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COX-2 expression in androgen independent prostate cancer
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·*Original Article*·

Cyclooxygenase-2 expression is dependent upon epidermal growth factor receptor expression or activation in androgen independent prostate cancer

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Abstract

Aim: To investigate the expression of cyclooxygenase-2 (COX-2) and epidermal growth factor receptor (EGFR) and the possible mechanism in the development in androgen independent prostate cancer (AIPC). **Methods:** Immunohistochemistry was performed on paraffin-embedded sections with goat polyclonal against COX-2 and mouse monoclonal antibody against EGFR in 30 AIPC and 18 androgen dependent prostate cancer (ADPC) specimens. The effect of epidermal growth factor (EGF) treatments on the expression of COX-2 and signal pathway in PC-3 and DU-145 cells was studied using reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. ELISA was used to measure prostaglandin E2 (PGE2) levels in the media of PC-3 and DU-145 incubated with EGF for 24 h. **Results:** COX-2 was positively expressed in AIPC and ADPC, which were predominantly in endochylema of prostate cancer (PCa) cells. Intense staining was seen in AIPC (80%) and in ADPC (55.5%), but there was no significant association between the two groups. EGFR expression was also positive in the two groups (61.8% in ADPC and 90% in AIPC, $P < 0.01$). A significant association was found between EGFR expression and a higher Gleason score ($P < 0.05$) or tumor stage ($P < 0.05$). The expression of PGE2 was increased in PC-3 and DU-145 cells after being incubated with EGF. Both p38MAPK and PI-3K pathway were involved in the PC-3 cell COX-2 upregulation course. In DU-145, only p38MAPK pathway was associated with COX-2 upregulation. **Conclusion:** EGFR activation induces COX-2 expression through PI-3K and/or p38MAPK pathways. COX-2 and EGFR inhibitors might have a cooperative anti-tumor effect in PCa. (*Asian J Androl 2008 Sep; 10: 758–764*)

Keywords: cyclooxygenase 2; epidermal growth factor receptor; prostatic neoplasms

1 Introduction

Cyclooxygenase-2 (COX-2) is an inducible enzyme stimulated by cytokines, growth factors, oncogenes, or tumor promoters during inflammation or malignancy. COX-2 expression is increased in association with de-

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creased apoptosis, increased tumor invasiveness, immunosuppression and angiogenesis. Furthermore, increased COX-2 expression correlates with poor differentiation, increased tumor size, increased nodal and distant disease, and decreased overall survival in a variety of cancers [1–4]. In addition, there is evidence that prostaglandin E2 (PGE2), a downstream product of COX-2 metabolism, can phosphorlyate epidermal growth factor receptor (EGFR) and trigger mitogenic signaling pathways in many cancer cell lines [5, 6]. Selective EGFR inhibitors and COX-2 inhibitors have been shown to have a co-operative antitumour effect against cancer xenografts in nude mice [7].

Epidemiological studies have shown that prolonged aspirin ingestion reduces the incidence of prostate cancer (PCa). This effect might result from, at least in part, COX-2 inhibition [8, 9]. Many other studies have shown over-expression of COX-2 in PCa and COX-2 over-expression has a good relationship with the development of androgen independent prostate cancer (AIPC) [10–13], but COX-2 level changes and regulation pattern in AIPC is unresolved. The relationship between COX-2 and EGFR is poorly understood in the pathogenesis of AIPC now. We therefore sought to determine the expression of COX-2 and EGFR in a series of surgically resected PCa specimens and two AIPC cell lines to examine the associations between these two factors and their impact on prognosis.

