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Urinary BTA: Indicator of Bladder Cancer or of Hematuria

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Abstract

Background—In this study, we investigated the influence of hematuria on the performance of the BTA tests in a clinical cohort and in an experimental model.

Materials and Methods—Analysis of urine samples from a cohort of 126 subjects (64 with BCa and 62 controls) were analyzed by ELISA for hemoglobin and BTA. The experimental model involved the spiking of urine with blood from the same subject, and hemoglobin, red blood cell count, and BTA levels (BTA stat© and BTA-TRAK©). BTA-TRAK© analyses were also performed on serum samples obtained from 40 subjects (20 with confirmed with BCa).

Results—In the 126-subject cohort, correlation between hemoglobin and BTA was 0.732. Of the 64 BCa samples, 72% had a positive BTA assay, but 47% of controls were also positive. The sensitivity and specificity of BTA to detect BCa was 72% and 53%, respectively. Hematuria, measured by urinary hemoglobin, was a better indicator of BCa with 75% sensitivity and 90% specificity. Spiking of BTA-negative urine samples with as little as 1μl/10ml was enough to produce positive a BTA test. High levels of BTA were found equally in the serum of subjects with, or without BCa (mean BTA levels 355,159 U/ml vs. 332,329 U/ml, respectively).

Conclusions—Rather than detecting a bladder tumor antigen, urinary BTA assays may be measuring serum cFH introduced by bleeding, a common presenting factor in BCa subjects. The presence of hematuria in subjects without malignant disease can result in false-positive BTA assays.

Keywords

complement factor H; hematuria; BTA; bladder cancer; diagnosis

Introduction

Early detection and post-surgical surveillance of bladder cancer (BCa) require invasive procedures for confirmed diagnosis. Non-invasive urine tests are highly desirable for both patient and healthcare system, and a number of urinary tests have been developed that can augment voided urine cytology. A commercial assay that have been used in this context is the bladder tumor antigen (BTA) test, which detects urinary complement factor H-related

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Conflict of Interest

The authors declare that they have no conflict of interest.

proteins, using monoclonal antibodies (1,2). Two assays, BTA statTM and BTA TRAKTM (Polymedco Inc. Cortlandt Manor, NY, USA), are FDA approved for the detection and surveillance of BCa in urine samples (3,4). Given the fact that the complement factor H proteins are serum factors, there is the possibility that BTA tests are monitoring the presence of serum proteins that would be concomitantly present with hematuria. In this study, we examined the presence of urinary and serum BTA in clinical cohorts, and designed an experimental model to investigate the nature of the influence of hematuria on BTA tests.

Materials and Methods

Clinical sampling and processing

Under IRB approval and informed consent, urine samples and associated clinical information were prospectively collected from three independent cohorts (Table 1). From the first cohort, (urinalysis) mid-stream urine samples were collected from 126 subjects (64 active newly BCa never treated before with intravesical therapy and 62 controls) prior to any intervention. Controls included individuals with microscopic hematuria (35%) or voiding symptoms (65%). In the second cohort (hematuria model), 50 mL of voided urine and 10 mL of whole blood were obtained from five healthy controls with no active BCa, no history of BCa, no current urinary tract infection, no voiding symptoms, urolithiasis or hematuria. Whole blood, serum and plasma from each subject were analyzed using the BTA-TRAK[©] assay. No difference in the median values of BTA in whole blood, serum or plasma (>300,000 U/ml) were observed, so whole blood was used in the experimental hematuria model. In the third cohort (serum analysis), 10 mL of whole blood (serum separated) and associated clinical information were collected from 20 men with no previous history of BCa and 20 men with newly diagnosed BCa. Serum was isolated, snap frozen and stored at -80°C until use. Serum aliquots were diluted 1/1000 with PBS for analysis.

Hematuria Model

Zero, 1, 5, 20 and 50 µl of whole blood were added to urine samples from each healthy control for a final dilution of 1/10000, 1/2000, 1/500 and 1/200, and zero. The number of red blood cells (RBC) in each urine sample was determined by microscopic examination before and after adding blood. In addition, urine samples were tested by Multistix Pro dipstick test for abnormalities. The precise level of urinary hemoglobin was determined by ELISA assay. Urinary BTA levels were quantified by ELISA assay. The BTastat[©] assay was performed on each urine sample by adding 3 drops of urine into the well of the point-of-care test device. As recommended by the manufacturers, results were considered to be positive if any band, no matter how faint, appeared in the test zone within 5 minutes of adding the urine to the well.

