

A Viral Vaccine Encoding Prostate-Specific Antigen Induces Antigen Spreading to a Common Set of Self-Proteins in Prostate Cancer Patients

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Abstract

Purpose: We previously reported a randomized phase II clinical trial combining a poxvirus-based vaccine encoding prostate-specific antigen (PSA) with radiotherapy in patients with localized prostate cancer. Here, we investigate whether vaccination against PSA induced immune responses to additional tumor-associated antigens and how this influenced clinical outcome.

Experimental Design: Pretreatment and posttreatment serum samples from patients treated with vaccine + external beam radiation therapy (EBRT) versus EBRT alone were evaluated by Western blot and serologic screening of a prostate cancer cDNA expression library (SEREX) to assess the development of treatment-associated autoantibody responses.

Results: Western blotting revealed treatment-associated autoantibody responses in 15 of 33 (45.5%) patients treated with vaccine + EBRT versus 1 of 8 (12.5%) treated with EBRT alone. SEREX screening identified 18 antigens, which were assembled on an antigen array with 16 previously identified antigens. Antigen array screening revealed that 7 of 33 patients (21.2%) treated with vaccine + EBRT showed a vaccine-associated autoantibody response to four ubiquitously expressed self-antigens: DIRC2, NDUFS1, MRFP1, and MATN2. These responses were not seen in patients treated with EBRT alone, or other control groups. Patients with autoantibody responses to this panel of antigens had a trend toward decreased biochemical-free survival.

Conclusions: Vaccine + EBRT induced antigen spreading in a large proportion of patients. A subset of patients developed autoantibodies to a panel of four self-antigens and showed a trend toward inferior outcomes. Thus, cancer vaccines directed against tumor-specific antigens can trigger autoantibody responses to self-proteins, which may influence the efficacy of vaccination. *Clin Cancer Res*; 16(15); 4046–56. ©2010 AACR.

Prostate cancer is the most frequently diagnosed cancer in North American men and remains the second leading cause of cancer-related death in men despite the improvement of early detection using prostate-specific antigen (PSA) screening. Choice of curative treatment for localized disease is challenging and is based on several factors, including stage and grade of disease, PSA value, comor-

bidity, and patient preference. In general, localized disease is treated with either surgery or radiation therapy (external beam or brachytherapy), with the addition of androgen deprivation therapy (ADT) for higher risk patients. Unfortunately, ~35% of patients fail definitive local therapy within 10 years as evidenced by an increase in PSA, often due to occult metastatic disease (1). Most recurrences are treated with ADT, which, while initially efficacious, inevitably develops into castration-resistant disease. Although chemotherapy can provide modest benefit at this stage, recurrent cancers are incurable. Thus, there is a clear need for more effective therapies for both primary and recurrent disease.

There is considerable evidence that the immune system recognizes prostate tumors in a significant proportion of patients. Serum antibody responses to several different antigens have been detected in patients with prostate cancer (2–9). The presence of tumor-infiltrating lymphocytes provides additional evidence that prostate tumors are recognized by the immune system. Vesalainen et al. (10) found that tumors with lower densities of tumor-infiltrating

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

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doi: 10.1158/1078-0432.CCR-10-0948

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Translational Relevance

Although cancer vaccines offer promise as a new form of treatment, the results of most cancer vaccine trials to date have been disappointing. To improve on these results, we require a better understanding of the effect of vaccination on the patient's immune system. This report is focused on patients with localized prostate cancer who were treated as part of a phase II clinical trial with a recombinant poxvirus-based vaccine targeting prostate-specific antigen (PSA). In addition to inducing immunity to PSA, as intended, vaccination induced autoantibody responses to a panel of widely expressed self-antigens in a significant proportion of patients. Moreover, these vaccine-induced autoantibody responses seemed to be associated with inferior outcomes. Therefore, tumor-specific vaccines can induce immune responses to self-proteins in patients, which we hypothesize may compromise therapeutic efficacy by provoking regulatory responses by the host to minimize autoimmune damage.

lymphocyte were associated with higher risk of tumor progression and poor prognosis; however, in another study, the presence of CD4+, CD8+, and CD20+ tumor-infiltrating lymphocytes in primary prostate tumors was associated with inferior outcomes (11). Thus, the immune system responds to prostate tumors, but it is unclear whether these responses are beneficial or detrimental to patients.

Although much is known about immune responses at the time of diagnosis, far less is known about how tumor immunity changes when patients undergo treatment. We have recently shown that ADT and radiation therapy induce autoantibody responses to a variety of tumor-associated antigens in 17% to 30% of prostate cancer patients (12). Although we do not yet know the influence of these treatment-associated immune responses on clinical outcomes, we examined this issue in a mouse model. Using the androgen-dependent Shionogi carcinoma model, we found that castration (the experimental equivalent of ADT) induced autoantibody and T-cell responses to the self-antigen PABPN1 in ~50% of animals. Remarkably, mice that developed autoantibody and T-cell responses to PABPN1 showed a higher rate and shorter latency of tumor recurrence (13). Thus, castration-induced autoantibody responses are associated with inferior outcomes in the Shionogi tumor model, consistent with a growing number of studies indicating that antitumor immunity can sometimes promote tumor growth (14–17).

