Serum Antibodies to Huntingtin Interacting Protein-1: A New Blood Test for Prostate Cancer

Sarah V. Bradley,1 Katherine I. Oravec-Wilson,1 Gaelle Bougeard,1 Ikuko Mizukami,1 Lina Li,1 Anthony J. Munaco,1 Arun Sreekumar,1 Michael N. Corradetti,1 Arul M. Chinnaiyan,2,3 Martin G. Sanda,1 and Theodora S. Ross1

Departments of Internal Medicine, Pathology, and Urology, University of Michigan Medical School, Ann Arbor, Michigan

Abstract

Huntingtin-interacting protein 1 (HIP1) is frequently overexpressed in prostate cancer. HIP1 is a clathrin-binding protein involved in growth factor receptor trafficking that transforms fibroblasts by prolonging the half-life of growth factor receptors. In addition to human cancers, HIP1 is also overexpressed in prostate tumors from the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model. Here we provide evidence that HIP1 plays an important role in mouse tumor development, as tumor formation in the TRAMP mice was impaired in the Hip1null/null background. In addition, we report that autoantibodies to HIP1 developed in the sera of TRAMP mice with prostate cancer as well as in the sera from human prostate cancer patients. This led to the development of an anti-HIP1 serum test in humans that had a similar sensitivity and specificity to the anti-AMACR test and when combined with the anti-AMACR test yielded high sensitivities (9–11).

Because HIP1 is specifically up-regulated in prostate cancer relative to benign prostatic epithelia (12) and is a cytoplasmic protein, we hypothesized that HIP1 autoantibody formation could, like AMACR, yield a useful blood test for prostate cancer. In addition, because overexpression of HIP1 is associated with advanced prostate cancer (12) and HIP1 directly transforms fibroblasts (13), we hypothesized that HIP1 may be necessary for in vivo tumor cell survival or progression.

To experimentally evaluate these two questions in mice, we employed the transgenic adenocarcinoma of the mouse prostate (TRAMP) model (14) and Hip1 mutant mice generated in our laboratory (15). TRAMP mice express SV40 T antigen under the control of the probasin promoter. This targets transgene expression to the epithelial cells of the prostate and leads to prostate cancer. Although many of the tumors in these mice are more representative of a neuroendocrine rather than epithelial cancer (16), the progression of these cancers in the TRAMP model is similar to human prostate cancer in that the prostates of these mice develop hyperplastic epithelia, in situ carcinoma, locally invasive cancers followed by metastases to the liver, lung, lymph nodes, and bone. In addition to providing evidence here that HIP1 may indeed be necessary for tumorigenesis in the TRAMP prostate, we have discovered that both TRAMP mice and men with prostate cancer produce autoantibodies to HIP1 more frequently than control individuals. Using both immunoblot and ELISA tests, described herein, we have found that the sensitivity and specificity of this novel prostate cancer blood test is similar to that of PSA, and when combined with AMACR, has the exciting potential to surpass the specificity of the PSA test.

Materials and Methods

Animals. The Hip1null/null mice (15) and TRAMP mice (14) were maintained on a C57BL/6J129svJ background. SV40 T antigen “homozygous” TRAMP male mice were intercrossed with Hip1null/null females to generate T antigen transgenic (TRAMP/Hip1null/null) mice that were heterozygous for the Hip1 mutation (TRAMP/Hip1+/null). The latter were intercrossed to make TRAMP littermates containing either wild type or knockout Hip1 alleles. Mouse tail DNA was genotyped for the SV40 T antigen by PCR (14) or for the Hip1 null allele by southern blot of tail tissue and Western blot of tumors for the presence or absence of HIP1 and T antigen protein bands. Mouse care followed established institutional guidelines.

Evaluation of transgenic adenocarcinoma of the mouse prostate tissue. Sixteen TRAMP/Hip1+/+/null and eight TRAMP/Hip1null/null littermate mice were analyzed for tumor extent at 6.5 months of age. Prostate and

Note: S.V. Bradley and K.I. Oravec-Wilson contributed equally to this work.

