**NEFL** is overexpressed and it modulates invasion and migration in neuroendocrine-like PC3-ML2 prostate cancer cells

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**Abstract**

Prostate cancer clinical outcomes are varied, from non-aggressive asymptomatic to lethal aggressive neuroendocrine forms which represent a critical challenge in the management of the disease. The neurofilament light (**NEFL**) is proposed to be a tumor suppressor gene. Studies have shown that expression of the gene is decreased in various cancers. We have used quantitative RT-PCR, immunoblotting, methylation specific PCR, siRNA knockdown followed by migration/invasion assays to determine associations between **NEFL** expression and disease phenotype in a panel of prostate cells. We demonstrate that **NEFL** is overexpressed and it modulates invasion and migration in PC3-ML2 prostate cancers cells which have an aggressive neuroendocrine-like phenotype.

![Figure 1. NEFL is overexpressed in prostate cancer cell lines with neuroendocrine like phenotypes and knockdown cells have reduced migration and invasion capacity.](image)

(A) Semi-quantitative mRNA expression of **NEFL** in 10 prostate cell lines. cDNA was synthesized from total mRNA extracted from 10 prostate cell lines and used for RT-PCR to evaluate the expression of **NEFL** in the cells. GAPDH was used as a control for normalization. (B) Western blot detection of NEFL protein in 10 prostate cancer cell line and GAPDH was used as a loading control. (C) Expression fold change of NEFL protein and transcript are based on normalization by individual
Prostate cancer (PCa) is the most common cancer in men in USA. For the year 2022, current estimates predict the diagnosis of 268,490 new cases and 34,500 deaths in the country (Siegel et al., 2022). Prostate cancer has diverse disease pathogenesis and clinical outcomes. Some patients develop insignificant indolent disease whereas others develop highly aggressive forms of the disease with lethal consequences. PCa pathogenesis is associated with the progression to a neuroendocrine phenotype at the late stages (Aggarwal and Small, 2014; Kanayama and Luo, 2021). This is usually lethal and most patients die in less than one year. The mechanisms that mediate aggressive disease progression have not been completely characterized and understood. Several factors including tumor suppressors and oncogenes play a central role in malignant transformation and progression to aggressive disease. The presence of inactive tumor suppressor genes (TSGs) often characterize aggressive cancers.

Neurofilaments proteins (NF) are type IV intermediate filament heteropolymers composed of light, medium, and heavy chains. These proteins are constituents of the axoskeleton and they functionally maintain the neuronal caliber. The neurofilament light (gene name NEFL Synonyms: NF68, NFL), has been proposed to be a tumor suppressor gene in different cancers. NEFL is located at chromosome 8p21 loci where mutations heterozygous and homozygous and loss of heterozygosity are observed in several cancers (Macoska et al., 1995; Vocke et al., 1996; Häggman et al., 1997; Takimoto et al., 2001; Coon et al., 2004; Burke et al., 2006; Schmidt et al., 2007). Methylation which results in the silencing and inactivation of TSGs has been reported for NEFL in different cancers, and this may play a role in cancer progression and aggressive disease (Calmon et al., 2015; Dubrowskija et al., 2014; Kang et al., 2013; Revill et al., 2013; Li et al., 2005). Neurofilament proteins are mostly abundant in most abundant in the nervous system. Functionally, NEFL protein maintains neuronal caliber and is involved in the transportation of neurotransmitters to axons and dendrites (Herrmann et al., 2009; Eriksson et al., 2009; Liem et al., 2009; Steinert et al., 1988; Friede et al., 1970). The role of NEFL gene in prostate cancer initiation and progression is still incompletely understood. Published data demonstrate that neurofilament genes are differentially expressed in multiple cancers and that the NEFL gene likely plays a role in cancer development and progression (Calmon et al., 2015; Dubrowskija et al., 2014; Kang et al., 2013; Li et al., 2012; Huang et al., 2014; Shen et al., 2016; Capasso et al., 2014; Chen et al., 2021; Fan et al., 2022). To further understand the role of NEFL in prostate cancer pathogenesis, we have determined the expression profile of NEFL gene in a panel of 10 cell lines that include a normal epithelial prostate cancer cell line and nine prostate cancer cell lines with different malignancy phenotypes. The origin and other characteristics of the cells are provided in the Table. We have used RT-PCR, Western blot, and methylation specific PCR (MSP) to determine the expression profile of NEFL in a panel of 10 prostate cell lines.

