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Urinary oncofetal ED-A fibronectin correlates with poor prognosis in patients with bladder cancer

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Abstract

The extracellular matrix protein fibronectin (FN) contributes to the structural integrity of tissues as well as the adhesive and migratory functions of cells. While FN is abundantly expressed in adult tissues, the expression of several alternatively spliced FN isoforms is restricted to embryonic development, tissue remodeling and cancer. These FN isoforms, designated ED-A and ED-B, are frequently expressed by cancer cells, tumor-associated fibroblasts and newly forming blood vessels. Using a highly sensitive collagen-based indirect ELISA, we evaluated the correlation of urinary ED-A and ED-B at time of cystectomy with overall survival in patients with high-grade bladder cancer (BCa). Detectable levels of total FN as well as ED-A and ED-B were found in urine from 85%, 73% and 51% of BCa patients, respectively. The presence of urinary ED-A was a significant independent predictor of 2-year overall survival (OS) after adjusting for age, tumor stage, lymph node stage, and urinary creatinine by multivariable Logistic Regression ($p=0.029$, OR=4.26, 95% CI, 1.16–15.71) and improved accuracy by 3.6%. Furthermore, detection of ED-A in the urine was a significant discriminator of survival specifically in BCa patients with negative lymph node status (Log-Rank, $p=0.006$; HR=5.78, 95% CI, 1.39–24.13). Lastly, multivariable Cox proportional hazards analysis revealed that urinary ED-A was an independent prognostic indicator of 5-year OS rate for patients with BCa ($p=0.04$, HR=2.20, 95% CI, 1.04–4.69). Together, these data suggest that cancer-derived, alternatively spliced FN isoforms can act as

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prognostic indicators and that additional studies are warranted to assess the clinical utility of ED-A in BCa.

Keywords

fibronectin; oncofetal; bladder cancer; biomarker; ED-A; ED-B

Introduction

Fibronectin (FN) is a critical and abundant glycoprotein of the extracellular matrix. Although it is expressed as a soluble 500 kDa globular homodimer, after secretion from the cell, it can be assembled into a fibrillar matrix by a variety of cell types through integrin-dependent mechanisms. FN can be alternatively spliced into at least 20 different isoforms [1]. In general, FN can be divided into two groups according to its origin: 1) plasma FN, which is produced in the liver and distributed systemically through the circulation, and 2) cellular FN, which is produced by other tissues and deposited locally [2]. Plasma FN is expressed primarily by hepatocytes and is present in plasma at very high levels (0.3–0.4 mg/ml) [3, 4]. Cellular FN, including the oncofetal isoforms, is expressed by embryonic cells, tumor cells and somatic cells engaged in growth and tissue remodeling, such as activated fibroblasts and angiogenic endothelial cells [3, 5, 6].

Oncofetal FN isoforms can be identified by the presence of the alternatively spliced regions designated extra domain-A (ED-A), extra domain-B (ED-B), and the IIICS domain. While these extra domain segments are expressed by somatic cells engaged in growth and remodeling, they are not produced in healthy liver and, thus, are not found in plasma FN [7]. Although both ED-A and ED-B expression is abundant in fetal tissues, particularly in the brain, thymus, lung, and gastrointestinal tract, their expression is greatly reduced in most tissues during postnatal development [8, 9] and only retained at low levels in the adult ovary, myometrium, and coelomic epithelium [8]. However, these oncofetal isoforms are re-expressed during wound healing, vascularization and oncogenesis where they function to support tissue remodeling. These alternative splice variants of FN can be expressed by the tumor cells themselves [7, 8, 10, 11] or by reactive stroma, such as endothelial cells involved in tumor-associated angiogenesis [10, 12, 13]. These observations suggest that FN switching or the reactivation of oncofetal FN expression is associated with oncogenesis [6].

Ectopic ED-A/ED-B expression has been found to activate physiological and pathological phenotypes [6, 10, 14–16] that are distinct from plasma fibronectin. ED-A demonstrates a unique, enhanced binding to integrins $\alpha 4\beta 7$ and $\alpha 5\beta 1$, which alters adhesion and influences both cell proliferation and differentiation [14, 17, 18] and leads to changes in migration, tissue repair and inflammation [19–21]. ED-B is more closely linked to angiogenesis [22, 23] but has also been shown to promote adhesion and remodeling in several tissues [24, 25]. Studies have also demonstrated that ED-B influences structural availability of domains within FN [26] and can, therefore, promote fibril formation [27].

Significant research has been conducted looking at total FN as a diagnostic and prognostic biomarker in a variety of cancers [28–31]. In addition, several studies have demonstrated

that ED-A and ED-B are highly expressed in cancerous tissue but rarely observed in normal adult tissues [16, 32], suggesting that oncofetal FN isoforms have potential utility as cancer biomarkers. However, little attention has been paid to oncofetal FN isoforms until recently when isoform specific antibodies and an ELISA were developed to detect FN isoform levels in various biofluids, including plasma and cerebral spinal fluid [33]. Previously, primary detection of ED-A and ED-B had been restricted to mRNA, which often lacks direct correlation with protein expression [8, 28]. The absence of oncofetal FN in saliva, cervicovaginal secretions, and urine from healthy individuals [33] emphasizes the possible utility of biofluid-derived oncofetal FN isoforms in cancer diagnosis and prognostication. Despite this, very few clinical studies have evaluated the diagnostic and/or prognostic strength of ED-A, ED-B and/or III-CS FN isoforms in cancer [16, 28, 32, 34, 35].

