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ORIGINAL ARTICLE

New gene expressed in prostate: a potential target for T cell-mediated prostate cancer immunotherapy

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Abstract New gene expressed in prostate (NGEP) is a prostate-specific gene encoding either a small cytoplasmic protein (NGEP-S) or a larger polytopic membrane protein (NGEP-L). NGEP-L expression is detectable only in prostate cancer, benign prostatic hyperplasia and normal prostate. We have identified an HLA-A2 binding NGEP epitope (designated P703) which was used to generate T cell lines from several patients with localized and metastatic prostate cancer. These T cell lines were able to specifically lyse HLA-A2 and NGEP-expressing human tumor cells. NGEP-P703 tetramer binding assays demonstrated that metastatic prostate cancer patients had a higher frequency of NGEP-specific T cells when compared with healthy donors. Moreover, an increased frequency of NGEP-specific T cells was detected in the peripheral blood mononuclear cells of prostate cancer patients post-vaccination with a PSA-based vaccine, further indicating the immunogenicity of NGEP. These studies thus identify NGEP as a potential target for T cell-mediated immunotherapy of prostate cancer.

Keywords NGEP · Prostate cancer · T cell · Vaccine · Epitopes

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Abbreviations

APC	Antigen-presenting cells
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
EBV	Epstein Barr virus
EST	Expressed sequence tag
E:T	Effector:target
HLA-A2	Human leukocyte antigen-A2 allele
IFN	Interferon
IVS	In vitro stimulation
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
NGEP	New gene expressed in prostate
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin
PSA	Prostate-specific antigen
TRICOM	Triad of costimulatory molecules (B7.1, ICAM-1, LFA-3)

Introduction

Prostate cancer is one of the most common cancers in men in the United States, with 186,320 new cases estimated in 2008, and one of the leading causes of cancer death among males, with approximately 28,660 deaths estimated in 2008 [1]. Despite recent advances in androgen-deprivation therapy and chemotherapy, there is currently no curative treatment for metastatic prostate cancer. With current therapies being unable to completely eliminate androgen-independent prostate cancer cells that remain after androgen ablation [2], novel approaches for the treatment of prostate cancer are essential. Specific immunotherapy, either alone or in combination with standard definitive radiation therapy or chemotherapy, is one such novel approach [3, 4]. In the

past few years, immunotherapy employing several different prostate cancer vaccines has shown promising evidence of clinical benefit in various patient populations [5, 6]. Tissue-specific antigens, which are expressed in both normal prostate and prostate cancer cells, can be targeted for prostate cancer-specific immunotherapy.

Several prostate cancer-associated antigens have been identified [7–16]. Some of these antigens, however, are also expressed in some normal tissues [17, 18].

New gene expressed in prostate (NGEP) was identified by analysis of expressed sequence tag (EST) databases. The NGEP gene, also known as TMEM16G, is located on chromosome 2 at 2q37.3. There are two spliced forms of NGEP mRNA; the smaller transcript (NGEP-S) encodes a 179-amino acid cytoplasmic protein and the larger transcript (NGEP-L) encodes a 933-amino acid polytopic membrane protein that is a member of the TMEM16 protein family [19]. It has been previously reported that many of the human TMEM16 genes are overexpressed in cancer and could be valuable tumor markers, especially in profiling gene expression with microarrays [20]. RNA analysis and Western blot analysis have detected NGEP-L in prostate tissue samples (normal, benign prostatic hyperplasia, and prostate cancer) but not in other tumors or essential normal tissues [19, 21, 22]. In a recent study, 91% of prostate tissue samples from 123 patients with localized prostate cancer (Gleason scores 4 and 5) were shown to be strongly positive for NGEP-L in the cancerous regions by immunohistochemistry. In addition, NGEP-L was found to be highly expressed in lymph nodes from two patients with metastatic prostate cancer [22]. NGEP has previously been shown not to be secreted into culture supernatant fluid (I. Pastan, unpublished data).

The examination of NGEP-L by confocal microscopy using anti-NGEP-L antibody on NGEP-L-transfected LNCaP cells has previously demonstrated that NGEP-L is located in the plasma membrane with a higher concentration detected at cell-contact regions. It has also been shown that as the cell density in culture increases, large aggregates are formed in the presence of NGEP-L and that siRNA for NGEP-L prevents the formation of these large aggregates. These observations suggested an important role for NGEP-L in promoting cell:cell interactions and thus may play a role in prostate cell adhesion [21]. It is not known at this time, however, whether NGEP is a true prostate differentiation antigen that is expressed in higher levels of more differentiated tumors, nor is it known whether it is found in higher levels on androgen-independent versus androgen-dependent prostate cancers.

