Nuclear vs Cytoplasmic localization of Filamin A in Prostate Cancer: Immunohistochemical Correlation with Metastases

Supplementary Materials

Immunohistochemistry: Sections were heated at 60°C, then cleared and dehydrated in xylene and graded alcohols. Antigen retrieval was performed with 0.01M/L sodium citrate buffer at pH 6.0 for 10 minutes in a 121°C pressure chamber. Slides were allowed to cool for another 30 minutes followed by sequential rinsing in TBS-T[50mmol/L Tris-HCL (pH 7.4) 150mmol/L NaCl, Tween 20(0.1%)Endogenous peroxidase activity was quenched by incubation in TBS-T containing 3% hydrogen peroxide. Each incubation step was carried out at room temperature (22 to 25°) and followed by three sequential washes in TBS-T. The tissues were then incubated for 20 min. in TBS-T containing 10% bovine serum albumin (BSA)to block non-specific proteins. Sections were incubated in the primary antibody at a 1:25 dilution in TBS-T containing 1% bovine serum albumin for 2 hours at room temperature followed by incubations with biotinylated secondary antibody for 15 min, peroxidase- labeled streptavidin for 15 min (LSAB2 System, DAKO Cytomation Corp) and diaminobenzidine-substrate for peroxidase-based immunohistochemistry (DAKO Cytomation). Slides were counterstained with hematoxilyn (DAKO Cytomation) dehydrated and mounted with a xylene based medium.

Protein Extraction from Frozen Tissue: The tissues were flash frozen in liquid nitrogen and stored in liquid nitrogen. At least 25 µg of tissue was used. The frozen tissue was powdered on dry ice and placed in the following buffer: 50 mM Tris, pH 7.5, 120 mM NaCl, 0.5% Nonidet p-40, 1µM calpeptin, 1 mM EGTA and a protease inhibitor cocktail (1/100 dilution) (Sigma, St. Louis, MO.). Tissue was homogenized at 4ºC prior to lysing. Cells were lysed for 1 h on ice, centrifuged for 10 min. at 4ºC in a microfuge. Supernatants were removed and quantitated using a Bradford assay. The lysates were aliquoted and stored at -80ºC.

In Vitro Assays:

Motility/Migration Assay. Falcon 24 well cell culture inserts were used (BD Biosciences Durham NC) The inserts have a polyethylene terephthalate track-etched (PET) membrane with pores 8.0 micron in size. Under sterile conditions 0.5 ml of RPMI 1640 medium with 5%FBS serum were added to the wells as chemoattractant. The inserts were placed in the wells, and cells suspended in RPMI 1640 without serum were seeded at $3X10^4$/insert to a total volume of 0.5 ml, then incubated at 37ºC in 5% CO_2 atmosphere for 48 hr. The cells were fixed with 0.5 ml/well of 75% ethanol for 15 minutes, washed with PBS and stained with 0.5 ml/well of Crystal violet for 15 minutes then further washed with PBS and ddH_2O. The cells on top of the PET membrane were then removed with a q-tip dipped in PBS. **Quantification.** Cells were counted under the microscope at 40X magnifications at an average of 3 individual fields, and the average/membrane obtained by mean calculations.

Invasion Assay Cell invasion ability was measured by QCM ECMatrix Cell Invasion Assay kit (Chemicon, Inc Temecula, CA, USA). 300 µl media was added to the interior of the inserts to re-hydrate the ECM layer which has a polyethylene terephthalate track-etched (PET) membrane with pores 8.0 micron in size for 30 min. After removing the media from the inserts without disturbing the membrane, 500 µl of media was added to the lower chamber. 300 µl of prepared cell suspension containing 50,000 cells were plated on the invasion chamber. Cells were incubated at 37ºC in 5% CO_2 atmosphere for 48 hours and fixed with 0.5 ml/well of 75% Ethanol

for 15 minutes, washed with PBS and stained with cell stain provided from the kit for 15 minutes and washed with H_2O. The cells on top of the PET were removed with a cotton-tipped swab dipped in PBS, invasive cells stained on the lower surface of the membrane were and quantified under the microscope at 40X magnifications.

