

Effect and mechanism of PAR-2 on the proliferation of esophageal cancer cells

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Abstract. – OBJECTIVE: Esophageal Cancer (EC) is a common malignant tumor occurred in the digestive tract. In this study, we investigated the mechanism of Protease Activated Receptor 2 (PAR-2) on the proliferation of esophageal cancer cell.

MATERIALS AND METHODS: Transfected esophageal cancer (EC) cell (PAR-2shRNA EC109) was established with low stable PAR-2 expression. EC109 cell was treated with PAR-2 agonist, PAR-2 anti-agonist and MAPK inhibitor respectively; Untreated EC109 cell (blank control) and PAR-2shRNA EC109 cell were used for analysis also. The mRNA expressions of PAR-2, ERK1, Cyclin D1, and c-fos in each group were detected by reverse transcript and polymerase chain reaction. Western blot was used to detect the protein expressions in each group. The cell growth curves were drawn to compare the cell growth.

RESULTS: Compared with the blank control, the mRNA and protein expressions of PAR-2, Cyclin D1, and c-fos in PAR-2 agonist group increased significantly ($p < 0.05$), while decreased significantly in PAR-2shRNA EC109 cell and MAPK inhibitor group ($p < 0.05$). The mRNA expression of ERK1 and protein expression of p-ERK1 increased in PAR-2 agonist group, decreased in PAR-2shRNA EC109 cell and MAPK inhibitor group when compared with blank control ($p < 0.05$). The growth of cells was upward in PAR-2 agonist group at cell growth phase when compared with blank control, while decreased in PAR-2 shRNA EC109 cell and MAPK inhibitor group with statistical difference ($p < 0.05$).

CONCLUSIONS: PAR-2 regulate cell proliferation through the MAPK pathway in esophageal carcinoma cell, and Cyclin D1, c-fos are involved in this process.

Key Words:

Protease activated receptor-2 (PAR-2), Esophageal carcinoma cell, Cell proliferation, Extracellular signal-regulated kinases (ERKs).

Introduction

Esophageal cancer (EC) is a common malignant tumor occurred in the digestive tract, and the incidence and mortality of EC are increasing in China over the past decades^{1,2}. The EC progresses rapidly and often lead to lymph node metastasis. Due to its high malignant biological behavior, the prognosis of EC is poor. Researchers³ believe that the abnormal epithelial hyperplasia is the early stage of EC. The epithelial hyperplasia includes basal cell hyperplasia (BCH), dysplasia and carcinoma in situ (CIS)^{4,5}. These lesions were considered as precancerous lesions of the esophagus. Despite the mechanism of EC is not very clear, the abnormal expression of tumor suppressor genes and (or) the oncogenes are believed to cause the regulation disorder of cell proliferation, thus lead to abnormal cell proliferation and carcinogenesis^{6,7}. Invasion and metastasis are the most representative characters of the cancer cell, explore the underlying molecular mechanisms of invasion and metastasis in EC cell could provide potential treatment target and has important significance for the treatment of EC.

Protease-activated receptor 2 (PAR-2) is a single-chain transmembrane G-protein coupled receptors with the molecular weight of 40 KDa^{8,9}. PAR-2 is one of the four protease-activated receptors (PAR-1, PAR-2, PAR-3, PAR-4)¹⁰ and is expressed in different tissues of the human body, such as the epithelial cells of the digestive system, respiratory system, and the eyes. PAR-2 plays important roles in the intestinal secretion, tumor cell expression, phagocytosis, skin allergies and repairing body tissue^{11,12}. There were re-

searchers^{13,14} indicated that PAR-2 is expressed in a variety of digestive system tumors and expressed higher than normal tissues. Han et al¹⁵ indicated trypsin or tumor-associated trypsin (TAT) could activate PAR-2, promote tumor cell proliferation in esophageal adenocarcinoma, and ERK/MAPK pathway is involved in the proliferation of esophageal adenocarcinoma cells (EACs).

Our early research showed activation of PAR-2 could promote the invasion and migration ability of EC109 cell, an esophageal carcinoma cell. To study further the related mechanism of PAR-2 in EC, we constructed PAR-2 targeted shRNA plasmids, and transfected them into EC109 cell to silence the expression of PAR-2 in human EC109 cell; in addition, the PAR-2 agonist, PAR-2 anti-agonist, and MAPK inhibitor were also used to compare the expressions of PAR-2, ERK1, Cyclin D1, c-fos and the cell-proliferation of EC109 under different treated conditions.

