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Profiling Bladder Cancer Using Targeted Antibody Arrays

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Bladder cancer is a common malignancy requiring a high degree of surveillance because of the frequent recurrences and the poor clinical outcome of invasive disease. To date, serum biomarkers for bladder cancer lack optimal sensitivity and specificity to assist in diagnosis and disease categorization. Here, we designed antibody arrays for bladder cancer by selecting antibodies against targets differentially expressed in bladder tumors. Serum protein profiles measured by an antibody array containing 254 antibodies discriminated bladder cancer patients from controls (n = 95) with a correct classification rate of 93.7%. A second independent antibody array containing 144 antibodies revealed that protein profiles provide predictive information by stratifying patients with bladder tumors (n = 37) based on their overall survival (P = 0.0479). In addition, serum proteins, such as c-met, that were top ranked at identifying bladder cancer patients were associated with pathological stage, tumor grade, and survival when validated by immunohistochemistry of tissue microarrays containing bladder tumors (n = 173). This study provides experimental evidence for the use of several integrated technologies strengthening the process of biomarker discovery. Serum protein profiles obtained by antibody arrays represent comprehensive means for bladder cancer diagnosis and clinical outcome stratification, which could potentially assist in selection of cancer patients who would benefit from early, individualized therapeutic intervention. ***(Am J Pathol 2006, 168:93–103; DOI: 10.2353/ajpath.2006.050601)***

Bladder cancer is a common malignancy requiring a high surveillance because of the frequent recurrences and the poor clinical outcome when tumors progress into invasive disease. The diagnosis and follow-up of patients with bladder tumors is based on the information provided by cystoscopy in combination with urinary cytology.[1] Noninvasive procedures can assist in early detection, cancer patient surveillance and risk assessment. Availability of cancer biomarkers to be measured in body fluids is critical for the management of these patients. Many tumor markers have been evaluated in body fluids for the detection and monitoring of the disease.[2,3] However, none of the biomarkers evaluated in serum to date has provided sufficient sensitivity and specificity for the early detection of superficial bladder cancer or favorable efficacy for predicting relapses and response to chemotherapy in patients with advanced disease. Thus, the development of alternative serum biomarkers for diagnostic and prognostic stratification is of clinical importance for the management of patients with bladder cancer.

The genetic and resulting protein alterations are primary determinants steering neoplastic transformation and tumor progression. The advent of high-throughput DNA microarrays is accelerating the discovery of cancer targets.[4–7] These targets cannot only assist at characterizing the biology underlining tumorigenesis and progression but can also identify biomarkers for the clinical management of cancer patients. Direct and comparative fluorescent labeling techniques can measure the relative abundance of gene sequences. Moreover, they can also estimate the presence of antigens by antibody solutions printed on derivatized surfaces.[8–11] Antibody arrays are feasible for clinical applications, such as detecting neoplastic or autoimmune diseases, using tissues and body

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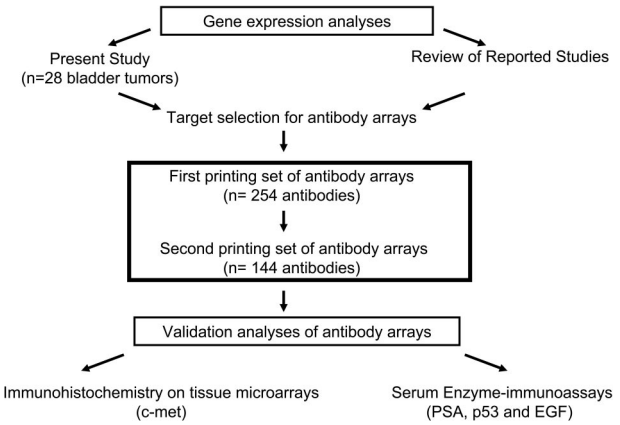

Figure 1. Experimental design. Gene expression analyses were performed to identify targets differentially expressed in bladder cancer. These analyses comprised the transcript profiles of 56 bladder tissues combined with review of reported gene-profiling studies of bladder cancer. The design of the antibody arrays was based on availability of antibodies against these targets. A first exploratory antibody array set included 254 antibodies. A second confirmatory antibody array included 144 antibodies. Two types of validation studies of the protein profiles measured with these antibody arrays were performed. Immunohistochemical patterns were analyzed on tissue arrays containing 173 tumors. Enzyme immunoassays measured protein levels of several proteins on serum specimens.

fluids.[11–16] The main goal of this study is to test whether targets characteristic of bladder tumors obtained by gene expression analyses, can detect and stratify bladder cancer using specific custom-made antibody arrays on serum specimens of patients with uroepithelial tumors (see experimental design, Figure 1).

Materials and Methods

Gene Profiling Using U133A GeneChips

Tissue Samples and RNA Extraction

Tissues from 56 bladder tissues belonging to two patients with superficial bladder cancer (pT1 lesions) and 26 patients with invasive bladder tumors (pT2+) and their corresponding normal urothelium were collected by cystectomy or cystoprostatectomy under institutional review board approval at Memorial Sloan-Kettering Cancer Center. The clinicohistopathological features of these 28 patients with bladder cancer are available as Supplementary Table 1A at *http://ajp.amjpathol.org*. Macrodissection of OCT-embedded tissue blocks was performed to ensure a minimum of 75% of normal urothelial and tumor cells for each type of specimen, respectively. Total RNA was extracted using TRIzol (Life Technologies, Rockville, MD), and purification with RNeasy columns (Qiagen, Valencia, CA). RNA quality was evaluated based on 260/280 ratios of absorbances and by gel analysis using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

Labeling and Hybridization

Complementary DNA of the 56 analyzed specimens was synthesized from 1.5 μg of total RNA using a T7 promoter-tagged oligo-dT primer. RNA target was synthesized by *in vitro* transcription and labeled with biotinylated nucleotides (Enzo Biochem, Farmingdale, NY). Labeled target was hybridized on GeneChip test 3 arrays (Affymetrix, Santa Clara, CA) to assess the quality of the sample before hybridizing onto the human genome U133A arrays including 22,283 probes representing known genes and expressed sequence tags (Affymetrix), as previously reported.[17]

GeneChip Analysis

Scanned image files were visually inspected for artifacts and analyzed using Affymetrix Microarray Suite 5.0 (MAS 5.0). Expression values of each array were multiplicatively scaled to have an average expression of 500 at least across the central 95% of all genes on the array. Signal was used as the primary measure of expression level, and detection was retained as a complementary measure.

Gene Ranking

Final ranking to obtain genes differentially expressed among paired normal urothelium and bladder tumors was determined using *t*-test, estimating also the false-positive rates.[18] Only probes providing *P* values lower than 0.001 were considered for further analyses related to antibody selection for antibody arrays.

