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Proteomics and
Protein Markers

Discovery of Candidate Tumor Markers for Prostate Cancer via Proteomic Analysis of Cell Culture–Conditioned Medium

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Objective: **Prostate-specific antigen measurement, widely used for early detection of prostate cancer (CaP), suffers from low specificity. Additional tumor markers are needed for the early detection of clinically relevant CaP. Our objective was to perform a qualitative proteomic analysis of conditioned medium (CM) from the CaP cell line PC3(AR)$_6$.**

Methods: **We used a roller bottle culture system to culture the PC3(AR)$_6$ cell line in chemically defined serum-free medium for 14 days. By using strong anion-exchange chromatography, we fractionated the CM and trypsinized the fractions. The tryptic peptides were further fractionated by reversed-phase C-18 chromatography before being subjected to electrospray ionization tandem mass spectrometry. We used MASCOT software to search the mass spectra generated and organized identified proteins based on their genome ontology classification of cellular location. We used an immunoassay to measure a newly identified secreted protein, Mac-2BP, and kallikreins 5, 6, and 11 in serum samples from CaP patients and healthy men.**

Results: **We classified 262 proteins according to cellular location; the sample was found to contain a significant proportion of secreted (23%) and membrane (16%) proteins. In a proportion of cancer patients compared with healthy men, we determined by ELISA that serum concentrations of a novel candidate biomarker Mac-2BP were increased.**

Conclusions: **These identified proteins, and possibly many others found in the CM, may have utility as novel CaP biomarkers.**

Prostate cancer (CaP)[5] is the most common malignancy in men and the 2nd leading cause of cancer-related deaths *(1)*. Early diagnosis of cancer improves clinical outcomes, but detection methods for clinically relevant preclinical CaP are limited *(2)*. Tumor markers can be used to detect cancer, determine prognosis, or monitor treatment *(3)*. The established CaP tumor marker prostate-specific antigen (PSA) has low diagnostic specificity *(4)*; increased concentrations are also seen in benign prostatic hyperplasia and prostatitis *(5)*. The detection limit and specificity of the PSA test for CaP have been improved by modifications such as measuring PSA concentrations over time (PSA velocity) and measuring the ratio of free to total PSA or human kallikrein (hK) 2 in addition to PSA *(6–8)*. Other methods for detecting CaP are not highly specific for CaP and are uncomfortable to patients *(9)*. Serum proteomic profiling has emerged as a new method for detecting CaP *(10, 11)* but has not been adequately validated *(12, 13)*.

Determining the clinical significance of a prostate tumor is a major concern of CaP testing *(14)*. An autopsy study of men who died of other causes revealed CaP or precursor lesions in 29% of men 30 to 40 years of age and in 64% of those >60 years of age *(15)*. Because treatments for CaP (androgen ablation, radical prostatectomy, radiation, and chemotherapy) have serious side effects, there is

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[5] Nonstandard abbreviations: CaP, prostate cancer; ACN, acetonitrile; CM, conditioned medium; FPLC, fast performance liquid chromatography; hK5, human kallikrein 5 protein; hK6, human kallikrein 6 protein; hK11, human kallikrein 11 protein; MS/MS, tandem mass spectrometry; PSA, prostate-specific antigen; SAX, strong anion exchange; TBST, Tris-buffered saline with Tween 20.

a need to differentiate patients who require treatment from those who do not. Mass spectrometry (MS) for biomarker discovery *(16–18)* has generated large databases of protein sequences. We used a cell culture–based proteomic approach to search for novel candidate prostate tumor markers in the proteins secreted into the conditioned medium (CM) of the CaP cell line $PC3(AR)_6$.

Methods

ROLLER BOTTLE CELL CULTURE

We grew the CaP epithelial cell line $PCR(AR)_6$, kindly provided by Dr. Theodore Brown (Toronto Ontario Canada) in a humidified incubator at 37 °C and 5% CO_2 in RPMI 1640 (Gibco) with 80 mL/L fetal calf serum (Hyclone) to confluence (20×10^6 cells/flask) in 2 175-cm^2 tissue culture flasks (Nunc). The cells were trypsinized and transferred to an 850-cm^2 roller bottle flask with a vented cap (Corning), placed on a roller culture apparatus (Wheaton Science Products), and incubated for 2 days in 150 mL RPMI with 8% fetal calf serum to allow the cells to adhere (Fig. 1). Afterward, the medium was discarded, and the interior was rinsed twice with 150 mL phosphate-buffered saline (137 mmol/L NaCl, 10 mmol/L phosphate, 2.7 mmol/L KCl, pH 7.4). Next, 400 mL of chemically defined Chinese hamster ovary medium (Gibco) supplemented with glutamine (8 mmol/L) (Gibco), was added, and the roller bottle was incubated for 14 days. During the culture period, we measured total protein by the Coomassie (Bradford) assay (Pierce Biotechnology) and hK5 protein (hK5) and 6 protein (hK6) by ELISA *(19, 20)*. The CM was collected, spun down (3 000*g*) to remove cellular debris, and frozen at −20 °C for later use. We processed 2 replicates for MS analysis; these replicate cultures are referred to as batch 1 and batch 2.

