Abstract. – OBJECTIVE: To determine whether the combination of prostaglandin E2 (PGE2) and EP2, the subtype receptor of PGE2, could trans-activate the epidermal growth factor receptor (EGFR).

PATIENTS AND METHODS: In this experiment, we selected epithelial cells from normal esophageal mucosa as the negative control group, and the ESCC EC109 and TE-1 cell strain as the observation group. Real-time PCR and Western-blotting were used to detect the expression of EP2, EGFR and phosphorylated EGFR (p-EGFR). The pre-treatment of ESCC cell strains was carried out using Butaprost (special agonist of PGE2 and EP2) and RNAi of EP2, and we observed the expression of EP2, EGFR, and p-EGFR. WST-8 (CCK-8) was applied for the detection of the cell proliferation rate. The transwell invasion experiment was conducted for the detection of the invasion capability of cells. The expression of MMP-9 (matrix metalloproteinase-9), VEGF (vascular endothelial growth factor), pro-inflammatory factors (IL-6 and TNF-α) in the cell supernatant were detected using ELISA.

RESULTS: The high mRNA and protein expression of EP2, EGFR, and p-EGFR were found in the EC109 and TE-1 cell strains in the observation group, which were higher than those in the control group (p < 0.05). After the intervention of PGE2 and Butaprost, great increases were seen in the cell proliferation rate, invasion capability, and the expression of MMP-9, VEGF, IL-6, and TNF-α in EC109 and TE-1 cell strains (p < 0.05), however, the intervention of RNAI could reduce above indexes (p < 0.05). CONCLUSIONS: Through cell experiments, we verified that the combination of prostaglandin E2 (PGE2) and EP2, the subtype receptor of PGE2, could trans-activate the epidermal growth factor receptor (EGFR) to regulate the proliferation and invasion capability of esophageal squamous cell carcinoma (ESCC) cells, and secrete and express multiple cytokines, thus discovering the pathological mechanism of inflammation to carcinoma transition in the occurrence of ESCC, and providing the experimental evidence for the search of new target in the treatment of ESCC. ESCC cells can highly express the receptor subtype EP2 of PGE2 that can transactivate the EGFR, through which PGE2 is involved in the transition mechanism from inflammation to cancer.

Key Words: Prostaglandin E2, EP2 receptor, Epidermal growth factor receptor, Esophageal carcinoma.

Introduction

In China, the incidence rate of esophageal carcinoma is relatively high, and the patients that were killed by esophageal carcinoma account for almost 1/5 of the deaths related to malignant...
Investigation on the regulatory effect of PGE2 on ESCC cells

Patients and Methods

Materials

Isolation and Culture of Epithelial Cells of Normal Esophageal Mucosa

The esophageal mucosa was rinsed and disinfected using phosphate-buffered saline (PBS) containing 200,000 u/L penicillin and 200 mg/L streptomycin. Then, the mucosa was digested using 2.5 g/L neutral proteinase at 4°C for 18-24h. After the mucosal layer was dissected, the remaining tissues were put into 2.5 g/L trypsin for digestion at 37°C for 5-10 min, in which Dulbecco’s modified eagle medium (DMEM) in equal volume containing 100 mL/L fetal bovine serum (FBS) was added to terminate the digestion. A single-cell suspension was prepared through blowing the cells and, then, centrifuged at 2000 g for 15 min, followed by washing using PBS and inoculation. The inoculated cells were placed in an incubator (37°C and 50 mL/L CO2) for culture. After 24-48h, we observed that the cell adherence to the wall and for cells that did not adhere to the walls, the original medium was replaced by fresh medium for continuous culture. When 75% or more of the bottom area of the culture dish was covered by epithelial cells, 2.5 g/L trypsin containing EDTA was added into the cells for digestion followed by passage. The study was approved by the Ethics Committee of Anhui Provincial Hospital Affiliated to Medical University of Anhui

Culture of ESCC Cell Strains

The ESCC EC109 and TE1 cell strains were selected in this experiment. The regularly frozen-thawed cells were cultured in an incubator (37°C and 5% CO2) using the medium high-sugar DMEM containing 10% (v/v) fetal bovine serum, 1×105 U/L penicillin, and 100 g/L streptomycin. 2×10⁷/L cell resuspension was prepared using PBS after several rounds of digestion using 0.25% trypsin and passage.

