Humoral Immune Response to \( \alpha \)-Methylacyl-CoA Racemase and Prostate Cancer

Arun Sreekumar, Bharathi Laxman, Daniel R. Rhodes, Srilakshmi Bhagavathula, Jason Harwood, Donald Giacherio, Debasish Ghosh, Martin G. Sanda, Mark A. Rubin, Arul M. Chinnaiyan

**Background:** Although prostate-specific antigen (PSA) is a prototypic biomarker for prostate cancer, it has poor specificity. Expression of \( \alpha \)-methylacyl-CoA racemase (AMACR), which is involved in the conversion of R-stereoisomers of branched-chain fatty acids to S-stereoisomers, has been shown to be specifically increased in prostate cancer epithelia. However, attempts to detect AMACR in circulation have not been successful. Hence, we determined whether an immune response to AMACR could be used as a serum biomarker for prostate cancer. **Methods:** Sera from patients with biopsy-proven prostate cancer and from control subjects were screened for a humoral immune response to selected tumor antigens, including AMACR, by using protein microarrays (46 patients, 28 control subjects). Humoral immune response to AMACR was then validated using high-throughput immunoblot analysis (151 patients, 259 control subjects) and enzyme-linked immunosorbent assay (ELISA) (54 patients, 55 control subjects). Receiver operating characteristic curves were used to determine the sensitivity and specificity of the immune response to AMACR to detect prostate cancer. **Results:** Immunoreactivity against AMACR was statistically significantly higher in sera from patients with prostate cancer than in control subjects by all three techniques \((P_{\text{protein microarray}} = .009, P_{\text{immunoblot}} < .001, P_{\text{ELISA}} = .011)\). High-throughput immunoblot analysis revealed that, in subjects with intermediate PSA levels \((4–10 \text{ ng/mL})\), the immune response against AMACR was more sensitive and specific than was PSA in distinguishing sera from prostate cancer patients relative to control subjects \((77.8\% \text{ versus } 80.6\% \text{ versus } 45.6\% \text{ and } 50\%, \text{ respectively; area under the curve of } 0.789 \text{ versus } 0.492; P < .001\). **Conclusion:** Assays to detect a humoral immune response against AMACR may have the potential to supplement PSA screening in identifying patients with clinically significant prostate cancer, especially those with intermediate PSA levels. [J Natl Cancer Inst 2004;96:834–43]

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See “Notes” following “References.”

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Prostate cancer is the second-leading cause of cancer-related death in American men (1). Although the use of prostate-specific antigen (PSA) screening has led to the earlier detection of prostate cancer, the impact of PSA screening on cancer-specific mortality is still unknown, pending the results of prospective randomized screening trials (2–4). Interpretation of the serum PSA test is made on the basis of PSA levels, with levels of 0–4 ng/mL being considered normal and levels of greater than 4 ng/mL being considered clinically significant for prostate cancer screening. A major limitation of the serum PSA test is its lack of specificity for prostate cancer, especially in the intermediate range of PSA levels (4–10 ng/mL). In this range, the specificity of the PSA test to detect prostate cancer has been reported to be only 20% at a sensitivity of 80% (5). This poor specificity is, in part, associated with the fact that serum PSA levels can be increased in patients with nonmalignant conditions such as benign prostatic hyperplasia or prostatitis and that PSA is highly expressed in both benign prostatic epithelia and prostate cancer cells.

Coincident with increased serum PSA testing, the number of prostate needle biopsies performed has increased dramatically (6). Although a recent study (7) suggests lowering the threshold of PSA for recommending prostate biopsy from 4.1 ng/mL to 2.6 ng/mL to correct for verification bias (i.e., the bias introduced in the sensitivity and specificity of a screening test when the disease status has not been confirmed in all the subjects and when the results of the screening test itself are used for confirmation) and potentially improve the clinical value of the PSA test, lowering the threshold will lead to a further increase in the number of prostate biopsies performed. Thus, additional serum and tissue biomarkers to supplement PSA are needed.

