SPANDIDOS PUBLICATIONS

Cyclooxygenase-2 expression in invasive transitional cell carcinoma of the urinary bladder

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Abstract. Cyclooxygenase-2 (COX-2) activity is reported to increase apoptosis, inhibit angiogenesis and reduce metastasis. We analyzed COX-2 expression in patients with invasive bladder cancer to evaluate the feasibility of selective COX-2 inhibitor treatment targeting COX-2. Forty patients with pathologically diagnosed invasive transitional cell carcinoma of the urinary bladder (pT2-pT4) were evaluated. Immunohistochemical staining was used to evaluate COX-2 expression, and cases with staining of ≥10% of tumor cells were defined as positive. In 2 patients, 0% of the primary tumors stained for COX-2, while 1-5% was stained in 16 patients, 5-10% in 3 patients and ≥10% in 19 patients (19/40, 47.5%). In terms of grade, 2 patients with grade 2 (2/3, 66.6%) and 17 patients with grade 3 (17/37, 45.4%) were COX-2 positive. When categorized by stage, 11 patients with pT2 (11/22, 50.0%), 6 with pT3 (6/13, 46.1%) and 2 with pT4 (2/5, 40.0%) were positive. Lymph node metastasis was observed in 10 patients; 2 of them, with pN2, were COX-2 positive. Those with COX-2-positive metastatic lymph nodes had grade 3 primary tumors, which were also COX-2 positive. In addition, COX-2-negative metastatic lymph node patients also had negative primary tumors. The results of this study suggest that 47.5% of patients with invasive bladder cancer may benefit from treatment with selective COX-2 inhibitors targeting COX-2, and that treatment efficacy can be expected in patients with lymph node metastasis when their primary tumors are COX-2 positive.

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Introduction

Recent molecular biological studies have revealed that cancer becomes invasive and metastatic, increasing in malignancy, through the multi-step accumulation of genetic or epigenetic anomalies of cancer-related genes or cell cycle-related molecules (1). On the other hand, a significantly lower risk of colon cancer has been reported in patients who take non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, for cancer prevention. The chemopreventive effect of NSAIDs has attracted much attention (2). Other reports indicate that administration of the NSAID sulindac decreases the size and number of adenomas in patients with familial adenomatous polyposis, who are considered to be a high risk group for colon cancer (3), and that NSAID users have a decreased risk of developing urinary bladder cancer (4). One of the most common targets of NSAIDs is cyclooxygenase (COX). COX is an enzyme in the arachidonic acid pathway that produces prostaglandins and thromboxane, among others. It is rarely expressed under normal conditions and is transiently strongly expressed in macrophages, fibroblasts, synovial cells, endothelial cells and neurons when stimulated by various growth factors, carcinogens, tumor promoters, endotoxins and hormones. The enzyme is believed to be involved in the inflammation, proliferation and differentiation of cells. Recently, selective COX-2 inhibitors, which only inhibit COX-2, have been developed. Their anti-proliferative effect is drawing a great deal of attention.

In this study, we retrospectively evaluated COX-2 expression by immunohistochemical (IHC) staining to investigate the possible effectiveness of selective COX-2 inhibitor treatment in patients with invasive bladder cancer.

Materials and methods

Patients. We evaluated 40 patients with pathologically diagnosed invasive transitional cell carcinoma of the urinary bladder (pT2-pT4) who were examined at Aichi Medical University Hospital from January 2001 to December 2004. Thirty-three patients underwent radical cystectomy and 7

Table I. Patient characteristics.

No. of patients	40
Age	
Range	47-80
Average	65.6±8.35
Median	65
Gender	
Male	37
Female	3
T classification	
pT2	29
pT3	5
pT4	6
N classification	
pN0	23
pN1	4
pN2	6
pNx	7
Grade	
G2	3
G3	37

TUR-Bt for diagnosis. Patient age ranged from 47 to 80 years (median 65, mean 65.6±8.4 years). Thirty-seven were male and 3 were female. In terms of pathological grade, 3 patients were grade 2 and 37 were grade 3. Regarding T classification, 29 patients were pT2, 5 were pT3 and 6 were pT4. Regarding N classification, 23 were pN0, 4 were pN1, 6 were pN2 and 7 were pNx. The World Health Organization classification system (5) was used for the evaluation of histopathological grade, and TNM classification was used for the evaluation of the primary tumor and lymph node metastasis (6).

This study was approved by the Institutional Review Board, Aichi Medical University (no. 275). All patients, after receiving sufficient information, provided informed consent prior to the analysis of COX-2 expression. None of the patients were administered any medication influencing the expression of COX-2. Table I shows the characteristics of the 40 patients.

