-ori 3-1 cells, $p<0.05$; **B**, Expression of RET gene in different cell transfection groups, *compared with blank group, $p<0.05$.

protein expression of N-cadherin and Vimentin, EGFR, p-Src and p-FAK, but decreased E-cadherin mRNA and protein expression (all $p<0.05$). However, there was no statistical difference when compared blank group with si-RET + NSC 228155 group ($p>0.05$). In addition, no statistical difference was found between oe-RET group and NSC 228155 group, as well as between si-RET group and AG-490 group (both $p>0.05$).

Experimental Results of Cell Migration and Invasion by Cell Scratch Assay and Transwell Assay

As shown in Figures 3 and 4, compared with the blank group, the ability of migration and invasion of cells in the negative control group did not change significantly ($p>0.05$). oe-RET group and NSC 228155 group had significantly increased migration and invasion of cells (both $p<0.05$), whereas they were significantly decreased in si-RET group and AG-490 group (both $p<0.05$). Meanwhile, compared with si-RET group, si-RET + NSC 228155 group showed increased migration and invasion of cells (both $p<0.05$), but it did not change significantly when compared with the blank group (both $p>0.05$). In addition, no statistical difference was found in the migration and invasion of cells between oe-RET group and NSC 228155 group, as well as between si-RET group and AG-490 group (both $p>0.05$).

Experimental Results of Cell Proliferation by CCK8 Assay

In Figure 5, it could be found that compared with the blank group, the proliferation ability of cells in negative control group did not change significantly ($p>0.05$). There was significant increase in the cell proliferation ability in oe-RET group and NSC 228155 group (both $p<0.05$), which, however, was markedly decreased in si-RET group and AG-490 group (both $p<0.05$). Furthermore, compared with si-RET group, si-RET + NSC 228155 group had increased cell proliferation ability ($p<0.05$); but no significant difference when compared with blank group ($p>0.05$). In addition, no statistical difference was found in the proliferation of cells between oe-RET group and NSC 228155 group, as well as between si-RET group and AG-490 group (both $p>0.05$).

Experimental Results of Cell Apoptosis by Flow Cytometry

No significant difference was found in cell apoptosis between blank group and negative control group ($p>0.05$). There was significantly decreased cell apoptosis in oe-RET group and NSC 228155 group (both $p<0.05$), which it was significantly increased in si-RET group and AG-490 group (both $p<0.05$). Meanwhile, compared with si-RET group, si-RET + NSC 228155 group had decreased cell apoptosis ($p<0.05$); while it had no significant change in si-RET + NSC 228155 group when compared with blank group ($p>0.05$). No significant difference was detected between oe-RET group and NSC 228155 group, as well as between si-RET group and AG-490 group (both $p>0.05$), as shown in Figure 6.

Discussion

At present, there is insufficient research on PTC to fully elucidate its carcinogenesis mechanism.