Materials and Methods

Cell Line

EC109 cell and *Escherichia coli* (*E. coli*) Top10 strain were purchased from cell bank of Chinese Academy of Sciences (CCAS, Shanghai, China); the pGFP-V-RS shRNA cloning plasmid was purchased from OriGene (TR30007, Rockville, MD, USA).

Construction of Plasmid Vector

The mRNA sequence of PAR-2 gene was searched in GenBank (GeneID: 55065), 2 specific oligonucleotide sequences with lengths of 29 bp was synthesized: TTCCTAACTCTGGC-CTTGGTGTGGCAAT and GTGTTCTCATATGTGAAGGTGGCTGCAAG. A nonspecific nucleotide sequence was also synthesized. The stem-loop structure of shRNA was TCAAGAG. BamH I and Hind III restriction sites in the 5' and 3' ends, were introduced in synthesized single-stranded oligonucleotide. The complementary strand was synthesized, and connected with hairpin DNA into the plasmid vector. The recombinant plasmid was transformed into DH5 α competent cell for amplification and then was extracted. The concentration and purity were detected. Recombinant plasmids were named as PAR-2 shRNA-1, PAR-2 shRNA-2 and nonspecific sequence. The sequences were identified.

Cell Culture and Transfection

Roswell Park Memorial Institute-, 1640 (RP-MI-1640) culture medium (Gibco, Paisley, UK) containing 10% fetal bovine serum (FBS, PAN-Biotech, Aidenbach, Germany) was used for EC109 cell culture. Cells were cultured in 37°C incubator containing 5% CO₂. The cells were digested when they covered 70%-80% of the bottoms of the culture bottle, the logarithmic growing cells were used in the following experiment. Logarithmic growing EC109 cells were transfected by PAR-2 shRNA-1, PAR-2 shRNA-2 and nonspecific sequences for 24 h. The expression of the green fluorescent protein was calculated for transfection efficiency measurement. Puromycin (1 μ g/mL) was used for cell selection. Multiple passaged stable cells were obtained. The same batch untreated EC109 cells without transfections were selected as blank control. The transfected cells were named as PAR-2 shRNA-1, PAR-2 shRNA-2 and nonspecific sequence cell respectively.

Reverse Transcript (RT)-PCR for Transfection Analysis

Cells were washed by phosphate buffered saline (PBS) and the lysates solution was added. The cell suspension was moved to a 1.5 ml RNase-free centrifuge tube. Phenol/chloroform was used to deproteinization; ethanol was used to precipitate RNA. The total RNA was extracted by RNeasy pure cell kit (Qiagen Biotech, Beijing, China). DEPC water was added to dilute these RNAs. The purity of extracted RNA was tested by ultraviolet spectrophotometer. The OD260/OD280 ratio value should with 1.8-2.0. The cDNA was synthesized. The transcription conditions were: 30°C for 10 min, 42°C for 30 min, 99°C for 5 min and 5°C for 5 min. The designed primers' sequences were:

PAR-2: FP, 5'-AGAAGCCTTATTGGTAAG-GTT3' RP, 5'-AACATCATGACAGGTCGT-GAT-3'; the amplified fragment is 582bp

β -actin: FP, 5'-TGTTTGAGA CCTTCAACAC-CC-3' RP, 5'-AGCACTGTGTTGGCG TACA GG-3'; the amplified fragment is 540bp.

PCR reaction conditions were: PAR-2: 94°C for 45 s, 51°C for 45 s, 72°C for 1 min (35 cycle); β -actin: 94°C for 45 s, 55°C for 60 s, 72°C for 45 s (35 cycle). 2% agarose gel electrophoresis was used for separation of PCR amplified products; β -actin was used for amount correction.

Western Blot Analysis

The cells were cultured with the same process described above. The culture medium was discarded, washed in 4°C PBS for 2 times. Radioimmunoprecipitation assay (RIPA) lysate solution containing 100 mM PMSF buffer was added for lysis and incubated on the ice for 60 minutes, and then centrifuged at 12000 g 25 minutes at 4°C. The supernatants were collected. The concentrations of proteins were calculated by BCA assay with protein concentration curve (standard curve).

