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Urinary Levels of Bcl-2 are Elevated in Ovarian Cancer Patients

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Abstract

Objective(s)—The poor prognosis associated with ovarian cancer is due to the lack of overt early symptoms and the absence of reliable diagnostic screening methods. Since many tumors overexpress anti-apoptotic proteins, the purpose of this study was to determine whether elevated levels of the anti-apoptotic protein Bcl-2 were present in urine from patients with ovarian cancer.

Methods—Bcl-2 was assayed by ELISA in urine samples from two cohorts consisting of a total of 77 healthy women, 161 women with benign gynecologic disease and 150 women with ovarian cancer, 13 with early and 137 with late stage disease, respectively. Wherever possible, parallel serum samples were measured for CA125 levels by ELISA.

Results—Urinary levels of Bcl-2 from healthy individuals or women with benign disease averaged 0.59 ng/ml $\pm$ 0.61 and 1.12 ng/ml $\pm$ 0.79, respectively. In contrast, urinary levels of Bcl-2 averaged 2.60 ng/ml $\pm$ 2.23 and 3.58 ng/ml $\pm$ 1.55 from women with early (N=13) and late (N=137) stage ovarian cancer. Further, urinary levels of Bcl-2 were elevated in ovarian cancer patients regardless of tumor grade, stage, size, histologic subtype, creatinine levels or patient age, but appeared to complement CA125 measurements.

Conclusion(s)—Levels of Bcl-2 are elevated in the urine of patients with ovarian cancer and may be of diagnostic and/or prognostic clinical importance. Further studies of urinary Bcl-2 as a biomarker for ovarian cancer alone or in combination with other markers are warranted.

Introduction

Ovarian cancer is the fourth leading cause of cancer death among women after lung, breast and colorectal cancer and is associated with a 1.7% lifetime risk [1]. The lack of early, overt symptoms and the absence of a reliable screening test result in over 70% of women being diagnosed after the disease has spread beyond the ovary so that the prognosis is poor (5-year

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survival is no better than 37%). For women at high risk for developing ovarian cancer, annual pelvic examination, transvaginal ultrasound and/or measuring blood levels for CA125 are the generally recommended screening methods available for detection of ovarian cancer. However, use of these techniques for screening purposes is limited [2].

To safely and economically detect cancer with no or minimal invasiveness, previous studies have examined biological fluids for detectable cancer biomarkers. These include examining serum levels of soluble interleukin-2 as a marker for hematological malignancies [3], prostate-specific antigen and insulin-like growth factor binding protein-3 in nipple aspirate fluid from breast cancer patients [4] and urine levels of angiostatin and plasminogen or survivin for the detection of bladder cancer and sarcoma, respectively [5,6]. In the ovary, in addition to elevated serum CA125, lysophosphatidic acid (LPA) is also found in high levels in plasma and ascites of ovarian cancer patients and is considered a potential diagnostic marker for ovarian cancer [7]. Likewise, Hazelton et al [8] showed high levels of VEGF in cysts from ovarian cancers compared to cystic fluid from benign, borderline or functional cysts. However, the invasive nature for cystic fluid collection prohibits widespread use for general screening/diagnostic purposes. Strategies using multiple serum markers may increase sensitivity for ovarian cancer detection, but have yet to provide useful and cost-effective screening [9]. Proteomic analysis can distinguish ovarian cancer sera from those of healthy women. Individual proteomic biomarkers still require validation and the use of protein profiles has been hampered by the difficulties in defining optimal algorithms and inter-assay variability [10]. Recently, elevated serum and urinary levels of mesothelin have also been associated with ovarian cancer [11].

Deregulation of apoptosis due to overexpression of anti-apoptotic proteins contributes to the malignant phenotype by supporting cancer cell growth and therapeutic resistance. Since the anti-apoptotic protein, Bcl-2, is overexpressed in ovarian cancers [12], we sought to assess whether elevated urinary levels of Bcl-2 are associated with ovarian cancer.