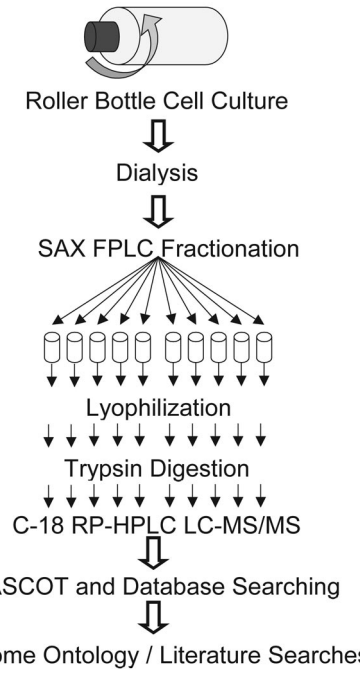

Fig. 1. Schematic representation of the workflow for proteomic analysis of roller bottle CM.

CM from roller bottles was collected and dialyzed overnight. The dialyzed medium was directly loaded onto a SAX column and eluted by FPLC. Ten fractions were collected, lyophilized, and trypsin-digested. The resulting peptides were ZipTip-desalted and separated by reversed-phase C-18 chromatography coupled online to an ion-trap mass spectrometer. The acquired MS/MS data were searched by Mascot, and identified proteins were manually categorized by Genome Ontology and literature searches through NCBI.

DIALYSIS

The thawed CM was dialyzed in tubing with a molecular weight cutoff of 3.5-kDa (Spectra/Por) in 10 L of 20 mmol/L diethanolamine (Sigma-Aldrich), pH 8.9, overnight at 4 °C. A sample aliquot was taken after dialysis.

FAST PERFORMANCE LIQUID CHROMATOGRAPHY OF CM

We loaded the dialyzed CM onto an HR10/10 column (GE Amersham) containing SOURCE15 strong anion exchange (SAX) beads (GE Amersham). We used an AKTA fast-performance liquid chromatography system (FPLC) running Unicorn v4.12 software equipped with a P-960 sample pump and Frac-900 fraction collector (GE Amersham), at a flow rate of 1 mL/min followed by a 2-stage elution gradient at a flow rate of 3 mL/min (0% to 60% elution buffer within 40 min followed by a ramp from 60% to 100% within 10 min) using 20 mmol/L diethanolamine, pH 8.9 running buffer, and 1 mol/L NaCl elution buffer. Absorbance was monitored at 214 nm. We collected the first 10 fractions of 10 mL, taking sample aliquots from each and from the flow-through. A SAX protein standard (Bio-Rad) was run each time to evaluate the quality of the column before each sample loading.

LYOPHILIZATION AND DIGESTION OF FRACTIONS

The collected fractions were lyophilized overnight to dryness, resuspended in 1 mL dH_2O, and trypsin-digested using a 10× digest buffer [5% acetonitrile (ACN), 200 mmol/L urea, and 50 mmol/L tricine, pH 8.8] to digest ~100 μg protein from each lyophilized fraction; 1 μg trypsin (Promega) was used per digest. The digests were incubated overnight at 37 °C and decreased the following day with dithiothreitol (1 mmol/L) for 1 h at 25 °C. We added a final 1 μg trypsin, and the samples were incubated at 37 °C for ~3 h.

LIQUID CHROMATOGRAPHY–MS ANALYSIS

Digested samples were collected on a C-18 ZipTip (Millipore) to purify and desalt the peptides. The ZipTip was primed with 50% ACN in 0.1% acetic acid and washed with 0.1% acetic acid before the digested samples were passed through the ZipTip. The peptides were eluted from the ZipTip with 2 μL of 0.1% acetic acid in 65% ACN, and dH_2O was added to give a final volume of 20 μL. The desalted peptides were injected at 2 μL/min onto a C-18 reversed-phase chromatography column (Vydak 300 μm × 15 cm) via an Agilent 1100 series HPLC system

coupled to a Bruker HCT ion-trap electrospray ionization mass spectrometer (Bruker Daltronics) via a metal electrospray needle. The sample was injected in 5% ACN in 0.1% acetic acid and, after loading for 5 min, a 1-min gradient to 12.5% ACN was followed by a 90-min gradient to 65% ACN in 0.1% acetic acid. We analyzed the eluted peptides by tandem MS (MS/MS), and data were acquired and deconvoluted with the software supplied by Bruker. The instrument was standardized with a tryptic digest of alcohol dehydrogenase, cytochrome C, and glycogen phosphorylase to assess mass accuracy and detection limit of the instrument before and after each set of runs.

