The role of Cyclooxygenase-2 Expression in Chemoresistance and Invasiveness of Urothelial Carcinoma

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Abstract: To investigate associations between cyclooxygenase-2 (COX-2) expression and chemoresistance, as well as invasiveness of human urothelial carcinoma (UC) cell lines and UC specimens in Taiwanese patients. Expression levels of COX-2 mRNA and protein in two human UC cell lines (T24 and NTUB1) and four chemoresistant UC cell lines (T24/A, NTUB1/P, NTUB1/As, and NTUB1/T) were evaluated and compared using reverse transcription polymerase chain reaction and Western blot analysis. Expression levels of COX-2 were investigated by immunohistochemical analysis of paraffin-embedded cancer tissue specimens from 102 UC patients and five healthy controls. The chemosensitivity for COX-2 inhibitor combining with other chemotherapeutic agents were tested using MTT array and Chou-Talalay method. Kaplan-Meier analysis and log-rank test were applied to calculate the differences in recurrence-free survival between invasive and non-invasive UC patients. Forty of the 102 UC patients (39.2%) were diagnosed as COX-2 positive expression cases. T24 cells but not NTUB1 cells expressed COX-2. All four chemoresistant UC cell lines expressed COX-2. COX-2 expression was significantly associated with local tumor invasion (p < 0.043) in human UC tissue specimens but not associated with other clinical and pathological characteristics, such as source of specimen, sex of the patient, and tumor grade. Rofecoxib at 1 μM enhanced the chemosensitivity of all 6 UC cell lines. There was no difference in recurrence-free survival between COX-2 positive and negative expression in both invasive and non-invasive UC cohorts. High COX-2 expression was detected in T24 UC cells but not in NTUB1 UC cells. COX-2 was expressed in chemoresistant UC cells. COX-2 overexpression was associated with tumor invasiveness in Taiwanese patients with UC.

1. Introduction

Cyclooxygenase (COX) is an essential enzyme in the conversion of arachidonic acid to prostaglandins and other eicosanoids. Two COX isoforms, COX-1 and COX-2, have been characterized. COX-1 is constitutively expressed in many tissues, whereas COX-2 is only inductively expressed in response to factors such as cytokines, growth factors, and tumor promoters.

COX-2 has been reported in the progression of human cancers [1]. In breast cancer cells, COX-2 modulates the chemoresistance mechanism involving the NF-κB pathway [2]. COX-2 is upregulated by galectin-1 in lung cancer progression and chemoresistance [3]. The COX-2/PGE2 signaling pathway is associated with cisplatin resistance and tumor recurrence in advanced head and neck squamous cell carcinoma [4]. COX-2 is inductive and can be overexpressed in human erythroleukemia cells in response to concurrent treatment with cycloamine and jervine [5]. These findings indicate that COX-2 is involved in chemoresistance.

COX-2 expression is upregulated via the MEK/ERK signaling pathway during progression of HER2-induced non–small-cell lung cancer [6]. MMP-7 is upregulated by COX-2 in invasive lung adenocarcinoma cells [7]. COX-2 overexpression increases the invasiveness of breast cancer cells [8]. These findings suggest that COX-2 is involved in cancer cell invasion. In addition, the presence of COX-2 in human bladder cancer tissues suggests it has a role in the development and progression of bladder cancer [9-13].
Bladder cancer accounts for about 5% of all new cancers in the U. S. and there are more than 76000 estimated new cases and 16000 deaths in 2016 [14]. Bladder cancer is about 4 times more common in men than in women [15]. In Taiwan, bladder cancer killed more than 900 people in 2015 [16]. Urothelial carcinoma is the dominated subtype of bladder cancer [17]. COX-2 was found to be overexpressed in high-grade bladder UC [18] and there was also a significant correlation among COX-2 expression, local invasion, [19] cellular differentiation, and tumor invasiveness in bladder UC. [20]. The COX-2 inhibitor showed its apoptosis-inducing anti-tumor effects in invasive UC [21] and also potentially played a cytotoxic role by inhibition of autophagy [22].

