



Bladder Cancer

Stress Proteins and Cytokines are Urinary Biomarkers for Diagnosis and Staging of Bladder Cancer

David Margel ^{a,b,1}, Meirav Pesvner-Fischer ^{a,1}, Jack Baniel ^a, Ofer Yossepowitch ^a, Irun R. Cohen ^{b,*}

^a Institute of Urology, Rabin Medical Center, Beilinson Campus, Petach Tikva, Israel

^b Department of Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

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Abstract

Background: Cancer often involves inflammatory processes. We hypothesized that immune mediators in urine may serve as biomarkers for bladder cancer (BCa).

Objective: To investigate whether BCa might be marked by urinary levels of heat shock proteins (HSPs; HSP60, HSP70, or HSP90) or cytokines (interferon [IFN]- γ , tumor necrosis factor [TNF]- α , tumor growth factor [TGF]- β , interleukin [IL]-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, or IL-13).

Design, setting, and participants: This was a case–control study with a discovery and validation phase. We examined urine from 106 consecutive patients: healthy controls ($n = 18$); hematuria with no evidence of BCa ($n = 20$); non–muscle-invasive BCa ($n = 50$); and muscle-invasive BCa ($n = 18$). The concentrations of HSPs and cytokines were assessed by enzyme-linked immunosorbent assay. In the validation phase, independent urine samples from 40 patients were analyzed (controls [$n = 19$] and BCa [$n = 21$]).

Measurements: We used the area under the curve (AUC) of a receiver operating characteristic analysis to determine the ability of HSPs and cytokines to mark BCa and applied a multivariate logistic regression to create a formula able to diagnose BCa. The formula was applied to the validation set without recalculation, and positive and negative predictive values were calculated.

Results and limitations: Urinary concentrations of IL-8, IL-10, and IL-13 were significantly elevated in BCa; IL-13 was the most prominent marker (AUC: 0.93; 95% confidence interval [CI], 0.85–0.99). The multivariate regression analysis highlighted HSP60 (odds ratio [OR]: 1.206; 95% CI, 1.041–1.397, $p = 0.003$) and IL-13 (OR: 1.020; 95% CI: 1.007–1.033, $p = 0.012$).

The validation assay was performed using HSP60 and IL-13. The overall positive predictive value was 74% (95% CI, 64–84%); and the negative predictive value was 76% (95% CI, 66–86%). Since we examined a small number of patients, the results need to be confirmed in a larger cohort.

Conclusions: These results suggest that it might be possible to develop a urinary biomarker for BCa and raise the possibility that expression of anti-inflammatory cytokines and HSPs might allow BCa to evade immune surveillance.

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¹ D. Margel and M. Pesvner-Fischer contributed equally to the manuscript.

* Corresponding author. Department of Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel. Tel. +972 8 9342911; Fax: +972 8 9344103.

E-mail address: irun.cohen@weizmann.ac.il (I.R. Cohen).

1. Introduction

The gold standard for detection of bladder cancer (BCa) is cystoscopy. However, cystoscopy is invasive and costly. Currently, voided urine cytology is the only established noninvasive adjunct to cystoscopy. While cytology is sensitive (70–80%) and highly specific (90–95%) for diagnosis of high-grade BCa, its sensitivity is as low as 6–38% for low-grade tumors [1]. The need for more accurate biomarkers for BCa is evident.

Numerous urine tests have been developed for detection of BCa, including bladder tumor antigen (BTA), BTA stat (Polymedco Inc, Cortlandt Manor, NY, USA), fibrin degradation products, nuclear matrix protein 22, ImmunoCyt (Scimedx Corp, Denville, NJ, USA), and fluorescence in situ hybridization (FISH; Urovysion; Vysis-Abbot Laboratories, Downers Grove, IL, USA) [2]. While most of these assays have a higher sensitivity compared to urine cytology, their specificity is generally lower [2]. Urinary tract infection, benign prostatic hypertrophy, and renal calculi can reduce their accuracy. At present, there is no consensus regarding their role in enhancing or replacing cystoscopy [3].

