Semaphorin 4F as a Critical Regulator of Neuroepithelial Interactions and a Biomarker of Aggressive Prostate Cancer

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Abstract

Background: Semaphorin 4F (S4F) has roles in embryologic axon guidance and is expressed in adults. S4F is involved in cancer-induced neurogenesis.

Methods: Prostate cells were transfected with S4F retrovirus. Cells and controls were used for a bromodeoxyuridine (BrdUrd) incorporation assay (proliferation) and in vitro scratch and Matrigel Transwell chamber invasion assay (migration). Monoclonal antibodies were developed using baculovirus-expressed recombinant GST-S4F and used to immunostain tissue microarrays. Slides were imaged using deconvolution and analyzed using tissue segmentation. Data were correlated with clinicopathologic parameters, other biomarkers and survival analysis conducted. Heterogeneity of S4F expression was analyzed with unsupervised clustering algorithms.

Results: Proliferation rates measured by BrdUrd incorporation were higher in all S4F-transfected cells. S4F overexpression was associated with increased motility of the cancer cells. S4F expression was overexpressed in high-grade prostatic intraepithelial neoplasia/prostate cancer than normal epithelium. S4F expression correlated with seminal vesicle invasion. Patients with high values of S4F in prostate cancer cytoplasm are at significantly higher risk of biochemical recurrence, by univariate and multivariate analyses. S4F cytoplasmic expression in prostate cancer cells also correlates with nerve density in prostate cancer and perineural invasion diameter. Correlations were identified with NF-kB and inversely with apoptosis in perineural invasion.

Conclusion: These data show that S4F is significantly involved in human prostate cancer progression. S4F is a key regulator of the interactions between nerves in the tumor microenvironment and cancer cells. Because of the importance of cancer nerve interaction in the biology of cancer and its clinical implication, S4F can be considered a major therapeutic target.

Introduction

Nerves and cancer cells interact at many levels. Invasion of the nerve sheath by cancer cells, termed perineural invasion, is a key feature of human prostate cancer. Perineural invasion is the process by which cancer cells wrap around nerves and the best described interaction between cancer and nerves. Perineural invasion is also a key route for prostate cancer metastasis. Our perineural invasion model (1) showed specific interactions between prostate cancer cells and nerves, which lead to costimulation of growth with reduced rate of apoptosis and an increased rate of proliferation through caveolin 1 and NF-kB–based mechanisms (2, 3). This phenomenon was validated in human tissues.

We have also recently described a novel biologic phenomenon, cancer-related axonogenesis, and neurogenesis (4). Our studies show that axon density is increased in cancer areas as well as in preneoplastic lesions compared with controls. Two- and 3-dimensional reconstructions of entire prostates confirmed axonogenesis in human tumors. Finally, 2 in vitro models confirmed that cancer cells, particularly when interacting with nerves in perineural invasion, induce neurite outgrowth in prostate cancer. Axonogenesis is correlated with features of aggressive prostate cancer and with recurrence in prostate cancer. In addition, the number of neurons in the ganglia of patients with cancer was significantly higher than in controls. This was the first description of cancer-related axonogenesis and neurogenesis (4). Accordingly, it is becoming more apparent that the biology regulated by nerves in cancer tissues is critical for the...
Translational Relevance

Interaction between nerves and cancer is key to cancer progression. Our studies have shown that perineural invasion delivers pro-growth conditions resulting in cancer cell decreased apoptosis and increased proliferation. Prostate cancer also induces axonogenesis/neurogenesis, and this process is regulated in part by semaphorin 4F (S4F). In this article, we show that S4F is the key critical regulator for neuroepithelial interactions, from cancer-induced neurogenesis to perineural invasion. S4F induces cancer growth and migration. Our data also indicate that S4F regulatory biology also is present in human samples and predictive of progression. As such, targeting the critical regulator of the interaction that defines prostate cancer aggressiveness is key to detaining prostate cancer progression.

