Membranous Expression of Secreted Frizzled-Related Protein 4 Predicts for Good Prognosis in Localized Prostate Cancer and Inhibits PC3 Cellular Proliferation in Vitro

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ABSTRACT

Purpose: Activation of the Wnt-signaling pathway is implicated in aberrant cellular proliferation in a variety of cancers. Secreted frizzled-related protein 4 (sFRP4) is a secreted protein with putative inhibitory activity of the Wnt-signaling cascade through binding and sequestering Wnt ligands. Because sFRP4 mRNA is overexpressed in prostate cancers (PCs), the aim of this study was to define the pattern of sFRP4 protein expression in normal and malignant human prostate tissue and to determine whether changes in expression were associated with disease progression and prognosis, as well as to define the phenotype of sFRP4-overexpression in an in vitro model of PC.

Experimental Design: Polyclonal antibodies were raised against a COOH-terminal peptide of sFRP4, characterized and used to assess sFRP4 protein expression in benign prostate tissue and 229 patients with clinically localized PC (median follow-up 77 months, range 1–156). In vitro studies of the function of sFRP4 overexpression were performed using PC3 cells transfected with sFRP4.

Results: Benign and malignant prostate tissue demonstrated cytoplasmic sFRP4 immunoreactivity, but there was a decrease in the expression of membranous sFRP4 in PCs compared with the hyperplastic lesions (P < 0.0001). Kaplan-Meier analysis revealed that patients whose PC expressed membranous sFRP4 in >20% of cells had improved relapse-free survival compared with those with ≤20% membranous expression (P = 0.002). Moreover, membranous sFRP4 expression (P = 0.04) was an independent predictor of relapse when modeled with Gleason score (P = 0.006), pathological stage (P = 0.002), and pre-operative prostate-specific antigen levels (P = 0.004). In addition, in vitro studies demonstrated a decrease in the proliferation rate of PC3 cells transfected with sFRP4 when compared with the control PC3-empty vector cells (P < 0.0001). Decreased levels of phosphorylated glycogen synthase kinase 3β in PC3-sFRP4 cells suggested that this phenotype is mediated by the “Wnt/β-catenin” pathway.

Conclusions: These data suggest that sFRP4 expression may be prognostic for localized PC, potentially as a consequence of an inhibitory effect on PC cell proliferation.

INTRODUCTION

Prostate cancer (PC) is the most commonly diagnosed cancer and a major cause of cancer death in men in Western countries (1, 2). Assessment of prognosis is one of the most important issues in localized PC as it forms the basis of clinical decision-making as to whether or not to proceed to radical prostatectomy (RP). This has been improved in recent years by the advent of a number of preoperative models which use clinicopathological parameters to assess the risk of recurrence in patients with localized PC (3, 4). However, the conventional parameters such as clinical stage, Gleason score and serum prostate-specific antigen (PSA) levels have limitations, in particular their inability to account for differences in outcome observed between cancers with similar clinicopathological features. More accurate markers of prognosis for clinical decision-making are required, in particular, new molecular markers of outcome.