Cancer often develops with an associated local inflammatory response [4]. Because the urine of patients with BCa is in close contact with tumor cells and adjacent inflamed urothelium, we hypothesized that immune mediators in urine may serve as biomarkers for BCa. We therefore examined the urine for three heat shock proteins (HSPs; HSP60, HSP70, and HSP90), and 11 cytokines (interferon [IFN]- γ , tumor necrosis factor [TNF]- α , tumor growth factor [TGF]- β , interleukin [IL]-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and IL-13).

The primary aim of our study was to evaluate whether the immune mediators discussed above can serve as markers for BCa. The secondary aim was to associate these markers with BCa stage.

2. Patients and methods

The study was approved by the Rabin Medical Center Institutional Review Board. It included two phases. In the discovery phase, we assessed various immune-system biomarkers for their ability to diagnose BCa. The validation phase applied the results to a separate group of patients.

2.1. Discovery phase

2.1.1. Patients

Banked serum and urine samples were analyzed from 106 consecutive patients. Intravesical treatments can affect urinary cytokine levels [6], thus only patients with no previous BCa were included. All patients signed an informed consent prior to sample collection. The samples were obtained immediately before cystoscopy, and stored at -20°C . All analyses were performed within 6 mo of collection. The study cohort was stratified as follows: healthy controls (group 1: $n = 18$); hematuria work-up with no evidence of malignancy (group 2: $n = 20$, including 12 patients with renal calculi, 4 with benign bladder lesions, and 4 with benign prostatic enlargement). Hematuria work-up included: white-light cystoscopy, cytology, and computed tomography urography. The four patients with benign bladder lesions also had a transurethral resection of bladder tumor, non-muscle-invasive BCa (NMIBC; group 3: $n = 50$), and muscle-invasive BCa (MIBC; group 4: $n = 18$). Patients with a positive urine culture were excluded.

Table 1 presents the clinical and pathologic characteristics of the subjects. There were no significant differences between the groups in age, gender, smoking habits, baseline renal function, or type 2 diabetes. All specimens were analyzed by a single genitourinary pathologist according to a standardized protocol. Pathologic staging was reported in accordance with the 2003 TNM classification and assigned a grade according to the World Health Organization classification.

2.1.2. Reagents

Human HSP60 was prepared as described [5]. HSP70 and HSP90 were purchased from StressGen Biotechnologies (Victoria, British Columbia, Canada). Antibodies for detection of HSP60 and HSP90 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and for HSP70 from StressGen (Victoria, British Columbia, Canada). Enzyme-linked immunosorbent assay (ELISA) antibody for detection of IFN- γ , TNF- α , IL-1 β , and IL-2 were purchased from BioSource (Camarillo, CA, USA); for IL-6, IL-10, and IL-8, from BioLegend (San Diego, CA, USA); and for IL-4, IL-13, and TGF- β , from R&D Systems (Minneapolis, MN, USA).

2.1.3. Heat shock protein measurements

A direct ELISA was used to quantify HSP concentrations in urine and serum. Assays were done in triplicate according to manufacturer's instructions. Minimal detection concentrations were 20 ng/ml for HSP60 and HSP70, and 5 ng/ml for HSP90.

2.1.4. Cytokine urine measurements

Sandwich ELISA was used to quantify cytokine concentrations in urine. The assays were done in triplicate according to manufacturer's instructions. Minimal detection concentration was 30 pg/ml for all cytokines. Urine from 72 subjects was available for cytokine analysis.

Table 1 – Subject characteristics

	Healthy	Hematuria	NMIBC	MIBC	<i>p</i> value
Age, yr					
Mean \pm SD	65.9 \pm 12.3	66 \pm 15.2	69.3 \pm 10.21	71.5 \pm 10.1	NS
Gender, %					
M	100	89	86	80	NS
F		11	14	20	
Smoking, %	53	44	55	55	NS
Creatinine, %	0.9 \pm 0.4	1.3 \pm 0.22	1.02 \pm 0.3	1.2 \pm 0.16	NS
Diabetes, %	40	50	32	53	NS

MIBC = muscle-invasive bladder cancer; NMIBC = non-muscle-invasive bladder cancer; SD = standard deviation.

Subjects were stratified to four groups as previously described: 18, 13, 26, and 15 patients in groups 1–4, respectively.

2.1.5. Statistical analysis

Univariate analysis using one-way analysis of variance (ANOVA) was performed to assess the differences in HSP and cytokine concentrations between groups. When the ANOVA test demonstrated a significant value, post hoc least significant difference analysis was used to determine statistically significant differences between groups.

