Identification of Differentially Expressed Proteins in Direct Expressed Prostatic Secretions of Men with Organ-confined Versus Extracapsular Prostate Cancer

Yunee Kim†, Vladimir Ignatchenko§, Cindy Q. Yao‡¶, Irina Kalatskaya¶, Julius O. Nyalwidhe¶**, Raymond S. Lance**‡‡, Anthony O. Gramolini§§, Dean A. Troyer‖**, Lincoln D. Stein¶, Paul C. Boutros¶, Jeffrey A. Medin‡¶¶, O. John Semmes**, Richard R. Drake***, and Thomas Kisling‡§*

Current protocols for the screening of prostate cancer cannot accurately discriminate clinically indolent tumors from more aggressive ones. One reliable indicator of outcome has been the determination of organ-confined versus nonorgan-confined disease but even this determination is often only made following prostatectomy. This underscores the need to explore alternate avenues to enhance outcome prediction of prostate cancer patients. Fluids that are proximal to the prostate, such as expressed prostatic secretions (EPS), are attractive sources of potential prostate cancer biomarkers as these fluids likely bathe the tumor. Direct-EPS samples from 16 individuals with extracapsular (n = 8) or organ-confined (n = 8) prostate cancer were used as a discovery cohort, and were analyzed in duplicate by a nine-step MudPIT on a LTQ-Orbitrap XL mass spectrometer. A total of 624 unique proteins were identified by at least two unique peptides with a 0.2% false discovery rate. A semiquantitative spectral counting algorithm identified 133 significantly differentially expressed proteins in the discovery cohort. Integrative data mining prioritized 14 candidates, including two known prostate cancer biomarkers: prostate-specific antigen and prostatic acid phosphatase, which were significantly elevated in the direct-EPS from the organ-confined cancer group. These and five other candidates (SFN, MME, PARK7, TIMP1, and TGM4) were verified by Western blotting in an independent set of direct-EPS from patients with biochemically recurrent disease (n = 5) versus patients with no evidence of recurrence upon follow-up (n = 10). Lastly, we performed proof-of-concept SRM-MS-based relative quantification of the five candidates using unpurified heavy isotope-labeled synthetic peptides spiked into pools of EPS-urines from men with extracapsular and organ-confined prostate tumors. This study represents the first efforts to define the direct-EPS proteome from two major subclasses of prostate cancer using shotgun proteomics and verification in EPS-urine by SRM-MS. Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.017889, 1870–1884, 2012.

Prostate cancer is the most common malignancy to affect men in the Western world, but only 15–20% of these men will present with aggressive, lethal disease (1, 2) whereas the majority of patients will die of other causes. Although the implementation of large-scale screening for prostate cancer using serum prostate-specific antigen (PSA) has dramatically improved early detection of disease, unnecessary biopsies and patient overtreatment are becoming increasingly evident (2, 3). Consequently, there has been a shift in emphasis away from detection of prostate cancer and toward identification of lethal disease. Currently, Gleason grading is considered to be one of the best outcome predictors; however, patients with Gleason 7 tumors are in the clinical “gray zone,” whereby the predictive ability of Gleason grading is mixed (4, 5). A recent study constructed a 157-gene signature based on the comparison of Gleason score ≤6 and ≥8 patients, and could show that their panel could predict lethality in the cohort of Gleason 7 patients (5). Nonetheless, the development and large-scale implementation of prognostic markers of prostate cancer has been hampered by numerous factors owing, in part, to the heterogeneous and multifocal nature of the...
disease (6). Although the widely used Gleason grading system attempts to control for heterogeneity of the glands and multifocality of cancerous lesions by summing the 2–3 most commonly observed histological patterns via inspection of multiple (typically 8–12) core biopsies, cancerous foci are still often missed (2, 6) providing only partial information that can lead to imprecise diagnoses and prognoses. Pathologic staging remains the gold standard for disease staging and risk assessment (7, 8); however, this process lacks timeliness in discriminating organ-confined from extracapsular disease. Indeed, one-third of individuals with nonorgan-confined disease are identified only after surgery (9). Furthermore, ~35% of men treated with radical prostatectomy with curative intent subsequently develop biochemical recurrence (10–13) and the mean time from surgery to recurrence is 3.5 years (4). Significant risk factors for time to prostate-specific mortality following biochemical recurrence after radical prostatectomy are PSA doubling time, pathological Gleason score, and time from surgery to biochemical recurrence (4). Estimates place the percent of lethal cases at 20–25% of all patients that show biochemical recurrence, suggesting that nearly 75–80% of patients in this group may be overtreated (14).

There is an emerging trend toward recruitment of men with perceived low-risk disease to an “active surveillance” monitoring approach. This is based on the supposition that most prostate cancers are slow growing, and that the more aggressive forms can be identified during a period of observation with little increased risk of death. Although a consensus may not exist for defining the disease stage where active surveillance is warranted, there is considerable agreement that men who have a PSA level less than 10 ng/ml, impalpable disease (clinical stage T1c) and only 1 biopsy core out of 12 or more that show Gleason 6 cancer are most likely to harbor indolent disease (15). Even so, these candidates for active surveillance will still contain individuals who will have disease progression and die from their cancer. Thus, despite efforts to recruit individuals to active surveillance protocols, overtreatment of prostate cancer is fueled by the lack of reliable means to accurately discriminate between men with clinically indolent prostate cancer from those with more aggressive disease (16, 17). This inability to accurately predict prostate cancer aggressiveness based solely on standard clinicopathologic features clearly underscores the need to explore the ability of additional biomarkers to enhance outcome prediction for men with prostate cancer. Furthermore, it is important to acknowledge that a single biomarker alone is unlikely to have sufficient prognostic power; rather, the integration of a panel of biomarkers hold the promise for improved prostate cancer detection and prognosis (2).