In the present study we describe for the first time the identification and characterization of NGEP CTL epitopes. One of these epitopes, designated P703, was used to expand NGEP specific T cells from the blood of prostate cancer patients. These NGEP-specific T cells were shown to produce both high levels of IFN-γ and the chemokine lymphotactin after peptide-specific stimulation, and demonstrated an ability to lyse NGEP-expressing tumor cells. An increase in NGEP-specific T cells was also observed in the peripheral blood mononuclear cells (PBMC) of prostate cancer patients after vaccination with a PSA-based vaccine, further demonstrating the immunogenicity of NGEP in prostate cancer patients. These studies form the rationale for the use of vaccines targeting NGEP in patients with prostate cancer.

Materials and methods

Cell cultures

The human prostate cancer cell lines LNCaP, 22rV1, MDA-PCA-2b, DU145, PC3; the human breast cancer cell line MCF-7; and the human pancreatic adenocarcinoma cell line AsPC-1 were purchased from American Type Culture Collection (Manassas, VA) and maintained as recommended by ATCC [16]. MCF-7-pNGEP-L and PC3-pNGEP-L were full-length NGEP-L gene transfected cell lines [21] maintained in the presence of 0.75 mg/mL G-418 (Cellgro; Mediatech, Inc.). The human prostate cancer cell line PR-22 [23] was provided by Dr. Hyman I. Levitsky, Johns Hopkins University School of Medicine, Baltimore, MD. The T2 cells transfected with the HLA-A2 gene [24] were provided by Dr. Peter Cresswell, Yale University School of Medicine, New Haven, CT, and maintained as previously described [16]. The C1RA2 cells [25] were obtained from Dr. William E. Biddison, National Institute of Neurological Disorders and Stroke, National Institutes of Health (NIH), Bethesda, MD, and maintained in the presence of 0.7 mg/mL of G-418 (Cellgro; Mediatech, Inc.). The human chronic myelogenous leukemia cell line K562 expressing HLA-A*0201 (K562/A*0201) [26] was obtained from C. Britten (Johannes Gutenberg-University of Mainz, Mainz, Germany) and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin solution (Invitrogen) and 0.7 mg/ml of G418. All of the above cell lines were mycoplasma-free.

Peptides

The amino acid sequence of NGEP was scanned for matches to consensus motifs for HLA-A2-binding peptides, using the computer algorithm developed by Parker et al. [27]. The HLA-A2 allele was chosen because it is the most commonly expressed class I allele. A panel of NGEP peptides (Table 1) with a purity >95%, PSA peptide

Table 1 Binding of NGEP peptides to HLA-A2 molecules

Peptides	AA position	Sequence	T2 binding (fold increase)
P703 (L)	703–712	GLFDEYLEMV	565 (6.4)
P215 (L)	215–223	VLLEVVPDV	464 (5.2)
P113 (S)	113–121	LVWEEDLKL	367 (4.1)
P356 (L)	356–365	WLLPAAVVGT	354 (4.0)
P258 (L)	258–266	ILFEILAKT	280 (3.1)
P10 (S)	10–19	GLGGPPLPTL	212 (2.4)
P855 (L)	855–864	IVFEHVVFSV	203 (2.3)
P143 (S)	143–152	FLDNIRAAGL	203 (2.3)
P170 (L)	170–178	ALLSASWAV	185 (2.1)
P557 (L)	557–565	ILILSKIYV	162 (1.8)
P427 (L)	427–435	SLFMALWAV	153 (1.7)
P846 (L)	846–854	LLAIRLWAV	126 (1.4)
MUC-1 (P1240)	Positive control	SLSYTNPAV	452 (5.1)
CAP-7	Negative control	HLFGYSWYK	88.0

Peptides were used at a concentration of 25 μg/mL. *L* long form, *S* short form. Amino acids are shown by the single-letter code. MUC-1 peptide is an HLA-A2-binding peptide and CAP-7 is an HLA-A3-binding CEA peptide. Results are expressed in mean fluorescence intensity. Values in parentheses are fold increases as compared with the negative control. This is a representative of three experiments

(VISNDVCAQV) and Flu peptide (GILGFVFTL) were synthesized by American Peptide Inc. (Sunnyvale, CA) [3]. MUC-1 peptide [28] and CAP-7 [16] peptide were made by Biosynthesis Inc. (Lewisville, TX), with purity >95%.

Flow cytometric analysis

Cells were fixed and permeabilized for 7 min in 70% methanol, blocked for 30 min with 10% normal goat serum in PBS, then incubated at 4°C for 1 h with 1:500 diluted polyclonal antibody for NGEP-L [21]. Cells were washed, then stained with FITC-conjugated goat anti-rabbit IgG (Dilution 1:5,000; Invitrogen) for 1 h at 4°C and washed once with PBS. Cells (1×10^5) were acquired on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and data were analyzed using CellQuest software (BD Biosciences). An appropriate isotype-matched control was used, and dead cells were excluded from the analysis based on scatter profile.