Materials and Methods

Generation of S4F retrovirus

S4F was cloned as described previously (4). The retroviral expression system was developed in Dr. Garry Nolan’s laboratory. Retroviral vector pBMN-I-GFP was purchased from Addgene, and retroviral packaging cell line Phoenix-A was obtained from ATCC Safe Deposit. To generate pBMN-I-GFP-4F, S4F cDNA was first inserted into pBMN-I-GFP EcoRI site, then a hemagglutinin (HA) tag with N-terminal S4F cDNA obtained by real time polymerase chain reaction (RT-PCR) was inserted into BamHI site (S4F N-terminal has a BamHI site); Forward primer: 5’-CCGGAATCCATGTTACCCGATACGGCCTCCCCAGATCCGCTCCTCCAAAAGATCCGCGCCCTCG (contain an BamHI site); reverse primer: 5’-CCAAATCAAAGTGTGTGG. Max Efficiency stbl2 Competent cells (Invitrogen) was used for produce pBMN-I-GFP-4F plasmid. pBMN-I-GFP-4F was then transduced into Phoenix-A cells by Calcium Phosphate Transfection kit (Invitrogen) according to Dr. Nolan’s laboratory protocol (http://www.stanford.edu/group/nolan/). Retrovirus containing media was harvested posttransfection 48 h and 72 hours, filtered with 0.45-μm filter, and stored in –80°C.

Retroviral infection and expression of S4F

To study the autocrine effects of S4F on the prostate cancer cells, we transduced S4F into Du145, PC-3, LnCaP, and PNT1A cells. These were retrovirally transduced using an expression system developed in Dr. Garry Nolan’s laboratory. Du145 and LnCaP cells were planted in T25 flask at a density of 30% to 50%. The next day, cell growth media were removed, 1 mL viral supernatant was mixed with 1 mL fresh cell growth media contain 8 μg/mL polybrene (final concentration) and added to cells. Three hours later, cells were washed with PBS, and 5 mL fresh growth media were added to cells. After 48 hours, cells were separated for scratch, proliferation, and invasion assays. S4F expression was confirmed by Western blotting (LnCaP cells) using anti-HA antibody (Abcam) or quantitative PCR (Du145 and LnCaP).

Proliferation assay

Du145, PC-3, LnCaP, and PNT1A cells with or without virus infection were seeded in 4-well chamber slide (Lab-Tek II, Fisher Sci.) at a density of 2 × 10⁴ to 3 × 10⁴, 1.5 × 10⁴ to 3 × 10⁴, and 2 × 10⁴ to 3 × 10⁴cells per well. Three days later, cell proliferation was assessed by the bromodeoxyuridine (BrdUrd) incorporation assay (Roche), which was conducted according to manufacturer’s instruction.

In vitro scratch assay

Du145 and PC-3 cells were infected with S4F or vector retrovirus, then Du145-V, Du145-4F, or PC3-V and PC3-4F cells were seeded in 60-mm plates. After cells were confluent, a “scratch” was created by using a p200 pipette tip. To remove the debris and smooth the edge of the scratch, cells were washed once with PBS and replaced with fresh growth medium. To obtain the same field during the image acquisition, markings were made as reference points close to the scratch. Photos were taken at 0 and 24 hours (Du145-V, Du145-4F) or 0, 24 and 48 hours (PC3-V, PC3-4F) under microscope.

Invasion assay

Du145, PC-3, LnCaP, and PNT1A cells with or without virus infection were resuspended in serum-free media and seeded into the upper Matrigel chamber of a Transwell system (Fisher Sci.) at a density of 1.5 × 10⁴ to 3 × 10⁴ cells per well. The bottom wells were filled with complete media containing 30 μg/mL of laminin. After incubating for 72 hours, the cells that had invaded the Matrigel membrane were fixed with 4% formaldehyde and stained with 4’,6 diamidino-2-phenylindole (DAPI). The cells were then counted under a fluorescence microscope.
**Generation of monoclonal S4F antibodies**