Although the foregoing discussion concerns the effects of standard treatments on tumor immunity, many groups are attempting to use vaccines to deliberately induce an immune response to prostate cancer. We conducted a clinical trial in which patients with localized prostate cancer were treated with a combination of external beam radiation therapy (EBRT; with or without neoadjuvant ADT)

and a poxvirus-based vaccine encoding PSA (18, 19). Initially, 30 patients were randomized to receive EBRT with PSA vaccine (vaccine + EBRT; $n = 19$) or EBRT alone ($n = 11$; ref. 18). Patients in the vaccine arm also received granulocyte-macrophage colony-stimulating factor (GM-CSF) and low-dose interleukin 2 (IL-2) as immunologic adjuvants. The primary end point was immunologic response specific to PSA. We found that 13 of 17 patients treated with vaccine + EBRT had significantly increased PSA-specific T-cell responses compared with 0 of 8 patients treated with EBRT alone. However, most patients on the vaccine arm were not able to receive the full dose of IL-2 due to significant adverse events. Therefore, we treated an additional 18 patients with vaccine + EBRT and lowered the dose of IL-2 from 4 MIU/M²/d for 5 days to 0.6 MIU/M²/d for 14 days (19). Use of very low-dose IL-2 significantly reduced the number of adverse events while still promoting vaccine-induced T-cell responses.

In addition to the significant increases in PSA-specific CD8+ T cells seen in the majority of vaccinated patients, there was also evidence of antigen spreading in 9 of 13 vaccinated patients, as manifested by the development of T-cell responses to antigens not contained in the vaccine, including PSMA, PAP, PSCA, MUC-1, XAGE-1, and PAGE-4 (18, 19). Such responses were also seen in one of six evaluated patients receiving EBRT alone. These positive results with a limited set of known prostate cancer antigens led us to hypothesize that antigen spreading may have extended to a broader repertoire of prostate cancer antigens. To test this hypothesis, we used Western blotting and SEREX immunoscreening to broadly assess the development of autoantibody responses to prostate antigens in patients from this trial.

Materials and Methods

Subjects and treatment schedule

This study included 41 prostate cancer patients from a clinical trial (00-C-0154) conducted at the National Cancer Institute (18, 19). The clinical trial protocol was approved by the Institutional Review Board of the National Cancer Institute, and the current immunologic study was approved by the University of British Columbia/British Columbia Cancer Agency Research Ethics Board. On this trial, men with previously nontreated, biopsy-confirmed prostate cancer were treated with EBRT or vaccine + EBRT. ADT was also given to most patients ($n = 34$) at the discretion of the attending radiation oncologist. The first vaccination cycle consisted of a priming dose of vaccinia PSA admixed with vaccinia B7.1, given on day 2. Patients then received seven boosts with fowlpox PSA on a 28-day cycle. GM-CSF was given at 100 µg/d at the vaccination site on days 1 to 4 of each cycle. IL-2 was given s.c. in the abdomen either at 4 MIU/M² on days 8 to 12 ($n = 17$) or at 0.6 MIU/M² on days 8 to 21 ($n = 16$). EBRT was administered after the third cycle of vaccine and consisted of a total dose of 70 Gy, with 1.8 to 2.0 Gy fractions given over 2 months (over cycles 4 and 5 of vaccine). In the vaccine

arm, blood samples were collected before vaccine treatment was initiated, after every vaccine cycle and after all treatment was completed. In the EBRT arm, blood samples were collected before EBRT, immediately after, and 3 months after completion of EBRT.

An additional 24 prostate cancer patients receiving standard treatment at the BC Cancer Agency in Victoria, British Columbia, Canada served as controls. Of these, 15 patients were treated with ADT + EBRT, whereas 9 patients chose active surveillance. Fifteen age-matched men with no personal history of prostate cancer served as an additional control group. Blood samples were collected with informed consent and approval from the University of British Columbia/British Columbia Cancer Agency Research Ethics Board. Patient characteristics and treatment categories are summarized in Table 1.

Western blot assay

Western blots were done as previously described (12). Briefly, 400 μ g of protein isolated from LNCaP and PC3 cells was separated by standard PAGE and transferred to nitrocellulose. Sera were diluted 1:500 in Blotto (5% dry milk powder, 0.1% Tween 20, 50 mmol/L Tris, 150 mmol/L NaCl) and incubated for 1 hour at room temperature using the Mini Protean II MultiScreen multichannel immunoblotting device (Bio-Rad). The membrane was then incubated for 1 hour at room temperature with horseradish peroxidase-conjugated goat anti-human IgG secondary

antibody (H+L; Jackson ImmunoResearch), diluted to 1:10,000, and visualized by enhanced chemiluminescence. Pretreatment and posttreatment sera were run against both LNCaP and PC3 protein lysate a minimum of two times each.

SEREX screening and antigen arrays

SEREX screening of a previously described prostate cancer phage cDNA expression library (12) was done using posttreatment serum samples from six patients who showed treatment-associated serologic changes by Western blot. To make screening more efficient, sera were pooled in pairs, with each serum represented at a 1:200 dilution. Approximately 3.0 to 4.8×10^4 clones were screened with each patient serum. Screening of the prostate cancer library and antigen arrays was carried out as previously described (12, 20). Antigen arrays were screened with a 1:200 dilution of pretreatment and posttreatment serum from the 33 vaccine + EBRT, 8 EBRT without vaccine, 15 ADT+EBRT, and 9 active surveillance prostate cancer patients as well as 2 serial serum samples from 15 cancer-free controls.