Requests for reprints: Theodora S. Ross, University of Michigan, 6322 CC GC, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0942. Phone: 734-615-5309; E-mail: tsross@umich.edu.

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tumor samples were fixed in 10% (v/v) buffered formalin, embedded in paraffin, serially sectioned and stained by H&E. The slides of prostatic tissue were evaluated for the presence of hyperplasia, adenoma, or invasive adenocarcinoma as described previously (18).

**Acquisition of serum samples from transgenic adenocarcinoma of the mouse prostate mice.** TRAMP mice and T antigen–negative control mice were initially bled between the ages of 2 and 4 months from the saphenous vein of the hind leg. Approximately 100 to 200 μL of blood was collected into Microvette CB 300 serum separation tubes (Starstadt, Nümbrecht, Germany) and 30- to 40-μL aliquots were stored at −20°C until analyzed.

**Human patient cohort and samples.** This study was approved by the University of Michigan Medical School Institutional Review Board. At the time of diagnosis and before prostatectomy, sera from 97 biopsy-proven clinically localized prostate cancer patients were collected and stored in the University of Michigan Prostate Specialized Programs of Research Excellence (University of Michigan, Ann Arbor, MI). Sera were stored in aliquots at −80°C for mouse sera and 20°C for human sera. All sera were stored in aliquots at −20°C until analyzed.

**Preparation of HIP1 antigen.** A glutathione S-transferase-3HIP1 (GST-3HIP1) fusion construct was used to generate 3HIP1 antigen. Briefly, GST was fused in frame to the COOH-terminal half of HIP1 amino acid sequence starting at the sole internal EcoRI site (nucleotide 1250) and ending at the native stop codon (nucleotide 3010; ref. 19). Expression of antigen was driven by the CMV promoter in the pETDuet expression plasmid. Recombinant 3HIP1 antigen was purified by using protein G-agarose (Pierce) columns.

**Immunoblot analysis of anti-HIP1 antibodies in mouse or human serum.** 3HIP1 protein (10 μg for mouse sera and 20 μg for human sera) was separated on a 10% preparative gel, transferred to nitrocellulose, and blocked overnight at 4°C in TBST (mouse sera) or TBS (human sera) with 5% milk and 5% goat (mouse samples) or donkey (human samples) serum ("blocking solution"). A Miniblotter 28-duall unit system (Immunetics, Inc., Cambridge, MA) was used to make 25 incubation chambers for serum samples, diluted 1:50 in 1:10 blocking solution (human sera) or 1:15 in TBST/5% milk (mouse sera). Membranes were incubated with the serum samples for 2 hours at room temperature and washed with TBST. For blots of TRAMP sera, goat antimouse horseradish peroxidase (HRP)–conjugated secondary antibody (Sigma, St. Louis, MO) was used at 1:5,000 dilution in TBST/5% milk for 1 hour at room temperature. The blots were washed for 1 hour with TBST and HRP developed with enhanced chemiluminescence (ECL). For analysis of human sera, a donkey anti-human biotin conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) was used at a 1:50,000 dilution in 1:10 "blocking solution" for 1 hour. After washing with TBST, HRP-conjugated streptavidin was incubated with the blots (1:25,000 dilution in 1:10 blocking solution) for 1 hour, and the blots were subjected to a final wash. Super-Signal ECL (Pierce, Rockford, IL) was used to develop the HRP for the human samples and generic ECL was used for mouse samples (20).

ELISA test for HIP1 autoantibodies. MaxiSorb immunoplates (Nalge Nunc International, Rochester, NY) were coated with 5 μg/mL of the 3HIP1 antigen by incubating 50 μL per well overnight at 4°C. The plates were washed twice with TBST. Plates were blocked with 200 μL of 5% milk in TBST overnight at 4°C, washed twice with TBST, and stored at 4°C for a maximum of 2 weeks. Serum samples (50 μL per well) diluted 1:100 in blocking solution were assayed in duplicate and incubated with the antigen-coated plates at room temperature for 1 hour. The plates were washed five times with TBST and incubated with 1:10,000 goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) at room temperature for 3 hours. After washing five times with TBST, the plates were incubated with avidin-biotin complex reagent (Pierce) for 30 minutes and washed. 100 μL of the 1-Step Ultra TMB (Pierce) was incubated on the plates for 30 minutes for color development and quenched with 100 μL of 1 M H2SO4. Absorbance was measured at 450 to 550 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA).