Fluids that are proximal to the prostate are attractive sources of potential prostate cancer biomarkers (2, 18), as they house secreted proteins and sloughed cells that provide a presumably more comprehensive assessment of the organ and extent of disease. Further, fluids such as urine are clinically favorable for their ease of collection, the volume and frequency at which they can be obtained, and their adaptability to routine clinical assays. Prostate-proximal fluids include seminal fluid, semen, and expressed prostatic secretions (EPS)1. Here, we focus on the analysis of EPS as our biological specimen, using direct-EPS samples for the discovery of candidate prognostic biomarkers and both direct-EPS and pooled EPS-urines derived from independent sets of patients for candidate biomarker verification. Direct-EPS is a prostatic fluid that is collected from patients undergoing prostatectomy by massaging the organ and expelling 0.5–1 ml of the fluid just prior to surgical removal. It was chosen as our discovery fluid as it is expected to house prostate-secreted proteins at a higher concentration and purity, and we have developed a workflow for the in-depth proteomic analysis of this fluid (19). Following discovery proteomics in 16 clinically stratified direct-EPS samples, verification studies were performed using independent sample sets of direct-EPS. Next, we focused our attention on the verification and quantitative analysis of candidate proteins in pooled EPS-urines. Before EPS-urine collection, men undergo digital rectal examination (DRE), often as part of a routine procedure, which causes direct-EPS to be expelled from the prostate and subsequently voided in urine. Because EPS-urine can be collected with substantial ease and in greater volumes and frequencies than direct-EPS, much attention has been paid to this fluid as a valuable resource of prostate cancer biomarkers amenable to routine clinical analysis. Following the recent FDA approval of the EPS-urine assay for prostate cancer gene 3 (PCA3), standardized clinical collection protocols will be widely implemented and easier access to this fluid is expected. Moreover, 1 The abbreviations used are: EPS, expressed prostatic secretions; ADAMTS1, a disintegrin and metalloproteinase with thrombospondin motifs 1; ANXA, annexin A; Ob, previously proposed cancer biomarker; EC, extracapsular; ELISA, enzyme-linked immunosorbent assay; FDR, false discovery rate; Fl, functional interaction; fmol, femtomole; GO, gene ontology; GSTP1, glutathione S-transferase P; HPLC, high performance liquid chromatography; iTRAQ, isotopic tags for relative and absolute quantitation; KCRB, creatine kinase B-type; LC-MS/MS, liquid chromatography and tandem mass spectrometry; MME, membrane metallo-endopeptidase; MudPIT, multi-dimensional protein identification technology; MYH9, myosin 9; NR, nonrecurrent; OC, organ-confined; PAP, prostatic acid phosphatase; PARK7, Parkinson protein 7; PCA3, prostate cancer antigen 3; PEDF, pigment ocularismotin protein; PTEN, phosphatase and tensin homolog; PTPRS, receptor-type tyrosine-protein phosphatase S; R, biochemically recurrent; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SFN, stratatin; SISCAPA, stable isotope standards and capture by anti-peptide antibodies; SP, signal peptide; SPEG, solid-phase extraction of glycopeptides; SRM-MS, selected reaction monitoring-mass spectrometry; SSR, sequence specific retention; TF, transferrin; MMP, matrix metalloproteinase; TGM4, transglutaminase 4; TIMP1, tissue inhibitor of metalloproteinase 1; TMD, transmembrane domain.
we have recently identified a number of prostate-enriched proteins in EPS-urine by comparing its proteome to a urine background (20).

The present study used multidimensional protein identification technology (MudPIT) coupled with bioinformatics to first catalog and comparatively analyze the direct-EPS proteomes from a small cohort of patients with extracapsular versus organ-confined prostate cancers. A semiquantitative algorithm based on spectral counts (QSpec) (21) and an integrative data mining strategy led to the selection of a number of putative biomarkers that were verified by Western blotting in direct-EPS. Lastly, to demonstrate accurate quantitative measurements of verified candidates in EPS-urine, a pilot study utilizing SRM-MS was undertaken as a proof-of-concept.

**EXPERIMENTAL PROCEDURES**

**EPS Sample Properties**—Direct-EPS samples were obtained from patients following informed consent and use of Institutional Review Board approved protocols at Urology of Virginia and Eastern Virginia Medical School between 2007 and 2009 and the Research Ethics Review Board at the University Health Network. Sample collection and handling has been previously described (19). Briefly, an aggressive prostate massage was performed in confirmed prostate cancer patients under anesthesia prior to undergoing prostatectomy to collect 0.2–1 ml of fluid. Samples were diluted with saline to 5 ml and stored on ice for a maximum of 1 h. Particulates were sedimented by low-speed centrifugation and the resulting supernatants were aliquoted to 1 ml and stored at −80 °C. The clinical data linked to the patients enrolled in the study are outlined in Tables I and II.

**Organ-confined disease** is defined as the condition of patients with tumors not beyond stage T2c that are margin, seminal vesicle and...
lymph node negative. Extracapsular disease is differentiated from organ-confined by evidence of margin involvement, transcapsular extension, or positive for tumor in seminal vesicles or lymph node. Biochemical recurrence is defined as patients with rising serum PSA values over 0.2 ng/ml at 3 months or longer post-prostatectomy. EPS-urines were collected from men reporting to the clinic for a prostate biopsy via prostate massage with three strokes on each side of the median sulcus of the prostate followed by collection of voided urine (10–20 ml), as previously described (18). Pools of EPS-urines were derived from 17 patients classified as having organ-confined prostate cancer and 17 from nonorgan-confined, extracapsular cancers using 1 ml per patient sample (Table III).

Tumor grades, staging, prostate volumes and percent of cancer positive needle biopsy cores were determined using standard pathological procedures. Patient information was recorded, including demographics, medical history, pathology results, and risk factors, and stored in a Caisis database system. All personal information or identifiers beyond diagnosis and laboratory results were not available to the laboratory investigators.

### Table III

**Clinical information of patient samples used to generate EPS-urine pools**

| Sample # | serum PSA (ng/ml) | EPS PSA (µg/ml) | T-stage | Gleason |
|----------|------------------|----------------|---------|---------|
| **Extracapsular tumor group** | | | | |
| 1        | 5.8              | 67             | T2b     | 4 + 5   |
| 2        | 24.5             | 28             | T2a     | 5 + 4   |
| 3        | 500              | 12             | N/A     | N/A     |
| 4        | 3.2              | 75             | T2a     | 4 + 4   |
| 5        | 0.1              | 1              | T3a     | 4 + 4   |
| 6        | 0.2              | 12             | T2b     | 4 + 5   |
| 7        | 65.7             | 77             | T3b     | 5 + 5   |
| 8        | 2                | 2              | T3b     | 5 + 5   |
| 9        | 4.6              | 150            | T1c     | 3 + 5   |
| 10       | 7.2              | 160            | T1c     | 4 + 4   |
| 11       | 3.1              | 120            | T1c     | 4 + 4   |
| 12       | 9.7              | 24             | T2a     | 3 + 5   |
| 13       | 3.8              | 14             | T1c     | 3 + 5   |
| 14       | 6.3              | 100            | T2b     | 4 + 5   |
| 15       | 7.6              | 120            | N/A     | 4 + 5   |
| 16       | 69.7             | 2              | T1c     | 4 + 5   |
| 17       | 59.1             | 11             | T3b     | 5 + 4   |
| **Organ-confined tumor group** | | | | |
| 1        | 4.6              | 200            | T1c     | 3 + 3   |
| 2        | 3.6              | 19             | T1c     | 3 + 3   |
| 3        | 5.2              | 165            | T1c     | 3 + 3   |
| 4        | 8.5              | 33             | T1c     | N/A     |
| 5        | 5.7              | 5              | T1c     | 3 + 3   |
| 6        | 12.7             | 25             | T1c     | N/A     |
| 7        | 6.6              | 90             | T1c     | 3 + 3   |
| 8        | 4.3              | 1              | N/A     | N/A     |
| 9        | 1                | 1              | T1c     | 3 + 3   |
| 10       | 5.9              | 160            | T1c     | 3 + 3   |
| 11       | 2.7              | 39             | T1c     | 3 + 3   |
| 12       | 9.8              | 115            | T1c     | 3 + 3   |
| 13       | 6.6              | 4              | N/A     | 3 + 3   |
| 14       | 10               | 63             | T1c     | 3 + 3   |
| 15       | 3.6              | 140            | T1c     | 3 + 3   |
| 16       | 3.3              | 35             | T1c     | 3 + 3   |
| 17       | 5.6              | 30             | T1c     | 3 + 3   |
Prognostic Prostate Cancer Markers in Prostatic Fluids

Immuno-assays in Prostatic Fluids—The Human Neprilysin (MME) DuoSet® ELISA Development System (R&D Systems, Minneapolis, MN) was used to measure MME in direct-EPS. Total protein concentration corresponding to 140 μg was obtained from each sample. Samples and standard were diluted in 1% BSA in PBS, pH 7.4 and assayed in duplicate following the protocol outlined by the manufacturer. PSA and PAP ELISAs were performed as previously described (18, 41).