One such prostate cancer biomarker is α-methylacyl-CoA racemase (AMACR), an enzyme that catalyzes the racemization of R-stereoisomers of branched-chain fatty acids to S-stereoisomers and plays an important role in peroxisomal β-oxidation of branched-chain fatty acids (8–10). Differential display and expression array analyses have identified the AMACR gene as a gene whose expression is higher specifically in prostate cancer epithelia relative to benign prostatic epithelia (11–15). AMACR is a highly specific and sensitive marker for cancer cells within the prostate gland (16–18). In addition, a recent study demonstrated that sequence variants of AMACR may be associated with prostate cancer risk (19). Although AMACR may be potentially useful in the diagnosis of prostate cancer from tissue specimens, it would have considerably more utility as a tumor marker if it could be detected in serum.

Not all tumor markers are secreted into the circulation in concentrations high enough to detect easily. Thus, for tumor markers or antigens that are secreted or leaked into the circulation at low levels and are difficult to detect, it may be possible and preferable to monitor the immune response to the tumor marker. Recent data support the notion of a connection between cancer and inflammation and the role of inflammatory cells in orchestrating the development and progression of tumors (20). Autoantibodies to various antigens such as PSA, prostatic acid phosphatase, HER-2/neu, p53, and glucose-regulated protein 78 kD (GRP78) have been observed in the sera of prostate cancer patients (21–23). However, such studies have not shown that the presence of autoantibodies has a high degree of sensitivity or specificity in the early detection of organ-confined disease, nor have they shown the usefulness of autoantibodies in predicting the risk of disease progression.

In this study, we used protein microarrays consisting of 12 distinct antigens, including AMACR, that are known to be expressed in tumors or during inflammatory conditions, as a “discovery tool” to evaluate the humoral immune response in serum samples from patients with prostate cancer and from control subjects. Because AMACR has been shown to be an excellent tissue biomarker of prostate cancer, we hypothesized that monitoring the endogenous humoral immune response to AMACR may be useful in identifying prostate cancer patients, especially those with intermediate levels of PSA.

**Subjects and Methods**

**Subjects and Samples**

This study was approved by the Institutional Review Board of the University of Michigan Medical School. From January 1995 through January 2003, sera from 151 men with biopsy-proven clinically localized prostate cancer were collected at the time of diagnosis with written informed patient consent. The sera were stored in the University of Michigan Prostate SPORE tissue/serum bank. Sera were collected from these patients before radical prostatectomy. The average patient age was 59.8 years (range = 41–83 years). Clinical and pathology data from the patients with clinically localized prostate cancer used in this study are provided in Table 1. From May 2001 through May 2003, sera from 259 control subjects with no known history of prostate cancer were analyzed.

**Table 1. Demographic, clinical and pathology characteristics for prostate cancer patients used in the study**

<table>
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<th>Characteristic</th>
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<tr>
<td>Mean age (y) ± SD</td>
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<td>Mean gland weight (g) ± SD</td>
<td>55.8 ± 49.8</td>
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<tr>
<td>Mean gland size (cm) ± SD</td>
<td>1.6 ± 1.3</td>
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<tr>
<td>PSA§</td>
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</tr>
<tr>
<td>Mean (ng/mL) ± SD</td>
<td>8.1 ± 8.0</td>
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<tr>
<td>0–2.5 ng/mL (%)</td>
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<td>66.9</td>
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<tr>
<td>T2 (%)</td>
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<td>Unknown (%)</td>
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</table>

*Data were available for 142 patients only. SD = standard deviation.
†Data were available for 120 patients only.
‡Data were available for 132 patients only.
§Data were available for 146 patients only.
¶Data were available for 121 patients only.
#Data were available for 121 patients only.
cancer (average age = 58.3 years; range = 27–83 years) were collected in the University of Michigan Clinical Pathology laboratories. All sera were stored in aliquots at −20 °C until used. In a pilot study, sera from 46 patients with prostate cancer and from 28 control subjects were randomly selected from the total serum pool (151 prostate cancer patients and 259 controls) and were assessed by using protein microarrays consisting of 12 tumor-associated antigens, including AMACR. All serum samples (from 151 patients and 259 control subjects) were validated for humoral immune response to AMACR by high-throughput immunoblot analysis. We also tested a subset of these sera (from 54 patients and 55 control subjects) were randomly selected from the pool and analyzed by enzyme-linked immunosorbent assay (ELISA) as an alternate assay amenable to clinical implementation.