Protein Profiling Using Antibody Microarrays

Serum Samples

Sera were collected from 95 individuals representing 58 controls and 37 patients with bladder cancer. Control specimens were selected to evaluate the specificity of the protein profiles in a variety of healthy, benign urological conditions and other solid and hematological tumors. These included healthy donors (H, $n = 18$), pregnant women (P, $n = 2$), patients with benign conditions such as benign prostatic hyperplasia (BPH, $n = 8$), kidney calculi (KC, $n = 3$), urinary tract infections (UTI, $n = 5$), and patients with other malignancies such as prostate (PC, $n = 8$), breast (BRC, $n = 6$), colon (CC, $n = 2$), and ovarian (OC, $n = 1$) carcinomas, as well as multiple myeloma (MM, $n = 3$) and lymphoblastic leukemia (LL, $n = 2$). Clinicopathological features of the 37 patients with bladder cancer are available in Table 1, and information about the diagnosis of the controls in Supplementary Table 1B at *http://ajp.amjpathol.org*. Review of clinical reports of control individuals revealed that none of them had bladder cancer or industrial exposures. Serum samples belonging to patients with bladder cancer and controls were collected at Memorial Sloan-Kettering Cancer Center and the "Hospital Universitario de Salamanca," Spain, following institutional review board requirements of these institutions.

Construction of Antibody Arrays

Antibodies were obtained by purchasing from different companies and as a result of contributions from collabo-

Table 1. Clinical Information of the Bladder Cancer Patients Utilized for Serum Profiling Using Custom-Made Antibody Arrays

Case	Set 1 ID	Set 2 ID	Status	Time	Age	Sex	Stage	Grade	Node	VI	CIS	SQ
1000-B	59669A	59705A	NED	24	64	M	PT2	High	No	No	Yes	No
1001-B	59669B	59705B	NED	6.7	42	M	PTA	High	No	No	No	No
1002-B	59668A	59706A	DOD	4	58	F	PT3B	High	Yes	Yes	Yes	Yes
1003-B	59668B	59706B	NED	20.2	64	M	PT3B	High	Yes	No	Yes	No
1004-B	59764A	59707A	NED	11.7	68	M	PT1	High	No	No	No	No
1008-B	59764B	59707B	NED	21.8	43	M	PTIS	High	No	No	Yes	No
1009-B	59763A	59708A	NED	20	69	M	PTIS	High	No	No	Yes	No
1011-B	59763B	59708B	NED	22.3	78	F	PT3B	High	No	No	Yes	Yes
1012-B	59762A	59709A	NED	19.8	69	M	PTA	High	No	No	No	No
1013-B	59762B	59709B	NED	19	75	M	PT1	High	No	No	No	No
1014-B	59667A	59710A	NED	22	73	M	PT1	High	No	No	Yes	No
1015-B	59667B	59710B	NED	19	69	F	PT2	High	No	No	Yes	No
1016-B	59666A	59711A	NED	21.2	66	F	PT4	High	Yes	Yes	No	Yes
1017-B	59666B	59711B	NED	21	65	F	PTIS	High	No	No	Yes	No
1018-B	59665A	59712A	NED	22.2	61	M	PTA	Low	No	No	No	No
1019-B	59665B	59712B	NED	19.8	68	M	PT3B	High	No	No	No	No
1020-B	59761A	59713A	NED	13.4	75	M	PT1	High	No	No	No	No
1021-B	59761B	59713B	NED	16	70	F	PT1	High	Yes	No	Yes	No
17161-B	59752A	57850A	DOD (#)	38.2	71	M	PTA	High	No	No	Yes	No
17162-B	59757A	59718A	DOD	52	75	M	PT1	High	No	No	No	No
17168-B	59753B	59722B	DOD	8.7	74	F	PT3B	High	Yes	Yes	Yes	No
17195-B	59759B	59716B	DOD	31.7	49	M	PT1	High	No	No	No	No
17199-B	59755B	59720B	NED	25.3	63	M	PTIS	High	No	No	Yes	No
17208-B	59754B	59721B	DOD	38.7	79	M	PTIS	High	No	No	Yes	Yes
17224-B	59754A	59721A	DOD	6.8	80	M	PTIS	High	Yes	No	Yes	Yes
17229-B	59755A	59720A	NED	96.6	64	M	PT3A	High	No	No	No	No
17230-B	59753A	59722A	NED	56.1	62	M	PTA	High	No	No	No	No
17231-B	59756B	59719B	DOD	5.5	67	M	PT3B	High	No	Yes	Yes	Yes
17237-B	59757B	59718B	DOD (*)	36	79	M	PTA	Low	No	No	No	No
17238-B	59756A	59719A	DOD	29.2	53	F	PTIS	High	No	No	Yes	No
17240-B	59758B	59717B	DOD	2	60	M	PT1	High	Yes	Yes	Yes	Yes
17242-B	59758A	59717A	DOD	24.3	74	M	PT1	High	No	No	Yes	No
17246-B	59759A	59716A	NED	93.6	67	M	PTA	Low	No	No	No	No
17251-B	59746B	59715B	NED	36	70	M	PTA	Low	No	No	No	No
17267-B	59746A	59715A	DOD	9.4	50	M	PT4	High	Yes	Yes	Yes	Yes
17270-B	59760A	59714A	NED	87.2	75	M	PTIS	Low	No	No	Yes	No
17280-B	59760B	59714B	DOD	24	52	M	PTIS	High	No	No	Yes	No

The table includes the identification of the bladder cancer cases in each set of antibody arrays (set 1 ID and set 2 ID), their overall survival status (NED, no evidence of disease; DOD, dead of disease), follow-up time (months), age (years), sex (F, female; M, male), histopathological stage, tumor grade, as well as the presence of lymph node metastases, vascular invasion (VI), carcinoma *in situ* (CIS), and squamous differentiation (SQ). Two patients with Ta lesions died of disease; one of them (#) presenting carcinoma *in situ*, tumor progression, and dying of bladder cancer. The other patient (*) had a high grade Ta, recurred, and died of the disease.

rators. The specific antibodies and suppliers printed on each set of arrays are listed in Supplementary Table 2 at *http://ajp.amjpathol.org*. Antibodies that were supplied in ascites fluid or antisera were purified using Protein A beads (Affi-Gel Protein A MAPS kit; Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Antibody solutions (10 to 15 μl each) of 100 to 200 μg/ml in 1× phosphate-buffered saline (PBS) were prepared in polypropylene 384-well microtiter plates (Genetix, Boston, MA). The replicate printing pattern was differentially designed in each set of antibody arrays. Antibodies were spotted at least in duplicate in the first printing set of arrays designed for screening purposes and at least in triplicate in the second set of arrays designed for validation purposes. Antibodies against proteins commonly expressed in serum, such as immunoglobulin isotypes, albumin, or C-reactive protein, were used as internal controls. Several antibodies against different epitopes for certain targets were printed onto the microscope slides using a custom-built robotic arrayer.

The first set of antibody arrays included 254 antibodies against 183 targets with 768 spots. The selection of the antibodies for the first printing set was based on the top differentially expressed genes between bladder tumors and normal urothelium, given by the U133A expression arrays. Availability of antibodies to differentially expressed transcripts restricted the inclusion of reagents against genes of interest. To compensate for the potential issue of field effect given by the comparison of bladder tumors and paired normal urothelium, other gene profiling analyses were examined as well.[6,7] Information regarding the differentially expressed targets given by the U133A gene expression analyses and related antibodies selected for the antibody arrays is provided in Supplementary Table 3 at *http://ajp.amjpathol.org*. The second set of antibody arrays included 144 antibodies against 114 targets with 366 spots. The selection of these antibodies was based on the presence of signal in the first printing set of arrays in at least 50% of the sera from patients with bladder cancer.

