Autoantibody Landscape in Patients with Advanced Prostate Cancer

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ABSTRACT

Purpose: Autoantibody responses in cancer are of great interest, as they may be concordant with T-cell responses to cancer antigens or predictive of response to cancer immunotherapies. Thus, we sought to characterize the antibody landscape of metastatic castration-resistant prostate cancer (mCRPC).

Experimental Design: Serum antibody epitope repertoire analysis (SERA) was performed on patient serum to identify tumor-specific neoepitopes. Somatic mutation–specific neoepitopes were investigated by associating serum epitope enrichment scores with whole-genome sequencing results from paired solid tumor metastasis biopsies and germline blood samples. A protein-based immunome-wide association study (PIWAS) was performed to identify significantly enriched epitopes, and candidate serum antibodies enriched in select patients were validated by ELISA profiling. A distinct cohort of patients with melanoma was evaluated to validate the top cancer-specific epitopes.

Results: SERA was performed on 1,229 serum samples obtained from 72 men with mCRPC and 1,157 healthy control patients. Twenty-nine of 6,636 somatic mutations (0.44%) were associated with an antibody response specific to the mutated peptide. PIWAS analyses identified motifs in 11 proteins, including NY-ESO-1 and HERVK-113, as immunogenic in mCRPC, and ELISA confirmed serum antibody enrichment in candidate patients. Confirmatory PIWAS, Identifying Motifs Using Next-generation sequencing Experiments (IMUNE), and ELISA analyses performed on serum samples from 106 patients with melanoma similarly revealed enriched cancer-specific antibody responses to NY-ESO-1.

Conclusions: We present the first large-scale profiling of autoantibodies in advanced prostate cancer, utilizing a new antibody profiling approach to reveal novel cancer-specific antigens and epitopes. Our study recovers antigens of known importance and identifies novel tumor-specific epitopes of translational interest.

Introduction

The role of adaptive immunity in cancer is of great translational interest given the recent development of novel, clinically effective immunotherapies that focus on generating T-cell responses to tumor antigens. While the T-cell landscape of numerous cancer types has been explored in some depth, the role of humoral immunity in cancer is much less well-characterized. Several studies have demonstrated that a distinct antibody signature may be detectable in the serum of patients with breast (1), prostate (2), and lung (3) cancer and may thus be useful for cancer detection. In addition, studies have demonstrated that B-cell infiltration into the tumor microenvironment is associated with prolonged patient survival and enhanced response to immunotherapy in melanomas, renal cell carcinomas, and sarcomas (4–9), with several studies suggesting that B-cell autoantibodies may play a direct role in mounting an antitumor response (10, 11). In the setting of cancer vaccines, preclinical data indicate that IgG antitumor antibody responses to neoantigens in a mouse model of breast cancer can predict corresponding T-cell responses to the same epitopes (12). Furthermore, in a completed phase III trial that led to approval of the autologous cellular vaccine sipuleucel-T, one of the first immunotherapies approved by the FDA for solid tumors, productive antibody responses to the immunogen were correlated with longer overall survival (OS) in retrospective analysis (13). Finally,

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Clin Cancer Res 2020;26:6204–14

doi: 10.1158/1078-0432.CCR-20-1966

Translational Relevance

Autoantibodies have been shown to inform treatment response and candidate drug targets in various cancers. We present the first large-scale profiling of autoantibodies in advanced prostate cancer, utilizing a new next-generation sequencing-based approach to antibody profiling to reveal novel cancer-specific antigens and epitopes.

antitumor immune responses can also be stimulated by proteins ectopically expressed outside of immune-privileged sites in somatic tumor tissues, the prototype of which is cancer/testis antigen, NY-ESO-1. The prevalence of autoantibodies to the NY-ESO-1 peptide and putative conservation of B- and T-cell epitopes have led to more than 30 NY-ESO-1 T-cell receptor immunotherapy clinical trials, at various stages of completion, in diverse cancer types (14, 15). Altogether, these findings support the notion that a patient's antibody repertoire may reflect a specific immune response to the patient's cancer and may have potential diagnostic and therapeutic implications.

Tumor-associated antibodies detectable in patient serum are traditionally profiled using microarray-based methods (16–18), phage display approaches (19–21), or techniques incorporating principles of the two (22–24). One key limitation of candidate protein–based approaches is the throughput and subsequently limited number of antigens that can be profiled, and the inability to detect patient- or tumor-specific sequence variants generated by mutation. The serum epitope repertoire analysis (SERA) tool leverages a randomized bacterial display library paired with next-generation sequencing (NGS) to identify peptides binding to serum antibodies (25). By leveraging the randomized library, SERA is able to examine both wild-type and mutant sequences without any modification to the experimental protocols. Protein-based immunome-wide association study (PIWAS) builds on top of the SERA assay to identify proteome-constrained antigenic signals from the SERA assay. PIWAS calculates, for each sample and protein, a smoothed log-enrichment value across a window of overlapping kmers to identify a protein (gene)-level enrichment score while retaining epitope-level resolution for the signal source. By comparing PIWAS values between cohorts using the outlier sum, PIWAS is able to identify autoantigens against the human proteome (26).

While autoantibody enrichment has previously been demonstrated in prostate cancer, these studies were limited by smaller discovery cohorts (27, 28) or relatively restrictive peptide libraries (29, 30). It also appears that autoantibody enrichment may be context specific. For example, one large study that leveraged a phage display approach developed a signature for prostate cancer screening, but found that this signature could be found only in a minority of patients with castration-resistant disease (2). Thus, the autoantibody landscape for patients with mCRPC has yet to be elucidated.

Given that mCRPC represents one of the leading causes of cancer-associated death in men, we sought to characterize the autoantibody landscape of this disease. Utilizing SERA, PIWAS, and IMUNE (25, 31), we performed an unbiased analysis of autoantibodies enriched in the serum of patients with mCRPC compared with healthy controls. Specifically, we leveraged DNA sequence–level information from the assay to identify not only the proteins, but also the specific epitopes (subpeptides) within the full-length proteins that were putatively antigenic in mCRPC. We also integrated the serum antibody profiling results with whole-genome sequencing performed on metastatic tumor biopsies and peripheral blood (germline) specimens from the same patients to assess the immunogenicity of antigens resulting from somatic mutations. We validated our top candidate antigen in *NY-ESO-1*, a known immunogenic tumor marker across cancer types, using an independent cohort of patients with melanoma. We further validated the PIWAS-based seropositive results of our top motifs using ELISA experiments performed on the same serum specimens. In total, our study both recovered previously identified cancer antigens and identified novel, putative cancer-specific antigens in mCRPC.