For Western blotting, anti-SFN (ab14123), anti-MME (ab951), anti-PARK7 (ab11251), anti-PSA (ab46976) were obtained from Abcam (Cambridge, MA), anti-TGM4 (sc55791) was from Santa Cruz Biotechnology (Santa Cruz, CA), anti-TIMP1 (RP1T1) antibody was from Triple Point Biologics (Forest Grove, OR). Direct-EPS from patients with biochemical recurrence and no evidence of recurrence were quantified by Bradford assay and a corresponding 30 μg of total protein were resolved on Criterion 10–20% Tris-HCl SDS-PAGE gels (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). The membranes were blocked with 5% milk in TBS-T for 1 h at room temperature. Primary antibodies were diluted in blocking solution and incubated at 4 °C overnight, washed with TBS-T, and incubated with secondary antibody diluted in blocking solution for 1 h at room temperature. Signals were developed using SuperSignal West Pico or secondary antibody diluted in blocking solution for 1 h at room temperature. Signals were developed using SuperSignal West Pico or secondary antibody diluted in blocking solution for 1 h at room temperature, washed with TBS-T, and incubated with secondary antibody diluted in blocking solution for 1 h at room temperature. Signals were developed using SuperSignal West Pico or secondary antibody diluted in blocking solution for 1 h at room temperature, washed with TBS-T, and incubated with secondary antibody diluted in blocking solution for 1 h at room temperature. Signals were developed using SuperSignal West Pico or secondary antibody diluted in blocking solution for 1 h at room temperature, washed with TBS-T, and incubated with secondary antibody diluted in blocking solution for 1 h at room temperature.

Relative Quantification by SRM-MS in EPS-urines—For quantification by SRM-MS, EPS-urine pools were prepared as follows: concentrated EPS-urines were precipitated in 100% methanol (1:20 v/v ratio) overnight at −20 °C. Protein pellets were recovered by centrifuging at 15,000 g of total protein were resolved on 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and assayed as described above.

Statistical Analysis—Following candidate protein selection, we verified their differential expression by Western blot densitometry and/or ELISA. To determine if the protein levels differ between organ-confined tumor groups and extracapsular tumor groups, we applied the Mann-Whitney U test to assess if the difference is statistically significant. A p value ≤0.05 was considered significant.

SRM-MS Development—A spectral library was built using the Skyline software tool (version 0.5 and higher) (42) from the LTQ Orbitrap XL data obtained from the 16 direct-EPS samples. All spectra had scores that passed a stringent peptide score as determined by X!Tandem target decoy search criteria (i.e. 0.5% decoy spectral matches). In addition, two publicly available consensus libraries were used: NIST_human_QTOF (v 3.0 05/24/2011) and GPM human_ipi_cmp_20. Protein sequences were converted to FASTA format and uploaded into Skyline for the prediction of signature peptides. Peptides were chosen based on previously reported specifications (43): predicted tryptic digests of 7–30 amino acids in length and containing zero possible missed cleavage sites or cysteine and methionine residues, and matching the spectral libraries. Four to 6 of the most intense y-ions with a precursor charge of ±2 and a fragment ion charge of ±1 were selected from the libraries, resulting in several possible peptides for each of the candidates. The GPM (http://www.gpm.org) was used to narrow down the list of peptides to those that have been observed experimentally with a high number of ±2 charge states. Furthermore, no possible missed cleavage sites within 3-amino acids before or after the peptide, no acidic residues on cleavage sites, and uniqueness to the gene (43) were additional requirements. Other favorable conditions included no possible post-translational modification sites, no N-terminal Q, E, no W, and SSR hydrophobicity 5–30 (43–45). Based on these criteria, 21 signature peptides for six candidates (SFN, MME, PARK7, TIMP1, TGM4, and PSA) were chosen for SRM-MS experimentation (Supplemental Table S4).

Lyophilized, unpurified 13C- and 15N-labeled arginine and lysine peptides (SpikeTides_L™, JPT Peptide Technologies, Berlin, Germany) were obtained in a 96-well format and solubilized in 80% 0.1 M ammonium bicarbonate and 20% acetonitrile. A heavy isotope labeled peptide standard stock containing all 21 SpikeTides_L™ at equal concentrations was made for subsequent experimentation.

For optimization studies, the heavy peptide standard stock corresponding to 500 fmol on-column was added to BSA digests and automatically loaded from a 96-well microparticle autosampler using the EASY-nLC system. Analysis was performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) interfaced with the EASY-nLC system (Proxeon Biosystems, Odense, Denmark), as described previously (19, 22). A 40 min HPLC gradient running at a flow rate of 400 nl/min was used. The gradient conditions were as follows: starting with 100% buffer A (water/0.1% formic acid) followed by an increase to 60% buffer B (acetonitrile/0.1% formic acid) for 36 min, followed by a steep increase to 95% buffer B for 2 min, and finally to 100% buffer B for 2 min. For compound optimization, the thermo TSQ Tune software (Thermo Fisher Scientific, San Jose, CA) was used. The XCalibur data system (Thermo Fisher Scientific, San Jose, CA) was used to input the following SRM-MS method parameters: one scan event over a 40 min method time at a fixed scan width of 0.02 m/z, scan time of 0.010 s, 0.2 fwhm Q1 and 0.7 fwhm Q3 resolution, and data collection in profile mode. For each peptide, 3–4 most intense transitions for each eluting peptide were selected and their accurate retention times were recorded using Skyline (42). These transitions were grouped into two separate methods to maintain a cycle time of 1 s.

Integrative Data Mining—Differentially expressed proteins were compared with transcriptomic profiles based on gene mapping using an in-house relational database with mRNA microarrays for 22 normal tissues, including the prostate, available through Human Gene Atlas (34). The median mRNA expression level of each gene product was calculated across all 22 tissues and compared with the level of expression in the prostate alone, as detected by a total of 218 probe IDs. In this way, genes that demonstrate a propensity toward elevated expression in the prostate compared with other tissues at the transcriptomic level were derived. To assess whether the differentially expressed proteins had predicted transmembrane domains (TMDs) and signal peptides (SPs), their assigned UniProt IDs were uploaded onto the Protein Center bioinformatics software (Thermo Fisher Scientific), which uses TMAP (http://emboss.sourceforge.net/apps/emboss/apps/tmap.html) and PrediSi (http://www.predi si.de/) tools for predictions. The Human Protein Atlas (35) was used to determine whether a given candidate was previously proposed as a cancer biomarker (Cb; from the Plasma Proteome Institute) and to assess the immunoreactivity of antibodies directed against candidate proteins in normal (nonneoplastic and morphologically normal) and cancerous prostate tissue sections. Comparisons across various proteomics data sets (direct-EPS, seminal plasma, urine, cell lines (19, 41), bladder, prostate tissues, including the prostate, available through Human Gene Atlas (34)) were additional requirements. Other favorable conditions included no possible post-translational modification sites, no N-terminal Q, E, no W, and SSR hydrophobicity 5–30 (43–45). Based on these criteria, 21 signature peptides for six candidates (SFN, MME, PARK7, TIMP1, TGM4, and PSA) were chosen for SRM-MS experimentation (Supplemental Table S4).
16,000 × g for 30 min at 4 °C. Two additional washes with methanol were performed to obtain clean protein pellets. The pellets were subsequently resolubilized in 50 μl of 8 M urea, 2 mM DTT, 50 mM Tris-HCl, pH 8.5, and incubated at 37 °C for 1 h. Carbamidomethylation was performed by incubating samples with 8 μM of iodoacetamide for 1 h at 37 °C in the dark. Samples were then diluted to −1.5 M urea using 100 mM ammonium bicarbonate, pH 8.5. Calcium chloride was added to a final concentration of 2 mM and the protein mixture was digested with 4 μg of trypsin at 37 °C overnight. The digested peptide mixture was purified with C18 Macrospin™ columns and concentrated by vacuum centrifugation and reconstituted to a volume of 100 μl with 5% acetonitrile, 0.1% formic acid. Samples were incubated at 37 °C for 30 min to allow peptides to go into solution and subsequently centrifuged at 16,000 × g for 30 min at 4 °C to remove any remaining debris.