Bladder cancer (BCa) is currently diagnosed and monitored using urinary cytology, cystoscopy, and CT urography. While these techniques have high diagnostic sensitivity and specificity for BCa, prognostic indicators remain more elusive [36, 37]. Recently, the detection of oncofetal FN in plasma has been explored as a potential biomarker and prognostic factor for bladder cancer. It was observed that levels of ED-A in plasma were significantly higher in BCa patients than normal controls as well as correlated with stage [34]. Though this study was able to show a clear distinction between cancer cases and normal controls, urine offers a particular advantage in examining potential prognostic biomarkers due to its proximity to the primary tumor site and ease of collection. Secretion of soluble FN and the release of proteolytic fragments during remodeling into the adjacent urine make for an attractive biomarker. Previous studies examining total FN and the III-CS oncofetal FN isoform in urine have detected elevated levels of both in BCa patients compared to individuals with benign urological diseases or normal controls [29, 38–42]. Unfortunately, the detection of total FN in the urine of BCa patients is quickly compromised by plasma FN entering the urine through bleeding from the bladder wall, a common occurrence in BCa. In contrast, the oncofetal FN isoforms, ED-A, ED-B and III-CS, are released solely by malignant cells and the tumor microenvironment, making their detection in urine a specific indication of cancer.

Although these previous studies have been able to consistently show increases in expression and biofluid detection of oncofetal FN isoforms in bladder cancer, there has been a particular focus on III-CS and little exploration into fluid-derived ED-A and ED-B. Therefore, in our study, we sought to assess the prognostic value of urinary ED-A and ED-B compared to total FN. Using a highly sensitive, collagen-based indirect ELISA, we were able to demonstrate that urinary ED-A, but not ED-B or total FN, is an independent predictor of overall survival after surgical resection in patients diagnosed with high-grade BCa. Moreover, discrimination of outcome with urinary ED-A was specific to lymph node negative disease.

Methods

Cell Culture and Conditioned Media Collection

The colorectal cancer cell line Caco-2 (ATCC, HTB-37) and the fetal lung cell line WI-38 (ATCC, CCL-75) were obtained from the American Type Cell Collection. Human infant

foreskin fibroblasts (HFFF) were generously donated by G. Lenk (Washington State University, Pullman, WA). All cells were cultured at 37°C, 5% CO₂ and maintained in DMEM (Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum (Atlas Biologicals, Inc., PS-0500-A) and 1% penicillin-streptomycin, MEM amino acids, HEPES, L-glutamine, and sodium pyruvate (Life Technologies). When cells were 80–90% confluent, they were gently washed and incubated in serum-free DMEM for 1 hour at 37°C. Following incubation, the serum-free media was aspirated and replaced with fresh serum-free DMEM to ensure that the subsequent conditioned media would contain little albumin. Conditioned media was collected after 48 hours and spun at 1000 rpm for 5 minutes to pellet cellular debris, then the supernatant passed through a sterile 0.45 µm filter, aliquoted and stored at –80°C.

Western Blots

Conditioned media collected from Caco-2, WI-38, and HFFF cell cultures was prepared for electrophoresis by first concentrating it approximately 4-fold using Amicon Ultra centrifugal filters (Millipore, Billerica, MA). Concentrated conditioned media was diluted with 4X Laemmli sample buffer then boiled at 95°C for 5 minutes and run on a 10% polyacrylamide gel. Following electrophoresis, protein was transferred to a PVDF membrane (Millipore) and blocked with 5% milk for 1 hour at room temperature. Membranes were incubated with primary antibodies for ED-B (1:1000, S-FN4 clone BC-1, Sirius Biotech), ED-A (1:2000, clone FN-3E2, Sigma F6140), or total FN (1:1000, clone IST-4, Sigma F0916) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated anti-mouse secondary antibody for 1 hour at room temperature (GE Healthcare, UK). Protein expression was detected by chemiluminescence (cat. no. 34080, Thermo Scientific, Waltham, MA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Creatinine ELISA—The creatinine ELISA was performed according to manufacturer protocol with urine diluted 1:25 in assay buffer, a linear range from 0.3125 to 20 mg/dL, and colorimetric detection at 490 nm (MBL International Corp., JM-K635-100).

Fibronectin ELISA

Construction of Standard Curves for Fibronectin (FN) Isoforms: A standard curve for each FN isoform was developed using Caco-2 48-hour conditioned media based on elevated expression/secretion of both ED-A and ED-B [38]. The concentration of total FN in Caco-2 conditioned media was determined prior to experimental runs using known concentrations of human plasma fibronectin (Life Technologies, cat. no. 33016-015) as an initial standard. The levels of ED-A and ED-B FN in the Caco-2 conditioned media were set at one-tenth and one-one hundredth of total FN, respectively, as published by other groups [29, 39, 43, 44].

Indirect ELISA with Gelatin: FN has been extensively described as binding to collagen with high affinity [45]. As collagen I is a main component of gelatin and has been shown to bind all isoforms of FN, we coated high-affinity binding 96-well plates with sterile filtered 0.5% gelatin (Sigma-Aldrich, St. Louis, MS) and incubated at 37°C for 4 hours [46]. Gelatin

was then aspirated, the wells washed with phosphate buffered saline plus 0.05% Tween-20 (PBSt) and blocked with 3% BSA (Sigma-Aldrich). Coated plates were stored at 4°C.

