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ORIGINAL ARTICLE

Antibody responses to galectin-8, TARP and TRAP1 in prostate cancer patients treated with a GM-CSF-secreting cellular immunotherapy

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Abstract A critical factor in clinical development of cancer immunotherapies is the identification of tumor-associated antigens that may be related to immunotherapy potency. In this study, protein microarrays containing >8,000 human proteins were screened with serum from prostate cancer patients ($N = 13$) before and after treatment with a granulocyte–macrophage colony-stimulating factor (GM-CSF)-secreting whole cell immunotherapy. Thirty-three proteins were identified that displayed significantly elevated ($P \leq 0.05$) signals in post-treatment samples, including three proteins that have previously been associated with prostate carcinogenesis, galectin-8, T-cell alternative reading frame protein (TARP) and TNF-receptor-associated protein 1 (TRAP1). Expanded analysis of antibody induction in metastatic, castration-resistant prostate cancer (mCRPC) patients ($N = 92$) from two phase 1/2 trials of prostate cancer immunotherapy, G-9803 and G-0010, indicated a significant ($P = 0.03$) association of TARP antibody induction and median survival time (MST). Antibody induction to TARP was also significantly correlated ($P = 0.036$) with an increase in prostate-specific antigen doubling time (PSADT) in patients with a biochemical (PSA) recurrence following prostatectomy or radiation therapy ($N = 19$) from in a previous phase 1/2 trial of prostate cancer immunotherapy, G-9802. RNA and protein encoding TARP and TRAP1 was up-regulated in prostate cancer tissue compared to matched normal controls. These preliminary findings suggest that antibody induction to TARP may represent a possible biomarker for treatment response to GM-CSF secreting cellular immunotherapy in prostate cancer patients and demonstrates the utility of using protein microarrays for the high-throughput screening of patient-derived antibody responses.

Keywords Immunotherapy · Tumor antigen · Autoantibody · Protein microarray · Prostate cancer · Biomarker

Introduction

GVAX® immunotherapy for prostate cancer is a whole cell cancer vaccine comprised of two allogeneic prostate carcinoma cell lines, LNCaP and PC-3, modified to secrete GM-CSF. The LNCaP and PC-3 cell lines were originally isolated from a lymph node and bone metastasis, respectively, of prostate cancer patients [1, 2]. Together the two cells lines provide a comprehensive prostate tumor antigen source for priming the immune system in prostate cancer patients. GM-CSF is a potent cytokine that improves the function of APCs by the maturation, activation, and recruitment of dendritic cells (DCs), and/or macrophages and monocytes [3, 4].

GVAX Immunotherapy for prostate cancer has previously been investigated in 3 phase 1/2 studies coded G-9802, G-9803 and G-0010. The G-9802 trial [5] investigated the safety and clinical activity of GVAX prostate in non-castrate prostate cancer patients ($N = 19$) with biochemical (PSA) recurrence following prostatectomy or radiation therapy. Clinical activity was determined by the change in PSA

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velocity and PSA doubling time (PSADT). The G-9803 trial [6] evaluated the safety and clinical activity of GVAX immunotherapy for prostate cancer in CRPC patients with radiological metastasis ($N = 34$) or PSA-rising disease only ($N = 21$). The G-0010 trial [7] investigated GVAX immunotherapy for prostate cancer in metastatic CRPC patients ($N = 80$) only. Results from G-9803 and G-0010 demonstrated safety and feasibility, as well as preliminary evidence of immunologic activity [6, 7].

GVAX immunotherapy for prostate cancer has also been examined in a Phase 3 clinical trial (VITAL-1) compared to Taxotere® (docetaxel) chemotherapy plus prednisone and enrolled 626 advanced prostate cancer patients with asymptomatic castrate-resistant metastatic disease. VITAL-1 was terminated based on the results of a futility analysis which indicated that the trial had less than a 30% chance of meeting its predefined primary endpoint of an improvement in overall survival. However, the final Kaplan–Meier survival curves for the two treatment arms suggest a late favorable effect of GVAX immunotherapy on patient survival compared to chemotherapy [8], with the curve for GVAX patients crossing above the chemotherapy curve at approximately the same time median survival was reached in both treatment arms (21 months).

The objective of these research studies was to retrospectively investigate immune based biomarkers from the G-9802, G-9803 and G-0010 studies that may allow patient selection and prediction of a response to GVAX immunotherapy. Protein microarrays containing >8,000 human proteins were screened with serum from prostate cancer patients ($N = 13$) before and after immunotherapy to identify potential tumor associated antigens that are related to immunotherapy response. Thirty-three target proteins to which antibodies were significantly induced over the course of immunotherapy treatment were identified. A literature-based search indicated that three of these proteins, galectin-8, TARP and TRAP1, had an association with prostate cancer and they were thus selected for further study. An association of TARP antibody induction and improved clinical outcome was observed in patients from the G-9802 and G-9803/G-0010 trials. In addition, expression of TARP RNA and protein was up-regulated in prostate cancer compared to normal tissue controls. These findings suggest that antibody induction to TARP may represent a candidate biomarker of response to a GM-CSF secreting cellular immunotherapy in prostate cancer patients.

Materials and methods

Clinical protocol and patients

Serum was obtained from patients treated on protocol G-9802 [5], G-9803 [6] and G-0010 [7] according to Institutional Review Board and the National Institute of Health (NIH) containment guidelines for recombinant DNA. All patients provided signed, written consent. G-0010 patients ($N = 13$) were selected for protein microarray analysis (Online Resource 1) because their observed survival exceeded that predicted by the Halabi nomogram [9]. Normal age- and sex-matched donor sera ($N = 25$) were obtained from SeraCare (Milford, MA, USA). Serum samples from consenting mCRPC patients receiving docetaxel chemotherapy were obtained from T. Higano (University of Washington, Seattle, WA, USA). Serum samples were also obtained from consenting patients treated in an autologous GVAX lung carcinoma phase 1/2 clinical trial ($N = 20$) and a GVAX chronic myeloid leukemia (CML; $N = 19$) study (provided by Hy Levitsky, John Hopkins University, Baltimore, MD, USA).

