ORIGINAL ARTICLE

Autoantibody Signatures in Prostate Cancer

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ABSTRACT

BACKGROUND

New biomarkers, such as autoantibody signatures, may improve the early detection of prostate cancer.

METHODS

With a phage-display library derived from prostate-cancer tissue, we developed and used phage protein microarrays to analyze serum samples from 119 patients with prostate cancer and 138 controls, with the samples equally divided into training and validation sets. A phage-peptide detector that was constructed from the training set was evaluated on an independent validation set of 128 serum samples (60 from patients with prostate cancer and 68 from controls).

RESULTS

A 22-phage-peptide detector had 88.2 percent specificity (95 percent confidence interval, 0.78 to 0.95) and 81.6 percent sensitivity (95 percent confidence interval, 0.70 to 0.90) in discriminating between the group with prostate cancer and the control group. This panel of peptides performed better than did prostate-specific antigen (PSA) in distinguishing between the group with prostate cancer and the control group (area under the curve for the autoantibody signature, 0.93; 95 percent confidence interval, 0.88 to 0.97; area under the curve for PSA, 0.80; 95 percent confidence interval, 0.71 to 0.88). Logistic-regression analysis revealed that the phage-peptide panel provided additional discriminative power over PSA ($P<0.001$). Among the 22 phage peptides used as a detector, 4 were derived from in-frame, named coding sequences. The remaining phage peptides were generated from untranslated sequences.

CONCLUSIONS

Autoantibodies against peptides derived from prostate-cancer tissue could be used as the basis for a screening test for prostate cancer.

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Limitations of the prostate-specific antigen (PSA) test for the early detection of prostate cancer[1] indicate the need for other means of screening for this neoplasm. The finding that patients with cancer produce autoantibodies against antigens in their tumors[2-7] suggests that such autoantibodies could have diagnostic and prognostic value.[4,5,8-10] For example, mutant forms of the p53 protein elicit anti-p53 antibodies in 30 to 40 percent of patients with various types of cancers.[11] Recently, we found that patients with prostate cancer produce antibodies against α-methylacyl-coenzyme A racemase,[12] an overexpressed protein in epithelial cells in prostate cancer.[13-15] This autoantibody had 72 percent specificity and 62 percent sensitivity in detecting prostate cancer.[12] The use of additional prostate-cancer antigens could improve the sensitivity and specificity of an autoantibody-based screening test for prostate cancer.

Here we report the use of a technique that combines phage-display technology with protein microarrays to identify and characterize new autoantibody-binding peptides derived from prostate-cancer tissue. A similar approach has been used to identify selected antigens for the diagnosis of breast cancer.[16] This emerging area of research, termed "cancer immunomics," allows a global analysis of the autoantibodies against antigens in a neoplasm (see the Glossary for definitions of terms).

Methods

Populations and Samples

This study, which was approved by the institutional review board of the University of Michigan Medical School, started in March 2003 and ended in December 2004. It had discovery, training, and validation phases. All serum samples, unless otherwise indicated, were obtained from patients in the University of Michigan Health System. Written informed consent was obtained from all patients.

The tissue and serum bank at the University of Michigan Specialized Research Program in Prostate Cancer has collected more than 2000 serum samples since 1995. Of these, 331 samples, which were collected from 1995 to 2004, met the following eligibility criteria: they were obtained immediately before surgery from patients with biopsy-proven, clinically localized prostate cancer who were at least 40 years old and who had received no previous prostate-cancer therapy. Of the 331 sam-

Glossary

Autoantibody signature: A molecular "fingerprint" of autoantibodies produced against a disease state.

Biopanning: An iterative process of affinity purification used in the identification and isolation of phages carrying peptides with high affinity and specificity to a given target molecule. Briefly, a library of phage-displayed peptides is incubated with a plate (or bead substrate) coated with the target. Unbound phage particles are washed away, and the bound phages eluted. This process is then repeated several times.

Dendrogram: A treelike diagram that summarizes the process of clustering. Similar cases are joined by links whose position in the diagram is determined by the level of similarity between the cases.

Epitope: A molecular region on the surface of an antigen that is capable of eliciting an immune response and of combining with the specific antibody produced by such a response. The epitope is also known as an antigenic determinant.

Genetic algorithm: An optimization algorithm based on the mechanisms of Darwinian evolution that uses random mutation, crossover, and selection procedures to breed better models or solutions from an originally random starting population or sample.

Heatmap display: A method of visualizing results of a microarray analysis.

Hierarchical clustering: A computational method that uses similarity among data to group genes or samples into clusters. The result is typically represented by a cluster tree or dendrogram.

Humoral immune response: An immune response in which the body mounts a defense against microorganisms, viruses, and substances that it recognizes as foreign and potentially harmful. The defense involves antibodies that are secreted by B cells. The B cells are activated when a specific antigen binds to the antibody, which is located on the surface of the B cells. Plasma B cells release antibodies specific for the antigen.

Leave-one-out cross-validation: A validation method in which one sample is withheld, and a model is built only on the remaining samples and used to predict the class of the withheld samples. The process is repeated for each sample, and the cumulative error rate is calculated.

Mimotope: A sequence of amino acids (or other chemical entities) that specifically binds to the antigen-binding region of an antibody and in sequence is unrelated to the sequence of the antigen that the antibody was raised against.

Nearest-neighbor analysis: A method of searching expression patterns of genes. Briefly, an "idealized expression pattern" is defined, corresponding to a gene that is uniformly high in one class and uniformly low in the other. The analysis determines whether there is an unusually high density of genes "nearby" (that is, similar to) this idealized pattern, as compared with equivalent random patterns.

