Humoral Response Profiling Reveals Pathways to Prostate Cancer Progression*


There is considerable evidence for an association between prostate cancer development and inflammation, which results in autoantibody generation against tumor proteins. This immune system-driven amplification of the autoantibody response to intracellular antigens can serve as a sensitive tool to detect low abundance serum proteomic tumor markers for prostate cancer as well as provide insight into biological processes perturbed during cancer development. Here we examine serum humoral responses in a cohort of 34 patients with either benign prostatic hyperplasia or clinically localized prostate cancer (PCa). The experimental strategy couples multidimensional liquid-phase protein fractionation of localized and metastatic prostate cancer tissue lysates to protein microarrays and subsequent mass spectrometry. A supervised learning analysis of the humoral response arrays generated a parsimonious predictor having 78% sensitivity and 75% specificity in distinguishing PCa from benign prostatic hyperplasia in a cohort of American males with elevated prostate-specific antigen levels. Enrichment analysis of the PCa-specific humoral signature revealed large scale immune reprogramming mediated by STAT transcription factors and the generation of autoantibodies to enzymes involved in nitrogen metabolism. Meta-analysis of independent prostate cancer gene expression data validated the presence of STAT-induced immunomodulation. Concomitant validation of elevated levels of the nitrogen metabolism pathway was obtained by direct measurement of metabolic levels of glutamate and aspartate in prostate cancer tissues. Thus, in addition to functioning as markers in prostate cancer detection, humoral response profiles can serve as powerful tools revealing pathway dysregulation that might otherwise be suppressed by the complexity of the cancer proteome. Molecular & Cellular Proteomics 7:600–611, 2008.

Although prostate carcinoma is the leading cancer diagnosis in American men, its early detection facilitates effective treatment modalities and improved mortality (1). Although the advent of prostate-specific antigen (PSA) screening has led to earlier detection of prostate cancers (2), its lack of specificity for neoplasms has resulted in a dramatic increase in the number of subsequent prostate needle biopsies (3). As the population of men 65 years and older is expected to increase from 14 million in the year 2000 to 31 million by 2030 (4), it will be increasingly important to distinguish men with benign prostatic hyperplasia from those having neoplastic disease warranting clinical intervention. Thus, there is a compelling need to define additional clinical markers for accurate detection of prostate cancers.

This situation has spawned a wide range of serum-based early detection methodologies, including protein microarrays (5). Complicating this approach is the fact that potentially viable tumor biomarkers are embedded among a bounty of proteomic noise. This noise includes housekeeping and other highly abundant proteins, whereas the relatively low abundance of protein biomarker candidates confounds their detection. This span in protein concentration in a complex biofluid such as plasma or serum requires that effective detection methodologies bridge as many as 7–10 orders of magnitude in dynamic range to reliably detect those lowest concentration markers (6). No such existing technology or platform offers such a broad dynamic range without implementing prefractionation strategies that may result in the loss or suppression of important biomarkers; also many high abundance proteins subjected to depletion act as carriers for low abundance biomarkers (7). Here we show that the immune system-driven amplification of the autoantibody response to intracellular antigens can yield higher sensitivity, specificity, predictive value, and reproducibility in detecting low abundance biofluid tumor markers (8).

Early efforts have identified many gene products eliciting humoral response, including somatic alterations in p53 in

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30–40% of affected patients that have been shown to predetermine cancer diagnosis (9). In other work, 60% of patients with lung adenocarcinomas exhibited humoral response to glycosylated annexins I and/or II, whereas none of the sera from non-cancer patients demonstrated such a response (10). Similarly, autoantibodies to the prostasome and to such antigens as PSA, prostatic acid phosphatase, HER-2/neu, p53, α-methylacyl-CoA racemase, and GRP78 have been observed in the sera of prostate cancer patients (11–14). Autoantibody signatures have also been identified using phage microarrays that can delineate prostate cancer patients from control individuals with >90% accuracy (15). However, one of the major caveats of this platform lies in the fact that most of the humoral targets identified are mimotopes that resemble the target protein in either the amino acid sequence or structure (15). Furthermore it is important to note that most of the proteins that elicit a humoral response are often differentiation antigens or antigens overexpressed or modified in cancer (13, 14). Additionally the humoral response elicited by cancers is heterogeneous. This is supported by studies from humoral response trials where among the large numbers of patients tested only a subset of patients with a specific tumor type develop a response to a specific antigen. This heterogeneity in humoral response generation necessitates the use of a multiplex panel of protein targets as autoantibody biomarkers to detect tumors with broad coverage for a large number of people. This requirement motivates our strategy of coupling comprehensive two-dimensional liquid-phase fractionation of the prostate cancer proteome to protein microarray analysis of patient sera and then mass spectrometry for the identification of proteins contributing to a discriminating multiplex humoral response to prostate cancer antigens (see Fig. 1). In this work we used the humoral response signature not only for prostate cancer detection but also for extensive analysis of pathways particularly dysregulated in prostate cancer development and progression.