Our findings demonstrate a unique increased expression of NEFL in the highly metastatic androgen independent PC3 cell derivatives, which have a neuroendocrine-like phenotype (Figures 1A-C). These two syngeneic cell lines PC3N2 and PC3-ML2 have different tumorigenic phenotypes. Unlike PC3-ML2 cells which are highly tumorigenic in vitro and with skeletal bone metastases in vivo, the N2 cells do not migrate through matrigel-coated membrane in vitro and they do not induce skeletal metastases in SCID mice. NEFL expression is significantly upregulated (>3 fold) in the PC3-ML2 versus the non-metastatic PC3-N2 cells. Although there is an mRNA signal for the NEFL transcript for RWPE-1 cells which is a normal epithelial prostate cancer cell line (Figure 1A), the protein is translated and expressed at extremely low levels (Figure 1C), and with >5 fold upregulation in PC3-ML2 versus RWPE1-1. NEFL is not expressed in the other cell lines which include the androgen dependent LNCAP and its derivative C4-2 cell lines. In LNCAP and C4-2 cells, which have a common origin and background, the more aggressive C4-2 cells exhibit lower methylation as compared to LNCAP cells. In syngeneic PC3-N2 and
PC3-ML2 cells, the methylation status of these regions decreases with increased aggressiveness with the lowest levels of methylation observed in the aggressive PC3-ML2 which have higher invasive capacity and metastatic potential amongst the two cell lines (Figure 1D). In addition, siRNA mediated NEFL knockdown inhibits the migration capacity of PC3-ML2 Cells in a scratch/wound healing assay (Figures 1E-G). There are slight differences in the wound healing assay result between control siRNA transfection and Transfection reagent but both are significantly different from the NEFL siRNA transfection experiment.

Overall, we show that NEFL expression occurs in a subset of prostate cancer cells with neuroendocrine-like phenotype that is associated with aggressive prostate cancer and siRNA silencing of NEFL inhibits the migration and invasion potential of PC3-ML2 cells.

Methods

Cell Lines and Culture Conditions: A panel of one normal epithelial prostate cell line and nine prostate cancer cell lines were used in the study. Four cell lines RWPE-1, LNCAP, C4-2, DU-145 and MDAPCa2b were directly obtained from the American Type Culture Collection (ATCC). Three cell lines RC92A, RC77N-E and RC77T-E were obtained from Dr. John S. Rhim (USUHS) and they have been described previously (Theodore et al., 2010). Two cell lines PC3-N2 and PC3-ML2 were obtained from Dr. Stearn and their development has been described (Wang et al., 1991). The characteristics of 10 cell lines are shown and summarized in reagents.

RT-PCR Analysis and Western Blot Analysis: Total RNA was extracted from the 10 prostate cell lines using (RNasey; Qiagen), and used for cDNA synthesis using SuperScript II reverse transcriptase kit according to manufacturer’s instructions (Invitrogen). NEFL and GAPDH specific transcripts were targeted using primers designed to generate PCR products with approximately the same number of base pairs. GAPDH was used as a housekeeping gene control for normalization. The primers for NEFL were as follows: forward, 5′-ATGAGTTCCTTCAGCTACGA -3′ and reverse, 5′-TCAATCTTTCTTCAGTGC-3′, and GAPDH forward, 5′-CAGCCCTCAAGATCATCACGA-3′ and reverse, 5′-ACGTCCTTCTGGGTCGACTGGA-3′. The NEFL and GAPDH expression profiles as determined by obtained RT-PCR were further validated in the 10 cell lines using immunoblots as we have previously described (Burch et al., 2013; Burch et al 2015). The anti-NEFL antibody (sc-20012, Santa Cruz Biotechnology) was used to determine the expression levels of the protein in the 10 cell lines. GAPDH (sc-25778, Santa Cruz Biotechnology) was used as the protein loading control.