Results

Serologic changes associated with vaccination against PSA

To broadly assess vaccine-induced immune responses, patient sera were first analyzed by Western blot against

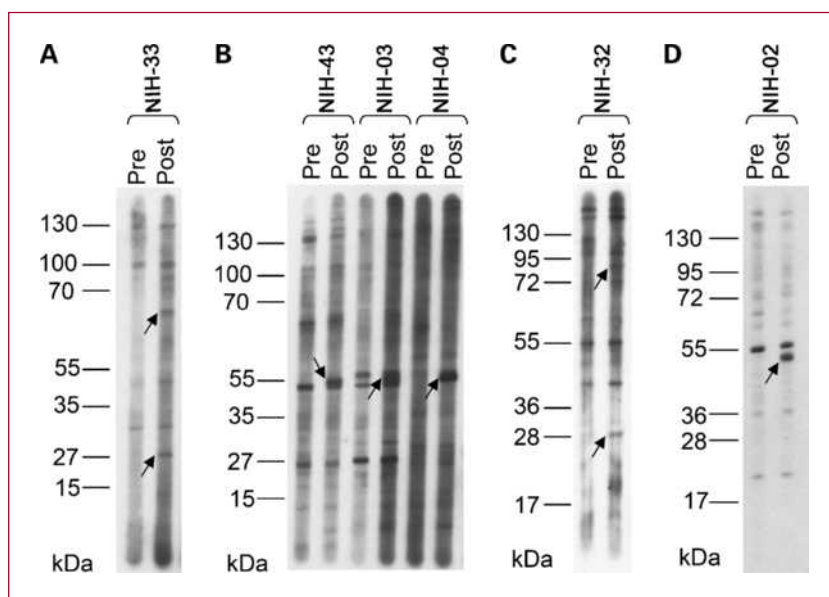
Table 1. Patient characteristics and treatment categories

	Vaccine + EBRT*	EBRT (no vaccine)	ADT + EBRT	WW
No. patients	33	8	15	9
Age, median (range)	61 (50-76)	70 (61-80)	69 (57-79)	67 (60-80)
Gleason score, <i>n</i> (%)				
5	2 (6.1)	0 (0)	0 (0)	1 (11.1)
6	8 (24.2)	2 (25)	5 (33.3)	7 (77.8)
7	9 (27.3)	5 (62.5)	10 (66.7)	1 (11.1)
8	7 (21.2)	1 (12.5)	0 (0)	0 (0)
9	7 (21.2)	0 (0)	0 (0)	0 (0)
Median	7	7	7	6
Stage, <i>n</i> (%)				
T ₁	12 (36.4)	4 (50)	2 (13.3)	7 (77.8)
T ₂	15 (45.5)	1 (12.5)	7 (46.7)	2 (22.2)
T ₃	6 (18.2)	3 (37.5)	6 (40)	0 (0)
PSA at diagnosis (ng/mL)				
Median (range)	14.7 (3.84-206)	9.66 (5.5-23)	11.3 (3.1-100)	6.2 (3.1-18)
Risk group, <i>n</i> (%)				
Low	5 (15.2)	1 (12.5)	3 (20)	7 (77.8)
Intermediate	7 (21.2)	2 (25)	3 (20)	2 (22.2)
High	21 (63.6)	5 (62.5)	9 (60)	0 (0)

Abbreviation: WW, watchful waiting = active surveillance.

*The vaccine + EBRT and EBRT (no vaccine) patients were part of the NIH 00-C-0154 clinical trial. The ADT + EBRT and WW patients were treated by standard therapy at the BC Cancer Agency Vancouver Island Centre.

Fig. 1. Treatment with vaccine + EBRT or EBRT without vaccine induces autoantibody responses to a variety of tumor antigens. Western blot analysis of serum from six patients probed against LNCaP (A-C) or PC3 (D) cell lysates. A, treatment with vaccine + EBRT in patient NIH-33 is associated with the development of autoantibody responses against antigens of approximately 65 and 28 kDa. B, vaccine + EBRT treatment in patients NIH-43, NIH-03, and NIH-04 is associated with the development of autoantibody responses against a ~55-kDa antigen in all three patients (arrows). C, vaccine + EBRT treatment in patient NIH-32 is associated with development of autoantibody responses to two antigens of approximately 90 and 28 kDa. D, patient NIH-02, treated with EBRT without vaccine, developed an autoantibody response to a ~53-kDa antigen.



lysates from the prostate cancer cell lines LNCaP and PC3, as per Nesslinger et al. (12, 21). Two groups of patients were assessed as follows: those who received vaccine + EBRT ($n = 33$) and those who received EBRT without vaccine ($n = 8$). Of the patients receiving vaccine + EBRT, 15 of 33 (45.5%) showed a serologic change when comparing the prevaccine and postvaccine serum samples (Fig. 1). In contrast, only 1 of 8 (12.5%) patients who received EBRT without vaccine showed a serologic change during EBRT, in accord with our previous study of an independent cohort of patients receiving EBRT (12). The difference between the vaccine + EBRT versus EBRT without vaccine groups was not statistically significant ($P = 0.12$, Fisher's exact test), likely due to the low number of patients in the EBRT without vaccine group. Indeed, if we increase the sample size of the EBRT without vaccine group by including patients from our prior study (12), the trend reaches significance ($P = 0.003$, Fisher's exact test; 15 of 33 vaccine + EBRT versus 5 of 40 EBRT without vaccine). Thus, vaccination seems to induce serologic changes beyond those induced by EBRT.

A wide variety of antigens seemed to underlie the vaccine-associated serologic changes, as evidenced by the diversity of protein sizes seen on Western blot (Fig. 1). Although the majority of serologic changes were seen with both LNCaP and PC3 protein lysates, four serologic changes were seen with one protein lysate but not the other, indicating these two cell lines have somewhat distinct antigen repertoires.