Protein microarray screening of patient serum

Patient serum samples pre and post-GVAX immunotherapy for prostate cancer were profiled on ProtoArray® Human Protein Microarrays v4.0 (Invitrogen, Carlsbad, CA, USA) containing approximately 8,000 human proteins and analyzed according to manufacturer's instructions. For detailed methods, see Online Resource 2.

Cloning and protein production of candidate antigens

Full-length *LGALS8*, *TARP* and *TRAP1* cDNAs were obtained from Origene (Rockville, MD, USA), PCR cloned with a C-terminus Flag-tag and transfected into 293 cells for protein production. Protein was purified using antibody-affinity purification according to manufacturer's procedures (Sigma-Aldrich, St. Louis, MO, USA).

ELISA development

For ELISA analysis of patient antibodies to selected antigens, 96 well MaxiSorp (Nunc, Rochester, NY, USA) plates were coated with 200 ng/well of protein, blocked and patient serum added at a 1:100 dilution. Wells were then incubated with a donkey-anti Human IgG/IgM HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and detected using TBM substrate (KPL, Gaithersburg, MD, USA). To determine induction of an antibody response, the post-therapy OD value was divided by the pre-therapy OD to determine a fold induction. Fold induction levels ≥ 2 were considered significant. Tetanus toxoid (Calbiochem, San Diego, CA, USA) and prostate specific antigen (PSA; AbD Serotec, Raleigh, NC, USA) were employed as controls in ELISA assays.

RNA analysis

RNA from normal ($N = 8$) and prostate cancer tissue ($N = 40$) was obtained from Origene (TissueScan Prostate Cancer Tissue qPCR arrays). Cell line RNA was extracted from PC-3, LNCaP, 293, K-562 and HeLa cells using RNeasy (Qiagen). Gene expression for *LGALS8, TARP and TRAP1* determined using gene specific primers and probes obtained from Applied Biosystems (Foster City, CA, USA). Samples were run on an ABI Prism 7700 Sequence detector (Applied Biosystems). All samples were normalized for β-actin (*ACTB*) expression.

Immunohistochemistry

Immunohistochemical detection of selected antigens was performed on normal and cancerous prostate tissues using tissue microarrays (Pantomics, San Francisco, CA; US Biomax, Rockville, MD, USA). Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. Primary mouse anti-human TARP and TRAP1 monoclonal antibodies were purchased commercially from eBioscience (San Diego, CA, USA) and BD Biosciences (San Jose, CA, USA), respectively. Primary antibody was incubated with tissue microarrays and bound antibody detected using MACH 3 mouse/rabbit polymer detection followed by DAB chromogen (Biocare medical, Concord, CA, USA) incubation. Semi-quantitative staining scores were generated by three independent observers by grading of tissue immunoreactivity by microscopy from 0 to 3, with 0 representing negative, 1 representing low expression, 2 representing intermediate and 3 high-expression of the antigen within the tissue sample.

Statistical analysis and data presentation

The PSADT was calculated using Ln 2 divided by the slope. The PSA data within 20 weeks of first treatment and all post-treatment PSA data before initiation of new prostate cancer therapy were included. Wilcoxon signed rank test was used to compare PSADT. Exploratory analysis was conducted to evaluate the association of survival with antibody induction while controlling for prognostic variables. Survival data between groups was analyzed by the log-rank test. The observed median survival time (MST) was compared with the median of predicted survival times calculated for each patient on the basis of baseline characteristics using a validated pretreatment prognostic model [9]. Version 9.1 of SAS software (SAS Institute, Cary, NC, USA) was used for PSADT and survival data analysis. Comparisons of patient antibody reactivity OD and cycle threshold (C_t) mRNA expression were analyzed using GraphPad Prism Software (La Jolla, CA, USA) and considered significant if $P = <0.05$.

Results

Identification of immunotherapy-induced antibody responses in prostate cancer patients by protein microarray screening

To identify antibodies induced in mCRPC patients during the course of GVAX immunotherapy for prostate cancer treatment and their associated target antigens, pre- and post-treatment patients' sera ($N = 13$) selected from a previous phase 1/2 trial of GVAX immunotherapy for prostate cancer (G-0010; [7]) were analyzed by protein microarray screening. The signals arising from the 8,000 proteins present on the microarrays profiled with serum samples from 13 patients before and after GVAX immunotherapy for prostate cancer treatment were evaluated for significant increases in pixel intensity in post-treatment sera relative to the arrays profiled with the pre-treatment sera. Thirty-six proteins, representing 33 individual proteins, exhibited elevated interactions with serum autoantibodies in post-GVAX treatment sera compared to donor-matched pre-treatment sera that met the threshold criteria ($P < 0.05$; Table 1). In comparison, antibody response to the influenza A antigen, to which all tested patient serum samples were immunoreactive, remained unchanged between pre- and post-treatment (data not shown). Several of the antibody target proteins identified in post-treatment sera have reported associations to prostate cancer. Two independent preparations of the galectin-8 were identified as having an elevated antibody response in post-treatment patients compared to patient sera before therapy. Galectin-8 was originally designated prostate tumor-associated antigen-1 [10]. Expression of galectin-8 is correlated with a variety of cancers including prostate [11]. Nine patients had an increase in autoantibody reactivity to T-cell alternative reading frame protein (TARP). TARP represents an androgen-regulated tumor antigen that appears to have a role in prostate tumor cell growth and gene regulation [12–14]. In addition, five patients developed an autoantibody response to TNF receptor-associated protein 1 (TRAP1) post-treatment. Expression of TRAP1 is significantly up-regulated in primary and metastatic prostate cancer tissue and displays an anti-apoptotic function [15]. Additional experiments specifically investigated the role of galectin-8, TARP and TRAP1 antibodies in patient immunotherapy response given their pre-established involvement in prostate cancer as defined by the literature.