Promoter Region Methylation Analysis: DNA was isolated based on the protocol for kit P-1018, FitAmp Blood and Cultured Cell DNA Extraction Kit (Epigenetek, Inc.). DNA was eluted in TE buffer in a total volume of 30 µl and the DNA was quantified by Nanodrop spectroscopy. Bisulfite modification was performed using 100 ng of purified DNA based on the protocol for the P-1001 Methy1amp DNA Modification Kit (Epigenetek, Inc.). Methylated HeLa CpG DNA was included as a control. QPCR was performed in duplicate using 1 µl of modified DNA and gene-specific primers designed for modified DNA template. For the NEFL promoter region (Acc#: L04147.1), two genomic regions were targeted: Distal region (1319-1400) covered by primer pairs un-methylated 1 (UM1) or methylated 1 (M1) and Proximal region (1957-2047) covered by primer pairs un-methylated 2 (UM2) or methylated 2 (M2). The primer sequences are as follows: UM1 forward 5′-GTAAAAGTTATTTGTGTTTT-3′, reverse 5′-TACAACAAATATCCACCT-3′; M1 forward 5′-ACGTTAAGTTATATTGCGGT-3′, reverse 5′-TTACAAACATATCCIACGT-3′; UM2 forward 5′-TTTGGGTGTAGTTTGAATTT-3′, reverse 5′-CACAAACACACCACAAATAA-3′; M2 forward 5′-TTCCCGGTGTGTTGATTTT-3′, reverse 5′-ACGAACGCACGCGAATAA-3′. Real time quantitative PCR amplification was performed for 40 cycles of 96 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with 5 min at 95 °C for initial denaturation and 5 min at 72 °C for final elongation as described by Shen et al 2016. Beta-actin was used as an internal control to determine bisulfite conversion efficiency. Data analysis was performed using QPCR Ct values to calculate CpG methylation % of each target region using the following expression: % methylation = \{1-[2(Ct M- Ct UM) / (2(Ct M- Ct UM) +1)]\} x 100%.

Transfection and Silencing of NEFL by siRNA: The expression of the NEFL gene in PC3-ML2 cells was silenced using small interference RNA specific to the gene (sc-36048, Santa Cruz Biotechnology Inc.). Scrambled non-targeting siRNA (control siRNA-A, sc-37007, Santa Cruz Biotechnology Inc.) were used in negative control experiments. The control siRNA consists of a scrambled 20-25 non-targeting sequence negative control that do not lead to the specific degradation of any cellular message. The transfections were performed using 20 nM of the siRNA duplexes according to the manufacturer’s protocol from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The transfection reactions with NEFL and control siRNA were done on six well plates and allowed to proceed for 72 hours before further biochemical and imaging analyses. The cells were harvested and processed for Western blot analysis and for immunofluorescence detection of NEFL by confocal microscopy to validate efficient silencing of NEFL gene expression upon siRNA transfection. Another batch of transfected cells were used in
scratch/wound healing assays to determine the effects of the knockdown on the migration and invasive properties as we have previously described (Burch et al., 2013).

Confocal Microscopy Analysis: NEFL protein expression in PC3-ML2 cells was determined using confocal microscopy as we have previously described (Burch et al., 2013).

Scratch-Wound Healing Invasion Assay: The effects of NEFL silencing on the migration and invasion capacity of PC3-ML2 cells was determined using a scratch/wound healing assay as previously described (Burch et al., 2013) Transfections were performed in triplicate experiments using non-targeting and NEFL specific siRNA as described in the preceding section using 2 x 10^5 cells per well. To determine the rates of invasion, images of the scratch were taken immediately after the scratch, at 0 hours, and then after every 6 hours to a maximum of 48 hours. The migration distances in the wounds were measured, and the mean and standard deviation values in the triplicate experiments were calculated and the differences compared between the control-non-transfected, scrambled control siRNA transfected and NEFL siRNA transfected PC3-ML2 cells.

Statistical Analysis: All the experiments were performed in triplicate. Differences between experimental variables of the groups was determined using two two-tailed Student t-tests to determine differences with a p-value threshold of < 0.05 being significant. Statistical analyses were performed using SPSS (SPSS Incorporated, Chicago, USA).