Methods and Materials

Patient cohort

With prior institutional approval, urine and blood samples were collected from an initial cohort of healthy controls (N=19), women with benign gynecologic disorders (N=38) and patients with early (EOC, N=4) and late (LOC, N=31) stage ovarian cancer at the H. Lee Moffitt Cancer Center at the University of South Florida (USF). All except 8 specimens were collected prior to initial surgical cytoreduction while the latter 8 specimens presented with recurrent disease at the time of enrollment in this study. The cancer category consisted of women diagnosed with ovarian cancer and primary peritoneal cancer, which is often related to ovarian cancer. The samples collected from women with benign gynecologic disease consisted of broad range of non-malignant gynecologic disorders. The second blinded urine sample cohort collected with IRB approval through the tissue bank at the M.D. Anderson Cancer Center (MDA) consisted likewise of healthy controls (N=58), women with benign gynecologic disease (N=123) and women with EOC (N=9) and LOC (N=106) stage ovarian cancer. Though these cohorts comprise a small pilot study (Table 1), they are representative of a typical clinical practice with regards to histology, grade and stage distribution.

Paraffin blocks were identified, where possible, and the slides reviewed to confirm the histologic diagnosis according to FIGO scores. Anonymized information regarding patient age, tumor type, stage, grade, size and CA125 values were also obtained as per availability.

Sample preparation

Urine and blood samples were collected from patients, anonymized and decoded to protect patient identity, and released from the tissue banks for this research project. All samples were kept in ice following collection. USF urine samples were treated with a standard protease inhibitor cocktail (80 µg/ml 4-(2 aminoethyl)-benzene sulfonyl fluoride, 200 µg/ml EDTA, 0.2 µg/ml leupeptin, 0.2 µg/ml pepstatin, Sigma Scientific, St. Louis, MI) and centrifuged at 3000 × g. Urinary supernates and plasma samples were then aliquoted and stored at −20°C. Urine samples from MDA were likewise centrifuged, but without addition of protease inhibitors and the supernatants aliquoted and frozen at −80°C.

Enzyme-linked immunosorbant assay

To measure Bcl-2 levels in patients' urine, samples were assayed using the quantitative sandwich enzyme-linked immunosorbant assay (ELISA; Bender MedSystems, Burlingame, CA) according to the manufacturer's instructions. In accordance with previous studies [11], urinary Bcl-2 threshold levels were set to include 95% of Bcl-2 values of urine samples from healthy women. When not derived from clinical data, CA125 levels in subjects' plasma were assayed by ELISA (Bio-Quant, San Diego, CA) according to the manufacturer's instructions. The enzymatic reactions were detected at 450 nm using a Dynex MRX plate reader (Dynex Technologies, Chantilly, VA) and Bcl-2 results expressed as the mean absorbance of triplicate samples ± S.E, while CA125 results were expressed as the mean of duplicate samples.

Statistical analysis

Samples for Bcl-2 ELISA were run in triplicate and the data subjected to descriptive, one way ANOVA, Tukey and ROC-AUC analyses.

Results

Urinary levels of Bcl-2 are elevated in patients with ovarian cancer from the USF cohort

Since a standard cutoff threshold value for urinary Bcl-2 has not been established, we used the 95th percentile of Bcl-2 values for healthy controls as the threshold as previously employed to determine threshold values for urinary mesothelin [11]. The amount of urinary Bcl-2 was generally negligible (average 0.30 ng/ml) in healthy control samples (Fig. 1A, Table 2) with no samples above the determined threshold of 1.7856 ng/ml. Similarly, urinary Bcl-2 levels from women with benign gynecologic disease were low averaging 0.74 ng/ml (Fig. 1B, Table 2). In contrast, urinary Bcl-2 associated with ovarian and primary peritoneal cancer was generally >10× that found in normal control. Interestingly, only two ovarian cancer samples exhibited Bcl-2 levels below the threshold level (1.12 ng/ml and 1.78 ng/ml), but it is noteworthy that these samples were derived from patients with mucinous ovarian cancer. Further of note, a single benign sample and a single ovarian cancer sample patient demonstrated extremely elevated urinary Bcl-2 levels in the absence of other notable clinical symptoms. Since these two extreme values were greater than the mean + 2.58 SD, they were omitted from the summarial box plots (Fig. 2A). Because ANOVA tests revealed significant differences among the four ovarian samples (F value = 64.48, p<0.001), pair-wise comparisons were used in further Tukey analyses (Table 3). These latter analyses indicated significant differences in urinary Bcl-2 between healthy controls and benign samples, between healthy controls and cancer samples as well as between benign samples and cancer samples. ROC-AUC analyses indicated that every cancer sample (both EOC and LOC) were above the threshold (Table 3). Lastly, we did not detect any significant change in urinary Bcl-2 levels when representative samples were

tested following repeated freeze thaws (up to 3 times), following storage at -20° or -80° C (> 3 months for each temperature) or at 4° C for one week (data not shown).