Antibodies were printed on nitrocellulose FAST slides (Schleicher & Schuell, Keene, NH) using a robotic arrayer at room temperature with ~45% humidity. The slides were then incubated in a humidified chamber with satu-

rated NaCl solution overnight at room temperature and then rinsed with PBST 0.5% (1× PBS, 0.5% Tween-20). Block of the slides was performed with 1% bovine serum albumin in PBST 0.5% before rinsing with PBST 0.5% and spinning dry.

Serum Labeling

Sample labeling was performed using a rolling circle amplification protocol, detailed below.[14] In both sets of antibody arrays experiments, the reference, labeled with Cy5, consisted of two pools containing equal amounts of each of the serum specimens used in each of the printing sets. One aliquot from each of the serum samples was labeled with digoxigenin (Molecular Probes, New Haven, CT), and another aliquot was labeled with biotin (Molecular Probes). Each serum aliquot was diluted 1:20 with 200 mmol/L carbonate buffer at pH 8.3, and a 1/20 vol of 6.7 mmol/L *N*-hydroxysuccinimide ester-linked biotin or digoxigenin in dimethyl sulfoxide was added. After the reactions proceeded for 2 hours on ice, a 1/20 vol of 1 mol/L Tris-HCl (pH 8.0) was added to each tube to quench the reactions, and the solutions were allowed to sit 20 minutes. The unreacted dye was removed by passing each solution through a size-exclusion chromatography spin column (Bio-Spin P6, Bio-Rad) using 6000 d as the molecular weight cutoff. Digoxigenin-labeled samples were pooled, and equal amounts of the pool were transferred to each of the biotin-labeled samples. Each dye-labeled protein solution was supplemented with nonfat milk to a final concentration of 3%, Tween-20 to a final concentration of 0.1%, and 1× PBS to yield a final serum dilution of 1:100.

Processing of Antibody Microarrays

Each labeled serum sample mix (100 μl) was incubated on a microarray with gentle rocking at room temperature for 2 hours. The microarrays were rinsed briefly in PBST 0.1% to remove the sample, washed three times for 10 minutes each in PBST 0.1%, and dried by centrifugation. The following reagents specific for RCA detection were kindly provided by Molecular Staging, Inc. (New Haven, CT): anti-biotin antibody covalently conjugated to a 22-base oligonucleotide (primer 1), anti-digoxigenin antibody covalently conjugated to a different 28-base oligonucleotide (primer 4.2), 81-base circular DNA (circle 1) with a portion complementary to primer 1, and 80-base circular DNA (circle 4.2) with a portion complementary to primer 4.2. The microarrays were incubated for 1 hour at room temperature with a solution containing 75 nmol/L circle 1, 75 nmol/L circle 4.2, 1.0 μg/ml primer 1-conjugated anti-biotin, and 1.0 μg/ml primer 4.2-conjugated anti-digoxigenin in PBST 0.1% with 1 mmol/L ethylenediaminetetraacetic acid and 5 mg/ml bovine serum albumin. The microarrays were rinsed briefly in PBST 0.1% and washed at room temperature with gentle rocking for 10 minutes in PBST 0.1%. Phi29 DNA polymerase (TempliPhi; Amersham Biosciences Corp, Piscataway, NJ) in 1× Tango buffer (Fermentas, Hanover, MD) solution with 0.1% Tween-20 and 1 mmol/L dNTPs was incubated on the arrays at 37°C for 30 minutes. The microarrays were rinsed briefly in 2× standard saline citrate (SSC)/0.1% Tween-20, washed twice for 5 minutes each at room temperature with gentle rocking in 2× SSC/0.1% Tween-20, and dried by centrifugation. A Cy3-labeled 18-bp oligonucleotide (decorator 1) complementary to the repeating DNA strand from primer 1 and a Cy5-labeled 27-bp oligonucleotide (decorator 4.2) complementary to the repeating DNA strand from primer 4.2 were prepared at 0.2 μmol/L each in 2× SSC with 0.1% Tween-20 and 0.5 mg/ml herring sperm DNA. This solution was incubated on the microarrays for 1 hour at 37°C with gentle rocking. The microarrays were briefly rinsed in 2× SSC/0.1% Tween-20, washed for 10 minutes at room temperature in 2× SSC/0.1% Tween-20, and dried by centrifugation.

Data Acquisition and Normalization

The slides were spun dry before scanning at 543 nm and 633 nm using a ScanArray microarray scanner (Packard Bioscience, Meriden, CT). GenePix Pro 3.0 (Axon Instruments, Union City, CA) software program was used to quantify the image data. Normalization was performed based on an intensity-dependent algorithm as follows.[19] The local background in each color channel was subtracted from the signal at each antibody spot, and spots having obvious defects, no detectable signal by GenePix, or a low net fluorescence in either color channel were removed from the analysis. The ratio of net signal from the sample-specific channel to the net signal from the reference-specific channel was calculated for each antibody spot, and ratios from replicated antibody measurements in the same array were averaged. It is common to plot a red (Cy5) versus green (Cy3) channel scatter plot to examine distribution of intensities; however, we found that transforming to fold change versus average intensity displayed the data in a more easily viewed form. If Ired is the background subtracted red channel intensity, and Igreen is the background subtracted green intensity, then the following variables were created: R = Ired/Igreen and $A = \sqrt{(\text{Ired} \times \text{Igreen})}$, where R is simply the fold change ratio and A is the average intensity (the geometric mean, which is equivalent to averaging the log intensity). The curvature in the scatter plot indicated a dependence of the ratio R on the overall intensity. This curve is then used to normalize the data: logIred/Igreen->log (Ired/Igreen) − $c(A)$, where $c(A)$ is the fit. This is equivalent to multiplying the green channel intensity (or dividing the red) by an intensity-dependent normalization constant $k(A)$ where $\log[(k(A)] = c(A)$. Optimal normalized data should be horizontal and centered.

Clustering

The hierarchical clustering algorithm was used to evaluate the association of protein profiles and individuals under study taking the Pearson correlation (p) as the

distance metric (distance = $(1 - p)/2$) and the average linkage method.[20] The application of bootstrapping techniques to hierarchical clustering using the *R* statistical package estimated the robustness of the associations of the relationships among the samples and protein expression patterns.[21] Resampling and replacing on the original data are performed to generate replicated datasets. This is done 1000 times, and for each new dataset a clustering tree is generated. A consensus tree is then constructed from the bootstrap trees where at each node the number of times that subgroup appeared in the 1000 trees. The closer this number is to 1000 the more robust that subgroup is. The sensitivity and specificity of antibody arrays was evaluated based on the distribution of patients within the clusters.[22] The sensitivity of the antibody arrays was tested on the patients with bladder cancer and the specificity of the antibody arrays was tested on the controls comprising benign and malignant conditions. Cystoscopic evaluation together with the histopathological report was considered as the gold standard for classification of bladder tumors. Inclusion of controls was based on clinical reports.