| Reagents | Cell Line |
|----------|-----------|
| RWPE-1   | Immortalized (HPV) prostate epithelial cell line from a normal 54 year old Caucasian male | Non-malignant, non-metastatic, does not form tumors, androgen sensitive, expresses PSA |
| RC92A    | Telomerase-immortalized malignant cells from a 57 year old Caucasian American male | Primary adenocarcinoma |
| DU145    | Adenocarcinoma brain metastatic tumor from a 69 year old Caucasian male | Moderately metastatic, not hormone sensitive, does not express PSA |
| LNCAP    | Adenocarcinoma supraclavicular metastatic lymph node tumor from a 50 year old Caucasian male | Low metastatic potential develops tumors in mice, hormone sensitive, expresses PSA |
| C4-2     | LNCAP subline | Tumorigenic and metastatic, androgen insensitive, lower PSA expression, reduced AR expression |
| PC3-N2   | PC3 subline. PC3 is an Adenocarcinoma bone metastatic tumor from a 62 year old male | Less aggressive compared to PC3-ML2, hormone refractory, does not form metastases when injected in SCID mice |
| PC3-ML2  | PC3 subline. PC3 is an Adenocarcinoma bone metastatic tumor from a 62 year old male | More aggressive compared to PC3-N2, hormone refractory, form metastases when injected in SCID mice |
| RC77N/E  | Immortalized (HPV) non-malignant cells from a normal 62 year old African American male | Non-malignant |
| RC77T/E  | Immortalized (HPV) prostate adenocarcinoma cells from a normal 62 year old African American male | Primary adenocarcinoma |
| MDAPCa2b | Adenocarcinoma metastatic bone tumor from a 63 year old African American male | Adenocarcinoma metastatic bone tumor, hormone refractory |
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References

Aggarwal RR, Small EJ. 2014. Small-cell/neuroendocrine prostate cancer: a growing threat? Oncology (Williston Park) 28: 838-40. PubMed ID: 25323608

Burch TC, Isaac G, Booher CL, Rhim JS, Rainville P, Langridge J, Baker A, Nyalwidhe JO. 2015. Comparative Metabolomic and Lipidomic Analysis of Phenotype Stratified Prostate Cells. PLoS One 10: e0134206. PubMed ID: 26244785

Burch TC, Watson MT, Nyalwidhe JO. 2013. Variable metastatic potentials correlate with differential plectin and vimentin expression in syngeneic androgen independent prostate cancer cells. PLoS One 8: e65005. PubMed ID: 23717685

Burke B, Sebire NJ, Moss J, Hodges MD, Seckl MJ, Newlands ES, Fisher RA. 2006. Evaluation of deletions in 7q11.2 and 8p12-p21 as prognostic indicators of tumour development following molar pregnancy. Gynecol Oncol 103: 642-8. PubMed ID: 16806440

Calmon MF; Jeschke J; Zhang W; Dhir M; Siebenkäs C; Herrera A; et al.; Ahuja N. 2015. Epigenetic silencing of neurofilament genes promotes an aggressive phenotype in breast cancer. Epigenetics 10: 622-32. PubMed ID: 25985363

Capasso M; Diskin S; Cimmino F; Aciero G; Totaro F; Petrosino G; et al.; Maris JM. 2014. Common genetic variants in NEFL influence gene expression and neuroblastoma risk. Cancer Res 74: 6913-24. PubMed ID: 25312269

Chen R, Masuo K, Yogo A, Yokoyama S, Sugiyama A, Seno H, Yoshizawa A, Takaishi S. 2021. SNAIL regulates gastric carcinogenesis through CCN3 and NEFL. Carcinogenesis 42: 190-201. PubMed ID: 33313663

Coon SW, Savaer AT, Zarbo RJ, Benninger MS, Chase GA, Rybicki BA, Van Dyke DL. 2004. Prognostic implications of loss of heterozygosity at 8p21 and 9p21 in head and neck squamous cell carcinoma. Int J Cancer 111: 206-12. PubMed ID: 15197772

Dubrowinskaja N; Gebauer K; Peters I; Hennenlotter J; Abbas M; Scherer R; et al.; Serth J. 2014. Neurofilament Heavy polypeptide CpG island methylation associates with prognosis of renal cell carcinoma and prediction of antivascular endothelial growth factor therapy response. Cancer Med 3: 300-9. PubMed ID: 24464810

Eriksson JE, Dechat T, Grin B, Helfand B, Mendez M, Pallari HM, Goldman RD. 2009. Introducing intermediate filaments: from discovery to disease. J Clin Invest 119: 1763-71. PubMed ID: 19587451

Fan ZL; Yang LY; Zhang N; Feng D; Guo J; Chang C; et al.; Hao JJ. 2022. NEFL promotes invasion and migration of esophageal squamous carcinoma cells via the EGFR/AKT/S6 pathway. Yi Chuan 44: 322-334. PubMed ID: 35437240

Friede RL., Samorajski T. 1970. Axon caliber related to neurofilaments and microtubules in sciatic nerve fibers of rats and mice. Anat Rec 167: 379-87. PubMed ID: 5454590