DATABASE, GENOME ONTOLOGY, AND LITERATURE SEARCH

We searched the resulting MS/MS spectra in MGF format from batches 1 and 2 by using the Mascot algorithm search engine (version 2.1) with default variables and trypsin specified. The database we used was a custom-built nonredundant compilation of human, mouse, and rat sequences from GenBank, Ensembl, and Swiss-Prot, compiled January 2005. We used a bioinformatics program through Protana Inc. to identify peptides from the MS/MS spectra present from each fraction, giving each peptide a score. The identified peptides from all the fractions within each respective batch were clustered with other peptides common to a particular protein, and each group of peptides was then given a cluster score. We removed from the data any peptide with a Mascot score <20 and any protein with a score <40. We manually analyzed and classified the identified proteins by their genome ontology cellular component classification and conducted PubMed literature searches on each protein.

We measured the false-positive rate of protein identification by searching a random database, in which every sequence entry from the "normal" database was randomly shuffled. The number of hits from each search was categorized based on score, and for each scoring interval, the false-positive rate was calculated as number of random hits/(number of random hits + number of normal hits).

GEL ELECTROPHORESIS AND WESTERN BLOT

Aliquots of the CM taken at days 0, 2, 4, 7, 9, 11, and 14 were centrifuged in 1.5-mL tubes, and 25 μL of the supernatant was used for Western blotting. Gradient gels (1.0 mm $\times$ 10 well 4% to 12% NuPAGE Bis-Tris Invitrogen) were run under reducing conditions. The cell pellet was prepared by trypsinizing PC3(AR)$_6$ cells from a T-75 flask, washing the cells with phosphate-buffered saline, centrifuging, and resuspending the cell pellet in 1 mL dH_2O. The cells were lysed by 5 cycles of freeze/thaw and centrifuged, and the supernatant was measured for total protein. The amount of supernatant used for Western blotting was matched with respect to total protein to be the same as the day 9 aliquot. Proteins were electroblotted

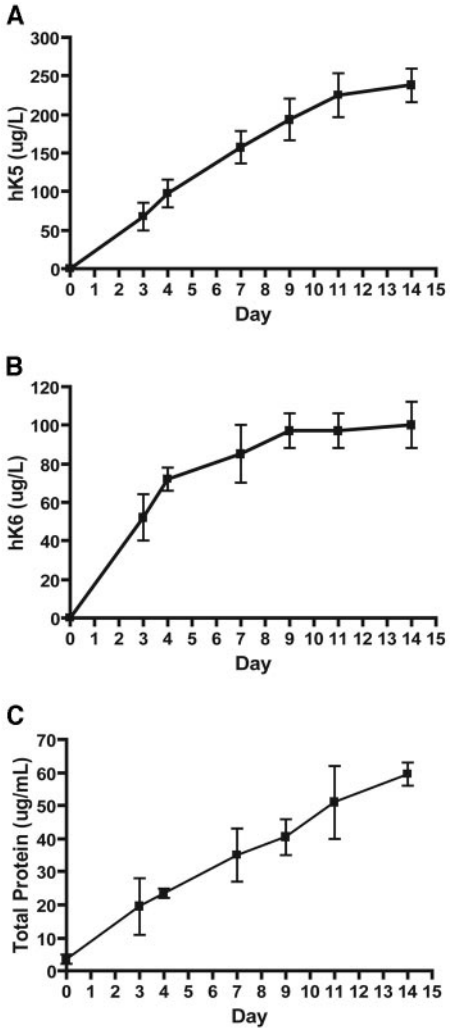

Fig. 2. hK5 *(A)*, hK6 *(B)*, and total protein *(C)* concentrations over time in CM of the PC3(AR)$_6$ roller bottle culture.