Table 1 Target antigens in immunotherapy treated patients ($N = 13$) identified by autoantibody profiling of patients serum using protein microarray analysis

Database ID	Pre-GVAX patients Ab +ve	Post-GVAX patients Ab +ve	*P* value	Protein name (gene name)
BC015818.1	0	12	<0.001	Galectin 8 (*LGALS8*)
BC014001.1	0	10	<0.001	UBX domain-containing 8 (*UBXD8*)
NM_016467.1	1	13	<0.001	ORM1-like protein 1 (*ORMDL1*)
BC017085.1	0	9	<0.001	Serine incorporator 2 (*SERINC2*)
NM_014613.1	0	9	<0.001	UBX domain-containing 8 (*UBXD8*)
NM_181689.1	0	9	<0.001	Neuronatin (*NNAT*)
NM_139280.1	1	10	<0.001	ORM1-like protein 3 (*ORMDL3*)
NM_001803.1	0	8	<0.001	Campath-1 antigen (*CD52*)
NM_014182.2	0	8	<0.001	ORM1-like protein 2 (*ORMDL2*)
NM_138820.1	0	8	<0.001	HIG1 domain family, member 2A (*HIGD2A*)
BC001120.1	1	9	0.0018	Galectin 3 (*LGALS3*)
BC014975.1	1	9	0.0018	FAM136A (*FAM136*)
BC053667.1	4	12	0.0018	Galectin 3 (*LGALS3*)
NM_138433.2	0	9	0.0018	Kelch domain-containing 7B (*KLHDC7B*)
CARDIOLIPIN	2	10	0.0024	Cardiolipin
BC005840.2	0	7	0.0026	Selenoprotein S (*SELS*)
BC016486.1	0	7	0.0026	Galectin 8 (*LGALS8*)
BC021701.1	0	7	0.0026	UPF0445 transmembrane protein C14orf147 (*C14orf147*)
NM_001234.3	0	7	0.0026	Caveolin-3 (*CAV3*)
NM_007022.1	0	7	0.0026	Cytochrome b-561 domain-containing 2 (*CYB561D2*)
NM_007107.2	0	7	0.0026	Translocon-associated protein subunit gamma (*SSR3*)
BC015596.1	1	8	0.0056	UPF0601 protein FAM165B (*FAM165B*)
PV3846	1	8	0.0056	Ribosomal protein S6 kinase alpha-2 (*RPS6KA2*)
BC042179.1	0	6	0.0075	Fat-inducing protein 1 (*FIT1*)
XM_096472.2	0	6	0.0075	Hypothetical protein LOC143678 (*LOC143678*)
NM_172341.1	2	9	0.0077	Gamma-secretase subunit PEN-2 (*PSENEN*)
NM_001003799.1	3	12	0.015	TCR gamma alternate reading frame protein (*TARP*)
NM_138501.3	0	7	0.015	Synaptic glycoprotein SC2 (*GPSN2*)
BC018950.2	0	5	0.02	TNF receptor-associated protein 1 (*TRAP1*)
NM_032318.1	0	5	0.02	Hippocampus abundant gene transcript-like protein 2 (*HIATL2*)
NM_139161.2	0	5	0.02	Crumbs protein homolog 3 (*CRB3*)
NM_181555.1	7	13	0.02	CKLF-like MARVEL domain-containing protein 3 (*CMTM3*)
BC005807.2	0	6	0.037	Acyl-CoA desaturase (*SCD*)
NM_139348.1	0	4	0.048	Myc box-dependent-interacting protein 1 (*BIN1*)
PV3879	0	4	0.048	Serine/threonine-protein kinase 2 (*PKN2*)
BC015749.1	2	7	0.049	Syntaxin binding protein 1 (*STXBP1*)

Galectin-8, TARP and TRAP1 antibody response in CRPC patients treated with GVAX immunotherapy for prostate cancer

Specific ELISAs were developed for determining galectin-8, TARP and TRAP1 serum autoantibodies. ELISA analysis of antigen specific pre- and post-treatment serum antibodies employing the 13 patients originally profiled by protein microarray analysis demonstrated comparability between the two assays (Online Resource 3). Monitoring of serum antibody responses to galectin-8, TARP and TRAP1 over the course of therapy in two individuals, patients 057 and 202, demonstrated that antibody response increased over the course of treatment (Fig. 1). Patient 057 demonstrated a significant increase in antibody reactivity to TRAP1 between vaccinations 5 and 6 (Fig. 1a), which remained stable up to 185 days from the start of therapy despite the patient receiving his last vaccination on day 85. Galectin-8 and TARP antibody reactivity in patient 057 reactivity remained relatively stable over the course of dosing. In comparison, patient 202 demonstrated a steady rise in