Antibody Ranking

The Wilcoxon test, together with the Bonferroni correction, was applied to rank the most discriminatory antibodies between patients with bladder cancer and controls and between high-risk and low-risk patients with bladder cancer.[22] This analysis was done based on the classification given by the hierarchical clustering together with bootstrapping analysis. Additionally, the association of protein profiles with outcome was evaluated using the log-rank test.[22]

Validation Analyses

Tissue Arrays and Immunohistochemistry

Three different tissue microarrays were constructed in the Division of Molecular Pathology.[23] They included a total of 173 primary transitional cell carcinomas (TCCs) of the bladder, belonging to patients recruited at Memorial Sloan-Kettering Cancer Center under institutional review board approved protocols. A total of 40 superficial and 64 invasive TCC tumors were analyzed in the first two microarrays. These tumors corresponded to 24 grade 1, 8 grade 2, and 82 grade 3 lesions. The third tissue microarray comprised 69 bladder primary high-grade TCC cases with annotated follow-up, including two superficial and 67 invasive lesions. Protein expression patterns of c-met were assessed at the microanatomical level on these tissue microarrays by immunohistochemistry using standard avidin-biotin immunoperoxidase procedures. We used a mouse monoclonal antibody against c-met (C-28) from Santa Cruz Biotechnology (Santa Cruz, CA) at 1:50 dilution.

Statistical Analysis

The consensus value of the representative cores from each tumor sample arrayed was used for statistical analyses. All TCCs ($n = 173$) were used for the analysis of association of c-met with histopathological stage and tumor grade, using the nonparametric Wilcoxon-Mann-Whitney and Kruskall-Wallis tests.[22] The association of c-met with outcome was evaluated using the 69 TCC cases contained in the third tissue microarray for which follow up was available. The log-rank test was used for this purpose taking the cutoff of 50% expression.[24] Overall survival time was defined as the months elapsed between transurethral resection or cystectomy and death from disease (or the last follow-up date). Patients who were alive at the last follow-up or who were lost to follow-up were censored. Survival curves were plotted using standard Kaplan-Meier methodology.[22] Associations between proteins' expression given by different techniques were analyzed using Kendall's tau b test using the SPSS statistical package (version 10.0).[22]

Enzyme Immunoassays

As part of the target verification strategy, enzyme immunoassays were used to validate the performance of the antibodies used on the immobilized arrays. The ratios given of several antigens including prostatic-specific antigen (PSA), p53, and epidermal growth factor given by the first printing set of antibody arrays in 35 men were compared to protein levels measured using enzyme immunoassays. Serum total PSA was measured by electrochemiluminescence using an autoanalyzer Elecsys 2010 (Roche Diagnostics, Berkeley, CA). Commercial immunoassays were also used to measure p53 (Zymed, South San Francisco, CA) and epidermal growth factor (American Laboratory Products Co., Windham, NH). Associations between proteins' expression given by different techniques were analyzed using Kendall's tau b test using the SPSS statistical package (version 10.0).[22]

Results

The transcript profiles of 28 normal urothelium and paired bladder tumors obtained by cystectomy were first analyzed using the U133A oligonucleotide microarrays. A *t*-test analysis allowed identification of differentially expressed genes between bladder tumors and their paired normal urothelium. The analysis revealed the relevance of cell cycle-related genes and members of p53 and RB networks in tumorigenesis and tumor progression. Antibodies related to these genes and the pathways where they are involved, especially to cell cycle, were selected for designing bladder cancer antibody arrays. Table 2 shows known differentially expressed genes given by these analyses that were considered for antibody selection for the protein profiling using antibody arrays. The complete list of probes differentially expressed between these pairs of normal urothelium and bladder tumors comprising both known genes and expressed se-

Table 2. Known Genes Differentially Expressed between Bladder Tumors and Paired Normal Urothelium Given by Gene Expression Analyses Using the U133A Gene Chip

Probe	*P* value	Gene name	Related antibodies
209875_s_at	1.27E-09	*Nephropontin 1 (osteopontin)*	Osteopontin, oncostatin, IL-11
204170_s_at	8.14E-08	*CDC28 protein kinase 2*	Cdc47, cdk2, cdk4, chk2, cyclin D 1–3/E/G
208694_at	1.09E-07	*DNA-dependent protein kinase (DNA-PKcs)*	DNA-PKcs
219295_s_at	1.45E-07	Procollagen C (PCOLCE2)	Collagen I, collagen IV, collagen X
220677_s_at	2.32E-07	Metalloprotease D1-thrombospondin 1	MMP9, MMP1, thrombospondin, cathepsins
208079_s_at	2.57E-07	*Serinethreonine kinase 6 (STK6)*	Akt, pakt, pten, waf1
823_at	2.69E-07	CX3C chemokine precursor	CXCR4, fusin, CCR7
214435_x_at	2.91E-07	*v-ral oncogene A (ras related) (RALA)*	v-h-ras, chras
216333_x_at	8.73E-07	Tenascin XA	Tenascin, gelsolin, zyxin
205489_at	9.13E-07	Crystallin, mu	Aquaporin, inhibin, filamin
202192_s_at	1.32E-06	Growth arrest-specific 7 (GAS7),	p53, mdm2, p16, p27, p33ING1, p63, p73, Rb
202350_s_at	1.33E-06	Matrilin 2	Fibronectin, laminin, claudin
209543_s_at	1.60E-06	CD34	Endostatin
202666_s_at	1.64E-06	BAF53	bax, bcl2, bcl6, bclx, caspases 1/3/ 9
206742_at	1.69E-06	c-fos-induced growth factor (VEGFD)	VEGF, VEGFR, PVEGFR
203661_s_at	1.96E-06	Tropomodulin	Tropomyosin
203362_s_at	1.99E-06	*MAD2*	MAD2, E2F1, E2F4
202796_at	2.78E-06	Synaptopodin	Synaptopodin, myopodin
209409_at	2.80E-06	Growth factor receptor-bound protein 10	akt, pakt
221928_at	3.29E-06	B-cell growth factor	CD106, cd117, cd147, cd29, IL-11
201202_at	3.69E-06	*Proliferating cell nuclear antigen (PCNA)*	PCNA
219728_at	4.05E-06	Titin immunoglobulin (myotilin) (TTID)	Ezrin, 14-3-3
212843_at	4.21E-06	Neural cell adhesion molecule 1	N-cadherin, E-cadherin
214612_x_at	4.22E-06	*Melanoma antigen, family A, 6*	MAGE1
221747_at	4.58E-06	Tensin	Tubulin, 14-3-3, desmin
220420_at	4.70E-06	ERGL protein	EGF, EGFR, CerbB3, CerbB4, FGF2, FGFR
219213_at	6.31E-06	Vascular endothelial junction (VE-JAM)	VEGF, VEGFR, PVEGFR, angiostatin
218486_at	6.40E-06	TGF-β inducible early growth response 2	wnt 5a, smad4, cadherins

Antibodies against these genes, and targets related to the pathways in which these top known genes are involved, were selected to design targeted antibody arrays for bladder cancer. Antibodies against other tumor antigens identified in previous gene profiling analyses were also considered for the design of antibody arrays (Supplementary Table 2 at *http://ajp.amjpathol.org*). Overexpressed genes in bladder tumors are in italics.

quenced tags are provided in Supplementary Table 3 at *http://ajp.amjpathol.org*.