The strategy of transcript profiling has been used to characterize the gene expression profiles of clinical samples of PC in an attempt to identify genes associated with both the progression and outcome of PC (5–7). One such candidate is secreted frizzled-related protein 4 (sFRP4), a secreted inhibitor of the Wnt-signaling pathway. Luo et al. (7) demonstrated by both transcript profiling and reverse transcription PCR that sFRP4 mRNA expression alone, out of the sFRP molecules, was increased 2-fold in PCs compared with normal prostate (NP), a finding validated by our own transcript profiling data (data not shown).
The Wnt-signaling pathway mediates a variety of cellular functions including cell polarity, tissue patterning, control of cellular proliferation and development of neoplasia (8–10). This pathway is initially activated by a Wnt ligand binding to a frizzled receptor, which subsequently transduces a signal through one of at least three distinct intracellular signaling pathways: the classical “Wnt/β-catenin” pathway, the “Wnt/Ca2+/CaM” pathway and the “Wnt/polarity” pathway (10, 11). The Wnt/β-catenin pathway, in particular, has been implicated in a number of cancers, including those of breast and colon (9, 10). When the Wnt/β-catenin pathway is inactive, glycogen synthase kinase 3β (GSK3β) forms a complex with axin and adenomatous polyposis coli, which phosphorylates β-catenin marking it for degradation. Activation of the pathway leads to inhibition of GSK3β activity by phosphorylation; β-catenin accumulates in the cytoplasm and subsequently translocates into the nucleus, where it binds and activates transcription factors, such as T-cell factor and lymphoid enhancer factor, to induce expression of proliferation targets, including cyclin D1 (10, 12) and c-myc (13). More recently, the Wnt/Ca2+/CaM pathway has been associated with cellular invasion in melanoma (14). When activated, the Wnt/Ca2+/CaM pathway results in intracellular Ca2+ release and activation of the Ca2+-sensitive enzymes Ca2+-calmodulin-dependent protein kinase II and protein kinase C in a β-catenin-independent manner (15, 16). sFRP4 is one of a family of molecules known to bind both Wnt ligands (17, 18) and the ligand-binding domain of the frizzled receptor (19) via the amino-terminal frizzled domain, thereby inhibiting activation of the Wnt pathway.

Although there is limited evidence for involvement of the Wnt pathway in PC, β-catenin has been implicated in prostate carcinogenesis (20–23), in particular, through activation of the phosphatidylinositol 3-kinase/Akt pathway (24). A number of studies have also demonstrated an interaction between β-catenin and the androgen receptor (20, 21), which results in up-regulation of androgen receptor-mediated transcription in vitro (21, 22). In addition, stabilization of β-catenin in the prostate of transgenic mice induces lesions similar to human prostatic intraepithelial neoplasia (PIN), suggesting a role for β-catenin and potentially the Wnt pathway in PC development (23). Wnt 5A is up-regulated at the mRNA level in PC (25), and although allelic loss at the adenomatous polyposis coli locus was initially implicated in metastatic PC (26), subsequent studies have demonstrated a very low adenomatous polyposis coli mutation rate (27). Hence, the aim of this study was to define the pattern of sFRP4 protein expression in normal and malignant human prostate tissue and to determine whether changes in expression were associated with disease progression and prognosis, as well as to define the phenotype of sFRP4-overexpression in an in vitro model of PC.

MATERIALS AND METHODS

Patient Population. A cohort of archival formalin-fixed, paraffin-embedded specimens (n = 229) were selected from a previously described group of 732 patients (28). This subset was the group of patients with the longest follow up and available tissue blocks. All patients (mean age, 64 years; range, 44–75 years) were treated with RP for localized PC at St Vincent’s Hospital (Sydney, Australia). All surgery was performed by one of six specialist urologists. Patients who received neoadjuvant hormonal therapy were excluded from the study. In addition, paraffin-embedded tissue from five patients with pelvic lymph node metastases (LNM) and seven patients who underwent a transurethral retrograde prostatectomy for PC in the setting of established bony metastases (Stage D2) were assessed. NP tissue was obtained from four men at the time of organ donation for transplantation (mean age, 25.6 years; range, 17–33 years). Patients were followed post-operatively by their surgeons on a monthly basis until satisfactory urinary continence was obtained and then at 3-month intervals until the end of the first year, at six-monthly intervals to 5 years and yearly thereafter. Relapse was defined by the following criteria: biochemical disease progression with a serum PSA concentration ≥0.4 ng/ml increasing over a 3-month period or local recurrence on digital rectal examination confirmed by biopsy or by subsequent rise in PSA. This project was approved by the St Vincent’s Campus Research Ethics Committee (H00/088).