The US National Cancer Institute recommends evaluating the performance of potential markers for cancer detection using receiver operating curves (ROCs) [7]. Therefore, we used ROC to calculate the area under the curve (AUC) and the 95% confidence interval (CI) for association with the presence of BCa and for an association with the stage of disease: MIBC and NMIBC.

We applied a multivariate, stepwise, binary logistic regression to produce a formula with the ability to detect BCa utilizing a minimum number of variables. Samples that included urinary HSP and cytokine measurements were analyzed; all patients had no previous BCa ($n = 72$). Subjects were divided into controls ($n = 31$) and patients with BCa ($n = 41$). The model included urinary HSP and cytokine concentrations, and age. We calculated the odds ratio (OR), the 95% CI of statistically significant markers, and the AUC \pm 95% CI of the entire model. We applied the same model for bladder cancer staging (MIBC vs NMIBC). Statistical analyses were carried out using SPSS statistical software v.12.0 (SPSS Inc, Chicago, IL, USA); $p < 0.05$ was considered significant.

2.2. Validation phase

In this phase, we applied the formula obtained from the discovery phase without recalculating. The sensitivity, specificity, and positive and negative predictive values of the formula in the validation cohort were calculated.

2.2.1. Patients

Banked urine samples from an independent group of 40 consecutive patients were analyzed within 6 mo of storage. The samples were obtained and stored as previously described. The cohort was divided into controls ($n = 19$) and 21 BCa patients (14 with NMIBC and 7 with MIBC).

2.2.2. Reagents and HSP60 and IL-13 urine measurement

Reagents were obtained and HSP60 measurements performed as described above. In these additional urine samples, we discovered that the test was

encumbered by the presence of inhibitors of IL-13. To overcome this problem, we spiked different urine concentrations with pure IL-13 and discovered that the inhibitors could be diluted with phosphate-buffered saline (PBS) at 1:8 (urine to PBS). To inactivate these inhibitory factors and thus restore the ability to detect the IL-13, we prepared the urine in the following manner: We first filtered the urine using Millipore Amicon Ultra with cut-off of 3 kDa (Billerica, MA, USA). We then reconstituted the urine volume with PBS, and subsequently diluted the urine $\times 3$. We then assayed the urine for IL-13 by ELISA (detection limit: 3–100 pg/ml). The kits were purchased from Orgenium Laboratories, Vantaa, Finland.

3. Results

3.1. Heat shock proteins

Fig. 1a–c depict the urinary concentrations of the HSPs in the different subgroups. Urinary concentrations of all HSPs were significantly elevated in patients with MIBC ($p < 0.01$ for all; Fig. 1a, Table 2). HSP levels did not distinguish patients with hematuria from those with NMIBC. We could not detect HSP in sera of any of the study participants.

3.2. Urinary cytokine concentrations

IFN- γ , TNF- α , IL-1 β , IL-2, IL-4, and IL-5 were not detected in the urine (data not shown), whereas IL-6, IL-8, IL-10, IL-13, and TGF- β were detected at various levels (Fig. 2a–e).

Urinary concentrations of IL-8, IL-10, and IL-13 (Fig. 2a–c, Table 2) were elevated in patients with BCa (NMIBC and MIBC) compared to those without BCa (healthy and hematuria, $p > 0.05$ for all). However, they did not distinguish between MIBC and NMIBC. Urinary levels of IL-6 and TGF- β (Fig. 2d–e, Table 2) were elevated in patients with MIBC.

3.3. Discovery phase

3.3.1. Heat shock protein molecules and cytokines as biomarkers for bladder cancer (all stages)

Fig. 3a–b demonstrate the ROC of the HSP and cytokine assays for BCa. IL-13 appeared to be the most prominent marker for BCa (AUC: 0.93; 95% CI, 0.85–0.99).