The initial protein profiling analyses using antibody arrays focused on technical issues, including design of replicate spots along each antibody array and optimization of normalization criteria (Materials and Methods). Two independent sets of antibody arrays were designed to evaluate the diagnostic and prognostic abilities of protein profiling of serum specimens from patients with bladder cancer and controls. The first set contained 254 antibodies against 183 antigenic targets and was used to evaluate differences between serum samples from bladder cancer patients ($n = 37$) and controls ($n = 58$). The associations of protein expression profiles considering all targets and cases under analysis was evaluated by means of hierarchical clustering and bootstrapping techniques. Two main clusters discriminating patients with bladder tumors and control individuals were identified (Figure 2). Protein profiles distinguished bladder cancer patients from controls with a sensitivity of 89.2% and a specificity of 96.5%. Strikingly, the correct classification rate was 93.7%. A *t*-test analysis was performed to rank the antibodies with better discriminatory properties between patients with bladder cancer and controls (Table 3). Antibodies included on the arrays were selected based on the comparison of the gene profiles of the bladder tumors versus paired normal urothelium, as well as other targets reported to be differentially expressed in bladder cancer (Figure 1). The presence of several antibodies against different epitopes for certain targets (Supplementary Table 2 at *http://ajp.amjpathol.org*) among these top ranked antibodies provided reliability to the protein profiles given by these antibody arrays. The lack of associations found between noncancer and cancer conditions among the individuals included in the control group supports the specificity of the protein profiles measured with this antibody array for bladder cancer.

The second set of antibody arrays contained a reduced number of antibodies ($n = 144$) against 114 antigenic targets. The antibodies were selected on the basis of having been expressed in at least 50% of the patients with bladder cancer in the first printing set. This strategy served to validate the reproducibility of the protein profiles obtained in the first set. Moreover, this independent analysis, performed taking only the patients with bladder cancer ($n = 37$), served to evaluate the prognostic ability of antibody arrays. The protein profiling results given by the second set of antibody arrays classified bladder cancer patients based on their clinical outcome. The application of bootstrapping on unsupervised hierarchical clustering identified two clusters associated with overall survival that were defined as high-risk and low-risk clusters. More specifically, 7 of 11 patients in the high-risk cluster died of bladder cancer (DOD), whereas only 6 of 26 cases in the low-risk group died of the disease (Figure 3A). Protein profiling of serum samples distinguished two subgroups of bladder cancer patients with different overall survival (log rank, $P = 0.0479$) (Figure 3B). Patients belonging to the high-risk cluster showed shorter survival as compared to those included into the low-risk cluster. The median follow-up time of the 37

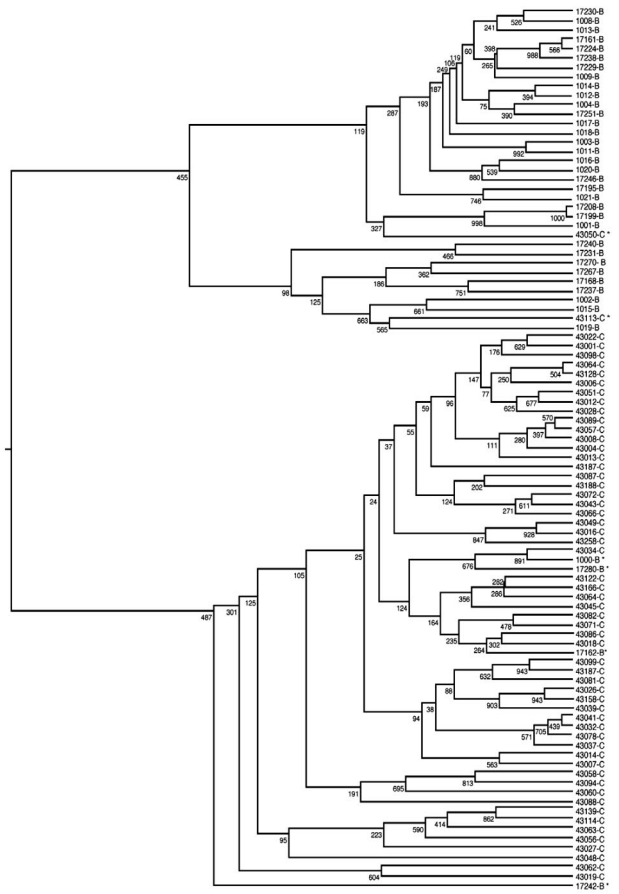

Figure 2. Diagnostic properties of bladder cancer-targeted antibody arrays: first set of antibody arrays. Hierarchical clustering and bootstrapping of the first set of antibody arrays on sera specimens of patients with bladder cancer (**B**) and controls (**C**) ($n = 95$). Two main clusters segregated bladder cancer patients (cluster 1) from controls (cluster 2). *Misclassified cases.

bladder tumors was 23.5 months. It is noteworthy that the definition of these overall survival risk clusters was based on unsupervised hierarchical clustering, analysis that could not have been predicted based solely on known histopathological data. An additional *t*-test analysis was performed to rank the antibodies with better discriminatory properties between groups of patients belonging to the high- and low-risk clusters (Figure 3C). Strikingly, the protein profiles measured using bladder cancer targeted antibody arrays predicted clinical outcome among patients with bladder tumors. This is of clinical relevance because selection of patients more likely to present a more aggressive behavior would benefit from earlier individualized therapeutic intervention. Overall, transcript profiling comprehensively identified differentially expressed targets involved in cell cycle, growth, differentiation, senescence, and apoptotic functional networks. In line with these observations, top ranked antibodies identifying patients with bladder cancer from controls and risk groups were associated with loss of cell-cycle regulation (eg, p33ING1, cyclin D2), angiogenic response (eg, angiostatin, epidermal growth factor), or metastatic risk (eg, osteopontin, CXCR4).[25–27] Alterations of these pathways in bladder cancer progression have already been identified both through low-throughput molecular analyses and high-throughput gene profiling.[4–7,25,28,29] However, this represents the first study in which they are detected in the serum of bladder cancer patients.

The use of immunohistochemical analyses on tissue arrays is a standard means for validating transcript profiling studies. Moreover, it can be used to evaluate the association with tumor progression of proteins detected in the serum using antibody arrays. c-Met was chosen as one of the top serum proteins at segregating patients with bladder cancer from controls, showing this ability using two different antibodies printed on the antibody arrays. An independent set of well-characterized bladder tumors contained in tissue arrays ($n = 173$) served to delineate associations of c-met with clinicopathological variables using an antibody printed on the arrays. The expression levels of c-met were increased in invasive bladder tumors when compared to superficial lesions (Figure 4, A and B), reaching a significant association with histopathological stage and grade (Mann-Whitney, $P < 0.001$). Moreover, when a subgroup of 69 bladder tumors with available follow-up (median follow-up time, 36.0 months) was studied, patients displaying a higher expression of c-met died earlier (Figure 4C), and c-met expression was inversely correlated with overall survival (log rank, $P = 0.0444$). Other top discriminatory targets identified by serum protein profiling with bladder cancer, such as p33ING1, cyclins, and p53/Rb were already found associated with bladder cancer staging using immunohistochemical analyses using other cohorts or even these specimens.[6,7,28,29] Thus, immunohistochemical analyses represent complementary proof of principle experiments supporting the association of the serum protein profiles with protein alterations present in bladder tumors. Serum verification analyses included enzyme immunoassays on the serum specimens used for antibody microarrays. Concordant results were obtained when comparing the Cy3/Cy5 ratios of the first printing set of antibody arrays and enzyme immunoassay measurements. Significant correlations were observed for antigens such as epidermal growth factor (Kendall's tau correlation coefficient $r = 0.836$, $P < 0.0005$, $n = 35$), p53 (Kendall's tau $r = 0.824$, $P < 0.0005$, $n = 35$), and PSA (Kendall's tau $r = 0.804$, $P < 0.0005$, $n = 35$). Because PSA is one of the most accepted tumor markers in sera, this observation supported the reliability of protein profiling using antibody microarrays to correlate with serum standard biomarkers used for the clinical management of patients with cancer.