Nonparametric-pattern-recognition approach: A pattern-recognition approach that does not require prior knowledge but only a large amount of data.

Phage display: A technique in which bacteriophages are engineered to fuse a foreign peptide or protein with their capsid proteins and hence expose or display it on their external surface. The immobilized phage may then be used as a screen to see which ligands bind to the expressed fusion protein displayed on the phage surface.

Phage-protein microarray: Phage lysates expressing diverse peptide fusion proteins are spotted in an arrayed format onto a coated substrate. These phage peptides can then serve as bait to capture specific autoantibodies in serum.

Supervised analysis: An analysis of the results of microarray profiling that takes external factors into account.

Unsupervised analysis: An analysis of the results of microarray profiling that does not take into account external factors, such as survival or clinical measures.

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ples, 150 were randomly chosen for the discovery, training, and validation phases of the study. Four samples were excluded because proteins had precipitated in the serum vials. Twelve samples that fulfilled the criteria were obtained from the Dana–Farber Cancer Institute for the validation phase in order to explore the robustness of the assay across institutions. These samples were chosen randomly from 236 samples that met the eligibility criteria.

The University of Michigan Clinical Pathology Laboratories provided 159 control samples of serum from men between the ages of 46 and 83 years who had no history of cancer. These samples were collected from 2001 to 2004 in three independent collection periods. An additional 55 samples (designated as the "other" category) were collected from patients after prostatectomy (14 samples) or from men with either advanced hormone-refractory prostate cancer (11) or lung cancer (30, randomly selected from 74 samples obtained from male patients). Details regarding the serum samples that were used in our "other" cohort are in the Supplementary Appendix (available with the full text of this article at www.nejm.org).

In the discovery phase (biopanning and 2304-element microarrays), 39 prostate-cancer samples and 21 control samples were used. The training phase involved the use of 59 prostate-cancer samples and 70 control samples. To evaluate the phage-peptide detectors that we developed in the discovery and training phases, we used an independent validation set of 60 prostate-cancer samples (48 from the University of Michigan and 12 from the Dana–Farber Cancer Institute) and 68 control samples. In addition, 55 samples in the "other" category were assessed exclusively in the validation phase.

In the 257 prostate-cancer samples and control samples (which included the training and validation sets [Table 1]), the median levels of PSA were 6.3 ng per milliliter (range, 0.1 to 46.3) and 1.7 ng per milliliter (range, 0.1 to 24.5), respectively.

AUTOANTIBODY PROFILING

By iterative biopanning (see Glossary) of a phage-display library derived from prostate-cancer tissues, we developed phage protein microarrays and used them to develop an autoantibody signature to distinguish samples with prostate cancer from those of controls. Details concerning the construction of phage-display libraries and preparation of the phage-protein microarrays are given in the Supplementary Appendix and shown in Figure 1.

Figure 1 (facing page). Schematic Representation of the Development of Phage-Protein Microarrays to Characterize Autoantibody Signatures in Prostate Cancer (Discovery Phase).

A cDNA library was constructed from a pool of total mRNA isolated from prostate-cancer tissue obtained from six patients. After digestion, the cDNA library was inserted into the T7 phage vector. The T7 fusion vectors were then packaged into T7 phages to generate a prostate-cancer cDNA T7 phage-display library. To enrich the library with clones of peptides reacting with human serum from patients with clinically localized prostate cancer and not with serum from controls, several cycles of affinity selections (biopanning) were performed. Briefly, the phage libraries were preadsorbed onto purified IgGs from the control pool of serum samples (from 10 patients) to remove nonspecific clones. Next, the precleared phage libraries were enriched for cancer-specific peptides with the use of a pool of IgGs purified from the serum of 19 patients with prostate cancer. The bound phages were eluted and propagated by infecting bacterial cells. After five rounds of biopanning, enriched prostate-cancer–specific peptide clones were cultured onto LB agar plates. A total of 2304 single colonies, including T7 empty phage clones as negative spots and antihuman IgG as positive spots, were randomly picked and propagated into 96-well plates. Phage-clone lysates were then printed onto coated glass slides with the use of a robotic spotter to create a phage-protein microarray. Cy5 (red fluorescent dye)–labeled antihuman antibody was used to detect IgGs in human serum that were reactive to peptide clones, and a Cy3 (green fluorescent dye)–labeled antibody was used to detect the phage capsid protein in order to normalize for spotting. Thus, if a phage clone carries a peptide that is reactive to human IgG, after scanning, this spot will be yellow in color; otherwise, the spot will remain green, representing an unreactive clone. A total of 31 samples (20 from patients with cancers and 11 from controls) were tested on the 2304 phage-peptide microarray. Analysis of these 31 samples identified 186 phage peptides with the highest level of differentiation between cancers and controls, which were then used to develop focused microarrays for analyses in the subsequent training and validation phases.