Immunohistochemistry for PSA and AMACR

Histologic sections of prostate cancers were graded using the Gleason grading system (by M. A. Rubin) (24,25). For immunohistochemical analysis, consecutive tissue sections containing adjacent benign and malignant glands were selected. The sections were subjected to antigen retrieval by steaming for 10 minutes in sodium citrate buffer (10 mM, pH 6) in a microwave oven at high power for 10 minutes. The sections were blocked in peroxidase blocking solution (DAKO Cytomation, Carpinteria, CA) at room temperature for 5 minutes and incubated with primary antibody (polyclonal rabbit anti-AMACR antibody [a gift from R. Wanders, University of Amsterdam, Amsterdam, The Netherlands] at a 1:1500 dilution) or polyclonal rabbit anti-PSA antibody (DAKO Cytomation) at a 1:3000 dilution, both in antibody diluent (DAKO Cytomation) for 30 minutes at room temperature. Immunoreactivity was detected by incubation with EnVision+ rabbit peroxidase detection system (DAKO Cytomation) for 30 minutes at room temperature. Immunoreactivity was detected by incubation with EnVision+ rabbit peroxidase detection system (DAKO Cytomation) for 30 minutes at room temperature. The chromogen was 3,3′-diaminobenzidine. The sections were counterstained with hematoxylin. Information regarding how the sections were scored for immunoreactivity has been described elsewhere (17).

Protein Microarray Analysis

Recombinant proteins heat shock protein 47 kD (Hsp47), heat shock protein 27 kD (Hsp27), heat shock protein 25 kD (Hsp25), heat shock protein 40 kD (Hsp40), heat shock protein 60 kD (Hsp60), heat shock cognate protein 70 kD (Hsc70), and Escherichia coli K-12 oxidoreductase (DsbA) were purchased from Stressgen Biotechnologies (Victoria, British Columbia, Canada); MBP was purchased from New England BioLabs (Beverly, MA); and PSA and carcinoembryonic antigen (CEA) were purchased from Alpha Diagnostic International (San Antonio, TX). A fusion protein of AMACR and MBP (AMACR–MBP) was expressed and purified from E. coli transformed with AMACR–MBP cDNA cloned into the pMALc2 propharyctic expression vector harboring IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter (a gift from R. Wanders, University of Amsterdam). The fusion protein was tested for purity and immunoreactivity by silver staining and immunoblotting, respectively (data not shown). Recombinant yeast cytosine deaminase (yCD) was a gift from Drs. Mukesh Nyati and Theodore S. Lawrence (University of Michigan, Ann Arbor).

Proteins were suspended in sample buffer containing 125 mM Tris–Cl (pH 6.8), 0.4% sodium dodecyl sulfate (SDS), and 2% β-mercaptoethanol (Sigma, St. Louis, MO) in a final volume of 25 μL. The final protein concentration was approximately 1 mg/mL, with the exception of PSA and CEA, for which the final concentrations were 60 ng/mL and 30 ng/mL, respectively. All protein samples were denatured by heating at 70 °C for 2 hours and at 100 °C for 10 minutes in a thermal cycler (Perkin-Elmer Life Sciences, Boston, MA). Cy5-conjugated streptavidin (1:100 and 1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA) and antibodies against total human serum (serial dilutions from 1:100 to 1:800 and 1:1000; Jackson ImmunoResearch Laboratories) were included as control proteins. The samples were transferred to a 96-well microtiter plate and printed on FAST slides (Schleicher & Schuell, Keene, NH) by using a GMS 417 microarray printer (Genetic MicroSystems, Woburn, MA). Each spot measured approximately 300 μm in diameter, and the distance between spots was 1200 μm. Each protein was printed a minimum of four times and a maximum of sixteen times on each slide. A total of 12 recombinant proteins and two control proteins were used, and each slide contained either 96 or 192 spotted elements. The slides were used immediately or stored at 4 °C.

Protein microarray slides were first blocked in Tris-buffered saline (TBS) containing 1% nonfat powdered milk, 5% normal donkey serum (Sigma), and 0.1% Tween 20 (Sigma) for 1 hour at room temperature. The slides were rinsed with TBS and incubated with patient or control serum diluted 1:50 in dilution buffer (TBS containing 0.1% nonfat powdered milk, 0.5% normal donkey serum, and 0.01% Tween 20) in incubation chambers (Schleicher & Schuell) for 2 hours at room temperature. The slides were washed six times with TBS containing 0.1% Tween 20 (TBS–T), with each wash lasting 5 minutes. The slides were then incubated with biotin-conjugated anti-human immunoglobulin G (IgG; Jackson ImmunoResearch Laboratories) diluted 1:200 in dilution buffer for 30 minutes at room temperature, washed with TBS–T, and incubated with Cy5-conjugated streptavidin diluted 1:250 in dilution buffer for 30 minutes at room temperature. The slides were washed extensively in TBS–T, dried by spinning at 500g for 10 minutes at room temperature, and analyzed by scanning at 565 nm using a microarray scanner (Axon Instruments, Foster City, CA). The intensity of each protein spot was calculated using the software package (Genepix 4.0) recommended by the manufacturer of the scanner. Images of scanned microarrays were gridded and linked to a protein print list.