ELISA Protocol: PBSt and PBSt with 1% BSA were used as wash buffer and sample/antibody diluent, respectively. Urine samples were diluted at 1:4, 1:8, and 1:16 for ED-B, ED-A, and total FN, respectively, and incubated overnight at room temperature. Following incubation, wells were washed 3 times and incubated with primary antibodies for ED-B (1:2000, S-FN4 clone BC-1), ED-A (1:10000, clone FN-3E2), and total FN (1:5000, clone IST-4) overnight at room temperature. Wells were again washed and incubated with the biotin-conjugated secondary antibody (1:10000, anti-mouse, Vector Laboratories, Burlingame, CA) at 37°C for 2 hours. Lastly, wells were washed and incubated with streptavidin-HRP (1:200, Vector Laboratories) for 20 minutes at room temperature. ELISA plates were developed with SuperSignal Western Blot Pico ECL detection substrate (Thermo Scientific). Plates were read on a BioTek Synergy2 (Winooski, VT) using endpoint luminescence detection with an integration time of 2 seconds and sensitivity of 100. Concentrations were calculated based on a standard curve of 0.005–5 pg/ml for ED-A FN, 0.06–60 pg/ml for total FN and a 0.02–20 pg/ml standard curve for ED-B FN using a two-fold serial dilution (11 points). The linear range for total FN was 125 fg/ml to 4 pg/ml, for ED-A FN was 2 pg/ml to 32 pg/ml, and for ED-B FN was 2.5 to 20 pg/ml. All samples were diluted separately for each isoform to fall within the linear range. Samples below the limit of detection were run in a repeated assay at various lower dilutions to verify absence of signal.

Immunofluorescence

Immunofluorescence was performed on representative BCa and adjacent normal tissue from the VUMC cohort. Sections (5µm) were deparaffinized and rehydrated. Antigen retrieval was performed in a steamer either for 30 minutes in pre-warmed modified citrate retrieval buffer (pH 6.0; S2369; Dako) for the total FN or for 12 minutes in Tris-EDTA (TE) buffer (pH 8.0) for ED-A FN. Sections were then blocked in 20% Aquablock/PBS (East Coast Biologics) plus 0.05% Tween-20. The sections being stained for total FN were also treated with an avidin-biotin blocking kit (SP-2001; Vector Laboratories) prior to addition of primary antibody. Primary antibodies were biotinylated rabbit anti-FN (ab6584; Abcam; 1:50) and mouse anti-FN [ED-A] (Clone FN-3E2; 1:200). Detection antibodies were Texas Red conjugated Avidin D (A-1100, Vector Laboratories) and Alexa-647 conjugated goat anti-mouse (Life Technologies). Hoechst 33342 was added to the final incubation step to mark nuclei. Slides were mounted in ProLong Gold Antifade (Invitrogen) and imaged on a BX61 Olympus microscope equipped with a digital camera (Orca ER, Hamamatsu) and controlled with Volocity image acquisition software (PerkinElmer).

Specimen Collection

All specimen collections were performed in accordance with the 1964 Helsinki Declaration and approved by the Vanderbilt Institutional Review Board (IRB). Patient confidentiality was protected according to the U.S. Health Insurance Portability and Accountability Act (HIPAA). Fluid was stored at –80°C and had undergone one round of freeze-thaw for aliquoting at time of use.

Bladder Cancer Urine Cohort

The analysis of urinary FN isoforms was performed on a retrospective cohort of patients with high-grade bladder cancer undergoing radical cystectomy at VUMC from 2001–2006, which included pathological stages from pT0 to pT4 and excluded patients who had received chemotherapy prior to surgical resection (n=110). Urine collection was right before surgery via foley/catheter. Details of this cohort can be found in Table 1. Last follow-up was performed in June of 2015.

Control Urine Cohorts

Age-matched, control clean-catch and catheter-derived urine specimens were collected at VUMC by the Cooperative Human Tissue Network. Non-cancer control urines (Normal Controls) were collected from patients with no history or current diagnosis of cancer undergoing non-urologic surgeries including cardiac bypass, gastric bypass, thyroidectomy, esophagomyotomy, knee replacement, and hernia repair.

Statistical Analysis

Statistical analyses were performed at a two-tailed significance of 0.05. Kruskal-Wallis followed by Mann-Whitney U tests were used to assess the differences in means. Overall survival was defined as time from date of cystectomy to date of death or last follow-up with 2 and 5-year cutoffs. Kaplan-Meier curves and log-rank tests were utilized for univariable overall survival analysis. Receiver operating characteristics (ROC) curves and the area under ROC curve (AUC) were used to assess the prognostic value and accuracy of the multivariable logistic regression models. For multivariable analysis of censored survival, Cox proportional hazards regression was performed with all variables treated as continuous covariates except ED-A and ED-B, which were dichotomized into present or absent because, in the initial statistical analysis, no additional benefit was observed when using them as continuous variables. Statistical analyses and graphing were performed with SPSS (IBM) and GraphPad Prism (GraphPad Software, Inc.). To address potential over-fitting, where over-fitting is defined as more than 1 covariate for every 10 events, statistical validation with bootstrap was performed in all multivariable regression analyses. Bootstrap validation was performed via SPSS with a two-tailed significance, 1000 iterations and a Mersenne twister of 2,000,000.

Results

Total and ED-A fibronectin expression is upregulated in invasive urothelial carcinoma

Immunohistochemical analysis of total FN protein expression in human tissue, available through the Human Protein Atlas [www.proteinatlas.org], demonstrated a notable increase in expression in high-grade, invasive BCa compared to normal bladder urothelium (Fig. 1a). The greatest increase in signal is located predominantly in the reactive tumor stroma, but total FN expression could also be seen at elevated levels in the malignant urothelium (Fig. 1a, bottom panel). In addition, we were able to detect both total FN and ED-A by immunofluorescence in our VUMC cohort (Fig. 1b). Whereas total FN was predominantly expressed in the submucosa in normal bladder and tumor stroma, ED-A FN expression was

localized to the urothelium of normal bladder and appears to be elevated, specifically in the malignant cell compartment of the tumor (Fig. 1b).