STATISTICAL ANALYSIS

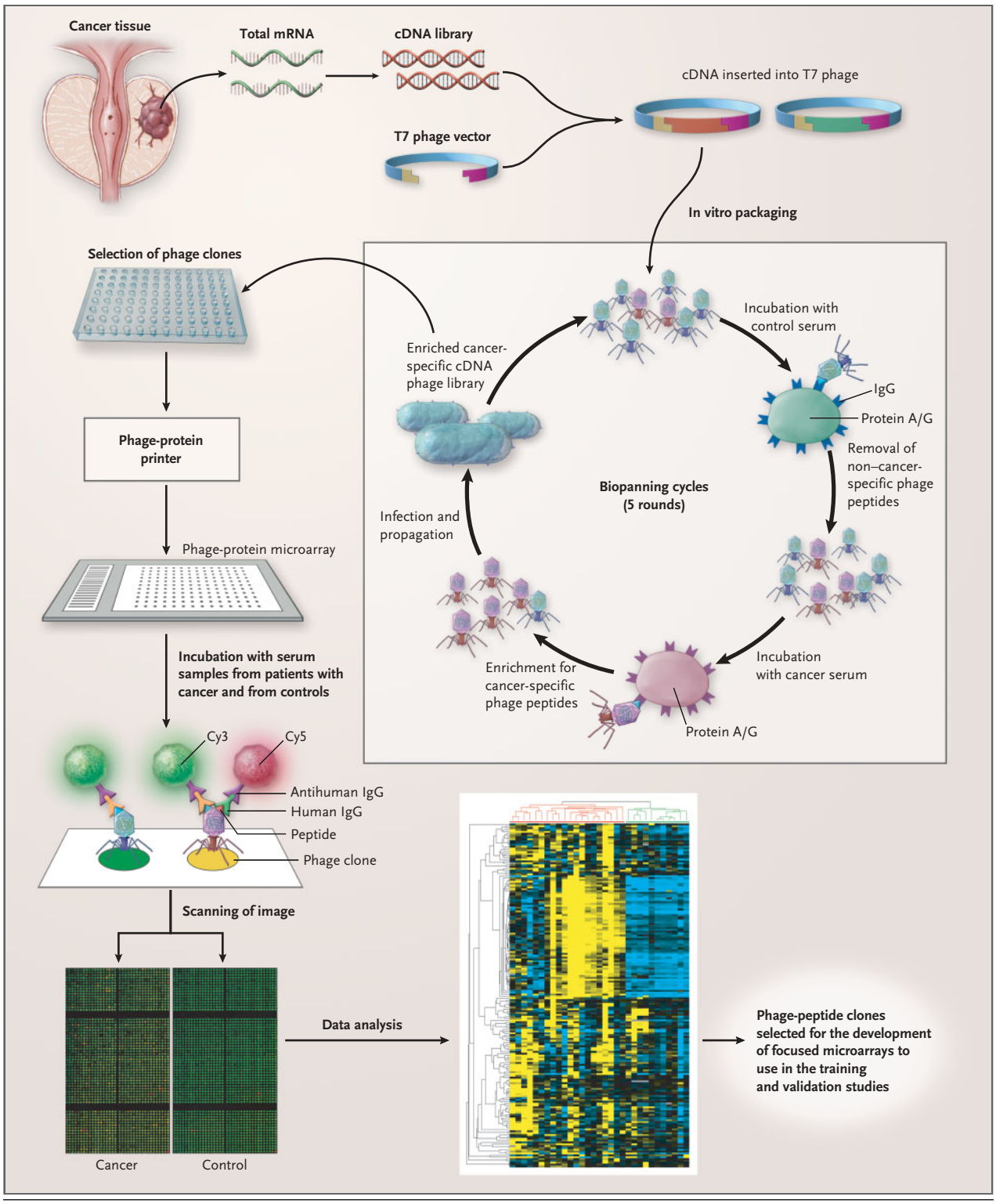
Statistical analyses were performed with SPSS software, version 11.5, and R 2.0. Details are given in the Supplementary Appendix.

RESULTS

DEVELOPMENT OF PHAGE-PROTEIN MICROARRAYS (DISCOVERY PHASE)

To develop a phage-display library of prostate-cancer peptides, we isolated messenger RNA (mRNA) from prostate-cancer tissue obtained from six pa-

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tients with clinically localized disease (Table 1 of the Supplementary Appendix). After the insertion of the complementary DNA (cDNA) fragments into the T7 phage system, peptides that were encoded by the prostate cancer cDNA were expressed and displayed on the surface of the phage fused to the C-terminal of the capsid 10B protein of the phage. This surface complex functioned as bait to capture autoantibodies in serum.

Table 1. Clinical and Pathological Information for the Training and Validation Samples.*

Variable	Training Set	Validation Set
Patients with clinically localized prostate cancer		
No. of patients	59	60†
Age (yr)	58.3±7.7‡	60.81±9.0‡
Gland weight (g)	49.55±17.17§	51.78±19.57¶
Diameter of maximal tumor (cm)	1.44±0.75§	1.62±0.97¶
PSA level		
Mean (ng/ml)	6.19±4.58‡	10.45±9.52‖
0–2.4 ng/ml (%)	17.2	7.7
2.5–10 ng/ml (%)	67.2	53.8
4–10 ng/ml (%)	50.0	42.3
>10 ng/ml (%)	15.5	38.5
Gleason grade (%)		
≤6	35.7**	37.3
≥7	64.3**	62.7
Primary tumor identification (%)		
T2a	29.8‡	43.7††
T2b	59.6‡	41.7††
T3a	3.5‡	2.1††
T3b	7.0‡	12.5††
Controls with no known history of cancer		
No. of patients	70	68
Age (yr)	62.8±8.6‡‡	63.6±9.3§§
PSA level		
Mean (ng/ml)	2.88±2.57	3.01±2.68¶¶
0–2.4 ng/ml (%)	61.4	59.7
2.5–10 ng/ml (%)	38.6	34.3
4–10 ng/ml (%)	32.9	29.9
>10 ng/ml (%)	0	5.9

* Plus–minus values are means ±SD. PSA denotes prostate-specific antigen.
† Number includes 12 prostate samples from the Dana–Farber Cancer Institute.
‡ Data were available for 58 patients.
§ Data were available for 54 patients.
¶ Data were available for 29 patients.
‖ Data were available for 52 patients.
** Data were available for 57 patients.
†† Data were available for 48 patients.
‡‡ Data were available for 69 patients.
§§ Data were available for 68 patients.
¶¶ Data were available for 67 patients.