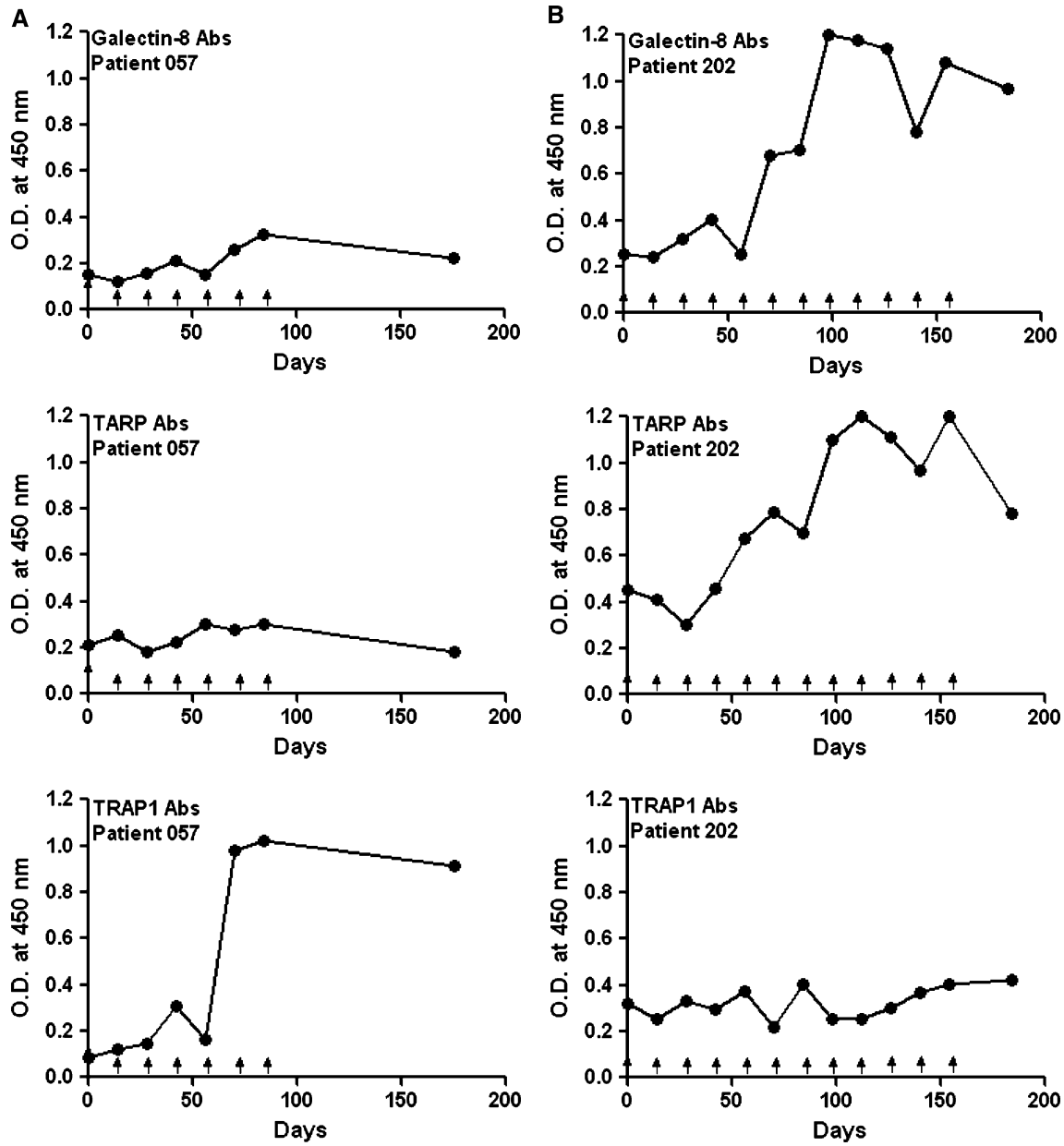

Fig. 1 Anti-human galectin-8, TARP and TRAP1 antibodies in patients treated with GVAX immunotherapy for prostate cancer. A longitudinal analysis of humoral reactivity to human galectin-8 (*upper panels*), TARP (*middle panels*) and TRAP1 (*lower panels*) in two G-0010 patients: 057 (**a**) and 202 (**b**). Serum samples at representative time points over the course of immunotherapy treatment were diluted 1:100 and incubated with 200 ng of the target antigen in an ELISA assay. An anti-human pan-IgG secondary antibody was used for detection. *Arrows* denote immunization with irradiated GM-CSF-secreting tumor cells

anti-TARP and a dramatic increase in anti-galectin-8 antibodies that peaked at vaccination 9 (Fig. 1b); however, anti-TRAP1 antibody induction was not observed.

Galectin-8, TARP and TRAP1 antibody induction and association with observed survival in CRPC patients

Association of autoantibody induction post-GVAX immunotherapy for prostate cancer and survival was then examined in all evaluable metastatic CRPC patients ($N = 92$) from the G-9803 and G-0010 phase 1/2 trials (Fig. 2a–c). ELISA analysis demonstrated that 71/92 (77%), 41/92 (45%) and 14/92 (15%) patients developed an induced response (post/pre OD $\geq$2-fold induction) against galectin-8, TARP and TRAP1, respectively. No patients showed induction of an antibody response against PSA or a tetanus control antigen (data not shown). The population of patients with galectin-8 antibody induction displayed a MST of 31.8 months, compared to an MST of

26.1 months for patients without galectin-8 antibody induction ($P = 0.24$; Fig. 2a). Patients with an induced antibody response to the TARP antigen had a MST of 38.2 months, compared to 24.0 months for those patients without TARP antibody induction (Fig. 2b). This difference in MST was significant ($P = 0.03$). Immunotherapy-treated CRPC patients with an induced antibody response to TRAP1 had an MST of 43.5 months (Fig. 2c). In comparison, patients without a TRAP1 response had an MST of 27.1 months, although this was not significant ($P = 0.08$).