Tetramer staining

Phycoerythrin (PE)-labeled NGEP-P703/HLA-A*0201 tetramer was prepared by the NIH/NIAID MHC Tetramer Core Facility (Atlanta, GA) and PE-labeled HIV gag (SLYNTVATL)/HLA-A*0201 tetramer (Beckman Coulter, Fullerton, CA) was used as a negative control. The staining was performed as previously described [16]. Cells (1×10^5) were acquired on a FACSCalibur flow cytometer (BD Biosciences) and LSRII (BD Biosciences), and data were analyzed using CellQuest software (BD Biosciences) and FlowJo software (BD Biosciences), respectively.

Binding of peptide to HLA-A2

Binding of NGEP peptides to HLA-A2 molecules was evaluated by the upregulation of HLA-A2 expression on T2 cells, as demonstrated by flow cytometry [29].

Culture of dendritic cells from PBMC

Peripheral blood mononuclear cells from HLA-A*0201$^+$ prostate cancer patients were obtained from heparinized blood. PBMC were separated using lymphocyte separation medium gradient (MP Biomedicals, Aurora, OH), according to the manufacturer's guidelines. Dendritic cells (DC) were prepared from PBMC, as previously described [16].

Generation of T cell lines

Peripheral blood mononuclear cells from two prostate cancer patients (patients A and B) were used to generate NGEP-specific T cell lines. Patient A was a 62-year-old male with a history of external beam radiation therapy for localized prostate cancer and rising PSA 4 years after treatment. However, he had no evidence of disease on whole-body scintigraphy or CT of chest, abdomen, and pelvis. A biopsy of his prostate confirmed recurrent disease, with a Gleason score 8, and he was enrolled on an intramural vaccine study [30]. After treatment with vaccine and androgen-deprivation therapy, his PSA declined from 10.7 ng/mL on study to <0.2 ng/mL. Patient B was a 52-year-old man with a history of metastatic prostate cancer at diagnosis. Following orchiectomy, his PSA initially declined, but then increased to 259 ng/mL, at which time he was enrolled on a phase II vaccine study [3]. Although he had extensive bone disease, he eventually sustained a >50% reduction in PSA and remained on study for 3.5 years with no evidence of progression. A modified version of the protocol described by Tsang et al. [31] was used to generate NGEP-specific cytotoxic T lymphocytes (CTL). Irradiated (3,000 rad) autologous DC were pulsed with 25 μg/mL peptides and used at an effector:antigen-presenting cell (APC) ratio of 10:1. After 3 days, human IL-2 (20 units/mL) was added to the cultures. Cells were restimulated after 7 days for a total of three in vitro stimulation (IVS) cycles. After IVS3, irradiated (23,000 rad) autologous Epstein Barr virus (EBV)-transformed B cells were used as APC (effector:APC ratio of 1:3).

Cytotoxicity assays

^{111}Indium release assays (6 or 16 h) were used to determine T cell-mediated killing [31]. A cytotoxic assay was performed to show the HLA-A*0201-restricted nature of the NGEP-specific lysis of the T cell lines, using as target cells PC3-pNGEP-L and PC3 cells plus or minus transiently transfected with HLA-A*0201 gene. Briefly, 1×10^6 PC3-pNGEP-L were transfected with 1 μg of purified pcDNA3.1-HLA-A2.1 (Protein Expression Laboratory, Advanced Technology Program, SAIC-Frederick, MD), using the nucleofactor device and technology according to the manufacturer's recommendations (Amaxa Biosystem, Gaithersburg, MD, USA). After 48 h, transiently transfected cells were first selected using FITC-conjugated HLA-A*0201 monoclonal antibody (mAb) (One Lambda, Canoga Park, CA, USA), then incubated with anti-FITC microbeads for 15 min at 4°C and collected by eluting the cells twice through a magnetic separation (MS) column (Miltenyi Biotec, Auburn, CA, USA). The expression of HLA-A*0201 was analyzed by flow cytometry and the cells were used as targets in a 16-h ^{111}Indium release assay.

Detection of cytokines

Supernatants of T cells stimulated for 24 h with peptide-pulsed autologous DC or EBV-transformed B cells in IL-2-free medium at various peptide concentrations were screened for secretion of IFN-γ and IL-2 using ELISA kits (BioSource International, Camarillo, CA) and screened for lymphotactin using an ELISA assay [32].

Elispot assay

A modification of the procedure, described by Arlen et al. [3], was performed, using K562/A*0201 as APC, as previously reported [26].