Although some studies reported that COX-2 is involved in human UC, very little research have examined the role of COX-2 in UC cells or tissues derived from Taiwanese. In this study, we investigate the association between COX-2 expression and chemoresistance in Taiwanese-derived UC and drug-resistant UC cells. In addition, the relation between the tumor invasiveness of Taiwanese UC patients and COX-2 expression was also examined.

2. Materials and methods

2.1. Urothelial carcinoma cell lines and tissues

We selected two human UC cell lines — T24 (American Type Culture Collection, Rockville, Maryland, USA) and NTUB1 — to characterize the expression levels and role of COX-2 [23]. The NTUB1 cell line was derived from a surgical specimen obtained from a 70-year-old woman with high-grade UC. To study chemoresistance, we used the following chemoresistant UC cell lines: T24/A (doxorubicin-resistant), NTUB1/P (cisplatin-resistant), NTUB1/As (arsenic trioxide-resistant), and NTUB1/T (paclitaxel-resistant) [24-29]. Furthermore, 102 paraffin-embedded cancer tissue specimens (75 bladder UC from transurethral resection of the bladder tumor or radical cystectomy, 27 specimens of upper urinary tract UC) and healthy control specimens (from five benign prostatic hyperplasia patients) were examined to identify differences in COX-2 expression levels.

2.2. Western blot analysis

We used the LNCaP cell line as the negative control for COX-2 expression in Western blot analysis [30]. After all the experimental and control cell lines were incubated in Petri plates for 24 h, the cells were soaked in 1 ml of modified radioimmunoprecipitation assay buffer (50 mM Tris base, 150 mM sodium chloride, 1% NP-40, 0.25% sodium deoxycholate, and 1 mM EGTA). Protease inhibitors (1 mM PMSF, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatin A, 1 mM Na3VO4, and 1 mM NaF) were added to the cell lines, and the mixture was then incubated on ice for 30 minutes. The mixture was subjected to 30 minutes of centrifugation at 12,000 g and 4°C. Subsequently, 50 mg of the suspension extract was separated with 12% SDS-polyacrylamide gels and then transferred to an Amersham Hybond P PVDF blotting membrane (Amersham Laboratories, Buckinghamshire, England), on which it was subsequently stained with antibodies. We then treated the membrane with blocking reagent (5% milk, 10 mM NaN 3, and 10 mg/mL BSA) at room temperature for 1 h. The human anti-COX-2 antibodies (BD Biosciences, Los

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of patients</th>
<th>No. of COX-2-positives (%)</th>
<th>Odds ratio (95% CI)</th>
<th>P-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number 102</td>
<td>40 (39.2%)</td>
<td></td>
<td></td>
<td>0.259</td>
</tr>
<tr>
<td>Source of specimen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder UC*** 75</td>
<td>32 (42.7%)</td>
<td>1.00</td>
<td>(0.52–0.84)</td>
<td>0.505</td>
</tr>
<tr>
<td>Upper urinary tract UC 27</td>
<td>8 (29.6%)</td>
<td>0.69 (0.52–0.84)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td>0.216</td>
</tr>
<tr>
<td>Male 73</td>
<td>27 (37.0%)</td>
<td>1.00</td>
<td>(0.95–1.56)</td>
<td></td>
</tr>
<tr>
<td>Female 29</td>
<td>13 (44.8%)</td>
<td>1.21 (0.95–1.56)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/2 47</td>
<td>16 (34.0%)</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 55</td>
<td>24 (43.6%)</td>
<td>1.28 (1.01–1.52)</td>
<td></td>
<td>0.043</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ta-1 56</td>
<td>17 (30.4%)</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2-4 46</td>
<td>23 (50.0%)</td>
<td>1.64 (1.29–2.36)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*CI: confidence interval; **Fisher’s exact probability test; ***UC: urothelial carcinoma
Angeles, CA, USA) were then added to the membrane, and incubated at 4°C for 12 h. Finally, horseradish peroxidase–labeled secondary antibodies (Biosource, Camarillo, California, USA) were added to the membrane to measure protein expression levels by detecting the chemiluminescent signals captured on film (ECL Western Blotting System, Amersham Laboratories, Buckinghamshire, England).