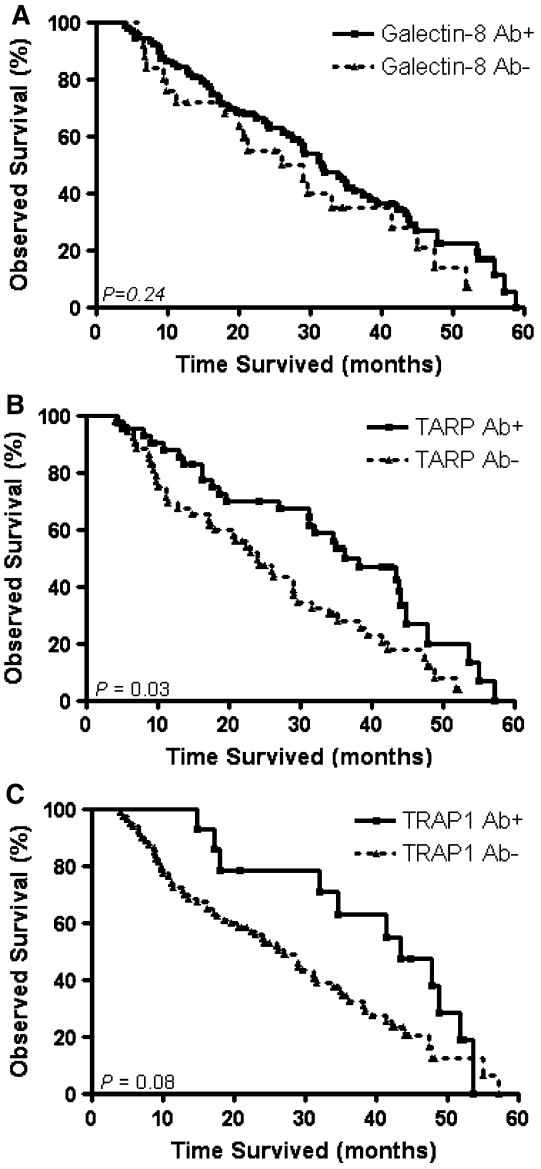

Fig. 2 Kaplan–Meier estimates of overall survival in G-9803/G-0010 mCRPC patients with (*solid lines*) or without (*dashed lines*) an induced antibody response to galectin-8 (**a**), TARP (**b**) or TRAP1 (**c**)

Galectin-8, TARP and TRAP1 antibody induction and association with PSA doubling time in non-castrate prostate cancer patients with biochemical recurrence treated with GVAX immunotherapy for prostate cancer

Antibody induction pre and post-GVAX immunotherapy for prostate cancer in non-castrate prostate cancer patients ($N = 19$) with biochemical (PSA) recurrence following prostatectomy or radiation therapy from the G-9802 trial was examined. ELISA analysis demonstrated that 14/19 (74%), 7/19 (37%) and 0/19 patients developed an induced response against galectin-8, TARP and TRAP1, respectively. No patients showed induction of an antibody response against PSA or a tetanus control antigen (data not shown). Signals of clinical activity in the G-9802 trial were assessed by changes in PSA doubling-time (PSADT) due to the longer survival time of patients with biochemical recurrence of prostate cancer compared to mCRPC patients. Exploratory analyses using the Wilcoxon signed rank test showed a positive association between induction of antibodies reactive against TARP protein and treatment-associated declines in PSADT. PSADT increased by a median of 182 weeks in patients with an anti-TARP response versus −10 weeks in those without ($P = 0.036$). PSADT for patients with an galectin-8 antibody response was 78 weeks compared to 15 weeks for patients without, although this difference was not significant ($P = 0.55$). No antibody-positive patients were observed for TRAP1 in G-9802 and therefore its relationship to PSADT was not analyzed.

Induction of galectin-8, TARP and TRAP1 autoantibodies in mCRPC patients treated with chemotherapy or in alternative GVAX immunotherapy indications

To determine the specificity of induced antibodies to galectin-8, TARP and TRAP1 in GVAX prostate immunotherapy-treated patients, an ELISA was used to evaluate antibody induction to these antigens in 20 patients treated with Taxotere® (docetaxel) chemotherapy according to standard dosing guidelines. Antibody evaluation on baseline and post-treatment serum samples showed that there was no induction of antibodies to any of the antigens tested (data not shown). Antibody induction to these antigens was also evaluated in patients treated with either GVAX immunotherapy for non-small cell lung carcinoma (NSCLC; $N = 20$) or GVAX immunotherapy for chronic myeloid leukemia (CML; $N = 19$) in pre- and post-immunization sera over the course of therapy. No induction of antibodies against TARP or TRAP1 was observed in any patient receiving either of the other two GVAX immunotherapies. Antibody induction to galectin-8 was observed in 1/19 patients (5%) from the GVAX CML trial.

Evaluation of serum antibodies to galectin-8, TARP and TRAP1 in mCRPC patients compared to normal donors

To further assess the significance of induced antibody responses to galectin-8, TARP and TRAP1 we compared antibody reactivity in mCRPC patients ($N = 60$) with normal age- and sex-matched donor serum samples ($N = 25$) to determine if a pre-existing antibody response could be observed in these patients before immunotherapy treatment (Fig. 3). Antibodies to the TARP antigen was significantly raised in mCRPC patients' sera compared to normal donor sera ($P = 0.016$; Fig. 3b). The level of antibodies specific for galectin-8 and TRAP1 were not significantly different between normal and CRPC patient serum samples (Fig. 3a, c).

Expression of target antigen RNA in normal and cancerous prostate tissue

To determine whether the RNA expression of the target antigens was up-regulated in prostate cancer compared to normal prostate tissue, quantitative PCR on a panel of normal prostate ($N = 8$) and prostate cancer ($N = 40$) cDNA samples (Fig. 4) was undertaken using gene-specific primers. The expression level of TARP (Fig. 4b) and TRAP1 (Fig. 4c) RNA was significantly increased ($P = <0.001$) in prostate cancer compared to normal prostate tissue as demonstrated by a decrease in the C_t. Expression of galectin-8 (*LGALS8*) and the control β-actin (*ACTB*) remained unchanged (Fig. 4a, d).

Expression of TARP and TRAP-1 protein in normal and cancerous prostate tissue

Given the potential association of TARP and TRAP-1 antibody induction with clinical performance in GVAX immunotherapy for prostate cancer clinical trials the expression of TARP and TRAP-1 protein was examined in prostate cancer tissue microarrays using antigen-specific monoclonal antibodies [15, 16]. Matched normal ($N = 9$) and prostate cancer ($N = 24$) tissue samples were evaluated for TARP or TRAP-1 immunoreactivity (Fig. 5). Enhanced expression of TARP and TRAP-1 in prostate cancer tissue samples (Fig. 5b, d) was seen relative to normal tissues (Fig. 5a, c). Staining scores for TARP and TRAP-1 in prostate cancer tissue samples were markedly higher than normal tissue controls (data not shown).

