

Study of NGEF expression in androgen sensitive prostate cancer cells: A potential target for immunotherapy

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Abstract

Background: Prostate cancer is one of the leading causes of cancer deaths among men. New gene expressed in prostate (NGEP), is a prostate-specific gene expressed only in normal prostate and prostate cancer tissue. Because of its selective expression in prostate cancer cell surface, NGEP is a potential immunotherapeutic target. To target the NGEP in prostate cancer, it is essential to investigate its expression in prostate cancer cells.

Methods: In the present study, we investigated NGEP expression in LNCaP and DU145 cells by real time and RT-PCR, flow cytometric and immunocytochemical analyses.

Results: Real time and RT-PCR analyses of NGEP expression showed that NGEP was expressed in the LNCaP cells but not in DU145 cells. The detection of NGEP protein by flow cytometric and immunocytochemistry analyses indicated that NGEP protein was weakly expressed only in LNCaP cell membrane.

Conclusion: Our results demonstrate that LNCaP cell line is more suitable than DU145 for NGEP expression studies; however, its low-level expression is a limiting issue. NGEP expression may be increased by androgen supplementation of LNCaP cell culture medium.

Keywords: DU145 cell line, LNCaP cell line, NGEP, Prostate cancer.

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Introduction

Prostate cancer is the second most common cause of cancer mortality and one of the leading cause of cancer deaths among men (1). It develops primarily in men over fifty, however, the onset of prostate cancer vary widely across the world population. The incidence of prostate cancer in Eastern countries is lower than the Western countries (2).

Standard treatments for localized prostate cancer include surgery, radiotherapy, and active monitoring. The current therapies for

advanced and metastatic cancer are hormone therapy and chemotherapy (3,4). However, these therapies are currently unable to completely eliminate androgen-independent prostate cancer cells that remain after androgen ablation therapy in metastatic prostate cancer. Androgen independent prostate cancer progression and metastasis are major causes of prostate cancer deaths (5,6). Therefore, novel approaches for the treatment of prostate cancer are essential. Previous studies have demonstrated that vaccine and targeted

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therapy could be promising efficacious treatments for this type of cancer (7). Thus, identification of the specific tumor markers for prostate cancer targeted therapy is crucial.

The recent studies have been shown that prostate specific antigen (PSA) in both prostate tissue and other normal tissues such as kidney, liver, esophagus, stomach, small intestine, colon, brain, and lung (8,9). These data showed that PSA cannot be considered as a specific target for immunotherapy of prostate cancer and researchers tend to identify particular markers for such idea.

Several studies have reported that NGEP that also called ANO7 is expressed specifically in prostate tissue. In an immunohistochemical analysis, it was shown that NGEP is highly expressed in low-grade prostate cancers (10,11). NGEP is a cell membrane protein of anoctamin/TMEM16 family that functions as calcium-activated chloride channels (12). Moreover, this antigen is the most attractive tumor marker for immunotherapy approaches because of its potent immunogenicity in addition to prostate tissue-restricted expression (13,14). There are two isoforms of NGEP due to different mRNA splicing. The smaller transcript encodes a 179-amino acid cytoplasmic protein (NGEP-S) and the larger transcript encodes a 933-amino acid polytopic membrane protein (NGEP-L) (10,13).

NGEP is important because expresses only in prostate tissue and therefore appropriate marker for prostate cancer immunotherapy. To target the NGEP in prostate cancer cells for future studies, it is essential to investigate NGEP expression in these cells. In the present study, we investigated NGEP expression in two standard prostate cancer cell lines; LNCaP and DU145, as androgen-dependent (15) and androgen-independent (16) cells, respectively.

Methods

Cell culture

The human prostate cancer cell lines, LNCaP and DU145, were purchased from

Pasteur Institute of Iran. These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Rockville, MD) supplemented with 10% fetal bovine serum (GIBCO) and 100 µg/ml penicillin/streptomycin (GIBCO). The cells were incubated in a 5% CO₂, 95% air humidified incubator at 37 °C.