Statistical analysis

Statistical analysis of differences between means was performed using a two-tailed paired *t* test (StatView statistical software; Abacus Concepts, Berkeley, CA).

Results

Until now, the NGEP-L protein has been considered a potential target for mAb-mediated prostate cancer immunotherapy [21]. In order to identify $CD8^+$ T cell epitopes of NGEP that could be of use in T cell-mediated immunotherapy, the primary amino acid sequence of human NGEP protein was analyzed for consensus motifs for HLA-A2-binding peptides. Seven 9-mer peptides and five 10-mer peptides were identified and investigated for their ability to bind to the HLA-A2 molecule in a T2 cell-binding assay. Two peptides, designated P703 and P215, demonstrated greater efficiency for binding to the HLA-A2 molecule (Table 1); the mean fluorescence intensity (MFI) for P703 and P215 was 6.4- and 5.2-fold, respectively, higher than the negative control. To examine the stability of peptide-MHC complexes, several peptides that exhibited the highest binding to HLA-A2 were incubated with T2 cells overnight. Unbound peptides were washed off and delivery of new class I molecules to the cell surface was blocked by the addition of brefeldin A. Cells were then analyzed for the presence of peptide-HLA-A2 complexes at various time points, as indicated by the degree of MFI (Fig. 1a). The P703- and P215-HLA-A2 complexes demonstrated the most stable complexes; therefore, these two peptides were used in subsequent studies. The ability of these two peptides to bind T2 cells was also evaluated at various peptide concentrations (Fig. 1b), and at all concentrations, peptide P703 bound to HLA-A2 at higher levels.

The immunogenicity of peptides P703 and P215 was then investigated by evaluating their ability to induce specific CTL in vitro. T cell lines were generated against both peptides from PBMC of a patient with locally recurrent prostate cancer (patient A). The T cell lines generated using peptides P703 and P215 were designated as T-A-P703 and T-A-P215, respectively. To evaluate the specificity of these T cell lines, an IFN-γ release assay was performed using irradiated, autologous DC pulsed with the corresponding NGEP and control peptides (Fig. 1c). The T-A-P703 cell line produced higher levels of IFN-γ compared to the T-A-P215 cell line. Note that no IFN-γ was produced employing a control peptide. It has previously been demonstrated [33] that peptide-specific T cells produce high levels of the chemokine lymphotactin after stimulation with agonist peptides. As shown in Fig. 1d, the T-A-P703 cell line produced higher levels of lymphotactin than the T-A-P215 cell line when stimulated with autologous DC pulsed with various concentrations of each corresponding peptide (Fig. 1d). Based on these data, we chose the P703 NGEP peptide for further analysis.

An additional T cell line was then established from PBMC of a patient with metastatic prostate cancer (patient B) using the P703 peptide; this cell line was thus designated as T-B-P703. This cell line also produced high levels of IFN-γ (876.5 pg/mL) in response to peptide-specific stimulation and undetectable levels of IFN-γ using the control HIV gag peptide. Studies were then conducted to investigate the frequency of NGEP-specific $CD8^+$ T cells in both the T-A-P703 and T-B-P703 cell lines, using an NGEP-specific P703/HLA-A*0201 tetramer and anti-CD8 antibodies. As shown in Fig. 2a and b, a higher frequency of

