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Urinary Thromboxane B₂ and Thromboxane Receptors in Bladder Cancer: Opportunity for Detection and Monitoring

Omar Moussa^{a,b}, Andrew Ciupek^{a,b}, Dennis K. Watson^{a,b}, and Perry V. Halushka^{c,d}

^aDepartment of Pathology and Laboratory Medicine, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC29425

^bHollings Cancer Center, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC29425

^cDepartments of Pharmacology and Medicine, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC29425

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 B₂, (TXB₂) 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.

Keywords

Bladder cancer; thromboxane synthase; Thromboxane B₂; thromboxane receptor; beta isoform

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 antiinflammatory drugs including aspirin has been associated with a decreased incidence of bladder cancer⁷. Both prostaglandins and thromboxane are products of the cyclooxygenase; thus, our previous studies were designed to determine specifically if thromboxane A₂ synthase [TXAS] and thromboxane A₂ [TP] receptors are altered in bladder cancer.

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^dCorresponding Author: Perry V. Halushka, PhD, MD, College of Graduate Studies, Room 101BEB, Medical University of South Carolina, 68 President Street, Telephone 843-876-2402, FAX 843-876-2416, halushpv@musc.edu.

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Using a microarray approach, we found that TXAS was overexpressed in bladder cancer relative to non-tumor tissue and bladder cancer cells⁸⁻⁹. This was found to be associated with an increased production of thromboxane A₂ as measured by its stable metabolite thromboxaneB₂ [TXB₂] 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 tissue⁸. 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 expression⁸.

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 cells¹¹ and the alpha for example is expressed in large amounts in platelets¹². Recent studies have shown that in some cancer cell lines, the alpha isoform is upregulated¹³. 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 tissue¹⁰. The overexpression of TPβ was associated with a poorer five year survival compared to the patients with an arbitrary lower expression¹⁰. Manipulation of the levels of TPβ expression also provided evidence for it playing a significant role in the cancer cell phenotype¹⁰.

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 TXB₂ and the TPβ receptor protein and mRNA.

Materials and Methods

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 3,000XG at 4°C. They were then stored at minus 20°C until thawed for assay. All subjects gave written informed consent. The study was approved by the institutional review board.

Real Time semi-quantitative PCR for mRNA TPβ and TPα

Urine sediments were washed twice with ice-cold PBS, resuspended in 1ml PBS and transferred to 1.5 ml microfuge tubes. Specimens were then centrifuged at 3,000XG at 4°C. Supernatants were aspirated and urine sediment was resuspended in 1ml 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 minutes and then 95°C for 2 minutes followed by 55 cycles of the following: 95°C for 10 sec., 56°C for 20 sec., and 72°C for 45 sec. 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 TP β and NTs1070-1091 on TP α] and 1356 Reverse [CACTGTCCATCCAGCA-CCC, complement to NTs1356-1338 on TP α , no complement on TP β] for TP α detection, 1070 Forward and 1378 Reverse [CAAAAGGAAGCAACT-GTACCCC, complement to NTs1399-1378 on TP β and NTs2039-2060 on TP α] for TP β detection, and HPRT forward [CTTGCTCGAGATGT] and HPRT Reverse [GTCTGCATTGT-TTTGCCAGTG] for HPRT detection [to be used as a normalization control].

Urinary Thromboxane B₂ levels

The urinary TXB₂ levels were measured using the Thromboxane B₂ ELISA kit [Neogen, Lexington, KY] according to the manufacturer's instructions. Duplicate 50 μ L aliquots of urine were used in the assays.

Dot blot assay for urinary TP β levels

Aliquots of urine [500 μ L] 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 hr. After blocking, the filters were incubated with the primary antibody for TP β [1:400 dilution in 5% milk] for 1 hour at RT. The TP β antibody was a gift from Dr. Anthony Ashton¹⁰. Following the incubation with the primary antibody, the sample was washed 5 times [5 minutes 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.

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 TXB₂ 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.

Results

Urinary Thromboxane B₂ levels

To determine if the increased levels of TXAS that we previously found were associated with increased production of thromboxane A₂, the stable metabolite of thromboxane A₂, TXB₂ 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 TXB₂ were significantly [p<0.01] greater in the bladder cancer patients compared to the normal volunteers or patients with other urologic disorders [Figure 1].

TP β mRNA and Protein Levels in Cancer Patient Urine Samples

To see how the levels of mRNA of TP α and TP β 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 TP α and TP β 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 TP α and TP β 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 TP β protein was elevated and not the mRNA⁸. TP β protein levels were measured in urine samples using a dot blot assay.