Discussion

The present study provides experimental evidence for the concept of using comprehensive tumor-specific biomarkers, by designing serum antibody arrays with targets differentially expressed in tumor specimens. Our results involve the use of serum protein profiling using targeted antibody microarrays specific for bladder cancer. The diagnostic abilities of protein profiling was initially performed on a first set of antibody arrays of 95 individuals comprising 37 patients with bladder cancer versus 58 controls. Importantly, protein profiling using these arrays successfully classified these two groups of individuals. A second set of antibody arrays focused on

Table 3. Top Ranked Antibodies Given by *t*-Test with the Most Discriminatory Properties to Segregate Bladder Cancer Patients from Controls

Antibody	Median cluster 1	Low range cluster 1	High range cluster 1	Median cluster 2	Low range cluster 2	High range cluster 2	*P* value
P33[1]	0.826771	−0.49619	4.704792	−0.69084	−2.27589	0.426	1.75E-21
IGG	0.778061	−1.02332	1.737738	−0.93929	−2.02767	0.518749	2.37E-19
ING1 CAB2[2]	−0.27167	−1.13245	0.485344	0.648367	−0.27763	1.861992	1.68E-18
E2F1 KH95 X	0.797734	−0.20314	2.273721	−0.43583	−2.01484	0.651882	3.30E-17
MET[2]	−0.598	−1.22674	1.060364	0.737387	−0.10018	1.429981	9.91E-17
A-IGA	1.07672	−0.17224	2.490754	−0.69416	−2.81228	2.059515	4.12E-16
ANGIOSTATIN	−0.24633	−0.9245	0.01194	0.017752	−0.45796	0.531193	1.12E-15
P63	−0.41828	−1.29076	0.308914	0.59604	−0.13249	1.451259	1.27E-15
ING1 CAB1[3]	−0.21494	−1.62127	0.452645	0.733282	−0.21271	1.963475	3.45E-15
A-IGM	0.799694	−2.2127	2.351215	−0.9489	−2.45375	0.620283	3.79E-15
MYOPODIN POOL AB1[3]	−0.14967	−1.16578	0.65802	0.73157	0.161324	1.628577	5.30E-15
19A211	−0.39529	−1.61263	0.76497	0.825256	0.091706	1.549763	6.64E-15
MTOR	−0.01431	−1.14206	0.728449	0.735319	0.246535	1.146621	1.60E-14
AP	1.346902	0.165885	2.903737	−0.00828	−1.26579	1.702453	2.85E-14
CXCR4	0.909902	−0.44466	1.790037	−0.38897	−1.89068	0.4052	5.49E-14
PSMA	1.466896	0.332234	3.192551	0.51111	−0.56503	1.966371	5.77E-14
MBD4	0.40681	−0.5677	1.303953	−0.90249	−2.89054	0.178085	6.87E-14
P53 AB3[4]	−0.06891	−0.64831	0.540043	0.505892	−0.10045	1.190142	2.02E-13
GELSOLIN	−0.33377	−2.79074	0.418368	0.355435	−0.42967	0.952798	7.81E-13
ONCOSTATIN M	0.672198	−0.28454	2.420902	−0.42375	−1.83057	0.753288	1.14E-12
LORICRIN	1.260256	−0.29838	2.651619	0.204449	−1.01212	1.399649	2.17E-12
A-VWF	0.316879	−2.13944	1.283145	−1.09845	−3.47999	0.683231	1.71E-11
CYCLIN D3	−0.0431	−0.46053	0.256379	0.1884	−0.28743	0.794247	3.10E-11
P16 AB1	0.071554	−0.30888	0.343288	0.31965	0.013749	0.643293	3.21E-11
M344	−0.41037	−0.77563	0.227785	0.541791	−1.75731	1.062779	3.51E-11
P53 AB7[4]	1.444611	0.543643	2.715114	0.35859	−0.49112	1.202275	8.20E-11
MYOPODIN E4 SII[3]	−0.17941	−1.51166	0.658046	0.727054	0.023417	1.794615	1.89E-10
FIBRONECTIN	0.601534	−0.69041	1.578943	−0.45372	−1.56445	0.774634	6.18E-10
BCLX	−0.23407	−0.71985	0.191889	0.045308	−0.53314	0.673991	1.01E-09
P27 F8	−0.1297	−0.53201	0.122159	0.138531	−0.43014	0.902908	2.05E-09
P73-GC15[5]	−0.35135	−1.10601	−0.08387	0.576424	−0.54297	1.141379	3.59E-09
JUN B	0.169028	−0.25008	1.546242	0.636509	0.15479	1.925726	3.73E-09
TENASCIN	0.038928	−0.91521	0.715137	0.421738	−0.0258	1.596131	7.26E-09
CDC47	−0.3739	−1.09284	0.193805	0.654337	0.006941	1.398326	8.21E-09
UNDERP-RB[6]	−0.36102	−1.13369	0.450144	0.309256	−0.41507	0.696602	1.23E-08
RB AB5[6]	−0.10479	−0.92598	0.67124	0.145206	−0.61442	0.454957	1.35E-08
P53 D01[4]	0.028437	−1.30839	0.477156	−0.15389	−0.9495	0.115623	3.58E-08
MDM2	0.407921	−0.40645	0.62535	0.155375	−0.1594	0.710622	7.12E-08
EGFR AB1	0.06739	−0.21791	0.422699	0.267661	0.036985	0.565729	7.39E-08
DCC AB1	−0.08547	−0.62073	1.23731	0.289029	−0.23351	1.184069	7.46E-08
P73[5]	−0.21908	−0.7613	−0.05317	−0.07383	−0.44075	0.23943	1.28E-07
OSTEOPONTIN	1.328634	0.409319	2.528758	0.002609	−0.73064	0.910498	2.91E-07
GM-CSFRA	−0.39609	−1.00925	0.310717	0.843076	0.085592	1.18916	3.16E-07
CHRAS AB1	−0.03102	−0.43555	0.279991	0.25065	−0.36412	0.813168	6.41E-07
P15 K18	0.14	−0.24913	0.291191	0.231645	−0.01652	0.467993	1.53E-06
WAF-1	−0.22513	−0.51163	−0.02052	−0.03459	−0.35525	0.425689	1.57E-06
MET[2]	−0.43963	−0.94327	−0.10253	−0.28762	−0.54402	0.06584	1.74E-06
PTEN[7]	−0.20643	−0.65829	0.021414	−0.37574	−1.18579	0.157148	2.42E-06
ATM	0.223235	−0.27717	0.478131	0.066096	−0.14388	0.364873	4.32E-06
CASPASE 9	−0.28539	−0.87945	0.517739	0.186016	−1.48712	0.799394	5.77E-06
CASPASE 3	−2.35187	−3.14027	−1.22445	−3.07539	−4.38301	−1.0822	5.95E-06
MYCTAG	0.78603	−0.21782	1.755111	0.033657	−1.16158	1.350244	6.30E-06
CHK2-CDS1	0.37568	−1.22071	1.228619	0.684497	−0.27405	1.293549	9.42E-06
CYCLIN D2 AB3[8]	−0.00904	−0.29109	0.296092	0.142004	−0.16629	0.502896	1.43E-05
CYCLIN D2[8]	−0.14103	−0.77588	−0.02671	−0.04973	−0.45911	0.211228	1.54E-05
P73A[5]	−0.19381	−0.62518	0.058046	−0.08685	−0.29868	0.459354	2.44E-05
PGLYP	0.803061	0.165194	2.72136	0.108987	−0.78411	0.99351	2.61E-05
CDK4 C22	0.030764	−0.37958	0.347106	0.135806	−0.12567	0.529905	7.87E-05
GLI-I	−0.27537	−0.58884	0.009222	−0.13689	−0.46988	0.164566	8.66E-05
PPTEN SER,THR[7]	−0.38769	−1.25892	−0.08534	0.501946	0.099984	1.154755	0.000175