30 µg protein of each sample were electrophoresed in 5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membrane. Nonspecific reactivity was blocked by 5% nonfat dry milk in TBS/T for 1 hour at room temperature. The membrane was then treated with primary antibodies of PAR-2 (1:200, Boster Biotech, Wuhan, China) at 4°C for 16 hours; then, it was cultured with horseradish peroxidase-labeled with a secondary antibody (1:2000, Boster Biotech, Wuhan, China) at 37°C for 1 hour. The protein was colored by ECL solution and captured by Gel imaging system (Amersham Bioscience, Santa Clara, CA, USA); the gray value was measured. β-actin served as control.

Activation and Inhibition of PAR-2 in EC109 Cell

After we obtained stable transfection PAR-2-shRNA EC109 cell, logarithmic growing EC109 cell was treated by following reagents respectively: PAR-2 agonist (50 nmol/l, SLIGKV-NH₂, Abcam, Cambridge, MA, USA), PAR-2 anti-agonist (50 nmol/l VKGILS-NH₂, R&D Systems, Minneapolis, MN, USA), and MAPK pathway inhibitor (EC109 cells were pre-treated by PD98059 (50 µmol/L, Abcam, Cambridge, MA, USA) 1 hour and then added SLIGKV-NH₂ (50 nmol/l)). The same batch of untreated EC109 cell and PAR-2-shRNA EC109 cell were also used for comparison analysis.

Reverse transcript Polymerase Chain Reaction (RT-PCR) after Activation and Inhibition of PAR-2

Logarithmic growing EC109 cells were transferred in culture flasks with a concentration of 6×10⁴/ml and cultured for 24 hours, then cultured for another 24 h in serum-free 1640 culture medium. The above reagents were added in cells according to the grouping and cultured for 24 h;

the total RNA was extracted same as described above. The primers of PAR-2, ERK1, Cyclin D1 and β-actin were designed by Omega 2.0 according to reported literatures, and synthesized by Dingguo Changsheng Biotech company (Beijing, China). Reverse transcription was performed with following condition: 95°C for 15 min, 95°C for 10 s, 60°C for 32 s (40 cycles) and fluorescence signal was detected at 60°C. The reaction solutions were added in 96 well-plate. The PCR was performed in PikoReal real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). β-actin served as a reference, the relative expressions of target genes were calculated as $2^{-\Delta\Delta Ct}$ ($\Delta Ct = Ct_{\text{targeted gene}} - Ct_{\text{reference gene}}$; $\Delta\Delta Ct = \Delta Ct_{\text{treatment group}} - \Delta Ct_{\text{control group}}$).

Western Blot Analysis after Activation and Inhibition of PAR-2

The process of Western blot was same as described above. The primary antibodies of PAR-2, ERK1, p-ERK1 and Cyclin D1 (1:200) were purchased from Boster Biotech (Wuhan, China).

Cell Growth Curve

Logarithmic growing EC109 cells were inoculated in 6 well culture plates with a concentration of 1.5 × 10⁴/ml, cultured in serum-free 1640 medium for 24 hours, then treated by different reagents as described above. Cell growth was observed continuously for 8 days. Cell numbers in 3 wells of each group were recorded and the average value was adopted to draw cell growth curve.

Statistical Analysis

SPSS18.0 statistical software (IBM, Chicago, IL, USA) was used to analyze all the data. The results data was showed mean ± SD. The comparison of single factor analysis of variance and independent t test was used for statistical analysis. $p < 0.05$ was considered as statistical significance.

Results

Plasmid DNA Sequence and Transfection Efficiency

The sequences of screened recombinant plasmid were completely consistent with designed oligonucleotide sequences of PAR-2. We could observe green fluorescence under the inverted