The intensity of immunoreactivity and various quality control parameters (e.g., intensity over local background) were determined for the individual proteins. The Genepix software auto-flagged spots with the following default criteria set by the manufacturer: 1) spot diameter was less than 300 μm, 2) less than 55% of the feature pixels of an individual spot were brighter than the median background intensity at both wavelengths, 3) median signal-to-background ratio was greater than 10, 4) mean of the median background intensity was less than 500 light units, 5) median of signal-to-noise ratio was greater than 10, 6) features with saturated pixels were less than 0.1%, 7) feature variation was less than 0.5%, and 8) background variation was less than 0.5%. Flagged spots were excluded from subsequent analyses. The mean intensity of reactivity was then calculated for each protein by using data from all the replicates on each
array. The mean values for each protein were normalized per array by subtracting the corresponding mean value of MBP. This normalization procedure was less sensitive to outlier values than subtraction of mean intensity for MBP from the mean intensity for each protein divided by the standard deviation for MBP.

**Conventional and High-Throughput Immunoblot Analysis**

For conventional immunoblot analyses, recombinant AMACR–MBP (5 μg/well) and MBP (5 μg/well) were subjected to electrophoresis through a precast 10% SDS gel (Bio-Rad, Hercules, CA) and transferred onto nitrocellulose membranes by using a semidy transfer method (Bio-Rad). The membranes were blocked in TBS (Bio-Rad) containing 5% nonfat powdered milk and 5% normal donkey or mouse serum (Jackson ImmunoResearch Laboratories) overnight at 4 °C. The membranes were then cut into two-lane strips (each strip containing AMACR and MBP) and probed with serum diluted 1:25 in dilution buffer (TBS containing 0.5% nonfat powdered milk and 0.5% normal donkey or mouse serum) from patients with prostate cancer or from control subjects for 2 hours at room temperature. Membranes were washed for 30 minutes with TBS-T and probed with either biotin-conjugated human IgG–specific antibody (Jackson ImmunoResearch Laboratories) diluted 1:100 000 or with biotin-conjugated human isotype–specific antibody (Zymed Laboratories, San Francisco, CA) diluted 1:500 in dilution buffer for 30 minutes at room temperature. The membranes were washed in TBS-T, probed with horseradish peroxidase–conjugated streptavidin (Jackson ImmunoResearch Laboratories), and diluted 1:25 000 in dilution buffer for 30 minutes at room temperature; the antigen–antibody complexes were then visualized by enhanced chemiluminescence (ECL-Plus; Amersham Biosciences, Piscataway, NJ). To test the specificity of the immune response, we quenched serum known to contain autoantibodies against AMACR (n = 3) overnight with recombinant AMACR–MBP (final concentration = 18 μg/mL) at 4 °C and used it as described above.

For high-throughput immunoblot screening, AMACR–MBP (50 μg) was subjected to electrophoresis on a preparative SDS–4 to 20% polyacrylamide gel and transferred onto a nitrocellulose membrane. A Miniblotter 28 dual system (Immunetics, Cambridge, MA) was used to create 56 incubation channels on two nitrocellulose membranes, which were sufficient to assess approximately 50 test and control conditions. Serum from a prostate cancer patient known to be consistently reactive for antibodies against AMACR was used as a positive control in every experiment. After sera and control samples were added to the wells, the membranes were incubated for 2 hours at room temperature, washed, and processed as described above. The films were scanned, and the bands were analyzed using NIH Image 1.61 software. The band intensities were tabulated, and AMACR immunoreactivity for each patient serum was then divided by the corresponding value for the positive control sera to obtain the normalized ratio for AMACR humoral response. We randomly selected 26 of 151 (17.2%) cancer sera and 61 of 259 (23.5%) control sera and tested them in duplicate to assess assay reproducibility. The ratios were averaged for samples from the same patient that were analyzed in duplicate.