Research Methods and Observation Indexes

Real-time PCR and Western-blot were used to detect the expression of EP2, EGFR and phosphorylated EGFR (p-EGFR). The pre-treatment of ESCC cell strains was carried out using Butaprost (special agonist of PGE2 and EP2) and RNAi of EP2, and we observed the expression of EP2, EGFR, and p-EGFR; WST-8 (CCK-8),
which was applied for the detection of cell proliferation rate. The transwell invasion experiment was conducted for detecting the invasion capability of cells, and the expression of MMP-9, VEGF, pro-inflammation factors (IL-6 and TNF-α) in the cell supernatant were detected using ELISA.

**Methods**

**RT-PCR**

The total RNA was extracted from cells using the normal TRIzol reagent, and the concentration and purification of RNA were detected using an ultraviolet spectrophotometer. The cDNA was synthesized using a reverse transcription kit, and the primer sequences were synthesized according to the sequences in Gene Bank by Sangon Biotech (Shanghai, China) Co., Ltd as follows: EP2: (F) 5'-GAGACGGACCACCTCATTTCT-3', (R) 5'-CGGGAAGAGGTGTCTTATC-3', 101 bp; EGFR: (F) 5'-GCCTCCAGAGGATGTTCAAT-3', (R) 5'-GACATAACCAGCCACCTCT-3', 119 bp; β-actin; (F) 5'-GGGAAATCGTGCGTGACATTAAGG-3', (R) 5'-CAGGAAGGAAGGCTGGAAGAGTG-3', 180 bp. The reaction system was as follows: 2 μl cDNA, 3 μl upstream primer/downstream primer, 0.5 μl Taq polymerase, 0.5 μl Taq polymerase, 1 μl dNTPs, 3 μl MgCl₂, 5 μl 10×Buffer, 2.5 μl ddH₂O. The reaction conditions were set as follows: 95°C for 5min, 95°C for 30s, 58°C for 30s, 72°C for 60s for a total of 30 cycles, and ended at 72°C for 10min. The PCR products were identified using 2% agarose gel electrophoresis, and the UV images were obtained using gel imaging analytic system. The grey value analysis was carried out using photo of digital camera, and the results were expressed using the 2-ΔΔCt method.

**Western Blots**

After the reaction was terminated, the cell lysis buffer was added to prepare the cell suspension, which was later centrifuged at 60,000 g for 45min at 4°C. The sediment was the membrane protein, and the supernatant was the cytoplasmic protein. The suspension was prepared using the sediment that was dissolved by the membrane protein buffer. The protein content was assayed via the BCA method. 30 μg of total protein was extracted and then isolated via 8% SDS-PAGE. The isolated strips were electrically transferred onto the polyvinylidene difluoride (PVDF) membrane, on which the rat anti-human monoclonal antibodies of EP2, EGFR and p-EGFR were added (1:2000, Sigma-Aldrich, St. Louis, MO, USA), followed by an overnight incubation. Then, rabbit anti-rat polyclonal antibodies, as secondary antibodies, were added onto the membrane (1:500, Sigma, St. Louis, MO, USA) for incubation at room temperature for 4h. Then, the membrane was washed with PBS, and the color development was performed using enhanced chemiluminescence (ECL). The results were scanned and preserved. Semi-analysis was carried out via Lab Works 4.5 gel image analysis software (Invitrogen, Carlsbad, CA, USA) and the results were presented as integral optical density (IOD).

**Proliferation Experiment of CCK-8**

The cells in each group were seeded onto the 96-well plate, 3 duplications were set in each group, and the average was taken as the result. 100μL of cell suspension was added into each well, and then the incubation of the plate was carried out at 37°C. Thereafter, 10 μL of the solution at a different concentration was assayed and added into the corresponding well for incubation at 37°C for 12h, and then 10μL of CCK-8 solution was added into each well for incubation at 37°C for 1-4h. The optical density (OD) at a wavelength of 450nm was assayed using a microplate reader, and the value of OD was positively correlated with the cell proliferation rate.

