

Preferential Humoral Immune Response in Prostate Cancer to Cellular Proteins p90 and p62 in a Panel of Tumor-Associated Antigens

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BACKGROUND. Cytoplasmic p90 autoantigen was recently cloned from a cDNA expression library using serum antibody from a cancer patient. The humoral immune response to p90 in prostate cancer and benign prostatic hyperplasia (BPH) was examined.

METHODS. An antigenic fragment of recombinant p90 protein and several other tumor-associated antigens (TAAs) were used in ELISA and Western blotting to detect antibodies in sera from patients with prostate cancer, BPH, and other controls.

RESULTS. Autoantibodies to p90 were detected in 30.8% of 133 prostate cancer patients versus 1.5% in 68 BPH patients. When a selected panel of six TAAs including p90 were used for immunoscreening, the cumulative positive reactions in prostate cancer sera reached 92.5%, significantly higher than in BPH and other control sera. Antibodies to p90 showed the highest frequency in prostate cancer (30.8%), followed by antibodies to p62 (22.6%).

CONCLUSIONS. A panel of six selected TAAs was shown to have high sensitivity and specificity as immunodiagnostic markers in prostate cancer. *Prostate* 63: 252–258, 2005.

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INTRODUCTION

In previous studies concerning the humoral immune response in hepatocellular carcinoma (HCC) patients, it was noted that there was concomitant presence of antibody to a 90 kDa cellular protein in addition to antibodies to p62 [1]. The p90 autoantigen was cloned from a cDNA expression library [2] using antibody from a cancer patient. A prominent feature of p90 is a coiled-coil domain of 255 amino acid residues at the C-terminus and several lines of evidence indicated that this region of p90 was immunogenic [2]. Several types of human cancer sera including gastric, esophageal,

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and colon cancer as well as HCC, were found to have antibodies recognizing this particular sequence of p90 [2]. Furthermore, it was observed that p90 was over-expressed in gastric cancer tissues [2]. These initial studies suggested that the p90 antigen-antibody system might also serve as a useful marker for other types of cancer.

Prostate cancer is a leading cause of cancer-related death in males and is second only to lung cancer [3]. Although effective surgical and radiation treatments exist for clinically localized prostate cancer, metastatic prostate cancer remains essentially incurable. Early diagnosis is key for treatment for prostate cancer. Prostate specific antigen (PSA) is quite sensitive for cancer detection and is feasible for screening purposes, since nearly 75% of prostate cancer patients have elevated levels of PSA [4]. However, certain healthy individuals also have elevated PSA determinations. Thus, extensive studies are being conducted towards the identification of new biological markers, which would add to current markers and increase the sensitivity and specificity for prostate cancer detection [5].

This study was initiated to investigate the potential use of the p90 antigen-antibody system in the detection of prostate cancer. We demonstrated that autoantibodies to p90 were detected in 30.8% of prostate cancer sera, but in only 1.5% of sera from patients with benign prostatic hyperplasia (BPH) ($P = 0.0085$). In addition, we demonstrated that the potential usefulness of using antibodies as immune markers for diagnosis of prostate cancer can be enhanced by addition of other selected tumor-associated antigens (TAAs).

MATERIALS AND METHODS

Patients and Controls

One hundred thirty three sera from prostate cancer patients treated at Loma Linda University Medical Center (Loma Linda, CA) were available for this study. For controls, 68 sera were drawn from individuals with BPH from Scripps Clinic (La Jolla, CA). The diagnosis of BPH was made on the basis of elevated PSA, enlarged prostate and absence of malignancy by further studies including prostate biopsy. Ninety-six other sera were drawn from a large epidemiological and genetic study of hemochromatosis in which "normal" sera were obtained from a random sample of non-hospitalized adults in San Diego (courtesy of Dr. E. Beutler, The Scripps Research Institute). Serum was prepared from blood collected into siliconized tubes containing inert barrier material (B.D. Vacutainer, Franklin Hills, NJ). After 30 min at room temperature, serum was withdrawn and frozen in aliquots at -70°C until used. In addition, as representative of patients with known immune reactivity to unrelated cellular antigens, sera

from 62 patients with systemic lupus erythematosus (SLE) and 41 patients with Sjögren's syndrome were obtained from the serum bank of the Autoimmune Disease Center (Scripps) and were evaluated with the same immunoassays for their reactivity to TAAs. This study was approved by the Institutional Human Subject Review Boards of Loma Linda University Medical Center and The Scripps Research Institute.