Materials and Methods

Data acquisition and sample processing

A prospective institutional review board (IRB)-approved study (NCT02432001) was conducted by a multi-institutional consortium that obtained serum, peripheral blood, and fresh-frozen, image-guided biopsy samples of metastases from patients with mCRPC. Serum samples for each patient were prospectively obtained at time of study enrollment, at 3-month follow-up, and at time(s) of cancer progression, if applicable. Blood was drawn at start of therapy, 3 months into therapy, and at clinically determined disease progression in serum separator tubes of 6 mL (BD #367815) or 10 mL (BD #367820). Tubes were spun within 90 minutes of collection (1,500 rcf for 10 minutes), aliquoted into 2-mL cryovials, and frozen on dry ice and shipped to a central laboratory at University of California, San Francisco (San Francisco, CA). Vials were stored upon arrival at −80°C until batch shipping on dry ice for processing at Serimmune. Solid tumor metastases biopsies were sequenced using whole-genome sequencing and RNA sequencing (RNA-seq) as described previously (32, 33). Serum samples from a control group consisting of 1,157 individuals without known history of cancer or other predicate disease were obtained from the Serimmune database of samples. A cohort of 106 patients with melanoma was used for validation of specific antigens. The prospective IRB-approved study (11-003254) of these patients was conducted at University of California, Los Angeles (Los Angeles, CA) that obtained peripheral blood and biopsy samples for various analysis from patients treated for advanced melanoma malignancies. Plasma samples for each patient were prospectively obtained at time of study enrollment, at approximately 3-month follow-up, and at further follow-up time(s) as prescribed. At baseline and after approximately 3 months of treatment, blood was collected in K3-EDTA lavender tubes of 9 mL (Greiner Bio-One# 455036) and so forth. Tubes were spun within 24 hours after collection (1,200 rcf for 10 minutes, brake off) and aliquoted at 500 μL into 2-mL cryovials for long-term storage at −80°C. A total of 106 subject aliquots were prepared as 120 μL and overnight shipped on dry ice for processing at Serimmune. The study was performed after approval by an IRB and was conducted in accordance with the ethical principles of the Declaration of Helsinki. All patients provided written informed consent.

Serum antibody-epitope profiling

An *E. coli* bacterial display library consisting of plasmids encoding random 12-mer peptides at a diversity of 8×10^9 was constructed and prepared as described previously (25). Serum samples were screened on this library as described previously (26). Briefly, serum samples, at a 1:25 dilution, were added to each well of a 96-well deep well plate containing 8×10^{10} (10-fold oversampling) induced library cells and incubated with orbital shaking at 4°C for 1 hour. Cells were washed once with PBS containing 0.05% Tween-20 (PBST) and then incubated with Protein A/G Sera-Mag SpeedBeads (GE Life Sciences, 17152104010350) for 1 hour at 4°C with orbital shaking. Cells displaying peptides bound to serum IgG antibodies were captured by

magnetic separation and washed five times with PBST. Selected cells were grown overnight in LB medium supplemented with 34 μg/mL chloramphenicol and 0.2% w/v glucose at 37°C with shaking at 250 rpm.

Amplicon preparation and NGS were performed as described previously (26). Briefly, plasmids were isolated from selected library cells using the Montage Plasmid MiniprepHTS Kit (Millipore, LSKP09604) on a MultiscreenHTS Vacuum Manifold (Millipore, MSVMHTS00) following the manufacturer's instructions. Next, DNA encoding the 12-mer variable regions was amplified and barcoded by two rounds of PCR. Finally, after normalizing DNA concentrations, pooled samples were sequenced using a NextSeq 500 (Illumina) and a High Output v2, 75 Cycle Kit (Illumina, FC-404-2005) with PhiX Run Control (Illumina, FC-110-3001) at 40% of the final pool concentration.

Identifying mutation-specific epitopes

Previously published results of whole-genome sequencing performed on fresh-frozen metastasis biopsies and paired peripheral blood samples of the same patients (32, 33) were analyzed to identify somatic protein-coding point and frameshift mutations present in each patient's tumor. Data from the SERA platform were broken into 5 mers and 6 mers for every sample and enrichments were calculated (25). Using the same approach as PIWAS, these enrichments were tiled against both the wild-type and mutated protein sequences (26). The enrichment values for the wild-type sequence were subtracted from the mutant sequence to identify differential signal. The maximum differential value was calculated for every mutated protein and the associated patient sample. The data were fit to an exponential distribution and the probability density function was used to estimate *P* values for every protein. *P* values were corrected for multiple hypothesis testing using the Benjamini–Hochberg procedure (34).

PIWAS approach

Using the patients with prostate cancer as cases and the individuals without known cancer as controls, we ran a PIWAS analysis against the human proteome (26). PIWAS was parameterized to have a window size of 5, the number of SD approach, and the maximum peak signal. The outlier sum FDR, as defined previously, was used to prioritize antigens (26). The reference human proteome was downloaded from UniProt on 28 February 2019.

The validation PIWAS was run using the same parameterizations with the melanoma cohort as cases and the individuals without known cancers as controls.

The PIWAS-IMUNE approach

For top antigens, *NY-ESO-1* and *HERV-K*, additional steps were taken to develop a motif panel for these antigens. In both cases, the PIWAS-IMUNE (PIWAS-I) algorithm was used to identify linear mapping motifs. In the initial PIWAS stage, prostate cancer samples with an antigen PIWAS score > 6 were identified. The positive prostate cancer samples and 30 random healthy controls were used as input to the second-stage IMUNE algorithm, which was parameterized with 20% sensitivity and 100% specificity (25). Motifs that mapped linearly to the target antigen were retained. For each retained motif, the mean and SD of enrichment scores were calculated using the 1,157 control specimens as a reference group. z-scores were calculated for every cancer specimen. Then, for each cancer specimen, enrichment scores for each motif were z-scored (on the basis of the enrichment score mean and SD of the control group) and summed to generate a composite score for each specimen. Thus, the final composite PIWAS-I "panel score" was defined as the sum of motif z-scores for each specimen. Thresholds for positivity on the panel were set at a 99% specificity.

ELISA

Briefly, *NY-ESO-1* recombinant protein (OriGene) at 0.5 μg/mL or control recombinant protein CENPA (OriGene) at 0.5 μg/mL, or *HERVK-5* recombinant protein (MyBiosource) at 1 μg/mL or control protein BSA (Sigma) at 1 μg/mL in PBS was coated onto flat bottom, 96-well plates (Nunc MaxiSorp), 50 μL per well at 4°C overnight. Plates were washed with PBS containing 0.1% Tween 20 and blocked with 5% nonfat milk in PBS for 2 hours at room temperature. Plates were then incubated with 100 μL of patient serum diluted 1/200 or 1/2,000 in 5% nonfat milk in PBS for 2 hours at room temperature. Following washing, plates were incubated with peroxidase-conjugated goat anti-human IgG secondary antibody (1/10,000 in 5% nonfat milk in PBS, Jackson ImmunoResearch) for 1 hour at room temperature. After a last wash step, the reaction was developed with 3,3′,5,5′-tetramethylbenzidine substrate solution (Thermo Fisher Scientific) for 1–10 minutes and stopped with 1 mol/L hydrochloric acid. Absorbance at 450 nm was measured on a plate reader. ELISA values were calculated as the mean difference between the testing recombinant protein and the control protein. Because of reagent availability constraints, sera reactivity to *HERVK-5* was assessed in lieu of reactivity of *HERVK-113* given the high sequence similarity of the HERVK-5 and HERVK-113 proteins (95.2% per BLAST analysis).