The concentrations of hK5 and hK6 were monitored by ELISA. Two replicates are shown.

with a Novex minicell apparatus (Invitrogen) onto Hybond-C Extra nitrocellulose membranes (GE Amersham). Membranes were blocked overnight at 4 °C in 5% powdered milk and Tris-buffered saline with Tween 20 (TBST) (25 mmol/L Tris, 150 mmol/L NaCl, 0.5% Tween 20, pH 7.8). Membranes were washed in TBST and probed with polyclonal anti-hK5 (1:2000) and hK6 (1:3000) primary antibodies for 1 h in 5% milk in TBST. The membranes were washed 3 times in TBST and incubated with secondary goat antirabbit antibody conjugated to alkaline phosphatase (1:5000) for 1 h. Membranes were washed again 3 times in TBST and visualized by chemiluminescence using chemiluminescent substrate and exposure to Hyperfilm ECL (GE Amersham).

ELISAS FOR KALIKREINS 5, 6, AND 11

We measured hK5, hK6, and hK11 by sandwich-type ELISA, 96-well plates, as described earlier *(19–21)* with

use of biotinylated detection antibodies, alkaline phosphatase-conjugated streptavidin and diflunisal phosphate substrate. Plates were read by time-resolved fluorescence *(22)*.

PROTEIN RECOVERY

To assay for sample recovery of proteins during dialysis and fractionation, we analyzed sample aliquots that had been taken during this procedure for hK5 and hK6 by the aforementioned ELISA assays.

Mac-2BP ELISA

We obtained the s90K/Mac-2BP ELISA reagent set from Bender Medsystems. Briefly, serum samples were diluted 1:500 and CM samples 1:10 in sample diluent buffer (provided by the manufacturer) and loaded onto 96-well strips precoated with anti–Mac-2BP antibody. Samples were incubated at 37 °C for 45 min with shaking at 100 rpm. The wells were washed 3 times with wash buffer (as provided), a detection antibody was added, and the plate was incubated for 45 min. The plate was washed and substrate solution (as provided) was added to each well, and the plate was incubated with shaking at room temperature for 10 min, following which a stop solution (as provided) was added to each well. Absorbance was measured at 490 nm by a Wallac–Victor^2 plate reader (Perkin-Elmer).

Results

TOTAL PROTEIN, hK5, AND hK6 CONCENTRATIONS IN ROLLER BOTTLE CULTURE OVER TIME

To demonstrate the accumulation of secreted proteins over time in the roller bottle culture, we measured 2 secreted proteins that are known to be produced by the PC3(AR)$_6$ cell line, namely hK5 and hK6 (our unpublished data), over the 14-day culture period (Fig. 2). The concentrations of hK5 and hK6 increased with time in culture and began to plateau after ~10 days. In addition, we performed Western blots of the aliquots (using different antibodies to that of the ELISA), with essentially the same results (data not shown). To show that the concentrations of hK5 and hK6 present in the CM were from secretion and not from cell death, we ran Western blots of cell pellets of PC3(AR)$_6$ cells. There was no detectable band in the cell pellet, confirming that because of cell death hK5 and hK6 were not present in the CM. We measured the total protein in the CM, which increased steadily throughout the culture period (Fig. 2C).

RECOVERY OF hK5 AND hK6 DURING SAMPLE PREPARATION

The recoveries of hK5 and hK6 were 25% and 17%, respectively (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/iss3). The major protein losses were seen after lyophilization. In addition, for hK5, a significant amount went in the flow-through after column loading.

PROTEINS IDENTIFIED BY MASS SPECTROMETRY

After LC-MS/MS and searching by Mascot from both batches, we identified 262 proteins from the SAX FPLC fractions that had a Mascot score of at least 40 (complete list in Data Supplement). Each protein identified was tabulated and cross-referenced with the genome ontology database for cellular components *(23)* (Fig. 3). A large percentage (39%) of all proteins identified were classified as extracellular (23%) or membrane (16%) proteins. Many identified proteins were classified as intracellular (50%), whereas 11% were unclassified. From the list of the 262