The monoclonal S4F antibodies 986 and 988 and the baculovirus-expressed recombinant GST-S4F ex protein were generated in the Dan L. Duncan Cancer Center Baculovirus/Monoclonal Antibody Shared Resource at Baylor College of Medicine (Houston, TX). We assume that Sema4F and Sema-W (an isoform of Sema-4F) have the same transmembrane domain (5), then the extracellular domain of S4F (S4F-ex) DNA was obtained by PCR: forward primer: 5′-CGGAATTCAGATGCCGCCCCCTGC (contain a EcoRI site); reverse primer: 5′-CGGAATTCTCACGGCGCTGGCCTGCC (contain a EcoRI site). The S4F-ex cDNA was inserted into the pAcSecG2T (BD Biosciences) baculovirus transfer vector (pAcSecG2T-S4F ex), and then pAcSecG2T-S4F ex and BD BaculoGold Linearized Baculovirus DNA (BD Biosciences) were cotransfected into Sf9-infected cells to obtain the recombinant baculovirus-expressing GST-S4F ex. Recombinant GST-S4F ex protein was produced by infecting high five cells with the recombinant baculovirus at a multiplicity of infection (MOI) of 1.0 for 48 hours. The purified GST-S4F ex fusion protein was used to immunize BALB/c mice, and the fusion was conducted with spleen cells from one mouse and the FOX-NY mouse myeloma to produce the S4F hybridomas/monoclonal antibodies. The monoclonal S4F antibodies 986 and 988 were produced as culture supernatants in spinner vessels, precipitated by ammonium sulfate, and purified using a standard Protein G Sepharose purification protocol. Antibodies have been tested successfully for use in Western blotting, immunohistochemistry in paraffin-embedded tissues and as neutralizing for axonogenesis. Antibody validation was conducted. LNCaP human prostate cancer cells were infected with vector (V) or S4F lentivirus, and cell lysate was extracted for Western blot analysis using the anti-S4F monoclonal antibody produced in our laboratory. Bands were identified in the expected distribution.

**S4F in human tissues**

**Prostate cancer progression microarray.** Two mm cores of areas of normal cancer, high-grade prostatic intraepithelial neoplasia (HGPIN), and cancer were cored from a total of 100 patients. They were included in 6 blocks and will be referred to as the progression tissue microarray (TMA). This set permits the study of HGPIN markers and comparison with normal prostate and cancer. The TAMs were constructed by using a manual tissue arrayer (Beecher Instruments).

**Outcomes TMA.** For this array, whole-mount slides were reviewed and mapped. The index tumor, defined as the largest and/or highest GS was identified on the slide, and areas representative of the highest GS were circled. Areas of tumor were circled and a 2-mm core were obtained from these areas and transferred to a recipient paraffin block.

**Clinical and pathologic characteristics.** The initial cohort consisted of 1,120 patients who underwent radical prostatectomy (RP) at Baylor College of Medicine–affiliated hospitals between 1983 and 1998. We qualified 640 cases for building TAMs based on the following criteria: (i) patients did not receive preoperative treatment, (ii) patients were operated on between 1983 and 1998, and (iii) sufficient prostate cancer tissue is available for building TMA. The full cohort patient characteristics have been published before (6–8). A total of 240 patients had analyzable S4F data for this study. Thirteen patients had lymph node metastasis, 90 extracapsular extension (ECE), 27 seminal vesicle invasion (SVI), 31 positive surgical margin, 28 patients had biochemical recurrence, and 12 died of prostate cancer. Tissue recruitment was in accordance with institutional review board approval.

**Immunohistochemistry**

Immunohistochemical staining of S4F on TMA slides was conducted by using an automated immunostainer (DAKO). Briefly, sections were deparaffinized in xylene, rehydrated through decreasing concentrations of alcohol ending in PBS, subjected to steam heat in 10 mmol/L citrate buffer (pH 6.0) for 40 minutes in a vegetable steamer, and then allowed to cool off at room temperature for an additional 10 minutes. After endogenous peroxidase activity was quenched in 3% hydrogen peroxide solution in distilled water, sections were incubated with rabbit polyclonal antibody against S4F (1:40, overnight at 48°C; cat no. 9462). Sections were washed and the bound antibody was detected by using a DAKO Envision Plus kit (DAKO) with diaminobenzidine (DAB) as chromogen. Finally, sections were counterstained with hematoxylin, dehydrated, and mounted. Negative controls were sections immunostained as above, but normal rabbit serum was used instead of primary antibody.

**Image procurement and interpretation**

An automated slide scanner (Bacus Laboratories) was used to digitize all the stained prostate cancer progression TAM slides to produce an image of every dot and also inform the dot coordinates on the slide. Each image was interpreted for immunoreactivity by using a 0 to 3+ semi-quantitation scoring system for both the intensity of stain and percentage of positive cells (percent labeling frequency). For the intensity, the grading scale ranged from no detectable signal (0) to strong signal seen at low power (3); 2 corresponds to moderate signal seen at low to intermediate power, and 1 corresponds to weak signal seen only at intermediate to high power. Labeling frequency was scored as 0 (0%), 1 (1%–33%), 2 (34%–66%), or 3 (67%–100%). To represent the intensity of hot spots, the highest intensity value was used. The staining index was obtained by multiplying the score of intensity with that of percentage. In the case of nuclear and cytoplasmic expression, both nuclear and cytoplasmic staining signals were interpreted and recorded separately.