Identification of antigens underlying vaccine-induced autoantibody responses

To identify the antigens underlying the above serologic changes, we performed SEREX immunoscreening of a cDNA expression library derived from the prostate cancer

cell lines LNCaP, PC3, and DU-145 (12). The library was screened with posttreatment serum samples from six patients who had shown vaccine-associated serologic changes by Western blot (patients NIH-04, NIH-32, NIH-33, NIH-37, NIH-43, and NIH-10). Eighteen antigens were identified, representing a wide range of structural and functional protein classes (Supplementary Table S1). To assess serologic responses to these antigens across the entire patient cohort, SEREX antigen arrays were constructed containing these 18 antigens plus 16 antigens derived from previous SEREX screens of the same prostate cancer library, a cancer-testis library, and an ovarian cancer library (ref. 12; Supplementary Table S1). Antigen arrays were screened with pretreatment serum (serum collected before initiation of vaccine or EBRT) and posttreatment serum (i.e., serum collected after completion of all eight cycles of vaccine and/or EBRT) from all patients from the clinical trial (33 vaccine + EBRT patients and 8 EBRT without vaccine patients). Arrays were also screened with pretreatment and posttreatment sera from an additional group of patients treated with standard ADT + EBRT. Finally, as additional controls, antigen arrays were screened with serial serum samples from prostate cancer patients undergoing active surveillance ($n = 9$), as well as age-matched men with no personal history of prostate cancer ($n = 15$).

This analysis revealed several patterns of autoantibody response. First, some antigens were recognized at baseline (i.e., pretreatment) by multiple cancer patients and cancer-free controls, suggesting they represent normal autoantigens (Supplementary Table S2). Second, some antigens showed baseline responses in prostate cancer patients only (Supplementary Table S2). Third, and of greatest interest, some antigens were associated with treatment-induced autoantibody responses. Specifically, 7 of 33 (21.2%)

patients in the vaccine + EBRT group (compared with 0 of 8 patients in the EBRT without vaccine group) showed treatment-induced autoantibody responses by antigen array (Table 2). Of these seven patients, five had shown a response by Western blot in the preceding section, whereas two had not. Thus, when the results of the Western blot and the antigen array were combined, the total number of treatment-induced autoantibody responses was 17 of 33 (51.5%) in the vaccine + EBRT group and 1 of 8 (12.5%) in the EBRT without vaccine group ($P = 0.059$, Fisher's exact test; Table 2). Again, this reached statistical significance when we included additional EBRT without vaccine patients from our previous study; 17 of 33 vaccine + EBRT versus 6 of 40 EBRT without vaccine; $P = 0.0009$, Fisher's exact test; ref. 12).

Remarkably, all seven patients from the vaccine + EBRT group who showed a treatment-induced autoantibody response recognized the same set of four antigens: DIRC2, NDUFS1, MRFAP1 and MATN2 (Figs. 2 and 3). These serologic responses were unique to vaccinated patients, as they were not seen in any other treatment or control group. This is the first time in our experience that multiple individuals showed treatment-induced responses to a common set of antigens; therefore, we hypothesized that these responses may have been induced by vaccination. Indeed, analysis of serial serum samples revealed that, in all seven patients, autoantibody responses to DIRC2, NDUFS1, MRFAP1, and MATN2 arose after three cycles of vaccination but before EBRT (Fig. 2B). Thus, this vaccine regimen induces autoantibody responses to a common set of autoantigens in a significant proportion of patients.

In an attempt to explain why patients might show autoantibody responses to a common set of antigens, we considered the possibility that the observed autoantibodies might be cross-reactive. To assess this, we performed sequence homology comparisons between the four antigens, as well as the viral vectors contained in the priming and boosting vaccines. Messenger RNA and protein sequences for DIRC2, NDUFS1, MRFAP1, MATN2, PSA, B7.1, GM-CSF, and IL-2 were obtained from the Genbank database, as were the complete nucleotide sequences of the vaccinia and fowlpox virus genomes. The sequence alignment program ClustalW2 was used to cross-compare all of these

sequences (22). There were stretches of homology between the viral vectors and each of the antigens; however, these were short (less than eight nucleotides) and were not shared between the antigens. Similarly, there was little homology between the four antigens and the vaccine components at either the nucleotide or amino acid level (less than eight nucleotides or three amino acids). Alignment between the four antigens themselves revealed no homology at the nucleotide or amino acid level, making it unlikely that the observed autoantibody responses resulted from cross-reactivity.

In our prior study, we reported that 9 of 13 vaccinated patients from this clinical trial had shown evidence of antigen spreading, as manifested by the development of T-cell responses to prostate-associated antigens not contained in the vaccine, including PSMA, PAP, PSCA, MUC-1, XAGE-1, and PAGE-4 (18, 19). Of these nine patients, three also showed treatment-associated autoantibody responses by either Western blot or antigen array in the present study. Conversely, of the 17 patients who showed autoantibody responses, 3 also showed antigen spreading by T-cell assays. Thus, although there was some overlap between autoantibody and T-cell responses, these seemed to be largely independent phenomena (Supplementary Table S3).

We considered whether the seven patients who showed treatment-induced responses against the four antigens had any clinical characteristics in common. Their ages (range, 51-73 y) and ADT use (five of seven patients) were typical of the entire cohort. Five of the patients were treated with IL-2 at the 4 MIU/M² dose, whereas the remaining two received the very low 0.6 MIU/M² dose. Finally, the seven patients showed no unusual features with respect to the severity of their prostate cancer: Gleason scores ranged from 6 to 8 (median 6.5); stages ranged from T1c to T3b; risk stratification ranged from low to high; and PSA values at diagnosis ranged from 5.7 to 206 (Supplementary Table S4). Thus, these patients had no obvious clinical characteristics that distinguished them from the rest of the cohort.