Expression of TARP and TRAP-1 RNA in cell lines

The expression of TARP and TRAP-1 RNA was evaluated in a panel of cell lines using quantitative PCR (Fig. 5e, f).

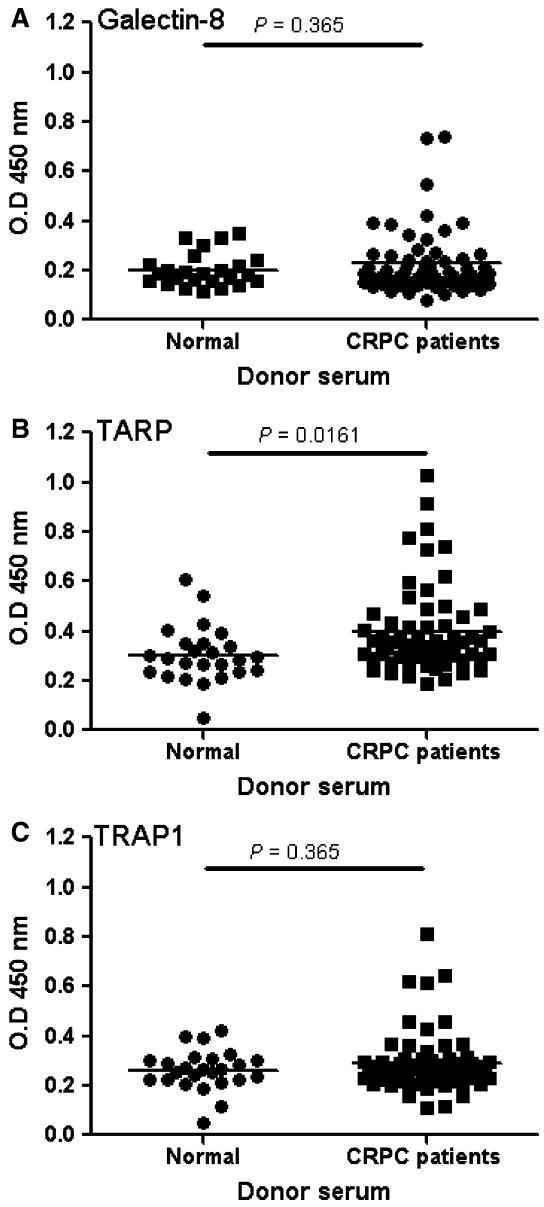

Fig. 3 Humoral responses to TARP are observed in CRPC patients before immunotherapy treatment. Comparison of antibody reactivity (OD at 450 nm) between normal ($N = 25$) and CRPC patients before the initiation of immunotherapy treatment ($N = 60$) to the galectin-8 (**a**), TARP (**b**) and TRAP1 (**c**) antigens. Serum samples were diluted 1:100 and incubated with 200 ng of the target antigen in an ELISA assay using an anti-human pan-IgG secondary antibody for detection

TARP expression in the two cell lines which comprise the cellular component of GVAX-immunotherapy for prostate cancer (PC-3 and LNCaP) was highly divergent. PC-3 cells had no detectable level of TARP expression (≥35 cycle threshold). In comparison, LNCaP cells displayed the highest level of TARP expression of the cell lines evaluated (22.3 cycle threshold). Expression of TARP within the K-562 cell line that is employed in the GVAX immunotherapy for CML vaccine was also un-detectable.

