Annals of Diagnostic Pathology 34 (2018) 50–55

Contents lists available at ScienceDirect

Annals of Diagnostic Pathology

journal homepage: www.elsevier.com/locate/anndiagpath

Overexpression of COX2 indicates poor survival in urothelial bladder cancer

Usha Agrawal[a,b], Nitu Kumari[a,c], Pawan Vasudeva[d], Nayan K. Mohanty[e], Sunita Saxena[a,*]

[a] National Institute of Pathology, Indian Council of Medical Research, New Delhi-110029, India
[b] Faculty of Health and Biomedical Sciences, Symbiosis International University, Lavale, Pune- 412115, India
[c] BITS, Pilani, Rajasthan, India
[d] Deptt of Urology, VMMC and Safdarjung Hospital, New Delhi-110029, India
[e] Saket City Hospital, Press Enclave Road, New Delhi -110017

ARTICLE INFO

Keywords:
COX2
Urothelial bladder cancer

ABSTRACT

Background: COX2 is a cyclo-oxygenase enzyme expressed in the tumor cells, inflammatory cells, stromal and non-epithelial cells. The study was conducted to evaluate the expression of COX2 in Urothelial carcinoma and find the association with progression and recurrence.
Methods: The expression of COX2 was evaluated by real-time PCR and immunohistochemistry.
Results: Gene expression of COX2 was found to be upregulated > 28-fold in urothelial cancer compared to adjacent normal bladder mucosa. Inflammatory cell expression of COX2 was found in 92% cases whereas only 37% cases showed COX2 overexpression in tumor cells. Tumor cell COX2 overexpression was significantly associated with invasion and recurrence.
Conclusion: COX2 expression is a marker of invasion, recurrence and poor survival and may have a role in predicting the cases which will benefit from additional treatment with COX2 inhibitors in urothelial carcinoma.

1. Introduction

Urinary bladder cancer is 7th common cancer globally, in males [1]. Among the various morphological subtypes, urothelial carcinoma accounts for 90% of bladder cancer [2]. The spectrum of urothelial cancer ranges from in situ lesions confined to the mucosa (pTa) to invasive lesions which penetrate the lamina propria (pT1), detrusor muscle (pT2) and extend perivesically (pT3). The morbidity of the disease is increased by frequent recurrences (~70%) in the pTa and pT1 stages [3]. The treatment of pTa and pT1 (Non-muscle invasive urothelial cancer or NMIUC) is conservative surgery with transurethral resection of bladder tumor (TURBT) and in the muscle invasive cancer (MIUC) stages, pT2 and pT3, it is by radical surgery such as cystectomy [4]. Further, to prevent recurrences in non-invasive stages immunomodulators such as BCG are instilled into the bladder directly (intravesical immunotherapy) [5]. Immunotherapy has been found to be of great help in reducing recurrences in about 50% of the cases but the rest recur [6]. At present there is no marker in clinical use to predict the progress or recurrence of disease and while morphologic grade is good at predicting behavior of the tumor it is not a good indicator of recurrence. It is also difficult to predict which cases will respond to immunotherapy and which will not. However, the response of at least 50% of the tumors to intravesical immunotherapy is an indication of the role the immune response plays in the tumor behavior.

A pro-inflammatory immune response is reported to be elicited in most tumors and is protective as the host tissue tends to limit damage by containing the tumor cells [7]. Most commonly reported is the cell-mediated immune response where tumor infiltrating lymphocytes (TILs), tumor associated macrophages and NK cells infiltrate into the tumor microenvironment [8]. These immune cells release a multitude of factors which include cytokines, chemokines and growth factors. The factors released have different roles, some promoting tumor cell destruction and some promoting their proliferation. Hence, the end effect on the tumor can only be decided by the way the balance is tipped. One of the pro-inflammatory factors released in tumors is COX2, which is an inducible enzyme belonging to the family of cyclo-oxygenases [9].