For quantification in EPS-urine pools, a total of 1 μg of peptides on-column (as determined by the micro BCA assay) were spiked with 500 fmol on-column of the heavy isotope-labeled peptide standard stock and assayed following the same conditions as above. Each sample was assayed in four technical replicates (Supplemental Table S5). Total peak area values and area ratios (light/heavy peptide) were obtained from Skyline (42). Relative quantitative values are shown for each technical replicate and as the average ratio of light/heavy peptide obtained from Skyline (42). Relative quantitative values are shown for each technical replicate and as the average ratio of light/heavy peptide.

RESULTS

Comparative Proteomics Characterization of the Extracapsular and Organ-confined Prostate Cancer Direct-EPS Proteomes—An overview of the study approach is illustrated in Fig. 1. Direct-EPS derived from 16 individuals who were clinically stratified as having extracapsular (n = 8) or organ-confined (n = 8) prostate tumors (Table I) were digested in-solution and analyzed by a nine-step MudPIT on a LTQ-Orbitrap XL mass spectrometer, in duplicate (32 MudPITS). A total of 624 unique proteins were identified by at least two unique peptides with a 0.2% FDR (1 total reverse proteins) (Fig. 2A, Supplemental Fig. S1, Supplemental Tables S1 and S2). Among these, 78 proteins (13%) were only identified in the extracapsular group and 216 (34%) were only identified in the organ-confined group, while the majority (330 proteins, 53%) were shared by both (Fig. 2B). A substantial overlap of our data set with other published prostate-proximal and urine proteomic data sets was observed (Supplemental Fig. S2). Furthermore, a large proportion of proteins had at least one predicted TMD and signaling peptide sequences (Supplemental Fig. S3). Proteins in this fluid spanned a wide range of functional roles, as determined via Gene Ontology enrichment (Fig. 2C). A number of Gene Ontology terms were significantly enriched in both tumor groups. They were implicated in proteolysis, regulation of programmed cell death, and cellular proliferation in the biological process category, as well as peptidase activity and cytoskeletal protein binding in the molecular function category. Notably, many proteins were localized in the extracellular region, as indicated by their over-representation in Gene Ontology.

Semiquantitative Comparison of Extracapsular and Organ-confined Prostate Cancer Direct-EPS Fluids—To identify differentially expressed proteins in both patient groups we employed the QSpec algorithm (21). Only proteins with a FDR of ≤ 0.05 and at least a twofold change in normalized spectral abundance factor between the extracapsular and organ-confined groups for each protein were considered. This analysis resulted in 133 differentially expressed proteins for further consideration (Fig. 3A), and 100 (77%) of these were present in a FI network. Following extraction of these proteins from the FI network and the addition of linker genes, a proteomics-associated prostate cancer network consisting of 161 genes, 61 of which are linkers, was obtained. To decompose this network into smaller independent pieces and run pathway annotation for every cluster separately, we used network community analysis to automatically identify network modules that contain genes and their co-regulators that are involved in common biological processes with high probability. We identified five modules of at least 10 proteins and pathway enrichment analysis was done for each cluster separately. The first module was significantly enriched in the “Glycolysis/gluconeogenesis” pathway; the second one was mapped to the cell cycle-related process “G2/M transition”; the functional annotation of the third and fifth modules showed that they were significantly enriched in hemostasis processes like “Complement and coagulation cascades” and “Further platelet releasate” and others (Fig. 3B, Supplemental Fig. S4, and Supplemental Table S3). The fourth module was not significantly associated with any known processes or pathways.
Generation of Candidate Shortlist—To select proteins for verification in a small, independent cohort of direct-EPS samples, we performed a systematic data-mining strategy using in-house and publicly available resources to minimize the panel of 133 proteins to a refined verification set (Fig. 4). Proteins were ranked initially based on six features by assigning a \( H_1 \) value to a given protein with the following annotations (0 if the term could not be assigned): 1) present by mass spectrometry in six out of eight samples corresponding to the risk group that the candidate was found to be higher in by spectral counting; 2) overexpression of candidates genes in the prostate as demonstrated by at least a threefold above median mRNA expression in the normal prostate tissue compared with 22 different normal human tissues; 3) presence of predicted TMDs; 4) presence of predicted SP sequences; 5) cellular localization assignment to “cell surface” (GO: 0009986) and “extracellular” (GO:0005576) by Gene Ontology annotation; and 6) cancer-associated protein as available from the Human Protein Atlas database. The rationale for each of the key features is as follows: as individual biological specimens can have a high level of variation, we set the criteria that a candidate protein should have been detected in at least six out of eight samples in its associated risk group. This would minimize the inclusion of proteins that are overrepresented by large numbers of spectra in just a small number of samples. The integration of mRNA microarray profiles from normal prostate and other human tissues with our proteomic data set was performed with the rationale that the

Fig. 2. A, Average number of unique proteins identified in the duplicate MudPIT runs for each discovery direct-EPS sample. B, Venn diagram depicting the total number of proteins exclusively expressed in the extracapsular or organ-confined groups. C, Gene Ontology enrichment of the extracapsular (EC) and organ-confined (OC) direct-EPS.
most promising biomarker candidates would be preferentially expressed in the prostate, and thus may have an important functional role in the organ that contributes to the outcome of the patient. Microarray data was therefore solely used to select proteins with a likely tissue-selective expression pattern. We also took into consideration the presence of pre-
dicted TMDs and SPs, as well as Gene Ontology annotations linked to the cell surface and extracellular region as they are characteristics of secreted and plasma membrane proteins, found in many currently known biomarkers. Sixty proteins had at least three out of six features; 32 of which are cancer-associated proteins (46). Because of limitations in antibody availability and resources, a small subset of proteins (14 candidates, Table IV) were chosen for verification. Also included in this list were two well-defined prostate cancer biomarkers, PSA and PAP, their levels were also evaluated by ELISA in a different set of 14 direct-EPS samples. As shown in Fig. 5A and Supplemental Fig. S6, the PSA and PAP concentrations determined by ELISA and Western blotting yielded a similar distribution as the mass spectrometry analysis, with an elevated level of expression being observed in the organ-confined group compared with the extracapsular group. Further verification of the expression of a subset of the short-listed candidate proteins was carried out in new sets of EPS fluids.