RNA isolation, RT-PCR and real time PCR

Total RNA isolation from LNCaP and DU145 cell lines were performed using TRIzol reagent (Invitrogen, Carlsbad, USA) followed by phenol-chloroform extraction and isopropanol precipitation. Extracted RNA was checked by spectrophotometry, and RNA integrity was confirmed by visualization of 28S and 18S bands on 1% of an agarose gel. The RNA was transcribed using the reverse transcription system (Transcriptor high fidelity cDNA synthesis kit; was obtained from Roche, IN, USA). The following primers were applied in PCR experiments forward NGEP: 5'-TTC CTG CCG CGT GC C TAC TA-3', reverse NGEP: 5'-GGT CTG GGA ATA ATG TCC ATC GTC-3'; forward GAPDH: 5'-TTGCCATCAATGACCCCTTCA-3', reverse GAPDH: 5'-CGCCCCACTTGATT TTGGA-3'; forward β2 microglobulin: 5'-CCTGAATTGCTATGTGTCTGGG-3', reverse β2 microglobulin: 5'-TGATGC TGCTTACATGTCTCGA-3'. Sizes of NGEP, GAPDH and β2 microglobulin mRNA detected by designed primers were 160, 174 and 244 base pairs, respectively. PCR was performed using HotStarTaq Plus DNA Polymerase (QIAGEN, CA, USA) and the mRNA expressions of the NGEP, GAPDH and β2 microglobulin were assayed using the following PCR cycling protocol: initial denaturation for 5 min at 95°C, 35 cycles of denaturation 10s at 94°C, annealing for 30s at 57°C, 30s at 72°C, and finally, elongation for 7 min at 72°C.

Real-time PCR was carried out using the AccuPower 2X Greenstar qPCR Master Mix (BIONEER, Korea). All PCR reactions were performed in a total volume of

20 μ l with the following sequence: one cycle of pre-denaturation for 5 min at 95 °C and 45 amplification cycles (5s of denaturation at 95°C and 35s of annealing and extension at 57°C and 72°C). The threshold cycle (Ct) values obtained from experiments were used to indicate the fractional cycle numbers at which the amount of amplified target reached a fixed threshold. The amount of NGEF mRNA in LNCaP cells was normalized to the internal control (GAPDH) and evaluated relative to DU145 cells. Relative expression levels were presented as the fold change that calculated using $\Delta\Delta C_t$.

Flow cytometric analysis

The cells were harvested by 0.25% Trypsin-EDTA solution (1X) (GIBCO), washed and resuspended in ice cold PBS, containing 5% BSA and 1% sodium azide. Two preparations of rabbit polyclonal antibodies (anti-NGEP-p1 and -p2 antibodies) against NGEF-L extracellular parts: anti-NGEP-P1 (specific for residues 669-684) and anti-NGEP-P2 (specific for residues 691-705) designed by an immuno-bioinformatics approach were produced in the Biotechnology laboratory of Iran University of Medical Sciences as primary antibodies, (data not shown). The antibodies were diluted in 3%BSA/PBS and added in parallel to cell suspensions. Cell suspensions were incubated for 30 min at room temperature. After washing 2 times with 3%BSA/PBS, the cells were stained with a solution of 1:160 FITC-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) in the same buffer for 30 min, and then washed 2 times. The cells which have not been treated with primary antibodies used as negative control. PBS and equal concentration of IgG fraction from pre-immunized rabbit serum applied as negative controls instead of the primary antibody.

Binding of the cells (1×10^5) to anti-NGEP-p1 and -p2 antibodies was investigated by flow cytometry (Partec, Germany) and data were analyzed using flomax software. The dead cells were excluded from

the analysis based on scatter profile.