Tissue Microarray Construction. Using the technique described by Kononen et al. (29), we constructed medium-density tissue microarrays using tissue core biopsies of 1.0 mm or 2.0 mm. H&E stained template sections of the RP paraffin donor blocks were marked for areas of PIN, nodular hyperplasia, and PC by a histopathologist (J. G. K., C.-S. L.) according to accepted criteria (30). Each patient case was represented by a mean of three biopsies (range, two to five biopsies) of PC representative of the primary, secondary, and tertiary Gleason grades, one biopsy of PIN where present, and one biopsy of nodular hyperplasia adjacent to cancer. In total, 901 elements representing 229 patients were placed in 14 tissue microarrays (627 PC, 251 nodular hyperplasia adjacent to cancer, 23 PIN). Verification of the pathology of each tissue element was performed by a histopathologist (J. G. K.). NP, LNM, and D2 cases were assessed using conventional sections. All tissues were serially sectioned at 4 μm and mounted on Superfrost Plus adhesive slides (Lomb Scientific, Australia).

In Situ Hybridization. sFRP4 mRNA expression was assessed using in situ hybridization (ISH) in a subset of the PC cohort (n = 47) and NP tissue (n = 2). A 425-bp probe for PSA (nucleotides 819-1243) was used as a positive control for prostate tissue and a section of prostate processed with no probe as a negative control. A 425-bp probe for sFRP4 was derived from sFRP4 cDNA (nucleotides 1136–1560). ISH was performed at a hybridization temperature of 60°C using the method described previously (31). Expression was scored on an intensity scale as negative, weak, moderate, or strong.

Polyclonal Antibody Generation. Two sheep were immunized with keyhole limpet hemocyanin-conjugated peptide (Mimotopes, Clayton, Victoria, Australia) corresponding to residues 292–304 (QEQRRTVQKKTA) of sFRP4 and immune sera harvested 3 months later (Chemicon Australia, Boronia, Victoria, Australia). Sera were affinity-purified on a resin column of the unconjugated peptide (Chemicon).

Expression of sFRP4. The cDNA for the open-reading frame of sFRP4 was cloned into the Gateway pDONR201 vector (Invitrogen, Rockville, MD) from a PC cDNA library constructed from six localized PCs (Life Technologies, Inc., Rockville, MD). After sequencing, the clone was subcloned into...
destination vectors, pDEST-ET 42 and pDEST 12.2 (Invitrogen), which produced an inducible bacterial expression vector with a COOH-terminal HIS-tag and a mammalian construct for native protein expression, respectively.

The pDEST-ET 42 construct was transformed into competent BL21 *Escherichia coli* and incubated at 37°C in Luria-Bertani media containing 50 µg/ml ampicillin and 170 µg/ml chloramphenicol until logarithmetic growth was reached. Cells were induced with 1 mM isopropyl-β-D-thiogalactoside (Progen Industries Ltd., Darra, Queensland, Australia) for 4 h at 30°C, centrifuged at 5000 rpm and lysed by sonication in PBS containing 10 µg/ml leupeptin, 10 mM phenylmethylsulfonyl fluoride, 0.2 mM DTT, and 0.6 mM NaCl. Both the soluble and the insoluble fractions were denatured using Laemmli buffer and boiled at 100°C for 3 min and 10 min, respectively.

**Development of PC3 Cells Expressing sFRP4.** PC3 cells (32) were grown in 10% FCS-supplemented RPMI 1640 medium and transfected for 48 h with pDEST12.2-sFRP4 and FuGENE (Roche, Mannheim, Germany). Control PC3 cells were transfected with the empty pDEST12.2 vector using the same protocol. The cells were selected for 12 days in 600 µg/ml Genetac (Invitrogen) and clones isolated and seeded to 96-well plates. These were expanded in RPMI 1640/10% FCS and Genetac 500 µg/ml Protein. Extracted using a lysis buffer containing 0.5% deoxycholate, 150 mM NaCl, 1% NP40, 50 mM Tris (pH 8.0), 0.1% SDS, 10% Glycerol, 5 mM EDTA, 200 mM NaF, 1 mM Na2VO4, and the following protease inhibitors: 10 µg/ml aprotonin, 10 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. To assess secretion of sFRP4 into the medium, transfected cells were incubated in serum-free RPMI 1640 medium for 24 h before harvest. Conditioned medium was subsequently collected and concentrated using YM-10 MW Centricon filters (Millipore, Bedford, MA). To assess glycosylation of sFRP4, we grew stable PC3-sFRP4 transfectants to 60% confluence and treated them for 24 h with 1 µg/ml tunicamycin (Sigma, St. Louis, MO) before harvest.