Expression and Purification of Recombinant TAAs

To produce recombinant p90, gel purified 1.2 kb EcoRI-XhoI insert from GC291 was excised from the pBK-CMV plasmid and subcloned into the pET28b expression vector (Novagen, Madison, WI) as previously described [2]. The pET construct was transformed into *E. coli* BL21 (DE3) (Stratagene, La Jolla, CA) for recombinant protein expression in the presence of 2 mM IPTG. After 4 hr incubation, recombinant proteins were extracted and purified using nickel-nitrilotriacetic acid (Ni-NTA) bead affinity columns (Qiagen, Valencia, VA) [2]. Purification of other TAAs has been previously described [6]. In brief, recombinant p62 was expressed from a clone derived from a cDNA expression library by immunoscreening with antibody from a patient with HCC [1]. p62 cDNA was subcloned into pET28a vector producing a fusion protein with NH-terminal 6 \times histidine and T7 epitope tags. The recombinant protein expressed in *E. coli* BL21 (DE3) was purified using nickel column chromatography. Koc cDNA cloned in the pcDNA3 vector [7] was similarly subcloned in pET28a vector and recombinant protein expressed as above. IMP1 construct pCMV5-IMP1 was kindly provided by F.C. Nielsen [8] and p53 clone (p53SN3) by Yuxin Yin of Columbia University (New York, NY) and subcloned in pET28a as described above. cDNA from *c-myc* was amplified by polymerase chain reaction from human fetal liver tissue and survivin cDNA was amplified from human survivin EST clone (BG258433) before subcloning in pET28a vector [6]. Cyclin B1, cyclin A, and cyclin D1 were cloned into pGEX, pRSET, and pGEX vectors, respectively, and corresponding proteins were purified as previously described [10]. Recombinant protein was examined by SDS-PAGE and Coomassie Blue staining to determine that adequate amounts of expression products with the expected molecular sizes were produced.

Enzyme-Linked Immunosorbent Assay (ELISA)

Purified recombinant proteins were diluted in PBS to a final concentration of 0.5 $\mu\text{g}/\text{ml}$ and 200 μl were pipetted into each well to coat Immulon 2 microtiter plates (Dynatech Laboratories, Alexandria, VA). All

human sera were diluted 1:200, incubated with antigen-coated wells at room temperature for 90 min followed by washing with PBS and developed with horseradish peroxidase-conjugated goat anti-human IgG (Caltag Laboratories, San Francisco, CA) using the substrate 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid; Boehringer Mannheim GmbH, Mannheim, FRG). Each serum sample was tested in duplicate and the average OD at 450 nm was used for data analysis.

The cut-off value designating positive reactions was the mean OD of the 96 normal human sera +3 SD. In addition, all positive sera were confirmed with repeat testing, as were some negative sera. The data were analyzed using the χ^2 -test and two significance levels ($P < 0.05$ and $P < 0.01$) were used.

Cancer sera were also tested against four unrelated cellular antigens, double-strand DNA (dsDNA), Sm, SS-A/Ro, and SS-B/La which are known targets of autoantibodies present in the prototype autoimmune diseases, systemic lupus erythematosus and Sjogren syndrome [11]. Enzyme immunoassay kits from Helix Diagnostics (West Sacramento, CA) were used for analysis.