Urinary levels of Bcl-2 are elevated in ovarian cancer patients from the MDA cohort

For validation purposes, urinary Bcl-2 levels were measured in a second sample cohort obtained from MDA. In agreement with the USF cohort, urinary Bcl-2 was low in healthy control or benign samples (average 0.69 ng/ml and 1.23 ng/ml, respectively) (Table 2). Urinary Bcl-2 associated with ovarian cancer was elevated and averaged 2.70 ng/ml and 2.30 ng/ml from EOC and LOC, respectively. In contrast to the USF cohort, however, some EOC and LOC samples fell below the determined threshold and may reflect differences in the cohorts especially as that may pertain to sample preparation and storage. Also as above, since a single EOC and LOC sample demonstrated extreme values were greater than the mean + 2.58 SD, they were omitted from the summarial box plots (Fig. 2B). Because ANOVA tests revealed significant differences among the four ovarian samples (F value = 40.84, $p < 0.001$), pair-wise comparisons were used in further Tukey analyses (Table 3). These latter analyses indicated significant differences in urinary Bcl-2 between healthy controls and benign samples and between healthy controls and cancer samples. While benign samples tended to be lower than EOC, they were not statistically significant and there were no differences between EOC and LOC. ROC-AUC analyses also indicated that over 50% of cancer samples were above the threshold (Table 3).

When the data from both USF and MDA cohorts was combined (Fig. 2C, Tables 2&3), urinary Bcl-2 levels in ovarian cancer remained elevated. That is, urinary Bcl-2 levels among healthy and benign samples (averages 0.59 ng/ml and 1.12 ng/ml, respectively) were statistically lower than those from EOC and LOC (averages 2.60 ng/ml and 3.58 ng/ml, respectively).

Surgical debulking reduces urinary levels of Bcl-2

Levels of urinary Bcl-2 were compared in 7 ovarian cancer patients immediately prior to (Fig. 3, black bars) and within 2 weeks following initial cytoreductive surgery (Fig. 3, white bars). Though this subset represents a small sample size, Bcl-2 levels decreased up to 100% following surgical removal of tumor further suggesting that presence of tumor correlates with elevated urinary Bcl-2 in ovarian cancer patients.

Patients at high risk for ovarian cancer may be predisposed to elevated urinary levels of Bcl-2

Though there was a tendency for elevated Bcl-2 levels with increasing tumor grade and stage, the differences were not statistically significant (Tables 2 & 3). Likewise, urinary Bcl-2 levels did not appear to vary between cancer histotypes and serum creatinine measured at the time of urine collection indicated that elevated urinary Bcl-2 levels in cancer patients were not related to renal dysfunction (data not shown). In addition, while elevated levels of Bcl-2 could be detected in the serum of some ovarian cancer patients (data not shown), serum levels of Bcl-2 did not detect ovarian cancer as accurately as urinary levels of Bcl-2 perhaps due to the presence of confounding proteins in serum compared to urine. Further, comparison of clinical parameters also indicated that urinary Bcl-2 levels did not relate with patient age (data not shown). Though the overall average age from normal controls and benign samples were somewhat less than that from ovarian cancer patients (Table 1), the age range among all samples was comparable. Similarly, urinary Bcl-2 levels did not correlate with tumor size measured at cytoreductive surgery (data not shown) and may reflect variation in tumor composition. However, initial analyses of benign gynecologic samples from the MDA cohort revealed a subset of 10 individuals with elevated urinary Bcl-2 above threshold levels (Fig. 4). Upon further examination, 8 of these were identified

as individuals at risk of developing ovarian cancer due to a familial history of breast and/or ovarian cancer, previous treatment for breast cancer or BRCA mutations carriers.