**Deconvolution imaging and Nuance**

Using our newly developed S4F antibody, we have combined deconvolution imaging (Nuance) and image segmentation technology (inForm) to quantitate biomarker expression more reliably. The Nuance multispectral deconvolution imaging system (CRI) was used to image the
outcomes TMA. The system uses an optimized high-throughput tunable filter and its spectral range is 420 to 720 nm. Each final image is composed of numerous component images that specifically target small portions of the spectrum. The final result is an image that does not look different from an image captured with a regular RGB camera but can be analyzed with greater detail because it carries significantly superior amounts of discriminating information.

The inForm advanced image analysis software permits tissue segmentation. It is based on learn-by-example automated image processing with object recognition and data analysis tools. inForm was successfully trained to find tissue types (cancer vs. stroma). Subsequently, it was used to automatically assess immunohistochemical staining levels, on a cell-by-cell and subcellular basis, for per-cell phenotyping.

**Statistical analysis**

The semiquantitative differences of S4F expression between normal, preneoplastic, and prostate cancer were compared between normal prostate and cancer specimens by using matched pair analysis (Wilcoxon rank tests). The S4F quantitative data from the outcomes cohort obtained using combined deconvolution imaging (Nuance) and image segmentation technology (inForm) required a different analysis. Data is provided on a cell per cell basis (nuclear vs. cytoplasmic), per tumor compartment, per patient. This created a statistical challenge that required new analytical methodology. The intensity span was divided into 10 intensity bins. Each bin was then populated with the percentage of cells that fell within such intensity span. Each bin was then weighted for the intensity of stain and the percentage of cells expressing the stain multiplied against the weight for each bin. Finally, a weighted average is obtained from each patient that more truthfully reflects the total protein concentration per tumor. Four values were obtained, epithelial nuclear and cytoplasmic and stromal nuclear and cytoplasmic.

For survival analysis, the endpoint was the biochemical recurrence of the cancer, defined as serum prostate-specific antigen (PSA) level higher than 0.4 ng/mL or clinical evidence of progression. Time to recurrence was defined as the interval between the date of surgery and the date of identification of biochemical recurrence. The predictive value of S4F for recurrence-free survival was evaluated using the Kaplan–Meier actuarial analysis and the log-rank test. Kaplan–Meier survival curves were constructed for patients with low and high levels of S4F expression. Actual cutoff value of S4F expression for division into high and low expression groups was selected using minimum P value method. The Cox univariate and multivariate proportional hazard models were used to determine the HRs. Cox multivariate analysis was applied to assess the prognostic value of S4F in presence of known predictors such as clinical stage, preoperative PSA (pre-PSA), ECE, SVI, margins, and Gleason grade. The HR and its 95% confidence interval (CI) were recorded for each marker. S4F expression was also tested for correlations with some other biomarkers in our database.

All analyses were conducted with statistical software (SPSS 11.0, SPSS).

**Results**

**Semaphorin effects on prostate cancer proliferation**

To study the autocrine effects of S4F on the prostate cancer cells, we overexpressed S4F using a retrovirus into Du145, PC-3, LNCaP, and PNT1A cells. Empty vectors and naïve cells were used as controls. Proliferation rates measured by BrdUrd incorporation were consistently higher in S4F-transfected cells. The percentage of BrdUrd-positive cells were increased with S4F overexpression in all 4 cell lines examined compared to naïve and empty vector controls (Du145: 33.8% ± 2.8% and Du145 ev: 32.7% ± 2.1% vs. Du145 S4F: 38.6% ± 2.8%; P < 0.05; Fig. 1A); (LNCAP: 26.2% ± 3.1% and LNCAP ev: 25.9% ± 3.1% vs. LNCAP S4F: 30.8% ± 1.5%; P < 0.05; Fig. 1B); (PC3: 45.6% ± 4.5% and PC3 ev: 44.3% ± 2.9% vs. PC3 S4F: 52.6% ± 4.9%; P < 0.05; Fig. 1C); (PNT1A: 41.8% ± 3.3% and PNT1A ev: 42.5% ± 2%; P < 0.05; Fig. 1D).