COX2 and its enzymatic product PGE2 have a key role in cancer progression [9]. COX2 is encoded by the PTGS2 gene (prostaglandin-endoperoxide synthase) and is involved in the conversion of arachidonic acid to prostaglandin H2 which is then converted to

Abbreviations: COX2, cyclo-oxygenase 2; NMIUC, Non-muscle invasive urothelial carcinoma; MIUC, muscle invasive urothelial carcinoma; TURBT, Transurethral resection of bladder tumor; BCG, Bacillus Calmette Guerin; TIL, Tumor infiltrating lymphocytes; NK, Natural Killer; NSAID, Non-steroidal anti-inflammatory drug; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, Real-Time Polymerase Chain Reaction; IHC, Immunohistochemistry; mRNA, messenger Ribonucleic acid; FFPE, Formalin fixed paraffin embedded; ANOVA, Analysis of variance

* Corresponding author.
E-mail addresses: uagrawal@instpath.gov.in (U. Agrawal), sunita_saxena@yahoo.com (S. Saxena).

https://doi.org/10.1016/j.anndiagpath.2018.01.008

prostaglandins (PGD2, PGE2, PGF2α), prostacyclin (PGI2), or thromboxane A2 by tissue-specific isomerases [10, 11]. COX2 expression has been found in the stromal inflammatory compartment as well as in the tumor cells of colorectal carcinoma, cervical carcinoma and others [12-15]. The expression of COX2 in tumor cells has been associated with a bad prognosis in cervical cancer and with invasion in breast cancer [14,16,17]. Newer modalities of cancer treatment targeting COX2 are being tried in cancer and include NSAIDs, aspirin and coxibs. There has been initiation of clinical trials testing COX-2 inhibitors for the chemoprevention of a wide variety of cancers such as colorectal, oral, skin, esophageal, and non-small-cell lung cancers and for the treatment of cervical, prostate, and metastatic breast cancers that overexpress COX-2 [18].

In the present study gene expression of COX2 in Urothelial carcinoma was compared to paired normal tissue. The results were validated by studying COX2 protein expression and localization in tumor and stroma by immunohistochemistry. An analysis of association with grade and stage showed increased expression in high grade and muscle invasive cases. Recurrence was found associated with tumor cell expression of COX2.

2. Materials and methods

The study included 87 tumor tissue samples and paired normal urinary bladder mucosa collected at the time of surgery in the Department of Urology, Safdarjung Hospital and VMMC, New Delhi, India and also formalin-fixed, paraffin-embedded blocks from 325 cases of urothelial bladder cancer from the archives of the National Institute of Pathology, New Delhi, India.

Surgical tissue samples (tumor and paired normal urinary bladder mucosa) was collected separately in 10% buffered formalin and RNAlater. Tissue samples collected in RNAlater were stored at −80 °C till further processing. Formalin-fixed paraffin embedded sections were taken, stained with hematoxylin and eosin and examined for confirmation of diagnosis and morphologic grading and staging according to the 2004 WHO/ISUP criteria [19]. The patients were kept on follow-up for 36 months and recurrence free survival was noted. The diagnosis on histopathology was 46 NMIUC (29 low grade, 17 high grade, 16 recurrent) and 41 MIUC (9 low grade, 32 high grade) cases. Of the 16 recurrent cases 9 were high grade and 7 were low grade.

Samples stored at −80 °C were thawed and 20 mg of tissue was weighed and total RNA was extracted from these samples using the RNeasy mini kit (Qiagen). Genomic DNA contamination was eliminated by DNAse treatment by using RNAse free DNAse kit (Qiagen GmbH, Hilden, Germany). Good quality RNA with readings of > 1.8–2.0 at 260/280 and 260/230 absorbance were utilized for further experiments.