Western Blotting

Western blotting was performed as described by Zhang et al. [1]. Cell extracts were electrophoresed on SDS-PAGE and transferred to nitrocellulose paper. After preblocking with PBS containing 0.05% Tween-20 and 5% nonfat milk for 30 min at room temperature, the nitrocellulose strips were incubated for 60 min at room temperature with 1:200 dilution of serum. As secondary antibody, horseradish peroxidase-conjugated goat anti-human IgG (Caltag Laboratories) was applied (1:2,000 dilution). The detection of immunoreactive bands was performed with an ECL kit (Amersham

Corp.) according to the manufacturer's instructions and followed by autoradiography.

Statistical Analysis

Fisher's test for 2×2 contingency tables were used to determine significant differences between cohorts of prostate cancer patients, disease controls and normal subjects.

RESULTS

Prevalence of Autoantibody to p90 in Prostate Cancer Sera

An enzyme-linked immunosorbent assay system was developed using p90 recombinant protein [2] as antigen to determine the prevalence of antibodies to p90. One hundred thirty three prostate cancer sera, 68 BPH sera and 96 control normal human sera (NHS) were analyzed. The normal range was taken from the mean + 3 SD of 96 NHS. As shown in Figure 1A and Table I, 41 of 133 prostate cancer sera had autoantibodies to p90 with optical density readings higher than mean + 3 SD of antibodies in NHS. Only one patient in the BPH group was over the cut-off line.

Twenty-nine of 41 with higher OD values for p90 from prostate cancer patients were also positive in Western blotting using MOLT-4 cell extract. Five of seven (71.4%) sera with OD values between 1.0 and 2.0 were positive in Western blotting and 24 out of 34 (70.5%) prostate cancer sera with OD values between 0.5 and 1.0 were positive in Western blotting. In general, higher O.D. values in ELISA gave stronger signals in Western blotting (Fig. 1B). The variations between the two different immunological assays might be related to the increased sensitivity of enzyme immunoassay over Western blotting, but other possibilities

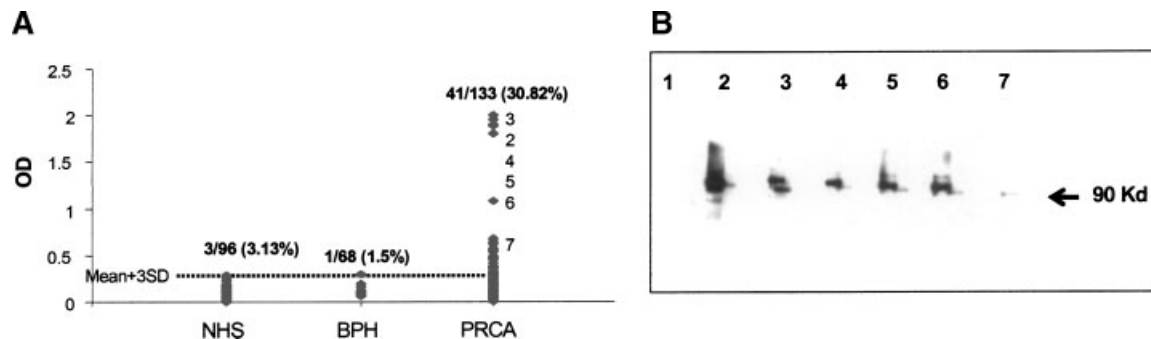


Fig. 1. **A:** Range of antibody titers to p90 in ELISA is expressed as optical density units. The mean + 3 SD of normal human sera are shown in relationship to prostate cancer and BPH sera. **B:** Western blot analysis shows antibody reactivity to p90 in strips of nitrocellulose membrane blotted with a human lymphocyte cell line (Molt-4) whole cell extract. **Lane 1:** Normal human serum. **Lanes 2–7** sera from prostate cancer patients. The corresponding OD values of the latter sera are shown in (A). In general, OD values in ELISA were related to intensity of signals in Western blotting with serum number 7 and others in somewhat similar O.D. range (A) giving weak immunoblotting signals.