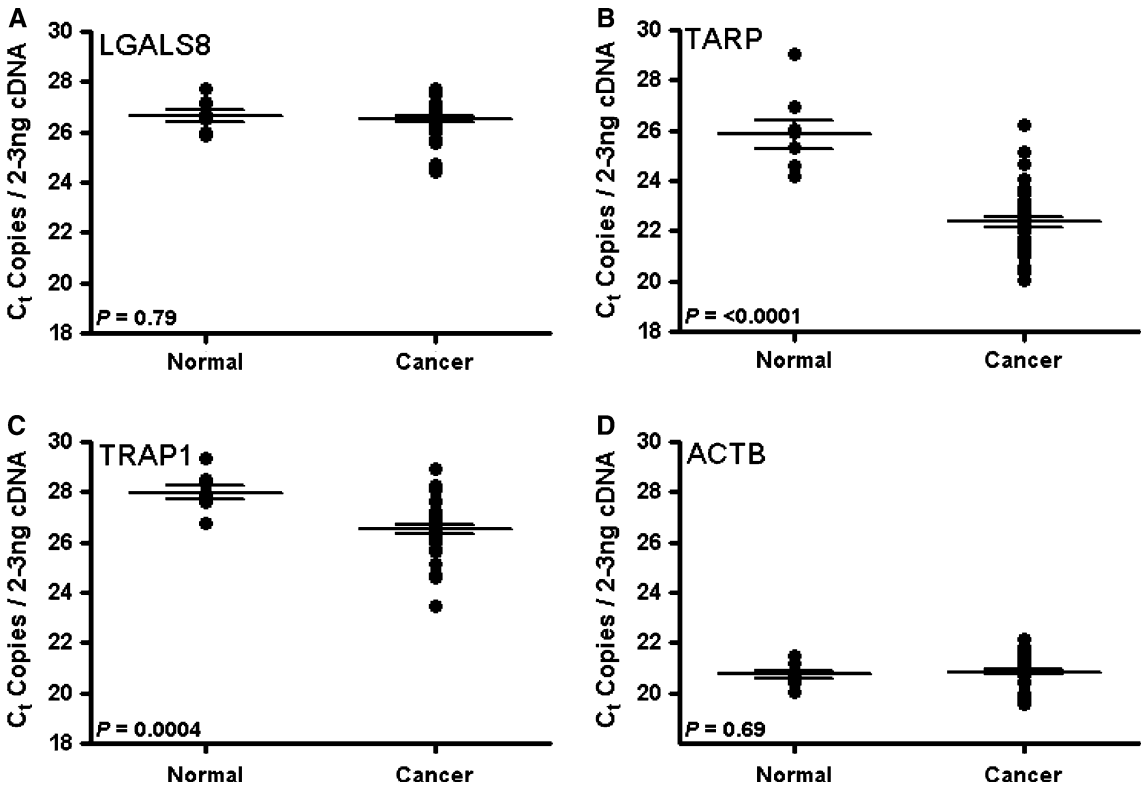

Fig. 4 RNA encoding the TARP and TRAP1 antigens shows enhanced expression in prostate cancer. RNA was extracted from normal ($N = 8$) and cancerous ($N = 40$) prostate tissue samples, cDNA was synthesized and then normalized for *ACTB* expression. Q-PCR primer and probe sets specific for *LGALS8* (**a**), *TARP* (**b**), *TRAP1* (**c**) and *ACTB* (**d**; control) were used to determine RNA transcript levels. C_t denotes cycle threshold. A decrease in C_t represents an increase in RNA transcript

Expression of TARP was not observed in 293 or HeLa cells. In comparison, expression of TRAP1 RNA was observed in all cell lines examined (Fig. 5f) with the highest level being observed in PC-3 cells.

Discussion

Thirty-three candidate antigens were identified by protein microarray screening as potential targets of an induced antibody response in 13 CRPC patients receiving GVAX immunotherapy for prostate cancer. The antigens that were identified in the post-GVAX treatment sera can be grouped into several protein categories that span diverse biological processes: (1) proteins with immunological function, including UBXD8 (two independent preparations) [17], CD52 [18], SELS [19], and IRF2 [20]; (2) proteins involved in signal peptide recognition or processing, including SSR3 [21]; (3) proteins are believed to have roles in either protein folding or the degradation of misfolded proteins, including the heat shock protein TRAP1 [22], and DERL1 [23] and three related endoplasmic reticulum (ER) membrane proteins, ORM-1 like 1, 2, and 3, that may also have a role in protein folding [24]; and (4) proteins that localize to mitochondria, including TRAP1 [25], TARP [16], and cardiolipin [26]. In addition, a number of proteins of unknown function including FAM136A, neuronatin, Kelch domain containing 7B, and UPF0445 transmembrane protein C14orf147 were also identified as possible target antigens in this study and may represent novel therapeutic targets or surrogate biomarkers for monitoring response to GVAX prostate treatment.

Over 50% of the 33 candidate antigens are membrane proteins, and of these at least 11 are ER membrane proteins. Interestingly, two of these proteins are known to interact. SELS (also known as VIMP) and DERL1 are ER stress-regulated components of a multiprotein complex that mediates ER retro-translocation and degradation of misfolded proteins [27]. The ER stress response is activated by conditions or agents that result in protein unfolding or misfolding, ultimately resulting in apoptosis. In addition, three proteins identified in post-treatment sera have ties to prostate cancer, including galectin-8, TARP and TRAP1 [10–15]. These proteins were selected for additional analysis of their potential association with clinical outcome in patients treated in two previous phase 1/2 trials of GVAX immunotherapy for prostate cancer.