2 Materials and methods

2.1 Tissue specimens

All PCa tissues included in the present study were from 48 adenocarcinoma of prostate cases that were diagnosed by two pathologists between 1999 and 2003. The median age of the patients was 67 years (range from 58 years to 83 years). History, transrectal ultrasound, computed tomography, magnetic resonance imaging and isotope scanning of the skeleton were combined to decide the clinical staging. These samples were obtained from 18 patients whose clinical tumor staging was T1 or T2 and who had received radical prostatectomies, and from needle-biopsies of the remaining 30 patients. The patients who had not been able to undertake radical prostatectomies had received neoadjuvant complete androgen ablation therapy based on luteinizing hormone-releasing hormone agonist and an antiandrogen treatment for 18–30 months (average 21.8 months), and had presented with continued rises in prostate specific antigen (PSA) levels or bone metastases. All patients were followed up after therapy: 14 patients who accepted radical prostatectomy did not have metastases and maintained very low PSA levels (below 0.2 ng/mL), with no relapse; and 4 patients who had had a radical prostatectomy had biochemical recurrences (average PSA level 1.9 ng/mL). Antiandrogen therapy was given intermittently to these 4 patients for a short period. We considered these 18 cases to be androgen dependent prostate cancer (ADPC). The other 30 patients presented with rises in PSA levels or bone metastases. These 30 patients were determined to have advanced hormone-refractory PCa. The PSA levels of 14 patients before treatment were ≤ 10 ng/L, and the others were > 10 ng/L. The Gleason scores of 16 patients were ≤ 7, and 32 patients' Gleason scores were > 7. The study was conducted with the approval of the ethical committee of Nanjing Medical University (Nanjing, China).

2.2 Immunohistochemistry

COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and EGFR (R&D Company, Minneapolis, MIN, USA) expression were analyzed in paraffin embedded tumor specimens from 48 patients. Sections (4 μm) were incubated overnight at 4ºC with the following antibodies (100:l per slide): COX-2: goat polyclonal IgG (1:100 dilution) and EGFR mouse monoclonal antibody (1:200 dilution). Following a phosphate buffered saline (PBS) wash, secondary antibody was applied (COX-2: biotinylated bovine anti-goat IgG/B [Santa Cruz] at a dilution of 1:400 in PBS with 0.1% bovine serum albumin; EGFR:biotinylated rabbit anti-mouse whole immunoglobulins at a dilution of 1:400) and slides incubated for 30 min (room temperature, RT) prior to a PBS wash. Avidin-biotin complex (ABC) solution (100:l) was applied to each slide (incubated for 30 min RT). Slides were mounted with a xylene-based mounting medium.

2.3 Specimen interpretation

Immunostained tissue sections were scored according to the percentage of tumor cells positive for COX-2 or EGFR. The median number of positive tumor cells stained was chosen as the cut-off point. The median number of tumor cells staining positive for COX-2 was 10% and for EGFR the median value was 10%. All slides were double interpreted at low power by individuals blinded to survival data. Where differences were

recorded, consensus was achieved using a dual-headed microscope. All slides were examined by trained pathologists in Nanjing First Hospital Affiliated to Nanjing Medical University (Nanjing, China).

2.4 Cell culture

PC-3 and DU-145 cell lines (American Type Culture Collection, Rockville, MD, USA) were routinely cultured in RPMI 1640-maintained media containing 10% fetal calf serum, 25 U/mL penicillin and 25 μg/mL streptomycin. In certain experiments, cells were treated with epideramal growth factor (EGF), the MAPK inhibitor SC203580, or phosphatidylinosito-3 kinase inhibitor LY294002 (BioSource, Camarillo, CA, USA). All experiments were repeated at least three times.

2.5 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from PC-3 and DU-145 cells was extracted using TRIzol (Gibco, Gaithersburg, MD, USA). RT-PCR testing was performed using an RT-PCR system according to the manufacturer's instructions (Takara, Shiga, Japan). The primers were as follows: up 5'-CGAGGTGTATGTATGAGTG TG-3' and down 5'-TCTAGCCAGAGTTTCACCGTA-3', and the length of the production was 582 bp. Thirty-five cycles of amplification were performed under the following conditions: melting at 95ºC; annealing at 56ºC; and extension at 72ºC. The PCR products were analyzed by electrophoresis on a 2% agarose gel.