Development of ELISA for detection of urinary levels of total FN as well as, ED-A and ED-B FN isoforms

Our goal was to develop an assay that could sensitively detect total FN, as well as the ED-A and ED-B oncofetal FN isoforms, in biofluids in order to assess their prognostic strength in BCa. Since full-length ED-A and ED-B FN are not available as purified or recombinant standards, we chose to develop the isoform standard curves for the ELISA based on the conditioned media from cells shown to express these isoforms. Western blot analysis of conditioned media from HIFF, WI-38, and Caco-2 cells revealed that although all 3 cell lines express FN, only Caco-2 express both the ED-A and ED-B isoforms at levels suitable for a standard curve (Fig. 2a). The lack of ED-A and ED-B expression in the HIFF and WI-38 conditioned media was not due to insufficient protein as shown by Coomassie blue staining (Fig. 2b). Therefore, we used the Caco-2 conditioned media as our standard curve for total FN and both FN isoforms.

To achieve high sensitivity, we took advantage of the high affinity of FN for collagen and used a thin coating of gelatin to capture all soluble isoforms of FN present in the biofluid of interest (Fig. 2c). Primary monoclonal antibodies specific for total, ED-A and ED-B FN provided isoform specificity (Fig. 2c). Utilizing biotin-streptavidin amplification and a chemiluminescent rather than a colorimetric substrate for quantification, we were able to detect the isoforms at high femtogram to low picogram concentrations (Fig. 2c). This indirect capture ELISA provides a uniform assay with isoform-specificity that has greater sensitivity than the currently utilized FN detection methods [47].

We determined the concentration of total FN in a large stock of Caco-2 conditioned media using known concentrations of human plasma fibronectin as the interim standard (Fig. 2c). Based upon previously published studies, we approximated and set the concentration of ED-A in our standard to be one-tenth of the total FN concentration, while the concentration of ED-B is approximately one-one hundredth of the total FN concentration [16, 29, 39, 44, 55, 56, 57]. Standard curves for ED-A, ED-B and total FN in the Caco-2 conditioned media were calculated using a four-parameter equation. Implementation of this assay not only improved the threshold of total FN detection, it also enabled the detection of low levels of the FN splice variants ED-A and ED-B in the urine of BCa patients.

Patients with BCa have elevated levels of urinary ED-A, ED-B and Total FN

Previous research has shown elevated levels of total FN in urine samples collected from patients with BCa [48, 49]. Using urine samples collected from BCa patients undergoing radical cystectomy (n=110) and normal age-matched controls (n=35), we not only validated these results with total FN, but revealed that both the ED-A and ED-B FN isoforms are present and elevated in the urine of these patients with BCa. The patients in the BCa cohort were 90% male and 95.5% white with an average age of 66 years (Table 1). All tumor stages were represented within the cohort, though 74.5% of the BCa patients were lymph node negative. Equal percentages of non-muscle invasive bladder cancer (NMIBC), organ-

confined muscle invasive (OCMIBC), extravesical muscle invasive (EVMIBC), and lymph node positive muscle-invasive (LN+) samples were obtained (Table 1), most having high grade disease. Of the 110 patients in the BCa cohort, 80 (73%) were ED-A positive and 56 (51%) were ED-B positive, while only 14/35 (40%) and 5/35 (14%) of normal controls were positive for these FN isoforms. Furthermore, patients with muscle invasive bladder cancer (MIBC) had significantly elevated levels of total FN (Mean=163,500 pg/ml, 95%CI=-77,910–404,900) compared to normal controls (Mean=51.01 pg/ml, 95%CI=29.61–72.40; Mann-Whitney U (MWU), $P<0.0001$) and an increased trend compared to NMIBC (Mean=43,610 pg/ml, 95%CI=-45,630–132,800; Mann-Whitney U (MWU), $P=0.052$) (Fig. 3a). Patients with MIBC also had elevated ED-A (Mean=39,860 pg/ml, 95%CI=-39,180–118,900) and ED-B FN (Mean=8.98 pg/ml, 95%CI=5.17–12.79) compared to normal controls (Mean=9.36 pg/ml, 95%CI=4.00–14.71; MWU, $p=0.0019$; Mean=1.88 pg/ml, 95%CI=0.22–3.54; MWU, $P=0.0005$) (Fig. 3b,c). Notably, ED-B FN is the only isoform significantly elevated in NMIBC compared to controls ($P=0.0094$). This data indicates that there is an overall increase in tumor-derived cellular FN in patients with invasive BCa compared to non-cancer controls and warrants further investigation into the prognostic utility of oncofetal FN splice variants in predicting tumor progression.

Patients with detectable urinary levels of ED-A FN have a decreased trend of overall survival by univariable analysis

Although, detection of total FN has been included in tumor biomarker panels in recent years [29, 50, 51], there are no published reports on the clinical utility of the oncofetal FN isoforms ED-A and ED-B. To determine the relative contribution of urinary total FN compared to contributions of the oncofetal FN isoforms ED-A and ED-B in the prediction of patient outcome, we performed univariable (Log Rank and Kaplan-Meier) analyses for 5-year overall survival (OS) in the BCa cohort. For univariable analysis, total FN was dichotomized above and below the median (120 pg/ml) while ED-A and ED-B were dichotomized as present or absent. Patients with high levels of urinary total FN show notable but not statistically significant reduction in 5-year OS compared to patients with low total FN (Log-Rank, $p=0.07$; Median OS, 42.53 months vs. not defined; Hazard Ratio (HR)=1.61, 95% Confidence Interval (CI)=0.96–2.71) (Fig. 4a). Patients with detectable urinary ED-A also exhibited a decrease in 5-year OS compared to those lacking urinary ED-A, with a minimum 30-month difference in median survival, though not quite reaching significance (Log-Rank, $p=0.051$; Median OS, 31.93 vs. not defined; HR=2.07, 95%CI=0.98–4.36) (Fig. 4b). The presence of urinary ED-B showed a similar trend as total FN and ED-A but with the least significance of the three (Log-Rank, $p=0.103$, Median OS, 35.67 months vs. not defined; HR=1.53, 95%CI=0.91–2.57) (Fig. 4c). Interestingly, when BCa patients were dichotomized by a combined ED-A and ED-B status, patients with both ED-A and ED-B present in their urine had significantly worse 5-year OS relative to patients with only one or neither detected (Log-Rank, $p=0.039$; Median OS, 31.07 months vs. not defined; HR=1.71, 95%CI=1.02–2.88) (Fig. 4d).