**Verification of Differentially Expressed Candidates in Direct-EPS**—For the verification of the short-listed candidates, we chose a small cohort of direct-EPS samples that could be linked with clinical outcome information related to whether the patient had biochemical recurrence or no evidence of recurrence within a two-year period post-prostatectomy (Table II). Using these samples, the expression levels of the 14 proteins in the verification set were evaluated by Western blotting. Five out of the 14 the candidates were successfully verified to correlate with the discovery data (Fig. 5, Supplemental Fig. S5). These are: stratifin (SFN), membrane metallo-endopeptidase (MME), Parkinson protein 7 (PARK7), tissue inhibitor of metalloproteinase 1 (TIMP1), and transglutaminase 4 (TGM4).

For SFN, QSpec analysis of the discovery data in direct-EPS revealed that it was threefold higher in the organ-confined group with a FDR of $\alpha = 0.002$. In the biochemically recurrent and nonrecurrent samples, the level of SFN expression was significantly higher in the nonrecurrent group ($p = 0.03$). For MME, it was found to be twofold higher in the organ-confined proteome with a FDR of $\alpha = 0.002$. In the comparison between biochemically recurrent and nonrecurrent samples, MME expression was found to be significantly higher in expression in the nonrecurrent group compared with biochemically recurrent patients ($p = 0.05$). In addition, we analyzed extracapsular ($n = 23$) and organ-confined ($n = 19$) direct-EPS samples for the expression of MME using a commercially available ELISA, and found a similar trend in this new set of samples ($p = 0.07$) (Supplemental Fig. S5B). PARK7 had a threefold difference by QSpec with a FDR of $<0.002$, with higher expression being observed in the organ-confined group. Simi-

| Protein | Direction | mRNA overexpressed in prostate | TMD | SP | Present in 6 of 8 | Cellular localization | Cb | Rank |
|---------|-----------|-------------------------------|-----|----|-------------------|----------------------|----|------|
| ADAMTS1 | Down | ✓ | ✓✓ ✓ | Mem, cyto, extracellular | ✓ | 5 |
| MME | Down | ✓ | ✓✓ ✓ | Mem, extracellular | ✓ | 5 |
| PAP | Down | ✓ | ✓✓ ✓ | Mem, cyto, extracellular, vacuole | ✓ | 5 |
| PSA | Down | ✓ | ✓✓ ✓ | Cyto, nuc, extracellular, organelle lumen | ✓ | 5 |
| TGM4 | Down | ✓ | ✓✓ ✓ | Mem | ✓ | 5 |
| PEDF | Up | | ✓✓ ✓ | Cyto, extracellular, mito | ✓ | 4 |
| PTPRS | Down | ✓ | ✓✓ ✓ | Mem, extracellular | ✓ | 4 |
| SFN | Down | ✓ | ✓✓ ✓ | Cyto, nuc, extracellular | ✓ | 4 |
| TF | Up | ✓ | ✓✓ ✓ | Mem, endosome, cytop, extracellular, mito | ✓ | 4 |
| ANXA1 | Down | ✓ | ✓✓ ✓ | Mem, cyto, nuc, cytoskel | ✓ | 3 |
| KCRB | Down | ✓ | ✓✓ ✓ | Mem, cyto, mito | ✓ | 3 |
| MYH9 | Down | ✓ | ✓✓ ✓ | Mem, cyto, nuc, cytoskel | ✓ | 3 |
| PARK7 | Down | ✓ | ✓✓ ✓ | Cyto, extracellular, mito | ✓ | 3 |
| TIMP1 | Down | ✓ | ✓✓ ✓ | Cyto, extracellular, organelle lumen | ✓ | 3 |
larly, this protein was found to be significantly higher in the nonrecurrent group compared with the biochemically recurrent group ($p = 0.008$). Both proteins TIMP1 and TGM4 were threefold different in expression in the discovery sample set, whereby their expression was higher in the organ-confined group with a FDR of $<0.002$. Upon subsequent analysis in biochemically recurrent and nonrecurrent patient direct-EPS samples, a similar trend was observed with heightened expression in the nonrecurrent group for TIMP1 ($p = 0.099$) but not for TGM4 ($p = 0.80$).

**Verification of Differentially Expressed Candidates in EPS-urines**—A major aim of the current study was directed toward the detection of our candidates in a clinically accessible fluid that can be collected with minimal invasiveness. EPS-urine samples obtained during a DRE meet these criteria. Therefore, EPS-urine samples were obtained from patients about to undergo a prostate biopsy procedure who subsequently were diagnosed with pathology-confirmed extracapsular and organ-confined prostate tumors. Pools of EPS-urines from both groups were prepared (Table III) and used to assess the presence of the candidate proteins in this bio-fluid by Western blot and a multiplexed SRM-MS approach. All candidates were quantifiable in the EPS-urine with at least one proteotypic peptide by SRM-MS (Supplemental Figs. S7, S8, Supplemental Tables S4, S5). The overall expression levels of MME, PARK7, and TIMP1 coincided with the Western blot analyses in the same pools (Fig. 6A). For SFN, one of the two peptides representing SFN (NLLSVAYK) was found to be lower in the organ-confined tumor group, whereas the other (VLSSIEQK) was not quantifiable. Similarly, both TGM4 peptides were down-regulated in the organ-confined tumor group, which is not in line with the Western blot analyses. PSA expression was found to be similar between the organ-confined and extracapsular tumor groups as measured by two peptides: peptide HSQPWQVLVASR was higher in the organ-confined group by an average of 1.7-fold and LSEPAELT-
DAVK was by an average of 1.3-fold. This trend was consistent with Western blot analysis of these same pools. ELISA was also performed on the individual EPS-urines that comprise the pools, whereby a notable difference between the two groups were not apparent (Fig. 6B, Supplemental Fig. S6).

**DISCUSSION**

Emerging advances in proteomics and genomics has led to the identification of a vast number of biomarker candidates for a plethora of malignancies; however, few see clinical application (47, 48). The potential of serum as a source of protein biomarkers is hampered by its immense complexity (49); thus we and others have looked to organ-proximal fluids for the discovery and targeted verification of putative biomarkers of various conditions (19, 37, 39, 40, 43, 50–54). Here, we interrogated a prostate-proximal fluid, EPS, for the identification of candidate biomarkers of aggressive prostate cancer by analyzing a cohort of patients with extracapsular versus organ-confined disease phenotypes. We followed up with verification of these biomarkers to identify aggressive disease through additional analyses of a cohort of biochemically recurrent and nonrecurrent patient direct-EPS. Efforts to adapt urine as a source of biomarkers for prostate cancer have been made using various approaches. For instance, DNA markers such as GSTP1 hypermethylation (55), RNA markers such as PCA3 (56), and protein markers such as ANXA3 (57), matrix metalloproteinases (58), and urinary versus serum PSA (59, 60). In light of the clinical relevance of urine samples, we accessed EPS-urine for verification and potential future assay development of our candidate biomarkers.