Immunohistochemistry method and evaluation. COX-2 was detected by immunostaining using the labeled streptavidin biotin method. Paraffin blocks of the specimens fixed with 20% formaldehyde were prepared from 4-μm sections. Slides were deparaffinized using xylene and hydrated with graded ethanol. Endogenous peroxidase was inactivated with 3% hydrogen peroxide in absolute methanol for 30 min at room temperature. Antigen retrieval was performed 4 times for 5 min each time using a microwave in a 1-mol/l concentration of EDTA (pH 8.0) followed by washing in deionized water. Staining was performed using an automated staining apparatus for IHC (Ventana NX System, Ventana Medical System, Inc., Tucson, AZ, USA) according to the manufacturer's guidelines.

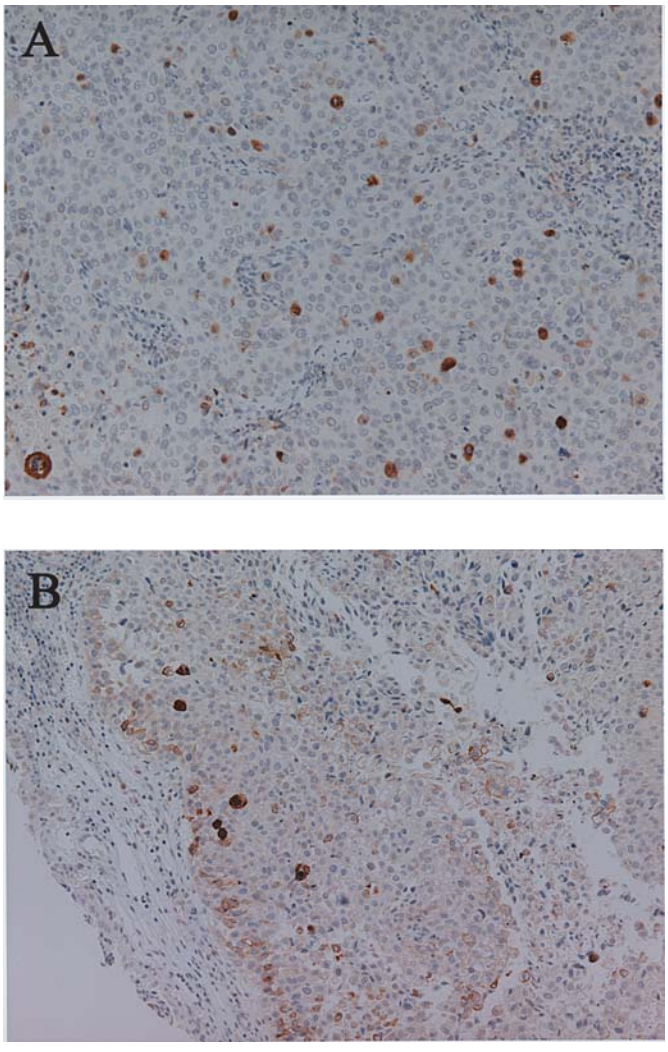

Figure 1. (A) COX-2 immunohistochemical staining in <5% of grade 3 pT3 tumor cells (COX-2-negative immunostaining) (x200). (B) Strong staining of cytoplasm in >70% of grade 3 pT3 tumor cells (COX-2-positive immunostaining) (x200).

Non-specific reactions were suppressed with the Endogenous Biotin Blocking Kit (Ventana Medical System, Inc.). Sections were treated with rabbit anti-human polyclonal COX-2 antibody (IBL Co., Ltd., Takasaki City, Gunma, Japan) diluted 1:25 in Tris-bovine serum albumin overnight at 4˚C. The sections were subsequently washed with phosphate-buffered saline (PBS). Biotin-labeled mouse anti-rabbit IgG antibody was allowed to react at 37˚C for 30 min, after which the specimen was washed in PBS, then allowed to react with horseradish peroxidase-labeled streptavidin at 37˚C for 30 min. After washing, color was developed using 0.5% diaminobenzidine and 0.01% hydrogen peroxide. The sections were counterstained with hematoxylin and mounted on slides. For negative controls, the primary antibody was omitted from the samples. Inflammatory lymphoid tissue was used as a positive control. Staining of the cytoplasm of <10% of the tumor cells was considered a COX-2-negative result, while staining of ≥10% cells was defined as COX-2 positive. Each tissue specimen was examined on two separate occasions by two experienced pathologists blinded to the stage and grade of the tumor (Fig. 1).

COX-2 expression by grade and stage.