**Immunoblotting.** The specificity of the anti-sFRP4 peptide antibody was confirmed by Western blots of lysates from BL21-sFRP4 cells +/− isopropyl-β-D-thiogalactoside induction and PC3-sFRP4 samples. Samples were fractionated on a 10% polyacrylamide gel and transferred to nitrocellulose membrane. After blocking with 5% skim milk powder, Tris-buffered saline and 0.01% Tween 20, the membrane was incubated with 1:12,000 sheep anti-sFRP4 polyclonal antibody and then 1:10,000 horseradish peroxidase linked Protein G (Zymed, Carlton, CA), followed by enhanced chemiluminescence detection (Perkin-Elmer, Boston, MA). Anti-sFRP4 peptide antibody was also incubated with the unconjugated blocking peptide (Mimotopes) and used for immunoblotting. Bacterial lysates were blotted with a 1:4,000 mouse monoclonal antibody against 6xHIS (Qiagen, Hilden, Germany) and 1:2,000 horseradish peroxidase linked antimouse antibody (Zymed). Apoptosis was assessed by immunoblotting for cleavage of poly(ADP-ribose) polymerase (PARP) with 1:1,000 rabbit polyclonal anti-PARP antibody (Cell Signaling Technology, Beverly, MA). Other antibodies used to assess signal transduction were phospho(Ser9)-GSK3β (Cell Signaling Technology), GSK3β (BD Transduction Laboratories, Lexington, KY), and β-actin (Sigma).

**Immunofluorescence.** Subcellular localization of sFRP4 was assessed using the stable transfectants PC3-sFRP4 and PC3-empty vector controls. Cells were plated onto chamber slides, fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and blocked with 2% BSA in PBS/0.2% Tween 20. Staining was performed using 1:500 anti-sFRP4 antibody followed by 1:100 FITC-conjugated-antisheep antibody (Vector Laboratories, Burlingame, CA). Cells were visualized using confocal microscopy. Cell surface expression of sFRP4 was further examined by flow cytometry using live cells stained with anti-sFRP4 antibody and FITC-conjugated-antisheep antibody as described above.

**Immunohistochemistry.** sFRP4 was detected using the polyclonal antibody described above. Samples of skeletal muscle (+/− antibody) and resting ovary were included as positive and negative tissue controls, respectively (33). The presence and absence of sFRP4 mRNA in these controls were confirmed by ISH. In addition, sFRP4 antibody pre-incubated with blocking peptide was used as a negative control. Sections were dewaxed and rehydrated before unmasking in target retrieval solution (pH 8.0; DAKO, Carpinteria, CA), initially incubated with avidin and biotin block (DAKO) for 10 min each, then incubated with 1:250 anti-sFRP4 antibody for 30 min, followed by a 15-min incubation with 1:200 biotinylated rat antisheep antibody (Vector Laboratories). Chromogenic detection and counterstaining was performed as described previously (31).

Immunostaining was initially assessed by one investigator (L. G. H.) and subsequently reviewed independently by a histopathologist (J. G. K.), both of whom were blinded to patient outcome. Both cytoplasmic and membranous (plasma membrane) staining patterns were observed. Membranous immunoreactivity was scored as a percentage of the total number of cells, whereas cytoplasmic expression was assessed according to an intensity scale as absent, low, moderate, or high. Each element in the tissue microarrays was scored separately and the results averaged across all of the scores present for individual patients to determine their score for hyperplasia, PIN, and cancer. The inter-observer Spearman rank coefficients for sFRP4 expression was 0.86. All discrepancies in scoring were reviewed and a consensus reached.

**Cell Proliferation Assays.** Proliferation rate was initially assessed using the Cell Titer 96 kit (Promega) to estimate total cell number. The parental cell line, PC3, PC3-empty vector (pooled transfectants and a clonal transfectant), and PC3-sFRP4 (three clonal transfectants) were plated into 96-well plates at four initial cell-seeding densities, 1500, 2000, 3000, and 4000 cells/well (six replicates per plating density) to a total volume of 100 µl/well. On days 1–7, 20 µl/well of Cell Titer 96 assay solution was added to the plates which were subsequently incubated for 4 h at 37°C in a humidified 5% CO2 atmosphere and 490-nm absorbance measured in a microplate reader. These experiments were repeated on two occasions.