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

An SV Total RNA Isolation kit (Promega, Madison, WI, USA) was used to extract the RNA from UC cell lines. We collected 2 mg of RNA from each sample for synthesizing cDNA. The primers for COX-2 cDNA were sense-(5ʹ-GCTCAAAACATGATTGTTCATTC-3ʹ) and antisense-(5ʹ-GCTGGCCCTCGCTTATGAT-3ʹ). The PCR running conditions were as follows: pre-denaturation, 95°C, 5 minutes; DNA denaturation, 95°C, 15 seconds; DNA annealing, 60°C, 1 minute; DNA elongation, 60°C, 1 minute and proceeded for 35 cycles. The amplified PCR products were mixed with a Tris-borate-EDTA buffer solution (Ambion, Austin, Texas, USA) and separated using gel electrophoresis (4% agarose for 45 minutes) and subsequently stained using ethidium bromide (Sigma Chemical Co., St. Louis, Missouri, USA) for 5 minutes. Electrophoresis images were recorded under UV light.

2.4. Patients and immunohistochemistry

Human UC and normal bladder epithelial tissues were obtained during surgery for patients with UC and then fixed in formalin and embedded in paraffin. The DAB-Plus kit (Dako Corporation, Carpinteria, CA, USA) was performed on the UC and normal bladder epithelial tissues. Briefly, paraffin sections were dewaxed, rehydrated and antigen retrieval. The sections were incubated with 3% H₂O₂ to eliminate endogenous peroxidase activity, incubated with normal serum to reduce nonspecific reactions and then incubated overnight with 1:300 diluted anti-COX-2 antibody (BD Biosciences, Los Angeles, CA, USA). The sections were incubated with secondary antibody (biotinylated anti-mouse IgG, Signet Pathology System, Dedham, MA, USA) and then signals were detected by adding chromogenic substrate (DAB). Sections were rinsed with deionized water, counterstained with hematoxylin and mounted for the subsequent histological analysis. The determination standard for COX-2 expression intensity was as follows: negative COX-2 expression, < 10% of cells with brown cytoplasm; positive COX-2 expression, ≥ 10% of cells with brown cytoplasm (Figure 1). The clinical and pathological characteristics of all patients were sealed before determination. In addition, the optimal antibody titer and concentration were tested on UC and normal bladder tissues before identification. Tumor invasiveness was defined as T2-4 stages. [31].

2.5. In-vitro chemosensitivity test for COX-2 inhibitor

The sensitivity of in vitro chemotherapeutic drugs was tested through assessment of the cell viability in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT, Sigma-Aldrich) assay [32-34]. Briefly, the UC cells were seeded in a 96-well microplate and incubated at 37°C for 24 h. To investigate chemosensitivity, 3 types of drug administration approaches, the single use of COX-2 inhibitor, the single use of a chemotherapeutic drug, and the combined use of both types of drugs, were applied to each of the 96 wells to determine whether the COX-2 inhibitor (rofecoxib) led to a synergistic toxic effect against the UC cells. At the end of treatments with chemotherapeutic drugs, 50 ml of MTT solution at 37°C was added to each cell line. The MTT crystals were evenly mixed with 150 ml of pure DMSO and the spectrophotometer values at 490 nm were then examined. The deeper the color was, the higher the mitochondrial activity in a well was, which is a direct indicator of the cells’ increased survival rate. Thus, the survival rates of the cell lines with different types and concentrations of drugs and their half maximal inhibitory concentration (IC50) could be calculated, and a chart displaying the proportion between the combination index (CI) of
each pair of drugs and their effect could be drawn, thereby verifying the synergistic toxic effect of the COX-2 inhibitors.