ELISA Assays for Hemoglobin and Bladder Tumor Antigen (BTA)

Commercially available ELISA assays for urinary hemoglobin (Cat#E88-135 Bethyl Laboratories Inc., Montgomery, TX, USA) and urinary BTA (BTA-Trak[©] Cat# 662150, Polymedco Inc. Cortlandt Manor, NY, USA) were conducted according to the manufacturer's instructions. Based on published literature, a cutoff value of 14 kilounit/L was used to define a positive BTA assay (5,6).

Data Analysis

The association between BTA or hemoglobin and BCa was tested using the Wilcoxon rank sum test. Nonparametric receiver operating characteristic (ROC) curves in which the value for sensitivity is plotted against false-positive rate (1-specificity) were generated to compare the performance of each diagnostic test. The optimal cutoff (Youden index) was calculated

from the ROC curves (7). Spearman rank correlation coefficients were used to examine the correlation between urinary BTA concentrations and urinary hemoglobin concentration. The association between serum BTA levels and BCa was tested using the Mann Whitney test. Statistical significance in this study was set at $p < 0.05$ and all reported p values were 2-sided. All analyses were performed with PRISM software version 5.00 (GraphPad Software, San Diego, CA).

Results

Comparison of BTA-TRAK® and hemoglobin in a 126-subject cohort

The mean urinary concentrations of BTA as determined by BTA-TRAK® assay in BCa subjects ($n = 64$) compared to control subjects ($n = 62$) were 1631 U/ml vs. 14.6 U/ml ($p < 0.0001$). The mean urinary levels of hemoglobin in BCa subjects compared to control subjects were 4451 ng/ml vs. 2.6 ng/ml, $p = 0.005$. BTA had a relatively high correlation coefficient with urinary hemoglobin (0.745). Of samples obtained from biopsy-confirmed cancer-bearing subjects, 72% had a positive BTA assay (>14.0 U/ml), whereas 47% of controls had a positive BTA assay. The sensitivity and specificity of BTA to detect BCa were 72% and 53%, respectively. Hemoglobin diagnostic accuracy outperformed BTA, achieving values of 75% sensitivity and 90% specificity (Figure 1). The mean hemoglobin level for the 46 of 64 BCa subjects that were positive by BTA test (true positives: 6192 ng/ml) was significantly ($p < 0.0001$) higher than the mean hemoglobin level for the 18 of 64 BCa subjects with a negative BTA assay (false-negatives: 2.3 ng/ml). Clinical hematuria tests use values <100 ng/ml as negative for hematuria.

Hematuria model

Five urine samples were obtained from healthy volunteer controls for analysis in a hematuria model. There was no evidence of gross hematuria, urinary tract infection or any biochemical abnormalities in any of these samples. Both urinary dipstick and urinary microscopy were negative for hematuria in the samples, however, urinary hemoglobin measured by ELISA assay revealed trace amounts in all samples (mean 5.00 ng/ml). With the addition of only 1 μ l of whole blood to 10 mL of test urines (1/10,000 dilution), the mean urinary hemoglobin level was 121.49 ng/ml, and microscopy revealed a median 1 RBC/hpf. In urinary dipstick, analyses of urine samples were reported as 'Trace'. At this level, 40% of BTAsat® assays were positive, and 40% of BTA-Trak® assays were positive. With the addition of 50 μ l of whole blood to 10 mL of urine, all urines had gross hematuria visibly, the mean urinary hemoglobin level was 4,422 ng/ml, and a median of 22 RBC/hpf was noted. At this level, 100% of urinary dipstick was positive for large blood, BTAsat® assays were positive, and 100% of BTA-Trak® assays were positive. As the concentration of whole blood added to the urine samples increased, the median urinary hemoglobin level, the extent of hematuria assessed by microscopy, the amount of blood detected by urinary dipstick and the number of positive BTAsat® and BTA-Trak® assays increased accordingly (Table 2). There was a high correlation coefficient between urinary hemoglobin and BTA (0.83).