Statistical methods and survival analysis

OS was measured from time of mCRPC diagnosis. Survival analyses were conducted using the Kaplan–Meier method with log-rank testing for significance. The χ^2 test was performed to assess the relationship between ELISA and PIWAS antibody enrichment results. All independence and hypothesis tests were performed using a two-sided significance level of 0.05. Multiple hypothesis testing correction was performed using the Benjamini–Hochberg procedure.

Results

Serum specimens were obtained from a cohort of 72 patients with mCRPC with a mean age of 72 years at time of mCRPC diagnosis (**Table 1**). The cohort was predominantly Caucasian (87%) with high-grade primary tumors in 54% of patients. Visceral metastases were observed in 15 of 72 (21%) patients. Sera obtained at more than

Table 1. Patient clinicopathologic characteristics.

Characteristic	No. of patients ($N = 72$)
Age, years (SD)	72 (8.4)
Race	
Asian	4 (5.9)
Black or African American	5 (7.4)
White	59 (86.8)
Missing	4 (5.9)
Gleason score at diagnosis	
8+	35 (53.8)
<8	30 (46.2)
Missing	7 (10.8)
Metastatic sites at time of biopsy	
Liver	7 (9.7)
Visceral metastases (non-liver)	8 (11.1)
Bone +/− lymph node	51 (70.8)
Lymph node only	6 (8.3)

Note: All clinicopathologic variables were measured at time of first solid tumor biopsy and are presented as "number (%)."

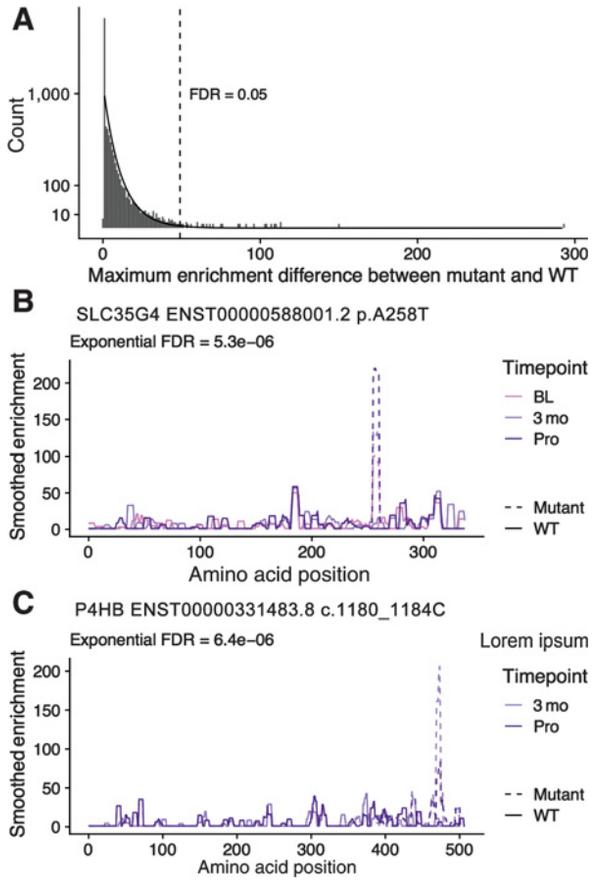

Figure 1.

Analysis of mutation-specific epitopes in the patients with prostate cancer. **A,** Enrichments were calculated for every mutated protein in the affected patients (*n* mutations = 6,636; *n* patients = 72). An exponential distribution (indicated by the solid line) was fit to the data to calculate significance of each mutation. Difference between enrichment values in the mutant sequence and the wild-type (WT) sequence in that patient are shown along the *x*-axis. **B,** A high scoring point mutation in SLC35G4 is shown for patient DTB-129. **C,** A high scoring frameshift mutation in P4HB is shown for patient DTB-102. (BL, baseline; 3 mo, 3 months after enrollment in study; Pro, disease progression). Note that no baseline serum specimen was available for patient DTB-102 at time of study.

one timepoint were available for 79% of patients (Supplementary Table S1).

Integrating serum antibody profiling results with whole-genome sequencing results, we first sought to assess whether somatic, protein-coding mutations were associated with an antibody response specific to the mutant peptide in mCRPC. Twenty-nine of the 6,636 protein-coding somatic mutations observed in our cohort were associated with a significant enrichment (exponential FDR < 0.05) in antibodies specific to the mutated peptide (**Fig. 1A**; Supplementary Table S2). These 29 mutations were approximately evenly distributed between frameshift and missense mutations (Supplementary Fig. S1). These events constituted the minority of mutations (0.44%), consistent with literature that suggests that most protein-coding mutations do not elicit an immune response (35). Each of the mutation-specific antibodies was enriched in only one patient. However, the somatic mutations that coded the epitopes were also private to individual patients. This suggested that the observed antibody response was specific to the individual in which the mutant antigen was available. In 11 of 20 mutant epitopes derived from patients with multiple serum specimens available, multiple independent serum samples obtained from the same individual at different timepoints confirmed the mutation-specific antibody enrichments (Supplementary Table S2). Across all patients with multiple serum specimens obtained at different timepoints, there did not appear to be a consistent trend in favor of either increasing or decreasing enrichment scores over time. Nine of 20 mutations were associated with progressively increasing and 11 of 20 mutations were associated with progressively decreasing enrichment values over time. We highlight an example of a patient with a point mutation and a patient with a frameshift mutation that demonstrated an enriched autoantibody response to the corresponding mutant epitope across multiple timepoints (**Fig. 1B** and **C**).

Next, to investigate cancer-specific autoantibodies resulting from nonmutant proteins, we performed a PIWAS. We found 11 proteins to be significantly enriched for antibodies in patients with mCRPC compared with healthy controls (**Fig. 2**; **Table 2**; Supplementary Table S3). The top two candidates were cancer/testis antigens, *NY-ESO-1* and *NY-ESO-2*, with the dominant epitope occurring in a conserved region between the proteins. Eight of 72 (11%) patients demonstrated PIWAS values >6, all of which mapped to amino acids 11–30 of *NY-ESO-1* (**Fig. 3A**; Supplementary Fig. S3A). PIWAS values for seven of these eight patients remained above the threshold at all timepoints (Supplementary Fig. S3B). Of note, this dominant B-cell epitope has been described in a previous study using a peptide approach and was found to be present in prostate cancer at a similar frequency (36). To identify additional *NY-ESO-1* antigenic regions, we applied the previously described IMUNE algorithm to identify peptide motifs that were significantly enriched in patients with prostate cancer relative to healthy controls (25). For this analysis, eight *NY-ESO-1* PIWAS-positive samples were analyzed by IMUNE using 30 healthy patient samples as controls. A total of nine cancer-specific motifs were identified that mapped to *NY-ESO-1* (**Fig. 3B**). While seven of the nine motifs aligned to the same portion of *NY-ESO-1* identified by PIWAS, two of the motifs aligned to a new epitope around the 100th amino acid that is additionally present in samples without the PIWAS epitope. Samples with composite panel scores greater than 6.6 (on the basis of a predefined 99% specificity threshold) were designated positive. Using this panel, nine of 72 patients (12.5%), including one patient without enrichment of the dominant epitope, were positive for *NY-ESO-1* at a specificity of 99% (**Fig. 3C**; Supplementary Fig. S3C).