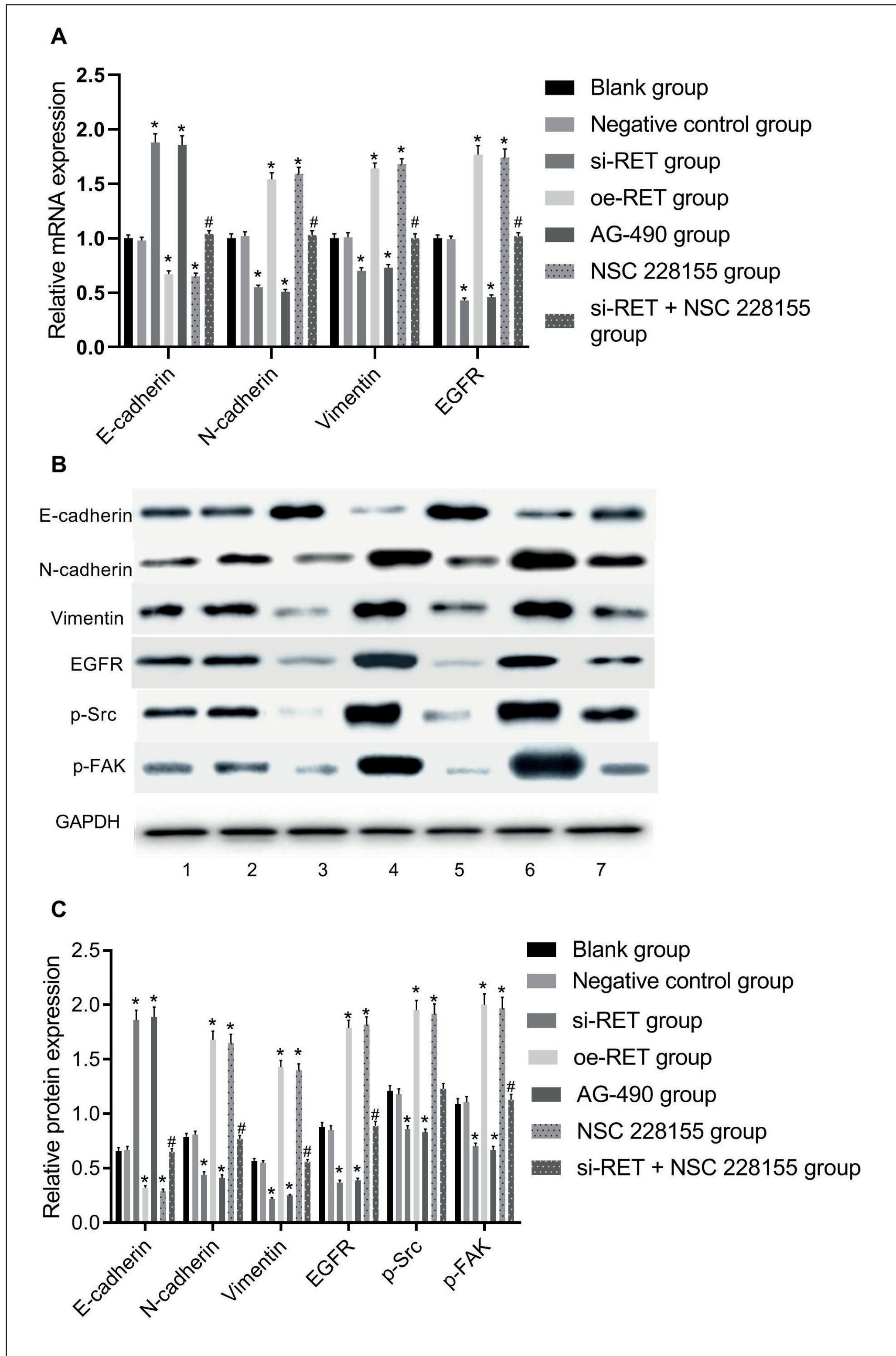

Figure 2. mRNA and protein expression of RET gene, EGFR and its downstream signaling pathway factors, E-cadherin, N-cadherin and vimentin related to EMT. Note: **A**, qRT-PCR detection of mRNA expression of RET gene, EGFR, E-cadherin, N-cadherin and vimentin; **B**, Western blotting detection of protein expression of RET gene, EGFR, p-Src, p-FAK, E-cadherin, N-cadherin and vimentin (1. Blank group; 2. Negative control group; 3. si-RET group; 4. oe-RET group; 5. AG-490 group; 6. NSC 228155 group; 7. si-RET + NSC 228155 group); **C**, Statistical chart of relative protein content; *compared with blank group, $p<0.05$; #compared with si-RET group, $p<0.05$.