Proliferation rate was also assessed by manual counting of cells on repeated days. Clones were plated out at a density of 0.9 × 105 cells/25-cm2 flask. Cell numbers were estimated in triplicate using a hemacytometer and repeated daily until the clone became confluent. Proliferation rates were calculated using the equation: doubling time = ln 2/k, where k is the constant of the slope of the exponential growth phase.
The rates of DNA synthesis were assayed by measuring the incorporation of 5'-bromo-2'-deoxyuridine (BrdUrd; BD Pharmingen, San Diego, CA). Cells were incubated with 10 μM BrdUrd for 4 h, harvested, and fixed in repeated washes of 3:1 methanol/ acetic acid. After treating with 1.5 M HCl, cells were incubated for 1 h with 12.5 ng/μL FITC-conjugated mouse anti-BrdUrd monoclonal antibody (Chemicon, CA). BrdUrd incorporation was assessed by flow cytometry.

**Statistical Analysis.** Disease-specific relapse was measured from the date of RP to the date of relapse or last follow-up. Kaplan-Meier and log-rank analyses evaluating disease relapse were performed on the raw membranous sFRP4 scores in a stepwise fashion (i.e., using a cutoff of 5%, then 10%, up to 95%). Assessment of these results revealed the natural split in the data. Further survival analysis was performed using univariate and multivariate analyses in a Cox proportional hazards model for sFRP4 status (as a continuous or a dichotomized variable) and other clinical and pathological predictors of outcome as previously described (28, 31). The multivariate model was produced by assessing sFRP4 status with other baseline covariates of clinical relevance, such as Gleason grade, pathological stage, and preoperative PSA, which were modeled as dichotomous or continuous variables as appropriate. The associations between sFRP4 expression and discrete categorical variables were tested using the \( \chi^2 \) test. Differences in doubling time between different cell lines/clones were analyzed using an unpaired \( t \) test. A value of \( p < 0.05 \) was required for significance. All reported \( p \) values were two-sided. All statistical analyses were performed using Statview 4.5 software (Abacus Systems, Berkeley, CA).

**RESULTS**

**ISH.** To confirm that sFRP4 mRNA is up-regulated in PC compared with NP, we examined sFRP4 mRNA expression by ISH in a cohort of RP specimens and NP tissue. Expression was observed exclusively in the epithelium of prostate tissue. Both NPs expressed sFRP4 mRNA weakly (Fig. 1A), whereas 81% (38 of 47) of PCs were positive. Moreover, of the 38 PC cases that were positive, 74% demonstrated a moderate-to-strong expression of sFRP4 (Fig. 1B). In addition, the majority of hyperplastic lesions adjacent to cancer (33 of 45) demonstrated sFRP4 mRNA expression.

**In Vitro Expression of sFRP4.** The polyclonal anti-sFRP4 antibody generated in sheep was characterized by assessing its ability to detect recombinant sFRP4 protein. A 6xHis and anti-sFRP4 reactive band of \(~45\) kDa, corresponding to the predicted size of the sFRP4-HIS fusion protein after isopropyl-β-D-thiogalactoside induction in sFRP4 transformed BL21 cells, is shown in Fig. 2A. PC3 cells transfected with a native sFRP4 construct expressed sFRP4 in the conditioned medium, lysate, and insoluble fraction, whereas there was no endogenous sFRP4 expression in PC3-empty vector controls (Fig. 2B). In addition, the peptide used to raise the antibody was able to block the ability of the anti-sFRP4 antibody to detect recombinant sFRP4 in PC3 cells (Fig. 2C).