**EXPERIMENTAL PROCEDURES**

**Patient Population and Sample Selection**

The Institutional Review Board of the University of Michigan Medical School approved this study. Serum samples from patients who visited the Urology Clinic for prostate cancer screening were collected prior to biopsy. The sera were banked at the University of Michigan Specialized Research Program in Prostate Cancer (Specialized Program of Research Excellence) tissue/serum bank. A total of 34 serum samples from patients who visited the clinic on 2 successive days were sequentially used for the experiments. Among these, 18 patients were biopsy-positive for prostate cancer (PCa), and 16 were negative for neoplasm but diagnosed with benign prostatic hyperplasia (BPH). The average age of all prostate cancer patients was 63.2 ± 12.8 years. For patients diagnosed with BPH, the average age was 64.8 ± 10.7 years. PSA values for the PCa and BPH groups were 7.81 ± 5.34 (2.9–20.4) and 6.79 ± 3.76 ng/ml (2.1–14.1), respectively. Detailed clinical and pathology data for this study are available in supplemental Table 1.

**Preparation of Reference Pools**

Tissue samples obtained postsurgery from clinically localized prostate cancer patients (n = 5) and metastatic prostate cancer patients (n = 5) were used for two-dimensional liquid-phase fractionation as described below. Five patients were selected in each group to account for individual variations in tumor proteome. All chemicals were purchased from Sigma unless otherwise mentioned. For protein extraction, the tissue samples were resuspended in lysis buffer consisting of 7 M urea, 2 M thiourea, 100 mM DTT, 0.5% Bio-Lyte 3–10 (Bio-Rad), 2% octyl glucoside, and 1 mM PMSF. Samples were lysed at room temperature for 30 min followed by centrifugation at 35,000 rpm at 4 °C for 1 h. The supernatant was stored at −80 °C for future use. Sample preparation for chromatococusing (CF) included a PD 10 column, equilibrated with 25 mM bis-tris in 6 M urea and 0.2% octyl glucoside, that was used to exchange the tissue lyse from the lysis buffer to the above buffer. We loaded 15 mg of tissue lyse in the first dimension as two separate pools; one was a mixture of the five primary disease samples, and the other was a mixture of the five metastatic disease samples described below.

**Two-dimensional Protein Fractionation**

**CF of Tissue Lysates**—The CF experiment used a start buffer of 25 mM bis-tris with pH 7.1 and an elution buffer consisting of a 10-fold dilution of Polybuffer 96 and Polybuffer 74 in a ratio of 3:7 with pH adjusted to 4.0. Both buffers were prepared in 6 M urea and 0.2% octyl glucoside. Imidazole acid was used to adjust the pH of both buffers. The PS-HPCF 1D column was equilibrated with the start buffer until the pH of the effluent was 7.1. Sample was applied to the column with multiple injections. Once a stable base line was achieved, the elution buffer was switched on to elute the proteins on the column in an isocratic mode. UV detection was performed at 280 nm, and the pH of the effluent was monitored using a flow-through on-line pH probe. The pH fractions were collected in 0.2 pH intervals, and 15 fractions in total were collected in the range of pH 7–4. The CF separation was stopped when the pH of the effluent reached 4. The flow rate in the CF experiment was 1 ml/min; and as fractionation was based on pH rather than time, fraction volumes ranged from 2 to 5 ml.