Immunocytochemistry

The cells were cultured in 96 well plates and grown for 24 h in 5% CO₂/95% air humidified incubator at 37°C. The cells were then washed with PBS, fixed for 20 min in 4% formaldehyde, washed and incubated with 3% BSA/PBS for 30 min at room temperature. Subsequently cells were washed and treated with primary antibodies (anti-NGEP-p1 and p2 antibodies) diluted 1:2 for 1 hour at room temperature. The cells were then washed and incubated at room temperature for 1 hour with a dilution of 1:160 FITC-conjugated goat anti-rabbit IgG (Sigma), washed and analyzed using an inverted fluorescence microscope (Olympus, Japan). PBS and equal concentration of IgG fraction from pre-immunized rabbit serum applied as negative controls instead of the primary antibody.

Results

Expression studies of the NGEF were performed by RT-PCR on cDNA preparation from the prostate cancer cell lines. Using RT-PCR, we showed NGEF expression in LNCaP, but it was not detectable in DU145 cells (Fig. 1). To compare relative NGEF expression of LNCaP to DU145 cells, we performed real time RT-PCR assay. The GAPDH normalized results showed that LNCaP cells express NGEF 26.13 fold higher than that of DU145 cells.

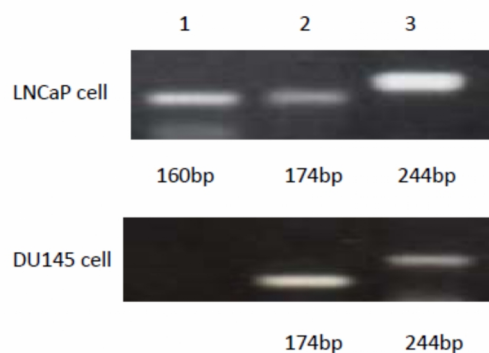


Fig. 1. RT-PCR analysis of prostate cancer cell lines; LNCaP and DU145 cells. Lane 1 shows NGEF expression, lanes 2 and 3 show GAPDH and b2 microglobulin expression respectively.

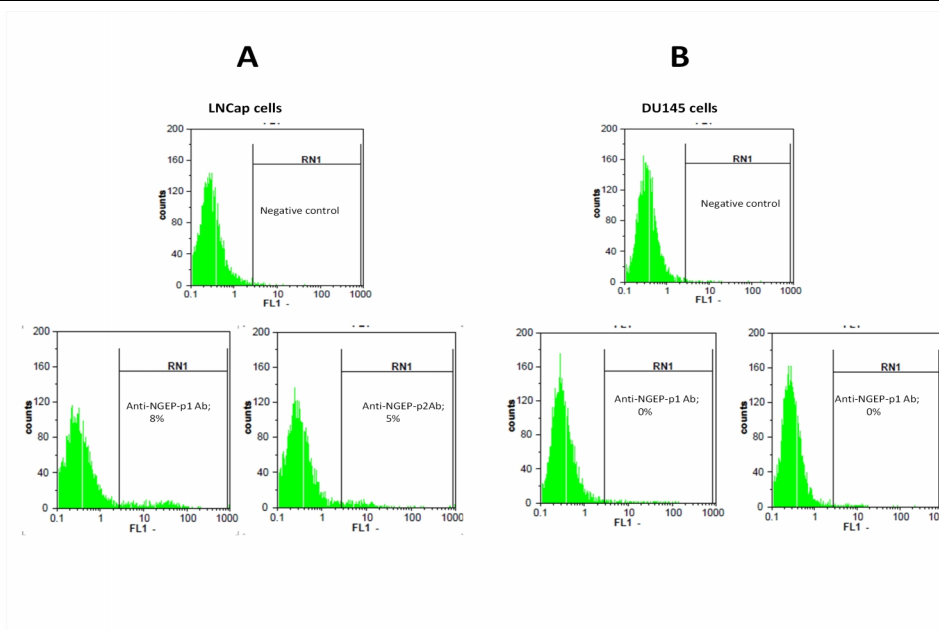


Fig. 2. Flow cytometric analysis of NGEp expression in prostate cancer cell lines. A: LNCaP cells and B: DU145 cells treated with anti NGEp-p1 and p2 antibodies. Negative control: the cells not treated with primary antibodies (anti-NGEP-p1 and or p2 antibody).