	COX-2 positive No. (%)	COX-2 negative No. (%)	Total	P-value
Grade				
G2	2 (66.6)	1 (33.3)	3	
G3	17 (45.4)	20 (54.6)	37	0.93
T classification				
T2	11 (50.0)	11 (50.0)	22	
T3	6 (46.1)	7 (53.9)	13	
T4	2 (40.0)	3 (60.0)	5	0.92
N classification				
N1	0 (0.0)	4 (100.0)	4	
N2	2 (33.3)	4 (66.7)	6	0.63

Statistical analysis. For statistical analysis, Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) software version 10.0 for Windows was used. The χ^2 test was used to detect statistically significant differences between groups, with a level of significance of $P<0.05$.

Results

The primary tumors of 2 patients had COX-2 staining in 0% of the cells, 16 in 1-5%, 3 in 6-10% and 19 in ≥10% (19/40, 47.5%). As shown in Fig. 1, COX-2 expression was observed as light brown staining in the cytoplasm of tumor cells, whereas no staining was observed in the nuclei. In terms of grade, 2 patients with grade 2 (2/3, 66.6%) and 17 with grade 3 (17/37, 45.4%) were COX-2 positive. According to stage, 11 patients with pT2 (11/22, 50.0%), 6 with pT3 (6/13, 46.1%) and 2 with pT4 (2/5, 40.0%) were COX-2 positive. Ten patients had lymph node metastasis. Of these, 2 had COX-2 staining of 0%, 6 of 1-5% and 2 of ≥10% (2/10, 20.0%). No patients with pN1 were COX-2 positive, and only 2 with pN2 were COX-2 positive (2/6, 33.3%). No statistically significant difference was observed between any of the groups (P=0.93, 0.92, 0.63) (Table II). However, COX-2-positive patients with lymph node metastasis also had positive primary tumors, and COX-2-negative patients with lymph node metastasis also had negative primary tumors. A statistically significant difference was observed between primary COX-2-positive patients and metastasis COX-2-positive patients (P=0.03). This finding is significant since it suggests that metastatic lymph nodes are likely to be COX-2 positive when the primary tumor is COX-2 positive (Table III).

Table III. COX-2 expression in primary tumors and lymph node metastases.

Primary tumors	Positive	Negative
Lymph node metastases (n=10)		
Positive	2	0
Negative	0	8
	P=0.03	

Discussion

To date, the standard treatment for invasive bladder cancer without metastasis is in most cases radical cystectomy. However, the results of treatment with radical cystectomy greatly depend on the pathological stage and degree of lymph node metastasis at the time of surgery. When invasion is limited to the muscle layer (pT2), the cause-specific survival rate is reported to be as high as ~70%. However, when peripheral fatty tissue is invaded (pT3), this decreases to 30-40%, and when lymph node metastasis is observed, it drops to ~20% (7,8). In addition, it is reported that most recurrences after radical cystectomy are of distant metastasis, with local metastasis comprising ~10% (9). Therefore, in order to improve the treatment results of radical cystectomy, it is more important to eradicate micrometastasis that cannot be identified by imaging than to improve the cure rate. Surgical resection is not sufficient for invasive bladder cancer with extramural spread or lymph node metastasis; consequently, additional treatment of some sort is required.

For progressive bladder carcinoma that is unresectable or metastatic, combination chemotherapy is usually the treatment of choice. M-VAC treatment is a common chemotherapeutic regimen, and has been the gold standard since Sternberg *et al* (10,11) reported a response rate of 72% and a complete remission rate of 36%. However, later studies have shown a lower response rate. In addition, due to the short duration of the response, long-term survival cannot be expected. Since most patients are elderly and require dose reduction, this regimen can cause problems in terms of dose intensity, and its high toxicity may be a great physical burden to patients with bladder carcinoma who are mostly advanced in age (12,13). In addition, a standard treatment for M-VAC-resistant carcinoma has not been established. Currently, clinical studies focusing on gemsitabine and taxane agents are being conducted to develop an alternative regimen, but their effectiveness is still under investigation (14-16).

On the other hand, the targeted treatment of cancer involves the administration of drugs targeting cancer-specific changes. Conventional chemotherapeutic agents mainly affect the nucleic acid synthesis process, DNA and microtubules, and demonstrate an antitumor effect. Since they affect both normal and tumor cells, their effects lack tumor selectivity. Therefore, the maximum tolerated dose is considered to be the optimal dose when administering conventional chemotherapeutic agents. However, targeted treatment agents generally have lower toxicity than conventional chemotherapeutic agents, and are thus better able to treat elderly patients safely - a benefit which has been emphasized. In addition, they can be co-administered with conventional chemotherapeutic agents and used as tailor-made treatment.