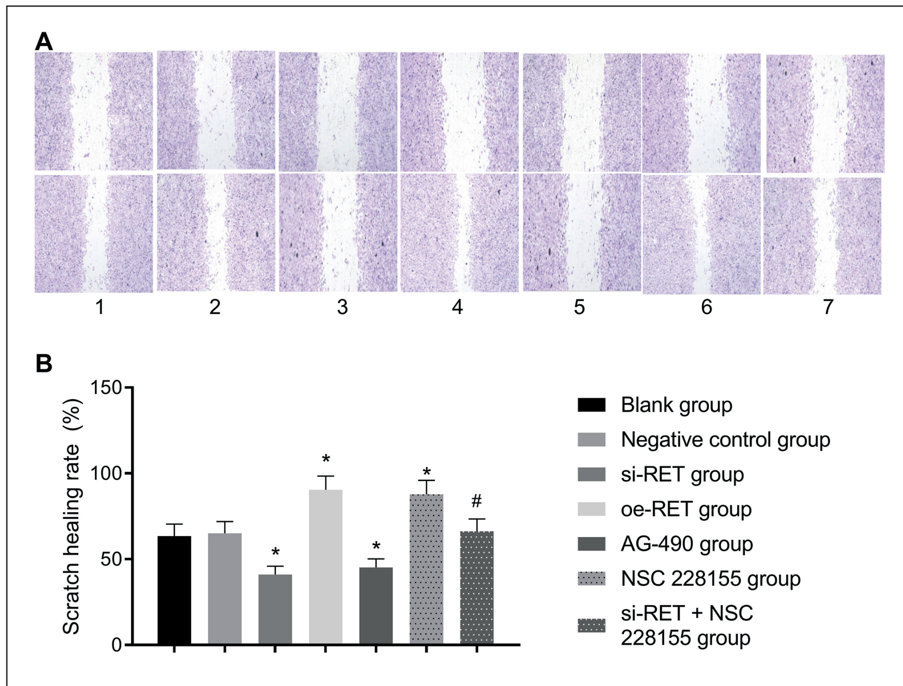

Figure 3. The migration ability of PTC TPC-1 cells detected by cell scratch assay. Note: **A**, Detection of the invasion ability of PTC TPC-1 cells by cell scratch assay (magnification 300X; 1. Blank group; 2. Negative control group; 3. si-RET group; 4. oe-RET group; 5. AG-490 group; 6. NSC 228155 group; 7. si-RET + NSC 228155 group); **B**, Statistical chart of scratch healing rate; *compared with blank group, $p<0.05$; #compared with si-RET group, $p<0.05$.

It is still a hot topic to study potential biomarkers involved in the proliferation, invasion, metastasis and apoptosis of PTC cells at the molecular level, and to understand the occurrence and development of PTC on the basis of mechanism exploration. The proliferation, invasion, migration and apoptosis of PTC is a complex process that involves multiple factors and pathways, among which RET gene and the EGRF signaling pathway were found in our study to have important roles in the process.

According to previous research, abnormal RET gene activity is the cause of TC[18]. Different forms of chromosomal aberrations and insertions lead to the formation of certain fusion gene, which is considered to be the driving form of PTC[19]. With the deepening of research, RET gene is discovered to be significantly involved in the occurrence of many diseases, including lung cancer, Hirschsprung's disease, etc.[20-22]. Moreover, abnormal expression of RET gene can regulate the proliferation, invasion, migration and apoptosis of

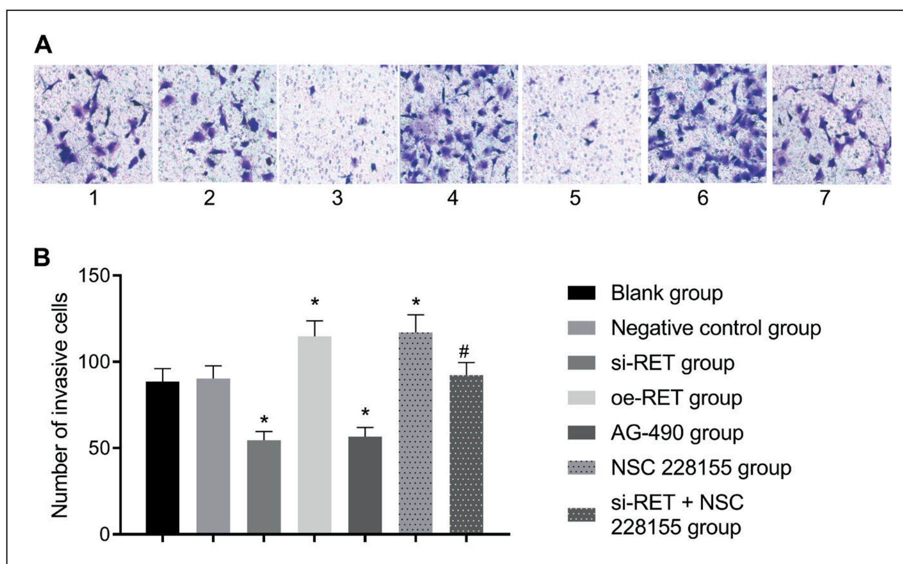

Figure 4. The proliferation ability of PTC TPC-1 cells was detected by transwell assay. Note: **A**, Detection of the proliferation ability of PTC TPC-1 cells by transwell assay (magnification 300X; 1. Blank group; 2. Negative control group; 3. si-RET group; 4. oe-RET group; 5. AG-490 group; 6. NSC 228155 group; 7. si-RET + NSC 228155 group); **B**, Statistical chart of cell invasion capacity; *compared with blank group, $p<0.05$; #compared with si-RET group, $p<0.05$.