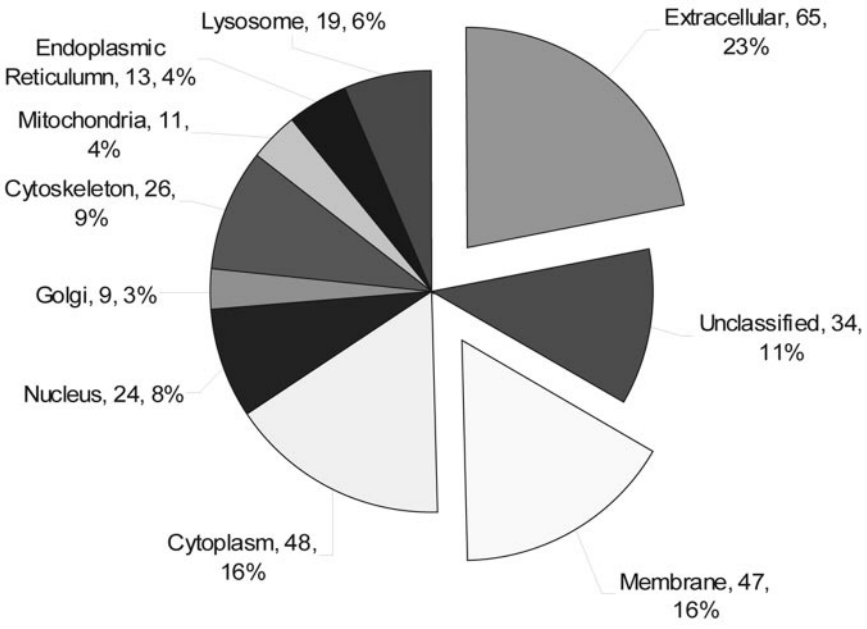

Fig. 3. Classification of proteins by cellular location from PC3(AR)$_6$ CM, batches 1 and 2.

Each protein identified after Mascot searching was classified by its cellular location using Genome Ontology classifiers (www.geneontology.org).

Table 1. Extracellular candidate tumor markers identified in culture medium of the PC3 $(AR)_6$ roller bottle culture.

Gene	Protein Description	Mascot Score	No. Peptides Identified
GDF15	Prostate differentiation factor; PTGF-β	40.81	1
COL2A1	Alpha 1 type II collagen, isoform 1, preproprotein; collagen II,	44.96	2
PAM [a]	Peptidylglycine α-amidating monooxygenase, isoform c, preproprotein	45.64	2
IGFBP2	Insulin-like growth factor binding protein 2 precursor (IGFBP-2) (IBP-2)	45.7	1
S100A8 [a]	S100 calcium-binding protein A8; cystic fibrosis antigen; calgranulin A	47.9	1
TLR9 [a]	Toll-like receptor 9, isoform A precursor	49.44	2
TPT1 [a]	Tumor protein, translationally controlled 1	50.73	1
CTSL [a]	Cathepsin L	52.09	1
PLG	Plasminogen	54.92	2
LGALS3	Lectin, galactoside-binding, soluble, 3 (galectin 3); Lectin, galactose-binding	55.53	1
LTBP2 [a]	Latent transforming growth factor β binding protein 2	65.36	2
MUC5B [a]	Mucin 5, subtype B, tracheobronchial	66.33	2
GALNT6 [a]	Polypeptide *N*-acetylgalactosaminyltransferase 6	66.35	2
IGFBP6	Insulin-like growth factor binding protein 6	72.26	1
INHBB	Inhibin β B subunit precursor; Inhibin, β-2	74.86	1
TIMP2	Tissue inhibitor of metalloproteinase 2 precursor	75.21	2
LAMC2	Laminin, γ 2, isoform a precursor; nicein (100 kDa)	75.96	1
CXCL3 [a]	Macrophage inflammatory protein-2-β precursor (MIP2-β)	80.8	2
AZGP1	α-2-glycoprotein 1, zinc; α-2-glycoprotein	82.29	2
NPC2 [a]	Niemann-Pick disease, type C2; Niemann-Pick disease, type C2 gene	82.8	2
MDK [a]	Midkine (neurite growth-promoting factor 2)	90.34	2
SERPINA1	Serine (or cysteine) proteinase inhibitor	93.92	3
KLK6 [a]	Kallikrein 6 (neurosin, zyme); protease M; protease, serine, 9 (neurosin)	95.71	2
SEMA3F [a]	Semaphorin 3F precursor (Semaphorin IV) (Sema IV) (Sema III/F)	100.2	2
C19orf10 [a]	Chromosome 19 open reading frame 10; interleukin 25; interleukin 27	103.18	2
LRG1	Leucine-rich α-2-glycoprotein	109.66	3
COL6A2	α 2 type VI collagen, isoform 2C2	109.73	3
GALNT2	Polypeptide *N*-acetylgalactosaminyltransferase 2; UDP-GalNAc transferase 2	114.5	3
IGFBP5	Insulin-like growth factor binding protein 5	120.38	3
RNASET2 [a]	Ribonuclease 6 (EC 3.10.27.—) precursor	124.53	4
FBLN1	Fibulin 1 isoform C precursor	137.3	3
DAG1 [a]	Dystroglycan 1 precursor; α-dystroglycan; Dystrophin-associated glycoprotein-1	145.92	3
S100A9	S100 calcium-binding protein A9; calgranulin B	152.1	3
TGFB2	Transforming growth factor, β 2	152.59	3
SPINT1 [a]	Hepatocyte growth factor activator inhibitor precursor	154.24	3
B2 mol/L	β-2-microglobulin	177.47	3
LCN2 [a]	Lipocalin 2 (oncogene 24p3)	181.58	5
KLK5 [a]	Kallikrein 5; stratum corneum tryptic enzyme	181.94	4
LCN7 [a]	P3ECSL; glucocorticoid-inducible protein; oxidized-LDL responsive gene 2	184.67	3
DF	Complement factor D preproprotein; adipsin; properdin factor D	187.96	4
SFN [a]	Stratifin	193.8	4
APP [a]	Amyloid β (A4) precursor protein (protease nexin-II, Alzheimer disease)	215.47	6
TIMP1	Tissue inhibitor of metalloproteinase 1 precursor; Erythroid-potentiating activity	227.55	4
C1S	Complement component 1, s subcomponent	254.89	4
GRN	Granulin	258.53	5
PLTP [a]	Phospholipid transfer protein	282.02	5
STC2 [a]	Stanniocalcin 2; stanniocalcin 2; stanniocalcin-related protein	303	4
PTX3 [a]	Pentaxin-related gene, rapidly induced by IL-1β; Pentraxin-3	308.44	5
CYR61 [a]	Cysteine-rich, angiogenic inducer, 61; cysteine-rich heparin-binding protein 61	327.5	6
IL6	Interleukin 6 (interferon, β 2)	336.16	6
PSAP [a]	Saposin precursor	359.65	9
PLAU	Plasminogen activator, urokinase	361.2	6
FSTL1 [a]	Follistatin-like 1 precursor; follistatin-related protein	367.11	7
P4HB [a]	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase)	375.33	8
AGRN [a]	Agrin	486.69	9