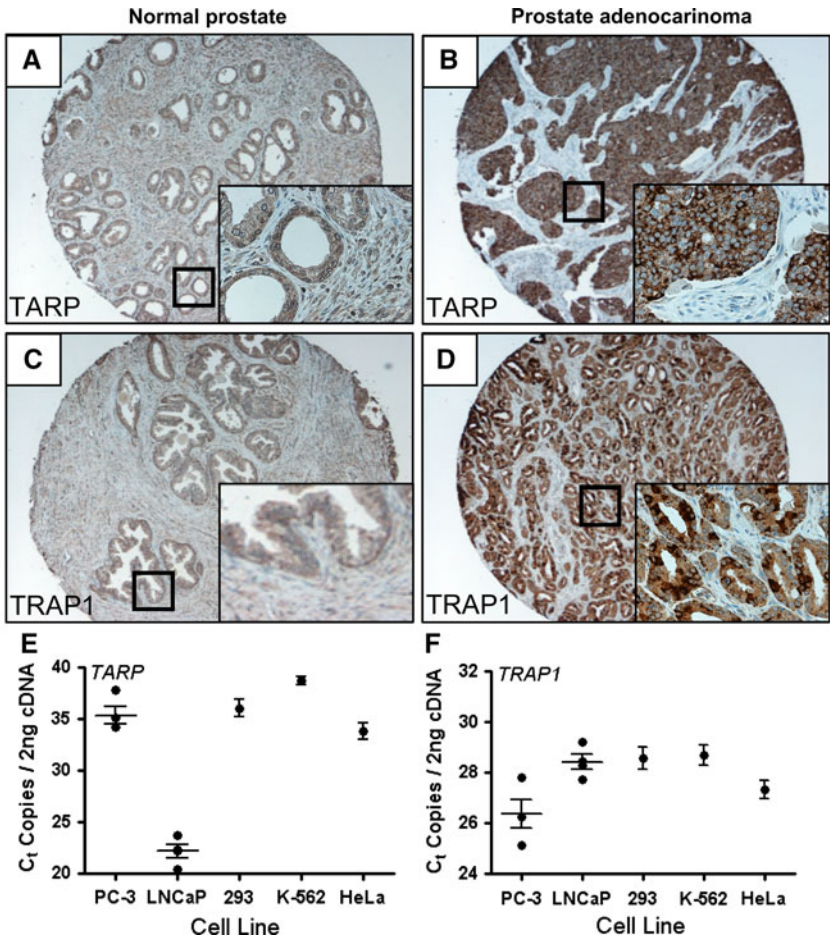

Fig. 5 TARP and TRAP1 protein is over-expressed in prostate cancer. Representative photomicrographs of normal prostate (**a**, **c**) and prostate adenocarcinoma (**b**, **d**) tissue stained with an anti-TARP (*upper panels*) or anti-TRAP1 (*lower panels*) antibody. Original magnifications: ×5 (main-panel) and ×40 (sub-panel). *Black boxed area* (×5 magnification) indicates the area examined under higher magnification (×40) presented below. **e**, **f** *TARP* and *TRAP1* RNA expression in a panel of cell lines as evaluated by quantitative PCR. RNA was extracted from PC-3, LNCaP, 293, K-562 and HeLa cells, cDNA synthesized and expression determined using gene specific Q-PCR primer and probe sets. C_t denotes cycle threshold. A decrease in C_t represents an increase in RNA transcript

Examination of TARP antibody response in prostate cancer patients with biochemical relapse (study G-9802) revealed a significant association of TARP antibody induction and increases in PSADT. An increase in MST was also observed in metastatic, CRPC patients in studies G-9803/G-0010. A significant increase in PSADT or MST was not observed for the galectin-8 or TRAP1 antibody response in studies G-9802 or G-9803/G-0010, respectively. For the galectin-8 antigen this was perhaps related to the high frequency of patient antibody response in both trials. Antibody responses to galectin-8 may represent a marker of immunotherapy administration rather then representing a correlate of clinical activity. In comparison, antibody responses to the TRAP1 antigen were relatively infrequent and only observed in the CRPC patients with metastatic disease in G-9803 and G-0010. Patients displaying a TRAP1 antibody response did display an increase in survival, although given the low N number of antibody positive patients this was not significant. Expression of TRAP1 is significantly up-regulated at the RNA and protein level in prostate cancer in agreement with recent studies by Laev et al. [15] and appears to display an anti-apoptotic function inhibiting chemotherapy induced cell death. Future studies should aim to expand the numbers of GVAX-treated CRPC patients examined, perhaps employing the VITAL-1 cohorts, to determine the impact of TRAP1 antibody induction on patient survival in more detail.

TARP was originally identified by expressed sequence tag (EST) mapping of transcripts highly expressed in human prostate and prostate cancer [12]. TARP expression is uniquely restricted to prostate tissue and prostate-derived cell lines, including LNCaP [13], which is one of the cell lines comprising GVAX immunotherapy for prostate cancer. The TRAP mRNA transcript is derived from the un-rearranged T cell receptor γ (TCRγ) locus, although it is truncated in comparison to the transcript normally detected in lymphoid tissues [12]. TARP expression is regulated by androgen levels related to the presence of an androgen-responsive element (ARE) in the TARP promoter [13, 14, 28]. Overexpression of TARP in PC-3 cells stimulates growth and is also associated with up-regulation of a number of genes associated with prostate cancer, including caveolin 1, caveolin 2 and amphiregulin [14]. In our studies, we observed the up-regulation of RNA transcript encoding TARP using Q-PCR in prostate cancer tissues

compared to normal matched controls, as previously reported in previous microarray studies of prostate cancer by Rhodes et al. [15] and Schlomm et al. [29]. In addition, we also correlated RNA up-regulation to overexpression of TARP protein in prostate cancer compared to normal prostate tissue employing a TARP-specific monoclonal antibody, TP-1 [28], on tissue microarrays. Up-regulation of TARP protein in prostate cancer tissue has not, to our knowledge, previously been reported. TARP protein expression was largely restricted to the neoplastic epithelium (Fig. 5a, b) although a low level of expression was observed in the normal prostate epithelium in agreement with earlier in situ hybridization mapping of the TARP RNA transcript [12].

In addition to its prostate-specific tissue expression pattern and up-regulation in prostate carcinogenesis, TARP appears to have a pre-established antigenic role in prostate cancer. T-cells targeting TARP have been identified in prostate cancer patients and MHC-class-I and -II restricted peptides have been detected [30–32]. TARP specific T-cells derived from prostate cancer patients have the ability to specifically lyse TARP expressing tumor cell lines, including the LNCaP cell line, indicating that TARP-derived MHC-class-I epitopes may be endogenously processed and presented by TARP-positive cells [30]. In support of the TARP T-cell data, the current studies show that an anti-TARP autoantibody response exists in prostate cancer patients prior to immunotherapy treatment (Table 1; Fig. 3b) and is boosted by administration of a GM-CSF-secreting whole cell immunotherapy. Pre-existing antibody responses before immunotherapy were not observed to galectin-8 or TRAP1 (Table 1; Fig. 3a, c), suggesting that these responses may represent more of a neo-antigen response. It would be interesting to examine the correlation of TARP autoantibody and T-cell response in the same patient; however, we were unable to monitor corresponding T-cell activities in the G-9803 or G-0010 trials because of absence of PBMCs from the patients.

GVAX immunotherapy for prostate cancer has been examined in a Phase 3 clinical trial (VITAL-1) [8]. It will be interesting to examine antibody induction to the antigens identified in this manuscript, such as TARP and TRAP1, in the VITAL-1 patient population to determine subgroups of patients which may receive benefit from the immunotherapy.

In summary, we have identified a group of proteins that are frequently targeted by an autoantibody response in prostate cancer patients post administration of a GM-CSF secreting whole cell immunotherapy. A sub-population of these proteins has an association with prostate cancer carcinogenesis. Retrospective analysis indicates that autoantibodies to TARP in particular correlate with clinical outcome in two phase 1/2 clinical trials of GVAX immunotherapy for prostate cancer. These antibody responses will serve as candidate biomarkers to be evaluated in additional GVAX clinical trials with the goal of identifying potential biomarkers of response and novel tumor-associated antigens.

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