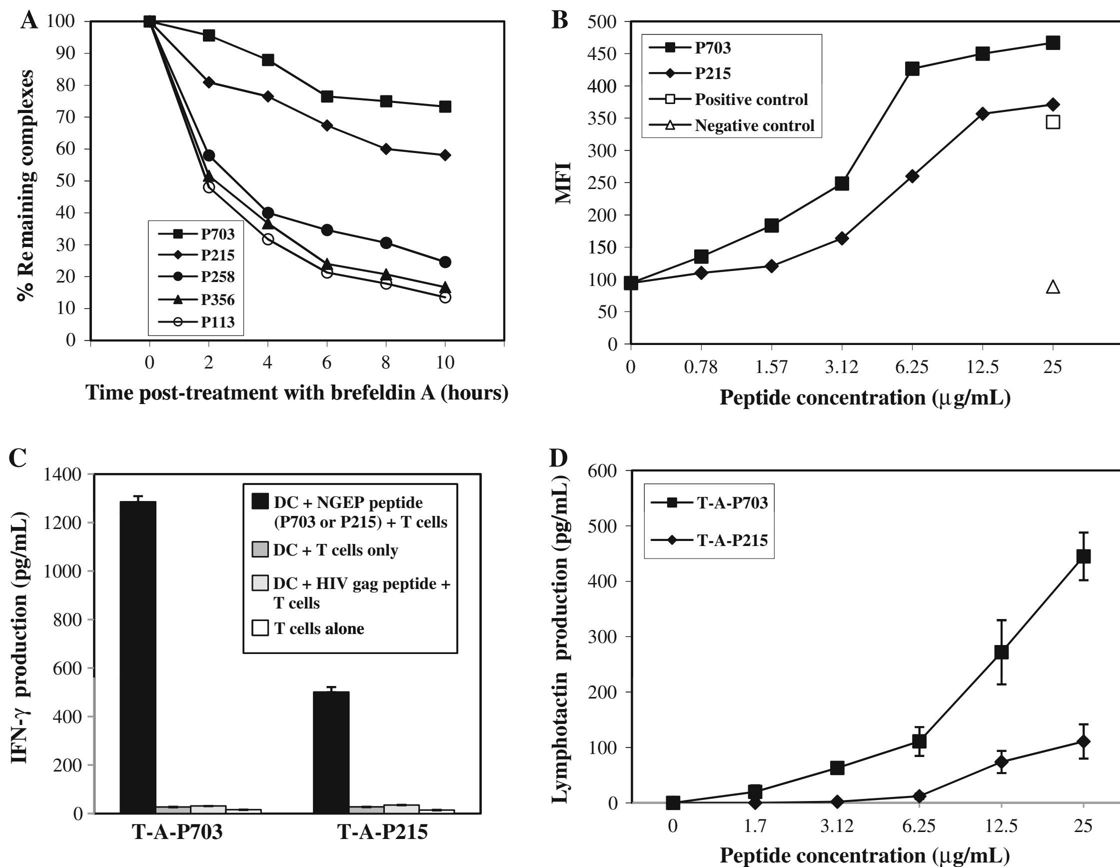

Fig. 1 Analyses of NGEP peptides. **a** Analysis of the stability of HLA-A2/NGEP complexes. T2 cells were incubated overnight with peptides P356, P113, P215, P703, and P258 at a concentration of 25 µg/mL. Results are expressed in relative percentage of binding compared to 100% at time 0. The experiments were repeated twice. **b** Binding of peptides P703 and P215 to HLA-A2 molecules at various peptide concentrations. Results are expressed in MFI. The positive control peptide was MUC-1 and the negative control peptide was CAP-7. The experiments were repeated twice. **c** IFN-γ production by NGEP-specific T cell lines. Isolated CD8$^+$ T cells were used as effectors at IVS-4, and stimulated with irradiated autologous DC pulsed with peptides as indicated at an effector:APC ratio of 10:1. Twenty-four hour culture supernatants were collected and screened for secretion of IFN-γ. Results are expressed in pg/mL. The experiment was repeated three times. *Bars* SD. **d** Lymphotactin production by NGEP-specific T cell lines at various peptide concentrations. Results from a representative experiment are expressed in pg/mL. *Bars* SD

NGEP-specific CD8$^+$ T cells was generated in the T-A-P703 T cell line (95.2%). Both cell lines were then tested for cytotoxic activity against peptide-pulsed HLA-A2$^+$ targets in a 6-h CTL assay (Fig. 2c). As expected, the T-A-P703 cell line specifically lysed C1RA2 cells pulsed with the P703 peptide at various effector:target (E:T) cell ratios with higher efficiency than the T-B-P703 cell line. As previously reported [19, 21, 22], 91% of over 100 prostate cancer biopsies and lymph node metastases from 2/2 patients were highly positive for NGEP expression. Examination of six established prostate cancer cell lines, however, showed very low expression in three lines and only moderate expression in the other three (Table 2). Unfortunately, the two lines showing the most expression of NGEP (22rV1 and MDA-PCA-2b) were devoid of HLA-A2 expression and were thus not suitable targets for the HLA-A2-directed NGEP T cell lines generated. To determine if these T cell lines, raised against the P703 peptide, could kill target cells endogenously expressing full-length processed NGEP- L, a CTL assay was performed using MCF-7 tumor cells transfected with the NGEP-L gene (HLA-A2$^+$, NGEP$^+$) and untransfected MCF-7 cells (HLA-A2$^+$, NGEP$^-$) as a negative control (Fig. 2d). The results showed that the T-A-P703 cells can specifically kill tumor cells endogenously expressing the NGEP-L gene. Studies were undertaken to determine if NGEP-specific T cells could specifically lyse the human prostate cancer cell line PC3 in an MHC-restricted manner. PC3 is devoid of the HLA-A2 allele. As seen in Fig. 3, two NGEP-specific T cell lines from two different prostate cancer patients both efficiently lysed the PC3 prostate cancer cells after transfection with HLA-A2.