ED-A FN predicts poor outcome specifically in lymph node negative patients

In urothelial cancer, dissemination to the lymph nodes as well as the number of lymph nodes involved, is negatively associated with survival [52]. To determine if FN had differential

predictive power among high risk (lymph node positive, LN+) and low risk (lymph node negative, LN-) patients, we performed a subgroup analysis comparing those patients with positive versus negative lymph node status for total FN and the oncofetal FN isoforms ED-A and ED-B.

Total FN was unable to discriminate 5-year OS in either LN- or LN+ patients (Log-Rank, $p=0.206$ and $p=0.485$) (Fig. 5a,b). On the other hand, the presence of ED-A in the urine significantly correlated with a decrease in 5-year OS in LN- patients (Log-Rank, $p=0.006$; Median OS, 46.10 months vs. not defined; HR=5.78, 95%CI=1.39–24.13) but not in LN+ patients (Log-Rank, $p=0.432$) (Fig. 5c,d). Notably, patients that were negative for both urinary ED-A and lymph node invasion achieved a greater than 80% survival at 5 years, which is twice the rate of survival of lymph node negative patients that had detectable levels of urinary ED-A (Fig. 5c). This highlights the fact that the discrimination in survival seen with ED-A when analyzing the entire cohort (Fig. 4b) was specifically due to discrimination in LN- patients. Therefore, the discrimination effect is greater in the LN- specific cohort (HR=5.78 vs. 2.07) (Fig. 5c). A similar trend was observed for LN- BCa patients with detectable urinary ED-B, albeit this did not reach significance (Log-Rank, $p=0.056$; Median OS, 46.10 months vs. not defined; HR=1.91, 95%CI=0.97–3.77) (Fig. 5e). Similar to ED-A, no survival difference was noted for the presence of urinary ED-B in lymph node positive patients (Log-Rank, $p=0.488$) (Fig. 5f).

ED-A FN is an independent predictor of overall survival by multivariable analysis

Although our initial univariable analyses revealed that urinary ED-A has potential as a prognostic biomarker in BCa, especially in lymph node negative patients, a marker must prove to be independently predictive beyond standard indicators in order to have clinical utility. In other words, it must improve upon standard predictors. To assess this possibility, we performed multivariable Logistic regression and Cox proportional hazards analyses for 2 and 5-year OS with ED-A dichotomized as present or absent and all other predictors treated as continuous. In addition, rather than normalize urine levels of ED-A to urinary creatinine to adjust for proteinuria, which would give ED-A in arbitrary units, we included urinary creatinine in the prediction model. Gender and race were excluded from the multivariable regression analyses because our current BCa cohort is, by nature, restricted to 90% male and greater than 95% white (Table 1).

Using multivariable Logistic regression, urinary ED-A was found to be a significant independent predictor of 2-year OS after controlling for standard predictors such as age, pathological tumor stage, lymph node stage and urinary creatinine (Odds Ratio=4.26; 95%CI, 1.16–15.71; $P=0.029$) (Table 2). Furthermore, the addition of urinary ED-A to these standard predictors improved prediction accuracy by 3.6% based on the area under the curve (AUC) of the receiver operating characteristic (ROC) curve (AUC, 81.7% vs. 78.1%) (Fig. 6a). As a result, the sensitivity and specificity of the prediction model that included urinary ED-A was 81.0% and 72.1%, respectively, compared to 81.0% and 57.4% without ED-A (Fig. 6a), an improvement of 15%. Furthermore, similar to the univariable results, if multivariable Logistic regression analysis is performed specifically on the LN- cohort, the presence of urinary ED-A indicates a much greater risk of death within 2-years following

surgery (Odds Ratio=10.81; 95%CI, 1.24–94.19; P=0.0031) (Table 2). This translates into a 6.3% increase in prediction accuracy and a 13.8% increase in specificity at 79.2% sensitivity with the addition of urine ED-A in LN– BCa patients (AUC, 83.0% vs. 76.7%) (Fig. 6b).

Although the sample size and number of events limits the number of predictors that can be incorporated into the prediction model for the current cohort study, we tested several other potential predictors one by one, as well as all together, for their effect on the prediction strength of urinary ED-A in the current Logistic regression model, with the understanding that this causes over-fitting and, thus, reduces generalizability (Table S2–S8). Predictors tested were time from last transurethral resection (TUR) to cystectomy (Table S2), prior treatment with Bacillus Calmette-Guerin (BCG) (Table S3), presence of carcinoma *in situ* (CIS) (Table S4), adjuvant chemotherapy (Table S5), number of lymph nodes removed (Table S6), lymphovascular invasion (LVI) (Table S7), and urinary hemoglobin (Table S8). Individually, LVI was the only predictor to reduce the prediction strength of urinary ED-A, which we speculate is mainly due to a decrease in the number of patients with information on LVI which was not routinely recorded in the pathology reports until 2004 and is reflected by the fewer number of patients and events included in the LVI containing model (Table S7). Furthermore, even when all these predictors were included together in the base model, the addition of urine ED-A was still able to improve prediction of 2-year OS, an effect most prominent in LN– patients where accuracy increased by 4.1% and specificity increased from 63.0% to 76.1% when sensitivity was set at 78.9% (Fig. S1).