Table 1. Continued

Gene	Protein Description	Mascot Score	No. Peptides Identified
HSPG2 [a]	Basement membrane-specific heparan sulfate proteoglycan core protein precursor	506.82	10
INHBA [a]	Inhibin β A subunit precursor; Inhibin, β-1; EDF	507.77	11
NUCB1 [a]	Nucleobindin 1 precursor (CALNUC)	631.69	12
LGALS3BP [a]	Galectin 3 binding protein; L3 antigen; Mac-2-binding protein; serum protein 90K	632.55	13
COL6A1	Collagen, type VI, α 1 precursor; collagen VI, α-1 polypeptide	766.46	13
CTSD	Cathepsin D (lysosomal aspartyl protease)	771.08	12
THBS1	Thrombospondin 1 precursor	865.57	14
BF	Complement factor B preproprotein; B-factor, properdin; C3 proactivator	1071.18	22
FN1	Fibronectin 1 isoform 2 preproprotein; cold-insoluble globulin	1978.92	37
C3	Complement component 3	3339.99	57

[a] Proteins linked to cancer in previous literature reports.

proteins (see Table 2 in the online Data Supplement) we selected candidate biomarkers (see Table 1 and Table 3 in the online Data Supplement) based on the following criteria: *(a)* Proteins were searched manually against the Genome Ontology database *(23)* for their cellular localization. Proteins that were classified as secreted and membrane-bound were selected. *(b)* Literature searches through the National Center of Biotechnology Information PubMed database were then performed to determine whether these proteins are novel molecules that have yet to be explored as potential biomarkers and whether these proteins are known to participate in critical pathways implicated in cancer initiation and progression. We determined the overlap of the proteins identified between the 2 batches through visual inspection of the Mascot data.

Proteins in these lists (Table 1 and online Supplemental Data Table 3) that are marked with a [a] in Table 1 and an asterisk in the Supplemental Data have not been evaluated previously as serum biomarkers for CaP as determined through PubMed literature searches specific for the protein.

We calculated false-positive protein identification rates based on a random database search. False-positive rates for specific scoring intervals were as follows: 40–50, 44%; 50–60, 35%; 60–70, 7%; 70–80, 8%; 90–150, 7%; and >150, 0%. The presence of a higher false-positive rate in the 70–80 scoring interval is the result of a statistical fluctuation attributable to additional protein identification in the random database search.

Overlap of proteins identified in batches 1 and 2

To determine the reproducibility of the method, the proteins identified from batches 1 and 2 were manually compared for overlap. We identified 145 proteins in both batches (55% overlap). Additionally, we identified 78 proteins only in batch 1 and 39 proteins only in batch 2. Combined, the total number of identified proteins was 262. The proteins identified in both batches are highlighted in the online Supplemental Data Table 2. As expected, the more abundant proteins were preferentially identified in both batches.