**Statistical analysis.** All statistical analyses were done with Excel, Medcalc, or SPSS. To test for the difference in tumor incidence and histologic appearances the MedCalc program was used to perform correlation and χ2 tests. To test for significant differences in HIP1 immune response between prostate cancer patients and control subjects, Pearson’s χ2 test as well as Student’s two-sided t test were done using SPSS. ROC curve analysis was achieved using the MedCalc program.

**Results**

Diminished prostate tumor development in transgenic adenocarcinoma of the mouse prostate/3Hip1null/null mice. To examine the in vivo necessity of HIP1 overexpression in prostate tumors, TRAMP mice (14) and 3Hip1null/null mice (15) were crossed to generate TRAMP mice deficient of HIP1 (TRAMP/3Hip1null/null) as well as control littermates (TRAMP/3Hip1+/− mice). This experiment was based on the previous observation that murine HIP1 is overexpressed in 50% of prostate tumors that develop in TRAMP mice (12) and that the loss of function mutation of Hip1 does not alter the development or maintenance of normal prostate tissue nor does it affect hormone levels in mice including testosterone (15, 17). In an initial study of these mice, we noted that TRAMP mice deficient for HIP1 did not develop as many palpable tumors as their wild-type HIP1 expressing littermates (data not shown). To quantitate this observation, we initiated a second experiment where TRAMP littermates without HIP1 expression (TRAMP/3Hip1null/null) and their controls (TRAMP/3Hip1+/−) were sacrificed at 6.5 months of age. We chose to analyze mice at this age, as by 6 months of age, all TRAMP mice develop prostate tumors (18). As seen in Fig. 1, the absence of HIP1 expression resulted in fewer grossly observed prostate tumors than littermate Hip1 wild-type controls (2 of 8 TRAMP/3Hip1null/null [25%] versus 13 of 16 TRAMP/3Hip1+/− [81%], respectively. P < 0.01).
The diminished tumor frequency observed in the Hip1<sup>null/null</sup> mice could be due to a reduced rate of tumor initiation. Alternatively, HIP1 may be required for tumor growth or progression to invasive carcinoma. To begin to distinguish between these possibilities, the histologic characteristics of the prostates and their tumors from these mice was scored using a previously described grading system of prostatic lesions (18). Briefly, serial tissue sections were characterized for their most advanced lesions. For example, "hyperplasia" was scored when the epithelial cells of the acini were crowded and formed foci in cribriform or papillary patterns but still followed the outline of the acini. "Adenoma" was scored when in some parts of the tissue the epithelial cells completely filled the lumen or distinct epithelial masses were found in the lumen of the acinus. "Invasive carcinoma" was scored when there was local invasion into and beyond the capsule of the acini or there were distant metastases. The TRAMP mice with "adenomas" or "invasive carcinomas" also contained multiple foci of hyperplasia.

Using this scoring system, we found that development of invasive cancers was diminished in TRAMP/Hip1<sup>+/+</sup> mice. At 6.5 months of age, most of the TRAMP mice with normal Hip<sub>t</sub> had adenomas or invasive cancers (8 of 8 observed TRAMP/Hip1<sup>+/+</sup> mice were found to have adenomas [75%] or invasive carcinomas [25%], as expected). In contrast, most of the TRAMP/Hip1<sup>null/null</sup> mice had only hyperplastic lesions (five of six, 84%). The differences in tumor incidence either by gross observation or by histology between control and TRAMP/Hip1<sup>null/null</sup> mice was significant (P < 0.01 and P < 0.025, Pearson’s χ², respectively). These data suggest that there is a delay in the ability of prostatic lesions from Hip1<sup>null/null</sup> mice to progress from hyperplasia to adenomas and invasive carcinomas. Previously, we reported that 50% of TRAMP prostate tumors overexpressed HIP1 by Western blot analysis of tumors (12). In contrast, we find here that at least 75% of the TRAMP prostates required HIP1 expression for invasive tumor formation (Fig. 1A, first column). This suggests that the sensitivity to detect HIP1 overexpression by Western blot analysis of prostate tumors may be limited.