As illustrated in Fig. 2, flow cytometric analysis of the LNCaP line with anti NGEp-p1 and -p2 antibodies against NGEp showed that only 8% and 5% of the cells were stained with these two antibodies, respectively. Whereas, no staining was seen in DU145 cells and expression of NGEp

protein was negative in DU145 cells.

We also investigated staining pattern of NGEp in prostate cell lines treated with anti NGEp-p1 and -p2 antibodies by immunocytochemistry. Immunocytochemical analysis of LNCaP cell line both anti NGEp-p1 and -p2 antibodies showed weak

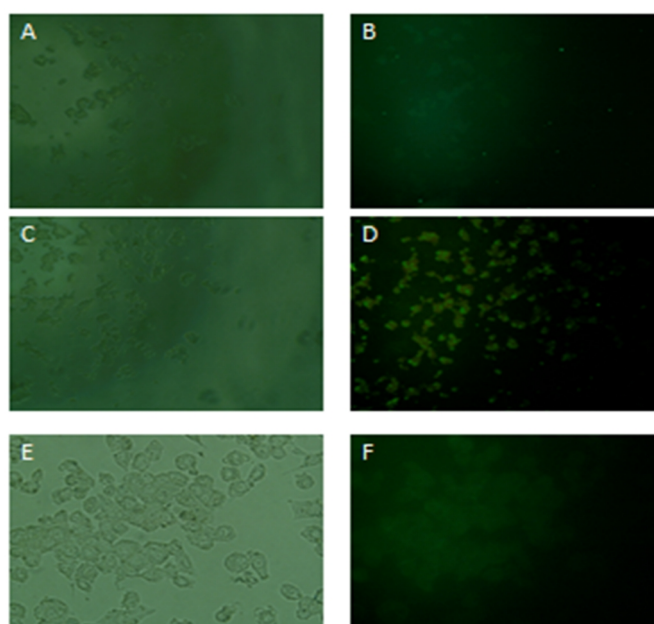


Fig 3: Images of LNCaP and DU145 cell lines taken by light microscopy (A,C,E) and immunofluorescence (B,D,E) when treated with anti-NGEP-p2 antibody. A and B; LNCaP cells not treated with anti-NGEP-p2 antibody (negative control), C and D; LNCaP cells treated with anti-NGEP-p2 antibody, D and E; DU145 cells treated with anti-NGEP-p2 antibody.

green fluorescence signals but in DU145 cells there were very weak or no signal of fluorescence and observed ghost of cells as shown at Fig. 3 by anti-NGEP-p2 antibody.

Discussion

In an immunohistochemical study, we demonstrated that NGEP expression level was decreased from low-grade to high-grade prostatic adenocarcinoma samples, indicating that NGEP can be a valuable potential prognostic tumor antigen and therefore an attractive target for antibody-based immunotherapy (11). Other previous studies have also shown that NGEP is an androgen-dependent and differentiation antigen that is highly expressed in well differentiated prostate cancer tissues in comparison to the poor differentiated prostate cancer cells (17). The LNCaP cell line is more differentiated than PC3 and DU145 cell lines as evidenced by production of PSA and its response to androgens (18). The RT-PCR analysis has shown NGEP expression in LNCaP cell line and prostate cancer specimens, however, NGEP mRNA not detected in the PC3 cell line (10). It has been shown that NGEP antigen was located in the plasma membrane and its concentration increased at cell contact regions which play an important role in cell-cell interactions (13).

Consideration to NGEP significance for prostate cancer immunotherapy, in the present study, we evaluate its expression at molecular and protein levels in prostate cancer cells using RT-PCR, flow cytometry and immunocytochemistry techniques.