Serum samples from 39 patients with prostate cancer and 21 controls were selected randomly from the University of Michigan serum cohorts for the discovery phase. To enrich the library for peptides that bind specifically to autoantibodies in patients with prostate cancer, we carried out successive rounds of selection (Fig. 1). The procedure entailed removal of irrelevant phages from the library with the use of protein A/G beads that were coated with a pool of IgG antibodies isolated from 10 randomly selected control serum samples (Fig. 1, and Table 2 of the Supplementary Appendix). These beads were incubated with the phage particles, and the supernatant, which contained unbound phage particles, was collected. These residual phages, now freed of phage particles that bind to irrelevant antibodies in normal serum, were enriched for phage particles that express prostate-cancer–specific peptides by incubation with protein A/G beads coated with a pool of IgG antibodies from 19 patients with prostate cancer who were chosen randomly from the University of Michigan serum collection (Fig. 1, and Table 2 of the Supplementary Appendix). Finally, the adherent phages were eluted from the beads and propagated in bacterial cells.

We carried out five such rounds of purification (biopanning). Phage clones, each bearing a single fusion peptide derived from the prostate-cancer cDNA library, were selected randomly from the purified library to generate protein microarrays on coated glass slides with the use of a robotic spotter. Once in a microarray format, the enriched phage clones were used to test serum for autoantibodies against prostate cancer peptides.

Initially, we selected 2304 individual phage clones, including 5 empty clones as negative controls, from the enriched phage library and constructed a high-density protein microarray (Fig. 3 of the Supplementary Appendix). To decrease the complexity of subsequent validation studies and develop a focused array, we randomly selected 20 serum samples from patients with cancer and 11 samples from controls from the respective University of Michigan collections (Table 3 of the Supplementary Appendix) and screened the high-density phage microarray. Of the 20 samples from patients with prostate cancer, 19 contained antibodies that reacted with phage-peptide clones on the microarrays, whereas only 1 of 11 controls had such antibodies (Fig. 3 of the Supplementary Appendix). After normalization of all values obtained by the scanner, we selected phage-peptide clones that

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yielded a ratio of Cy5 to Cy3 greater than 1.2 in at least one of the serum samples. This analysis identified 186 phage-peptide clones that reacted with serum samples from patients with prostate cancer. These clones, along with negative-control phage clones, were used to construct a smaller, focused protein microarray for subsequent screening of serum samples (training and validation phases).

IDENTIFICATION OF A PHAGE-PEPTIDE DETECTOR (TRAINING PHASE)

Figure 2 shows the training and validation phases of this study. A total of 257 serum samples from 119 patients with clinically localized prostate cancer and 138 controls, plus 55 samples in the "other" category, were tested on the 186-element focused arrays (Table 1 and Tables 4 through 9 of the Supplementary Appendix). In the training phase, we analyzed 59 samples from patients with prostate cancer and 70 control samples (Fig. 2). An algorithm[17] was used to evaluate whether the sample size we profiled was sufficient to build a classifier for clinical diagnosis. On the basis of this algorithm and with the use of the specific measures of our phage-protein microarray data, we found that a mean (±SD) of 46±40 samples from 1000 simulation runs would be sufficient to ensure with 95 percent confidence that the probability of the misclassification of a future sample would be less than 0.15. Figure 3 depicts representative scanned arrays. To create a "class detector," we used a nonparametric-pattern-recognition approach that consisted of a genetic algorithm combined with k-nearest neighbor to select a subgroup of "informative" phage peptides based on leave-one-out cross-validation on the training samples. We identified a panel of 22 phage-peptide clones that could best distinguish serum samples from patients with prostate cancer from those of controls, with 97.1 percent specificity (2 of 70 control samples were misclassified) and 88.1 percent sensitivity (7 of 59 prostate-cancer samples were misclassified) in the training set (Tables 10 and 11