**Transwell Invasion Experiment**

The transwell chamber was placed on the culture plate. The media for upper layer was added into the chamber (the upper chamber), and the media for lower layer was added in the plate (the lower chamber). The culture media of the upper and lower layers were separated using a polycarbonate membrane. The tumor cells were inoculated into the upper chamber, and FBS was added into the lower chamber, which would make the tumor cells invade to the lower chamber with a high concentration of nutrition. The polycarbonate membrane on the side of upper chamber was covered by a matrigel to simulate the extracellular matrix of the body. Thus, cells that could pass through the membrane should secrete the MMPs in order to dissolve the matrigel. The cell count in the lower chamber reflected the invasion capability of tumor cells.

The detailed procedures were as follows: 1. Preparation of matrigel: The BD matrigel was cryopreserved at -80°C and placed at 4°C overnight to thaw. 2. 300 μL of serum-free culture
medium was taken and mixed with 60 μL of matrigel in the ice bath at 4°C, and then the well-mixed solution was then added into the upper chamber at 100 μL/chamber. Then, the plate was placed into an incubator for incubation at 37°C for 4-5h, and the appearance of a “white layer” appeared to mean that the solution transformed into a solid phase. 3. The plate was washed using a serum-free culture medium 3 times, followed by a cell count and the preparation of cell suspension. 4. The matrigel was washed using a serum-free culture medium for once and, then, 100 μL cell suspension was added into each well. 5. 500 μL of conditioned culture medium containing 20% FBS was added into the lower chamber for incubation at 37°C for 20 to 24h. 6. The transwell chamber was taken out and washed using PBS 2 times, and then the cells were fixed in 5% glutaraldehyde at 4°C. 7. 0.1% of crystal violet was added to a slide for 5-10 min of staining, and then the slide was placed at room temperature for 0.5h. After the slide was washed using PBS 2 times, it was placed under a microscope for observation.

**Results**

**mRNA Expressions of EP2, EGFR, and p-EGFR**

The high mRNA and protein expression of EP2, EGFR and p-EGFR were found in the EC109 and TE-1 cell strains in the observation group, which were significantly higher than those in the control group \((p<0.05)\); no differences were identified in comparison between the EC109 and TE-1 cell strains \((p>0.05)\) (Figure 1).

**Protein Expressions of EP2, EGFR, and p-EGFR**

The high protein expression of EP2, EGFR, and p-EGFR were found in the EC109 and TE-1 cell strains in the observation group, which were significantly higher than those in the control group \((p<0.05)\). There were no differences identified in comparison between the EC109 and TE-1 cell strains \((p>0.05)\) (Figure 2).

**mRNA and Protein Expressions of EP2, EGFR, and p-EGFR After the Cell Strains Were Intervened**

In the EC109 and TE-1 cell strains of the observation group after the intervention of PGE2, EP2 expression was decreased and the p-EGFR expression was increased \((p<0.05)\).

**ELISA**

In this experiment, all reagents were purchased from Beyotime (Jiangsu) Biotechnology Co., Ltd, and the microplate reader from Bio-Rad Co., Ltd (Hercules, CA, USA). Experiments were conducted according to the instructions. The standard curve was firstly established, the experiment was repeated 3 times, and the average was taken as the result.

**Statistical Analysis**

The statistical analysis was carried out using SPSS 20.0. The measurement data was presented by mean ± standard deviation, and single-factor ANOVA was applied for intergroup comparison. The LSD-t-test was used to the comparisons between any two groups, and a paired t-test was used to the comparison before and after intervention. \(p < 0.05\) suggested that the difference was statistically significant.
There was no variation found in the expression of EGFR ($p>0.05$). After the cells were intervened using Butaprost, the expression of EP2 and p-EGFR were increased ($p<0.05$), and no changes were identified in expression of EGFR ($p>0.05$). After the intervention of RNAi, expression of EP2 and p-EGFR were decreased ($p<0.05$), and no changes were identified in expression of EGFR ($p>0.05$) (Figures 3 to 5).
Comparison of Cell Proliferation Rates

In the observation group, after intervention of PGE2 and Butaprost, a great increase was seen in the cell proliferation rate in EC109 and TE-1 cell strains (p<0.05), but the intervention of RNAi could reduce the proliferation rate (p<0.05) (Table I).