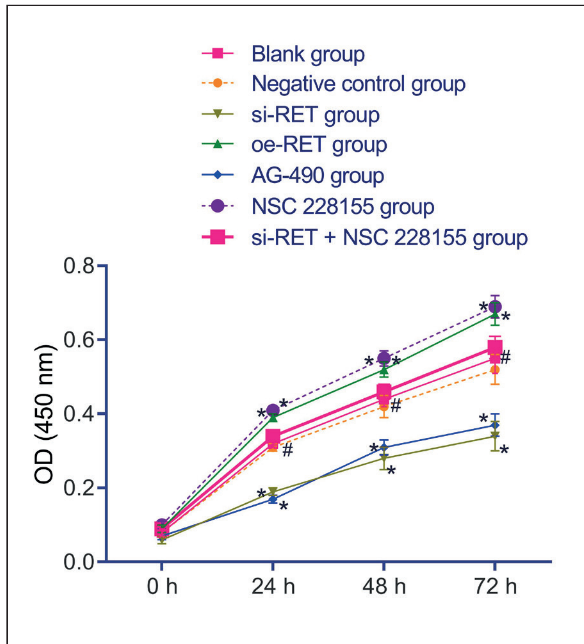

Figure 5. Detection of PTC TPC-1 cell proliferation by CCK8 assay. Note: *compared with blank group, $p<0.05$; #compared with si-RET group, $p<0.05$.

various tumor cells[23-25]. Significantly, it has been previously reported that inhibiting the expression of RET by naphthalene diimide mediated gene promoter G-quadruplex stabilization could have an anti-tumor effect on oncogene-addicted medullary TC[26]. Accordingly, in this study, RET gene was checked to be highly expressed in PTC cells, suggesting that RET gene may be an oncogene to participate in the development of PTC and inhibition of its activity may thus exert an anti-tumor effect. It has been previously identified that RET was an oncogene, which was discovered by Takahashi et al[27] for the first time. RET oncogene is located in the long arm of chromosome 10, which has 21 exons and at least 4 transcripts[28]. The content of RET oncogene is different in different tissues[29], which may highlight its different roles in different diseases. In our study, the abnormal high expression of RET might hint the occurrence of PTC, which provided a basis for subsequent cell transfection and experiments.

In the subsequent experiments, overexpression of RET and the activation of EGFR signaling pathway using NSC 228155 resulted in the activation of this pathway, accelerated EMT, increased abilities of cell migration, invasion and proliferation, while decreased apoptosis. These results not only proved our preliminary hypothesis that RET was an oncogene in the development of PTC, but also revealed that exogenous overexpression of RET and activation of the proposed signaling pathway both exert tumor promoting roles. It may suggest that besides RET, EGFR signaling pathway may also have a negative role in PTC. By contrast, silencing of RET gene expression and AG-490 (EGFR signaling pathway inhibitor) treatment inhibited the activation of the studied