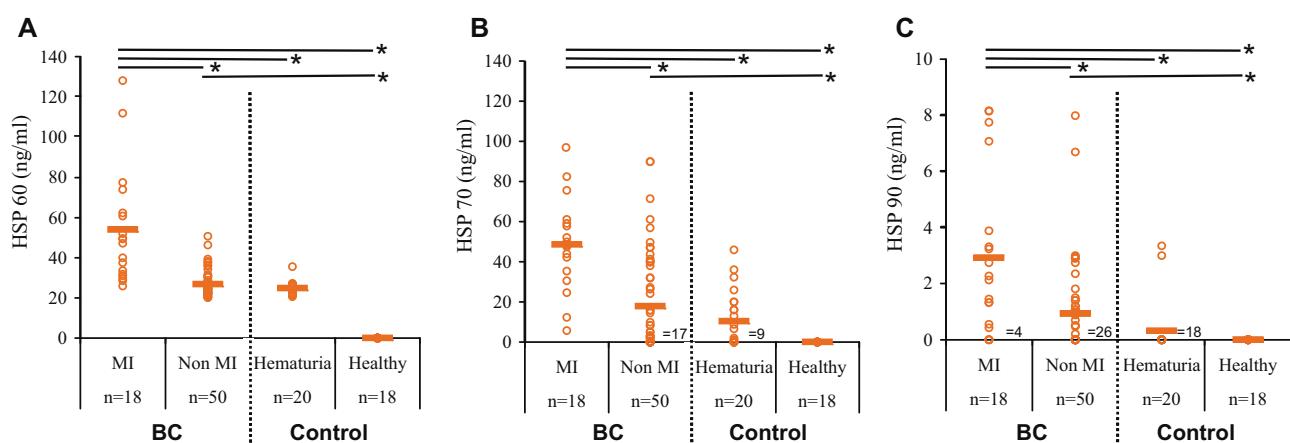


Fig. 1 – Urinary concentrations of heat shock protein (HSP) HSP60, HSP70, and HSP90. Urine was assayed by enzyme-linked immunosorbent assay for the presence of (a) HSP60, (b) HSP70, and (c) HSP90. The results are presented by a scatter plot, with each dot representing one patient. One dot is presented when urinary concentrations overlap and the number of overlapping patients is indicated. The mean of each group is presented by a line. BC = bladder cancer; MI = muscle-invasive; non-MI = non-muscle-invasive.

Table 2 – Urinary heat shock protein (HSP) and cytokine concentrations (mean plus or minus standard deviation)

	MIBC	NMIBC	Hematuria	Healthy	<i>p</i> value
HSP60, ng/ml	52.4 ± 7.9	26 ± 1.8	24.6 ± 0.6	0 ± 0	>0.001
HSP70, ng/ml	47.5 ± 8.2	18.6 ± 4.6	8.3 ± 2.6	0 ± 0	>0.001
HSP90, ng/ml	2.6 ± 0.71	1.8 ± 0.5	0.5 ± 0.3	0 ± 0	>0.001
IL-6, pg/ml	90 ± 42.5	8 ± 4.7	1.2 ± 2	0 ± 0	0.003
IL-8, pg/ml	214.3 ± 62.5	151 ± 47	9.6 ± 7.1	0.9 ± 0.5	0.002
IL-10, pg/ml	100.5 ± 34	152 ± 36	2 ± 1.1	2.1 ± 0.5	0.001
IL-13, pg/ml	172 ± 22	152 ± 15	39 ± 25	16 ± 15	>0.001
TGF-β, pg/ml	20.4 ± 7.8	0 ± 0	5.1 ± 5.2	0 ± 0	0.001

MIBC = muscle-invasive bladder cancer; NMIBC = nonmuscle-invasive bladder cancer; IL = interleukin; TGF = tumor growth factor.

The regression analysis highlighted HSP60 (OR: 1.206; 95% CI, 1.041–1.397; *p* = 0.003) and IL-13 (OR: 1.020; 95% CI, 1.007–1.033; *p* = 0.012).

The formula produced by the model was:

$$\frac{e^{(C+W_1 \cdot HSP60+W_2 \cdot IL13)}}{1+e^{(C+W_1 \cdot HSP60+W_2 \cdot IL13)}} = \begin{cases} < 0.5, & \text{Negative (No BCa)} \\ \geq 0.5, & \text{Positive (BCa)} \end{cases}$$

In this model, C is constant, with a value of 5.5 in our model; *W*₁ is the weight allocated for HSP60 concentration, and its value in our model is 0.187; *W*₂ is the weight

allocated for IL-13 concentration, and its value in our model is 0.02; HSP60 concentration is in nanograms per milliliter; IL-13 concentration is in picograms per milliliter.