Mac-2BP concentrations in CaP vs healthy men and CM

From the proteins identified, Mac-2BP was chosen as 1 novel biomarker candidate for further validation. The concentrations of Mac-2BP increased in the CM over time (Fig. 4A), as expected and in a similar fashion to hK5 and hK6 (Fig. 2). We measured serum concentrations of Mac-2BP from 26 men with CaP and 17 healthy men by using a Mac-2BP ELISA. The median Mac-2BP concentrations in CaP patients were almost twice as high as those in healthy men (Fig. 4B), with 50% of the CaP patients having increased Mac-2BP concentrations compared with the healthy men (at the 100th percentile of healthy men as a cutoff). The difference in medians of the 2 populations by Mann–Whitney test was highly significant ($P = 0.003$). The negative correlation between Mac-2BP and PSA in these 26 patients was also significant, with a Spearman correlation coefficient (r_s) of -0.63 ($P < 0.001$; Fig. 4C).

We further measured hK5, hK6, and hK11 protein (a previously identified prostate and ovarian cancer biomarker) *(24)* in the same set of patients and controls, as above. The data (Fig. 5) showed decreased concentrations of hK5 in CaP ($P < 0.0001$ by Mann–Whitney test), decreased concentrations of hK6 in CaP ($P = 0.03$ by Mann–Whitney test), and increased concentrations of hK11 in CaP ($P < 0.0001$ by Mann–Whitney test).

Spearman correlations for all pairs of measured concentrations (Mac-2BP, hK5, hK6, hK11, and PSA) included only 1 statistically significant negative correlation, between Mac-2BP and PSA (Fig. 4C).

Discussion

We used a proteomic method for identification of secreted proteins from the CM of the metastatic CaP cell line $PC3(AR)_6$ as a model to discover novel markers for CaP. Because large amounts of cells are needed for confident MS detection of low-abundance cellular proteins, we determined if a cell line could be grown in a large volume of serum-free medium for an extended period. The use of protein- and peptide-free chemically defined Chinese

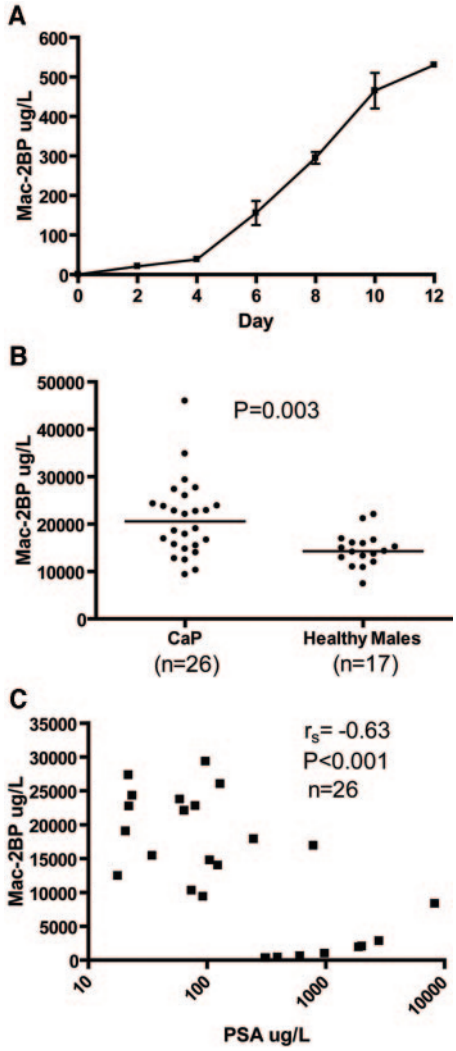

Fig. 4. Mac-2BP concentrations and the correlation between serum Mac-2BP concentrations and PSA.

(*A*), Mac-2BP concentrations in CM of PC3(AR)$_6$ cell line over time. (*B*), Mac-2BP concentrations in serum of 26 CaP patients and 17 healthy men. *Horizontal lines* indicate medians. *P* value was calculated with the Mann–Whitney test. (*C*), Correlation between serum Mac-2BP concentrations and PSA in the 26 cancer patients. r_s = Spearman correlation coefficient.