Urinary Bcl-2 complements CA125 measurements

To address the potential for urinary Bcl-2 to serve as a biomarker for ovarian cancer, urinary Bcl-2 was compared with CA125 values. Using 12 healthy controls and 23 patients with ovarian cancer from the USF cohort (Fig. 5), elevated urinary Bcl-2 ($>1.8\text{ng/ml}$) was associated with 95% ovarian cancer detection (Fig. 5A) correctly identifying 17/17 patients with serous adenocarcinoma, 4/4 patients with mucinous ovarian cancer and 1/2 patients with primary peritoneal cancer as ovarian cancer positive. None of the healthy controls had urinary Bcl-2 levels $>1.8\text{ ng/ml}$ and were, then, correctly classified as cancer-negative. In contrast, CA125 levels $>35\text{ U/ml}$ from matched samples identified 13/17 or 76% of patients with serous adenocarcinoma, 3/4 or 75% of patients with mucinous ovarian cancer (though CA125 levels in these patients ranged between 41-43 U/ml somewhat above threshold CA125 values), and 1/2 or 50% of patients with primary peritoneal cancer as cancer positive while 2/12 or 16% of healthy controls were false positive (Fig. 5B).

When urinary Bcl-2 levels were compared with CA125 values among EOC (N=17) and LOC (N=83) cancers collected from both USF and MDA cohorts, high urinary Bcl-2 was detected in 5 (29%) early and 60 (72%) late ovarian cancers compared with 13 (76%) and 76 (92%) CA125 measurements. However, combined urinary Bcl-2 and CA125 positivity in these groups increased to 14 (82%) and 82 (99%) detection of early and late cancer, respectively. In addition, comparison between urinary Bcl-2 and CA125 measurements revealed a Pearson correlation of 0.644, $p<0.0001$ indicative of strong complementarity.

Interestingly, 7/8 samples collected from women with endometriosis, often a cause for false positive CA125 values, tested negative for urinary Bcl-2 while the remaining sample tested just at, but not above, the threshold level for urinary Bcl-2 (data not shown).

Discussion

While apoptosis is an essential biological process for normal development and maintenance of tissue homeostasis, it is also involved in a number of pathologic conditions including tissue injury, degenerative diseases, immunological diseases and cancer [13]. Whether activated by membrane bound death receptors [14] or by stress-induced mitochondrial perturbation with subsequent cytochrome c release [15], activation of downstream caspases leads to stepwise cellular destruction by disrupting the cytoskeleton, shutting down DNA replication and repair, degrading chromosomal DNA, and, finally, disintegrating the cell into apoptotic bodies [16]. The key regulators of apoptosis include members of the bcl-2 protein family [17].

The bcl-2 protein family consists of both pro- and anti-apoptotic protein family members that act at different levels of the apoptotic cascade to regulate apoptosis. The bcl-2 family members contain at least one Bcl-2-homology (BH) domain [17]. Though all bcl-2 family members demonstrate membrane channel forming activity, Bcl-2 (the archetypal bcl-2 family member) channels are cation (Ca^{++}) selective and, owing to its exclusive endoplasmic reticulum (ER) and mitochondrial membrane localization [18], the anti-apoptotic function of Bcl-2 is at least partly mediated by its ability to prevent calcium release from the ER and subsequent mitochondrial membrane perturbation and cytochrome c release. Since Bcl-2 is overexpressed in many tumor types including ovarian cancer [12,19], it contributes to chemoresistance by stabilizing the mitochondrial membrane against apoptotic insults. Currently, preclinical studies focused on the development of agents to

inhibit Bcl-2 include antisense oligonucleotides such as G3,139 [20] and small molecular inhibitors of Bcl-2 [21].

In addition to promoting cancer cell survival by promoting resistance to apoptosis, recent studies indicate that Bcl-2 overexpression may also promote cancer cell survival through increased angiogenesis by upregulating VEGF expression [22]. Specifically, Bcl-2 mediates VEGF transcription in a Hif-1-dependent manner and enhances VEGF mRNA stabilization [23]. Consequently, targeting Bcl-2 for therapeutic intervention may also complement anti-angiogenic therapies.