**Reverse-phase HPLC on pI Fractions**—Reverse-phase (RP)-HPLC was performed using PS-HPCF 2D (4.6 × 33-mm) columns. Solvent A was 0.1% TFA (J. T. Baker Inc.) in water, and solvent B was 0.1% TFA in acetonitrile (Burdick and Jackson, Muskegon, MI). The gradient was run from 5 to 15% in 1 min, 15% B to 25% in 2 min, 25 to 31% in 2 min, 31 to 41% in 10 min, 41 to 47% in 6 min, 47 to 67% in 4 min, finally up to 100% B in 3 min and held for another 1 min, and then back to 5% in 1 min at a flow rate of 1 ml/min. The column temperature was 40 °C higher than the ambient temperature. UV absorptions were monitored at 214 nm. RP fractions were collected with fraction volumes ranging between 100 μl and 1 ml. The fractions were dried completely by SpeedVac at 75 °C and stored at −80 °C.

**Microarray Procedures**

**Printing the Fractionated Proteins**—The fractionated proteins were resuspended in 15 μl of buffer containing PBS at pH 7.4 and protease inhibitors (Roche Applied Science) at an average protein concentration of ~10 pg/well. The samples were transferred to a 96-well microtiter plate (MJ Research, Waltham, MA) and printed on nitrocellulose slides (Schleicher & Schuell) using a GeSiM Nanoplotter2, a non-contact ink jet printer. Each spot measured ~300 μm in diameter; spots were placed 1200 μm apart. The slides were dried for 1 h at room temperature and were either used immediately or stored at room temperature in a desiccation chamber.

**Hybridization of Slides**—Nitrocellulose slides containing spotted
proteins were hydrated in PBS for 10 min and blocked in PBS containing 1% BSA (Sigma) and 0.1% Tween 20 (Sigma) overnight at 4°C. The slides were then incubated with serum (1:400) either from prostate cancer patients or from patients with BPH in probe buffer (PBS, pH 7.4, containing 1% BSA, 5 mM MgCl2, 0.5 mM DTT, 0.05% Triton X-100, and 5% glycerol) for 2 h at 4°C. Slides were washed six times with probe buffer, each time for 5 min. They were then incubated with Alexa 647-conjugated anti-human IgG (1:2000, Invitrogen) for 1 h at 4°C, washed with probe buffer as above, dried, and analyzed.

Data Analysis

Primary analysis, including scanning and quantification of slides, was executed with the GenePix 4000B scanner (Axon Laboratories, Inc., Foster City, CA); gridding was completed according to the manufacturer’s instructions. The single red channel intensity values were calculated for each individual fraction spot. An initial round of spot checking was performed using GenePix default parameters. This was followed by a second round of curation wherein spots having any of the following characteristics were manually flagged: a diameter smaller than 300 μm, an irregular outline, localization in regions of high local background, or presence of any areas of the arrays with obvious defects. Flagged spots were seeded to −1 in raw intensity units in the subsequent analysis. The median minus background of the red channel was extracted from each array and normalized. The total feature set was filtered for dominantly negative fractions, and only those fractions with non-negative raw intensity in ≥50% of samples in the cohort were retained. Within-array standardization entailed median centering and scaling by their respective median absolute deviations. Quantile normalization was then executed to ensure the same empirical distribution across all arrays. Two-way average-linkage hierarchical clustering of an uncentered Pearson correlation similarity matrix was executed and figures were generated using Cluster and TreeView (16).

Development of a Predictor

A supervised analysis was completed to coalesce around a subset of fractions from the 2016-element (excluding control features) humoral response arrays most predictive for class distinction across the serum samples. Array data, normalized as described above, were applied to a test statistic-based feature selection procedure calculating the F-statistic between the 18 biopsy-proven PCs and 16 BPH samples across all 2016 fractions. Different counts of the best ranking fractions by F-statistic (every count of best fractions from 5 to 100) were used to build a support vector machine (SVM) prediction model. The SVM over multiple kernel test permutations was embedded in a finite grid search of paired values of exponentially growing sequences to build a support vector machine (SVM) prediction model.