Quantitative real-time PCR was performed for detection of gene expression levels of COX2 using TaqMan Universal Master Mix from Applied Biosystems in the ABI Prism 7000 Sequence Detection System. Total RNA was reverse-transcribed by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). Quantitative real-time PCR was performed with 22 ng of cDNA. Each sample was assayed in duplicate in independent reactions. The gene probe for COX2 was FAM labeled (Hs00153133_m1, Applied Biosystem). Target gene expression data were normalized with the expression of housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative quantitation was used to calculate Ct values by SDS software (ABI7000) and differential expression between tumor tissue and normal mucosa was identified with the cutoff of p value < .05 (Students t-test) and a mean difference ≥ 2-fold. The gene expression in association with grade and stage was analyzed by Students t-test.

For evaluating the immunohistochemical expression of COX2, formalin fixed paraffin embedded (FFPE) blocks of 325 cases of urothelial bladder cancer were retrieved. The demographic details are given in Table 1. The peak age incidence (46.5%) was in the age group 40–60 years. Of the 325 cases, 54 were females and 271 were males. Hematoxylin & eosin stained slides were re-examined to confirm the morphologic diagnosis and included 243 NMIUC (140 low grade, 103 high grade) and 82 MIUC (19 low grade, 63 high grade).

Table 1
Clinicopathological characteristics of the archival cases.

Characteristic	Label	N(%)
Age	< 40	28 (8.6%)
	41–60	151 (46.5%)
	> 61	146 (44.9%)
Gender	Male	271 (83.4%)
	Female	54 (16.6%)
Grade	Low grade	159 (48.9%)
	High grade	166 (51.1%)
Stage	Non-muscle invasive	
	pTa	56 (17.2%)
	pT1	187 (57.5%)
	Muscle invasive	
	pT2	78 (24.0%)
	pT3	4 (1.2%)
Recurrences	Absent	219 (67.4%)
	Present	106 (32.6%)

The intensity of peritumoral inflammation was estimated independently by two pathologists (UA and SS). The intensity was categorized into four groups (no inflammation = 0, sparse inflammation = 1, moderate inflammation = 2 and dense inflammation with or without formation of lymphoid follicles = 3).

FFPE sections (4 μm thick) were taken on poly-L-lysine coated glass slides and processed for immunohistochemistry by non-biotin polymeric technology using Super sensitive one-step polymer-HRP detection kit (Biogenex, Fremont, CA). Heat-induced antigen retrieval was performed with slides placed in citrate buffer (pH 6.0) and slides were incubated with the primary antibody to COX2 (SantaCruz Biotechnology, Dallas, Texas) at 4 °C overnight. DAB (diaminobenzidine) was used as the chromogen. The slides were observed for the expression of COX2 in the inflammatory and tumor cells and the estimated percentage of cells positive for the protein was noted by both pathologists independently and mean of the 2 values was taken for the final analysis. For those cases where the difference in observation between the two pathologists was > 10%, the slides were observed by them together to reach a consensus.

The number of cases positive and negative for each component, inflammatory and tumor was analyzed for association with grade and stage. The cases were further categorized as inflammatory only (expression in stromal inflammatory cells only), tumor only (expression in tumor cells alone) and both (expression in inflammatory and tumor cells in the same slide). Each component in the tissue was then scored for COX2 expression in a scale of 0–3 (no expression = 0, < 10% expression = 1, 11–40% expression = 2, and > 40% cells expressing COX2 = 3). COX2 expression in both inflammatory and tumor cells was compared in different grades and stages to analyze the association between them by using the χ^2 (Chi-Square) or Fishers' exact test as appropriate. Significance was taken at $p < .05$.

3. Results

3.1. Gene expression of COX2

COX2 mRNA expression was found upregulated in tumor tissue compared to normal mucosa with a mean fold change of > 28-fold. The expression was significantly more in non-muscle invasive tumors as compared to muscle invasive tumors (p = .003). The expression in different pT stages was found significant (p = .029) by ANOVA and multiple comparisons revealed that COX2 expression in pTa and pT1 were each significantly upregulated when compared to pT2 (p = .010