In contrast to therapeutic targeting, our data suggest that Bcl-2 overexpression may result in increased levels of Bcl-2 in bodily fluids that could represent a novel biomarker for ovarian cancer. Specifically, data from two separate cohorts indicated significantly elevated urinary Bcl-2 among ovarian cancer patients compared to healthy controls or women with benign gynecologic disease. While minor differences existed between urinary Bcl-2 levels in the two cohorts examined, these may reflect differences in cohort size, differences in initial sample storage temperatures, length of sample storage, number of repeated freeze thaws or necessity for addition of protease inhibitors for sample testing. However, the similarity of Bcl-2 values among samples in both cohorts (e.g. USF healthy 0.30 ng/ml & MDA healthy 0.69 ng/ml or USF cancer 2.54 ng/ml and MDA cancer 2.30 ng/ml) appears to validate our hypothesis. Interestingly, though the number of samples available from EOC was small, the lack of statistical differences in urinary Bcl-2 levels between EOC and LOC suggests that urinary Bcl-2 may be useful for early detection of ovarian cancer. Further, the most apparent clinical features related to elevated urinary Bcl-2 are high risk for disease and complementarity with CA125. While the former represents an important target group, the latter suggests potentially important diagnostic and prognostic roles for urinary Bcl-2. By helping to discern between ovarian cancer and false positive CA125 values arising from other conditions, urinary Bcl-2 measurements may improve detection of early stage ovarian cancer. Given the only association between urinary Bcl-2 and cancer reported to date describes the presence of methylated urinary Bcl-2 DNA in urine sediments associated with bladder cancer [24,25], development of urinary Bcl-2 protein testing could provide a convenient and non-invasive strategy for detection/screening of ovarian cancer. Clearly, further studies are warranted to verify the potential for urinary levels of Bcl-2 to serve as a biomarker alone or in combination with other markers, especially as that may pertain to screening for ovarian cancer.

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Figure 1. Urinary Bcl-2 levels are elevated in ovarian cancer patients

As representative, urinary samples are shown from the USF cohort examined by ELISA for Bcl-2 levels in normal healthy controls and ovarian cancer patients (A) and in benign samples (B). Samples were examined in triplicate and the data expressed as the average ng/ml of Bcl-2 $\pm$ S.E. Cancer samples were subdivided into histologic subtype: endometrial (white bars), mucinous (striped bars), primary peritoneal (checkered bars) and serous carcinomas (grey bars) and the latter further subdivided by tumor grade. Urinary sample #41 from an ovarian cancer patient was included in (B) to serve as a positive control.

Figure 2. Urinary Bcl-2 levels are elevated in ovarian cancer patients

Urinary samples from the USF (A), MDA (B) and combined USF + MDA (C) cohorts were examined by ELISA for Bcl-2 levels in normal healthy controls, women with benign disease and early and late stage ovarian cancer patients. Samples were examined in triplicate, the data expressed as the average ng/ml of Bcl-2 $\pm$ S.D. and graphed as box plots.

Figure 3. Urinary Bcl-2 decreases after cytoreductive surgery

Urinary levels of Bcl-2 were measured in duplicate by ELISA from seven patients of the USF cohort prior to initial surgery (black bars) and within 2 weeks following their initial cytoreductive surgery (white bars). The data are expressed as the mean ng/ml Bcl-2.

Figure 4. Patients at high risk may demonstrate elevated urinary Bcl-2 levels

Urinary samples from the MDA cohorts were examined by ELISA for Bcl-2 levels in normal healthy controls or in individuals at high risk for ovarian cancer and plotted as a scatter plot.

Table 1
Histologic diagnoses and clinical characteristics of the study population

	Years (mean $\pm$ SD; range)	Sample number
USF Cohort		
Normal	54.49 $\pm$ 12.7; 29-81	<i>n</i> = 19
Benign	55.90 $\pm$ 13.9; 28-84	<i>n</i> = 38
Cysts		10
Serous cystadenoma		10
Leiomyoma		9
Adenofibroma		4
Mucinous cystadenoma		2
Teratoma		2
Polycystic disease		1
Ovarian Cancer	66.21 $\pm$ 13.8; 26-92	<i>n</i> = 35
Histotype		
Endometriod		1
Mucinous		7
Peritoneal		2
Serous		25
Stage		
I		3
II		1
III		23
IV		8
Grade		
1		8
2		10
3		17
MDA Cohort		
Normal	63.31 $\pm$ 6.56; 51-77	<i>n</i> = 58
Benign	49.48 $\pm$ 14.19; 19-81	<i>n</i> = 123
Cysts		31
Serous cystadenoma		19
LMP		14
High risk		13
Leiomyoma		12
Teratoma		11
Endometriosis		8
Serous cystadenofibroma		4
Fibroma		3
Endometrial hyperplasia		3
Mucinous cystadenoma		2