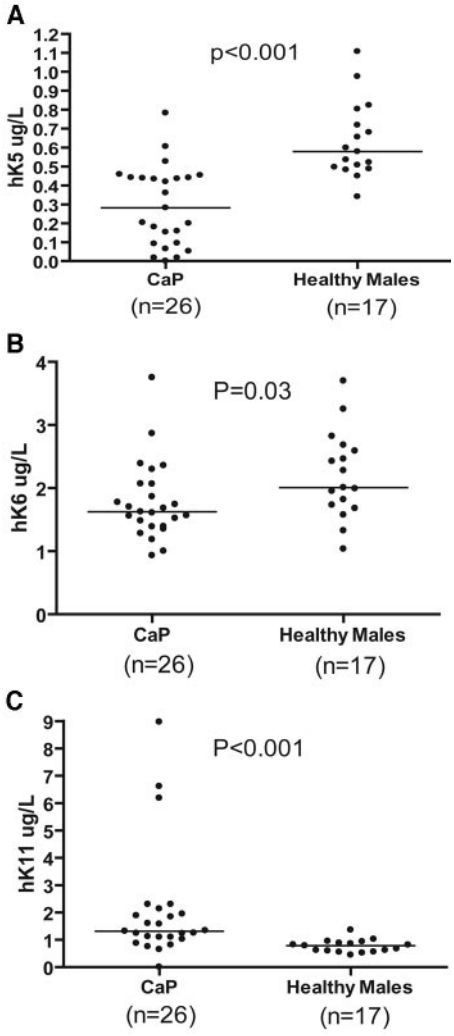

Fig. 5. Concentrations of hK5, hK6, and hK11 in serum of 26 CaP patients and 17 healthy men.

Horizontal lines indicate medians. *P* value was calculated with the Mann–Whitney test.

hamster ovary serum-free medium simplified analysis, providing a distinct advantage over fetal calf serum–containing medium, which would contaminate the CM.

The loss of hK5 in the flow-through during SAX FPLC was attributable to incomplete capture of hK5 by the SAX column. The incomplete capture is consistent with its relatively high pI of ~8. Appreciable losses of both hK5 and hK6 also occurred after lyophilization of the FPLC fractions, possibly attributable to incomplete solubilization of the freeze-dried protein *(25)*.

Independent MS detection of hK5 and hK6, proteins known to be secreted by PC3(AR)$_6$, was confirmed for batches 1 and 2 in the expected fractions. The complex mixture of proteins present at various concentrations in CM necessitated fractionation before MS to increase the depth of identification. However, not all proteins in a mixture can be ionized and detected in 1 run, with the lower abundance proteins not being identified in both batches, as highlighted in the online Supplemental Data Table 2 *(26)*.

Fifty percent of the proteins identified were intracellular. Their presence in the CM is to be expected because of cell death and their high abundance within cells. Because we were primarily interested in investigating proteins that are secreted or shed from CaP cells in vivo, we selected the extracellular and membrane proteins for further evaluation. Each protein was examined to establish if it had been previously evaluated as a CaP biomarker or if it had any link to cancer. The selected candidates are listed in Table 1 and the online Supplemental Data Table 3.

We performed preliminary validation of Mac-2BP by ELISA. Mac-2BP has been a serum prognostic marker in

lymphoma *(27)*, and lung *(28)*, breast *(29)*, hepatocellular *(30)*, ovarian *(31)*, and colon *(32, 33)* carcinoma. Serum concentrations have not been evaluated in CaP, however, despite the correlation of immunohistochemical staining for Mac-2BP with Gleason grade *(34)*. Serum Mac-2BP was increased in 50% of the CaP patients (Fig. 4). The correlation of Mac-2BP concentrations and PSA concentrations in these patients was weak and negative (Fig. 4C). Mac-2BP may be implicated with aggressiveness of CaP tumors and could serve as a prognostic marker because its concentrations seem to decrease as the tumor progresses. Two kallikreins (hK5 and hK6) were present at lower concentrations in CaP, whereas hK11 was present at increased concentrations. These data support the theory that secreted proteins are adjunct biomarkers for CaP, although with less diagnostic accuracy than PSA. No correlation was seen between any pairs of these markers in serum.

The results of this and other similar studies *(35, 36)* suggest that a wealth of knowledge is obtainable by analyzing the CM of cell lines. We observed minimal overlap of our data with those of Martin et al. *(36)* and Lin et al. *(37)*, who studied the proteins secreted and present in the LNCaP cell line, with 67 proteins from the Martin et al. study and 27 proteins from the Lin et al. study overlapping. This highlights the heterogeneity of cell lines and the data that can be derived from each. Obviously, much work is needed to further evaluate the identified candidate biomarkers (Tables 1 and 3), but this study will form the basis of future communications.

In total, after LC-MS/MS and searching by Mascot from both batches, 262 proteins were identified from the SAX FPLC fractions that had a Mascot score of at least 40 (the complete list of proteins is presented in the online Supplemental Data). This material is available free at http://pubs.acs.org. This work was supported by a University-Industry grant from the Natural Sciences and Engineering Council of Canada (NSERC) and YYZ Pharmatech, Toronto, ON, Canada. Bioinformatics support was provided by Dr. Rob Ewing of Infochromics, Toronto, ON, Canada.

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