Studies were then undertaken to determine if prostate cancer patients recognized the NGEP-P703 epitope. PBMC from four additional patients with metastatic prostate cancer and from four healthy donors were analyzed for the presence of CD8$^+$ T cells reactive with the NGEP-P703

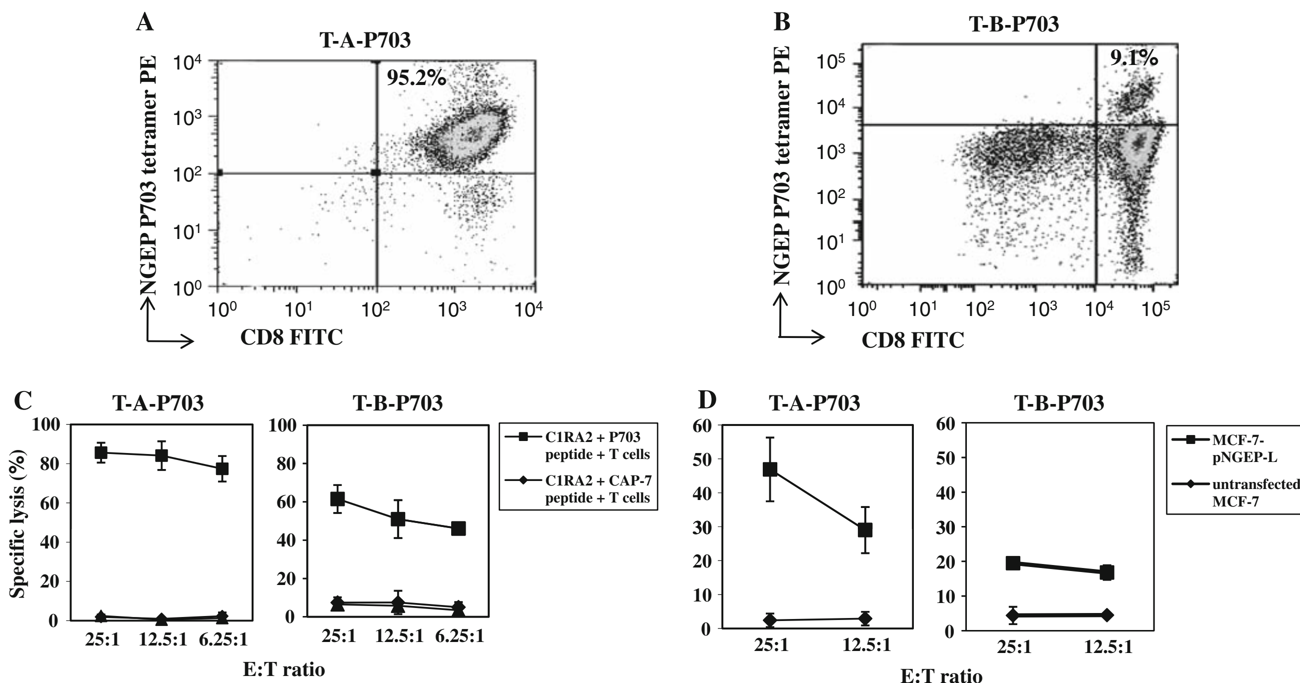

Fig. 2 Characterization of NGEP-specific T cell lines. Frequency of NGEP-specific $CD8^+$ T cells in the T-A-P703 cell line (**a**), and in the T-B-P703 cell line (**b**), by staining T cells with PE-labeled NGEP-P703/HLA-A*0201 tetramer and anti-CD8 antibody (1×10^6 cells/20 µL), at IVS-3. Indicated is the % of tetramer positive NGEP-specific T cells in $CD8^+$ T cell population. **c** Cytotoxic activity of the T-A-P703 and T-B-P703 cell lines on C1RA-2 pulsed with NGEP or control peptides as indicated. The experiment was performed in triplicate and repeated twice. *Bars* SD. **d** Cytotoxic activity of the T-A-P703 and T-B-P703 cell lines on MCF-7 cells transfected with the NGEP gene (MCF7-pNGEP-L). MCF-7-pNGEP-L cells were 69% positive for NGEP and 76% positive for HLA-A2 by FACS analysis. Untransfected MCF-7 cells were negative for NGEP and 85.9% positive for HLA-A2. The experiment was performed in triplicate and repeated twice. *Bars* SD

Table 2 Expression of NGEP and HLA-A2 in established prostate cancer cell lines

	NGEP expression	HLA-A2 expression
Prostate cancer cell lines		
PR-22	2.68% (13.10)	72.29% (17.22)
DU145	11.42% (20.02)	Neg.
22 rV1	42.47% (17.80)	Neg.
MDA-PCA-2b	71.42% (39.43)	Neg.
LNCaP	34.92% (18.93)	16.9% (16.21)
PC3	7.32% (16.43)	9.46% (14.73)
Non-prostate cancer cell lines		
AsPC-1	Neg.	Neg.
MCF-7	1.2% (11.9)	85.79% (43.80)