Häggman MJ, Wojno KJ, Pearsall CP, Macoska JA. 1997. Allelic loss of 8p sequences in prostatic intraepithelial neoplasia and carcinoma. Urology 50: 643-7. PubMed ID: 9338751

Herrmann H, Strelov SV, Burkhard P, Aebi U. 2009. Intermediate filaments: primary determinants of cell architecture and plasticity. J Clin Invest 119: 1772-83. PubMed ID: 19587452

Huang Z, Zhuo Y, Shen Z, Wang Y, Wang L, Li H, Chen J, Chen W. 2014. The role of NEFL in cell growth and invasion in head and neck squamous cell carcinoma cell lines. J Oral Pathol Med 43: 191-8. PubMed ID: 23992471

Kang S, Kim B, Park SB, Jeong G, Kang HS, Liu R, Kim SJ. 2013. Stage-specific methylome screen identifies that NEFL is downregulated by promoter hypermethylation in breast cancer. Int J Oncol 43: 1659-65. PubMed ID: 24026393

Kanayama M, Luo J. 2021. Delineating the Molecular Events Underlying Development of Prostate Cancer Variants with Neuroendocrine/Small Cell Carcinoma Characteristics. Int J Mol Sci 22: . PubMed ID: 34884545

Li LC, Carroll PR, Dahlia R. 2005. Epigenetic changes in prostate cancer: implication for diagnosis and treatment. J Natl Cancer Inst 97: 103-15. PubMed ID: 15657340

Li LX, Li L, Xiao CH, Feng YM. 2012. NEFL mRNA expression level is a prognostic factor for early-stage breast cancer patients. PLoS One 7: e31146. PubMed ID: 22319610

Liem RK, Yen SH, Salomon GD, Shelanski ML. 1978. Intermediate filaments in nervous tissues. J Cell Biol 79: 637-45. PubMed ID: 83322
Macoska JA, Trybus TM, Benson PD, Sakr WA, Grignon DJ, Wojno KD, Pietruk T, Powell IJ. 1995. Evidence for three tumor suppressor gene loci on chromosome 8p in human prostate cancer. Cancer Res 55: 5390-5. PubMed ID: 7585607

Revill K; Wang T; Lachenmayer A; Kojima K; Harrington A; Li J; et al.; Powers S. 2013. Genome-wide methylation analysis and epigenetic unmasking identify tumor suppressor genes in hepatocellular carcinoma. Gastroenterology 145: 1424-35.e1-25. PubMed ID: 24012984

Shen Z; Chen B; Gan X; Hu W; Zhong G; Li H; et al.; Chen J. 2016. Methylation of neurofilament light polypeptide promoter is associated with cell invasion and metastasis in NSCLC. Biochem Biophys Res Commun 470: 627-634. PubMed ID: 26801564

Siegel RL, Miller KD, Fuchs HE, Jemal A. 2022. Cancer statistics, 2022. CA Cancer J Clin 72: 7-33. PubMed ID: 35020204

Schmidt H, Semjonow A, Csiszar K, Korsching E, Brandt B, Eltze E. 2007. [Mapping of a deletion interval on 8p21-22 in prostate cancer by gene dosage PCR]. Verh Dtsch Ges Pathol 91: 302-7. PubMed ID: 18314628

Steinert PM, Roop DR. 1988. Molecular and cellular biology of intermediate filaments. Annu Rev Biochem 57: 593-625. PubMed ID: 3052284

Takimoto Y, Shimazui T, Akaza H, Sato N, Noguchi M. 2001. Genetic heterogeneity of surgically resected prostate carcinomas and their biopsy specimens is related to their histologic differentiation. Cancer 91: 362-70. PubMed ID: 11180083

Theodore S; Sharp S; Zhou J; Turner T; Li H; Miki J; et al.; Rhim JS. 2010. Establishment and characterization of a pair of non-malignant and malignant tumor derived cell lines from an African American prostate cancer patient. Int J Oncol 37: 1477-82. PubMed ID: 21042716

Vocke CD; Pozzatti RO; Bostwick DG; Florence CD; Jennings SB; Strup SE; et al.; Linehan WM. 1996. Analysis of 99 microdissected prostate carcinomas reveals a high frequency of allelic loss on chromosome 8p12-21. Cancer Res 56: 2411-6. PubMed ID: 8625320

Wang M, Stearns ME. 1991. Isolation and characterization of PC-3 human prostatic tumor sublines which preferentially metastasize to select organs in S.C.I.D. mice. Differentiation 48: 115-25. PubMed ID: 1773917

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