To validate this finding with an orthogonal serum profiling approach, the composite panel score results were benchmarked against a NY-ESO-1 ELISA experiment performed on the same prostate cancer serum samples. We found that the panel score and ELISA results were strongly associated (Cohen kappa = 0.57; **Fig. 3D**). RNA-seq expression data revealed that NY-ESO-1 was expressed in the metastases of six of nine patients demonstrating *NY-ESO-1* antibody enrichment at time of initial metastatic tumor biopsy, confirming antigenic availability in these patients (Supplementary Table S4). Altogether, these findings demonstrated the ability of the joint PIWAS-I approach to identify disease-specific epitopes in prostate cancer.

While cancer specific, the dominant *NY-ESO-1* epitope was previously known to be a tumor marker not only in advanced prostate cancer, but also other cancers including melanoma. Specifically, a prior study found autoantibodies to the dominant *NY-ESO-1* epitope to be enriched in 12.5% of melanoma samples (36). To assess whether a similar finding would be observed using the PIWAS-I approach, we profiled the serum antibody repertoires of an independent cohort

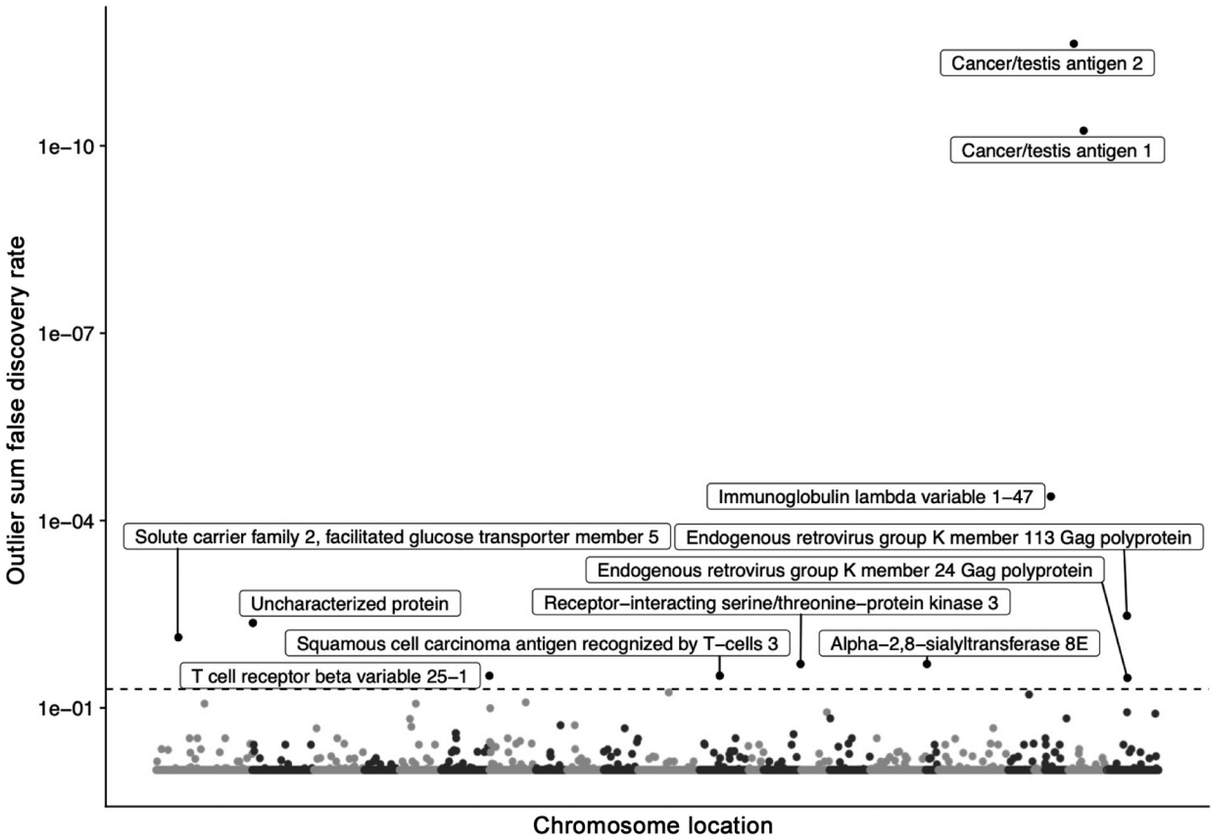

Figure 2.

Manhattan plot of PIWAS results highlighting antigens significantly enriched in patients with prostate cancer compared with healthy control patients. Outlier sum FDRs are shown for every protein in the human proteome. Labels are shown for all proteins with an FDR < 0.05. Copositivity of proteins with FDR < 0.05 are shown in Supplementary Fig. S2.

consisting of 106 patients with melanoma. We observed the dominant *NY-ESO-1* epitope enriched in eight of 106 (7.5%) samples (**Fig. 3A** and **C**), consistent with the prior report. This finding further validated the robustness of the PIWAS-I approach.

In addition to NY-ESO-1, the HERV (HML-2) family of proteins were also found by PIWAS to be significantly enriched for autoantibodies in mCRPC. Nine of 72 (12.5%) patients demonstrated significant enrichment of autoantibodies to HERVK-113, with recurrent epitopes identified near the 155th amino acid and C-terminus of the protein (**Fig. 4A**; Supplementary Fig. S4A). The presence of autoantibody enrichment to HERVK-113 was consistent across different timepoints in patients with multiple independently sampled serum specimens (Supplementary Fig. S4B). A motif panel for HERVK-113 was generated as described above using nine PIWAS-positive samples for IMUNE-based motif discovery (**Fig. 4B**). The panel scores were enriched in 16 of 72 (22.2%) patients (**Fig. 4C**; Supplementary Fig. S4C). The panel scores were highly concordant with confirmatory ELISA testing (Cohen kappa = 0.91; **Fig. 4D**). In addition, RNA-seq expression of HERVK-113 was observed in all patients with panel score positive for HERVK-113, suggesting antigenic availability (Supplementary Table S4).