Also, it is possible that urinary ED-A is just a surrogate marker of tissue remodeling, bladder injury or hematuria since it does have a significant correlation with both urine hemoglobin levels (R=0.273, P=0.005) and time from TUR to cystectomy (R=0.290, P=0.002) (Table S1). However, as already stated, the addition of either of these predictors to the multivariable Logistic regression model failed to abrogate the significant contribution of urinary ED-A (Tables S2 & S8). Furthermore, urinary hemoglobin is not able to substitute for or recapitulate the improved prediction seen with urinary ED-A as represented by 2-year OS ROC curves for both the entire or the LN– cohorts (Fig. S2).

Lastly, after adjusting for age, pathological tumor stage, lymph node stage and urinary creatinine in a Cox proportional hazards regression model, which takes into account time, urinary ED-A remained a significant independent predictor of 5-year OS in the entire cohort (HR=2.20; 95%CI, 1.04–4.69; P=0.040) and, especially, in the LN– cohort (HR=6.77; 95%CI, 1.61–28.44; P=0.012) (Table 3). Of note, neither total FN nor ED-B showed significant correlation to overall survival by multivariable Logistic and Cox regression analyses (total FN: p=0.125 & 0.118; ED-B: p=0.243 & 0.089).

In summary, if all other factors are equal, patients with detectable levels of urinary ED-A are 4 times more likely to die within 2 years following radical cystectomy and die at twice the rate as their ED-A negative counterparts over 5 years. Furthermore, this effect is pronounced in lymph node negative disease where patients with detectable levels of urinary ED-A are 10 times more likely to die and die at 6 times the rate as ED-A negative counterparts. Taken together, these results suggest that the presence of the oncofetal FN isoform ED-A in the urine is not just a surrogate marker of tissue injury but is, rather, a relevant prognostic

indicator of post-cystectomy survival in patients with BCa and has the potential to discriminate risk in patients with negative lymph node disease.

Discussion

In this study, we demonstrate that the presence of total FN and the oncofetal FN isoforms ED-A and ED-B are negatively associated with survival in BCa patients undergoing a radical cystectomy. Our findings support previously published reports identifying a modest negative correlation between total FN and patient survival with levels in muscles invasive disease averaging at 117–170 ng/ml compared to a mean of 160 ng/ml reported here [43, 48, 53]. However, the clinical value of total FN is statistically not significant. This is likely due to the fact that plasma FN is readily introduced into the urine through bleeding from the bladder wall. Consequently, total FN measurements could be a proxy for hemoglobin measurements, which have already been shown to have limited prognostic value [54]. In contrast, the oncofetal FN isoforms are produced within the tumor and are more likely to report specifically on the presence and nature of the cancer. Indeed, we show the prognostic significance of ED-A FN is not dependent on proteinuria, hematuria, bladder injury or wound-healing (supplemental materials). Our finding that urinary ED-A is an independent prognostic indicator for BCa suggests that it could be useful in the identification of BCa individuals with a poor prognosis.

Urine can be an effective source of clinical indicators because of its proximity to the tumor, its availability, its abundance, and ease of collection. Consequently, we developed a highly sensitive and isoform-specific ELISA protocol for the detection of total, ED-A, and ED-B FN in urine samples. This assay is based on the immobilization of soluble FN onto collagen-coated ELISA plates followed by detection using a monoclonal antibody that recognizes all isoforms (Total FN) or monoclonal antibodies that are isoform-specific (ED-A and ED-B). This ELISA enabled detection of FN in urine at very low pg/ml quantities (Fig. 2c,d & 3). Levels of oncofetal FN isoforms were detected over a considerable range in the BCa patient urines (ED-A: 0–3290 pg/ml; ED-B: 0–276.7 pg/ml), but were mostly undetectable in urine from healthy individuals. Although quantified as a continuous variable, in the statistical analysis, ED-A and ED-B were dichotomized across absent/present as no additional benefit was observed when using them as a continuous variable.

While all three FN measurements had a negative correlation with patient outcome, only ED-A contributed significantly to the multivariable prediction models. Although the weak association of total FN with patient outcome was expected for reasons discussed above, the detection of ED-B could still be a valuable measurement but was present in such small quantities that it could not be detected in the majority of samples. Since ED-B was able to contribute to our analysis when combined with ED-A, we suspect that ED-B will be able to improve prognostic accuracy in larger cohorts. Interestingly, this observation implies that these two FN isoforms contribute independently to cancer progression. Nevertheless, in this study, it is the detection of urinary ED-A that provides valuable prognostic information in patients with urothelial BCa undergoing radical cystectomy. More importantly, the clinical relevance of urinary ED-A is pronounced in those patients with negative lymph node status; whereby, patients with detectable urinary ED-A have almost 11 times the risk of death and

die at over 6 times the rate as those whose urinary ED-A is undetectable by multivariable analyses (Table 2 & 3). Within our retrospective BCa cohort, we were able to show that the addition of urinary ED-A to clinically relevant indicators such as age, tumor stage, lymph node stage, and urinary creatinine, increased the prediction accuracy of the model by 3.6% in the entire cohort and 6.3% when the analysis was restricted to patients with negative lymph node status (Fig. 6). To our knowledge, this is the first study to show the prognostic implications of ED-A in this context.

Though the results of this study reveal the clinical value of ED-A as a prognostic biomarker for BCa, limitations remain. Detection of the FN isoforms could be improved with a faster and more sensitive assay. Moreover, we limited this study *a priori* to the detection of ED-A and ED-B, but other isoforms of FN should be considered as they may have value in the assessment of BCa. We have applied this analysis to a cohort of cystectomy patients where the prognostic value of these biomarkers can be useful in identifying patients that need accelerated or more aggressive intervention. However, it will be important to assess patient progression and outcome along the full disease spectrum to determine if these biomarkers could also be useful in monitoring patients with low grade and/or non-invasive disease. Lastly, it will be important to validate these studies in a larger prospective cohort to further define their clinical value.