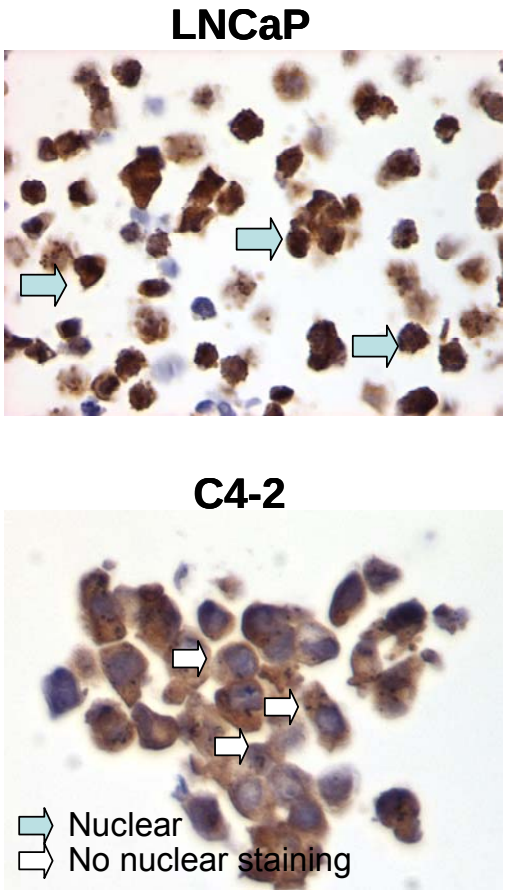
Subcellular fractionation: Cells were separated into cytoplasmic and nuclear fractions as described earlier (1): the plates were washed with PBS twice and the cells collected in 0.5 ml of Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT) containing 200 µl of 10% IGEPAL and protease inhibitors (0.1 mM benzamidine, 1 mM PMSF, 10 µg/ml each phenathroline, leupeptin, aprotinin, and pepstatin A). Following 10 min incubation at room temperature, the lysate was transferred to ice and centrifuged at 4°C at 1500 rpm in a benchtop refrigerated centrifuge (Eppendorf 5417R) for 5 min, and the supernatant collected as the cytosolic fraction. The pellet containing the nuclei was resuspended in 150 ml of Buffer B (20 mM HEPES, pH 7.9, 0.4M NaCl, 1 mM EDTA, 10% Glycerol) containing the same protease inhibitors and solubilized by vigorous shaking at 4°C for 2h. The suspension was then centrifuged at 4°C as before for 5 min and the supernatant collected as the nuclear fraction.

***AR transcriptional activity*:** AR activity was determined by luciferase assay as previously described (1). LNCaP and/or C4-2 cells were transfected with 2 µg of hPSA-luc using Lipofectamine PLUS (Invitrogen, Grand Island, NY) according to the manufacturer's recommendations. After 24 h, cells were trypsinized, and 100,000 cells plated in 6-well tissue culture plate. Cells grown in phenol-free medium containing charcoal-stripped serum for 24 h were treated as required for an additional 24 h. Cells were harvested, and cell lysates prepared for performing luciferase assays using a luciferase enzyme assay system (Promega Corp., Madison, WI). Each transfection experiment was performed in duplicate or triplicate on at least three separate occasions. Results represent an average of independent experiments with data presented as relative luciferase activity using means of untreated controls as standards.

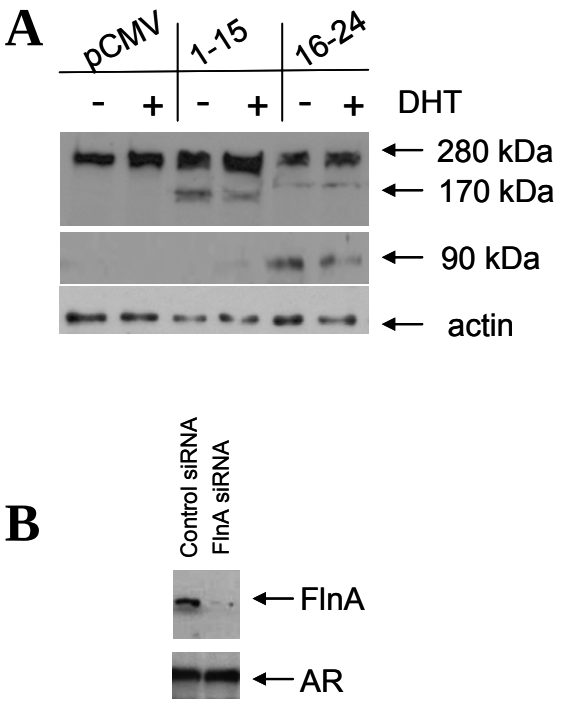
***Flow cytometry*:** The fraction of cells in S-phase was determined by flow cytometry in propidium iodide stained ethanol-fixed cells. Cells were grown under desired conditions in 100 mm dishes at 0.5 X 10^6 cells /dish as described previously (2). Flow cytometry was conducted on FACStar Plus (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cells were illuminated with 200 mW of 488 nm light produced by an argon-ion laser. Fluorescence was read through a 630/22 nm band-pass filter (for propidium iodide) or a 530/30 nm band-pass filter (for Annexin V-FITC). Data was collected on 20,000 cells as determined by forward and right angle light scatter and stored as frequency histograms; data used for cell cycle analysis was further analyzed using MODFIT (Verity software, Topsham, ME). Experiments were repeated three times, unless otherwise specified. Proliferation rates are normalized to the percentage of cells in the S-phase for the control population, which was considered 100.