Additional mCRPC-specific epitopes were identified using the PIWAS approach. These included epitopes in the protein products of *SART3, RIPK3, ST8SIA5, IGLV1-47, TRBV25-1*, and *SLC2A5* (**Fig. 2**; Supplementary Table S3). Seven of 72 (9.7%) patients demonstrated significant enrichment of autoantibodies to *SART3*, 6 of 72 (8.3%) demonstrated enrichment of autoantibodies to *RIPK3*, 5 of 72 (6.9%) demonstrated enrichment of autoantibodies to *ST8SIA5*, and four of 72 (5.6%) demonstrated enrichment of autoantibodies to *IGLV1-47* (Supplementary Fig. S2). To assess autoantibody coenrichment patterns, we assessed pairwise correlation between autoantibody enrichment scores of the 11 putative mCRPC-specific epitopes using our cohort of 72 patients (Supplementary Fig. S2A and S2B). Enrichment of autoantibodies to NY-ESO-1 and HERVK-113 was mutually exclusive on PIWAS analysis, as no patients demonstrated significant antibody enrichment to both NY-ESO-1 and HERVK-113 (Supplementary Fig. S2A). Presence of autoantibody enrichment to epitopes in NY-ESO-1 or HERVK-113 was not prognostic of OS (Supplementary Figs. S3D and S4D). We also examined the association between individual autoantibody enrichments and clinical variables including Gleason Grade Group, site of metastasis, prior abiraterone therapy, and prior enzalutamide therapy (Supplementary Figs. S5–S8). None of these variables were significantly associated with observed autoantibody enrichments, at least partially because of limited statistical power, given relatively low enrichment prevalence and modest cohort size.

PSA and PSMA are of particular interest given their utility as prostate cancer biomarkers and potential therapeutic targets. Thus, although our unbiased PIWAS analysis did not identify autoantibodies to PSA or PSMA as being significantly enriched in mCRPC, we

Table 2. Top epitopes from prostate cancer PIWAS.

Protein	Outlier sum FDR	Top epitopes
Cancer/testis antigen 2 (NY-ESO-2)	2.3E-12	GIPDGPGGNAG, PDGPGGNAGGP, SPMEAELVRRI
Cancer/testis antigen 1 (NY-ESO-1)	5.7E-11	GIPDGPGGNAG, PDGPGGNAGGP, PPSGQRR
Immunoglobulin lambda variable 1-47 (IGLV1-47)	4.1E-05	YWYQQLPGTAP
Endogenous retrovirus group K member 113 Gag polyprotein (HERVK-113)	0.0033	NDWAIIKAALE, VIYPETLKLEG, IQPFVPQGFQG, QGFQGQQPPLS, GFQGQQPPLSQ, PLSQVFQGISQ
Uncharacterized protein	0.0043	YDPKEYDPFYM, FYMSKKDPNFL, SKKDPNFLKVT, ISNSRHFITPN
Solute carrier family 2, facilitated glucose transporter member 5 (SLC2A5)	0.0074	DQSMKEGRLTL, PLVNKFGRKGA, FFPESPRYLLI, VAEIRQEDEAE, AIYYYADQIYL, YYADQIYLSAG, IEINQIFTKMN
Receptor-interacting serine/threonine-protein kinase 3 (RIPK3)	0.02	HPPPVGSQEGP
Alpha-2,8-sialyltransferase 8E (ST8SIA5)	0.02	GPFEYNSTRCL, QEIFRMFPKDM
T-cell receptor beta variable 25-1 (TRBV25-1)	0.03	YQQDPGMELHL
Squamous cell carcinoma antigen recognized by T-cells 3 (SART3)	0.03	MGPAWDQQEEG, DVEPPSKQKEK, MDGMTIKENII
Endogenous retrovirus group K member 24 Gag polyprotein (HERVK-24)	0.033	PEQGTLDLKDW, NDWAIIKAALE, VIYPETLKLEG, QGFQGQQPPLS, GFQGQQPPLSQ, PLSQVFQGISQ

performed a tiled enrichment analysis to directly assess the potential antigenicity of these proteins. We confirmed that no autoantibody enrichment to subpeptides of PSA or PSMA was observed (Supplementary Fig. S9).

Discussion

Herein, we have characterized the autoantibody landscape of mCRPC. We observed cancer-specific enrichment of antibodies to mutant peptides in select genes and to nonmutant peptides in the NY-ESO-1 and HERVK-113 proteins among others.

Previous reports demonstrated that disease-specific neoepitopes may include defective gene products resulting from somatic alterations such as mutations (37) and errors in protein translation (38). The extension of this principle to cancer is supported by prior studies in lung and colorectal cancers, which found that tumors with missense mutations in *TP53* and frameshift mutations in select genes were associated with autoantibodies to the mutant protein products (39, 40). We performed the first comprehensive assessment of cancer-specific B-cell neoantigens to date and observed several examples of this phenomenon in genes such as *SLC35G4* and *P4HB*. However, the majority (99.6%) of somatic mutations did not result in antibody-specific epitopes in our cohort. This finding is consistent with prior T-cell studies, which found that only a minority of mutations stimulate a specific T-cell response (35, 41). In this study, mutations generating the strongest detected responses were approximately equally distributed between missense and frameshift mutations.

The mutations that were observed to be associated with an epitope-specific humoral immune response tended to be private to individual patients, rather than shared among multiple individuals. This observation too is consistent with a prior report in colorectal cancer (41) and may be due to the fact that somatic mutations themselves (and hence, the resulting aberrant mutant protein) tend not to be recurrent across patients with mCRPC. Nevertheless, we observed that the autoantibodies to mutant peptides were often present in multiple serum specimens collected independently from the same patient. These data validate the specificity of the epitope profiling and PIWAS approaches and support the notion that select mutations may induce a humoral immune response in mCRPC.

We observed enrichment of autoantibodies to not only mutant but also wild-type epitopes. This is supported by prior studies, which suggest that cancer-specific overexpression of nonmutant antigens may comprise the majority of tumor-associated antigens (42–45). *NY-ESO-1*, or cancer/testis antigen 1B (*CTGAG1B*), has been well-characterized as an antigen that elicits humoral immune responses in various cancers including melanoma and breast, lung, bladder, ovarian, and prostate cancers (46–48). In addition, given its cancer-specific expression pattern outside of the testes (48–51), *NY-ESO-1* has shown great promise as a potential target for T-cell immunotherapies in various cancers (46). In our unbiased approach to identify immunogenic antigens, we found that *NY-ESO-1* was the top candidate. Moreover, by experimental design, we were able to identify the specific recurrent motif that has been previously demonstrated to be immunogenic in multiple cancer types (36, 52). These findings, along with empiric validation via the ELISA approach, support the notion that PIWAS-I can be used to reliably recover immunogenic motifs in cancer.