The AUC plus or minus 95% CI of the ROC of the multivariate model was 0.95 (95% CI, 0.87–0.98).

3.3.2. Elevated heat shock protein and cytokine measurements are associated with muscle-invasive bladder cancer

Fig. 4a–b demonstrate the association between the stage of BCa and urinary HSP and cytokines levels. HSP60 (AUC: 0.95; 95% CI, 0.91–0.99) showed the closest association with BCa stage.

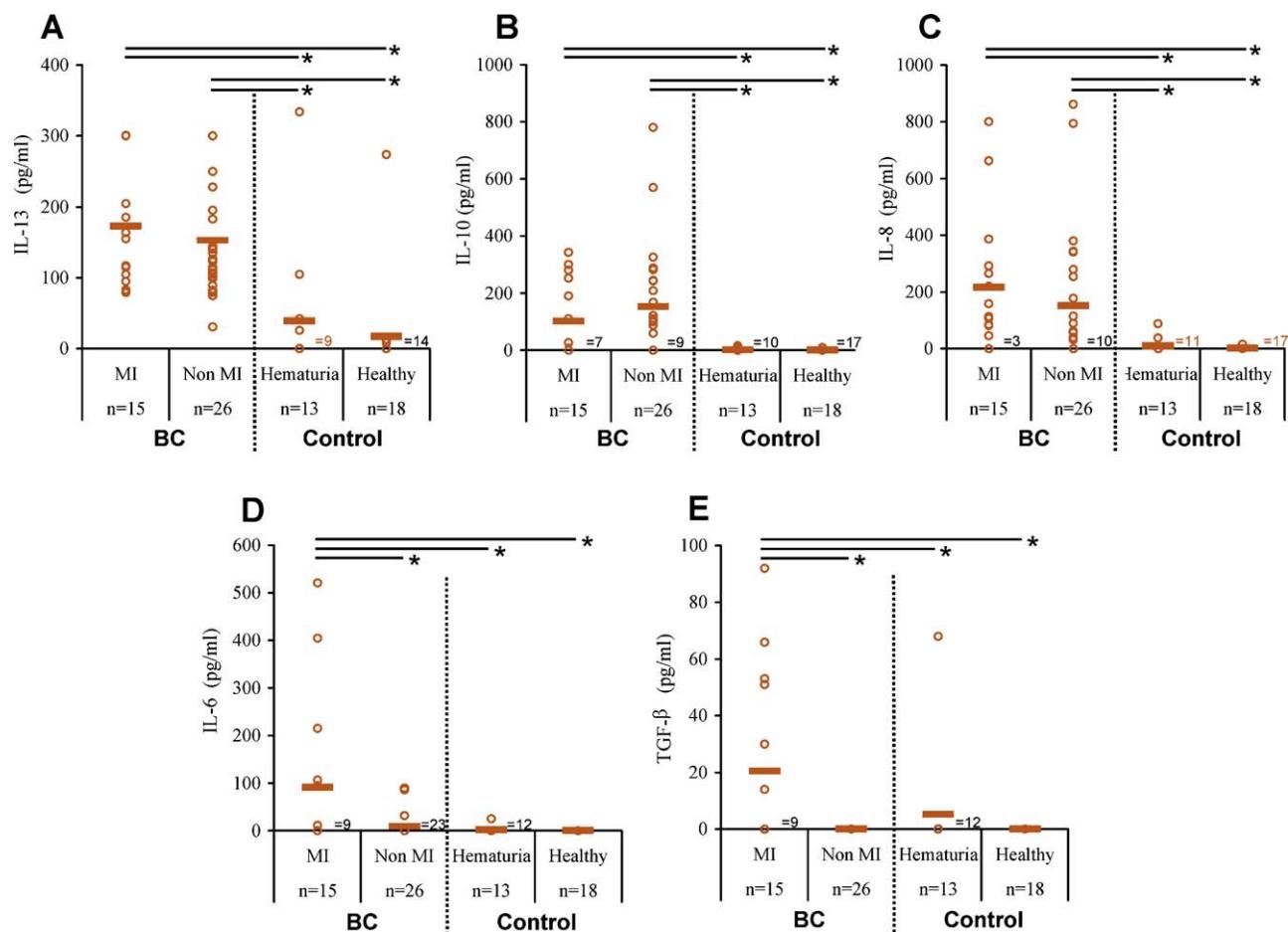


Fig. 2 – Urinary concentrations of cytokines. Urine was assayed by enzyme-linked immunosorbent assay for the presence of (a) interleukin (IL)-13, (b) IL-10 (c) IL-8, (d) IL-6, and (e) tumor growth factor (TGF)-β. The results are presented by a scatter plot. Each dot represents one patient. One dot is presented when urinary concentrations overlap and the number of overlapping patients is indicated. BC = bladder cancer; MI = muscle-invasive; non-MI = non-muscle-invasive.