Further *in vitro* studies of PC3-sFRP4 stable transfectants and the corresponding PC3-empty vector controls were performed to better characterize the molecule. Immunofluorescence studies localized sFRP4 expression to the cytoplasm of transfected cells (Fig. 2, D and E), with minimal expression at the cell surface (data not shown). Interestingly, PC3-sFRP4 cells were slightly larger and more epithelioid in appearance compared with the PC3-empty vector controls, which appeared more mesenchymal in morphology (Fig. 2, F and G). Treatment of PC3-sFRP4 cells with tunicamycin resulted in a shift of the sFRP4 reactive band to 40 kDa, the predicted size of the protein based on the predicted amino acid sequence (Fig. 2H). This provided evidence for posttranslational glycosylation of sFRP4. Taken together, these data suggest that our polyclonal antibody is specific for sFRP4 and that sFRP4 is glycosylated before secretion into the conditioned medium.

**Immunohistochemistry.** To assess the association of sFRP4 with PC progression and the potential utility of sFRP4 in predicting outcome in localized PC, expression of sFRP4 was assessed in benign tissue, a well-defined cohort of patients after RP, and a group of patients with metastatic disease. Assessment of the large RP cohort was facilitated by the use of tissue microarray technology (Fig. 3, A and B). Both cytoplasmic and membranous morphological patterns of sFRP4 staining were observed. Membranous immunoreactivity is a pattern of immu-
nohistochemistry protein expression that occurs for secreted extracellular molecules as well as membrane-bound proteins, but it is beyond the sensitivity of immunohistochemistry to detect which. The physiological correlates of the expression pattern must be established by more sensitive in vitro systems, which, in this case, demonstrated that sFRP4 was secreted but not membrane-bound.

Expression of sFRP4 in NP Tissue. All four of the NP specimens expressed sFRP4 in the epithelial compartment of the tissue. Expression was demonstrated exclusively in the cytoplasm, with no expression at the epithelial cell membrane (Fig. 3C). The stroma was negative for sFRP4 expression.

Expression of sFRP4 in Prostatic Hyperplasia. Of the 229 PC cases, 204 had adjacent hyperplastic lesions that were assessed for sFRP4 expression. In keeping with the NP specimens, all cases demonstrated cytoplasmic expression, but the majority of hyperplastic lesions also had membranous expression (Fig. 3D). In fact, 93% (190 of 204) of the hyperplastic lesions had ≥80% of cells demonstrating membranous expression of sFRP4 (Fig. 4A).

Expression of sFRP4 in Localized PC. sFRP4 expression was assessed in a cohort of 229 patients treated with RP for localized PC. The majority (77%) were treated with surgery alone, whereas the remaining received a variety of adjuvant therapies, including 35 who were treated with hormones alone, 11 who received radiotherapy alone, 6 who received hormones/radiotherapy, and 2 who received orchietomy. Pathological assessment of the RP specimens revealed 48% (109 of 229) had organ-confined tumors, 19% (43 of 229) had seminal vesicle involvement, 1% (3 of 229) had pelvic LNM and 54% (123 of 229) had surgical margins involved with tumor. The median Gleason score was 6 (range, 4–10), whereas the median preoperative PSA concentration was 10.2 ng/ml (n = 228; range 1–182 ng/ml). At a median follow-up of 77 months (range, 1–156 months), 67 of 229 (29%) had relapsed. The median time to relapse was 56 months (range, 0.2–127 months). Only four patients (1.7%) died of PC during the study period.

All PC cases expressed sFRP4 protein in the cytoplasm, but there was considerable variation in the expression of sFRP4 at the membrane (Figs. 3, E, and F, and 4B). Patients whose cancers had ≥20% of cells expressing membranous sFRP4 had a decreased biochemical relapse-free survival (median, 45 months; range, 1–127 months), as assessed by Kaplan-Meier survival analysis (Fig. 4C), compared with those who had <20% malignant cells demonstrating sFRP4 membranous expression (median relapse-free survival, 65 months; range, 0.2–120 months; P = 0.002). Although the survival curves crossed at ≥8 years, this difference in relapse-free survival was both statistically and clinically significant. Despite the two groups