The statistical model for testing and storing the results of associations between independent molecular concepts is as described previously (20). Protein identifications from the humoral response signature were converted to Entrez Gene identifiers and batch-loaded to MCM (Oncomine) for analysis. As a control during concept enrichment, we similarly analyzed 14 of 27 fractions selected as a non-differential negative control that were sequenced as described above and their protein content was culled with identical criteria. Any concept enriching both the differential predictor and negative control was excluded from the analysis. We extended the analysis one level with concept-to-concept enrichments for five promoter binding site concepts (see Fig. 3A, boxed). Each seeded a subset MCM enrichment on its gene list rather than on the original humoral target list. The five resulting enrichment networks were sequentially merged into a single common network. Orphaned concepts from single concept enrichment were removed. To generate an immune program under STAT control for meta-analysis with public gene expression studies, we downloaded the immune response in silico repository of 1622 genes expressed in, and classified by, multiple immune cell lineages (21). There is a 179-gene overlap between the immune response in silico compendium and those genes under STAT control, the union of either STAT1, STAT3, or STAT5B (homodimer). This seeds the metapmap analysis described in the text.
RESULTS

Development of Arrays via Proteome Fractionation—An overview of the approach we took in identifying humoral targets in prostate cancer is depicted in Fig. 1. To develop a protein microarray for prostate cancer progression, we independently fractionated proteins from clinically localized and metastatic cancer tissues \( (n = 5, \text{ each}) \) in two dimensions using a combination of chromatofocusing and reverse-phase chromatography. The fractionated proteins were spotted on nitrocellulose-backed glass slides and served as bait to capture potential autoantibodies found in serum. This process included pooling samples from each group separately and loading and printing separately on the same slide. This was done to maintain both a localized cancer-specific and metastatic cancer-specific proteomic signature as they may individually produce two different antigen populations, one to each signature. Thus, we believed humoral response could either target antigens from early or aggressive disease, so we maintained distinct populations. Proteins that reacted with prostate cancer sera but not the control were identified using an ion trap mass spectrometer, database search, and downstream protein informatics (see “Experimental Procedures”). The list of proteins obtained was used both to characterize the predictor and to conduct a “molecular concept” analysis for their involvement in disease processes (supplemental Fig. 2 and supplemental Tables 4–7).

Approximately 2300 fractions were used to generate protein microarrays. Using this 2300-feature protein microarray, we evaluated sera from prostate cancer patients and controls. In this discovery approach we evaluated 34 serum samples consisting of 18 sera from prostate cancer patients (biopsy-positive, high PSA) and 16 from individuals with BPH (biopsy-negative for cancer, high PSA). Critically these samples constitute the clinically challenging distinction between cancer-negative
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We sought the pattern of differential autoantibody response that could discriminate between the benign and prostate cancer groups that was generated against specific tumor antigens represented by the cancer proteome spotted on the microarrays. Cross-validated supervised analysis implementing the non-parametric SVM was performed using the 34 samples as a training set, looking for humoral response correlates of the two-class distinction between BPH and PCa (see “Experimental Procedures”). Of the 1522 features remaining after filtering for dominantly negative fractions as the result of hybridization (see “Experimental Procedures”), a subset demonstrated differential reactivity patterns. Embedded feature reselection during LOOCV produced a 20-fraction predictor having 75% specificity (four of 16 BPH samples were misclassified) and 78% sensitivity (four of 18 prostate-cancer samples were misclassified) in discriminating between the group with BPH and that with PCa (supplemental Table 2). This was an especially encouraging result as this is a particularly challenging classification; all members of the cohort have a base-line inflammatory physiological condition that is eliciting a humoral response accompanying their biopsy-proven clinical condition. The 20-fraction predictor was chosen as the minimum fraction count producing maximum accuracy in classification without overfitting the predictor and with highest recurrence over left-out iterations during cross-validation, otherwise interpreted as highest tolerance in statistical significance to the leaving out of any given sample in hybridization.
the cohort (see Fig. 2A, “Experimental Procedures,” and supplemental Tables 2 and 3). A heat map of the reactivity profile generated by the 20-fraction predictor reveals a distinct bipartite pattern as would be expected by real reactivity to class-dependent antigens in lieu of silence in reactivity (Fig. 2, B and C). The heterogeneity of the reactivity profile is likely attributable to many causes including loss of humoral response. The latter has been reported previously in patients diagnosed with breast cancer where loss of autoantibodies to mucin have been shown to be indicators of poor prognosis (22). Additionally because inflammation can induce a higher immune response, we used an already immunologically active cohort in the case of BPH by virtue of its hyperplastic and inflammatory status. We expect this group to harbor an increased immune response as a physiologic start point, and this is the base-line condition from which we are interested in identifying the gain in humoral response upon progression to prostate cancer. Finally some heterogeneity in reactivity is certainly due to the aforementioned issues of the consistency of response across patients to specific intracellular antigens.