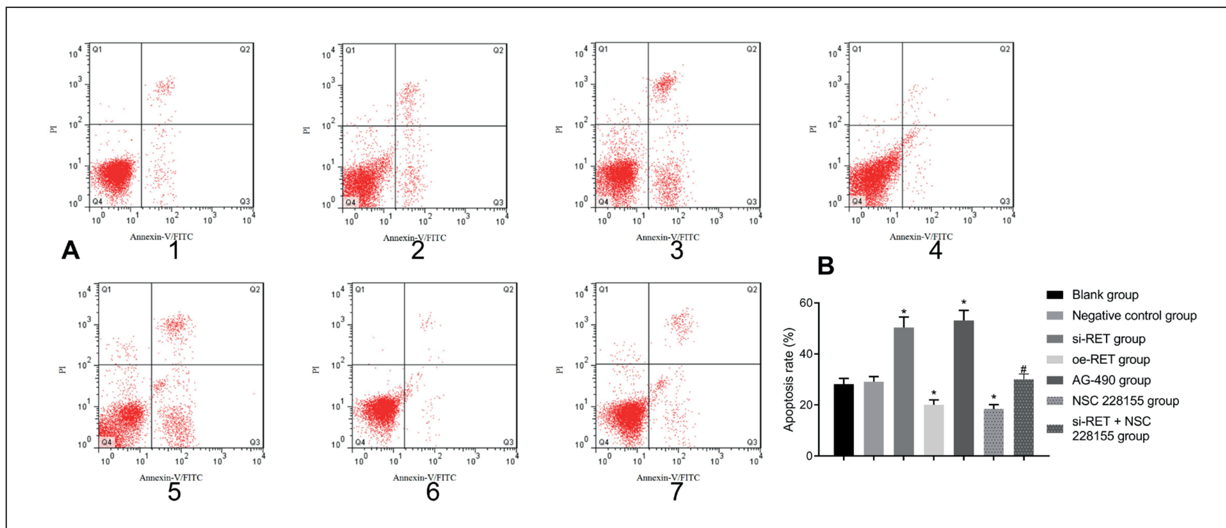

Figure 6. Detection of PTC TPC-1 cell apoptosis by flow cytometry. Note: **A**, Flow cytometry detection of PTC TPC-1 cell apoptosis (1. Blank group; 2. Negative control group; 3. si-RET group; 4. oe-RET group; 5. AG-490 group; 6. NSC 228155 group; 7. si-RET + NSC 228155 group); **B**, Statistical chart of cell apoptosis capacity; *compared with blank group, $p<0.05$; #compared with si-RET group, $p<0.05$.

pathway, suppressed EMT, decreased abilities of cell migration, invasion and proliferation, while increased apoptosis. It in turn confirmed the beneficial roles of RET and EGFR in PTC and identified the aforementioned results. More significantly, no significant difference was found after the combined treatment of RET gene silencing and pathway activation, suggesting that the expression of RET might have a regulatory effect on this pathway. Its activation via NSC 228155 exogenously reversed the positive role of RET gene silencing, and thus promoted the development of PTC. In our study, it was observed that in relative to silencing of RET gene, the additional treatment with NSC 228155 caused activated pathway, accelerated EMT, increased abilities of cell migration, invasion and proliferation, while decreased apoptosis, which clarified our speculation.

The encoded RET protein belongs to the receptor tyrosine kinase (RTK) protein family, which can regulate cell growth and differentiation[30]. It has the classical structure of RTK, including an extracellular domain rich in cysteine, a transmembrane domain and an intracellular domain catalyzing tyrosine kinase[31]. Theoretically, as for its potential mechanism in tumor development, after the binding of RET receptor and its ligand, tyrosine kinase phosphorylates in the intracellular area, then induces RET dimerization, autophosphorylation and substrate phosphorylation, thus activating multiple downstream intracellular signaling pathways[32]. As a bridge connecting the interior and exterior of cells, the signal transduction function of tyrosine kinase is enhanced that is transmitted through the phosphorylation of RET receptor, further promoting the transformation of oncogene[33]. RET oncogene can fuse with various genes, reconstitute a new gene (fusion gene) generally by breaking itself and then joining with another gene, so as to escape the regulation of receptor binding ligand, promote autophosphorylation and automatic signal transmission, and induce tumor formation eventually[34]. In our study, with the silencing of RET gene, EGFR and its downstream Src/FAK signaling pathway, as well as the progression of EMT were all inhibited, which might be the mechanism of RET gene involved in regulating cell biological characteristics of PTC. Among them, the abnormal activation of EGFR pathway can change the growth characteristics of cells and promote the malignant transformation and tumorigenesis of cells in a ligand dependent manner such as epidermal growth factor[35]. Meanwhile, Src/FAK signaling pathway in the downstream of EGFR has been proved to mediate the migration and invasion of cells in various tumors[36]. EMT is an essential factor for epithelial tumors to gain the ability of invasion and metastasis[37]. Through EMT, the epithelial cells lose their polarity and the connection with basement membrane and other epithelial phenotypes, and obtain a higher ability of migration and invasion, anti-apoptosis as well as degradation of extracellular matrix and other interstitial phenotypes[38]. It manifests primarily in the decreased or absent expression of E-cadherin, while increased expression of N-cadherin and vimentin[39,40]. In this study, the ability of invasion and metastasis of PTC cells could be further reduced by reversing the progression of EMT.

Conclusions

To sum up, RET gene is highly expressed in PTC, and silencing RET gene expression can inhibit the invasion and promote the apoptosis of PTC cells by inhibiting the activation of EGFR signaling pathway, and mediates the process of EMT. It shall be highlighted that our study for the first time identifies the role of silencing RET expression in exerting a tumor-suppressor function to inhibit the tumorigenesis and development of PTC via regulating the biological properties of PTC cells from the perspective of reversing phenotype from EMT and inhibiting the activation of EGFR signaling pathway, suggesting that RET may offer the possibility of a promising therapeutic target for the treatment of PTC. In view of the fact that RET may play a role as an oncogene through multiple signaling pathways in tumor progression, it is of great significance to broaden the study of RET gene in the growth, apoptosis, invasion and metastasis of PTC and its regulatory mechanism for targeted treatment of PTC.

Conflict of Interests

The authors declare that they have no conflict of interests.

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