The presence of different antibodies (superscript) against the same targets provides reliability of their classificatory relevance.

144 of the 254 antibodies analyzed on the first printing set of antibody arrays. All serum samples belonging to patients with bladder cancer used on the fist printing set of arrays were repeated in the second set of experiments. Antibody reduction was based on the presence of detectable signal in the first printing set of arrays in at least 50% of the sera from patients with bladder cancer. This set of experiments served to provide reproducibility of the

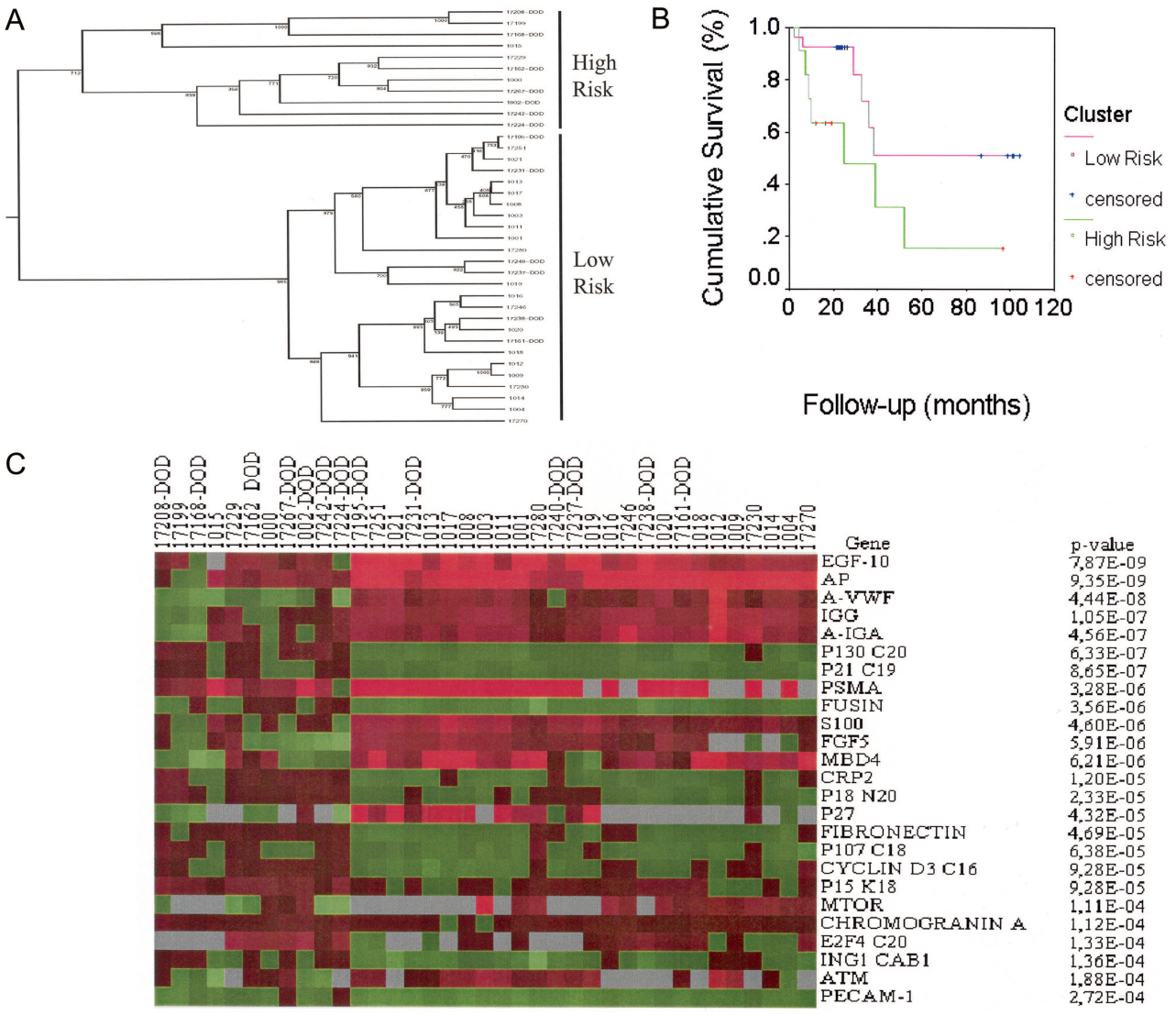

Figure 3. Prognostic properties of bladder cancer-targeted antibody arrays: second set of antibody arrays. **A:** Hierarchical clustering of the protein profiles given by the second set of antibody arrays measured on the sera specimens belonging to patients with bladder cancer ($n = 37$). Two clusters grouping high-risk and low-risk bladder tumors based on overall survival segregated patients with bladder cancer based on their clinical outcome. The majority of bladder cancer patients who died of the disease (DOD) were grouped into the high-risk cluster whereas the majority of patients grouped within the low-risk cluster showed no evidence of disease during follow-up. **B:** Kaplan-Meier survival analysis of patients with bladder tumors stratified by protein profiles measured using the second set of antibody arrays. Protein profiles measured using bladder cancer-targeted antibody arrays were significantly associated with overall survival ($P = 0.0479$). **C:** Top ranked antibodies given by *t*-test at stratifying bladder cancer patients into these clusters related to overall survival status.

detection of these proteins using the same serum specimens. Moreover, they allowed testing the prognostic abilities of antibody arrays, given the follow-up availability of the 37 patients with bladder cancer. Strikingly, serum protein profiling using bladder cancer-targeted antibody arrays not only diagnosed patients with bladder tumors but also stratified them based on their clinical outcome.