**Autoantibodies to HIP1 in transgenic adenocarcinoma of the mouse prostate mice.** Because HIP1 was overexpressed in prostate tumors of both humans and mice (12), we attempted to measure HIP1 levels in mouse serum by Western blot analysis using anti-HIP1 polyclonal (UM323) and monoclonal (1B11) antibodies. Our goal was to determine if HIP1 antigen quantitation could be used as a novel serum biomarker of prostate cancer. As one might expect for a cytoplasmic protein, we were not able to detect the HIP1 antigen in sera (data not shown). Because of this limited sensitivity, we decided to test the hypothesis that a humoral immune response to overexpressed HIP1 marks prostate cancer presence. If such a response was detected, we hypothesized that it could be used as a potential blood test for prostate cancer detection and prognosis.

To begin to test this, recombinant HIP1 (19) was purified (Fig. 2A, left) and immunoblot with specific HIP1 monoclonal antibodies, 4B10 and 1B11, confirmed its identity (Fig. 2A, right). The lower of the two bands on the Western blot was variably seen in different preparations of the purified antigen and was likely the result of degradation during antigen preparation. In an initial pilot Western blot study of mouse sera and 3’ HIP1 antigen, we found there was immune reactivity to the HIP1 antigen in sera from prostate tumor-bearing TRAMP/Hip1<sup>+/+</sup> mice but not control (T antigen negative) or TRAMP/Hip1<sup>null/null</sup> mice (data not shown). Serial serum samples from TRAMP mice and control mice were loaded in a miniblot apparatus (8) to determine the developmental time course and maintenance of autoantibodies to HIP1 in TRAMP mice (Fig. 2B). Remarkably, we found that there was an antibody response to HIP1 that varied in its time of onset (Fig. 2B) but was detected as early as 4 months of age in the TRAMP mice, all of which were expected to have developed prostatic lesions by 6.5 months of age. Twelve of the 22 (55%) TRAMP mice developed sustained immunity. In contrast, none of the 14 (0%) control (T antigen negative) littermates showed sustained presence of autoantibodies to HIP1.

**Serum antibodies to HIP1 in human prostate cancer patients.** In light of the presence of autoantibodies to HIP1 in prostate cancer-bearing TRAMP mice, we tested if there was an immune response to HIP1 in sera from human prostate cancer patients. Because one gel was only able to assay 25 distinct sera at a time and we had sera from 308 men available for testing, we used the same positive and negative HIP1 reactive sera on each blot as a reference point. This allowed us to quantitate and normalize signals between different blots. Results of one such screen using sera from prostate cancer patients and controls (n = 23 for each) are shown in Fig. 3A. Ultimately, the sera from

![Figure 1. HIP1 deficiency impairs tumorigenesis in the TRAMP model of prostate cancer.](https://example.com/figure1.png)
Serum Antibodies to HIP1 in Prostate Cancer Patients