**Fig. 6.** Verification of differential expression of candidates in EPS-urines pooled from patients with extracapsular (EC) or organ-confined (OC) prostate tumors. A, Five candidates were measured by Western blot and SRM-MS using heavy isotope-labeled peptide standards. Relative quantitative values are shown for each technical replicate and as the average ratio of light/heavy peptide in the organ-confined tumor group divided by the light/heavy peptide in the extracapsular tumor group ± standard deviation for each peptide. B, PSA measurements in individual EPS-urines from patients with extracapsular and organ-confined prostate tumors by ELISA (NS denotes insignificant p value). The same patient samples were pooled and assayed by Western blot for PSA as well as SRM-MS.
Our 624 EPS protein data set contained a number of previously proposed urinary prostate cancer biomarkers, including PSA, ANXA3, and matrix metalloproteinases (MMP7 and 9), all of which demonstrated differential expression between the extracapsular and organ-confined groups. Furthermore, a large proportion of the proteome of EPS was predicted to have TMDs and SP sequences that are characteristic of plasma membrane and secreted proteins, including our five candidates. MME, a cell-surface glycoprotein, has been proposed to have an inhibitory effect on prostate cancer cell growth and migration through its association with PTEN, PI-3 kinase (61) and its loss has been shown to be associated with PSA relapse (62). The observation in the current study was that this protein is significantly higher in expression in the organ-confined and nonrecurrent groups compared with the extracapsular and biochemically recurrent groups in direct-organ-confined and nonrecurrent groups compared with the extracapsular and biochemically recurrent groups in direct-EPS. In general, there was a trend of decreased expression of most proteins detected in the extracapsular groups relative to the organ-confined groups. However, at the protein network most proteins detected in the extracapsular groups relative to EPS. In general, there was a trend of decreased expression of extracapsular and biochemically recurrent groups in direct-organ-confined and nonrecurrent groups compared with the organ-confined and nonrecurrent groups compared with the extracapsular and biochemically recurrent groups in direct-EPS. In general, there was a trend of decreased expression of most proteins detected in the extracapsular groups relative to the organ-confined groups. However, at the protein network level, proteins in the complement and coagulation cascade were significantly higher in expression in the extracapsular tumor group. There are clearly a high abundance of immune system-related proteins identified in the EPS fluids, which is consistent with the chronic inflammation associated with development and progression of prostate cancer (63). Lymphocytes and macrophages present in the prostate are most often associated with this chronic inflammation, and less frequently plasma cells, eosinophils, and neutrophils (63, 64). The presence of tumor-associated macrophages have been reported as potential biomarkers of poor prognosis in prostate cancers (65, 66), and we have evidence that these macrophages can be detected by flow cytometry of the low speed cell pellets of EPS urine samples in a subset of prostate cancer patients (data not shown). A better understanding of the role of the many detectable biomarkers of immune system activity, like the members of the complement pathway overexpressed in nonorgan-confined EPS samples, is critical for improving diagnostic targeting of aggressive prostate cancers.

The complexity of the EPS proteome could be reduced using depletion columns, specifically targeted for high-abundance proteins such as albumin, before digestion. This method may facilitate the quantification of lower-abundance proteins although sample recovery may be a caveat if starting concentrations are low. Advances in nanoparticle technology are also showing promise in their application in proteomics to deepen proteome coverage and enhance recovery of low-abundance proteins. Nanoparticles, functionalized using high-affinity baits to selectively capture desired classes of proteins, have been shown to enrich otherwise, low-abundance proteins, by orders of magnitude (67–69). Yet other strategies focus on the analysis of subproteomes by extraction of desired organelles or exploiting post-translational modifications to selectively enrich for desired classes of proteins. For instance, glycosylation is the most common post-translational modification and N-linked glycosylation is particularly common in secreted or plasma membrane proteins (70). Enrichment of this class of proteins can be achieved by the selective capture of N-linked glycosites to a solid support via hydrazide chemistry followed by enzymatic release by peptide N-glycosidase F (71, 72), or various lectin affinity approaches. Quantification can be achieved by stable isotope labeling and tandem mass spectrometry. Zhao and colleagues have extended the technique of stable isotope labeling by amino acids in cell culture, by generating stable isotope labeled secretome standards for the quantification of soluble proteins in direct-EPS (52). Alternatively, more elegant techniques such as SISCAPA® (73, 74) can selectively enrich for target peptides using anti-peptide antibodies and coupled to SRM-MS for targeted quantification, albeit the pipeline from generation of monoclonal antibodies and their validation may be a significant limiting factor. Analyzing urinary sediments, which accounts for ~50% of the total protein content by weight (75), may offer interesting markers of prostate cancer that may be missed by solely investigating the soluble fraction. Mataija-Botelho et al. identified 60 proteins in the sediment of urine, many of which were also identified in the soluble fraction, suggesting that simply discarding the sediment may alter abundance ratios of different proteins (76). EPS fluids are also an abundant source of exosomes, a subfraction that is also being targeted for discovery of prostate cancer biomarkers (77, 78). Initial proteomic pilot studies of EPS-urine exosome fractions have indicated readily detectable and abundant proteins by mass spectrometry. This exosome fraction is a subcomponent of the proteins analyzed in the present study, and we are currently pursuing more detailed analyses of these EPS-derived exosomes from samples representative of the disease spectrum of prostate cancers.

Our pipeline attempts to bridge discovery proteomics in direct-EPS with validation in a more clinically obtainable fluid, EPS-urine. As such, we observed some discrepancies that can be attributed to differences in direct-EPS and EPS-urine samples, the translation of shotgun proteomics to targeted SRM-MS, and inherent challenges in SRM-MS quantification. In the current study, discrepancies in SFN and TGM4 by Western blotting and SRM-MS are reported. Although a moderate-good correlation between antibody-based assays (namely, ELISA) and targeted proteomics approaches have been reported (79–81), discordance between the two methods can be conceptualized by the differences in the targeting approaches. Therefore, it may be possible that the epitope for the SFN and TGM4 antibodies used in the current study may be modified thus resulting in inaccurate measurements, or the antibodies may be also targeting other isoforms of the proteins as both are members of larger 14-3-3 and transglutaminase protein families. The quantification of SFN and TGM4 thus remains inconclusive at this point of time.
Prognostic Prostate Cancer Markers in Prostatic Fluids

Recently, Hüttenhain and colleagues demonstrated a high-throughput pipeline for the development of SRM-MS assays for a large number of cancer-associated proteins in human urine and plasma (46). In their study, 408 proteins were detectable by SRM-MS in urine, of which 169 were previously undetected in the data sets from Human Protein Atlas and Adachi et al. (38). In another study, the serum and prostate glycoproteome of Pten-null and wild-type mice were comparatively assessed using label-free quantitative proteomics followed by SRM-MS of 39 protein orthologs in sera of prostate cancer patients and controls (82), demonstrating a useful platform for biomarker discovery. Similarly, the current study demonstrates the adaptation of a discovery-driven global proteomics experiment in a prostate-proximal fluid to a targeted quantitative assay in EPS-urine. Although a large proportion of the SRM-MS optimization process for peptides can be achieved in a simpler matrix, such as BSA, yeast, or cell lysate background, finer optimization in the highly complex clinical specimen is an important requirement. General caveats associated with protein biomarker studies in urine are low protein abundance, the presence of cellular material, high concentrations of salts and interfering substances such as urea, urobiolin and other metabolites, and intra- and interindividual variability (83). In our hands, initial optimization studies were performed in a BSA background, whereby lower concentrations of heavy isotope labeled peptide standards were easily identifiable by at least three transitions. However, once introduced to the EPS-urine background, we observed large amounts of ion suppression and interference of the heavy isotope-labeled peptide standards that were easily identifiable in BSA. Most peptides showed good linearity within 1–500 fmol on-column range but equimolar concentrations of peptides showed variable responses. Therefore, in the end we used a heavy peptide standard concentration of 500 fmol on-column, which allowed all peptides to be readily detectable in EPS-urine. Using heavy isotope-labeled peptide standards, we quantified PSA, SFN, MME, PARK7, TIMP1, and TGM4 in pooled EPS-urines of extracapsular and organ-confined prostate cancers by 1 or 2 peptides. The SRM-MS experiments presented in this proof-of-concept study primarily serves to demonstrate the applicability of EPS-urines to SRM-MS assay platforms and to support its value as an important clinical bio-fluid. By no means do pooled EPS-urines represent the heterogeneity of the patient population. The only way to verify the relevance of our proposed candidates would be to quantitatively measure them in large cohorts of individual EPS-urines and thus future work will be dedicated to extending the verification phase of the current study.