BTA as a potential serum biomarker for bladder cancer

Given the correlation of BTA detection with the presence of hematuria, and the known function of complement factor H, it is likely that the source of BTA is the serum that is introduced through bleeding. Using BTA-TRAK® assay, we investigated the level of BTA in a panel serum samples, and compared levels between patients with or without BCa to evaluate whether BTA could be a serum marker for BCa. Serum samples from 20 Caucasian men with BCa and from 20 age-matched Caucasian controls were analysed using the BTA-Trak® assay. Very high levels of BTA were found in both groups of men. Mean serum

BTA levels were not significantly different in men with BCa (355,159 U/ml) and controls (332,329 U/ml).

Discussion

The correlation between a positive BTA-TRAK© test and the presence of hematuria was significant ($r^2 = 0.745$, $p < 0.0001$), and the levels of hemoglobin in the positive and negative BTA test for the cancer-bearing subjects supported this fact. When hemoglobin levels were below levels routinely used to class samples as having hematuria, then the BTA test was negative. These findings prompted us to design a model of hematuria that used the spiking of BTA-negative urine samples with autologous blood. Although the model does not mimic the actual physiological situation exactly, the model can test whether the BTA analyte is present in the blood of and at what level of hematuria this would produce a positive BTA test. The level of spiked blood required was surprisingly low, causing some positive BTA tests with as little as 121 ng/ml hemoglobin, or 1 RBC/hpf. These are levels that registered as trace on urinary dipstick, which are frequently used in the clinic. When spiked hemoglobin levels reached 400 ng/ml or 3 RBC/hpf, levels that would only be classed as small amount of hematuria, then 100% of BTA tests were positive. Furthermore, hemoglobin diagnostic accuracy outperformed BTA, achieving values of 75% sensitivity and 90% specificity (Figure 1).

The analysis of serum BTA was designed to answer two questions. What was the level of BTA in serum, and was the serum level indicative of bladder disease status? The levels detected were very high (~350,000 U/ml), which supports the idea that very small amounts of serum protein incursion through bleeding could have a major impact on BTA assay data given the recommended cut-off for a positive test is in the order of 14.0 U/ml. Comparison of the data between serum samples from subjects with and without bladder cancer did not reveal a correlation of serum BTA levels with disease status.

In combination, these findings raise the question of whether the BTA tests are detecting a *bona fide* bladder tumor antigen in urine samples. In order to further investigate, we reexamined some of our own data and queried publicly available resources to see if there is any evidence showing that complement factor H is differentially expressed in bladder tumor tissues. We have previously demonstrated the feasibility of comprehensive gene expression profiling of naturally exfoliated urothelia in order to identify cancer-associated gene signatures (8), and we have recently expanded the profile to cases to 92 cases. Validation of selected candidate biomarkers has been performed in an independent cohort of 81 subjects and a highly accurate biomarker panel is under further validation in multi-institutional studies. There are advantages to analyzing urothelial samples; the material is available non-invasively, the cellular component can be isolated away from the urinary protein milieu, and there is no problem in obtaining comparable control samples as there is with solid tissue. If cFH is differentially expressed in malignant urothelial cells, our genome-wide transcriptome analysis may have been able to confirm it. Probes for both cFH and cFHR1 were present on the Affymetrix U133 2.0 Plus chips (47,000 transcripts) used in our study. Both targets were classed as 'absent' in about 50% of cases, with no specific distribution of this classification between tumor and normal cases. In the remainder, low levels of expression were detected, and no statistical significance was evident between groups. We also examined publicly available bladder tissue gene expression profiles for evidence of an association between cFH transcript levels and bladder cancer. Solid tissue profiling studies have the disadvantages of homogenizing samples of heterogeneous cellular content, and often have no comparable normal tissue to compare against, but if bladder tumor cells express high levels of cFH then it might be expected that this would have been identified in the wealth of tissue analyses performed to date. A search of the datasets in the GEO database (9) that included some

normal bladder tissue samples revealed that cFH transcripts were present in normal tissues at levels similar to tumor tissues. Furthermore, querying the Oncomine database (<https://www.oncomine.org>) for comparative expression between normal and cancer tissues suggested that the range across samples in a category was similar, and cFH was often under-expressed in tumor sample series. There is less information available on the expression of CFHR1 (the other protein recognized by the BTA TRAK assay©, but there is no evidence from profiling studies that bladder tissue CFHR1 expression is associated with cancer.