TABLE I. Frequency of Antibodies to Ten Tumor-Associated Antigens (TAAs)

Subject	Number	Numbers and percent of autoantibodies to									
		<i>c-myc</i>	p53	Koc	IMP1	Survivin	p62	p90	Cyclin B1	Cyclin D1	Cyclin A
Prostate	Ca 133	5 (3.7)*	6 (4.5)*	11 (8.3)*	11 (8.3)*	3 (3.0)*	30 (22.6)**	41 (30.8)**	19 (14.3)**	15 (11.3)*	10 (7.5)*
NHS	96	1 (1.1)	2 (2.2)	2 (2.2)	2 (2.2)	1 (1.1)	3 (3.1)	3 (3.1)	2 (2.2)	2 (2.2)	3 (3.1)
BPH	68	1 (1.5)	0 (0)	2 (3.0)	1 (1.5)	1 (1.5)	0 (0)	1 (1.5)	0 (0)	1 (1.5)	0 (0)
SLE	62	3 (4.8)	0 (0)	1 (1.6)	2 (3.2)	1 (1.6)	1 (1.6)	2 (3.2)	1 (1.6)	ND	ND
S.S.	41	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND

Cut off value: mean + 3 SD of NHS. ND, not done.

* $P < 0.05$.

** $P < 0.01$ compared to NHS.

could be related to recognition of different epitopes on the same antigen in the two assays.

Autoantibody Responses to Other TAAs Including mRNA Binding Proteins, Cyclins, p53, and *c-myc*

In addition to p90, we have extended the study to a number of other TAAs. p62, Koc, and IMP1 are three proteins containing similar RNA-binding motifs and their mRNAs are either over-expressed in cancer or antibodies have been identified to the protein products [1,6–8,12]. Other TAAs selected were anti-apoptotic cellular protein Survivin [9], oncogene product of *c-myc* [13] and tumor suppressor gene product p53 [14]. Cyclin B1 was chosen because autoimmunity to this protein has been reported both in humoral and cell-mediated immune response in cancer patients [10,15]. Similarly, involvement of other cyclins including cyclin A and cyclin D1 in tumorigenesis have also been reported [16,17].

Table I and Figure 2 show that prostate cancer sera also contained autoantibodies to nine other TAAs in addition to p90. Different frequencies of antibody reactivity to these TAAs are more clearly demonstrated in bar graph form in Figure 2. Of note was that the frequency of antibodies to each antigen varies, with the lowest to survivin (3%) and highest to p62 (22.6%) and to p90 (30%). It appears that prostate cancer sera have preferential humoral immune responses to p90 and p62 over the other TAAs examined. Antibodies to *c-myc*, p53 and survivin showed low frequencies and barely reached statistical significance while antibodies to Koc, IMP1, cyclins D1, A, and B1 were in the range of 7%–14%. With the exception of anti-*c-myc*, antibody frequencies in SLE patients were in similar ranges as normal human sera and in Sjogren's syndrome, no antibodies to eight TAAs were detected (Table I).

In addition, it was important to ascertain that the positive antibody reactions observed in prostate cancer sera were not the result of some unusual reactivity to

intracellular proteins antigens. There are several well-recognized intracellular autoantigens such as double-strand DNA, Sm, SS-A/Ro, and SS-B/La in rheumatic autoimmune diseases and autoantibodies to these antigens have an established role in clinical diagnosis in these conditions [11]. All 133 prostate cancer sera were analyzed for antibodies to these four antigens using enzyme immunoassay kits (see Materials and Methods), and none of the cancer sera showed reactions above the cut-off O.D. reading whereas SLE and Sjogren's syndrome sera showed the expected percentage of positive reactors. In this limited analysis, it appears that the reactions of prostate cancer sera to TAAs were not due to non-specific reactions with cellular proteins.