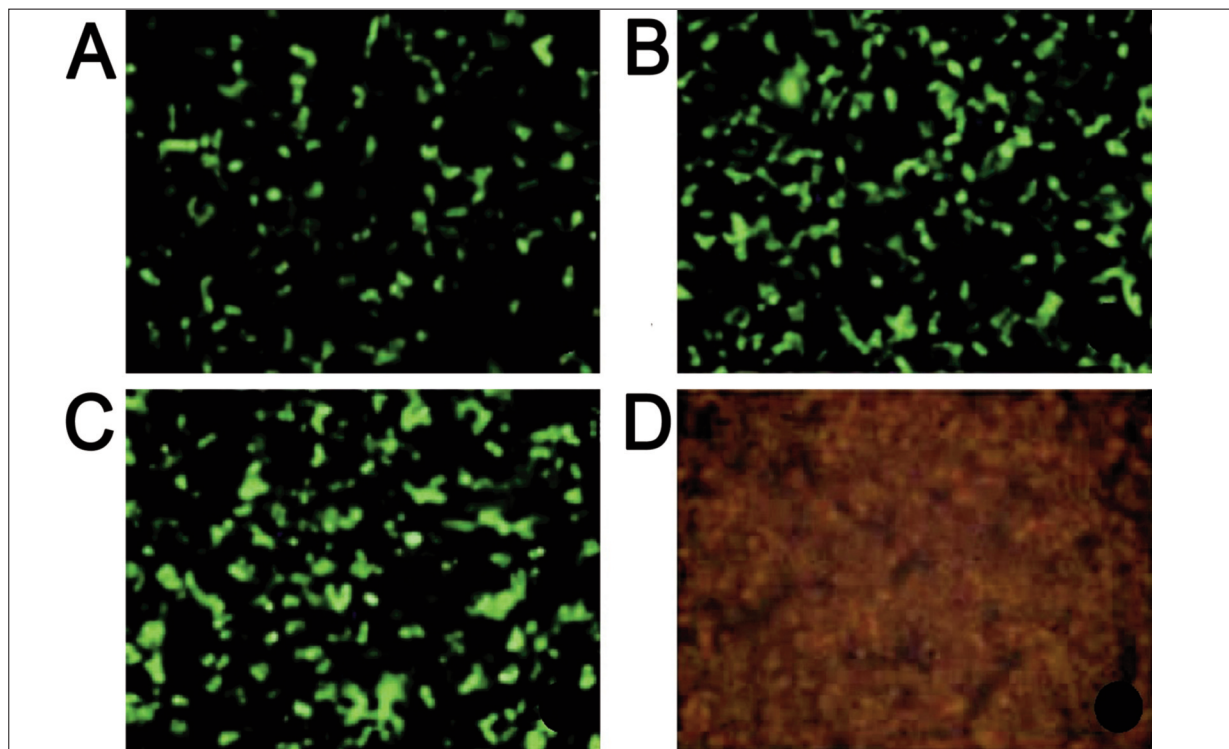


Figure 1. Green fluorescence of transfection under inverted fluorescence microscope ($\times 100$). Groups: **A**, PAR-2 shRNA-1; **B**, PAR-2 shRNA-2; **C**, Nonspecific sequence; **D**, Blank control.

fluorescence microscope after 24 h of transfection (Figure 1). The transfection efficiency was calculated as a percentage of green fluorescent cells to total cells. Results showed the average transfection efficiency of PAR-2shRNA-1, PAR-2shRNA-2 and the nonspecific sequence was 67.6%, while blank control cells have no green fluorescence cell.

PAR-2 Expressions in PAR-2-shRNA EC109 Cells

The RT-PCR result (Figure 2A) showed absorbance ratio value of PAR-2 were: 0.35 ± 0.03 , 0.43 ± 0.04 , 0.44 ± 0.04 and 0.45 ± 0.41 in PAR-2 shRNA-1, PAR-2 shRNA-2, nonspecific sequence transfected group, and blank control (EC109 cells), respectively. The PAR-2 shRNA-1 has lowest mRNA expression compared with the other 3 groups ($p < 0.05$). The relative PAR-2 mRNA expression in PAR-2 shRNA-2, nonspecific sequence transfected group, and blank control have no obvious difference ($p > 0.05$). The Western blot analysis (Figure 2B) showed same pattern: the gray ratio in 4 groups were: 0.96 ± 0.01 , 1.04 ± 0.02 , 1.05 ± 0.04 , and 1.09 ± 0.04 , respectively. PAR-2

shRNA-1 group has lowest PAR-2 protein expression compared with the other 3 groups ($p < 0.05$). The expressions in other 3 groups have no statistical difference ($p > 0.05$).