Fig. 2  Secreted frizzled-related protein 4 (sFRP4) expression in Escherichia coli and PC3 prostate cancer cells. Panel A, the insoluble fraction of BL21 cells (+/− isopropyl-β-D-thiogalactoside (IPTG) induction) expressing an sFRP4-HIS fusion protein immunblotted with an 6xHIS antibody and anti-sFRP4 antibody. Panel B, lysate (L), insoluble fraction (I), and concentrated conditioned medium (C) from PC3 cells overexpressing sFRP4 and PC3-empty vector controls immunblotted with anti-sFRP4 antibody. Panel C, lysate from sFRP4-PC3 clones 13 and 19 immunblotted with anti-sFRP4 antibody and anti-β-actin antibody pre-incubated with blocking peptide. Panel D, lack of sFRP4 expression in PC3-empty vector cells compared with cytoplasmic expression of sFRP4 in PC3 cells transfected with an sFRP4 construct by immunofluorescence (panel E). F, inverted phase contrast microscopy of PC3-empty vector control cells (×100). G, inverted phase contrast microscopy of PC3-sFRP4 cells (×100). H, lysate (L) and insoluble fraction (I) from PC3-sFRP4 and PC3-empty vector cells treated with tunicamycin to assess for glycosylation of sFRP4.
having the same median follow up, 97% of relapses in the group who had ≤20% sFRP4 membranous expression occurred in the first 5 years after RP, whereas those patients who had >20% sFRP4 membranous expression not only had a decreased relapse rate (24% versus 41%), but the spread of the relapses across time was more even, with 22% of patients relapsing between 5 and 10 years after surgery. Cytoplasmic expression of sFRP4 was not related either to progression or prognosis in PC.

sFRP4 membranous expression was assessed for potential relationships with other tumor characteristics and known prognostic factors. χ² tests performed for Gleason score, surgical margin status, pathological stage, pre-operative PSA levels, seminal vesicle involvement, and adjuvant therapy demonstrated no correlation between sFRP4 expression and any of these parameters. In addition, there was no correlation between the primary Gleason grade of each cancer biopsy on the tissue microarrays and membranous sFRP4 expression. Univariate analysis using a Cox proportional hazards model demonstrated that Gleason score, pathological stage, seminal vesicle involvement, preoperative serum PSA levels, surgical margin involvement, adjuvant therapy, and sFRP4 expression were all significant predictors of PSA-relapse (Table 1). Lymph node involvement was not significant because of the small number of patients (data not shown). Multivariate analysis was performed using factors that were predictive of outcome on univariate analysis. Seminal vesicle involvement was not included in the analysis because it is incorporated into the pathological staging system. Membranous sFRP4 expression was an independent predictor of relapse (P = 0.03) when modeled with Gleason score (P = 0.002), pathological stage (P = 0.001), pre-operative PSA level (P = 0.005), and adjuvant therapy (P = 0.8; Table 1). From a biological point of view, it is interesting to note that when membranous sFRP4 expression was considered as a continuous variable in the Cox proportional hazards model, it remained an independent predictor of relapse on both a univariate (P = 0.01) and multivariate analysis (P = 0.04) when modeled with Gleason score (P = 0.0006), pathological stage (P = 0.002), pre-operative PSA level (P = 0.004), and adjuvant therapy (P = 0.7).

Expression of sFRP4 in Advanced PC. Advanced PC was assessed by examining prostate tissue from patients with D2 disease and LNM. As in the case of localized disease, the majority of cases had cytoplasmic expression of sFRP4, but there was little membranous immunoreactivity. All of the seven D2 tumors had ≤20% sFRP4 membranous immunoreactivity (mean, 3%; range, 0–10%) whereas four of five of the LNM had...
membranous expression (20% or /H11022 survival in patients treated for localized PC stratified for sFRP4 mem-

<math>\text{survival in patients treated for localized PC. (mean, 20%; range, 0–20% of cells exhibiting sFRP4 membranous expression} /H11349\text{plastia lesions adjacent to PC (n cells expressing sFRP4 at the membrane in each case of nodular hyper-}

\text{Fig. 4 Membranous secreted frizzled-related protein 4 (sFRP4) expres-

A with radical prostatectomy. A, frequency histogram of the percentage of cells expressing sFRP4 at the membrane in each case of nodular hyperplasia lesions adjacent to PC (n = 204). B, frequency histogram of the percentage of cells expressing sFRP4 at the membrane in each case of localized PC (n = 229). C, Kaplan-Meier analysis of relapse-free survival in patients treated for localized PC stratified for sFRP4 membranous expression (>20% or ≤20%; log rank, } P = 0.002). \text{C}

≤20% of cells exhibiting sFRP4 membranous expression (mean, 20%; range, 0–75%).