In conclusion, our findings implicate a meaningful role for the detection of urinary ED-A in prognosis for patients with BCa. We have shown that total FN expression is significantly elevated in patients with BCa compared to normal controls. However, total FN is not sufficient at predicting survival. Additionally, we have shown that the oncofetal FN isoform ED-A is an independent prognostic indicator for BCa when one controls for age, tumor stage, lymph node stage, and urinary creatinine. Our results are important in the search for identifying new clinically relevant tumor biomarkers to assess overall patient prognosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Impact Statement

Despite improvements in the detection and treatment of bladder cancer (BCa), few advances have been made in predicting outcome and non-invasively monitoring disease progression. We show that the ED-A isoform of fibronectin and not total fibronectin is a prognostic urinary biomarker in BCa. Furthermore, the greatest discrimination with urinary ED-A occurs in lymph node negative patients where those with undetectable levels of urinary ED-A are 10 times more likely to survive 2 years following cystectomy compared to those with any detectable level of urinary ED-A.

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Figure 1. Total Fibronectin Expression in Bladder Cancer

a) Immunohistochemical (IHC) staining of total fibronectin in two cases of normal bladder (top) and two cases of invasive bladder cancer (bottom) courtesy of the Human Protein Atlas (www.proteinatlas.org). * urothelium, black dotted lines separate urothelium from the lamina propria/submucosa, white dotted lines separate tumor from stroma, white arrows point to tumor stroma. b) Immunofluorescence expression of total and ED-A FN in normal urothelium, the urethra, and an invasive tumor. Scale bars (white) are indicated in µm. * urothelium, white dotted lines separate urothelium from the lamina propria/submucosa in the

normal urothelium and the urethra, white dotted lines separate tumor from stroma, white arrows point to tumor stroma.

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Figure 2. Development of Fibronectin Isoform Specific ELISAs

a) Immunoblot analysis of total, ED-A, and ED-B fibronectin in conditioned media collected from HIFF, WI-38, and Caco-2 cells. b) Coomassie blue stain of conditioned media for the 3 cell lines showing that there was sufficient protein in all samples. c) Schematic representation of the indirect gelatin-capture ELISA protocol used to detect total, ED-A, and ED-B fibronectin. d) ELISA standard curves for total, ED-A, and ED-B fibronectin developed from Caco-2 conditioned media. Total fibronectin concentration in the Caco-2 conditioned media was determined using an initial standard curve of human plasma

fibronectin as described in methods shown as the mean $\pm$ the standard deviation for two replicates at each concentration. The concentrations for the fibronectin isoforms in the Caco-2 conditioned media were then assumed to be 1/10th (ED-A) and 1/100th (ED-B) of the total fibronectin based on previous literature [16, 29, 43, 44, 55, 57, 58].

Figure 3. Urinary ED-A, ED-B and Total Fibronectin Levels in Patients with Bladder Cancer Urinary total (a), ED-A (b), and ED-B (c) fibronectin in patients undergoing surgical resection of all stage, high-grade bladder cancer (BCa) compared to age-matched normal controls as measured by the isoform specific indirect ELISA. Patients with non-muscle invasive bladder cancer (NMIBC) were graphed separately from those with muscle invasive bladder cancer (MIBC). The dotted grey line indicates the y-axis at 1. Specimens that were undetectable by ELISA were given a value of zero. In order to plot the zero values on a log scale, 1 was added to all values. Statistics were run on the original unaltered values. Mann-Whitney U tests were used to assess differences in means. Mean and 95% CI is graphed (red). Two-tailed non-parametric significance (P) and fold-change of the medians (Fold) are given. Median values are also given. ND designates the fact that fold-change is unable to be calculated since the median of the normal controls is zero, where any number divided by zero is undefined.

Figure 4. Correlation of ED-A, ED-B and Total Fibronectin with Overall Survival in Bladder Cancer

Univariable correlation of urinary total, ED-A, and ED-B fibronectin with overall survival in patients with BCa. Kaplan-Meier survival curves were plotted, dichotomizing urinary total FN expression around the median of 120 pg/ml (a) or by the presence or absence of urinary ED-A (b), ED-B (c), or ED-A and ED-B double positive (d) fibronectin. Log-Rank test was used to determine median overall survival (OS) and significance, as well as univariable Cox proportional hazards regression was used to calculate the hazard ratio (HR) and 95% confidence intervals (CI), as given.

Figure 5. Correlation of ED-A, ED-B and Total Fibronectin with Overall Survival in Lymph Node Negative and Lymph Node Positive Bladder Cancer

Univariable correlation of urinary total, ED-A, and ED-B fibronectin with overall survival in patients with either lymph node negative (LN-) (a, c, e) or lymph node positive (LN+) (b, d, f) BCa. Kaplan-Meier survival curves were plotted, dichotomizing urinary total FN expression around the median of 120 pg/ml (a, b) or by the presence or absence of urinary ED-A (c, d) and ED-B (d, e) fibronectin. Log-Rank test was used to determine median overall survival (OS) and significance, as well as univariable Cox proportional hazards

regression was used to calculate the hazard ratio (HR) and 95% confidence intervals (CI), as given.

Figure 6. Receiver Operating Characteristic Curves for Overall Survival

Receiver operating characteristic (ROC) curves plotting 1-specificity versus sensitivity for demonstrating accuracy of prediction for 2-year overall survival of patients with bladder cancer. The accuracy, sensitivity and specificity of the standard multivariable Logistic regression prediction model (age, tumor stage, lymph node stage, and urinary creatinine) are compared to this same model with the addition of urinary ED-A FN (present/absent). This analysis is performed in the entire cohort (a), as well as specifically in lymph node negative (LN-) disease (b). The area under the curve (AUC), which is a measure of accuracy, along with its 95% confidence interval (CI) is shown. Also included is the specificity at a fixed 79.2% sensitivity. Standard Model Entire Cohort, N=113, 43 events; +ED-A FN Entire Cohort, N=110, 42 events; Standard Model LN- Cohort, N=85, 25 events; +ED-A FN LN- Cohort, N=82, 24 events.