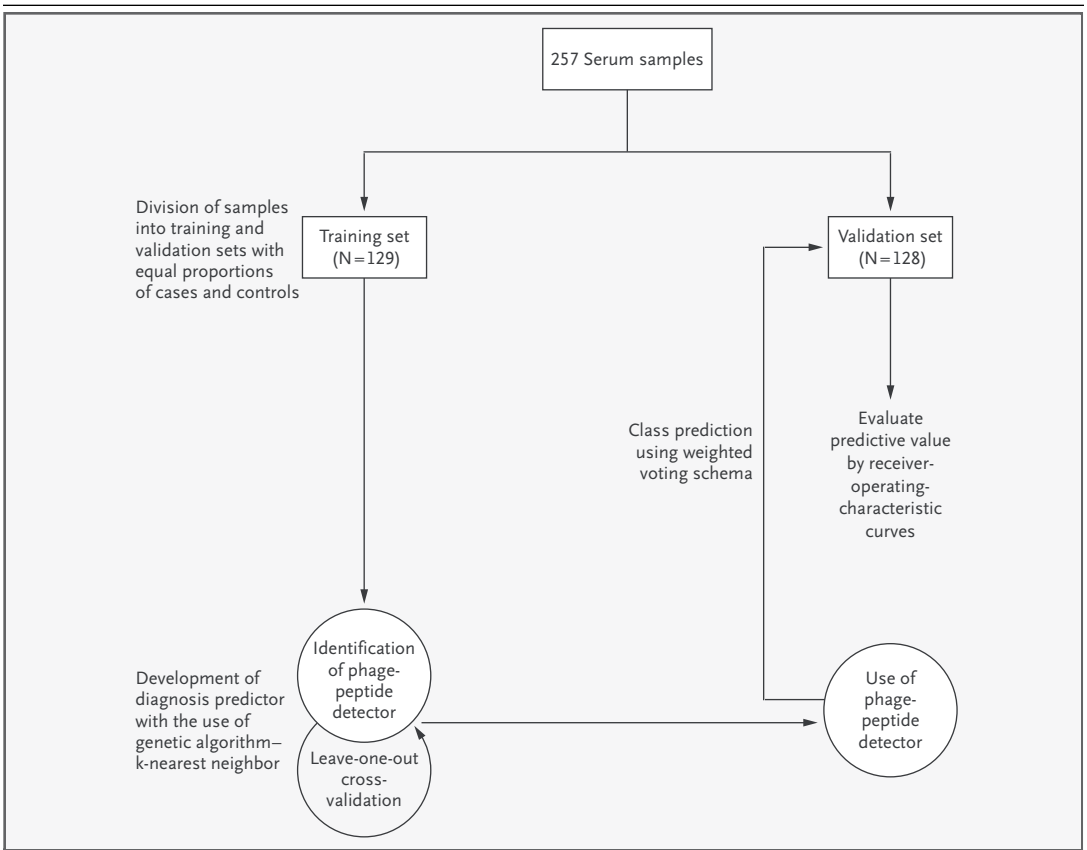


Figure 2. Overview of the Strategy Used for the Development and Validation of Autoantibody Signatures to Identify Prostate Cancer (Training and Validation Phases).

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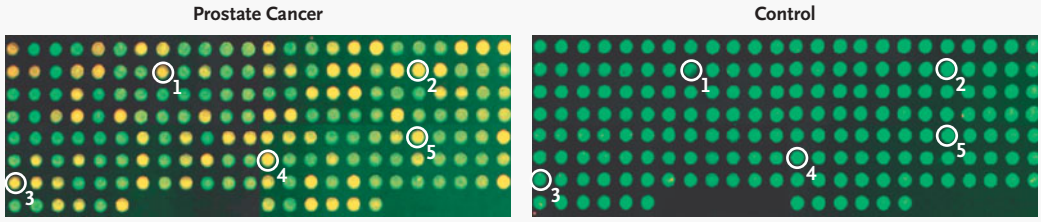


Figure 3. Autoantibody Signatures in Prostate Cancer.

Representative images of phage-protein microarrays demonstrate the difference in immunoreactivity between samples from patients with prostate cancer and those from controls. Yellow spots represent immunoreactivity in serum samples, and green spots no reactivity. Circled spots represent five phage-peptide clones: 5'-UTR_BMI1 (1), eIF4G1 (2), RPL13a (3), BRD2 (4), and RPL22 (5).

of the Supplementary Appendix). Figure 4A shows a heatmap of the results with the 22 phage-peptide clones in the training set.

VALIDATION OF THE PHAGE-PEPTIDE DETECTOR

With the use of a 22-phage-peptide detector derived from the training phase, we applied a weighted voting scheme to classify samples in the independent validation set (128 patients) as either prostate cancer or control (Fig. 2). In total, 8 of 68 serum samples from controls and 11 of 60 samples from patients with prostate cancer were misclassified in this validation set (Fig. 4B, and Table 12 of the Supplementary Appendix). These results yielded a specificity of 88.2 percent (95 percent confidence interval, 78 to 95) and a sensitivity of 81.6 percent (95 percent confidence interval, 70 to 90). Similar performance criteria were observed with the use of different class-prediction models and a second randomization of the data set (Section II of the Supplementary Appendix).

We next calculated receiver-operating-characteristic curves for the 22-phage-peptide detector and PSA levels in the validation set. Different cutoff values of weighted voting scores were used as threshold points to plot the true positive rate against the false positive rate for the prediction model. The ability of the panel of 22 phage peptides to discriminate between prostate-cancer samples and control samples was significant ($P<0.001$), with an area under the curve equal to 0.93 (95 percent confidence interval, 0.88 to 0.97) (Fig. 4C). The area under the curve for PSA was 0.80 ($P<0.001$; 95 percent confidence interval, 0.71 to 0.88). This result was expected, since these patients were identified primarily by elevated PSA levels. Among patients with PSA levels of 4 to 10 ng per milliliter in the validation set, the phage-peptide detector had significant discriminatory power ($P<0.001$) as compared with PSA ($P=0.50$) in distinguishing serum samples from patients with prostate cancer from those of controls. The area under the curve was 0.93 (95 percent confidence interval, 0.86 to 1.00) for the phage-display method and 0.56 (95 percent confidence interval, 0.38 to 0.74) for PSA (Fig. 4D). When the lower limit of PSA was decreased to 2.5 ng per milliliter, the discriminatory power of the phage-peptide profile was maintained ($P<0.001$), with an area under the curve of 0.94 (95 percent confidence interval, 0.88 to 1.00), whereas that for PSA decreased slightly to 0.50 (95 percent confidence interval, 0.33 to 0.66) (Fig. 4E, and Section III of the Supplementary Appendix).

We also used the 22-phage-peptide detector to test the 55 serum samples in the "other" category. Among the samples obtained from patients after prostatectomy, 5 of 14 samples were classified as prostate cancer, and 3 of 11 samples from patients with hormone-refractory disease were classified as prostate cancer (Table 13 of the Supplementary Appendix). Of the 30 samples from patients with lung adenocarcinoma, 9 were classified as having prostate cancer, which suggests some cross-reactivity of autoantibodies across tumor types.