Notably, there was no significant difference in antibody responses between BPH and normal human sera (Table I). It has been observed in recent studies [6,18] that although no single TAA was uniquely associated with a particular type of cancer when panels or

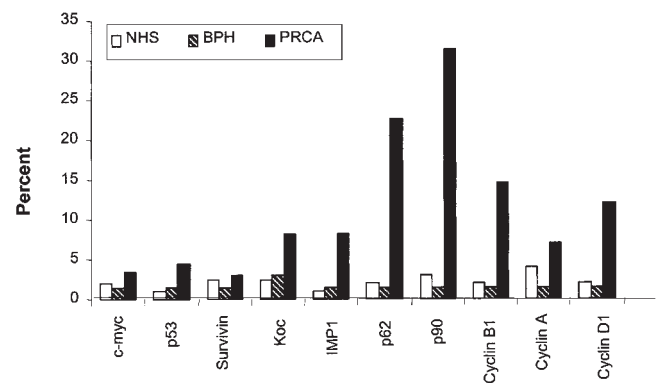


Fig. 2. Percentage of autoantibody responses to tumor-associated antigens (TAAs). The percentages refer to antibody titers exceeding the mean + 3 SD of normal human sera from enzyme immunoassays. Open bars represent reactions of normal human sera, hatched bars reactions of BPH sera and closed bars reactions of prostate cancer sera. In this panel of ten TAAs, the most reactive antigens were p62 and p90.

mini-arrays of TAAs were used in antigen platforms, some types of cancer were more reactive to certain specific TAAs. In this study, p90 and p62 appeared to be the most reactive antigens in prostate cancer and an analysis was performed to determine whether a subset and not necessarily all the ten TAAs (Table I) was sufficient to achieve optimal antibody frequency. Table II shows the stepwise increase in cumulative antibody frequency in prostate cancer versus normal controls. Only six TAAs (p90, p62, Koc, IMP1, cyclin B1, and cyclin D1) were sufficient to raise antibody frequency to 92.5% versus 14.8% in normal controls ($P = 0.021$). Addition of *c-myc*, p53, survivin, and cyclin A did not further increase the cumulative antibody frequency.

Relationship Between Gleason Scores, PSA, and Antibodies to TAAs

To investigate the possible relationship between Gleason scores, PSA levels and antibodies, we analyzed the clinical characteristics of 70 of 134 prostate cancer patients who had Gleason scores, which were available for analysis. The prostate cancer patients were divided into two groups. Group 1 consisted of 25 patients with Gleason scores between 4 and 6. Group 2 consisted of 45 patients with Gleason scores between 7 and 9. Table III illustrates Gleason score, numbers and percent of antibody reaction to the more reactive TAAs, p90, p62, cyclin B1, and cyclin D1. No significant difference was observed for antibody reaction to p62 and cyclin B1 in prostate cancer patients with Gleason scores between 4–6 and 7–9. However, frequencies of positive reactions to p90 and cyclin D1 were significantly higher in patients with Gleason scores of 7–9 than patients with Gleason scores of 4–6. Overall, 13/25 (52%) patients with lower Gleason scores had antibody reactions to one of the four antigens analyzed (over mean + 3 SD), compared to 20/45 (70%) in patients with higher Gleason scores. Antibody reaction to multiple antigens in patients with Gleason scores 4–6 (16%) were also lower than patients with Gleason scores 7–9 (29%).

TABLE II. Stepwise Increase in Rate of Antibody Positivity With Successive Addition of Antigens

Antigens	Number/percent of autoantibodies in prostate cancer	Number/percent of autoantibodies in NHS
p90	41 (30.8)	3 (3.1)
p90 or p62	70 (53.4)	6 (6.2)
p90 or p62 or Koc	81 (61.7)	8 (8.4)
p90 or p62 or Koc or IMP1	92 (70.0)	10 (10.6)
p90 or p62 or Koc or IMP1 or cyclin B1	111 (84.3)	12 (12.6)
p90 or p62 or Koc or IMP1 or cyclin B1 or cyclin D1	122 (92.5)	14 (14.8)

In 25 patients with Gleason scores of 4–6, PSA was available in only eight patients and the median range was 13.4. In 45 patients with Gleason scores of 7–9, PSA was available in 14 patients and the median range was 16.7. With the limited information on PSA levels, it was not feasible to determine if there was any correlation between PSA levels and antibodies.