2.6. Statistical analysis

We used the Fisher’s exact test or Chi-square test to calculate the associations of clinical and pathological characteristics (sources of tumor specimens, sex, tumor grade, and stage of tumor invasion) with COX-2 protein expression level in patients with UC. A two-tailed p-value of <0.05 was considered to indicate statistical significance. We applied the Kaplan-Meier method to calculate the recurrence-free survival rates and used log-rank test to analyze their statistical significance. To measure the synergistic toxic effect of the COX-2 inhibitor and other chemotherapeutic agents, we individually calculated their combination index (CI) by Chou-Talalay method [35] which provided quantitative definition for synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1) in drug combinations, respectively.

3. Results

The results of Western blot analysis of COX-2 are shown in Figure 2. Among the human UC cell lines, T24 but not NTUB1 expressed COX-2. All four chemoresistant UC cells expressed COX-2. As compared to the parental NTUB1, which is negative for COX-2 expression, all three NTUB1 drug-resistant cell lines were positive for COX-2 expression.

![Western blot of COX-2 in UC cell lines.](image)

Figure 2. Western blot of COX-2 in UC cell lines.
LNCaP is the negative control for COX-2 expression. T24 and NTUB1 are UC cell lines. NTUB1/P, NTUB1/As, NTUB1/T, and T24/A are drug-resistant UC cell lines against cisplatin, arsenic trioxide, paclitaxel, and doxorubicin, respectively. The molecular weight of COX-2 is 70 kDa.

Figure 3 shows RT-PCR results for COX-2 mRNA expression. All UC and drug-resistant cells, except the NTUB1 cell line, expressed COX-2 mRNA, which is compatible to the results of Western blot analysis (Figure 2). Both Western blot and RT-PCR results indicated that NTUB1 cells did not express COX-2, but other UC cells did express COX-2.

![RT-PCR of COX-2 in UC cell lines.](image)

Figure 3. RT-PCR of COX-2 in UC cell lines.
LNCaP is the negative control for COX-2 mRNA. NTUB1/P: cisplatin-resistant. NTUB1/T: paclitaxel-resistant. NTUB1/As: arsenic trioxide-resistant. T24/A: doxorubicin-resistant.

The associations between clinical and pathological characteristics for UC patients and COX-2 expression are shown in Table 1. The results of immunohistochemical staining are shown in Figure 1. No COX-2 expression was detected in specimens from normal controls; however, 40 of 102 (39.2%) specimens from UC patients were positive for COX-2 expression. Nine (22.50%) and 31 (77.50%) of the UC specimens were weakly (the cytoplasm of approximately 10%–50% of the UC cells turned brown) and strongly positive (the cytoplasm of > 50% of the UC cells turned brown), respectively (Figure 1). Local tumor invasiveness was significantly associated with the COX-2 expression (p < 0.05), showing that tumors of T2-T4 stage had higher COX-2 expression than Ta or T1 tumors; however, all other clinical and pathological characteristics were not associated with COX-2 expression.