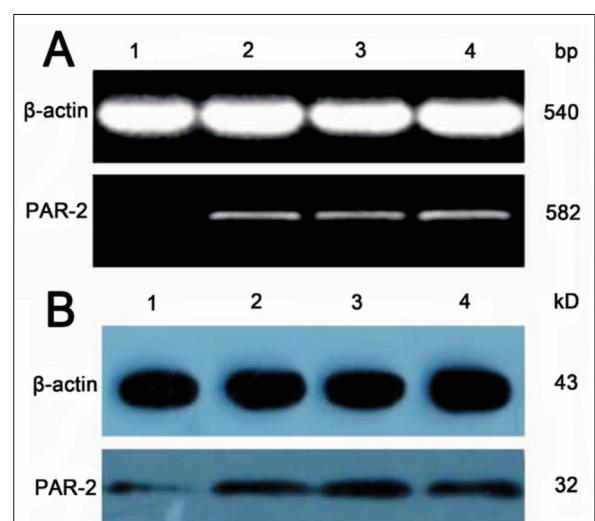


Figure 2. Expression of PAR-2 after transfection. **A**, mRNA expression. **B**, Protein expression. Groups: 1, PAR-2 shRNA-1; 2, PAR-2 shRNA-2; 3, nonspecific sequence; 4, blank control.

mRNA Expressions after Activation and Inhibition of PAR-2

PAR-2 shRNA-1 EC109 cell was adopted in further analysis (and was described as PAR-2 shRNA group). The expression of PAR-2 in PAR-2 agonist group was 2.651 times of the blank control ($p < 0.05$), and was 0.385 times in PAR-2 shRNA group when compared with blank control ($p < 0.05$, Figure 3A); The expression of ERK1 mRNA in PAR-2 agonist group was 2.58 times of the blank control ($p < 0.05$), and was 0.376 times in PAR-2 shRNA group when compared with blank control ($p < 0.05$); the expression of ERK1 mRNA in MAPK inhibitor group was 0.497 times of the blank control ($p < 0.05$, Figure 3B). Compared with blank control, the expression of Cyclin D1 mRNA in PAR-2 agonist group was increased to 2.012 times ($p < 0.05$), and decreased to 0.318 times in PAR-2 shRNA group ($p < 0.05$), the expression of Cyclin D1 mRNA in MAPK inhibitor group was 0.422 times of blank control ($p < 0.05$, Figure 3C); In addition, the expression c-fos mRNA in PAR-2 agonist group increased to 2.837 times compared with the blank control group ($p < 0.05$), and de-

creased to 0.265 times and 0.168 times in PAR-2 shRNA group and MAPK inhibitor group respectively (both $p < 0.05$, Figure 3D).

Protein Expressions after Activation and Inhibition

The PAR-2 protein expression (Figure 4A) in PAR-2 agonist group was higher than blank group, while the expression in PAR-2 shRNA group was lower than blank control; p-ERK1 protein expression (Figure 4B) was higher in PAR-2 agonist group but lower in PAR-2 shRNA group and MAPK inhibitor group compared with blank control. However, the ERK1 protein expression (Figure 4C) in each group did not change significantly. We observed same tendencies of cyclin D1 (Figure 4D) and c-fos protein expression (Figure 4E) in each group.

Cell Growth Comparison

The growth of cells (Figure 5) were upward in PAR-2 agonist group at cell growth phase when compared with blank control, while decreased in PAR-2 shRNA and MAPK inhibitor group with statistical difference ($p < 0.05$).