We next calculated receiver-operating characteristic curves for the 20-fraction predictor and measured PSA levels in the sample cohort (Fig. 2D). A variety of cutoff values of the SVM decision scores were used as thresholds to plot the true against false positive rates for the prediction model. The ability of the 20 fractions to discriminate PCa serum samples from BPH samples was significant (p = 0.013) with an area under the curve of 0.75 (95% confidence interval, 0.58–0.92; Fig. 2D). On the other hand, the area under the curve for measured PSA was 0.49 (p = 0.94; 95% confidence interval, 0.29–0.70). This result is significant as all benign patients in this cohort have elevated PSA levels associated with an early inflammatory condition, again a more challenging clinical distinction than with a healthy normal control. Furthermore a permuted rank-based test of significance of the difference in area under the curve between the SVM-derived predictor and that of measured PSA was also significant (p = 0.05; see supplemental methods and supplemental Table 8 for association to additional pathologic parameters).

Characterization of the 20-fraction Predictor—Although demonstrating promising results and certainly superior to the current PSA-based clinical standard, this result falls short of the accuracy of a desired diagnostic platform. Nevertheless as these tumor-associated antigens are derived from the cancer proteome and we expect them to be perturbed in cancer development given their loss of immunological tolerance, we hypothesized that their identification could form part of a larger alteration underlying a systematic biological process. To arrive at such a systems perspective, it was critical to identify these humoral targets. Consequently each of the 20 informative high stability fractions from classification was taken for mass spectrometry (see “Experimental Procedures” and supplemental Table 4). In addition, 27 non-informative fractions were sequenced as a negative control for downstream analysis.

A total of 359 unique proteins were identified from sequencing the 20-fraction predictor at probabilities of correct assignment greater than 0.90 (17). The immunoreactivity profile of a given fraction is a complex combination of potential interactions. It may be the case that a fraction is composed of as few as five to eight proteins, all of which elicit an autoantibody response to patient serum. Alternatively fractions of equal or higher protein content with similar reactivity may be a combination of isolated proteins eliciting a response, a cooperative humoral response between complexes of proteins, “bleed-over” of proteins common to adjacent fractions, and proteins that may appear in several fractions and represent cellular machinery having nothing to do with eliciting the class-dependent immunoreactivity of the fraction. We subsequently created a compendium of proteins from this sequencing that met criteria making them the mostly likely subset of proteins eliciting the humoral response signature. This included several rounds of subtraction for proteins considered either nonspecific or noise to the reactivity profile (see supplemental methods, supplemental Tables 5 and 6, and supplemental Fig. 2). The final compendium of likely humoral response targets numbered 248 (supplemental Table 7). This repository of humoral response targets was defined heuristically, and as such, it is possible that it contains small numbers of potential false positive and negative targets. Nevertheless as we will demonstrate, this limitation is tempered as the list is widely enriched for known targets of autoantibody response, and many are further supported by integration with other data modalities including gene expression and metabolomics data.

A total of 29 proteins were found to constitute a humoral signature that was prostate cancer-specific (supplemental methods and supplemental Tables 7 and 9). Among these, 12 proteins were solely identified using single peptides in one or more fractions. The mass spectra for these single peptide identifications are given in supplemental Figs. 3–8. Among the 29 PCA-specific humoral targets were proteins that have been previously shown to elicit humoral response; these may be considered positive controls for the method. Foremost among them was PSA (KLK3; supplemental methods). Others included known androgen-regulated proteins such as prostatic acid phosphatase and hypoxia up-regulated 1 (HYOU1); proteins involved in the fibroblast growth factor receptor signaling pathway like synaptotagmin-binding and cytoplasmic RNA-interacting protein (SYNCRIP); regulators of actin cytoskeletal reorganization including calponin 1 (CNN1), Was/was-interacting protein family member 2 (WICH2), and valosin-containing protein; and finally tumor suppressors including a novel ring finger B box, coiled-coil family member (HLS5) and synaptotodin (SYNO2).