Comparison of Cell Invasion Capability

In the observation group, after the intervention of PGE2 and Butaprost, a great increase was seen in the invasion capability in the EC109 and TE-1 cell strains (p<0.05), but the intervention of RNAi could reduce cell invasion capability (p<0.05) (Table II).

Comparison of the Expressions of Cytokines

In the observation group, after the intervention of PGE2 and Butaprost, a great increase were seen in the expression of MMP-9, VEGF, IL-6, and TNF-α in EC109 and TE-1 cell strains (p<0.05). However, the intervention of RNAi could reduce above expressions (p<0.05) (Figure 6).

Discussion

There are 4 subtypes of EPs, i.e., the EP1-EP4. The different subtypes of EP exhibits vary in the distribution and effects in different tumors. PGE2 can enhance the invasion and proliferation of bile duct cancer cells and hepatocellular cancer cells mainly through the EP1 receptor, while the EP2 mainly exerts its tumor-promoting effects in colorectal and ovarian cancer. These four subtypes of the EP receptor can be found in esophageal cancer, predominantly the high expression of EP2 in EAC and ESCC. Piazuelo et al. indicate that Butaprost, a selective agonist of EP2, can sufficiently inhibit the butyrate-induced apoptosis of OE33 cells in the EAC cell strain to facilitate the migration of OE33 cells. Kuo et al. detected the tumor samples of 226 ESCC patients and found that the high expression of EP2 is closely associated with prognosis of tumor. The in-vitro culture experiment of ESCC strain carried out by Yu et al. confirmed that the HKESC-1 tumor cell proliferation that was mediated by EP2 receptor is associated with the activation of PGE2/EP2/
PKC/ERK signal pathways. The biological function of PGE2 of autocrine or paracrine pathway is exerted through its interaction with a G-protein-coupled EP receptor. For instance, EP1 can couple with Gαq to activate phospholipase C, thus activating the release of Ca²⁺ from the cells\(^{17}\). The coupling of EP2 and EP4 with Gαs can activate the adenylate cyclase to augment the cAMP levels in cells\(^ {18}\).

In comparison of EGFR expression between the ESCC tissues and tissues of healthy population, the mRNA and protein expression are significantly elevated, which can be applied as an important marker in early diagnosis, efficacy, and judgment of prognosis of esophageal cancer. The EGFR is positively correlated with pathological classification, invasion and lymphatic metastasis of ESCC\(^ {19}\). EGF can combine with and activate EGFR, which can initiate a series of cell reactions, including cell proliferation, as well as resistance to apoptosis, invasion, and metastasis, which provokes neovascularization\(^ {20,21}\). Research has confirmed that the close relation between COX-2 and EGFR, i.e., the latter can induce the protein expression of COX-2/PGE2 in multiple tumor cells, which might be associated with the “cross-talk” in the downstream signal transduction pathway of EGFR and EP receptor\(^ {22}\).

**Conclusions**

The preliminary studies of this center have successfully completed the *in-vitro* culture of ESCC cell strains, and this study further concludes that the high mRNA and protein expressions of EP2, EGFR, and p-EGFR are found in the EC109 and TE-1 cell strains, and after the intervention of PGE2, EP2 expression was decreased and the p-EGFR expression was increased, which indicates that PGE2 might combine with EP2 in order to facilitate the activation of p-EGFR. After the cells were intervened using Butaprost, expression of EP2 and p-EGFR were increased, while the intervention of RNAi reduced the expression of EP2 and p-EGFR, which suggests that p-EGFR might be the target molecule of PGE2-EP2. After the intervention of PGE2 and Butaprost, a great increase was seen in the cell proliferation rate, invasion capability, and the expression of MMP-9, VEGF, IL-6, and TNF-α in EC109 and TE-1 cell strains. However, the intervention of RNAi could reduce the above indexes, which shows that PGE2 could transactivate EGFR through EP2 to participate in the transition mechanism from inflammation to cancer, which might serve as a new target for diagnosis and intervention in the early stage of ESCC. The next study will be an
in-vivo clinical test to verify the existence of the above target, and to search for specific drugs that can act on the above target, and, thus, increase the efficacy of treatment.

Acknowledgements
Funded by Natural Science Foundation of Anhui Province (No. 1408085MH179).

Conflict of Interest
The Authors declare that they have no conflict of interests.

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