Table 1

Bladder cancer total, ED-A, and ED-B fibronectin cohort descriptors and frequencies.

	N=	Minimum	Maximum	Mean	Median	95% CI
Age (Years)	120	35	89	68	69	66–69
Urine total fibronectin (ng/ml)	110	0	10136.77	142.55	0.13	27.63–347.04
Urine ED-A fibronectin (ng/ml)	110	0	3298.17	30.26	0.002	0.10–90.47
Urine ED-B fibronectin (pg/ml)	110	0	276.73	10.59	0.54	5.73–16.84
Urine Creatinine (mg/dl)	113	0	421.40	132.28	114.60	115.22–151.34
Urine Hemoglobin (ug/ml)	108	0	20290.00	1285.10	341.00	780.96–1855.44
TUR to Cystectomy Time (Days)	120	11	220	53	53	49–59
# LN Removed	120	1	37	10	8	9–11
Follow-up (Months)	120	<1	104	38	38	33–43
Time to Recurrence (Months)	33	<1	82	23	14	16–32
Time to Death (Months)	71	<1	97	23	15	18–28

	N=	Frequency	Percent
Gender	120		
Female		12	10.0%
Male		108	90.0%
Race	120		
White		115	95.8%
Black		3	2.5%
Unknown		2	1.7%
Recurrence	120	33	27.5%
Death (Full Follow-up)	120	71	59.2%
Death (2 years)	120	46	38.3%
Death (5 years)	120	64	53.3%
Pathological Tumor Stage	120		
pT0		5	4.2%
pTa		8	6.7%
pTis		9	7.5%

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pT1	8	6.7%
pT2a	20	16.7%
pT2b	11	9.2%
pT3a	31	25.8%
pT3b	9	7.5%
pT4a	19	15.8%
Lymph node Stage	120	
N0	90	75.0%
N1	6	5.0%
N2	24	20.0%
Group	120	
NMIBC	30	25.0%
OCMIBC	29	24.2%
EVMIBC	31	25.8%
LN+	30	25.0%
Grade	115	
Low	5	4.3%
High	110	95.7%
Prior BCG	119	41
Adjuvant Chemotherapy	118	23
CIS Present	120	55
Lymphovascular Invasion (LVI)	107	53

Description of the bladder cancer patient cohort used for prognostic assessment of total, ED-A, and ED-B fibronectin in urine. CI = confidence interval, NMIBC = non-muscle invasive BCa, OCMIBC = organ-confined muscle invasive BCa, EVMIBC = extravesical muscle invasive BCa, LN+ = lymph node positive muscle invasive BCa.

Table 2

Assessment of ED-A fibronectin as a predictor in a logistic regression analysis of 2-year overall survival in bladder cancer.

Entire Cohort (N=110, 42 events)	Odds Ratio	95% CI	Significance	Bootstrap Significance
Age (Years)	1.097	1.041–1.156	0.001	0.001
Tumor Stage	1.268	0.955–1.685	0.101	0.140
Lymph Node Stage	2.265	1.072–4.786	0.032	0.030
Urine Creatinine (mg/dl)	1.000	0.955–1.005	0.961	0.957
Urine ED-A Status	4.264	1.158–15.706	0.029	0.017
Lymph Node Negative (N=82, 24 events)				
Age (Years)	1.120	1.038–1.208	0.003	0.005
Tumor Stage	1.280	0.934–1.755	0.125	0.166
Urine Creatinine (mg/dl)	1.001	0.996–1.006	0.756	0.739
Urine ED-A Status	10.806	1.240–94.190	0.031	0.033

Assessment of urine ED-A fibronectin as a predictor of 2-year overall survival by multivariable logistic regression analysis. Top panel is analysis of entire cohort and bottom is analysis specifically in lymph node negative patients. Odds Ratio is the adjusted odds ratio, CI = confidence interval, Bootstrap Significance is two-tailed with 1000 iterations and a Mersenne twister of 2,000,000. Status refers to presence or absence.

Table 3

Assessment of ED-A fibronectin as a predictor in a multivariable Cox regression analysis of 5-year overall survival in bladder cancer.

Entire Cohort (N=110, 59 events)	Hazard Ratio	95% CI	Significance	Bootstrap Significance
Age (Years)	1.057	1.027–1.088	<0.001	0.002
Tumor Stage	1.239	1.047–1.468	0.013	0.012
Lymph Node Stage	1.578	1.100–2.263	0.013	0.014
Urine Creatinine (mg/dl)	1.000	0.998–1.003	0.767	0.758
Urine ED-A Status	2.204	1.036–4.688	0.040	0.029
Lymph Node Negative (N=82, 35 events)				
Age (Years)	1.073	1.026–1.124	0.002	0.004
Tumor Stage	1.186	0.984–1.429	0.073	0.070
Urine Creatinine (mg/dl)	1.001	0.998–1.004	0.647	0.657
Urine ED-A Status	6.767	1.610–28.438	0.009	0.012

Assessment of urine ED-A fibronectin as a predictor of 5-year overall survival by multivariable Cox regression analysis. Top panel is analysis of entire cohort and bottom is analysis specifically in lymph node negative patients. Hazard Ratio is the adjusted hazards ratio, CI = confidence interval, Bootstrap Significance is two-tailed with 1000 iterations and a Mersenne twister of 2,000,000. Status refers to presence or absence.