	Years (mean $\pm$ SD; range)	Sample number
Adenomyosis		2
Brenner tumor		1
Ovarian Cancer	60.51 $\pm$ 12.27; 33-88	$n = 115$
Histotype		
Serous		99
Endometrioid		12
Mucinous		3
Granulosa		1
Stage		
I		6
II		3
III		90
IV		16

Table 2
Elevated urinary Bcl-2 in cohorts as descriptive statistical information

Type	#	mean	S.D.	Median	Min	Max
USF						
Normal	19	0.30	0.39	0.15	0	1.26
Benign	38	0.74	0.66	0.68	0.1	3.99
EOC	4	2.38	0.75	2.10	1.86	3.48
LOC	31	2.54	1.83	3.17	1.12	9.80
MDA						
Normal	58	0.69	0.64	0.57	0	2.55
Benign	123	1.23	0.79	1.20	0	3.82
EOC	9	2.70	2.68	1.94	1.05	9.65
LOC	106	2.30	1.34	1.94	0	8.78
USF + MDA						
Normal	77	0.59	0.61	0.39	0	2.55
Benign	161	1.12	0.79	1.05	0	4.00
EOC	13	2.60	2.23	1.96	1.05	9.65
LOC	137	3.58	1.55	2.13	0	9.80

Table 3
Elevation of urinary Bcl-2 from healthy women and from patients with benign lesions, early (EOC) and late (LOC) stage ovarian cancer

	ROC-AUC	95%	Tukey mean difference	95% CI
USF				
Normal vs. benign	83%	15.8%	0.3586	0.4568 1.17 *
Normal vs. all cancer	100%	100%	2.9193	2.2938 3.55 *
Normal vs. EOC	100%	100%	2.0829	0.4936 3.67 *
Normal vs. LOC	100%	100%	3.0308	2.1838 3.88 *
Benign vs. all cancer	ND	ND	2.5607	2.0419 3.08 *
Benign vs. EOC	ND	ND	1.7243	0.2037 3.24 *
Benign vs. LOC	ND	ND	2.6722	1.9624 3.38 *
EOC vs. LOC	ND	ND	0.9478	-0.58898 2.48
MDA				
Normal vs. benign	71%	17.9%	0.5425	0.1608 0.92 *
Normal vs. all cancer	90%	50.4%	1.5219	1.1686 1.87 *
Normal vs. EOC	90%	44.4%	1.1355	0.2316 2.04 *
Normal vs. LOC	90%	50.9%	1.5513	1.1592 1.94 *
Benign vs. all cancer	ND	ND	0.9794	0.6944 1.26 *
Benign vs. EOC	ND	ND	0.593	-0.2815 1.47
Benign vs. LOC	ND	ND	1.0088	0.6904 1.33 *
EOC vs. LOC	ND	ND	0.4158	-0.4632 1.29
USF + MDA				
Normal vs. benign	71%	17.39	0.5061	0.0834 0.93 *
Normal vs. all cancer	93%	76.67%	1.8512	1.5286 2.17 *
Normal vs. EOC	92%	66.23%	1.4172	0.4714 2.36 *
Normal vs. LOC	93%	77.37%	1.8897	1.4545 2.32 *
Benign vs. all cancer	ND	ND	1.3451	1.0831 1.61 *
Benign vs. EOC	ND	ND	0.9111	-0.001 1.82

	ROC-AUC	95%	Tukey mean difference	95% CI
Benign vs. LOC	ND	ND	1.3837	1.0275 1.74 [*]
EOC vs. LOC	ND	ND	0.4725	-0.4455 1.39

*
p < 0.01

ND, not determined