In the MTT assay (Table 2), according to the proportional chart between the combination index (CI) and the drug effect, when combined with the chemotherapeutic drugs, rofecoxib exhibited a synergistic toxic effect against all 6 cancer cell lines at specific concentrations (CI < 1). The rofecoxib exhibited a synergistic toxic effect against T24 when rofecoxib:doxorubicin (30:1) and the concentration of the doxorubicin was between 0.1–10 μM. The rofecoxib exhibited a synergistic toxic effect against T24/A when rofecoxib:doxorubicin (10:1) and the concentration of the doxorubicin was between 0.3–3 μM. The rofecoxib exhibited a synergistic toxic effect against NTUB1 and NTUB1/P when rofecoxib:cisplatin (300:1) and the concentration of the cisplatin was between 3–30 μM to NTUB1 and between 1–100 μM to NTUB1/P. The rofecoxib exhibited a synergistic toxic effect against NTUB1 and NTUB1/T when rofecoxib:paclitaxel (300:1) and the concentration of the paclitaxel was between 0.03–0.3 μM to NTUB1 and between 0.01–0.3 μM to NTUB1/T. The rofecoxib exhibited a synergistic toxic effect against NTUB1 and NTUB1/As when rofecoxib:arsenic trioxide (30:1) and the concentration of the arsenic trioxide was between 0.1–3 μM.
The concentration ranges of the chemotherapeutic drugs required for triggering the synergistic toxic effect was then deduced for 1 μM rofecoxib. The peak concentration of human serum in the single chemotherapeutic drug application tests in previous studies [25-27, 33] showed it fell within the ranges of the drug concentrations required for the synergistic toxic effect against T24 (doxorubicin), T24/A (doxorubicin), NTUB1 (cisplatin), NTUB1/P (paclitaxel), and NTUB1/As (arsenic trioxide) in this study. In other words, with the exception of that of the NTUB1 (paclitaxel), the chemotherapeutic drug sensitivity of all other UC cell lines increased when the clinically suggested doses of the chemotherapeutic drugs were jointly applied with 1 μM of rofecoxib.

Neither the invasive UC group nor non-invasive UC group showed significantly differences of recurrence-free survival duration between positive and negative expressions of COX-2 (Figure 4).

4. Discussion

In this study, we examined COX-2 mRNA and protein expression levels of 6 UC cell lines using RT-PCR and IHC assays, respectively. T24 was used as the positive control of COX-2 expression [36] and LNCaP was used as negative control. The results showed 5 of those 6 UC cell lines expressed COX-2 mRNA and protein. T24, T24/A (doxorubicin-resistant), and NTUB1/As (arsenic trioxide-resistant) showed strong positive expression of COX-2 whereas NTUB1/P (cisplatin-resistant) and NTUB1/T (paclitaxel-resistant) exhibited weaker positive expression. Only NTUB1 had negative expression. COX-2 expression in cisplatin-resistant NTUB1/P was only slightly higher than that in NTUB1, which indicates that COX-2 expression may have a limited role in NTUB1/P cisplatin resistance. This finding is consistent with a previous study.

Figure 4. Recurrence-free survival rates between non-invasive UC and invasive UC patients.

Table 2. In-vitro chemosensitivity test for rofecoxib used in combination with chemotherapeutic agents

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Chemotherapeutic agents</th>
<th>Concentration chemotherapeutic agents CI&lt;1</th>
<th>Concentration of agents combined which IC50 (μM) of single chemotherapeutic agents</th>
<th>Peak concentration of single chemotherapeutic agents in serum (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T24</td>
<td>Doxorubicin</td>
<td>0.123-11.3</td>
<td>1μM Rofecoxib 0.0891-7.56 IC50=0.54±0.11 Rofecoxib=3μM IC50=5.51±0.78</td>
<td>1.63±0.49</td>
</tr>
<tr>
<td>T24/A</td>
<td>Doxorubicin</td>
<td>3.21-42.9</td>
<td>2.18-29.5 IC50=18.1±2.4</td>
<td>11.3±3.7</td>
</tr>
<tr>
<td>NTUB1</td>
<td>Cisplatin</td>
<td>5.79-50.5</td>
<td>4.72-35.6 IC50=20.1 IC50=5.51±0.78</td>
<td>11.3±3.7</td>
</tr>
<tr>
<td>NTUB1</td>
<td>Paclitaxel</td>
<td>0.414-0.956</td>
<td>0.225-0.401 IC50=0.054±0.067</td>
<td>0.087±0.004</td>
</tr>
<tr>
<td>NTUB1</td>
<td>Arsenic trioxide</td>
<td>0.362-4.98</td>
<td>0.204-1.78 IC50=0.79±0.21</td>
<td>0.41±0.09</td>
</tr>
<tr>
<td>NTUB1/P</td>
<td>Cisplatin</td>
<td>0.670-78.1</td>
<td>0.418-28.5 IC50=188.1±12.3</td>
<td>11.3±3.7</td>
</tr>
<tr>
<td>NTUB1/T</td>
<td>Paclitaxel</td>
<td>0.0489-0.276</td>
<td>0.0245-0.0910 IC50=0.14±0.023</td>
<td>0.087±0.004</td>
</tr>
<tr>
<td>NTUB1/As</td>
<td>Arsenic trioxide</td>
<td>0.406-4.37</td>
<td>0.266-2.85 IC50=2.84±0.47</td>
<td>0.41±0.09</td>
</tr>
</tbody>
</table>