Previous studies have suggested a correlation between hematuria and BTA test positivity (10,11), and ‘interference’ of the BTA assays has been reported using spiked hematuria models similar to ours (11-13). These model studies found that the BTA tests became positive in BTA-negative urine samples when spiked with whole blood at levels in line with ours (1/2000 dilution). In a response to the article by Oge *et al.*, investigators from BION Diagnostics Sciences pointed out that as part of the studies required for Food and Drug Administration clearance of the BTA tests, a number of interference factors were evaluated (14). Their studies revealed that erythrocytes, leukocytes, hemoglobin or albumin did not interfere with the BTA tests, however, neither whole blood, serum or plasma were mentioned as being tested in those studies. Hematuria can clearly affect urine-based assay in a number of ways, including the introduction of assay interference factors, but given the very high levels of cFH we found in the serum of 40 subjects, for the BTA tests it appears to be more likely that bleeding introduces the analyte to the urine, rather than introducing interference factors. With this said in daily clinical use, BTA analysis at best may only be used as a means to follow-up highly select patients, when hematuria (microscopic and macroscopic) is definitively excluded. However based on our results and the results of others, the lack of specificity of BTA assay then leads one to question the FDA approval of this assay.

Conclusion

The fact that the majority of patients with bladder cancer will present with hematuria, means that an assay that monitors an analyte that is normally at high concentrations in the blood/serum is likely to be susceptible to confounding false-positive tests.

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Figure 1.

ROC curve comparison of BTA and hemoglobin diagnostic performance. Receiver operator characteristic (ROC) curves were calculated from the analysis of urine samples from a cohort of 126 subjects (64 with confirmed bladder cancer). BTA and hemoglobin were measured by ELISA. AUROC, area under the ROC curve.

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Table 1
Demographic and clinicopathologic characteristics of study cohorts

	Urinary ELISA cohort		Hematuria model cohort		BTA serum cohort	
	Non-cancer N=62	Cancer N=64	Non-cancer N=5	Non-cancer N=20	Cancer N=20	
Median age (range)	60 (30-81)	69 (22-90)	33 (27-54)	61(50-75)	63.5 (50-74)	
Male (%)	87	86	80	100	100	
Race						
White (%)	41 (65)	58 (91)	4 (80)	20 (100)	20 (100)	
Gross hematuria	1 (2)	47 (73)	0 (0)	0 (0)	17 (85)	
Median follow-up (months)	11.5	12.0	6	9.0	11.5	
Clinical stage						
Tis	n/a	6 (9)	n/a	n/a	0 (0)	
Ta	n/a	15 (23)	n/a	n/a	2 (10)	
T1	n/a	9 (14)	n/a	n/a	4 (20)	
T2	n/a	31 (48)	n/a	n/a	14 (70)	
>T2	n/a	3 (6)	n/a	n/a	n/a	
Grade						
Low	n/a	9 (14)	n/a	n/a	1 (5)	
High	n/a	55 (86)	n/a	n/a	19 (95)	

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Table 2

BTA Assay Results in Hematuria Model

Dilution	Mean $\pm$ SD RBC/hpf	Mean $\pm$ SD Hemoglobin (ng/ml)	Mean $\pm$ SD BTA-TRAK (U/ml)	BTastat $\odot$	
				Neg. (%)	Pos. (%)
0	0	5.6 $\pm$ 4.9	8.9 $\pm$ 2.8	100	0
1/10,000	1.2 $\pm$ 1.0	199.7 $\pm$ 209.4	19.3 $\pm$ 4.7	60	40
1/2,000	3.6 $\pm$ 1.4	1179.4 $\pm$ 1288.8	70.9 $\pm$ 17.7	0	100
1/500	11.2 $\pm$ 3.3	2237.9 $\pm$ 1279.8	> 105	0	100
1/200	21.6 $\pm$ 5.0	4538.2 $\pm$ 1939.1	> 105	0	100

RBC/hpf, red blood cells/high power field