Our investigation on the NGEP gene expression in LNCaP and DU145 cells revealed that NGEP was expressed in LNCaP cell, while it not in DU145 cells. Our finding highlighted previous data showing that NGEP is expressed in more differentiated and predominantly androgen-dependent prostate cancer cells (11).

NGEP expression was examined by Cereda et al using flow cytometric analysis in six established prostate cancer cell lines including PR-22, DU145, 22rV1, MDA-

PCA-2b, LNCaP and PC3, demonstrating very low level of expression in three cell lines (PR-22; 2.68%, DU145; 11.42% and PC3; 7.32%), moderate expression in two cell lines (22rv1;42.47%, LNCaP; 35%) and high level of expression in one cell line (MDA-PCA-2b; 71.42%) (14). In the present study, we measured the expression of NGEP in LNCaP and DU145 using flow cytometric analysis and immunocytochemistry. The polyclonal antibodies against extracellular domains of NGEP-L were produced in our laboratory (data not shown). To show the specificity of polyclonal antibodies against designed peptides of NGEP-L (anti-NGEP-p1 and -p2 antibody) and to determine any nonspecific binding in prostate tissue by these antibodies, we were able to block completely the specific binding of antibodies to NGEP protein by appropriate concentrations of peptides. No background staining was observed using these antibodies in immunohistochemistry analysis, indicating that these polyclonal antibodies were specific for the regions to which they were produced (data not shown).

To target the tumor antigen, it is important to produce antibodies bound to extracellular domains of membrane protein but not intracellular domains. In previous studies antibody produced against NGEP was for intracellular domain (13,14), while in this study antibodies produced in our laboratory were for extracellular domains. Moreover, our finding was in controversy with previous studies in terms of NGEP protein expression level. Our data indicated that NGEP expressed at low levels in the cell membrane of intact LNCaP cells at 8% and 5% detected by anti-NGEP-p1 and p2 antibodies, respectively, and no expression was observed in DU145 cells. Whereas, the study performed by Cereda et al using antibody produced against intracellular domain showed the NGEP expression in cell membranes of both LNCaP and DU145 cells at 35% and 11.42%, respectively in permeable condition (14). Taken together, our result showed that LNCaP cells in native

condition could not be a good target for immunotherapy. Moreover, attempts to detect NGEP protein in LNCaP cells by western blot were unsuccessful (13).

On the other hand, our finding was in agreement with RT-PCR analysis of Bera and et al. (10) determining that NGEP gene was expressed only in LNCaP cell line, not in PC3 and DU145 cells. Our data also confirmed previous study (10) reporting that NGEP gene was not expressed in androgen-independent DU145 cell line, while it was detectable in sensitive androgen LNCaP cells.

It has been reported that the LNCaP prostate cancer cells express the androgen receptors and PSA (19). As PSA is almost exclusively expresses through the androgen receptor signaling pathway (19), it may suggest that NGEP gene expression also depends upon these receptor signaling pathways and can be regulated by androgen receptors.

On the other side, NGEP-L is a membrane antigen which exclusively detected in normal prostate, benign prostatic hyperplasia, high-grade prostatic intraepithelial neoplasia and prostatic adenocarcinomas with variety of levels (11). This relatively higher level of NGEP expression in prostate tissues (11,17) compared to prostate cell lines, can be due to repeated passages of cell lines in vitro and the growth of cells in androgen-free media which may finally decrease the NGEP expression in prostate cell lines.

The further studies are required to investigate low NGEP expression in androgen sensitive cells. It is suggested that LNCaP to be cultured in a medium containing androgens such as dihydrotestosterone and evaluated NGEP expression.

Conclusion

NGEP protein can be used as an attractive marker in immunotherapy of prostate cancer. Our results showed that LNCaP cell line is more suitable than DU145 for NGEP expression studies. However, the NGEP expresses in low level in LNCaP cell line;

hence further studies need to be conducted.

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