2.6 Western blotting

Total cell lysates were obtained from the PC-3 and DU-145. The cell lines were stimulated with EGF (10 μg/L) for 24 h in serum unsupplemented conditions. Equal amounts (35 μg) of protein were resolved by 5% and 10% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences, Uppsala, Sweden), which were incubated with the appropriate goat polyclonal COX-2 antibodies (Santa Cruz Biotechnology) with 1:100 dilution followed by incubation with peroxidase-conjugated secondary antibodies [10]. The level of β-actin expression was used as the internal control for equal loading. The bands were compared by densitometry of western blots using an Eastman Kodak Image Station 440CF (Kodak, New Haven, CT, USA), and the data were analyzed using Kodak ID V.3.5.4 (Scientific Imaging System, Rockville, MD, USA).

2.7 ELISA

PC-3 and DU-145 cells were cultured in serum-free medium incubated with 10 μg/L EGF, EGF and LY294002, EGF and SC203580, respectively. The standard was prepared by obtaining 1.5 mL microfuge tubes. The ELISA plate (Cayman Chemical, Ann Arbor, MI, USA), coated with goat antimouse IgG was loaded at 50 μL per well of standard. The plate was covered and placed in 4ºC for 16 h. After the incubation period, all the liquid from the wells were removed and the plate was washed with wash buffer (Cayman Chemical) five times. Next, 200 μL of Ellman's reagent (Cayman Chemical) was added to each well and the plate was covered and allowed to develop in the dark with low shaking at room temperature for 90 min. Following the developing step, absorbance in each well at 405 nmol/L was read using a microplate spectrophotometer (BMG Labtech FLUOStar Optima, Offenburg, Germany). Wells containing Ellman's reagent alone served as the blank for absorbance background.

2.8 Statistical analysis

Pearson's χ^2-test was used to analyze the relationship between COX-2 and EGFR, and associations with clinical-pathological features. The Kaplan-Meier method was used to generate survival plots and the log rank test was

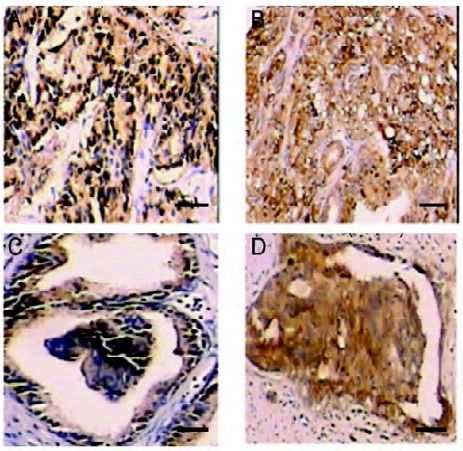

Figure 1. Immunohistochemical patterns of staining for cyclooxygenase-2 (COX-2) and epidermal growth factor receptor (EGFR) in prostate cancer (PCa) and benign prostatic hyperplasia (BPH) (brown denotes positive, × 400). (A): EGFR positive staining in PCa; (B): COX-2 positive staining in PCa; (C): EGFR positive staining in BPH; (D): COX-2 positive staining in BPH. Scale bars = 100 μm.

Table 1. The rate of epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2) positive expression in androgen dependent and independent prostate cancer (AIPC) groups, and the relationship of EGFR and COX-2 positive expression with different Gleason scores, clinical stages and prostate specific androgen (PSA) values.

	n	EGFR (+)		EGFR (–)	
		COX-2 (+)	COX-2 (–)	COX-2 (+)	COX-2 (–)
Gleason score					
≤ 7	16	2 (12.5)	5 (31.3)	2 (12.5)	7 (43.8)
> 7	32	14 (87.5)	13 (40.6)	4 (12.5)	1 (3.1)
T Stage					
≤ T2	18	6 (33.3)	5 (27.8)	4 (22.2)	3 (16.7)
> T2	30	22 (73.3)	5 (16.7)	1 (3.3)	2 (6.7)
PSA value					
≤ 10 ng/L	14	1 (7.1)	6 (42.9)	2 (14.3)	5 (35.7)
> 10 ng/L	34	15 (44.1)	12 (35.3)	4 (11.8)	3 (8.8)

used to assess statistical significance. $P < 0.05$ was considered significant.