Values are % cells expressing via FACS analysis. Numbers in parentheses are mean fluorescence intensity (MFI) values

tetramer. As can be seen in Table 3, three of the four patients with metastatic prostate cancer had a higher frequency of NGEP-P703 specific $CD8^+$ T cells as compared to the healthy donors. These same four prostate cancer patients were then analyzed for tetramer binding after receiving six monthly cycles of a PSA-based vaccine (PSA/TRICOM). As seen in Table 3, PBMC from all four patients showed higher tetramer binding post-vaccination as compared to pre-vaccination. These results provide evidence of cross-presentation of the NGEP epitope as a result of the vaccine therapy, and provide further evidence of the immunogenicity of NGEP in prostate cancer patients.

An additional four patients with metastatic prostate cancer were then analyzed pre- and post-vaccination with PSA/TRICOM for the generation of PSA-specific and NGEP-specific T cells in PBMC employing an ELISPOT assay for IFN production. As seen in Table 4, all four patients were negative for PSA-specific T cells prior to vaccination with increases in PSA-specific T cells in three of four patients post-vaccination. Interestingly, the three patients demonstrating increases in PSA-specific T cells also showed increases in NGEP-specific T cells. Taken together (Tables 3, 4), these studies demonstrate the immunogenicity of NGEP in six of eight prostate cancer patients.

Discussion

Recent clinical trials using various vaccines for prostate cancer have shown promising results in terms of survival

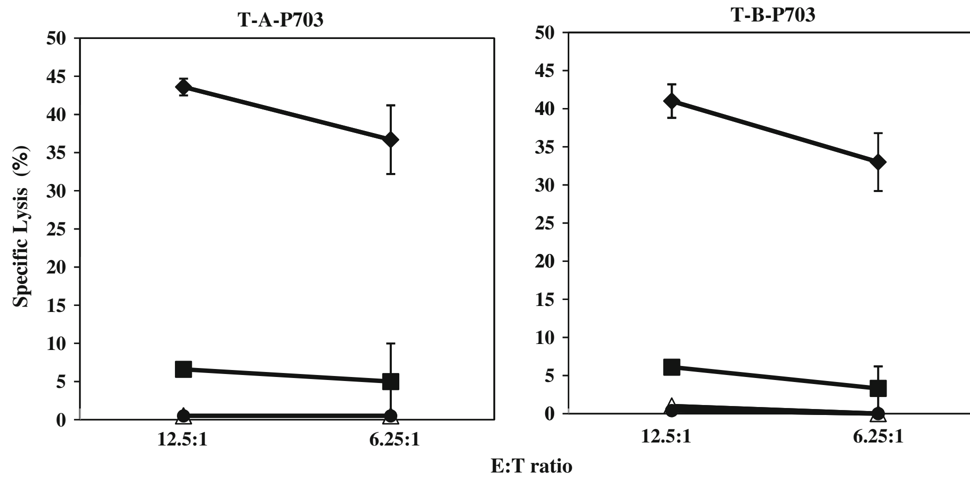

Fig. 3 Cytotoxic activity of the T-A-P703 and T-B-P703 cell lines on PC3-pNGEP-L cell line transfected with HLA-A*0201 gene (*filled diamond*), PC3-pNGEP-L cell line not transfected with HLA-A*0201 gene (*open triangle*, behind *filled circle*), PC3 cell line transfected with HLA-A*0201 gene (*filled square*) and PC3 cell line not transfected with HLA-A*0201 gene (*filled circle*). PC3-pNGEP-L cell line transfected with HLA-A*0201 gene was 80% positive for NGEP and 82% positive for HLA-A*0201. PC3 transfected with HLA-A*0201 gene was negative for NGEP and 85% positive for HLA-A*0201. As indicated, two T cells: target cells ratio were used (12.5:1 and 6.25:1). The experiment was performed in triplicate. *Bars* SD

Table 3 Identification of NGEP-specific CD8$^+$ T cells in healthy donors and prostate cancer patients using NGEP-P703/HLA-A* 0201 tetramer

Samples	NGEP-tetramer	HIV-tetramer
Healthy donors		
I	0.49	0.20
II	0.88	0.25
III	0.89	0.41
IV	0.71	0.27
Prostate cancer patients pre-vaccination		
Patient 1	0.85	0.58
Patient 2	1.77	0.16
Patient 3	2.04	0.29
Patient 4	1.52	0.52
Prostate cancer patients post-vaccination		
Patient 1	1.08	0.11
Patient 2	2.70	0.30
Patient 3	2.47	0.35
Patient 4	3.42	0.82