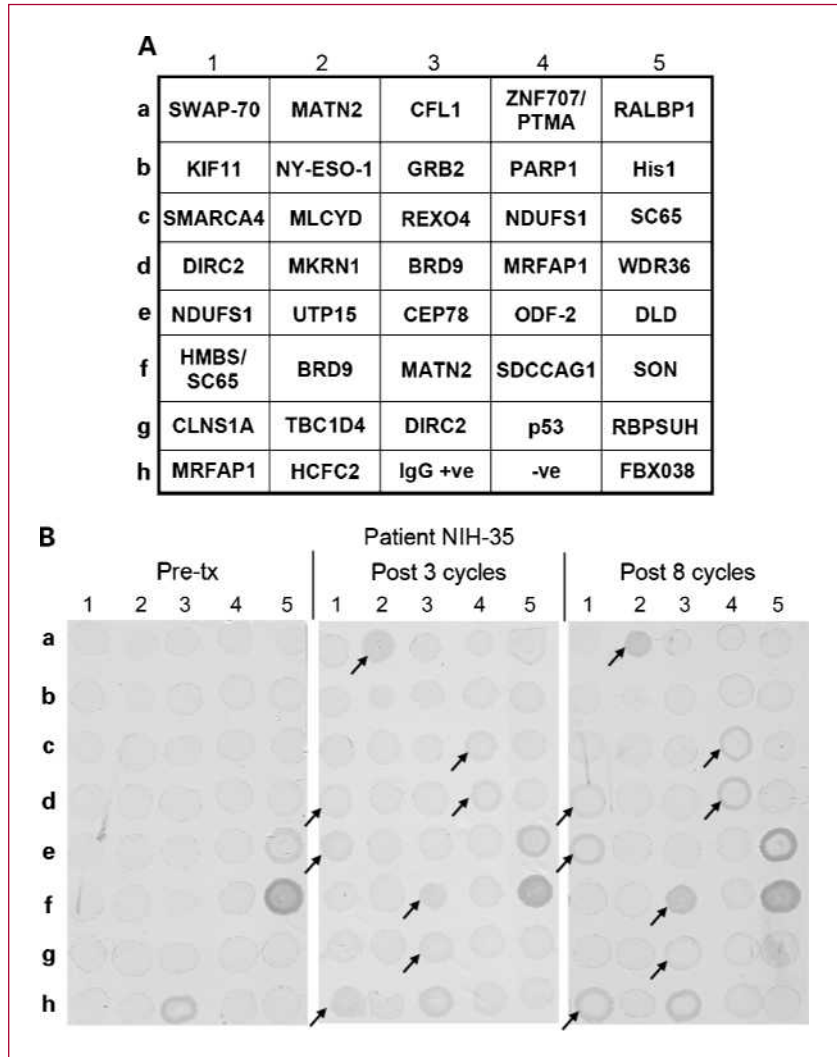
Kaplan-Meier analysis was done to compare the biochemical-free survival of patients in the vaccine + EBRT group who did or did not show treatment-induced autoantibody responses by Western blot or antigen array. There was no trend or significant difference between the two

Table 2. Frequency of treatment-induced autoantibody responses observed by Western blot, antigen array, or both

	Vaccine +EBRT (n = 33)	EBRT (no vaccine; n = 8)	ADT+EBRT (n = 15)	WW (n = 9)	Cancer-free controls (n = 15)
Western blot	15 (45.5%)	1 (12.5%)	3* (20.0%)	1 (11.1%)	0 (0%)
Antigen array	7 (21.2%)	0 (0%)	2* (13.3%)	1 (11.1%)	0 (0%)
Overall	17 (51.5%)	1 (12.5%)	3 (20.0%)	1 (11.1%)	0 (0%)

*The treatment-induced responses observed in the ADT + EBRT patients by Western blot and antigen array confirms our previously published results (Nesslinger et al. 2007).

Fig. 2. An example of SEREX antigen array analysis of pretreatment and posttreatment serum from patient NIH-35, treated with vaccine + EBRT, reveals treatment-associated autoantibody responses to a common set of tumor antigens. A, map of the antigen array containing the 18 antigens identified by SEREX screening (using serum from six patients with vaccine-associated autoantibody responses) plus 16 antigens derived from previous screens of prostate or ovarian cancer cDNA expression libraries. Note that several antigens are duplicated within the array. B, Sera collected from patient NIH-35 at three serial time points were used to screen the antigen array: pretreatment (pre-tx), after three cycles of vaccine (post 3 cycles; before EBRT), and after eight cycles of vaccine (post 8 cycles; after completion of EBRT). This revealed baseline autoantibody responses to two antigens (DLD and SON). In addition, autoantibody responses to four antigens (DIRC2, NDUFS1, MRFAP1, and MATN2) appeared only after treatment was initiated (arrows in post 3 and post 8 panels). These responses can be attributed to the vaccine as they are present in the post 3 serum sample, which was taken before the initiation of EBRT.



groups when autoantibody responses to all antigens were considered ($P = 0.9537$, log-rank test; Fig. 4A). Similarly, there was no significant difference between those patients that had a positive or negative T-cell response to PSA and biochemical-free survival ($P = 0.5339$, log-rank test). However, the seven patients who showed autoantibody responses to the panel of four common antigens (DIRC2, NDUFS1, MRFAP1, and MATN2) showed a trend toward decreased survival ($P = 0.1136$, log-rank test; Fig. 4B). Indeed, four of seven of these patients showed biochemical failure, compared with only one of eight nonvaccinated patients. Although not statistically significant, these results suggest that vaccination might not only be ineffective but actually detrimental to patients in those cases in which autoantibody responses to ubiquitous antigens are induced.