The analyses of both sets were initially performed by unsupervised hierarchical clustering, a standard means of displaying relationships among protein profiles and individuals. Bootstrapping of all of the antibodies under evaluation represents an appropriate tool to estimate the confidence of these associations without over-fitting the data. Later, a supervised approach was performed to identify top discriminatory markers by means of *t*-test analyses (Table 3 and Figure 4) and evaluation of the diagnostic and prognostic abilities using standard statistical methods. These two independent analyses robustly validated the target selection strategy. Furthermore, they served to test the reproducibility of the technology, especially on the most discriminatory antibodies, as well as the robustness of the clinical classificatory diagnostic ability of these targets.

The experimental design included two issues that we believe have been critical in obtaining the results presented above. First, the selection of the antibodies based on targets known to be differentially expressed in bladder tumors has increased the ability of antibody arrays to detect antigenic patterns characteristic of patients with bladder cancer. The concordance between gene and protein expression is proven by detecting more than 90% of selected targets in the serum of at least one bladder cancer patient in the first printing set. All of the 114

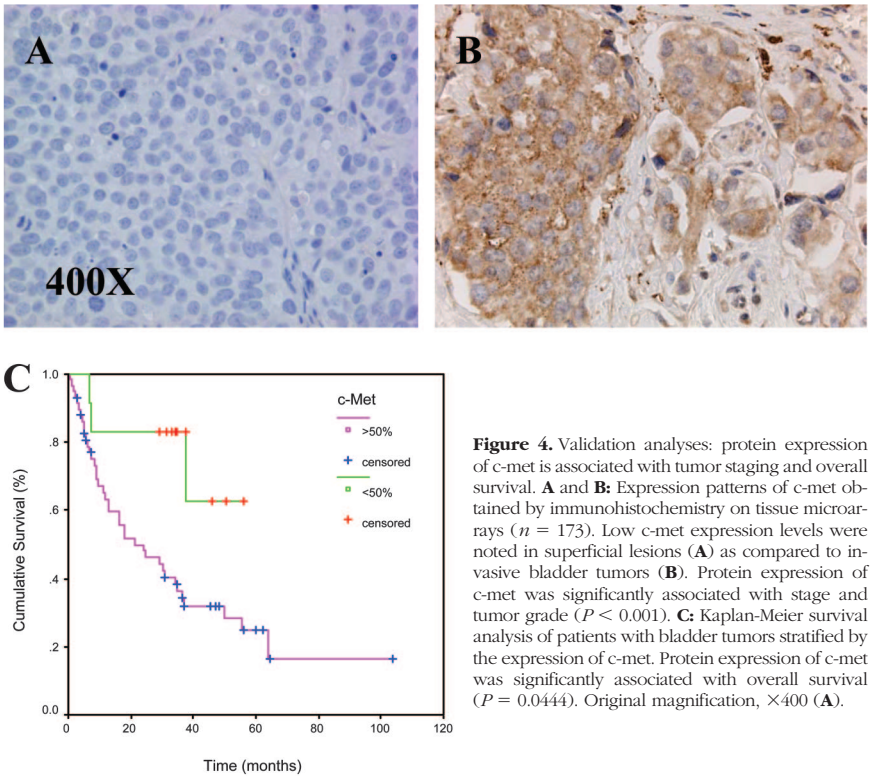

Figure 4. Validation analyses: protein expression of c-met is associated with tumor staging and overall survival. **A** and **B:** Expression patterns of c-met obtained by immunohistochemistry on tissue microarrays ($n = 173$). Low c-met expression levels were noted in superficial lesions (**A**) as compared to invasive bladder tumors (**B**). Protein expression of c-met was significantly associated with stage and tumor grade ($P < 0.001$). **C:** Kaplan-Meier survival analysis of patients with bladder tumors stratified by the expression of c-met. Protein expression of c-met was significantly associated with overall survival ($P = 0.0444$). Original magnification, ×400 (**A**).

targets used in the second set of experiments were detected in more than 50% of the bladder cancer patients. Although many of the reported serum biomarkers were cell cycle-related molecules rather than bladder-specific antigens, control specimens belonging to patients without the disease and presenting other malignancies showed differential protein patterns in these targets. This observation suggests that specific cell-cycle signatures might be associated to certain tumor types and points out the significance of the approach undertaken for the study of patients with carcinomas other than bladder cancer. Detecting the majority of the selected targets in the serum of patients with bladder cancer on both sets of experiments supports the relevance of the selection approach at improving the specificity of antibody arrays for bladder cancer stratification. Second, the use of rolling circle amplification has increased the sensitivity as compared to direct labeling protocols.

Antibody arrays represent a high-throughput feasible biomarker discovery platform with potential applicability in cancer diagnostics.[30,31] Construction and application of antibody arrays involves laborious complex processes and technical challenges.[31] In this study, verification of the antibody-antigen binding specificity was approached using various antibodies against the same target. Several antibodies against certain targets were found among the top discriminatory antibodies (Table 3). This reproducibility in the biological specificity of the antibody-antigen binding represents a robust validation by itself regardless of the differential affinity of each antibody. Detected products in serum from bladder cancer patients could have been synthesized either in tumor cells or in normal urothelium counterparts and stroma cells, as part of the host response mechanisms. In both instances, they could reach the blood stream by either secretion/excretion or by degradative mechanisms including apoptosis or tissue necrosis. Neovascularization, autocrine, and paracrine tumor cell networks are collaborative factors facilitating these antigenic targets reaching the blood stream, allowing multiplexed detection using antibody arrays. Because antibodies can detect antigenic epitopes carried by molecules other than proteins, antibody arrays should not be defined just as a proteomics exercise, rather a much more diversified attempt to elucidate the complexity associated with tumorigenesis and tumor progression. Although differences observed based on site of origin of samples have been reported using mass spectrometry proteomic techniques, our results show no differences associated with the site of origin of the samples. Care was taken on using the same collection serum tubes under the same centrifugation and storage protocols.

The results presented in this study reveal the clinical relevance of protein profiling using targeted antibody arrays as a comprehensive tool for the management of patients with bladder cancer. To date, no serum biomarker has shown clinical efficacy for the diagnosis of the disease using this noninvasive approach. The design of our study was not centered on specific bladder cancer stages or tumor grades. Further studies with specimens adequately selected will serve to generate an adequate multivariate model for addressing more specific clinical questions. The present study represents a proof of principle exercise for the use of the technology in bladder cancer diagnostics. Standardization of the internal reference and run-to-run controls will lead to surveillance of patients with cancer using this high-throughput approach. It is conceivable that in the

near future, individual biomarkers of diagnostic and/or prognostic utility will be progressively changed by the use of clusters of genes or protein profiles characteristic of bladder cancer. This strategy of evaluating numerous molecular endpoints in a test battery approach may provide, besides a more comprehensive approach, a more exhaustive outcome assessment. Identification of patients more likely to present severe clinical behavior will lead to more aggressive individualized therapeutic intervention. This innovative approach represents not only a clinically relevant exercise but also a potential paradigm shift in cancer patient management.

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