CHARACTERIZATION OF THE PHAGE-PEPTIDE DETECTOR

The panel of 22 phage-peptide clones was sequenced. Of these, five were in-frame and within known expressed sequences (Table 16 of the Supplementary Appendix). These five included bromodomain-containing protein 2 (BRD2), eukaryotic

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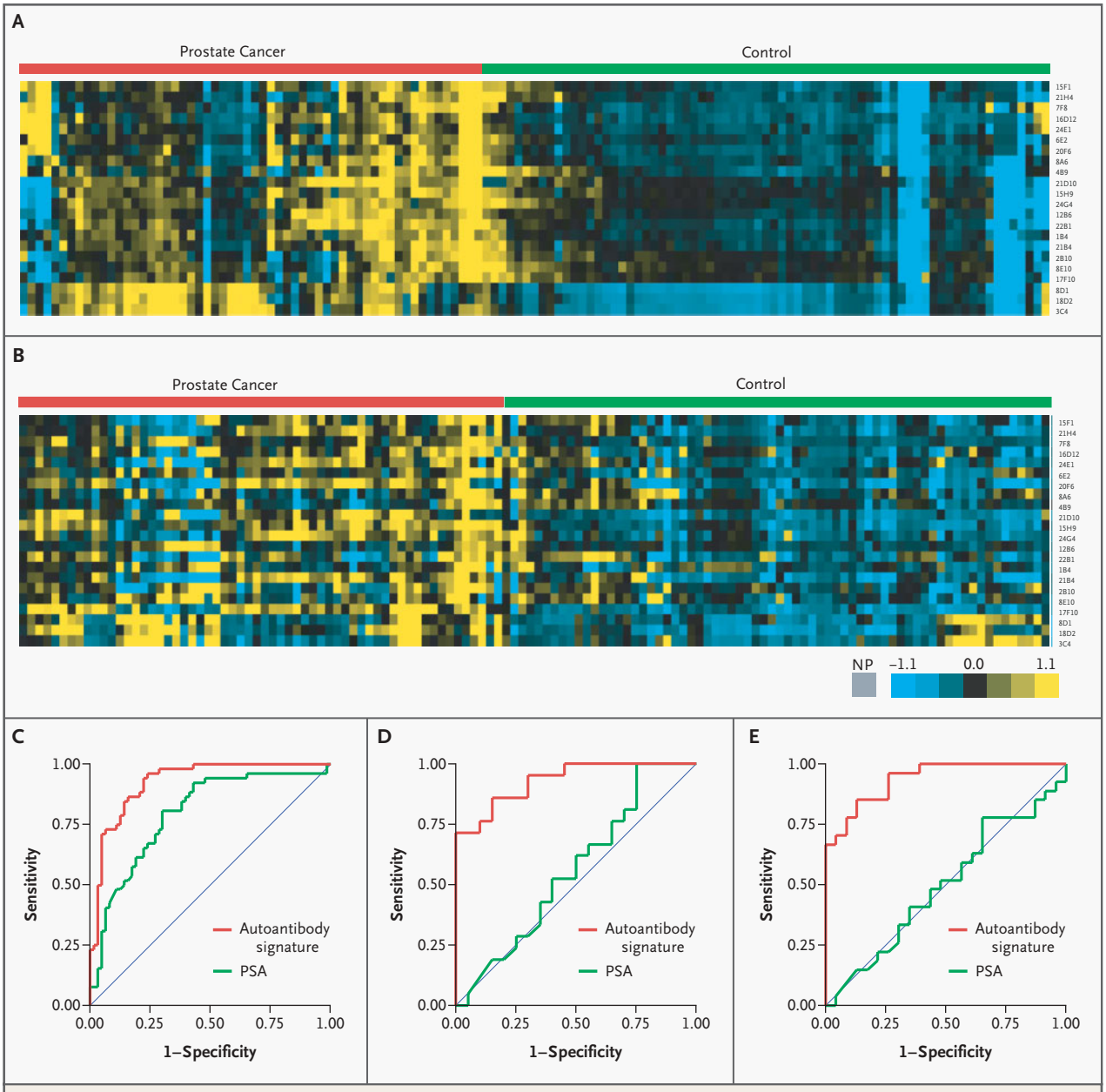


Figure 4. Supervised Analyses and Validation of Autoantibody Signatures in Prostate Cancer.

Representations are shown of heatmaps of 22 phage peptides analyzed for immunoreactivity across 129 training samples (Panel A) and for an independent validation set of 128 serum samples from patients with prostate cancer and from controls (Panel B). Individual peptide clones were represented in rows, whereas serum samples were represented in columns. Yellow indicates positive immunoreactivity, and black or blue no immunoreactivity. In Panel C, the performance of the 22-phage-peptide detector is compared with prostate-specific antigen (PSA) in the validation set. Receiver-operating-characteristic curves are based on multiplex analysis of the 22-phage-peptide biomarkers and serum PSA from a total of 128 samples (60 from patients with prostate cancer and 68 from controls). The red line indicates the 22-phage-peptide detector, and the green line indicates the PSA test. Panel D shows the performance of the 22-phage-peptide detector in patients with PSA levels between 4 and 10 ng per milliliter. The samples were a subgroup of the 128-sample validation group, with a total of 42 samples (22 from patients with cancer and 20 from controls). Panel E shows the performance of the 22-phage-peptide detector in patients with PSA levels between 2.5 and 10 ng per milliliter. The samples were a subgroup of the 128-sample validation group, with a total of 51 samples (28 from patients with cancer and 23 from controls).