T cells (5 × 105) were stained with 10 µl of PE-labeled NGEP-P703/HLA-A*0201 tetramer and anti-CD8 antibody. Tetramer PE-labeled HIV gag (SLYNTVATL)/HLA-A*0201 was used as a negative control. Results are expressed in % of tetramer positive cells in PBMC

Table 4 Precursor frequency of T cells specific for PSA and NGEP peptides from peripheral blood of PCa patients before and after six cycles PSA/TRICOM vaccination

Patient		PSA3a	NGEP P703
Patient 5	Pre	<1/200,000	<1/200,000
	D167	1/46,154	1/24,000
Patient 6	Pre	<1/200,000	1/150,000
	D171	1/150,000	1/75,000
Patient 7	Pre	<1/200,000	<1/200,000
	D174	1/75,000	1/31,579
Patient 8	Pre	<1/200,000	<1/200,000
	D174	<1/200,000	<1/200,000

PSA and NGEP peptides were used in the ELISPOT assay at a concentration of 25 µg/mL. Flu peptide was used at a concentration of 1 µg/mL. Peptide-pulsed K562/A2.1 cells were added to each well as APC at an effector:APC ratio of 1:2. Unpulsed K562/A2.1 cells were used as a negative control, and flu peptide was used as a positive control. Spots in each well were examined and counted using an ImmunoSpot Analyzer

benefit, reduced toxicities, and potential for use in combination therapies [5, 7].

NGEP-L is a membrane antigen detected exclusively in normal prostate, benign prostatic hyperplasia, and prostate cancer [19]. A previous report demonstrated that NGEP-L mediates cell contact-dependent interactions in prostatic epithelial cells [21]. In this study, we report for the first time the identification and characterization of a NGEP-derived HLA-A*0201-restricted CTL epitope (designated peptide P703) that was used successfully to generate NGEP-specific T cells from two different patients (one with localized prostate cancer and one with metastatic prostate cancer). As demonstrated here, both T cell lines released high levels of IFN-γ and were able to specifically lyse NGEP peptide-pulsed target cells. Analysis of six prostate cancer cell lines, however, showed only two with relatively high levels of expression (42 and 71%). Both of these lines, however, were negative for HLA-A2 and thus could not be

used in lysis assays. This relatively low level of NGEP expression in cell lines compared to biopsy specimens may be an "artifact" of the growth of cells in vitro and thus the loss of their epithelial phenotype. Studies are ongoing to investigate this phenomenon. To determine if the NGEP-specific T cells generated could specifically lyse tumor cells which endogenously express NGEP, we employed the NGEP negative MCF7 tumor cell line plus or minus transfected with NGEP. As seen in Fig. 2, both T cell lines specifically lysed the NGEP-expressing cells. In addition, we showed that both T cell lines can lyse the human prostate cancer cell line PC3-pNGEP-L at higher levels in an MHC-restricted manner (Fig. 3).

Other than conducting clinical studies, we believe there are two potential ways to define if a given antigen is immunogenic in humans. The first is to determine if cancer patients show any evidence of an endogenous T cell response to that antigen. As seen in Table 3, PBMC from three of the four prostate cancer patients showed higher levels of binding with NGEP-specific tetramer as compared to PBMC from normal donors. Another way to determine if there is evidence of immunogenicity for a given tumor-associated antigen is to look for evidence of antigen cascade or epitope spreading [3, 4, 34]. We have previously shown that prostate cancer patients who receive a PSA vaccine will mount immune responses to other prostate-associated antigens post-vaccination. This is most likely due to target cell destruction and cross-presentation of destroyed target cells to the immune system, thus activating T cells to tumor-associated antigens not in the vaccine. In the studies reported here we demonstrate that all four patients had increases in NGEP tetramer-positive T cells post-vaccination with a PSA-based vaccine (PSA-TRICOM). An additional four prostate cancer patients were also analyzed using an ELISPOT assay for IFN-γ production. Three of four of these patients vaccinated with PSA-TRICOM showed enhanced T cell responses to NGEP post-vaccination. The studies reported here demonstrate that human T cell lines can be generated from PBMC of patients with both localized and metastatic prostate cancer. Moreover, these T cell lines were shown to efficiently lyse prostate cancer cells. In addition, NGEP-specific T cells were detected by tetramer in the peripheral blood of prostate cancer patients post-vaccination with a PSA-based vaccine, indicating the potential immunogenicity of NGEP. These studies collectively thus provide evidence that NGEP-based vaccines may be of potential use in prostate cancer immunotherapy studies.

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Conflict of interest statement There are no potential conflicts of interest.

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