Molecular Concept Modeling of Humoral Response Targets—To determine the ability of our PCA-specific humoral signature to identify pathways that are dysregulated during prostate cancer development and progression, we performed
a molecular concept analysis (MCM) on the group of proteins that were identified to have cancer-specific autoantibody repertoire (see “Experimental Procedures”, Fig. 3A, and supplemental Table 7). Our laboratory’s recent work in the enrichment analysis of gene sets biologically related in a meaningful way allows for the discovery of patterns of shared behavior over a vast database of high throughput experimental data and biological annotation (20). The MCM analysis of the “increased humoral response in prostate cancer” signature identified an enrichment network containing metabolism concepts, including the Kyoto Encyclopedia of Genes and Genomes pathway for nitrogen metabolism ($p = 4.6 \times 10^{-8}$) as well as the mitochondrion cellular localization ($p = 3.4 \times 10^{-8}$), and multiple drug compounds, including a gene set up-regulated upon cyclosporin treatment ($p = 0.006$), which is an immunosuppressant, and a gene set down-regulated upon treatment with rosiglitazone ($p = 0.006$), which has a potent anti-inflammatory effect (Fig. 3A). Additional concepts enriched by the PCa-specific humoral targets included four promoter binding sites implicating a STAT-regulating transcriptome and Human Protein Reference Database interaction networks for three proteins, which in concert play a role in mRNA processing. Among these, several concepts were taken for further analysis (Figs. 3A and 4A, boxed).

Among the various aforementioned concepts, the nitrogen metabolism concept was intriguing in the context of earlier results from our laboratory that revealed a shift in the metabolism paradigm during prostate cancer development driven by increased protein biosynthesis (20). This in turn was thought to be regulated by a combination of androgen and overexpression of the fusion isofrom of the transcription factor ERG. Closer inspection of the nitrogen metabolism concept revealed three pathway components eliciting differential humoral response. These include two functionally similar glutamate dehydrogenase enzymes, GLUD1 and -2, and carbonic anhydrase II (Fig. 3B and supplemental methods). We next sought to validate this putative up-regulation of the metabolites glutamate and aspartate mediated by humorally re-
active GLUD1 and -2. Independently our laboratory generated high throughput quantitative profiling of relative levels of metabolites in 16 benign prostate tissues and 12 localized prostate cancer samples. Interestingly this analysis demonstrated increased levels of both glutamate and aspartate in the prostate cancer specimens (Fig. 3B). Furthermore the metabolomics data reveal elevated levels of additional metabolites in the urea cycle (data not shown), both results independently validating this model.

Thus, using MCM we were able to directly extend our interrogation beyond single molecule targets to examine global changes in biological pathways. Nevertheless there existed a nested set of significant concepts, including multiple STAT family promoter binding sites as well as c-Ets-1 68 (p68 splice variant) sites, that in isolation were not revealing. This was especially noteworthy as both have been shown to play an important role during prostate cancer development/progression (23–27). This is not unexpected as we are measuring autoantibody response, which unlike protein expression is an indirect measure of protein abundance or modification state and is highly variable depending on the immunogenicity of targets and their exposure to the immune system. Given this situation, one could imagine a scenario where a change in a group of proteins under the control of a common regulator can be attributed to a holistic change in the programming controlled by the regulator. In other words, change in levels of the master regulator could lead to a cascade of expression changes of its targets, only some of which will subsequently elicit a humoral response. Thus, it was important for us to understand the fate that results from dysregulation of such regulatory components (in this case, the five concepts enriched in our MCM analysis). In an attempt to interrogate this aspect, we executed individual MCM enrichment extensions for five concepts highlighted in Figs. 3A and 4A (see “Experimental Procedures”).

Interestingly extension of the four STAT concepts and the c-Ets-1 68 binding site concept revealed a common and systematic theme of immune modulation in a common enrichment network (Fig. 4A). The extended concepts reveal a high count of immunomodulatory events that included acute phase response, complement and coagulation cascades, chemokine activity, and more. There was a significant overlap of the STAT-controlled gene program with those regulated by ETS family transcription factors and androgen receptor, all of which have been critically implicated in prostate cancer (23, 24). This analysis also identified an overlap with gene expression profiles that were down-regulated upon treatment with a variety of anti-inflammatory drugs, further confirming the existence of an underlying immune modulation theme in the humoral signature (26).