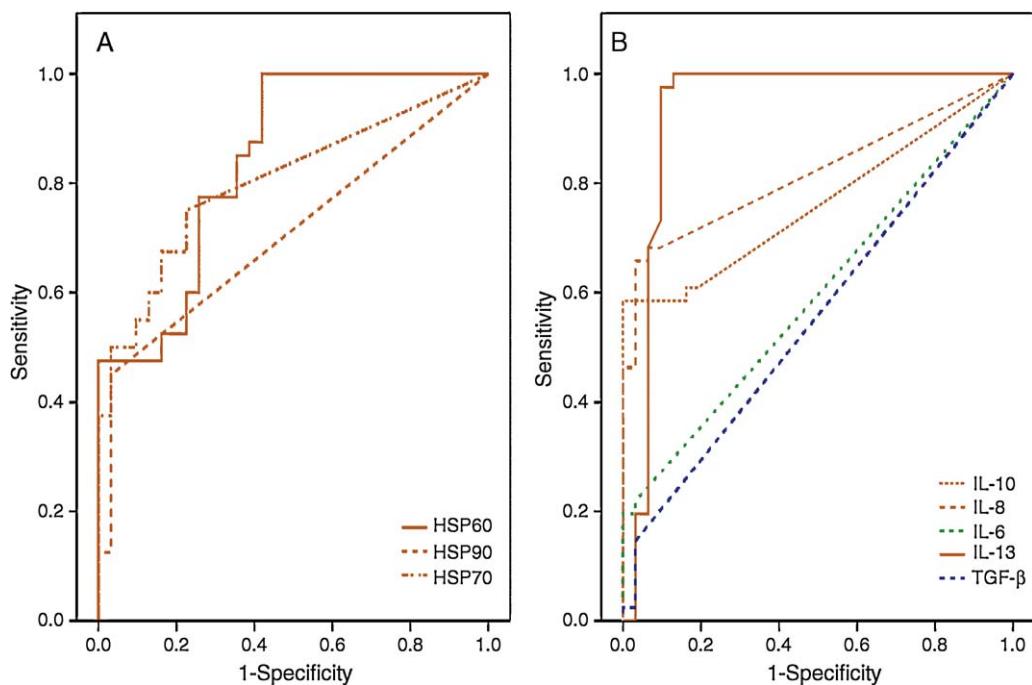


Fig. 3 – Receiver operating curve of (a) heat shock protein (HSP) molecules (a) and (b) cytokines in bladder cancer detection. IL = interleukin; TGF = tumor growth factor.

The regression analysis highlighted HSP60 (OR: 1.093; 95% CI, 1.013–1.179; $p = 0.022$); HSP70 (OR: 1.092; 95% CI, 1.09–1.128; $p = 0.004$); and HSP90 (OR: 2.404; 95% CI, 1.231–4.694; $p = 0.01$). The AUC plus or minus 95% CI of the ROC of the model was 0.96; 95% CI was 0.89–0.98.

3.4. Validation phase

We used the formula from the regression analysis on an independent set of patients ($n = 40$). Any value ≥ 0.5 was considered a positive test for BCa; a value < 0.5 was considered a negative test.