3 Results

3.1 COX-2 immunostaining

The cinnamomeous staining mean was positive. In our study, intense staining was seen in AIPC (80%) and in ADPC (55.5%), which were predominantly in endochylema of PCa cells. There was no significant difference between them ($P = 0.07$) (Figure 1). COX-2 was also seen in benign prostatic hyperplasia (BPH) (30%). A significant association was observed between COX-2 expression and higher Gleason scores ($P < 0.05$) and tumor stage ($P < 0.05$).

3.2 EGFR immunostaining

Eleven ADPC were positive for EGFR expression (61.1%). EGFR expression was increased to 27 of 30 (90%) samples in AIPC patients. This difference was statistically significant between the two groups ($P < 0.01$). A significant association was observed between EGFR expression and a higher Gleason score ($P < 0.05$) and between EGFR expression and tumor stage ($P < 0.05$) (Figure 1).

3.3 Relationship between EGFR and COX-2 expression and clinicopathological parameters in AIPC and ADPC

EGFR and COX-2 positive coexpression was found in 22 AIPC (73.3%), and only in 6 (33.3%) ADPC, and no in BPH. The positive rate in AIPC was significantly higher than that in ADPC and BPH; there was obvious correlation only in AIPC ($r = 0.5528$, $P < 0.001$). The 20 of 22 AIPC with coexperssion developed metastatic and had an obviously poor prognosis ($P < 0.05$) (Table 1).

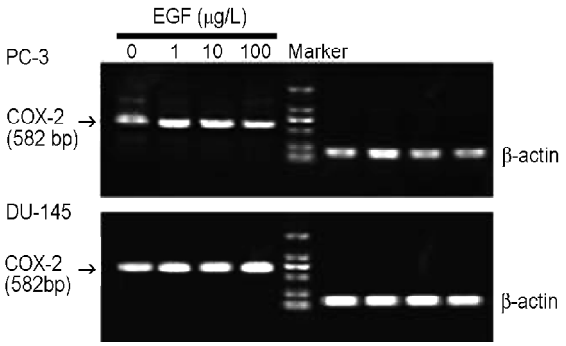

Figure 2. Serum-starved PC-3 and DU-145 cells were treated with epidermal growth factor (EGF) (0 μg/L, 1 μg/L, 10 μg/L, 100 μg/L) for 24 h. The β-actin was used as the control.

3.4 Effect of EGF stimulation on COX-2 levels signal pathway in PC-3 and DU-145 cells in serum free conditions

We analyzed in PC-3 and DU-145 cells the expression of COX-2 using RT-PCR and Western blot analysis. RT-PCR analysis revealed that COX-2 is obviously upregulated after EGF stimulation in a dose-dependent manner. Western blot analysis revealed that PC-3 and DU-145 cells was the same. One of the major targets for the therapy in PCa is EGFR that signals via the phosphoinositide-3 kinase/Akt and MAPK pathways. In