Acknowledgments—The views expressed do not necessarily reflect those of the OMohlTC.

* T.K. is supported through the Canadian Research Chairs Program. This work was supported in parts by grants from the Canadian Institute of Health Research (MOP-93772) to T.K., and in part by grants from the National Institutes of Health to R.R.D. (R01 CA135087, R21 CA137704) and O.J.S. (U01 CA085067). This research was funded in part by the Ontario Ministry of Health and Long Term Care.

This article contains supplemental Figs. S1 to S8 and Tables S1 to S5.

To whom correspondence may be addressed: Ontario Cancer Institute, TMDT Tower Room 9-807 101 College St., University Health Network, Toronto, ON M5R 2W7, Canada. Tel.: 416-581-7627; Fax: 416-581-7629; E-mail: Thomas.kislinger@utoronto.ca.

To whom correspondence may be addressed: Medical University of South Carolina, 173 Ashley Avenue, BSB 358 MSC 509, Charleston, SC 29425, USA. Tel.: 843-792-8975; Fax: 843-792-0481; E-mail: draker@musc.edu.

REFERENCES

1. Greene, K. L., Cowan, J. E., Cooperberg, M. R., Meng, M. V., DuChane, J., and Carroll, P. R. (2005) Who is the average patient presenting with prostate cancer? Urology 66, 76–82

2. Ploussard, G., and de la Taille, A. (2010) Urine biomarkers in prostate cancer. Nat. Rev. Urol. 7, 101–109

3. Schroder, F. H., Hugosson, J., Roobol, M. J., Tammela, T. L., Clatto, S., Nelem, V., Kwiatkowski, M., Lujan, M., Lilja, H., Zappa, M., Denis, L. J., Recker, F., Berenguer, A., Matttla, L., Bangma, C. H., Aus, G., Villers, A., Rebillard, X., van der Kwast, T., Blijenberg, B. G., Moss, S. M., de Koning, H. J., and Avounin, A. (2009) Screening and prostate-cancer mortality in a randomized European study. N. Engl. J. Med. 360, 1320–1328

4. Freedland, S. J., Humphreys, E. B., Mangold, L. A., Eisenberger, M., Dorey, F. J., Walsh, P. C., and Partin, A. W. (2005) Risk of prostate cancer-specific mortality following biochemical recurrence after radical prostatectomy. JAMA 294, 433–439

5. Penney, K. L., Sinnott, J. A., Fall, K., Pawitan, Y., Hoshida, Y., Kraft, P., Stark, J. R., Fiorentino, M., Perner, S., Finn, S., Calza, S., Flavin, R., Freedman, M. L., Settir, S., Sesso, H. D., Andersson, S. O., Martin, N., Kantoff, P. W., Johansson, J. E., Adami, H. O., Rubin, M. A., Loda, M., Golub, T. R., Andren, O., Stampfer, M. J., and Mucci, L. A. (2011) mRNA expression signature of Gleason grade predicts lethal prostate cancer. J. Clin. Oncol. 29, 2391–2396

6. Abate-Shen, C., and Shen, M. M. (2000) Molecular genetics of prostate cancer. Genes Dev. 14, 2410–2434

7. Chung, B. I., Tarin, T. V., Ferrari, M., and Brooks, J. D. (2011) Comparison of prostate cancer tumor volume and percent cancer in prediction of biochemical progression and prostate cancer survival. Urol. Oncol. 29, 314–318

8. D’Amico, A. V., Whittington, R., Malkowicz, S. B., Schultz, D., Kaplan, I., Beard, C. J., Tomaszewski, J. E., Renshaw, A. A., Loughlin, K. R., Richie, J. P., and Wein, A. (1998) Calculated prostate cancer volume greater than 4.0 cm3 identifies patients with localized prostate cancer who have a poor prognosis following radical prostatectomy or external-beam radiation therapy. J. Clin. Oncol. 16, 3094–3100

9. Yossepowitch, O., Eggheeser, S. E., Bianco, F. J., Jr., Carver, B. S., Serio, A., Scardino, P. T., and Eastham, J. A. (2007) Radical prostatectomy for clinically localized, high risk prostate cancer: critical analysis of risk assessment methods. J. Urol. 178, 493–499; discussion 499

10. Han, M., Partin, A. W., Pound, C. R., Epstein, J. I., and Walsh, P. C. (2001) Long-term biochemical disease-free and cancer-specific survival following anatomic radical retropubic prostatectomy. The 15-year Johns Hopkins experience. Urol. Clin. North Am. 28, 555–565

11. Roehl, K. A., Han, M., Ramos, C. G., Antenor, J. A., and Catalona, W. J. (2004) Cancer progression and survival rates following anatomical radical retropubic prostatectomy in 3,478 consecutive patients: long-term results. J. Urol. 172, 910–914

12. Hull, G. W., Rabbani, F., Abbas, F., Wheeler, T. M., Kattan, M. W., and Scardino, P. T. (2002) Cancer control with radical prostatectomy alone in 1,000 consecutive patients. J. Urol. 167, 528–534

13. Amling, C. L., Blute, M. L., Bergstrahl, E. J., Seay, T. M., Stezak, J., and Zinc, H. (2000) Long-term hazard of progression after radical prosa-
Molecular & Cellular Proteomics 11.12

Huang da, W., Sherman, B. T., and Lempicki, R. A. (2009) Systematic and bioinformatic analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57

Huang da, W., Sherman, B. T., and Lempicki, R. A. (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 37, 1–13

Wu, G., Feng, X., and Stein, L. (2010) A human functional protein interaction network and its application to cancer data analysis. Genome Biol. 11, R63

Gross, J. L., and Yellen, J. (1999) Graph theory and its applications. CRC Press, Boca Raton, Fla.