Discussion

The use of vaccines to augment the immune response to cancer has been the focus of intense investigation for sev-

eral decades. Although there have been occasional clinical successes, overall results have been disappointing. The reason for vaccine failure is not known in most cases; however, known barriers to success include tolerance to self-antigens, generation of antigen-loss variants, inadequate immunologic danger signals, and expression of immunosuppressive factors in the tumor microenvironment (23, 24). This study focused on patients with localized prostate cancer who were treated with a recombinant poxvirus-based vaccine containing PSA in combination with radiation therapy (18, 19). By serologic analyses, we found that 17 of 33 (51.5%) of patients developed autoantibody responses after vaccination. Remarkably, seven of these patients developed autoantibody responses to a common set of four antigens. This phenomenon seemed to be unique to vaccination, as it was not observed in patients receiving EBRT without vaccine in this study or our previous study (12). Patients who developed an autoantibody response to the panel of four antigens showed a trend toward reduced biochemical-free survival, suggesting the vaccine could

potentially have induced a detrimental immune response in these patients.

A priori, autoantibodies could play either a beneficial or detrimental role in cancer depending on several factors,

including the particular antigen. For example, one could imagine that autoantibody responses to tumor-specific antigens might be beneficial, as they could specifically target tumor cells for destruction by complement or

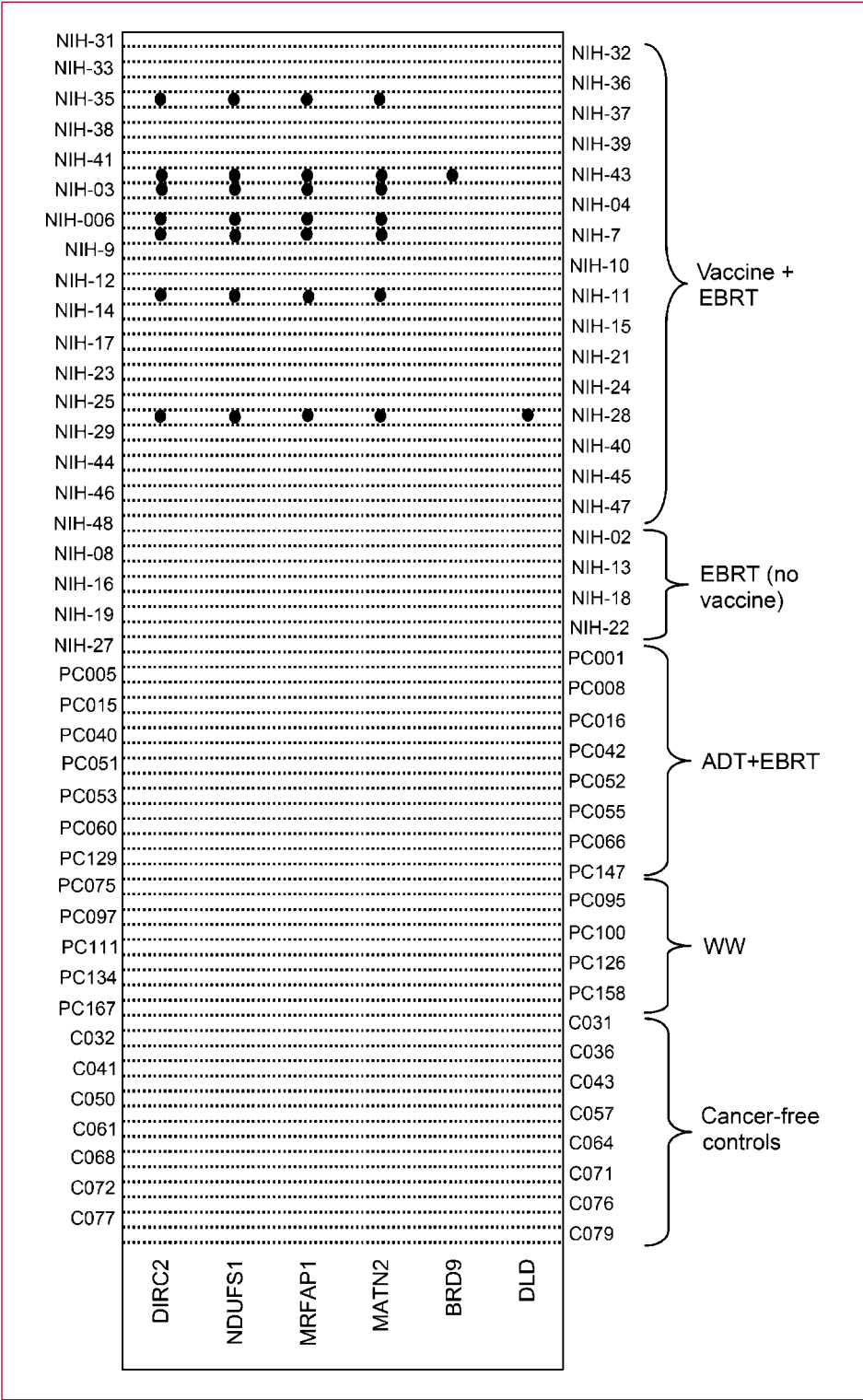


Fig. 3. Summary of all treatment-associated autoantibody responses detected by antigen array. ●, positive autoantibody responses against the indicated antigen. Of 33 patients treated with vaccine + EBRT, seven patients (NIH-35, NIH-03, NIH-43, NIH-006, NIH-7, NIH-11, and NIH-28) developed autoantibody responses to a common set of four antigens (DIRC2, NDUFS1, MRFAP1, and MATN2). Patients NIH-43 and NIH-28 showed an additional treatment-induced response to BRD9 and DLD, respectively. Responses to these antigens can be attributed to the vaccine, as they appeared after three cycles of vaccine but before initiation of EBRT. Additionally, they are not seen in patients treated with EBRT without vaccine ($n = 8$), ADT + EBRT ($n = 15$), watchful waiting (WW, $n = 9$), nor in cancer-free controls ($n = 15$).