Figure 2 is a representative dot blot, providing examples of positive and negative results. A total of 33 patients with a previous diagnosis of bladder cancer were evaluated. Cystoscopic evaluation was used to categorize the patients as having either active or inactive bladder cancer (e.g., no evidence of bladder cancer). The dot blot method had a sensitivity of 80% and specificity of 67%. The method correctly identified 12 of 15 patients with active cancer and apparently falsely identified 6 patients as having active disease. The assay correctly assigned 12 of the 18 patients with inactive disease (Table1).

Discussion

These studies demonstrated that the levels of TXB₂ and TP β protein are increased in the urine of a significant number of patients with bladder cancer compared to normal volunteers and patients with no evidence of bladder cancer. Previous studies by our group found an increase in the tumor tissue levels of TXAS mRNA and TP β protein compared to adjacent non-tumor tissue in patients with bladder cancer^{8, 10}. Those patients with increased levels above an arbitrary cutoff had a poorer five year prognosis compared to those with levels considered not significantly elevated^{8, 10}. These studies established a potential link between the overall prognosis for bladder cancer and the increases in the thromboxane pathway. Given these observations, the question arose as to whether these increases were paralleled by increases in the presence of TXB₂ and/or the TP β receptor protein in the urine of bladder cancer patients.

The exact amino acid sequence of the immunoreactive TP β receptor protein in the urine of the patients with bladder cancer is uncertain. Since the antibody is directed against the intracellular tail of TP β , it is presumed that the cancer cells are being lysed in order for it to be detected. It is also uncertain if the immunoreactivity represents the entire receptor protein or a proteolytic fragment of the cytoplasmic tail. Clearly, a proteomic analysis of the urine of the patients with bladder cancer is needed to determine the exact molecular structure of the protein material that is being analyzed. Another shortcoming of the current study was that there was no TP β receptor standard available for this study. Thus, the results reported in this study represent a qualitative result at this time.

These preliminary studies with a limited number of patients provide support for an additional larger study in which the patients with bladder cancer are stratified according to the severity of the disease. It is currently unknown if the increase in the components of the thromboxane A₂ signaling pathway is an early event in all forms of bladder cancer or a late event occurring in patients with advanced or invasive disease. Clearly, only with a larger trial will these questions be able to be answered. Furthermore, the specificity of the increase in TP β receptor in the urine as a marker for bladder cancer has not been validated. Indeed, there is evidence accumulating that renal cancers and prostate cancers¹⁴⁻¹⁵ are associated with increased TP receptor expression which may or may not be beta isoform specific. Also, it is possible that infiltrative diseases of the kidney may be associated with increased presence of TP β receptor¹⁶⁻²⁰.

However, these preliminary studies raise the possibility that the measurement of TP β receptor protein in the urine of bladder cancer patients may be a useful biomarker for two possible purposes. The first is to follow patients with superficial bladder cancer as they

receive Bacillus calmette-guerin (BCG) treatment and determine if they are in remission or have an exacerbation. The potential advantage of this approach would be the decrease in the need for routine follow up cystoscopies. In addition, it has the potential to be used to stratify patients to determine if they may respond to thromboxane receptor antagonists as an adjunctive therapy. However, before such approaches can be undertaken, a much larger clinical study must be conducted in which patients are stratified according to the diagnostic category of their bladder cancer.

The measurement of TXB₂ in the urine has some potential confounding factors. Although it is presumably coming from the kidney, it is possible that some may come from systemic sources or secondary to intrinsic renal diseases²¹⁻²⁴, thus raising the possibility that it may not represent a very specific marker for any increases in TXAS activity associated with bladder cancer.

In summary, human bladder cancer is associated with increased expression of TPβ receptor protein and TXAS. The former and/or the product of the latter measured in the urine may serve as useful biomarkers for the diagnosis, prognosis and/or stratification for treatment of patients with bladder cancer.

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Highlights

Increased urinary levels of thromboxane B₂ in bladder cancer patients

Increased urinary levels of thromboxane receptor beta isoform in bladder cancer patients.

Potential non-invasive diagnostic and prognostic test for bladder cancer.

Figure 1.

Urinary TXB₂ 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.

Figure 2.

Representative Dot-blot hybridization assay for the detection of TP β in urine samples.

Samples B2, C1, C3, G3, D4 represent true negative cases (negative cystoscopy and also negative for TP β). Cases E1, E3, and G4 represent true positive cases (positive cystoscopy and also positive for TP β). Cases B3, D3, and F2 represent false negative cases (positive cystoscopy and negative for TP β). Although more subjects were included on the dot blot only the cystoscopy patients were included in the analysis. Patients were entered into the study originally if they had a previous diagnosis of bladder cancer. Cystoscopy was not an inclusion criterion.

Table 1

Tabulation of the results obtained from the dot blot analysis of 33 patients that were either cystoscopy negative or positive.

Cystoscopy	Dot blot results		Total
	Negative	Positive	
Negative	12	6	18
Positive	3	12	15
Total	15	18	33