To confirm the existence of STAT-induced immune programming, we completed a meta-analysis, seeded by a STAT-regulated immune signature (see “Experimental Procedures”), with six prostate gene expression signatures profiling different comparisons between normal prostate, BPH, and prostate carcinoma using Oncomine (Fig. 4, B and C) (28–30). There is a dominant pattern of overexpression for this STAT-regulated immune signature in clinically defined carcinoma relative to the benign condition in each study (Fig. 4B). Additionally a subset of immune-regulatory genes under STAT control was significantly enriched in BPH compared with normal tissue, and the immune activity of PCs was found to be higher than BPH (Fig. 4C). It is important to note that our study design compares the immune response profile between two immunologically active cohorts and does not include profiling of normal sample due to the ambiguities of defining such a cohort of age-matched individuals for prostate disease. In an attempt to address the paucity of humoral targets in the cancer-specific signature and appreciating the caveat of low concordance between gene and protein expression, our aim was to identify a difference in expression among a set of immunomodulatory targets across a broad set of study data.

DISCUSSION

By coupling multidimensional protein fractionation with protein microarrays this proof-of-principle study demonstrates the power of immune system-driven autoantibody response in detection of prostate cancer. Through its ability to detect proteomic alterations, the autoantibody response can reveal dysregulated biological processes during cancer development and progression. Furthermore this study utilizes a clinically challenging population in which both the BPH and localized cancer patients have high amounts of circulating PSA, the current clinical standard for prostate cancer detection. Importantly only biopsy results were used to cull the cancer subgroup in this patient cohort. Needle biopsy by itself, in addition to being invasive, has a well documented false negative rate, missing 28% of existing prostate cancers (31). This adds to the complexity of the study cohort as a benign subset (as defined by negative biopsy) may very well harbor neoplasm that went undetected. Given this caveat, it was interesting that the 20-fraction tumor-specific humoral signature could classify with 75% specificity and 78% sensitivity, respectively. Interestingly in addition to known autoantibody targets like prostatic acid phosphatase and PSA, the PCA-specific humoral signature contained proteins that could play a role during tumor development and progression. For instance, the humoral signature included two proteins in the fibroblast growth factor receptor signaling pathway, which has been implicated in prostate cancer development and progression (32–34). Different predicted molecular phenotypes of increased FGF signaling include increased motility, invasiveness, proliferation, and androgen independence, all of which are ultimately thought to promote tumor progression (34). Notably elevated machinery regulating motility/invasion was evident in the PCa-specific compendium of humoral tar-

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2 A. Sreekumar, manuscript in preparation.
gets that included known regulators of actin cytoskeletal re-organization, namely calponin 1 (CNN1), Was/wasl-interacting protein family member 2 (WICH2), and valosin-containing protein. Also FGF is known to potentiate tumor progression by signaling through various pathways that include the STAT pathway (34), components of which were present in the cancer-specific humoral signature.

The ability to correlate the humoral signature with actual

**Fig. 4.** An extended molecular concept analysis of a cluster of concepts. A, the extension enrichment results (as described in the text) identifying the systematic immunomodulation and gene reprogramming of the differential immune response enriching the five promoter binding site concepts boxed in Fig. 3A. Edge color in this merged network reflects the original concept-to-concept enrichment from which it was derived: edges exclusive to STATx are pink, those from the STAT3 enrichment are blue, those exclusive to the STAT5 enrichment are gray, those from c-Ets-1 68 are green, and any edge shared among multiple enrichments from the original five are black. B, a metamap analysis of six gene expression signatures between benign prostate, BPH, or carcinoma and the immune program under STAT control. Genes are as indicated (rows); red reflects increasing statistical significance of the up-regulated expression of the given gene (see color bar) between either benign or BPH tissue and prostate carcinoma in each study (columns), whereas white indicates unchanged expression between tissue types. This demonstrates systematic overexpression of genes in either inflammatory BPH or cancer tissues relative to normal prostate. Highlighted is a study expressing base-line immune-specific expression in BPH relative to benign tissue. C, heat maps of the top 20 genes overexpressed in three comparisons between normal, BPH, and PCa (B. Laxman, unpublished data). Columns represent individual arrays, and rows represent the indicated feature. Red and blue indicate relative overexpression and underexpression, respectively, and gray indicates features excluded during normalization. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; N.S., not significant; AR, androgen receptor; PR, progesterone receptor; MSA, methylseleninic acid.