97 prostate cancer and 211 age-matched male control sera were screened by Western blot. The blots were analyzed by measuring the grayscale values of the reactive bands (Fig. 3A, arrows) and quantitated as a percent of the reference positive control (Fig. 3B). A positive score was assigned to bands with a value of ≥50% of the positive control, whereas those bands <50% of the positive control received a negative score. This cutoff was chosen because it yielded the highest values for specificity and sensitivity, as analyzed from ROC curves created from a randomly chosen subset of the prostate cancer and control subjects. All serum samples were validated for autoantibodies to HIP1 by this high-throughput immunoblot analysis. HIP1 antibodies were significantly more frequent in serum from prostate cancer patients compared with age-matched controls. HIP1 autoantibodies were significantly more frequent in serum from prostate cancer patients compared with age-matched controls (n = 4 replicates) were averaged. Figure 4A shows the average relative absorbencies for all of the prostate cancer patient sera and 81 of the control sera. A relative absorbance that was greater than the negative control (ELISA value, >1) was considered a positive score. The cutoff for this test was, like the high-throughput Western blot test, determined by using ROC curves on a subset of the patient sera and determining where the ELISA values yielded the highest specificity and sensitivity. All available serum samples were then tested for HIP1 antibodies by high-throughput ELISA. There were significantly more prostate cancer sera with positive scores (46% of sera from prostate cancer patients versus 27% of sera from age-matched controls; Fig. 4B). The ELISA test alone results in similar values for specificity and sensitivity as the Western blot analysis (Table 2). If both tests are required to be reactive for a positive test, only 24% of the prostate cancers are positive versus 12% of the controls. Although there is diminished sensitivity using the increased stringency (both tests necessarily reactive), it does raise the specificity to 88%. The observed decreased sensitivity with the combination of Western blot and ELISA tests is expected because the chance that both tests, which have distinct antigen presentations on either nitrocellulose membranes or plastic plates, would have accessible antigenic epitopes simultaneously in each patient is less likely than if only one were necessary. Hence, if only one of the two HIP1 reactivity tests is required for positivity, 69% of the prostate cancers are positive versus 44% of the controls (Table 2). It should be noted at this point that the control group did not undergo prostate biopsies or have close follow-up. Because of this limitation, the possibility of missed prostate cancer in the control group must be considered when evaluating this initial data. In addition, some of the “background” could be contributed by other occult malignancies such as melanoma, colon, or lung cancers.

Previously, we have found that using immunohistochemical analysis of HIP1 antigen in tissue sections, overexpression of HIP1 in prostate cancers predicted a poor outcome (12). It follows that the autoantibodies to HIP1 in prostate cancer patients might also contain prognostic information. In the current group of 97 prostate cancer patients, there were no statistically significant associations between HIP1 immune responses and linked clinical variables including initial PSA level, PSA recurrence, Gleason grade, tumor size, or stage.

In addition to assessing the relationship between linked clinical data and HIP1 autoantibody formation, we compared the HIP1 test...
to other serum tests such as the PSA and AMACR tests. In the initial study of the AMACR humoral response, a specificity of 71.8% and sensitivity of 61.6% were found (8). The samples used for this current study of H1P1 humoral response were also tested for their humoral immune response to AMACR and similar values for AMACR specificity and sensitivity were found as previously reported (67% and 64%, respectively; Table 2). The ROC curves for H1P1 and AMACR yielded similar values for area under the curve (data not shown).

As well as comparison with the AMACR test, it follows that the H1P1 antibody test could complement the PSA test. However, the comparison of the H1P1 test to the PSA test in the group of patients ($n = 90$) and controls ($n = 117$) for which PSA data was available was problematic. This was due to the availability of only a limited supply of banked serum samples from control patients with PSA values of >4.0 ng/mL. This resulted in an expected but skewed specificity and sensitivity (75% and 77%, respectively) for the PSA test (positive, >4.0 ng/mL). The reported 45% specificity and 50% sensitivity for PSA in a previous group of sera that were tested for AMACR are closer to expected (8). Because of this limited supply, a subgroup of 68 prostate cancer sera and 29 age-matched control sera that had PSA values of >4 ng/mL was analyzed separately for H1P1 autoimmunity (Table 3). There was again a significant difference in the numbers of H1P1-positive samples from prostate cancer patients versus control individuals, as determined by ELISA or Western blot ($P \leq 0.025$ and $P \leq 0.01$, respectively). The most significant difference was seen when a positive score by either ELISA or Western blot was required, giving a specificity of 64% and a sensitivity of 88% ($P \leq 0.001$) in a group that would all be considered positive by the PSA test. In addition, a combination of AMACR and H1P1 tests increased specificity dramatically (97%) suggesting that the combination of these two tests could lead to better predictions of cancer if added to the PSA test. Although further analysis of additional patient and control populations with prospective follow-up, serial sampling (as shown for the TRAMP mice in Fig. 2B) and from multiple different institutions is essential, these results suggest that the combination of the H1P1 test with PSA and AMACR tests results has the potential to yield a highly specific diagnostic test for prostate cancer.