SUPPLEMENTARY REFERENCES:

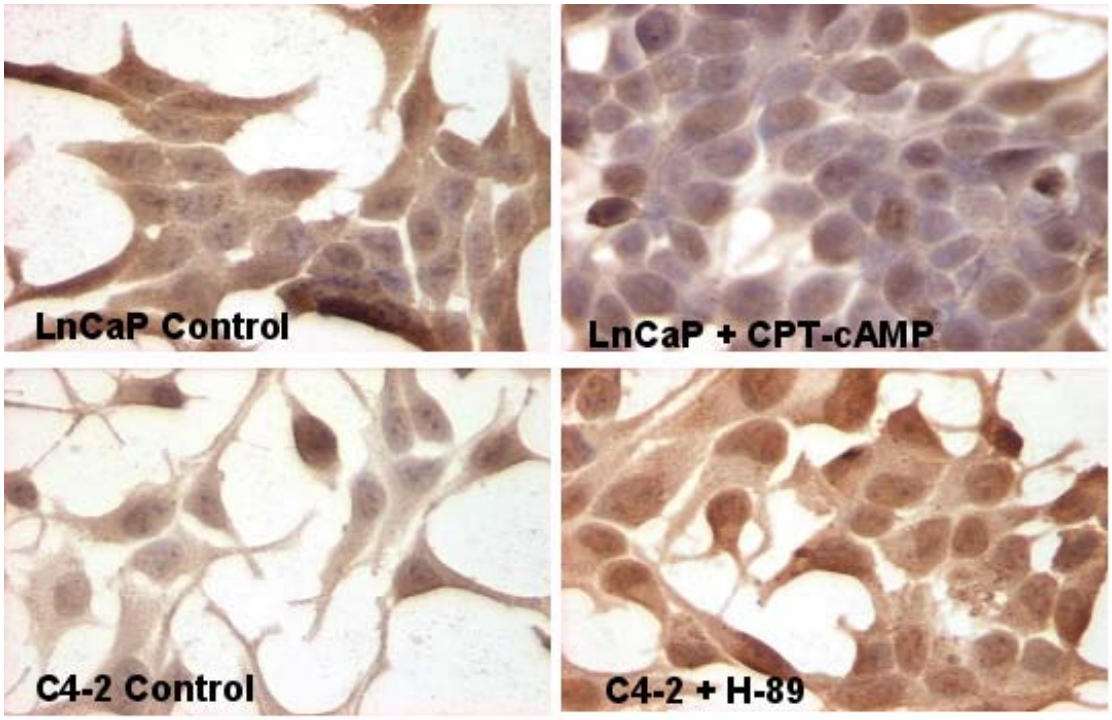
1. Wang Y, Kreisberg JI, Bedolla RG, Mikhailova M, deVere White RW, Ghosh PM. A 90 kDa fragment of filamin A promotes Casodex-induced growth inhibition in Casodex-resistant androgen receptor positive C4-2 prostate cancer cells. Oncogene 2007;26: 6061-70.
2. Ghosh PM, Bedolla R, Mikhailova M, Kreisberg JI. RhoA-dependent murine prostate cancer cell proliferation and apoptosis: role of protein kinase Czeta. Cancer Res 2002;62: 2630-6.



Supplementary Figure 1. Immunoperoxidase staining with anti-FlnA (C-terminal) antibody in LNCaP, and C4-2 cells. The C-terminal antibody detects both the 280 kDa and the 90 kDa proteins. Note that LNCaP cells display staining in both nuclei and in cell membranes (brown reaction product) with the C-terminal antibody. C4-2 cells show FlnA staining on cell membranes but not nuclei with both antibodies. Counter stain was with hematoxylin (blue). 40X original magnification.



Supplementary Figure 2. (A) Expression of an empty vector, FlnA(1-15) and FlnA (16-24). (B) Expression of FlnA siRNA downregulated total FlnA expression.



Supplementary Figure 3. Immunocytochemistry to examine nuclear localization of C-terminal FlnA in the presence of H-89 or CPT-cAMP. Increased cAMP levels prevented nuclear localization of FlnA in LNCaP cells, whereas H-89 induced FlnA nuclear localization in C4-2 cells.