Prostate cancer proliferates at a rate of approximately 5% in human tumors, as measured by Ki67, such that any increase might be clinically significant.

**Semaphorin effects on prostate cancer migration and invasion**

Motility was tested using invasion assay, scratch assays, and the perineural invasion model. In all 3 assays, S4F overexpression was associated with increased motility of the cancer cells. In the scratch assay, S4F-transfected cells completely covered the exposed area within 24 hours with PC3 cells and 48 hours with Du145 cells (Fig. 2A) and PC3 cells (Fig. 2B), whereas empty vector controls failed to do so at the same time points. Invasion assays confirmed that cancer cell motility was also increased as the number of migrating cells increased in S4F+ cells than controls (Du145: 77% ± 2.1% and Du145 ev: 75.3% ± 6% vs. Du145 S4F: 90.7% ± 11.9%; P < 0.05; Fig. 3A); (LNCAP: 75% ± 8.2% and LNCAP ev: 77% ± 6.3% vs. LNCAP S4F: 85.7% ± 7.1%; P < 0.05; Fig. 3B); (PC3: 77.7% ± 7.3% and PC3 ev: 78.8% ± 8.7% vs. PC3 S4F: 93.2% ± 6.8%; P < 0.05; Fig. 3C); (PNT1A: 71.3% ± 8.1% and PNT1A ev: 74.7% ± 7.3% vs. PNT1A S4F: 88% ± 9.3%; P < 0.05; Fig. 3D).

The effects are consistent in all cell lines studied. This increased in migration is corroborated by human prostate cancer data (see below). Increased S4F expression in prostate cancer, either cytoplasmic or nuclear of nucleus is associated with increased SVI, a measure of expansion of prostate cancer outside of the prostate, and an ominous predictive marker for aggressive disease (S4F prostate cancer cyto rho = 0.184, P = 0.004; S4F prostate cancer nucl, rho = 0.162, P = 0.011).

We also have a preliminary indication that S4F+ cells migrated directionally toward the dorsal root ganglia than empty vector controls, indicating that S4F might be involved in formation of perineural invasion (data not presented). Therefore, S4F effects in prostate cancer might not only be related to increased axonogenesis but also...
through an autocrine loop that increases growth, migration, and perineural invasion establishment.

**S4F in human prostate cancer progression**

S4F was initially identified through gene array studies comparing cancer cells in the perineural invasion *in vitro* model with and without DRG/nerves. Using visual semi-quantitation in the progression human TMA, S4F expression was localized in the cytoplasm as well as nuclei of epithelial components of normal tissue (Fig. 4A), HGPIN (Fig. 4B), and prostate cancer (Fig. 4C). On analysis, S4F was expressed more frequently in cytoplasmic location in HGPIN and prostate cancer than normal (4.17 and 4.27 vs. 0.12; *P* < 0.05; Fig. 4D). Stromal S4F expression was also identified.

**Identification of S4F in the prostate cancer and stromal compartments**

For this study, we used the progression array. After extensive testing with the deconvolution imaging method, we have established that hematoxylin and eosin gives the best discrimination of structure for image segmentation. Hence, instead of using hematoxylin as a background stain for the immunohistochemical stains, we use a combination of hematoxylin and eosin. The resulting image is difficult to interpret with the naked eye as can be seen in Fig. 5A. The inForm software was then trained to recognize and distinguish stroma and cancerous epithelium Figure 5B. shows the segmentation of the prostate cancer from the stroma. Every nucleus within each compartment is then recognized and the biomarker quantified within each cell and subcellular compartment (nuclear vs. cytoplasm) Figure 5C. shows each individual nuclei in the prostate cancer, circled in green, surrounded by their individual cytoplasm Figure 5D. shows the same for the stromal component. S4F expression levels for individual cell data per compartment were then quantified and used for subsequent correlation and survival studies.