The PIWAS-I approach also identified epitopes in *HERVK-113*. Human endogenous retroviruses (HERV) comprise a family of retroviruses whose genetic material has previously been integrated into the human germline and whose gene products have been implicated in cancer pathogenesis (53, 54). HERVs have been previously described as being transcriptionally activated and potentially antigenic in the context of cancer, prior studies of renal cancers and seminomas identified a cancer-specific IgG response to *ERVK-10* (22, 55, 56). Humoral responses to HERVs have similarly been reported in melanomas (57) and ovarian (58), breast (59, 60), and prostate (61) cancers. In addition, the gene product of HERV-K may be not only a biomarker of disease, but also a therapeutic target, as a preclinical model demonstrated that mAbs against the HERV-K env protein were associated with inhibition of tumor growth in breast cancer (62). Particularly in prostate cancer, detection of autoantibodies to the HERV-K gag protein has been shown to be enriched in advanced prostate cancer relative to early prostate cancer (21% vs. 1.4%) and associated with poor survival outcomes (63). We observed a similar prevalence of 22% for HERV-K antibody enrichment in our cohort of patients with advanced prostate cancer. However, we observed no significant difference in OS between patients with mCRPC with and

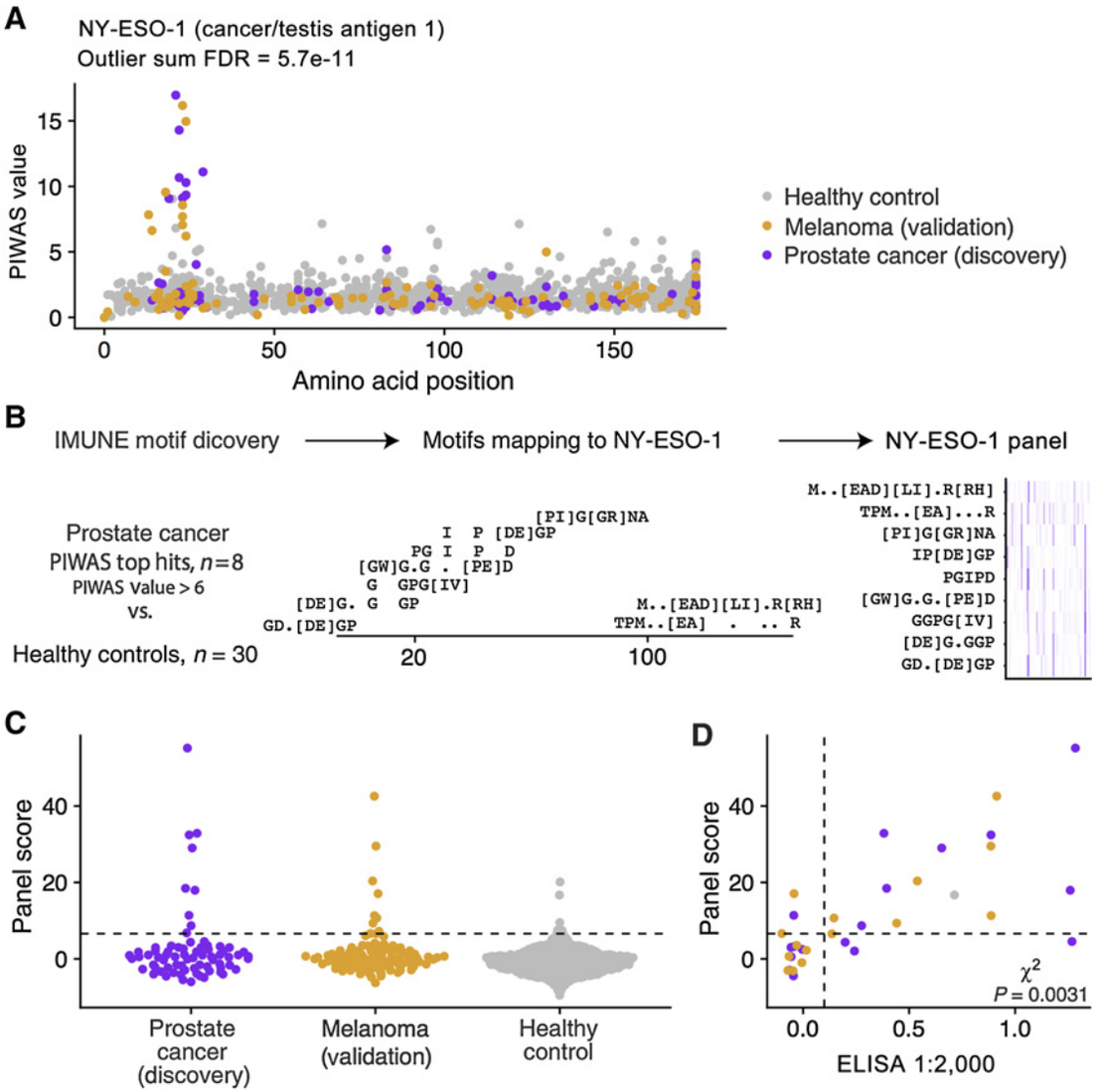

Figure 3.

Discovery and validation of *NY-ESO-1* antigenic signal. **A,** Manhattan plot visualizing PIWAS values for *NY-ESO-1*, with one point per patient being shown and colored by patient subgroup [purple, prostate cancer (discovery, $n = 72$); orange, melanoma (validation, $n = 218$); and gray, controls without known disease ($n = 1{,}157$)]. **B,** Prostate cancer samples that were positive by PIWAS are compared with healthy controls using IMUNE motif discovery algorithm. Motifs that mapped linearly to *NY-ESO-1* are included. A panel score was calculated by summing enrichment z-scores across all motifs. **C,** Dot plot of panel score for serum specimens stratified by patient subgroup. **D,** Scatterplot demonstrating concordance between *NY-ESO-1* panel score and ELISA results, assessed using the χ^2 test of independence. Points are colored on the basis of patient subgroup. Additional results are provided in Supplementary Fig. S3.

without HERV-K antibody enrichment. In contrast to the previously studied cohort, our cohort comprised exclusively of patients with advanced prostate cancer. Thus, our findings suggest that autoantibodies to the HERV-K protein may be associated with disease burden, but may not be prognostic of OS among patients with advanced disease. We also found that autoantibody enrichments to HERV-K and NY-ESO-1 were mutually exclusive on PIWAS analysis. This observation may reflect a difference in underlying tumor biology driving different patients' cancers, although the finding should be interpreted with caution because of the relatively modest size of our cohort. Additional prospective studies are needed to explore the prognostic value of HERV-K antibody enrichment in greater detail.