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cellular processes emphasizes the importance of using a screening platform containing proteins extracted from tumor, itself reflecting a physiologically realistic swath of the prostate cancer proteome. Such correlations to tumor function would be difficult to recapitulate using proteins fractionated from cell lines (35, 36) or using phage array platforms (15, 37). In addition, the multidimensional protein fractionation-coupled microarray retains post-translational modifications that are most indicative of the cellular phenotype and in most cases better reflects the reality of humoral response to cancer antigens. This is best illustrated by the observation of phosphorylated PSA as a target antigen in our data set (data not shown).

In addition to being sensitive to changes in protein levels or modifications, the humoral response generation could be driven by other factors like the presence of anti-idiotypic antibodies that constitute the causal mechanism behind the observation of humoral response against the autoantigen. Such is proven to be the case for Wegener granulomatosis autoantigen or PRNT3, autoantibodies to which are found in inflammatory vascular disease (38). These studies indicate that, during vascular inflammation, there occur complementary, or antisense, peptides that can bind to PRNT3, termed cPR-3, that are the initial targets for generation of autoantibodies. These cPR-3-specific idiotypic antibodies then elicit the anti-idiotypic response, antibodies originating from which can bind to the parent antigen PRNT3 (38). In our case, a similar concept may govern the presence of autoantibodies to GLUD1/GLUD2 for which HSP70-like protein has been shown to be the antisense gene pair in molds (39). Furthermore HSP70 has been shown to elicit autoantibodies to itself (40, 41). Accordingly one might predict that reactivity of prostate cancer serum to GLUD1/2 may potentially follow a mechanism similar to the one reported for PRNT3 (38). Alternately it is worth noting that both GLUD1 and GLUD2 are key regulators of nitrogen metabolism and downstream urea cycle activity. This gains importance in the context of our earlier gene expression-based analyses that revealed increased protein biosynthesis in localized prostate cancer (20). The breakdown of the resulting proteins could lead to increased accumulation of elemental nitrogen that will have to be eliminated through nitrogen breakdown and urea cycle pathways. Our humoral response data coupled with independent assessments showing increased levels of glutamate, aspartate, and constituent urea cycle metabolites in localized prostate cancer corroborate our gene expression-based hypothesis. Of course the metabolic flux of both GLUD1 and -2 as well their potential anti-idiotypic antibody response in prostate carcinoma requires further investigation.

In addition to drawing direct correlates between humoral targets and known pathway alterations in tumors, it was intriguing to observe proteins in the STAT pathway, which by themselves did not enrich for any known tumor-associated processes. Specificity of these STAT pathway-associated proteins to prostate cancer is validated by their absence in a random humorally nonreactive signature (see “Experimental Procedures”). Moreover it has been observed that STATs are regulated by FGF, the activity of which, according to our data and previous reports, is elevated in prostate cancer (34). Also elevation of STAT signaling has been reported in prostate cancer (42). Moreover it is known that proteins differ in their ability to generate an antibody response (43). Accordingly it was tempting to speculate that the existence of a group of proteins in a given pathway in our prostate cancer-specific humoral signature may signify global reprogramming of that pathway, in this case the global perturbation of the STAT pathway. We further interrogated the data set for enrichment of the STAT-regulated proteome with MCM, which revealed multiple immunomodulatory proteins as defined by an earlier gene expression study (21). This STAT-induced dysregulation of immunomodulatory components was validated by meta-analyses of independent prostate cancer data sets. The meta-analysis reveals the existence of high levels of basal immunoreactivity in BPH, which is further inflated during tumor development. This is not only consistent with reports of neoplastic development occurring on a background of focal inflammation (44) but also explains the challenge associated with discerning the two classes, namely BPH and biopsy-proven cancer, based on their humoral response profiles as reported in this study. Thus, we highlight the ability of autoantibody repertoires to uncover alterations in biological processes that might otherwise not be revealed by standard protein profiling platforms because of the vast dynamic range of the proteome.

In summary, this study for the first time uses autoantibodies generated against the tumor proteome to classify a clinically challenging cohort of patients comprising BPH and biopsy-proven PCa and, in the process, reveals multiple alterations in tumor-associated functional pathways that would otherwise not be discernable by conventional profiling strategies.

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REFERENCES


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