**Discussion**

Prostate cancer morbidity and mortality are due to its progression within the prostate as well as its metastatic spread beyond the prostate. Because of this, an understanding of the mechanism by which localized hyperplastic lesions progress to invasive and metastatic carcinomas is very important. In addition, obtaining blood tests that can provide for the earliest detection of prostate cancer will have important prognostic and therapeutic implications.

![Figure 3](image_url)

**Figure 3.** Prostate cancer patients have a specific humoral response to H1P1 overexpression. A, representative immunoblot of 46 sera assayed for reactivity to recombinant H1P1. Twenty-three of the 97 biopsy-proven prostate cancer patients and 23 of the 211 control individuals. Equal aliquots of all of the 308 serum samples were analyzed by immunoblot in at least two independent experiments and contained reference positive and negative controls (Positive and Negative lanes, respectively). B, bands were scanned from the developed blots and converted to grayscale values using Adobe Photoshop. Normalized grayscale values were converted to percentage of the positive control (Positive lane). Samples with band intensity of >50% of the positive control were given a positive score (above the dotted line). A negative score was given to samples <50% of positive control (below the dotted line). C, distribution of the values between prostate cancer and the control individuals was significantly different ($P < 0.001$, Pearson’s χ² test). Specificity of the test was 73% and calculated as those control samples with a negative test (153/153 + 58) and sensitivity was 46% and calculated as the percent of patient samples with a positive test (45/45 + 52).
Here we report in vivo genetic evidence for the necessity of the clathrin-binding protein, HIP1, in the prostatic hyperplasia-to-carcinoma transition. These experiments were initiated based on the fact that HIP1 expression is frequently elevated in human prostate cancer, and this overexpression predicts the progression of the disease in humans. In addition, because TRAMP mice have HIP1 up-regulated in their tumors (12), it was considered a relevant tumor model. We show that although all Hip1 null/null mice developed prostatic hyperplastic lesions in response to expression of T antigen, the development of bona fide tumors was significantly diminished compared with TRAMP mice with normal levels of HIP1.

Although the absence of HIP1 leads to testicular degeneration, it should be noted that the prostate glands from Hip1 knockout mice are normal histologically and serum testosterone levels are within normal limits (15, 17). This makes it unlikely that the effect of HIP1 deficiency on tumor development in this model is merely secondary to differences in the levels of testosterone or abnormalities in adult prostate epithelial cell maintenance. It should also be noted that the use of SV40 T antigen to induce prostate cancer is, in many ways, artificial in that T antigen does not seem to have a role in human prostate cancer. However, because HIP1 is overexpressed in TRAMP tumors, as it is in human tumors, and because T antigen does inhibit the human tumor suppressor gene products p53 and Rb, this model has significant validity for the purposes of initial studies of HIP1’s in vivo role in cancer biology.

It will be important to better understand the mechanism of how HIP1 could participate in the development of prostate cancer in humans. Previous work has shown that the HIP1 family of proteins is involved in the modulation of a variety of receptors such as the glutamate receptor (21), the epidermal growth factor receptor (EGFR), platelet-derived growth factor β receptor (PDGFβR; ref. 22), and transferrin receptor (23). This modulation of receptors leads to an increased survival and transformation of cells when HIP1 is overexpressed (12, 13). Although a direct regulatory effect of HIP1 on clathrin trafficking in prostate cancer remains to be shown, HIP1 could modulate signals from the EGFR and PDGFβR in prostate cancer as these receptors are clearly regulated by the clathrin trafficking network and are altered in prostate cancer. Determination if HIP1 can modulate other types of receptors that are not regulated by clathrin-mediated endocytosis but are involved in prostate cancer, such as the steroid hormone receptors (e.g., androgen receptor), will be important future experiments.