**Heterogeneity of S4F expression in human prostate cancer**

The progression TMA cohort was stained, imaged, and analyzed using image deconvolution (Nuance) and segmentation (inForm). This permits separation of compartments within the tumor (prostate cancer vs. stroma). Individual cells are then recognized within the prostate cancer and stroma and analyzed for nuclear versus cytoplasmic stain. Data are provided on a cell per cell basis. S4F was expressed more frequently in prostate cancer than stromal cells (median values; prostate cancer cyto 2.68 vs. stroma cyto 0.96; prostate cancer nucl 2.67 vs. stroma nucl 1.00).
To better visualize and understand the heterogeneity of expression of this biomarker within patients, we used an unsupervised clustering algorithm. Figure 6 shows the unsupervised clustering of S4F prostate cancer cytoplasmic expression (Fig. 6A), nuclear expression (Fig. 6B), and stromal cytoplasmic expression (Fig. 6C).

Each patient corresponds to a row, and the expression bins are columns. Color coding shows the percentage of cells present in each expression bin, with blue representing low percentage of expressing cells in the bin and orange high percentage of expressing cells. Results suggest that cell clusters with high S4F expressing in the cytoplasm is a major discriminator (Fig. 6A), corroborated by the survival data to follow. In contrast, the major clustering factor for nuclear expression of S4F in prostate cancer cells is the presence or absence of expression (Fig. 6B). This is an entirely new way of understanding biomarker expression in human cancer and will permit exploration of the value of heterogeneity in expression. New tools are needed to fully understand the biologic and predictive information obtained within this type of data.

Correlations

The expression levels in the cytoplasm and nucleus of prostate cancer cells were not correlated to each other. The same is true for the nuclear and cytoplasmic expression in the stromal cells. However, a correlation was identified between the cytoplasmic expression in prostate cancer and nuclear expression in stromal cells ($\rho = 0.644; P < 0.001$). S4F cytoplasmic expression in prostate cancer cells also correlates with nerve density in prostate cancer ($\rho = 0.135; P = 0.04$) and perineural invasion diameter ($\rho = 0.237; P = 0.004$). The higher the levels of expression within the cytoplasm of the prostate cancer cells, the higher the nerve density and the diameter of perineural invasion. These data corroborates involvement of this molecule in prostate cancer–induced axonogenesis and perineural invasion. While we did not identify a correlation between S4F expression and proliferation indices in prostate cancer as measured by Ki67, inverse correlations between S4F expression and apoptosis as measured by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) were identified, particularly with cells in the perineural space (S4F prostate cancer cytoplasmic rho = −0.362, $P = 0.027$; S4F prostate cancer nucleus rho = −0.334, $P = 0.043$).

S4F expression results were compared with previous biomarker data available at BCM in the same cohort of patients. Significant correlations were identified with nuclear expression of NF-kB (S4F prostate cancer cytoplasmic rho = 0.225, $P = 0.009$; S4F prostate cancer nucleus rho = 0.263, $P = 0.002$; S4F stroma nucleus rho = 0.220, $P = 0.01$). These data confirm that S4F is involved in prostate cancer–induced axonogenesis in human tissues, as well as in the development of perineural invasion and its subsequent survival advantage for the prostate cancer cells in perineural invasion. The correlations between S4F expression and NF-kB are consistent with perineural invasion mechanisms identified in prior publications (3).
S4F as a biomarker of aggressive prostate cancer

To test the predictive potential of S4F, we obtained a weighted average from each patient. This data point more accurately reflects the total protein concentration per tumor. S4F expression in the cytoplasm of prostate cancer cells is marginally significant for biochemical recurrence as a continuous variable when using the 95% CI (HR, 1.5; \( P = 0.056 \)). After extensive search for an optimal cutoff by using minimum \( P \) value method, a value of 4.35 was found to be the best for categorizing patients as high expression group (S4F \( \geq \) 4.35; 13 cases) or low expression group (S4F < 4.35; 227 cases). The difference in recurrence-free survival between patients with low level of S4F and those with high level of S4F is clearly illustrated with Kaplan–Meier survival curves (Fig. 6D). Univariate analysis showed that patients with high level of S4F had 5-fold increase in estimated risk of biochemical recurrence within follow-up time compared with those with low level of S4F (HR, 5.43; 95% CI, 2.19–13.40; \( P = 0.0002 \); Table 1).

In this model, Gleason, pre-PSA, lymph node status, and margins were also independently significant. However, ECE and SVI were dropped out of the model when assessed against S4F. Nuclear prostate cancer expression and stromal expression of S4F were not significant predictors.