*CI: combination index; CI<1: the combination treatment led to synergistic toxic effect against the cancer cells.
**The concentration ranges of the chemotherapeutic agents required for triggering the synergistic toxic effect was then deduced for when the rofecoxib concentration was 1 μM.

The peak concentration of single chemotherapeutic agents in serum was the pharmacodynamics measurement after administering single dosage.
which reported that the mechanism of NTUB1/P cisplatin resistance involved overexpression of antiapoptotic Bcl-2, antioxidant heme oxygenase-1, and cell cycle regulator p16INK4 rather than COX-2 overexpression [37]. COX-2 expression in paclitaxel-resistant NTUB1/T was slightly higher than that in NTUB1. Thus, COX-2 expression has only a limited role in NTUB1/T paclitaxel resistance. COX-2 expression was significantly higher in arsenic trioxide–resistant NTUB1/As than in NTUB1, which suggests that COX-2 expression has a crucial role in arsenic trioxide resistance. According to these findings, COX-2 was only expressed in the chemoresistant UC cell lines of NTUB1, which implies that COX-2 may involve in the chemoresistance mechanism in human UC. The chemoresistance circumstance to tumor cells conferred or mediated by COX-2 was also seen in lung cancer cells [3, 38-40], head and neck squamous cell carcinoma [4], colon cancer cells [41], acute myeloid leukemia (AML) [42], and breast cancer cell [43]. Our study showed compatible chemoresistance results of COX-2 in human UC cells.

In addition, COX-2 was also reported to be involved in reducing chemosensitivity in pancreatic cancer [44], laryngeal carcinoma [45] and cervical cancer [46]. We used MTT assay to examine the cell viability of UC cells when the COX-2 inhibitor, rofecoxib, are used in combination with commonly used chemotherapeutic agents, including doxorubicin, cisplatin, paclitaxel, and arsenic trioxide. Our finding revealed that COX-2 inhibitor combining with other chemotherapeutic agents increased chemosensitivity and should be a considerable therapy for UC patients in advanced clinical study.

COX-2 was also reported to be directly or indirectly involved in the invasiveness of human hepatocellular carcinoma [47], renal cell carcinoma [48], endometrial carcinoma [49], colorectal cancer [50], and breast cancer [51]. In our current study, we found that the tumors of T2-4 stage had higher COX-2 expression than Ta or T1 tumors. Therefore, local tumor invasiveness was significantly associated with COX-2 expression. But all other clinical and pathological characteristics, such as source of specimen, sex and tumor grade, were not significantly associated with COX-2 expression. Similar results were also found in other series [52-54].

In conclusions, COX-2 overexpression was detected in T24 UC cells but not in NTUB1 UC cells. COX-2 were expressed in chemoresistant UC cells. Furthermore, COX-2 overexpression has a role in tumor invasion but not prognosis of Taiwanese UC patients. Besides, there was no difference in recurrence-free survival if COX-2 expression is taken into account. COX-2 inhibitors effectively improved chemosensitivity of UC cell lines in vitro. Future studies should investigate the mechanisms responsible for the association of COX-2 expression with carcinogenesis, invasiveness, and chemoresistance in human UC. Clinical trials will also be necessary to determine whether COX-2 inhibitors provide a clinical benefit in treatment of invasive or chemoresistant UC patients.

5. Acknowledgements

This work was partly funded by National Taiwan University Hospital (NTUH 91-5023) and Taiwan Maple Urology Association.

6. References


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