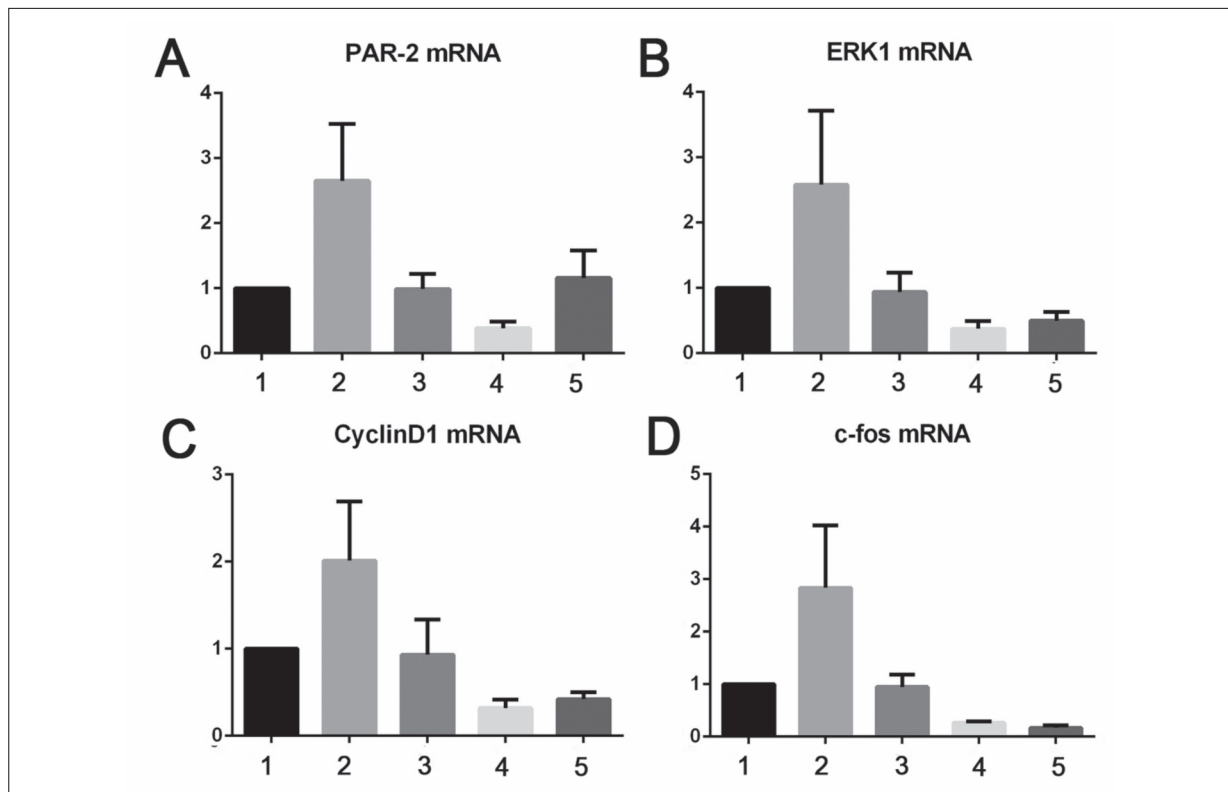


Figure 3. mRNA expressions after activation and inhibition of PAR-2. **A**, PAR-2; **B**, ERK1; **C**, Cyclin D1; **D**, c-fos. Groups: 1, blank control; 2, PAR-2 agonist group; 3, PAR-2 anti-agonist group; 4, PAR-2 shRNA group; 5, MAPK inhibitor group.