Receiver operator characteristic (ROC) curves were plotted by using the ratio of mean reactivity, and the threshold value for positive immunoreactivity was assigned at the point at which AMACR humoral response showed high specificity and sensitivity for detection of localized prostate cancer. A cutoff intensity ratio (i.e., intensity of the band in the test sample divided by intensity of the band in positive control serum) of 0.315 was assigned, which corresponded to a sensitivity and specificity of 61.6% and 71.8%, respectively.

**Development of an ELISA for AMACR Humoral Response**

Because recombinant AMACR was to be used as a target protein to screen for an AMACR-specific humoral response in sera from patients with prostate cancer, we generated a FLAG epitope–tagged version of AMACR in eukaryotic cells. AMACR was subcloned into the vector pcDNA3 (Invitrogen, Carlsbad, CA) in frame to generate an N-terminal FLAG-tagged fusion cDNA construct. FLAG-tagged AMACR cDNA was then transfected into COS cells by using FUGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s recommended protocol. After 48 hours, the FLAG-tagged AMACR protein was purified using anti-FLAG M1 affinity gel (Sigma) according to the manufacturer’s instructions.

All incubations for the ELISA were carried out in volumes of 200 μL/well unless otherwise specified. Recombinant AMACR–FLAG (500 ng/well) was added to anti-FLAG HS M2–coated 96-well plates (Sigma) and incubated overnight at 4 °C on an orbital shaker. The plates were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS–T) using an EL404 microplate autowasher (Bio-Tek Instruments, Winooski, VT). Sera (diluted serially from 1:50 to 1:400 in dilution buffer composed of PBS–T, 0.1% nonfat powdered milk, and 0.1% normal mouse serum) from patients with prostate cancer and from control subjects were added, and the plates were incubated for 2 hours at room temperature on an orbital shaker. Serum (diluted 1:1000) from a prostate cancer patient known to be consistently reactive against AMACR was used as a positive control in all experiments for purposes of interassay normalization. Between incubations, the plates were washed as described above. Biotin-conjugated human IgG2–specific antibody (1:500 in dilution buffer) was added, and the plates were incubated for 30 minutes at room temperature. After the plates were washed extensively, horseradish peroxidase–conjugated streptavidin (diluted 1:1000 in dilution buffer) was added, and the plates were incubated for 30 minutes at room temperature. The plates were washed extensively before the substrate 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma) was added. After 10 minutes, the reactions were stopped by the addition of 100 μL of 0.5 N H2SO4 per well, and the plates were read at 450 nm using an ELx 800 universal microplate reader (Bio-Tek Instruments). The absorbance values at 450 nm for the 1:50 dilution of the test sera were normalized against an AMACR-positive control serum used at a 1:1000 dilution in each ELISA plate. The normalized population means and 95% confidence intervals (CIs) were calculated for both the patient and control groups.

**Statistical Analysis**

All statistical analysis was performed with SPSS 11.1 (SPSS, Chicago, IL). The Student’s t test (two-sided) was used to test for statistically significant differences in the immune response to AMACR between patients with prostate cancer and control
subjects. No adjustment for multiplicity was made during the analysis. Associations between AMACR humoral immune response and various clinical and pathology parameters were determined using Pearson’s correlation test. The mean values for AMACR humoral immune response were presented as population mean values with 95% CIs. P values less than or equal to .05 were considered statistically significant. ROC analysis was performed to assess the sensitivity and specificity of AMACR humoral response for discriminating prostate cancer patient sera from control sera. Area under the curve (AUC) and the associated P values were used to assess statistical significance. ROC analysis was performed using AMACR humoral response or PSA as the test variable and prostate cancer and healthy controls as the state variable. Analysis was performed on the full patient cohort and also on subsets of patients with PSA values in the critical diagnostic range of either 2.5–10 ng/mL or 4–10 ng/mL. An odds ratio was used to test whether the AMACR humoral response could be used to distinguish between cancer and control groups in the critical PSA diagnostic range of 4–10 ng/mL.