These data, in aggregate, are confirmatory of the role of S4F in human disease, as a critical regulator of cancer nerve interactions and as a contributing factor in aggressive phenotypes.

Conclusion

S4F has been proposed as a molecule involved in prostate cancer–induced axonogenesis. New data presented here corroborate involvement in axonogenesis in human prostate cancer. In this article, we show novel biologic functions for S4F. We identified that S4F overexpression induces prostate cancer proliferation, migration and is possibly involved in the establishment of perineural invasion. Hence, S4F is a critical regulator of the interaction between cancer and nerves and the progression to aggressive prostate cancer. Using state-of-the-art quantitative methodology we...
corroborate the *in vitro* data in human prostate cancer and show that S4F is a potential biomarker for prostate cancer aggressive disease, measured by biochemical recurrence.

The nature of the interaction between tumor and host is a determinant of aggressive human prostate cancer. It is now accepted that prostate cancer requires the stimulatory effects of the host response to acquire an aggressive phenotype. The host response is a multicellular reaction. The most frequently component is reactive stroma, the myofibroblastic response to tumors. Angiogenesis and immune response are also accepted components of the host response. The function of nerves in the host response mechanisms been

Figure 4. Immunohistochemistry of S4F in human prostate cancer tissues. S4F in human prostate cancer progression, S4F expression was localized in the cytoplasm as well as nuclei of epithelial components of normal tissue (A), HGPIN (B), and prostate cancer (C). S4F was expressed more frequently in cytoplasmic location in HGPIN and prostate cancer than normal (D). Stromal S4F expression can also be identified.

Figure 5. Identification of S4F in the prostate cancer and stromal compartments. A cohort TMA was stained, imaged, and analyzed using image deconvolution (Nuance) and segmentation (inForm). This permits separation of compartments within the tumor (prostate cancer vs. stroma). Individual cells are then recognized within the prostate cancer and stroma and analyzed for nuclear versus cytoplasmic stain. A, S4F immunohistochemistry with DAB and hematoxylin and eosin as background stain. Image is difficult to interpret with the naked eye. B, the segmentation of the prostate cancer from the stroma. C, each individual nuclei only in the prostate cancer, circled in green, surrounded by their individual cytoplasm. D, the same exclusively for cells in the stromal component. S4F expression levels for individual cell data per compartment was then quantified and used for subsequent correlation and survival studies.
recently brought to the forefront. Our group has described a novel phenomenon, cancer-induced axonogenesis and neurogenesis. This phenomenon is regulated, at least in part, by a member of the semaphorin family, S4F.

Semaphorins are highly conserved molecules; classes III to VII are found in all mammals and are characterized by the presence of an acid N-terminal Sema domain (9). Classes IV–VII are transmembrane or membrane-anchored. The known function is embryologic axon guidance as collapsing factors and mediators of axon repulsion (10). Semaphorins also have roles in cardiovascular development and the immune response (11). M-sema H correlates with the metastatic ability of mouse tumor cell lines (12) and semaphorin E as a non-MDR drug resistance gene of human cancers (13). S4F has been shown to be involved in branch tubulogenesis in the developing breast (14).

Because of the effects of other semaphorins on cancer cells, we propose that the complex interactions between cancer cells and nerves result in elevated S4F expression by the cancer cells and that S4F overexpression by the cancer cells results not only in axonogenesis but also in elevated cancer cell proliferation, elevated cancer cell migration, and elevated perineural invasion owing to increased axon density and altered cancer cell biology.

There are several potential sources of S4F. Of note is that S4F has not been detected in neurons or nerves. S4F was originally identified in gene array studies of the perineural invasion in vitro model in cancer cells interacting with neurons than controls without neuronal input. The expression of S4F by prostate cancer cells was validated in human prostate cancer tissue.

Table 1. Univariate analysis showed that patients with high level of S4F had about 5-fold increase of risk for biochemical recurrence compared with those with low level of S4F (HR, 5.426; 95% CI, 2.19–13.40; P = 0.0002).

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<td>Univariate</td>
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<td>S4F Epi Cyto (groups)</td>
<td>5.43 (2.20–13.40)</td>
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<tr>
<td>S4F Epi Cyto (groups)</td>
<td>7.55 (2.35–24.31)</td>
<td>0.0007</td>
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NOTE: S4F was an independent indicator for biochemical recurrence. HR, 7.551; 95% CI, 2.345–24.312; P = 0.000.