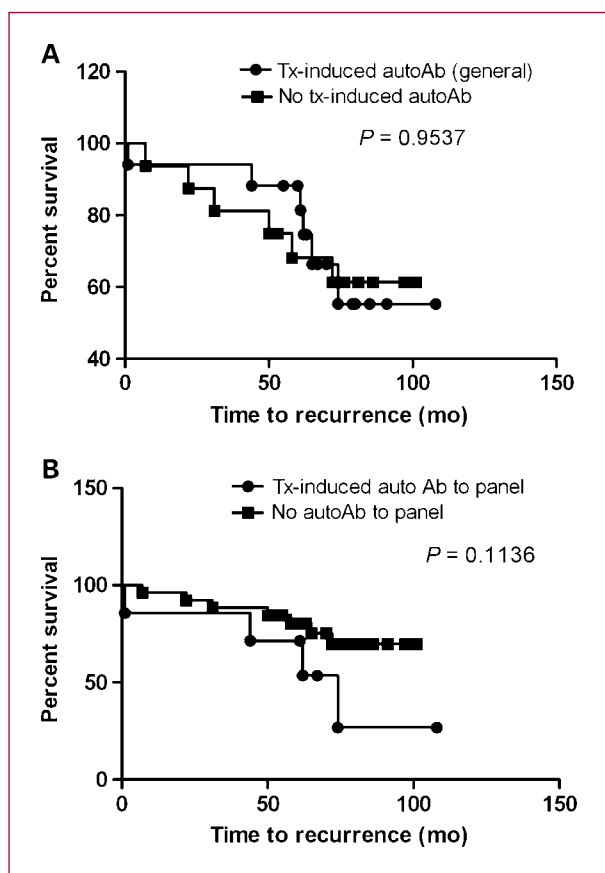


Fig. 4. Kaplan-Meier analysis of biochemical-free survival in relation to the development of autoantibody responses. A, patients were stratified according to the presence or absence of treatment-associated autoantibody responses to any antigen seen by Western blot or antigen array. The outcomes of the two groups were similar ($P = 0.9537$). B, patients were stratified according to the presence or absence of treatment-associated autoantibody responses to the panel of four antigens (DIRC2, NDUFS1, MRFAP1, and MATN2) as assessed by antigen arrays. There is a trend toward decreased survival in autoantibody-positive patients ($P = 0.1136$).

antibody-dependent cellular cytotoxicity. By contrast, autoantibody responses to broadly expressed self-antigens (as reported here) could potentially be detrimental, as they might provoke or reflect tolerizing immune responses that serve to minimize autoimmune damage. In reality, many tumor antigens lie somewhere between “tumor specific” and “self”-antigens, and there is no clear consensus on their association with clinical outcomes. The significance of autoantibodies against p53 has been most extensively studied, with some studies finding a positive correlation with clinical outcome (25, 26) and others a negative correlation with outcome (27–31). Autoantibodies to other antigens have been associated with improved prognosis in glioblastoma (32), melanoma (33), gastric cancer (34), and breast cancer (35). Although most of these studies examined the levels of autoantibodies at the time of diagnosis, some examined the development of autoantibodies during treatment. For example, in a clinical trial

of melanoma patients treated with IFN- α 2B, the development of autoantibodies and clinical signs of autoimmunity were associated with prolonged survival (36).

In the setting of prostate cancer, several groups have conducted phase II clinical trials using GVAX, an allogeneic vaccine composed of the LNCaP and PC3 prostate cancer cell lines engineered to secrete GM-CSF (37–39). By immunoblot analysis, vaccination induced the development of autoantibody responses to LNCaP or PC3 antigens in 79% of patients in one study (38) and 43% to 89% of patients (depending on dose) in a second study (39). In general, these responses were directed against a diverse set of antigens. However, a subset of patients exhibited new or enhanced autoantibody responses to the self-protein filamin-B, which was associated with favorable changes in PSA kinetics (37–39). By contrast, in the present study, the development of autoantibodies during vaccination seemed to be associated with inferior outcomes. Although this trend did not reach statistical significance, possibly due to insufficient sample size, it supports the idea that vaccine-induced autoantibody responses might be detrimental under some conditions.

There are several differences between the present study and the GVAX studies that could account for the different associations between autoantibodies and outcome. With the GVAX approach, autoantibodies are likely induced by a classic, direct immunization mechanism because the cell-based vaccine presented a full complement of human proteins in the context of a strong adjuvant. Thus, the development of autoantibody responses may reflect a successful vaccination overall, hence the correlation to favorable outcomes. By contrast, with the viral vectors used in our trials, autoantibody responses likely developed by an indirect mechanism because the target antigens were not contained within the vaccine. In this scenario, the autoantibody response might arise as a consequence of inflammation at the tumor site, secondary to T-cell recognition of tumor cells expressing the target antigen PSA. One could further speculate that this indirect form of autoantibody induction might be associated with ineffective T-cell responses, such as Th2 responses, rather than cytolytic Th1 responses, which are generally more effective against tumors.

An alternative explanation for the observed trend between autoantibody responses and inferior outcome is that tumors of higher malignant potential could potentially be more immunogenic. However, we saw no obvious differences between the relevant characteristics of patients who developed autoantibody responses to broadly expressed self-antigens and the remainder of the patients (Supplementary Table S4).