Needless to say, the targeted treatment of cancer requires identification of the target and is expected to be effective in only limited cases. In this study, we evaluated COX-2 expression to explore the possible effectiveness of treatment with selective COX-2 inhibitors targeting COX-2 for invasive bladder cancer.

The importance of COX-2 has mainly been studied in colon cancer, with one study showing the survival rate of patients with decreased COX-2 expression to be significantly higher than that of those with increased expression (17). Animal studies using mice and rats have revealed that the administration of high-dose selective or non-selective COX-2 inhibitors reduces the incidence of bladder cancer (18,19), and another study has reported a reduced risk of bladder cancer in NSAID users (4).

In this study, overall positive staining for COX-2 was found in 47.5% (19/40) of invasive bladder cancer patients and in 20% (2/10) of patients with lymph node metastasis. The frequency of COX-2 expression did not show a significant correlation with grade, pathological stage or lymph node metastasis. In addition, staining was limited to the cytoplasm of bladder carcinoma cells. We determined the presence of a clear immune response in 10% or more of COX-2-positive carcinomas, and a weak or absent immune response in COX-2-negative carcinomas. Thus, the effect of COX-2 on invasive bladder cancer may be clarified by selecting a lesion that is markedly influenced by COX-2. When considering selective COX-2 inhibitor treatment, an antitumor effect cannot be expected if 10% or more of the carcinoma is not affected.

Shirahama (20) reported the results of COX-2 immunostaining in 35 patients with transitional cell carcinoma of the bladder, and observed COX-2 expression in 20% of pT1 carcinomas and 45% of carcinomas with muscle layer invasion by immunoblotting, suggesting that invasive cancers have increased COX-2 expression. He additionally reported that 93% of carcinoma *in situ* (CIS) showed COX-2 expression. Komhoff *et al* (21) also reported that the expression of COX-2 increased with the grade and stage of bladder cancer. Yoshimura *et al* (22) evaluated COX-2 mRNA expression in normal bladder, bladder carcinoma and chronic cystitis using reverse transcription polymerase chain reaction. The results demonstrate a positive correlation between the frequency of COX-2 expression and the grade and stage of disease. Moreover, Mohammed *et al* (23) reported that 86% of invasive transitional cell carcinomas, 78% of non-invasive transitional cell carcinomas and 75% of CIS were COX-2 positive. In addition, in 53% of cases, morphologically normal epithelium adjacent to the cancer lesion was COX-2 positive. They reported that this indicates that morphologically normal epithelial cells can undergo acquired mutation and biological alteration like cancer cells, and may change into tumor cells due to a paracrine effect caused by increased cytokines and/or growth factors. It was suggested that this phenomenon occurred as an expression of the 'field effect', and that COX-2 expression may play a role in the pathogenesis of carcinoma. However, in our study COX-2 expression was not observed in normal cells surrounding the cancer lesions, and further examination is needed to prove what is only theoretically understood.

On the other hand, another study assessing COX enzyme activity in a transitional cell carcinoma cell line supported the opposing view, with an increase in enzyme activity of 70% of cells in the cell line being observed. In addition, low-grade and low-stage carcinomas exhibited high COX enzyme activity compared to high-grade and high-stage carcinomas (24). Ristimaki *et al* (25) detected COX-2 immunoreactivity in 66% of tumor cells in transitional cell carcinomas of the urinary bladder, compared to 25% in non-neoplastic samples. COX-2 immunoreactivity was localized in neoplastic cells. They reported that there was no significant difference in the rate of positivity between invasive and non-invasive carcinomas. Shariat *et al* (26) also measured COX-2 immunoreactivity in the cytoplasm of bladder carcinomas, and reported no association between COX-2 expression and clinical findings, pathological grade, stage or lymphatic involvement. This report is in accordance with our results, but we cannot exclude the possibility that the reason no significant difference was observed in our study was the low number, 3 cases, of patients with grade 2 disease.

In this study, it was not possible to analyze the relationship between COX-2 and prognosis. According to Shirahama *et al* (27), COX-2 expression is not a prognostic factor. However, Kim *et al* (28) reported that COX-2 expression could predict recurrence and progression of T1, grade 3 bladder carcinoma. Further evaluation with a greater number of cases would appear to be needed.

The results of our study suggest that 47.5% of invasive bladder carcinoma patients may benefit from treatment with selective COX-2 inhibitors, and that these drugs may be effective in patients with lymph node metastases when the primary tumor shows COX-2 expression. Further study leading to the establishment of effective treatment with selective COX-2 inhibitors for invasive bladder carcinoma is highly anticipated.

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