DISCUSSION AND CONCLUSIONS

Currently, there are no data available regarding the biological functions of the cytoplasmic protein p90. Our initial studies revealed that expression of p90 is developmentally regulated [2], similar to p62 [1]. Gastric cancer tissues overexpressed p90, and autoantibodies to p90 are present in sera of gastric cancer, esophageal cancer, and HCC [2]. The current study shows that autoantibodies to p90 were detected in 30.8% of prostate cancer sera which is significantly different ($P \leq 0.01$) from BPH (1.5%), or the autoimmune diseases SLE and Sjogren's syndrome and from normal control subjects.

The cellular requirements for the genesis of antibodies to TAAs and the regulation of such antibody production are not completely understood but many investigators have been interested in the use of autoantibodies as serological markers for cancer diagnosis,

TABLE III. Patient Characteristics and Antibodies to Major TAAs

Gleason score	Number	Age	Numbers and percent of autoantibodies to			
			p62	p90	Cyclin B1	Cyclin D1
4–6	25	46–100	7/25 (28%)	4/25 (16%)	4/25 (16%)	1/25 (4%)
7–9	45	50–100	13/45 (29%)	13/45 (29%)*	6/45 (13%)	7/45 (16%)**

Cut off value: mean + 3 SD of NHS.

* $P < 0.05$.

** $P < 0.01$.

especially because of the general absence of these antibodies in normal individuals and in non-cancer conditions [6,12]. Autoantibodies to p53 have been shown to be associated with p53 accumulation in the tumor due mostly to p53 missense mutations [14]. The specificity of anti-p53 for cancer was 96%, but, sensitivity was low with only 20% of patients with cancer having been found to have antibody against p53 [19–24]. The low frequency of positive reaction against any single antigen has precluded the use of individual autoantibodies as useful diagnostic markers [22]. To overcome this problem, a recent study has demonstrated that with successive addition of antigens to a larger panel consisting of seven TAAs, prostate cancer sera showed stepwise increase in percentage of positive reactions from 15% with one antigen to 59% with a seven TAA panel consisting of *c-myc*, p53, cyclin B1, p62, Koc, IMP1, and survivin [18]. In the current study, the TAA panel for detecting antibody markers in prostate cancer has been improved by eliminating redundant TAAs and reconstituting the panel to six antigens, p90, p62, Koc, IMP1, cyclin B1, and cyclin D1. Previously, we observed that breast, lung and prostate cancer patients showed their own distinct profiles of reactivity [6]. For breast cancer, it appears that *c-myc* and Koc antibodies are more frequently detected than other antibodies, but for lung cancer antibodies to cyclin B1, p62 and p53 are more frequently detected. In prostate cancer, there was higher frequency of antibodies to p90, p62, and cyclin B1 and in HCC anti-*c-myc* was predominant [6]. In a study reported recently, antibody to an intracellular protein called lens epithelium derived growth factor (LEDGF) or p75 was found in 18.4% of prostate cancer patients [25].

Currently, we do not understand why prostate cancer patients have preferential immune responses to p62 and p90, but this phenomenon may suggest a structural and/or a functional linkage between these two intracellular proteins. The practical value of the autoantibody responses to p90, p62, and cyclin B1 in prostate cancer might be further determined by longitudinal studies in prostate cancer patients. The timing of autoantibody occurrence to TAAs and whether antibody expression varies with progression or response to treatment also warrant further study.

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