The present results are reminiscent of our recent findings in the Shionogi mouse model (12, 13). The Shionogi tumor line is highly androgen-dependent and forms adenocarcinomas when implanted in male mice. Castration of host mice results in rapid regression of established tumors, similar to hormone therapy in human prostate cancer. In 50% to 75% of mice, castration-induced tumor regression is accompanied by the development of autoantibody and

T-cell responses to a self-antigen we identified as poly(A) binding protein N1 (PABPN1). Remarkably, mice that developed autoantibody and T-cell responses to PABPN1 showed inferior outcomes, as manifested by an increased rate of tumor recurrence (13). We are currently investigating whether B and/or T-cell responses to PABPN1 actively promote tumor recurrence in this model or simply serve as markers of other biological processes associated with tumor recurrence. In support of the former possibility, it has recently been shown in a mouse model of prostate cancer that androgen ablation results in infiltration of regressing tumors with leukocytes, including B cells (40). Importantly, tumor-infiltrating B cells were shown to produce cytokines such as lymphotoxin that activate IKK- α and signal transducers and activators of transcription 3 in prostate tumor cells, thereby enhancing hormone-free survival and the transition to androgen independence (40). Other studies have also shown a protumorigenic role for B-cell responses. For example, B cells (or serum antibodies) were shown to promote primary tumor formation in a transgenic mouse model of inflammation-associated carcinogenesis (17). Furthermore, B cells were shown to inhibit T cell-mediated rejection of thymoma, melanoma, and colon carcinoma cells in other murine studies (41, 42). Thus, there is abundant evidence that B-cell responses can promote the development or progression of cancer.

It remains unclear why the antigens DIRC2, NDUFS1, MRFAP1, and MATN2 were common targets of autoantibody responses. According to public databases, all four proteins are ubiquitously expressed in most normal and tumor tissues, and only DIRC2 seems to be overexpressed in prostate cancer tissue compared with normal prostate tissue. As mentioned, the four antigens do not share significant sequence homology with each other or with other coding sequences in the viral vectors. Nonetheless, it is formally possible that these antigens share conformational similarities that are not evident from their primary sequence. In our prior study of ovarian cancer, we found that autoantibody responses were biased toward gene products on chromosome 17 (20); however, the four antigens identified here are located on chromosomes 3, 2, 4, and 8, respectively. In addition, the four antigens have unique cellular functions. Disrupted in renal carcinoma protein 2 (DIRC2) is a membrane-bound protein with homology to members of the major facilitator superfamily of transporters. Intriguingly, it was identified as a breakpoint spanning gene on chromosome 3 in a constitutional familial case of a t(2;3)(q35;q21) translocation resulting in hereditary renal cell carcinoma (43). Mof4 family associated protein 1 (MRFAP1) is an intracellular protein that associates with members of the mortality factor on chromosome 4/MORF4-related gene (MORF4/MRG) family as well as the tumor suppressor protein Rb and thus may play a role in cell growth, immortalization, and/or senescence (44). Matrilin-2 (MATN2) is a secreted adaptor protein of the extracellular matrix that plays a role in cell growth and tissue remodeling (45). Interestingly, we initially cloned MATN2 from an ovarian cancer library using serum from

an ovarian cancer patient, suggesting that autoantibodies to this antigen are not unique to prostate cancer. Finally, NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75 kDa (NADH-coenzyme Q reductase; NDUFS1) is a subunit of respiratory complex I located at the mitochondrial inner membrane. Intriguingly, caspase-mediated cleavage of NDUFS1 is required for several mitochondrial changes during apoptosis (46), which fits with the general notion that caspase-mediated cleavage of proteins can generate neoepitopes that trigger autoantibody responses (47–49). We are currently investigating whether these four proteins serve as autoantigens in the context of other cancer vaccines.

In summary, our results provide further evidence that cancer vaccines targeting a single antigen such as PSA can induce broader immune responses involving multiple host antigens. Although this process of antigen spreading is generally considered a desirable outcome of vaccination, our findings suggest that this assumption may not be universally true. We propose that further studies involving other vaccine strategies are warranted. For example, we vaccinated patients in conjunction with EBRT and, in some cases ADT, both of which can contribute to the development of autoantibody responses, albeit not to the four antigens described here (12). Although the sample size is small, it is interesting to note that of the patients with treatment-induced immune response to the panel of antigens, all four patients who recurred had received prior ADT (Supplementary Table S4). We also included GM-CSF in the vaccine formulation, which several recent studies suggest might be a suboptimal adjuvant for cancer vaccines (50, 51). Finally, the use of IL-2 in the vaccine formulation may have played a role; notably, all four autoantibody-positive patients who recurred received the higher dose of IL-2 (Supplementary Table S4). To better understand the contribution of GM-CSF and IL-2 to the development of autoantibody responses, we intend to analyze samples from patients who received poxvirus-based vaccines without these adjuvants (52, 53). Going forward, if autoantibody responses are indeed detrimental to the efficacy of cancer vaccines, it may be possible to dampen or redirect these responses using other immunomodulatory agents that promote cytolytic over humoral immune responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

U.S. Department of Defense; grant no.: W81XWH-07-1-0259, British Columbia Cancer Foundation, The Capital Region Prostate Centre (Victoria, British Columbia), U.S. Army Medical Research and Materiel Command Prostate Cancer Research Program Grant DAMD17-02-1A-0004, Intramural Research Program of the Center for Cancer Research, National Cancer Institute, NIH.

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Received 04/12/2010; revised 06/07/2010; accepted 06/08/2010; published OnlineFirst 06/18/2010.

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