RESULTS

Immunohistochemical Analysis of PSA and AMACR in Prostate Cancer Tissue

To compare the specificity of AMACR with that of PSA in the context of prostate cancer, we first performed immunohistochemistry on consecutive tissue sections containing adjacent neoplastic and benign prostate glands (Fig. 1A). Antibodies specific for AMACR stained neoplastic prostate glands preferentially relative to benign prostate glands, in agreement with previous results (16–18). By contrast, antibodies specific for PSA stained benign epithelia with equal or greater intensity than that for prostate cancer epithelia.

Identification of the AMACR Humoral Response in Prostate Cancer Patients by Using Protein Microarrays

The difference in the specificity of PSA and AMACR expression prompted us to investigate whether AMACR could be used as a serum biomarker. In preliminary studies using conventional immunoblot analysis, low levels of AMACR protein were detected in the sera of three of 40 patients with prostate cancer selected randomly from the University of Michigan Prostate Spore Serum bank (A. Sreekumar, B. Laxman, and A. M. Chinnaiyan: unpublished observations). The low detection rate among patients with prostate cancer may have been the result of a lack of sensitivity of the method of analysis (immunoblot) or of minimal leakage or secretion of AMACR into the bloodstream by the majority of patients with prostate cancer.

We therefore examined whether an endogenous humoral immune response against AMACR could be detected in patients with prostate cancer. In a pilot study of the humoral immune response in patients with prostate cancer, we developed an antigen microarray (26) consisting of selected recombinant tumor antigens that would capture antibodies from sera. A total of 12 different recombinant proteins, prelabeled control proteins (i.e., Cy5-streptavidin), and antibodies against human serum were spotted in replicate on nitrocellulose-coated slides. Each protein microarray consisted of 96 or 192 spots. Here, in this proof-of-concept study, we used a protein microarray with AMACR–MBP and selected tumor antigens to screen sera from patients with prostate cancer. After incubating the protein microarrays with serum samples, we washed and incubated the microarrays with biotin-conjugated anti-human IgG and fluorescently tagged streptavidin (Fig. 1B). In a pilot study, a total of 74 serum samples (46 from patients with prostate cancer and 28 from control subjects) were assayed for humoral response by using protein microarrays. Representative protein microarrays incubated with sera from a control subject and a patient with prostate cancer are shown in Fig. 1C. Representative protein microarray spots from two controls (negative for AMACR humoral response) and four patients (positive for AMACR humoral response) with prostate cancer are shown in Fig. 1D. Specific immunoreactivity was observed in sera from patients with prostate cancer, regardless of PSA level (Fig. 1D). The immunoreactivity was specific for AMACR because immunoreactivity against MBP was not observed in sera from patients with prostate cancer or from control subjects. Immunoreactivity against PSA, however, was observed in all the control subjects and in 42 patients with prostate cancer (Fig. 1D and E). We quantified the intensity of the immunoreactivity and generated normalized mean intensities for all proteins on the microarray. AMACR immunoreactivity was statistically significantly higher (P = .009) in sera from patients with prostate cancer (mean = 2602.1 relative units; 95% CI = 1551.4 to 3652.6 relative units) than in sera from control subjects (mean = 1116.3 relative units; 95% CI = 759.1 to 1473.4 relative units) (Fig. 1E). Statistically significant differences in immunoreactivities between patients and control subjects were not observed for any of the other proteins on the microarray, including CEA and PSA (Fig. 1E and data not shown).

Validation of AMACR Humoral Response in Prostate Cancer by Using High-Throughput Immunoblot Analysis and ELISA

To validate the specificity of the humoral immune response to AMACR, we initially used conventional immunoblot analysis, in which we incubated 32 patient and 28 control subject sera that were randomly selected from the serum pool (151 prostate cancer patients and 259 controls) with membranes containing affinity-purified recombinant AMACR–MBP and MBP. A representative immunoblot of 11 samples (live from control subjects and six from prostate cancer patients) is shown in Fig. 2A. Compared with sera from control subjects, sera from patients with prostate cancer were immunoreactive against AMACR–MBP (86 kD) but not MBP (43 kD). AMACR reactivity was attenuated when the sera were presaturated or quenched with excess recombinant AMACR–MBP (Fig. 2B), confirming the presence of AMACR-specific autoantibodies in these patients.

We next screened sera from patients with prostate cancer and control subjects by using a miniblot apparatus that allows for simultaneous incubation with 48 distinct sera (Fig. 2C). From one such screening (representative data shown in Fig. 2D), 18 of 23 serum samples from patients with prostate cancer patients and three of 23 serum samples from control subjects were immunoreactive against AMACR. By using IgG subtype–specific antibodies, we found that the humoral immune response to AMACR involved an IgG2 subtype (Fig. 2E).