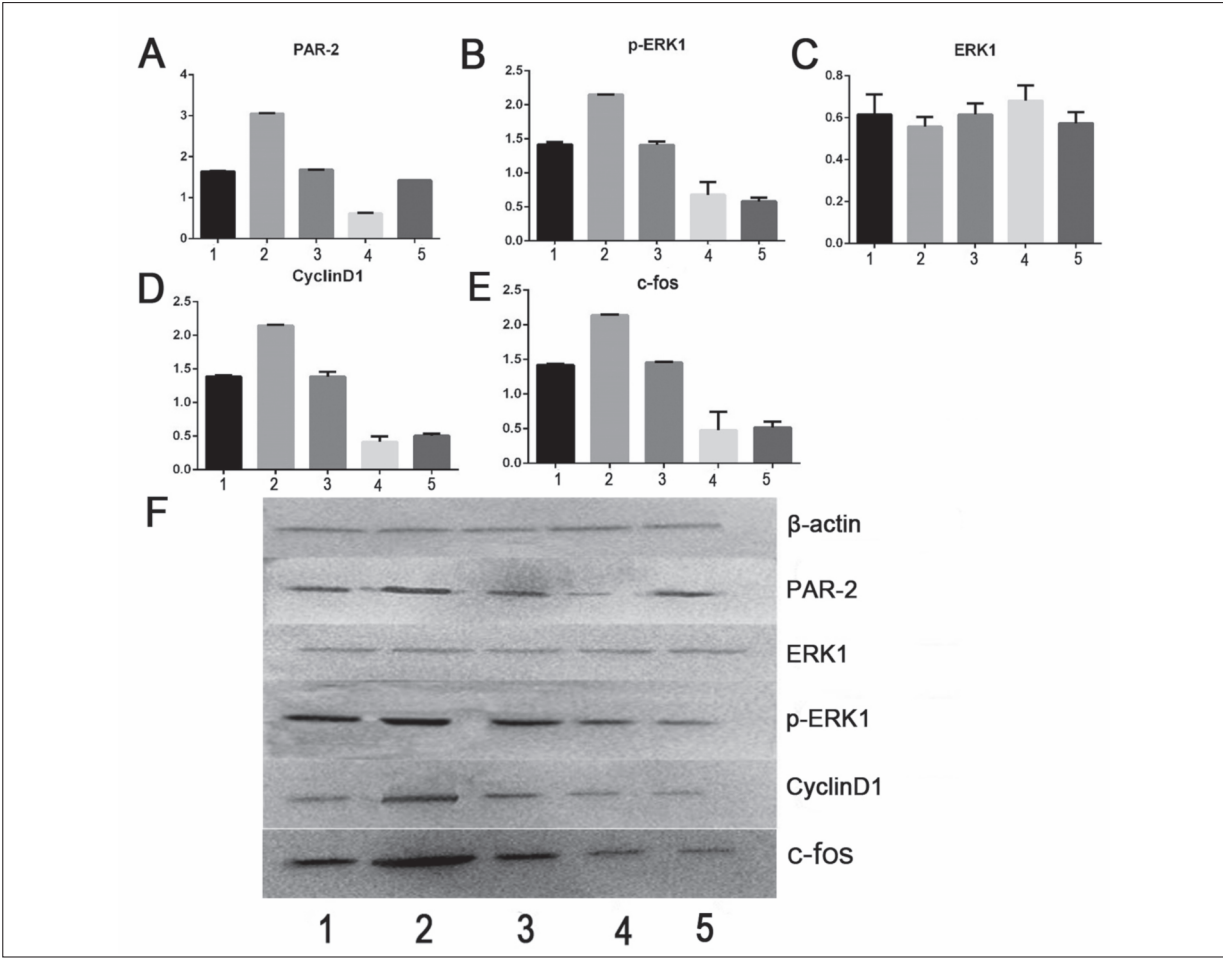


Figure 4. Protein expressions after activation and inhibition. **A**, PAR-2; **B**, p-ERK1; **C**, ERK1; **D**, Cyclin D1; **E**, c-fos; **F**, Western blot results. Groups: 1, blank control; 2, PAR-2 agonist group; 3, PAR-2 anti-agonist group; 4, PAR-2 shRNA group; 5, MAPK inhibitor group.

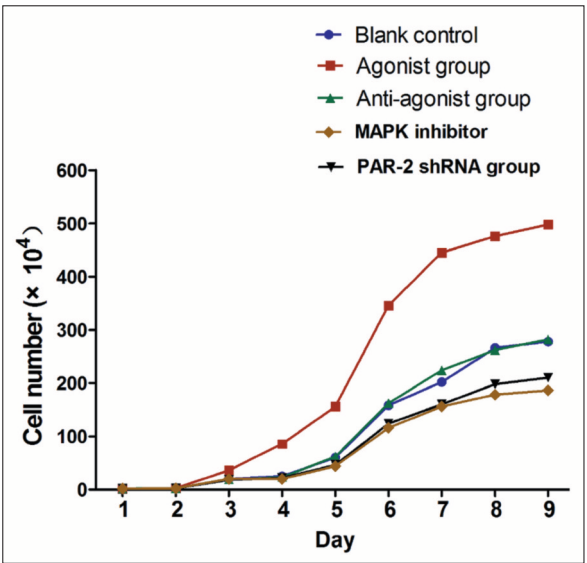


Figure 5. Cell growth curve in each group.

Discussion

All PARs family members contain 7 trans-membrane domains, 3 extracellular cyclic structure and 3 intracellular cyclic structure, and 1 intracellular C-terminus and 1 extracellular N-terminus^{16,17}. PAR-1, PAR-3 and PAR-4 are thrombin receptor, they can be activated by thrombin, and involved in blood coagulation, platelet activation and other physiological activities. Human PAR-2 protein is composed by 397 amino acid residues with molecular weight about 50-55kDa. PAR-2 gene is located on chromosome 5q13, includes 2 exons and 1 intron¹⁸. As a trypsin cell surface receptor, PAR-2 can be activated by multiple endogenous activators, such as trypsin, coagulation factor VII TF/VIIa compound, Xa, tryptase, plasmin,