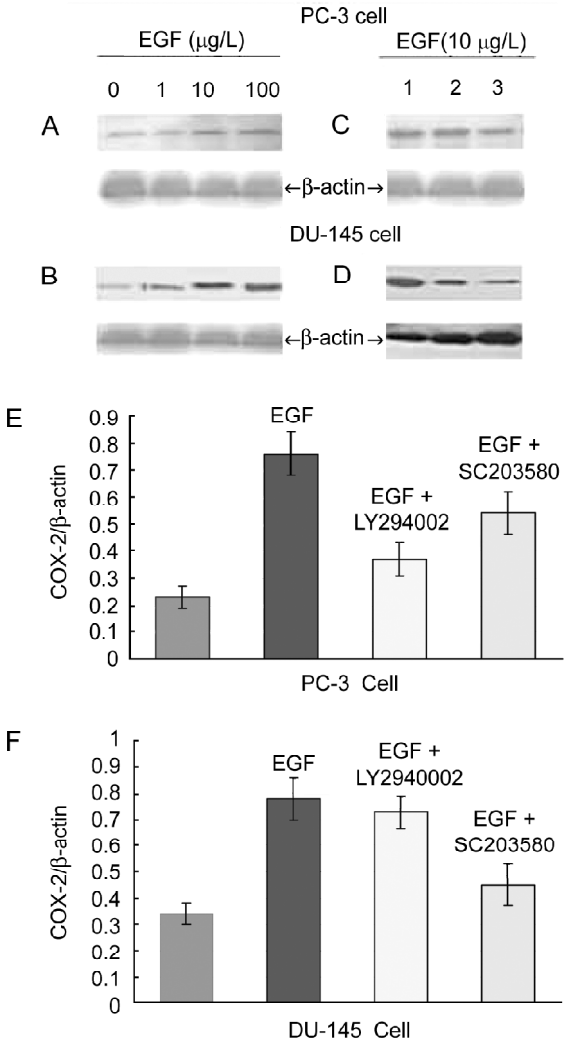

Figure 3. (A) and (B): Serum-starved PC-3 and DU-145 cells were treated with epidermal growth factor (EGF) (1 μg/L, 10 μg/L, 100 μg/L) for 24 h alone. Whole cell lysates were analyzed by Western blot using a specific antibody that recognized COX-2. (C) and (D): Serum starved PC-3 and DU-145 cells were stimulated with EGF (10 μg/L) alone (lane 1) and combination with either LY294002 (lane 2) or SC203580 (lane 3) for 24 h. (E) and (F): COX-2 and β-actin bands were subject to densitometry on an Eastman Kodak Co. Image Station 440 CF, and the ratio of COX-2 and β-actin was plotted for quantification of the blots. Representative results of three independent experiments.

this study we found that both p38MAPK and PI-3K pathways were involved in the PC-3 cells COX-2 upregulation course. Only p38MAPK pathway was associated with COX-2 upregulation in DU-145 (Figures 2 and 3).

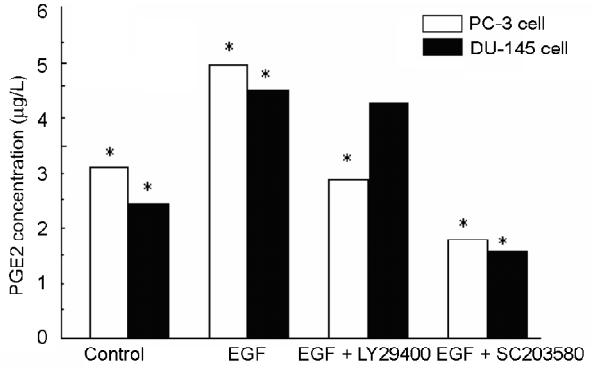

Figure 4. PC-3 and DU-145 cells were cultured in serum-free medium. prostaglandin E2 (PGE2) was significantly up-regulated after 10 μg/L epidermal growth factor (EGF) stimulation ($P < 0.05$). In PC-3 cell, both LY294002 and SC203580 reduce the production of PGE2 by EGF ($P < 0.05$); but PGE2 was only affected by SC203580 in DU-145.

3.5 ELISA

PGE2 was significantly increased after 10 μg/L EGF stimulation in both PC-3 and DU-145 cell lines ($P < 0.05$). In PC-3 cell lines, both LY294002 and SC203580 reduced the production of PGE2 by EGF ($P < 0.05$); but PGE2 was only affected by SC203580 in DU-145 (Figure 4).