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translation initiation factor 4 gamma 1 (eIF4G1), ribosomal protein L22 (RPL22), ribosomal protein L13a (RPL13a), and hypothetical protein XP_373908. Except for hypothetical protein XP_373908, these structures were derived from intracellular proteins involved in regulating either transcription or translation. The remaining 17 phage-peptide clones were either in untranslated regions of expressed genes or out of frame in the coding sequence of known genes. These clones may express peptides that are structurally similar to peptides in expressed proteins but are unrelated or weakly related at the protein-sequence level (Table 17 and section IV of the Supplementary Appendix).

To determine whether the four in-frame phage-peptide clones (Fig. 5A) are deregulated in prostate cancer, we performed a meta-analysis of publicly available data regarding gene expression in prostate cancer.[18-24] These analyses and a preliminary immunoblot analysis suggested that the four in-frame phage epitopes are overexpressed in prostate cancer (Fig. 5B and 5C, and sections V and VI of the Supplementary Appendix).

Figure 5 (facing page). Meta-Analysis of Gene Expression of Humoral-Immune-Response Candidates.

Panel A shows a heatmap representation of the immunoreactivity for four in-frame phage-peptide clones assessed across 257 serum samples. Yellow indicates immunoreactivity, and black or blue no immunoreactivity. Panel B shows relative levels of gene expression of in-frame phage-peptide clones assessed with the use of publicly available DNA microarray data on the Web site of Oncomine (www.oncomine.org). The first author of each DNA microarray study is provided, as are P values for each comparison — for example, benign versus localized prostate cancer (PCA) and PCA versus metastatic prostate cancer (MET). Panel C shows immunoblot validation of the overexpression of humoral-response candidates at the protein level in prostate cancer. Panel D shows expression of the humoral-response candidate eIF4G1 in prostate cancer by immunofluorescence staining. Subpanel 1 displays clinically localized prostate cancer (left) adjacent to a benign gland (right). Subpanel 2 displays magnifications of a single prostate-cancer gland. Stains for eIF4G1 (red), E-cadherin (green), and nuclei (blue) were used. The scale bar represents 5 μm. Panel E shows a histogram of staining intensity from immunohistochemical analysis. The open boxes represent benign tissue cores, and the black boxes represent tumor cores.

DISCUSSION

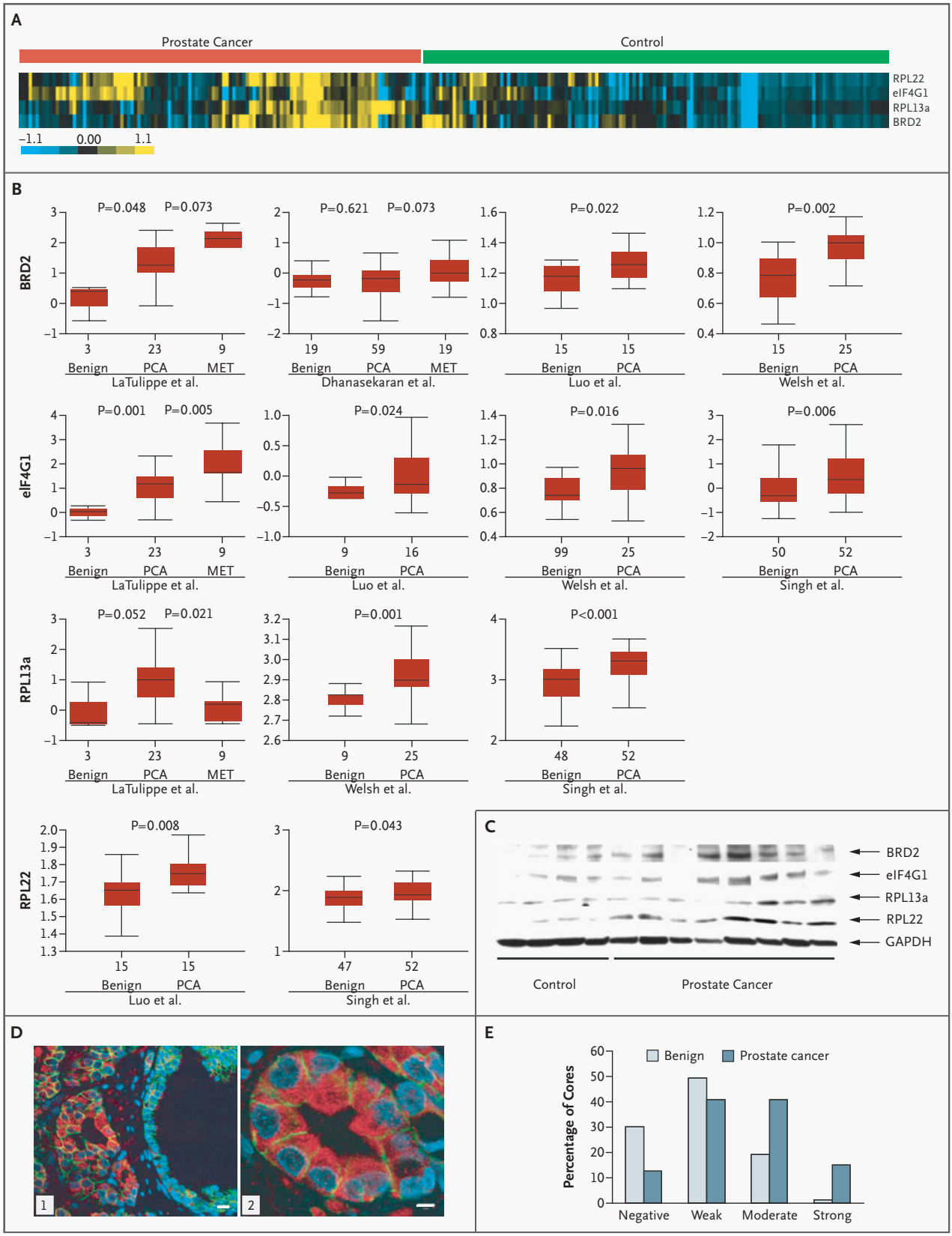
In this study, we used protein microarrays to identify autoantibodies against tumor antigens in patients with prostate cancer. Specifically, we constructed phage-protein microarrays in which peptides derived from a prostate-cancer cDNA library were expressed as a prostate-cancer–phage fusion protein. The phage-protein microarrays were screened to identify phage-peptide clones that bind autoantibodies in serum samples from patients with prostate cancer but not in those from controls.

