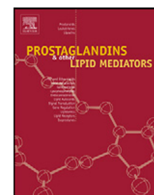
Prostaglandins & other Lipid Mediators 96 (2011) 41–44

Contents lists available at SciVerse ScienceDirect

Prostaglandins and Other Lipid Mediators

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



# Urinary thromboxane $B_2$ and thromboxane receptors in bladder cancer: Opportunity for detection and monitoring

Omar Moussa [a,b], Andrew Ciupek [a,b], Dennis K. Watson [a,b], Perry V. Halushka [c,d,*]

[a] Department of Pathology and Laboratory Medicine Medical, University of South Carolina, 173 Ashley Avenue, Charleston, SC 29425, United States
[b] Hollings Cancer Center Medical, University of South Carolina, 173 Ashley Avenue, Charleston, SC 29425, United States
[c] Department of Pharmacology, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC 29425, United States
[d] Department of Medicine, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC 29425, United States

ARTICLE INFO

*Article history:*
Received 29 April 2011
Received in revised form
20 September 2011
Accepted 22 September 2011
Available online 29 September 2011

*Keywords:*
Bladder cancer
Thromboxane synthase
Thromboxane $B_2$
Thromboxane receptor
Beta isoform

ABSTRACT

We have previously found increased expression of thromboxane synthase (TXAS) and thromboxane receptor (TP) beta isoform in the tissues of patients with bladder cancer. Studies in cell lines and mice have indicated a potential significant role of the thromboxane signaling pathway in the pathogenesis of human bladder cancer. This study was designed to determine if the changes observed in the tissues of patients with bladder cancer were mirrored by changes in the urine of these patients. We found increased levels of thromboxane $B_2$ ($TXB_2$) the major metabolite of TXAS and increased levels of the TPβ receptor. These results raised the possibility that patients with bladder cancer may be followed for progression or remission of their disease by quantitation of these substances in their urine.

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## 1. Introduction

Bladder cancer is the fifth most common cancer in the United States. It is estimated that there will be approximately 60,000 new cases each year with a 25% mortality rate [1,2]. Epidemiologic data have implicated a role for cyclooxygenase products in epithelial cell cancers, including bladder cancer [3–6]. Specifically, the chronic use of non-steroidal anti-inflammatory drugs including aspirin has been associated with a decreased incidence of bladder cancer [7]. Both prostaglandins and thromboxane are products of the cyclooxygenase; thus, our previous studies were designed to determine specifically if thromboxane $A_2$ synthase [TXAS] and thromboxane $A_2$ [TP] receptors are altered in bladder cancer.

Using a microarray approach, we found that TXAS was overexpressed in bladder cancer relative to non-tumor tissue and bladder cancer cells [8,9]. This was found to be associated with an increased production of thromboxane $A_2$ as measured by its stable metabolite thromboxane$B_2$ [$TXB_2$] in the cell line compared to a control cell line. This was followed up with additional studies validating the increase in TXAS in ∼70% of tumor tissue compared to adjacent normal tissue [8]. It was also discovered that the over-expression of TXAS was associated with a poorer 5 year survival rate compared to patients that had an arbitrary lower level of expression [8].

Because of previous reports of coordinate up regulation of the TXAS and the TP receptors, we also determined the level of message and protein for the TP receptor in both cell lines and human tissue [8,10]. The TP receptors are expressed as two isoforms, alpha and beta. They represent splice variants of the message and differ only in the carboxy terminal tail. Their distribution is unique in that the beta isoform is found predominately in endothelial cells [11] and the alpha for example is expressed in large amounts in platelets [12]. Recent studies have shown that in some cancer cell lines, the alpha isoform is upregulated [13]. However, it was not known if the beta isoform was also specifically or non-specifically upregulated in epithelial cell cancers. We found that the TPβ receptor isoform but not the alpha isoform was significantly upregulated in bladder cancer cell lines and tumor tissue [10]. The overexpression of TPβ was associated with a poorer 5 year survival compared to the patients with an arbitrary lower expression [10]. Manipulation of the levels of TPβ expression also provided evidence for it playing a significant role in the cancer cell phenotype [10].

Since urine may reflect what has been synthesized and/or released by bladder uroepithelial cells or may actually contain exfoliated cancer cells, we sought to analyze the urine of bladder cancer patients for the levels of $TXB_2$ and the TPβ receptor protein and mRNA.

* Corresponding author at: College of Graduate Studies, Room 101BEB, Medical University of South Carolina, 68 President Street, Charleston, SC 29425, United States. Tel.: +1 843 876 2402; fax: +1 843 876 2416.
*E-mail address:* halushpv@musc.edu (P.V. Halushka).

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doi:10.1016/j.prostaglandins.2011.09.002

## 2. Materials and methods

### 2.1. Urine samples

Urine samples were collected from patients already diagnosed with bladder cancer, patients exhibiting no evidence of bladder cancer, and healthy volunteers. After collection, the samples were kept on ice until they were centrifuged at 3,000 × *g* at 4 °C. They were then stored at −20 °C until thawed for assay. All subjects gave written informed consent. The study was approved by the institutional review board.

### 2.2. Real time semi-quantitative PCR for mRNA TPβ and TPα

Urine sediments were washed twice with ice-cold PBS, resuspended in 1 ml PBS and transferred to 1.5 ml microfuge tubes. Specimens were then centrifuged at 3,000 × *g* at 4 °C. Supernatants were aspirated and urine sediment was resuspended in 1 ml TRIzol reagent. TPα and TPβ mRNA levels were measured using semi-quantitative real time PCR with cDNA from urine samples obtained from patients and normal volunteers. cDNA was prepared using Invitrogen's Super Script III first strand synthesis System for RT-PCR as per manufacturer's protocol. Reactions were run using cDNA in a Light Cycler Thermocycler [Roche] with the following reaction conditions: 50 °C for 10 min and then 95 °C for 2 min followed by 55 cycles of the following: 95 °C for 10 s, 56 °C for 20 s, and 72 °C for 45 s. Product levels were measured from incorporation of fluorescent double stranded DNA binding dye SYBR Green [Invitrogen]. HPRT levels were measured for normalization of the results.