etc.¹⁹, and it mediates inflammatory reaction, affect the angiogenesis formation, growth, invasion, metastasis and survival of tumor²⁰. In this study, we constructed targeted shRNA for PAR-2 gene and obtained stable transfected PAR-2-shRNA EC109 cell, which expressed PAR-2 stably low. The further MTT, flow cytometry and transwell assay results showed the proliferation and invasion ability of PAR-2-shRNA EC109 cell decreased when compared with EC109 cell (data not show). Mitogen-activated protein kinase (MAPK) signaling pathway is a major downstream pathway of RAS, the ERK1 is one of serine/threonine kinase of MAPK pathways²¹. ERK1 dominated mitotic and anti-apoptotic signals play important roles in the growth and prognosis of the human malignant tumor cell. Kaufmann et al^{22,23} have demonstrated that PAR-2 influence the proliferation, invasion and metastasis of hepatoma cells through affecting ERK1/AP-1 way and Ca2 signaling pathways. Xie et al²⁴ also demonstrated PAR-2 enhances proliferation of human hepatoma cells and the ERK/AP-1 pathway is involved.

Our research demonstrates the mRNA expression of ERK1 increased in PAR-2 agonist group but decreased in PAR-2 shRNA group and MAPK inhibitor group when compared with blank control. However, the protein expressions of ERK1 have no change in each group, while the p-ERK1 protein expression increased in agonist group but decreased in PAR-2 shRNA group and MAPK inhibitor group when compared with blank control. P-ERK is the phosphorylated form of ERK, p-ERK is the active form of ERK and has specific biological effects. We can speculate that PAR-2 agonist can increase the expression of ERK1 gene in EC109 cell, but not ERK1 protein, the increased p-ERK1 expression indicated that there might exist ERK self-activation pathway beside MAPK pathway in the esophageal cancer cell. The activation of ERK is not dependent on the upstream pathway signal, it may exist KRAS like self-activation, and phosphorylation of ERK may be involved in carcinogenesis of the EC as an independent factor. Adjei et al²⁵ reported that abnormal activation of ERK, which did not rely on the KRAS signal and could transduce to the downstream pathway.

Cyclin D1 play a positive regulation role in cell cycle, Cyclin D1 will decompose rapidly in physiological state once cells develop into the S phase; the activation of related gene could lead to the sustained high expression of Cyclin D1,

shorten the G1 period of cell cycle and made the cells enter the S phase in advance; thus cause the out of control cell proliferation and the occurrence of tumor^{26,27}. *In vitro* studies showed that the Ras oncogene mainly acts on the CRE component to induce the over-expression of Cyclin D1 through activating MAPK/ERK pathway in tumors^{28,29}. Our results show the expression of Cyclin D1 increased in PAR-2 agonist group, decreased in PAR-2 shRNA group and MAPK inhibitor group compared with blank control group. PAR-2 agonist could upregulate Cyclin D1 expression in gene and protein level to accelerate the proliferation of EC109 cell through MAPK/ERK1 pathway. The running of cell cycle is precisely controlled by the network system composed of some gene groups and substrate groups, which mainly include Cyclin, Cyclin-dependent kinase (CDK) and Cyclin-dependent kinase inhibitor (CKI) factor, combine with Cyclin, they form active complexes, phosphorylate the corresponding substrate and drive the cell to complete the cell cycle.

C-fos is an oncogene-encoded nuclear protein. It is one of the immediate early gene members. Stimulated by multiple stimuli, these genes express immediately and produce the AP-1 transcription factor after combining to c-jun, thus regulate other genes' transcription and expression. Studies have shown the changed expression of c-fos and c-jun are correlated with the occurrence and development of a variety of tumor. Their expressions have varying degrees of correlation with the proliferative activity, differentiation, lymph node metastasis, and clinical outcomes of tumors^{30,31}. Our previous report²⁴ also demonstrated that over-expressed c-fos can promote the proliferation of cancer cells, which is consistent with the results of *in vivo* studies^{32,33}.

Conclusions

Our work showed that PAR-2 agonist could regulate the expression of ERK1 gene and ERK1 in its downstream pathway, accelerate the cell cycle progression and promote proliferation of EC109 cell. The agonist PAR-2 increase the downstream Cyclin D1 and c-fos expression in MAPK/ ERK1 pathway, but whether Cyclin D1 and c-fos could be regulated by other pathways in EC cells, and what pathways might be involved in the expression of Cyclin D1 and c-fos regulation still need in-depth research.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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