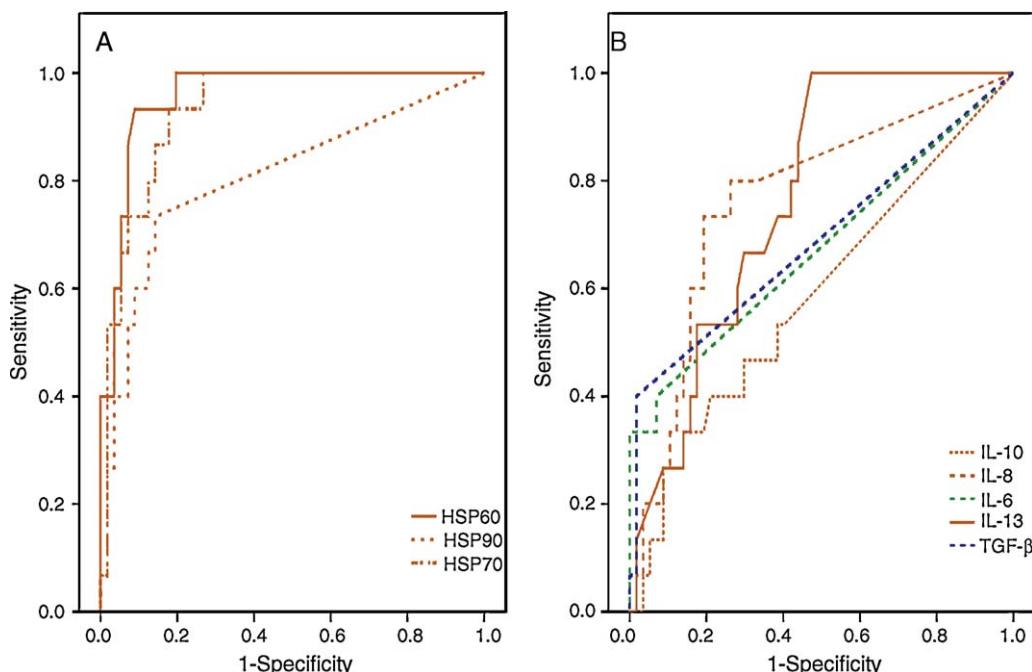


Fig. 4 – Receiver operating curve of (a) heat shock protein (HSP) molecules and (b) cytokines for bladder cancer stage prediction. IL = interleukin; TGF = tumor growth factor.

Table 3 – Urinary biomarker diagnostic yield in the validation phase

	Bladder cancer (n = 21)		No BC (n = 19)
	MIBC (n = 7)	NMIBC (n = 14)	
Positive test [*]	7	9	5
Negative test ^{**}	0	5	14

MIBC = muscle-invasive bladder cancer; NMIBC = non-muscle-invasive bladder cancer.
^{*} Positive test: formula ≥ 0.5 .
^{**} Negative test: formula < 0.5 .

The formula combining HSP60 and IL-13 was able to diagnose all patients with MIBC and 9 of the 14 with NMIBC. The sensitivity, specificity, and positive and negative predictive values were 76% (95% CI, 66–88), 74% (95% CI, 64–84%), 74% (95% CI, 64–84%), and 76% (95% CI, 66–86%), respectively (Table 3). No patient in the control group had detectable urinary concentrations of IL-13.

4. Discussion

We report that BCa is associated with increased urinary levels of HSPs and cytokines; they mark both NMIBC and MIBC.

Univariate analysis showed that urinary concentrations of IL-8, IL-10, and IL-13 were elevated in BCa compared to controls. IL-13 was the most prominent marker for BCa (AUC: 0.93; 95% CI, 0.85–0.99), but HSP60, HSP70, HSP90, IL-8, and IL-10 exhibited an AUC ≥ 0.70 (Table 3a). In addition to marking BCa, urine concentrations of all HSPs, IL-6, and TGF- β differentiated between MIBC and NMIBC (Figs. 1 and 2). Multivariate analysis showed that HSP60 and IL-13 together generated an AUC of 0.95 for BCa. However, an OR analysis showed that each measure alone was meaningful: The results indicated that an increase of 10 ng/ml of HSP60 or of 10 pg/ml of IL-13 was associated with more than 10 times the likelihood of a biopsy revealing BCa.

Our study included a validation phase in which we demonstrated the diagnostic yield of only two biomarkers—HSP60 and IL-13—in the diagnosis of BCa. The combination of these molecules was able to detect all MIBC and most of the NMIBC. In this study we did not attempt to form cut-off values; since the cohort was rather small, we preferred to use the regression analysis model for the validation phase. Note, however, that none of the controls had detectable concentrations of IL-13. This suggests that, with larger numbers of subjects, it will be possible to establish cut-off values and increase the specificity of our biomarker assay. In addition, false positives may, in fact, represent small upper-tract tumors yet to be diagnosed. Ongoing follow-up will eventually identify if upper-tract tumors indeed were present.