The use of PSA-based screening for prostate cancer has risen dramatically since its introduction in the late 1980s.[25,26] However, reliance on PSA for the detection of early prostate cancer is still unsatisfactory, especially because of a high rate of false positive results[27] — as high as 80 percent.[28,29] This rate results in many unnecessary prostate biopsies.[30] To circumvent this and other problems of screening for prostate cancer, we have begun to evaluate the use of autoantibody signatures to detect prostate cancer. By relying on multiple immunogenic prostate-cancer peptides, this approach may be an improvement over a single biomarker such as PSA.

Serologic analysis of recombinant cDNA expression libraries of human tumors with autologous serum (SEREX) has demonstrated that antibodies in the serum of patients with cancer can be used to isolate new tumor antigens.[3,4] This technique, however, relies on one-step screening without affinity-selection steps and requires a large volume of serum to screen phage clones blotted onto membrane filters. The SEREX approach has limited clinical use, since it is not conducive to the analysis of hundreds of serum samples of small amounts.

By taking advantage of combinatorial screening and high throughput analysis of autoantibody repertoires, we developed a technique that overcomes the disadvantages of SEREX for cancer diagnosis. However, like gene-expression profiling and pattern-recognition approaches with serum proteomics, our method may have the limitations of background signals, sample-selection bias, and limited reproducibility.[31] To minimize these problems, immunoreactivity for each phage peptide was measured in relation to an internal control signal detected by antibody against phage capsid proteins. The discriminatory power of autoantibody signatures was validated by reshuffling and analyzing the training and validation sets with the use of various class-prediction models. The reproducibility of the assay was investigated by the use of experimental assays both within and among the arrays. The difference between duplicate peptides in the same ar-

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ray was less than 5 percent for 98 percent of the spots. Analyses of repeated experiments with the use of the same serum samples revealed that the results were very consistent, with a correlation coefficient greater than 98 percent.

The autoantibody signature was detected in only 5 of 14 serum samples from patients who had undergone prostatectomy and in 3 of 11 serum samples from patients with hormone-refractory disease — a finding suggesting that the autoantibody profile is attenuated on removal of the "immunogen" or treatment with antiandrogens, chemotherapeutic agents, or both. Of 30 serum samples from patients with lung cancer, 9 were classified as prostate cancer. This result is in contrast to the more than 80 percent sensitivity for prostate cancer with the use of the phage-peptide system, suggesting that the autoantibody profile has relative specificity for prostate cancer ($P<0.001$ by the proportion test).

Our results were consistent across a range of clinical and pathological features, including PSA level, Gleason grade, stage, and presence or absence of PSA recurrence (Table 18 of the Supplementary Appendix), with sensitivities and specificities ranging from 80 to 90 percent in discriminating between patients with prostate cancer and controls. This diagnostic performance was maintained in the intermediate ranges of PSA (i.e., 4 to 10 ng per milliliter or 2.5 to 10 ng per milliliter).

Autoantibody signatures may be useful in combination with initial PSA screening; our data show that the 22-phage-peptide detector significantly adds to the diagnostic power of PSA alone ($P<0.001$). The use of such a "supplementary" autoantibody panel might be important at PSA levels of 10 ng per milliliter or less. This additional discriminatory power could improve the information used to make decisions about biopsy of the prostate.

The sequences in the phage display system are probably those of relatively short peptides rather than full-length proteins, since the cDNAs were enzyme-digested and fragmented before ligation into the phage vector. The average stretch of peptides in the 22-phage-peptide detector was 53±34 amino acids, with a maximum length of 134 residues and a minimum of 11 residues.

Four of the phage clones representing known proteins — BRD2, eIF4G1, RPL13a, and RPL22 — were substantially more reactive with serum from patients with prostate cancer than with that from controls (Fig. 5A). Both meta-analysis and immunoblot analysis of tissue extracts suggest that all four proteins are deregulated in prostate tumors. Immunofluorescence of tissue sections and immunohistochemical analysis of tissue microarrays showed that eIF4G1 was overexpressed in prostate-cancer epithelial tissue, as compared with benign epithelial tissue (Fig. 5D and 5E, and Fig. 5 and Section VI of the Supplementary Appendix).

Of the 22 phage peptides identified in this study, 17 are not present in peptide stretches in known proteins. These 17 peptides may be weakly homologous to known proteins or may have no distinct homology to the primary sequences of known proteins and thus may be "mimotopes" (i.e., stretches of amino acids that mimic an antigen but are not homologous at the sequence level).

We have not tested the phage-microarray system for screening for prostate cancer; this requires extension and confirmation in community-based screening cohorts. It will be important to evaluate the autoantibody signatures associated with prostate cancer in patients with prostatitis, autoimmune conditions, and other diseases. Although the technique is promising, how it will perform in prospective and multiinstitutional studies remains to be determined.

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