In addition to testing for HIP1 necessity in prostatic carcinogenesis, the previous observation of HIP1 overexpression in tumors

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**Figure 4.** Detection of HIP1 humoral response in human prostate cancer patients by ELISA. A, average (four replicates) relative absorbances (ELISA values) and their standard deviations are shown for 81 prostate cancer patient and 186 control sera. A relative absorbance of >1.0 (above the dotted line) was considered positive. B, numbers of positive prostate cancer and age-matched control sera. The specificity of this test was 73% and the sensitivity was 46%. The difference between prostate cancer patients and controls was significant (P < 0.01, Pearson’s χ²).

### Table 1

<table>
<thead>
<tr>
<th>Scores</th>
<th>Prostate Cancer patients (N=81)</th>
<th>Controls (N=186)</th>
</tr>
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<tbody>
<tr>
<td>Positive</td>
<td>37*</td>
<td>51</td>
</tr>
<tr>
<td>Negative</td>
<td>44</td>
<td>135</td>
</tr>
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</table>

* The frequency of a positive test in prostate cancer patients was statistically different than in control patients (p ≤ 0.01, Specificity (73%), Sensitivity (46%))
of TRAMP mice (12) prompted us to test if HIP1 could be detected in the serum of these mice. As expected for a cytoplasmic protein, we found that the circulating HIP1 antigen levels are low and therefore difficult to detect. However, we did find that TRAMP mice developed early and sustained levels of antibodies against HIP1 when measuring longitudinal samples. Interestingly, the T antigen–negative control mice also had samples of sera that tested positive randomly. However, sustained presence of anti-HIP1 antibodies were never observed in the control mice.

This led us to test if a humoral response to HIP1 could occur in humans with prostate cancer. The goal would be to find a novel blood test to substitute for or to complement the PSA test. Indeed, the test we describe herein for autoantibodies to HIP1 in prostate cancer has a relatively high specificity and improves the specificity of the PSA and AMACR tests, making it an attractive serum marker. Because we were able to show a sustained humoral response in TRAMP mice, we predict that future studies that are designed for prospective serial testing of humans for HIP1 antibodies will show an increase in the anti-HIP1 test’s sensitivity and specificity. Because prostate cancer is such a common cancer, markers with a greater specificity rather than sensitivity are needed to reduce unnecessary prostate biopsies or other invasive tests. For example, misdiagnosis with the PSA test may account for >30% of positive tests in a screened male population over the age of 55 (24), making reliance on the PSA test alone problematic. Finally, it is unlikely that any single marker for prostate cancer will have the desired high specificity and sensitivity, making it important to develop a collection of markers, which in combination could lead to accurate prostate cancer detection and prognosis.

### Table 2. Comparison of diagnostic tests and their combinations for all prostate cancer and control samples

<table>
<thead>
<tr>
<th>Test</th>
<th>Specificity (%)</th>
<th>Sensitivity (%)</th>
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<tbody>
<tr>
<td>HIP1 ELISA positive</td>
<td>73</td>
<td>46</td>
</tr>
<tr>
<td>HIP1 Western positive</td>
<td>73</td>
<td>46</td>
</tr>
<tr>
<td>HIP1 ELISA + HIP1</td>
<td>88</td>
<td>24</td>
</tr>
<tr>
<td>Western positive</td>
<td>56</td>
<td>69</td>
</tr>
<tr>
<td>HIP1 ELISA or HIP1 Western positive</td>
<td>86</td>
<td>50</td>
</tr>
<tr>
<td>AMACR positive</td>
<td>67</td>
<td>64</td>
</tr>
<tr>
<td>AMACR positive + HIP1</td>
<td>86</td>
<td>50</td>
</tr>
<tr>
<td>ELISA or HIP1 Western positive</td>
<td>75</td>
<td>77</td>
</tr>
<tr>
<td>PSA positive (≥4 ng/mL)</td>
<td>91</td>
<td>66</td>
</tr>
<tr>
<td>PSA positive + HIP1 ELISA or HIP1 Western positive</td>
<td>97</td>
<td>55</td>
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NOTE: There were 308 sera, except for the HIP1 Western, were assayed for every test listed. HIP1 ELISA values were available from 81 of the prostate cancer patients and 186 controls. AMACR Western values were available for 77 prostate cancer patients and 126 controls. PSA values were available for 90 prostate cancer patients and 117 controls. The increased frequency of a positive test in prostate cancer patients compared with controls was statistically different in all cases.