4 Discussion

PCa remains the most common cause of death among urologic malignances. The majority of PCa patients will develop AIDC after the initiation of androgen deprivation. Many of the biologic events leading to a predominantly hormone-independent state remain undefined up to now. There is no effective therapy for this disease today. Therefore, identification of new effective biology-based therapy is important. Epidemiological and clinical studies have found that COX-2 enzymes play a key role in the progression of PCa. Recently, much attention has been focused on the identification of COX-2 pathways involved in ADPC to AIPC to characterize potential therapeutic targets in cancer prevention and treatment [14, 15]. In our study, no difference was found in COX-2 expression between ADPC and AIPC, although COX-2 expression in PCa was significantly higher than in BPH. COX-2 expression in PCa was associated with recurrence and metastatic. A significant association was also observed between COX-2 expression and higher Gleason scores ($P < 0.05$), and between COX-2 expression and

tumor stage.

Two EGFR family members, Erb-B1 and Her2 (Erb-B2), are frequently overexpressed in PCa, which is associated with a more aggressive clinical outcome. The expression of EGFR increases during the natural history of PCa and is correlated with disease progression and hormone-refractory disease [16]. In addition, EGFR/Her2 and their ligands, EGF, play a critical role during tumourigenesis of the prostate gland and EGFR signaling has been linked to the progression of androgen-dependent responsive PCa to androgen-independent [17, 19]. Elevated expression of both EGFR and its ligands have been described in prostate tumors and in vitro studies have indicated that the growth of the androgen-independent prostate tumor cell line DU145 is regulated by the autocrine activation of the EGFR by EGF [16, 19]. This indicates that EGFR activation is associated with the development from ADPC to AIPC. In our study, we found that EGFR levels are overexpressed in AIPC and ADPC. A significant difference was found between them. This indicates that EGFR might be associated with AIPC development.

Despite *in vitro* data suggesting that COX-2 regulation is mediated, at least in part by EGFR signaling pathways, the evidence for such an association is not consistent in human tumors *in vivo*. Some studies have demonstrated that there is association between EGFR and COX-2 in hepatocellular and nasopharyngeal carcinoma cases [20, 21], and others have found coexpression of COX-2 and EGFR to be independently poor prognostic factors. However, no strong correlation has previously been found between COX-2 and EGFR immunopositivity [22, 23]. In our study, we used an immunohistochemical analysis to find an obvious association between EGFR and COX-2 in AIPC, but it did not exist in ADPC. EGFR and COX-2 staining were dependent on each other in AIPC, but not in ADPC. We also found that COX-2 levels were upregulated by EGF stimulation in AIPC cell lines (PC-3, DU-145). To the best of our knowledge, this is the first study to examine the relationship between COX-2 and EGFR in respect to the histological progression in ADPC and AIPC. Therefore, we thought that the regulation between EGFR and COX-2 might be involved in the development from ADPC to AIPC.

In numerous cell types, EGFR activation results in COX-2 expression. Our experiments determined the signal transduction pathways used by activated EGFR to rapidly induce COX-2 in PC-3 and DU-145 cells. EGFR activation can cause receptor autophosphorylation, which may trigger both PI3K-Akt and Ras-ERK signaling pathways, resulting in induction of COX-2 [24, 25]. In our study, EGF upregulated COX-2 in both PC-3 and DU-145 cells in a dose-dependent manner. P38MAPK pathway was involved in PC-3 and DU-145 cells COX-2 regulation, while PI-3K was only associated with PC-3 cell COX-2 regulation. PTEN expression was high in DU-145, while PTEN encodes a lipid phospyhatase that is a negative regulator of the phosphoinositide 30-kinase pathway: this might lead to inactivation of PI-3K in DU-145 cell, so LY294002 cannot block the COX-2 upregulation.

In summary, our study demonstrated that COX-2 and EGFR are overexpressed in PCa. There is obviously correlation between these two factors in either tumor samples or cell lines. EGFR activation induces COX-2 expression through PI-3K and/or p38MAPK signal transduction pathways. Thus both COX-2 and EGFR inhibitors might have a cooperative anti-tumor effect in PCa, the availability of agents able to specifically interfere with COX-2 and EGFR tyrosine kinase is of potential interest, and might lead to effective treatment in the future.

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