Semaphorins are highly conserved molecules; classes III to VII are found in all mammals and are characterized by the presence of an acid N-terminal Sema domain (9). Classes IV–VII are transmembrane or membrane-anchored. The known function is embryologic axon guidance as collapsing factors and mediators of axon repulsion (10). Semaphorins also have roles in cardiovascular development and the immune response (11). M-sema H correlates with the metastatic ability of mouse tumor cell lines (12) and semaphorin E as a non-MDR drug resistance gene of human cancers (13). S4F has been shown to be involved in branch tubulogenesis in the developing breast (14).

Because of the effects of other semaphorins on cancer cells, we propose that the complex interactions between cancer cells and nerves result in elevated S4F expression by the cancer cells and that S4F overexpression by the cancer results not only in axonogenesis but also in elevated cancer cell proliferation, elevated cancer cell migration, and elevated perineural invasion owing to increased axon density and altered cancer cell biology.

There are several potential sources of S4F. Of note is that S4F has not been detected in neurons or nerves. S4F was originally identified in gene array studies of the perineural invasion in vitro model in cancer cells interacting with neurons than controls without neuronal input. The expression of S4F by prostate cancer cells was validated in human prostate cancer tissue.

Figure 6. A–C, unsupervised clustering of the expression of S4F in the nucleus and stroma of the cancer and stroma. Each patient corresponds to a row, and the expression bins are columns. Color coding shows the percentage of cells present in each expression bin, with blue representing low percentage of expressing cells in the bin and orange high percentage of expressing cells. S4F prostate cancer cytoplasmic expression (A), prostate cancer nuclear expression (B), and stromal cytoplasmic expression (C). D, a Kaplan–Meier survival analysis of patients with prostate cancer with high or low S4F expression. The recurrence-free survival was significant higher in patients with low level of S4F (S4F < 4.35; 227 cases) than that in those with high level of S4F (S4F ≥ 4.35; 13 cases; P = 0.0002).
tumors. S4F was also identified in gene array studies of human prostate cancer reactive stroma versus normal host stroma. This expression was also detected in human samples. Hence, S4F could act in an autocrine fashion, derived from the prostate cancer cells and on the prostate cancer cells; and/or in a paracrine fashion, from the reactive stromal cells, on the prostate cancer cells. The data presented in this study suggest that the S4F derived from the prostate cancer cells has greater biologic significance, as measured by the correlations with nerve density, perineural invasion, with SVI and inversely with apoptosis, and the survival studies.

We have also identified that S4F has effects on the proliferation and migration of non-neoplastic prostate cancer cells (PNT1A). Although we cannot corroborate these data in human tissues at this point, it suggests that S4F can be involved in the homeostasis of non-neoplastic prostate, either directly on the epithelial cells or through the maintenance of the supporting neural network. Furthermore, S4F as well as other semaphorins have been involved in ductal morphogenesis of the breast. It is possible that S4F has similar functions in prostate organogenesis. Future studies will address this issue.

Another significant aspect of this article is the use of novel and state-of-the-art technology to test the predictive potential of biomarkers. True quantitation of protein expression has remained an elusive goal. The combination of image deconvolution and image segmentation gives us the ability to study the expression of S4F, and other proteins, selectively within different compartments of the tumor, in essence dissecting the effects on the cancer cells and the host response. Our combination of novel technology and the resources available permit this discrimination. The use of extensive tissue resources with significant follow, and a database with many other quantified biological correlates, permits a better understanding of the biologic and clinical significance of S4F. In this case, we have identified that S4F has an effect on both the cancer cells and the neural microenvironment, serving as a key regulator of the interaction between cancer and nerves. We were also able to dissect how S4F compartmentalization in the nucleus or the cytoplasm of prostate cancer or stromal cells can affect the development of an aggressive phenotype of prostate cancer. Another significant advantage is the ability to study the effects of heterogeneity of expression and the significance of clusters of high expressing cells. Furthermore, the methodology used to reach these conclusions is not only state of the art, but also will form the basis for reproducibility of S4F as a biomarker in validation studies.

We conclude through the use of human tissue–derived data that S4F is overexpressed in preneoplastic and neo-

References


Semaphorin 4F as a Critical Regulator of Neuroepithelial Interactions and a Biomarker of Aggressive Prostate Cancer

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