The increase in frequency of antibodies to HIP1 in prostate cancer compared with age-matched controls, together with the fact that we had previously found that HIP1 is overexpressed in many different epithelial cancers (12), will prompt us to investigate the potential for a specific humoral response in other cancers. This could also be a source of error in reducing the specificity of the HIP1 blood test for prostate cancer in our current control group, as the men could have had other occult or nonoccult malignancies. In fact, a specific humoral response to the HIP1-related protein, the only known mammalian relative of HIP1, has been reported to occur in colon cancer (25).

In conclusion, we have explored the role of HIP1 in in vivo tumorigenesis using the prostate cancer prone TRAMP mice and Hip1 knockout mice. Our data indicate that HIP1 may be necessary for tumorigenesis and that both mice and men with prostate cancer have autoantibodies to HIP1 in their serum. These data provide groundwork for further investigation into the functional involvement of HIP1 in other cancers and as a specific marker (especially in combination with AMACR) for other cancers. These data also pave the way for further prospective, longitudinal, and multi-institutional studies of how to best use the HIP1 Western blot and ELISA tests for improved care of patients with prostate cancer.

### Table 3. Comparison of diagnostic tests and their combinations for all prostate cancer and control samples with PSA values of ≥4 ng/mL

<table>
<thead>
<tr>
<th>Test</th>
<th>Specificity (%)</th>
<th>Sensitivity (%)</th>
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</thead>
<tbody>
<tr>
<td>HIP1 ELISA positive</td>
<td>76</td>
<td>49</td>
</tr>
<tr>
<td>HIP1 Western positive</td>
<td>82</td>
<td>54</td>
</tr>
<tr>
<td>HIP1 ELISA + HIP1 Western positive</td>
<td>93</td>
<td>28</td>
</tr>
<tr>
<td>AMACR positive</td>
<td>83</td>
<td>64</td>
</tr>
<tr>
<td>AMACR positive + HIP1 ELISA or HIP1 Western positive</td>
<td>97</td>
<td>55</td>
</tr>
</tbody>
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NOTE: There were 68 prostate cancer and 29 control sera that met the criterion of PSA of ≥4 ng/mL. Nine of the 68 prostate cancer patient sera were not available to test for HIP1 ELISA and AMACR Western. The increased frequency of a positive test in prostate cancer patients compared to controls was statistically different in all cases.

<table>
<thead>
<tr>
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<th>Sensitivity (%)</th>
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<td>PSA positive (≥4 ng/mL)</td>
<td>75</td>
<td>77</td>
</tr>
<tr>
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<td>91</td>
<td>66</td>
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We thank Teresa Hyun, Sean Morrison, Anj Dlugosz, Peter Lucas, Grant Rowe, and Mark Day for critically reading this article and Dan Normolle, Jason Harwood, Paul Nolan, June Escara-Wilke, Jenny Loveridge, and Melissa Rogers for their assistance during the course of this study.

**Acknowledgments**

Received 1/3/2005; revised 2/11/2005; accepted 2/18/2005.

**Grant support:** Komen Foundation predoctoral fellowship (S.V. Bradley), La Ligue Nationale contre le Cancer postdoctoral fellowship (G. Bougeard), NIH-R01 CA92963-01A1 (T.S. Ross), NIH-R01 CA98730-02 (T.S. Ross), and NIH-R01 CA82419-01 (M.G. Sanda).

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