The IgG2-specific secondary antibody was then used to validate the presence of AMACR immune response in all 151 patient and 259 control subject serum samples. The immunoblot
data were quantified by densitometric scanning and normalized among blots. Consistent with the protein microarray analyses, the population mean intensity for AMACR immunoreactivity was statistically significantly higher for sera from patients with prostate cancer (PCA) than for sera from control subjects (mean = 0.431; 95% CI = 0.382 to 0.481; P < 0.001) than for sera from control subjects (mean = 0.281; 95% CI = 0.242 to 0.319) (Fig. 3A).

To further characterize the reactivity of AMACR-specific autoantibodies in prostate cancer patients, we performed a titration of these AMACR-reactive sera from patients with prostate cancer. The AMACR humoral response in patients with prostate cancer showed appropriate attenuation with serum dilution (Fig. 3B and C), suggesting the presence of high-affinity autoantibodies against AMACR. However, the majority of AMACR-reactive sera from control subjects that reacted at low dilutions (1:50) did not show a correlation with serum dilution (Fig. 3B and C).

We next developed an ELISA to monitor the humoral immune response against AMACR because ELISAs may be more readily adaptable for use in clinical situations. Initial attempts to develop an ELISA with native AMACR fusion proteins expressed in bacteria (AMACR–MBP or histidine-tagged AMACR) were hampered by nonspecific reactivity of sera regardless of prostate cancer status (data not shown). Consequently, we developed an ELISA with FLAG-tagged AMACR purified from transfected mammalian COS cells and
immobilized anti-FLAG antibodies. This direct ELISA was then used to screen a random subset of the patients evaluated by immunoblot analysis (54 patients with prostate cancer and 55 control subjects) for a humoral immune response against AMACR. Consistent with results from protein microarray and immunoblot analyses, AMACR immunoreactivity detected by ELISA was statistically significantly higher in sera from patients with prostate cancer (mean = 0.823; 95% CI = 0.553 to 1.09; P = .009) than in sera from control subjects (mean = 0.460; 95% CI = 0.399 to 0.521) (Fig. 4A). Representative titration curves of serum samples from four patients with prostate cancer (positive for AMACR humoral immune response) and two control subjects are shown. The multiple bands that are smaller than that of AMACR–MBP likely represent degradation products. B) Specificity of the humoral response to AMACR was determined by incubating sera from randomly selected patients with prostate cancer (lanes marked PCA) who had earlier shown a humoral response to AMACR with 18 μg of purified recombinant AMACR–MBP (lanes marked quenched) per mL before immunoblot analysis as described in panel A. C) Schematic representing the high-throughput immunoblotting of sera for humoral response to recombinant AMACR. D) Representative high-throughput immunoblots of recombinant AMACR–MBP incubated with sera from 23 control subjects (control) and 23 patients with prostate cancer. The two panels show immunoreactivity against AMACR. E) Isotype analysis of the AMACR humoral response. Sera from five patients with an AMACR humoral response and three with no humoral response were incubated with immunoblots of recombinant AMACR–MBP and probed with anti-human IgG isotype-specific antibodies.

Fig. 3. Immunoblot-based validation and titration of the α-methylacyl-CoA racemase (AMACR) humoral response. A) Normalized population mean ratios for AMACR humoral immune response from 259 control subjects and 151 patients with prostate cancer (PCA) as assessed by high-throughput immunoblot analysis. Error bars represent 95% confidence intervals. B) Serial dilutions (1:25 to 1:400) of sera from four randomly selected patients with prostate cancer and two control subjects were analyzed for AMACR humoral response by using immunoblot analysis. C) The immunoblot band intensities for AMACR reactivity from five randomly selected patients with prostate cancer were subjected to densitometry and normalized to mean intensity of a positive control sera and plotted against serum dilution to generate a graphical representation of AMACR reactivity.
The purpose of this study was to examine whether AMACR could serve as an immune response biomarker that could be used to potentially supplement PSA for accurate detection of early-stage prostate cancer. The association of AMACR expression with prostate cancer was first highlighted by using gene expression analysis (13,14,27). Subsequent validation studies have shown its importance as a tissue biomarker for prostate cancer (16–18). Thus, these observations make AMACR an important target for development as a prostate cancer–specific serum biomarker. However, as is the case with many other biomarkers, the levels of circulating AMACR in most patients with prostate cancer appear to be very low, making the protein difficult to detect. Therefore, we explored the possibility of using an autoimmune response against AMACR as a means of detecting this antigen.