Three reactions were run for each sample changing only the primers each time [all primers are given in the 5 prime to 3 prime order and based on the following GenBank sequence numbers: 42518081 for TPβ and 117414146 for TPα]. Primers used were: 1070 forward [ACGGAGAAG-GAGCTGCTCATCT, complement to NTs1070–1091 on TPβ and NTs1070–1091 on TPα] and 1356 Reverse [CACTGTCCATCCAGCA-CCC, complement to NTs1356–1338 on TPα, no complement on TPβ] for TPα detection, 1070 Forward and 1378 Reverse [CAAAAGGAAGCAACT-GTACCCC, complement to NTs1399–1378 on TPβ and NTs2039–2060 on TPα] for TPβ detection, and HPRT forward [CTTGCTCGAGATGT] and HPRT Reverse [GTCTGCATTGT-TTTGCCAGTG] for HPRT detection [to be used as a normalization control].

### 2.3. Urinary thromboxane $B_2$ levels

The urinary $TXB_2$ levels were measured using the Thromboxane $B_2$ ELISA kit [Neogen, Lexington, KY] according to the manufacturer's instructions. Duplicate 50 μL aliquots of urine were used in the assays.

### 2.4. Dot blot assay for urinary TPβ levels

Aliquots of urine [500 μL] were pipetted onto nitrocellulose membranes in a 96 well plate. After filtering the sample, membranes were blocked with 5% milk in TBST [TBS with 0.05% Tween-20] at RT for 1 h. After blocking, the filters were incubated with the primary antibody for TPβ [1:400 dilution in 5% milk] for 1 h at RT. The TPβ antibody was a gift from Dr. Anthony Ashton [10]. Following the incubation with the primary antibody, the sample was washed 5 times [5 min each with TBST] followed by the addition of the secondary antibody [goat anti-rabbit, 1:500 Caltag, USA]. The secondary antibody was pre-absorbed against human albumin. After washing several times the chemiluminescent substrate [Super Signal Western Pico-Pierce] was added for 5 min at RT. After the elapsed time, the dot blot was analyzed. The individual responsible for analyzing the dot blot was blinded to patient diagnosis.

### 2.5. Statistical analysis

Raw data from real time PCR were analyzed using linear regression methods using the LinRegPCR computer program version 7.5 [program available upon request: email: bioinfo@amc.uva.nl; subject: LinRegPCR]. GraphPad Prism 4 software was used to perform a Mann–Whitney *t*-test on the data to check for statistical significance. Urinary $TXB_2$ levels were analyzed using an ANOVA followed by a post hoc Dunnet's test. The sensitivity and specificity were determined for the dot blot by analyzing the data that was put in table format.

## 3. Results

### 3.1. Urinary thromboxane $B_2$ levels

To determine if the increased levels of TXAS that we previously found were associated with increased production of thromboxane $A_2$, the stable metabolite of thromboxane $A_2$, $TXB_2$ was measured. Samples were obtained from normal volunteers, patients attending the urology clinic for non-bladder cancer problems and bladder cancer patients. The levels of $TXB_2$ were significantly [$p < 0.01$] greater in the bladder cancer patients compared to the normal volunteers or patients with other urologic disorders [Fig. 1].

### 3.2. TPβ mRNA and protein levels in cancer patient urine samples

To see how the levels of mRNA of TPα and TPβ compared in patient urine samples—QPCR was performed on the cDNA. Comparison was done between bladder cancer patients, healthy individuals, and others exhibiting other urologic disorders. The urinary levels of TPα and TPβ mRNA were not significantly different between healthy individuals, or those diagnosed with bladder cancer or those with no evidence of bladder cancer [data not shown].

In spite of the failure to see a significant difference in the mRNA levels for TPα and TPβ in the urine, we decided to determine if there were differences in the level of the respective proteins in the urine of the three different groups. Our previous studies found that only the TPβ protein was elevated and not the mRNA [8]. TPβ protein levels were measured in urine samples using a dot blot assay.

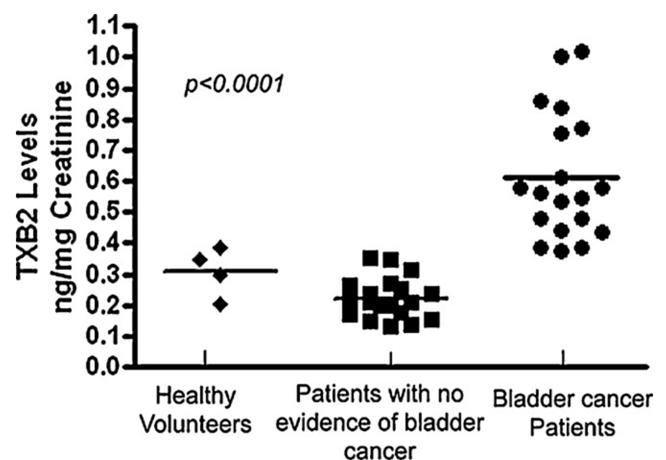


**Fig. 1.** Urinary $TXB_2$ levels in normal volunteers, patients with no evidence of bladder cancer and bladder cancer patients. The levels were measured using an ELISA assay. The results were analyzed using an ANOVA. The levels in the bladder cancer patients were significantly [$p < 0.01$] greater compared to the other two groups.

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