Girvan, M., and Newman, M. E. (2002) Community structure in social and biological networks. Proc. Natl. Acad. Sci. U.S.A. 99, 7821–7826

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504

Boorjian, S. A., Thompson, R. H., Tolleyson, M. K., Rangel, L. J., Bergstrahl, E. J., Blute, M. L., and Karnes, R. J. (2011) Long-term risk of clinical progression after biochemical recurrence following radical prostatectomy: the impact of time from surgery to recurrence. Eur. Urol. 59, 637–649

Stephenson, A. J., and Kattan, M. W. (2006) Nomograms for prostate cancer. BJU Int. 98, 39–46

Chodak, G. W., and Warren, K. S. (2006) Watchful waiting for prostate cancer: a review article. Prostate Cancer Prostatic Dis. 9, 25–29

Klotz, L. (2008) Active surveillance for prostate cancer: trials and tribulations. World J. Urol. 26, 437–442

Druce, R. R., White, K. Y., Fullerton, T. W., Fullerton, E., Clements, P., and Davies, L. (2010) Identifying patterns associated with invasive behavior by ovarian cancer cells using multidimensional protein identification technology (MudPIT). Proteomics 10, 941–953

Boorjian, S. A., Thompson, R. H., Tollefson, M. K., Rangel, L. J., Bergstralh, E., Rinsky, R., Smith, L., Sethi, A., Carapito, C., Heding, E., and Kristensen, K. (2012) Reproducible quantification of cancer-associated proteins in body fluids using targeted proteomics. Sci. Transl. Med. 4, 142–194

Anderson, N. L., and Anderson, N. G. (2002) The human plasma proteome: history, character, and diagnostic prospects. Mol. Cell. Proteomics 1, 845–867

Paulovich, A. G., Whiteaker, J. R., Hoofnagle, A. N., and Wang, P. (2008) The interface between biomarker discovery and clinical validation: The tar pit of the protein biomarker pipeline. Proteomics Clin. Appl. 2, 1386–1402

Rifai, N., Gillette, M. A., and Carr, S. A. (2006) Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nat. Biotechnol. 24, 971–983

Dowling, P., Woramal, R., Meadey, P., Henry, M., Curnan, A., and Clines, M. (2008) Analysis of the saliva proteome from patients with head and neck squamous cell carcinoma reveals differences in abundance levels of proteins associated with tumour progression and metastasis. J. Proteome Res. 7, 168–175

Hu, S., Yu, T., Xie, Y., Yang, Y., Li, Y., Zhou, X., Tsung, S., Loo, R. R., Loo, J. R., and Wong, D. T. (2007) Discovery of oral fluid biomarkers for human oral cancer by mass spectrometry. Cancer Genomics Proteomics
4, 55–64
52. Zhao, T., Zeng, X., Bateman, N. W., Sun, M., Teng, P. N., Bigbee, W. L., Dhir, R., Nelson, J. B., Conrads, T. P., and Hood, B. L. (2012) Relative quantitation of proteins in expressed prostate secretions with a stable isotope labeled secretome standard. *J. Proteome Res.* 11, 1089–1099
53. Addona, T. A., Shi, X., Keshishian, H., Mani, D. R., Burgessa, M., Gillette, M. A., Clauser, K. R., Shen, D., Lewis, G. D., Farnell, L. A., Fifer, M. A., Sabatine, M. S., Gerszen, R. E., and Carr, S. A. (2011) A pipeline that integrates the discovery and verification of plasma protein biomarkers reveals candidate markers for cardiovascular disease. *Nat. Biotechnol.* 29, 635–643
54. Whiteaker, J. R., Lin, C., Kennedy, J., Hou, L., Trute, M., Sokol, I., Yan, P., Schoenherr, R. M., Zhao, L., Votyavich, U. J., Kelly-Spratt, K. S., Krasnoselsky, A., Gafken, P. R., Hogan, J. M., Jones, L. A., Wang, P., Amon, L., Chodosh, L. A., Nelson, P. S., McIntosh, M. W., Kemp, C. J., and Paulovich, A. G. (2011) A targeted proteomics-based pipeline for verification of biomarkers in plasma. *Nat. Biotechnol.* 29, 625–634
55. Woodson, K. O., O’Reilly, K. J., Hanson, J. C., Nelson, D., Walk, E. L., and Tangrea, J. A. (2008) The usefulness of the detection of GSTP1 methylation in urine as a biomarker in the diagnosis of prostate cancer. *J. Urol.* 178, 508–511; discussion 511–502
56. Hessel, D., Klein Gunnewiek, J. M., van Oort, I., Karthaus, H. F., van Leenders, G. J., van Balken, B., Kiemeney, L. A., Witjes, J. A., and Schalken, J. A. (2003) DD3(PCA3)-based molecular urine analysis for the diagnosis of prostate cancer. *Eur. Urol.* 44, 8–15; discussion 15–16
57. Schostak, M., Schwall, G. P., Poznanovic, S., Groebe, K., Muller, M., Messinger, D., Miller, K., Krause, H., Pelzer, A., Horninger, W., Klocker, H., Hennenlotter, J., Feyerabend, S., Stenzl, A., and Schrattenthal, A. (2009) Annexin A3 in urine: a highly specific noninvasive marker for prostate cancer early detection. *J. Urol.* 181, 343–353
58. Roy, R., Louis, G., Loughlin, K. R., Wiederschain, D., Kilroy, S. M., Lamb, L., Chodosh, L. A., Nelson, P. S., McIntosh, M. W., Kemp, C. J., and Paulovich, A. G. (2011) A targeted proteomics-based pipeline for verification of biomarkers in plasma. *Nat. Biotechnol.* 29, 625–634
59. Anderson, N. L., Jackson, A. M., Haines, L. R., Hardie, D. B., Olafson, R. W., and Pearson, T. W. (2004) Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISIAPA). *J. Proteome Res.* 3, 235–244
60. Zhang, H., Li, X. J., Martin, D. B., and Aebersold, R. (2003) Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat. Biotechnol.* 21, 660–666
61. Heijde, D., Hoste, H., Saeys, W., Delaere, K., Vanhaecke, S., Vanhoegaerden, E., Vanhoegaerden, E., De Vos, K., Pauwels, R., Hens, P., and Cleghorn, J. (2003) An integrated methodology combining isoelectric focusing, Western blotting and mass spectrometry for the identification of potential serum biomarkers for breast cancer. *Eur. J. Cancer.* 39, 2105–2115
62. Gannon, P. O., Poisson, A. O., Delvoye, N., Lapointe, R., Mess-Masson, A. M., and Saad, F. (2009) Characterization of the intra-prostatic immune cell infiltration in androgen-deprived prostate cancer patients. *J. Immunol. Methods* 348, 9–17
63. Fredolini, C., Mealli, F., Luchini, A., Zhou, W., Russo, P., Ross, M., Pataranut, A., Tamburro, D., Gambara, G., Ornstein, D., Odicino, F., Ragnoli, M., Ravaggi, A., Novelli, F., Collura, D., D’Urso, L., Muto, G., Belluce, C., Pecorelli, S., Liotta, L., and Petricoin, E. F., 3rd (2010) Investigation of the ovarian and prostate cancer peptidome for candidate early detection markers using a novel nanoparticle biomarker capture technology. *AAPS J.* 12, 504–518
64. Cima, I., Schiess, R., Wild, P., Koni, M., Schuffler, P., Lange, V., Picotti, P., Ossola, R., Templeton, A., Schubert, O., Fuchs, T., Leipoldt, T., Wyler, S., Zehetner, J., Jochem, W., Buhmann, J., Cerny, T., Moh, C., Gillessen, S., Aebersold, R., and Krek, W. (2011) Cancer genetics-guided discovery of serum biomarker signatures for diagnosis and prognosis of prostate cancer. *Proc. Natl. Acad. Sci. U.S.A.* 108, 3342–3347
65. Thongboonkerd, V. (2007) Practical points in urinary proteomics. *J. Proteome Res.* 6, 3881–3890