A potential limitation of this study is that AMACR levels are elevated in certain cancers other than prostate cancer (28,29). Thus, the humoral response to AMACR needs to be evaluated in cohorts of patients with different epithelial cancers. Furthermore, it remains to be determined whether patients with autoimmune conditions produce an immune response to AMACR. In addition, because detection of the humoral immune response to AMACR depends on the presence of intact autoantibodies, care must be taken in storage of serum for purposes of AMACR humoral response. Multiple freeze–thawing of serum aliquots diminishes the immunoreactivity of AMACR autoantibodies (A. Sreekumar, B. Laxman, and A. M. Chinnaiyan: unpublished observations). Furthermore, development of ELISAs in which AMACR is expressed in eukaryotic cells rather than in bacteria is important because it appears that bacterial contaminating proteins can affect this assay format (unpublished data).

This study highlights the use of protein microarrays as discovery tools in translational research to characterize the humoral

**DISCUSSION**

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<th>Variable</th>
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<th>Pathological stage</th>
<th>Recurrence</th>
<th>Prostate weight</th>
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<td>.211</td>
<td>.369</td>
<td>.363</td>
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*Humoral response was assessed by high-throughput immunoblot analysis. PSA = prostate-specific antigen.

**Fig. 4.** ELISA-based validation of the α-methylacyl-CoA racemase (AMACR) humoral immune response. A) Normalized population mean ratios for AMACR humoral immune response from 55 control subjects and 54 patients with prostate cancer (PCA) as assessed by ELISA. Error bars represent 95% confidence intervals. B) Titration of AMACR humoral immune response by ELISA. Representative titration curves for four patients with prostate cancer (open circles) and two control subjects (closed circles) are shown. The absorbance values at 450 nm were normalized against a positive AMACR control sera used at 1:1000 dilution in each ELISA plate.
immune response against a tumor antigen. This initial survey approach led to the identification of autoantibodies to AMACR in patients with prostate cancer. In particular, we validated the AMACR immunoreactivity detected by protein microarrays by using high-throughput immunoblotting of the entire serum cohort, from 259 control subjects and 151 patients with biopsy-proven prostate cancer. To facilitate future potential clinical implementation, we developed an ELISA and tested a random subset of our serum cohort from 55 control subjects and 54 patients for AMACR reactivity.

Although our findings lend support for the use of AMACR humoral response for the early detection of prostate cancer (71.8% specificity and 61.6% sensitivity), a more promising use may be in combination with initial PSA screening. Use of such a “supplementary” biomarker becomes especially important because PSA, at levels lower than 10 ng/mL, has a relatively low specificity for organ-confined disease (30). The extensive overlap in levels of total PSA between healthy subjects and prostate cancer patients (30) leads to inaccurate discrimination between prostate cancer and control individuals in the clinically relevant PSA range of 4–10 ng/mL. The diagnostic value of anti-AMACR antibodies is most evident in this clinically significant PSA range, in which the assay for AMACR humoral response discriminated between cancer and noncancer populations, with sensitivities and specificities of approximately 80%. Because the low specificity of PSA for prostate cancer has resulted in a dramatic increase in the number of needle biopsies for unequivocal confirmation of cancer (6), our data suggest that detection of autoantibodies against AMACR in the patients with PSA levels in the critical diagnostic range of 4–10 ng/mL could improve the specificity of PSA for cancer detection. Thus, we found that, at a cutoff of 0.227 units of AMACR reactivity, the odds ratio of having a positive AMACR test and prostate cancer among patients with PSA levels between 4 and 10 ng/mL was 15.48 (95% CI = 5.87 to 40.71; P < .001). This additional discriminatory power could aid patients in considering whether to undergo a biopsy. Moreover, because prostate cancer is widespread among the general population (30,31), there is a need for markers having greater specificity rather than higher sensitivity. Although it is unlikely that any single marker for prostate cancer will have the desired high specificity and sensitivity, one could envision development of a multiplex panel of serum biomarkers that, in combination, would improve the diagnostic accuracy of prostate cancer screening.

References


NOTES

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