Additional antigens identified through the PIWAS-I approach included *IGLV1-47, TRBV25-1, SART3*, and *RIPK3*. Autoantibodies to *IGLV1-47* and *TRBV25-1* have been described as elevated in various inflammatory states, including in patients with systemic lupus erythematosus and rheumatoid arthritis (64–67). Thus, they are more likely to be nonspecific markers of chronic inflammation, rather than cancer-specific biomarkers. On the other hand, *SART3* and *RIPK3* have been previously implicated as biomarkers or potential regulators of cancer progression. *SART3* is a cancer/testis antigen that is expressed specifically in various cancer tissues (excluding normal testis; refs. 68, 69). *SART3* has been shown to induce both a humoral and cellular adaptive immune response in a vaccination study of

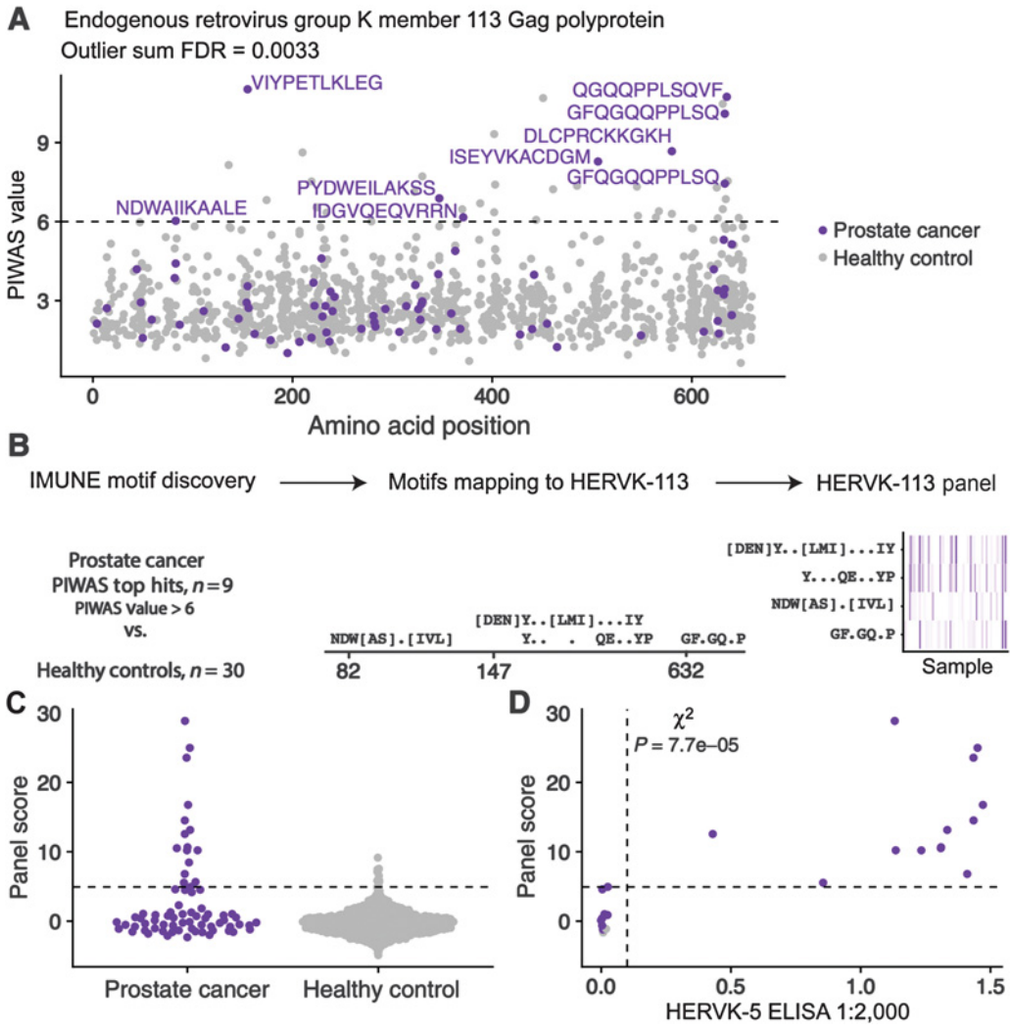

Figure 4.

Discovery and validation of *HERVK-113* antigenic signal. **A,** Manhattan plot visualizing antibody enrichment scores for *HERVK-113*. Epitopes associated with samples with a PIWAS value greater than 6 are labeled. **B,** Prostate cancer samples that were positive by PIWAS are compared with healthy controls using IMUNE motif discovery algorithm. Motifs that mapped linearly to *HERVK-113* are included. Panel score was calculated by summing enrichment z-scores across all motifs. **C,** Dot plot of panel scores for patients with prostate cancer compared with healthy controls. **D,** Scatterplot of *HERVK-113* panel score versus *HERVK-5* ELISA titer score. Additional results are provided in Supplementary Fig. S4.

patients with advanced colorectal cancer (70). *RIPK3* is a tumor suppressor whose downregulation has been associated with tumorigenesis, immunomodulation, and poor clinical prognosis in colorectal cancer (71, 72), although its exact role in the adaptive immunity is still under investigation. While confirmatory studies are needed, this study in conjunction with supporting studies in other cancer types nominates potential immune biomarkers and therapeutic targets in mCRPC.

In addition to recovering known and novel epitopes of interest in mCRPC, the findings of this study highlight potentially conserved B- and T-cell cancer-specific epitopes and a combined B- and T-cell response to cancer. *NY-ESO-1* has been found to elicit a high-titer IgG humoral response as well as a cellular immune response in patients with melanoma (46, 73). *SART3*, or "squamous cell carcinoma antigen recognized by T cells 3," was initially discovered as a T-cell epitope and was later found to also stimulate a correlated IgG response (68, 70). More generally, cancer vaccination studies have demonstrated how the humoral immune response to cancer-associated antigens may provide insights into targets of the endogenous cellular immune system (12, 70). Future work, profiling paired patient serum and T cells, may more definitively assess the overlap between B- and T-cell epitopes. In addition, profiling patients on clinical trials such as KEYNOTE-199 would be instrumental in identifying potential immune markers of exceptional response to immunotherapy (74).

This study is not without limitations. First, we acknowledge that our mCRPC cohort is of relatively modest size. Also, while the PIWAS-I approach has been successfully applied to immunologic (26) and now oncologic diseases to recover validated B-cell epitopes, ELISA-based validation experiments were performed on only the top two candidate epitopes (NY-ESO-1 and HERVK-113) in our mCRPC cohort. The patients described in this study should be considered a discovery cohort, and independent cohorts, ideally of larger size, are needed to

validate our findings. Finally, this study is limited by a lack of available paired peripheral blood mononuclear cell specimens and inability to investigate concomitant B- and T-cell responses. Additional studies are needed to further elucidate the frequency of epitopes on shared antigens among the humoral and cellular immune systems in mCRPC and the extent to which each contributes to antitumor activity.

In summary, we leveraged recently published epitope profiling techniques to characterize the autoantibody landscape of mCRPC and identify cancer-specific antigens and epitopes. By pairing patient serum profiling with whole-genome sequencing results of paired solid tumor biopsies, we identified 29 novel epitopes to mutant peptides generated by patient-specific somatic mutations. We also identified 11 conserved protein antigens, with several supported by prior reports in other cancer cohorts. Our findings and the presented NGS-based approach to autoantibody profiling provide insight into immune biomarkers and potential therapeutic targets in advanced prostate cancer.