Urinary biomarkers should be able to differentiate BCa from hematuria associated with benign conditions such as stones or benign prostatic hyperplasia. Thus, the control group in the present analyses included subjects presenting

with hematuria as well as healthy individuals. While urinary HSP levels could not differentiate control subjects with hematuria from patients with NMIBC, the addition of IL-13 to the model enhanced its performance to make this distinction. It is prudent that future studies measure cytokines as well as HSPs when considering their role as BCa urinary biomarkers.

Beyond serving as BCa biomarkers, we can consider the biology of the phenomenon: Why is BCa associated with increased amounts of HSPs and cytokines? HSPs are upregulated when cells are exposed to elevated temperature or other forms of stress [8]. In neoplasia, the expression of HSPs is implicated in the regulation of apoptosis [9] and tumor-cell growth [10]. In vivo, HSPs are overexpressed in many tumors [11,12].

Recently, the amounts of HSP60 and HSP90 detected by immunohistochemical staining were reported to be prognostic factors in BCa. Lebret et al showed that decreased expression of these HSPs in the tumor was associated with invasive characteristics [13]. Moreover, low tumor-cell expression of HSP90 was associated with failure to respond to immunotherapy [14]. Our findings of elevated urinary levels of HSP in BCa patients might seem paradoxical. The apparent discrepancy could be explained by the functional differences between static detection of HSP by immunohistochemistry and HSP accumulation in urine. It is conceivable that a BCa cell may contain less HSP because the molecules have been exported to the extracellular space, ultimately accumulating in the urine. Despite the fact that HSPs can upregulate the activity of dendritic cells and macrophages [15,16], HSPs outside the tumor might help the tumor evade immune surveillance: Administration of HSP60 [17,18], HSP70, and HSP90 [19] has been reported to abort Th1 effector immunity and enhance Th2 downregulatory immune responses. Thus, BCa tumor cells might thereby downregulate antitumor effector T cells.

The suppression of effector-type immune reactivity by HSPs is compatible with the predominance of the anti-inflammatory cytokines IL-10, TGF- β , and IL-13 we detected in the urine of BCa patients. Indeed, the secretion of immunosuppressive cytokines appears to be a strategy for immune evasion used by many malignancies, including BCa. Loskog et al. [20] demonstrated that BCa tissue is infiltrated by regulatory T cells expressing large amounts of TGF- β and IL-10 mRNA. They reported that circulating T cells of BCa subjects were unresponsive to polyclonal T-cell activation compared to healthy controls. Helmy et al. [21], using immunoelectromicroscopy, detected TGF- β in the urine of BCa patients.

The most informative urine cytokine in our study is IL-13. IL-13 was originally described as a cytokine that inhibits inflammatory cytokine production [22,23]. In cancer, IL-13 is involved in tumor immunosurveillance [24], contributes to tumor escape from apoptosis, and enhances tumor growth [25,26]. Upregulation of IL-13 has been linked to extracellular HSP60: The administration of an HSP60 peptide to patients with recent-onset type 1 diabetes led to the arrest of beta-cell destruction accompanied by increased IL-13 in the serum [27].

5. Conclusions

We have demonstrated a significant association between BCa and urinary levels of HSPs and anti-inflammatory cytokines. Since this study is limited by the small number of samples analyzed, it needs further validation in a larger cohort, perhaps utilizing a "high throughput" ELISA-based platform. Despite its small numbers, these findings provide a good foundation for the development of a urinary biomarker for BCa. In addition, they support a mechanistic insight: BCa cells might evade immune surveillance by producing, or inducing other cells to produce, HSP molecules and cytokines resulting in the suppression of effector immunity.

Author contributions: Irun R. Cohen had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Margel, Pesvner-Fischer, Baniel, Yossepowitch, Cohen.

Acquisition of data: Margel, Pesvner-Fischer.

Analysis and interpretation of data: Margel, Pesvner-Fischer, Baniel, Yossepowitch, Cohen.

Drafting of the manuscript: Margel, Pesvner-Fischer, Cohen.

Critical revision of the manuscript for important intellectual content: Scarpa. **Statistical analysis:** Margel.

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