Disclosure of Potential Conflicts of Interest

W.A. Haynes reports a patent titled immunome wide association studies to identify condition-specific antigens for PCT/US20/38856 pending, owned by Serimmune, not licensed yet, and employment with and shareholder at Serimmune. R. Waitz reports personal fees from Serimmune (employment and ownership of company shares) during the conduct of the study and outside the submitted work. K. Kamath reports other from Serimmune Inc (receives salary and stock options) outside the submitted work, as well as has an unlicensed patent for PCT/US20/38856 methods and compositions for assessing antibody specificities pending and a patent for pub. no.: US 2018/0267056 A1 issued to Serimmune Inc. M. Zhang reports other from Serimmune Inc (employee who receives equity compensation) outside the submitted work. S.G. Zhao reports patent applications for biomarkers unrelated to this work in prostate cancer with Decipher Biosciences, Celgene, and in breast cancer with PFS Genomics, and that a family member holds a leadership role in PFS Genomics. J. Chou reports grants from A.P. Giannini Foundation during the conduct of the study. T.M. Beer reports grants from Stand Up To Cancer—Prostate Cancer Foundation Dream Team during the conduct of the study, as well as grants from Alliance Foundation Trials, Corcept Therapeutics, Endocyte Inc, Harpoon Therapeutics, Janssen Research & Development, Medivation, Inc., Sotio, Theraclone Sciences/OncoResponse, and Zenith Epigenetics, personal fees and other from Arvinas (consulting and stock ownership), personal fees from AstraZeneca, Bayer, Bristol-Myers Squib, Clovis Oncology, GlaxoSmithKline, Janssen Biotech, Merck, Novartis, Pfizer, Tolero, and Sanofi, grants and personal fees from Astellas Pharma and Boehringer Ingelheim, and other from Salarius Pharmaceuticals (stock ownership) outside the submitted work. M. Rettig reports grants from Novartis, personal fees from Johnson & Johnson (speakers' bureau), Bayer (speakers' bureau), Ambrx (consultant), and Amgen (consultant), other from Constellation (consultant), nonfinancial support from Astellas (drug for clinical trial), Pfizer (drug for clinical trial), and Merck (drug for clinical trial) during the conduct of the study, as well as a patent for N-terminal androgen receptor antagonist for treatment of prostate cancer pending to no licensee yet, owned by UCLA. C.P. Evans reports grants from Stand Up To Cancer during the conduct of the study. K.N. Chi reports grants from Stand Up to Cancer during the conduct of the study, grants and personal fees from Amgen, Astellas, Janssen, Astra Zeneca, Sanofi, and Novartis, and personal fees from Merck, Daiichi Sankyo, and Point Biopharma. J.J. Alumkal reports grants from Stand Up to Cancer Foundation during the conduct of the study, grants from Zenith Epigenetics (research support to institution), Gilead Sciences (research support to institution), and Aragon Pharmaceuticals (research support to institution), grants and personal fees from Astellas (consulting and research support to institution) and Janssen Biotech (consulting and research support to institution), and personal fees from Merck (consulting) and Dendreon (consulting) outside the submitted work. A. Ashworth reports other from Tango Therapeutics (cofounder), Ovibio Corporation (cofounder), Azkarra Therapeutics (cofounder), SPARC (consultant), Bluestar (consultant), ProLynx (consultant), Earli (consultant), Cura (consultant), GenVivo (consultant), GlaxoSmithKline (consultant), Genentech (SAB), Circle (SAB), and Gladiator (SAB), grants from SPARC (research support) and AstraZeneca (research support), and other from Cambridge Science Corporation (board) outside the submitted work, as well as patents on the use of PARP inhibitors held jointly with AstraZeneca from which benefitted financially (and may do so in the future). R. Aggarwal reports grants from AstraZeneca, Janssen, Novartis, Amgen, Zenith Epigenetics, and Xynomic Pharmaceuticals, personal fees from Dendreon and Clovis, and grants and personal fees from Merck outside the submitted work. P.S. Daugherty reports a patent 8361933 issued, licensed, and with royalties paid from Serimmune and a patent 8293685 issued, licensed, and with royalties paid from Serimmune, both owned by the University of California and licensed to Serimmune, both on the topic of display of peptides on the outer surface of *E. coli* bacteria (i.e., *E. coli* surface display); this technology is part of the method Serimmune uses to generate datasets; and the author is an employee, stockholder, and director of Serimmune Inc. A. Ribas reports personal fees from Amgen, AstraZeneca, Checkmate, Merck, Novartis, Sanofi, Vedanta (honoraria from consulting), Advaxis, CytomX, Five Prime, RAPT, Isoplexis, Kite-Gilead (stock owner), 4C Biomed, Apricity, Arcus, Highlight, Compugen, ImaginAb, MapKure, Merus, Rgenix, Lutris, PACT Pharma, and Tango (scientific advisory board and stock owner) and grants from Agilent, Bristol-Myers Squibb (research support) outside the submitted work. D.Y. Oh reports grants from Prostate Cancer Foundation (Young Investigator Award) during the conduct of the study, other from Roche/Genentech (research support) and Merck (research support), and personal fees from Maze Therapeutics (consulting) outside the submitted work. F.Y. Feng serves as a scientific advisor for Serimmune and has received stock options from Serimmune; has also received consulting fees from Astellas, Bayer, Blue Earth Diagnostics, Celgene, Genentech, Janssen Oncology, Myovant, Roivant, and Sanofi, and is a founder and has an ownership stake in PFS Genomics; has a patent application with Decipher Biosciences; has a patent application licensed to PFS Genomics; and has patent applications with Celgene. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

This study was funded, in part, by a Stand Up To Cancer-Prostate Cancer Foundation-Prostate Dream Team Translational Cancer Research Grant. This

research grant was made possible by the generous support of the Movember Foundation. Stand Up To Cancer is a division of the Entertainment Industry Foundation. The research grant was administered by the American Association for Cancer Research. M. Sjöström was supported by the Swedish Research Council (Vetenskapsrådet) with grant no., 2018-00382 and the Swedish Society of Medicine (Svenska Läkaresällskapet). A. Ribas was funded by the Parker Institute for Cancer Immunotherapy, the Ressler Family Fund, support from Ken and Donna Schultz, and NIH grants R35 CA197633 and P01 CA244118. D.Y. Oh was supported by a Young Investigator Award from the Prostate Cancer Foundation and the following NIH grant: NIH/NIAID 5K08AI139375-02. F.Y. Feng was funded by Prostate Cancer Foundation Challenge Awards and the following NIH grants: NIH/NCI 1R01CA230516-01, NIH/NCI 1R01CA227025-01A1, NIH 2U10CA180868-06, and NIH P50CA186786. We thank the Serimmune team for supporting the processing and analysis of samples, including: Jack Reifert, Joel Bozekowski, Brian Martinez, Gregory Jordan, Timothy Johnston, Cameron Gable, Steve Kujawa, and Elisabeth Baum-Jones. Special thanks to Elizabeth Stewart for editing this article.

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Received May 20, 2020; revised August 3, 2020; accepted September 16, 2020; published first September 23, 2020.

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