\text{Proliferative Changes in Cells Overexpressing sFRP4.} Proliferation assays demonstrated that PC3 cells that overexpressed sFRP4 had a decreased growth rate compared with both the parent cell line and the empty vector controls. The growth curves for PC3, PC3-empty vector (both a pool of transfectants and a clonal cell line), and three stable clones of PC3-sFRP4 are illustrated in Fig. 5A. Expression of sFRP4 decreased the plating efficiency of cells, as evidenced by the decreased cell numbers in sFRP4 transfectants at day 1. Thereafter, cells grew exponentially, although growth rates were different. The slopes of the exponential growth phase for the parent cell line and the PC3-empty vector lines were superimposable, whereas the curves for all three PC3-sFRP4 clones demonstrated different slopes, all of which were less than the controls. Moreover, BrdUrd assays done in parallel with the growth curves showed a decreased percentage of cells in S phase during the exponential growth phase in the PC3-sFRP4 clones, as well as a prolonged growth phase compared with the PC3 and PC3-empty vector (Fig. 5B). After combining data from both the Cell Titer 96 assays and the manual counting of cells, it was apparent that the PC3-sFRP4 clones had longer doubling times compared with the PC3/PC3-empty vector lines (Fig. 5C). Furthermore, when data from multiple clones were pooled, there was a significant difference in the doubling time between the PC3-sFRP4 clones (mean, 2.8 days) and the PC3/PC3-empty vector clones (mean, 1.6 days; } P < 0.0001). Apoptosis was measured by cleavage of PARP in PC3, PC3-empty vector, and PC3-sFRP4 clones at all time points; however, there was no significant difference in the amount of PARP cleavage among the three groups of clones (Fig. 6A).

\text{Changes in Phosphorylated GSK3β in PC3 Cells That Overexpress sFRP4.} Because phosphorylation of GSK3β is a downstream effect of activation of the Wnt-signaling pathway, we tested whether overexpression of sFRP4, an inhibitor of the pathway, would decrease the levels of phosphorylated GSK3β in PC3 cells. When the data were adjusted for differences in protein loading using PARP expression (Fig. 6A), the total level of GSK3β was unaffected by sFRP4 expression (Fig. 6, B and D). However, immunoblots demonstrated a significant decrease (P = 0.002) in the ratio of phosphorylated GSK3β/total GSK3β in cells that overexpressed sFRP4 compared with both parental PC3 and PC3-empty vector (Fig. 6, C–E). These effects were reproducible in replicate experiments and demonstrated an ~20% decrease in GSK3β phosphorylation in PC3 cells overexpressing sFRP4 (Fig. 6E).

DISCUSSION

This study has identified sFRP4 as a potentially new prognostic marker in localized PC, as well as implicating it in PC progression. Although originally identified as being up-regulated at the mRNA level in localized PC compared with NP, the proportion of malignant cells that secrete sFRP4 (i.e., the number of cancer cells expressing membranous pattern sFRP4) is associated with outcome. Increased expression of membranous sFRP4 to a level >20% of malignant epithelial cells predicts for a longer biochemical relapse-free survival in patients with localized PC. This is consistent with biological data from other sFRP family members suggesting they inhibit the Wnt pathway by binding extracellular Wnt ligands. Furthermore, in advanced PCs, we observed minimal membranous expression suggesting that loss of secreted sFRP4 is associated with PC progression, which is compatible with the higher growth rates often seen in the advanced tumor setting, although a potential role for sFRP4 in inhibiting invasion and metastasis cannot be excluded.

\text{In vitro studies demonstrated a decrease in the proliferation rate of PC3 cells, which overexpress sFRP4, compared with the parent cell line and the PC3-empty vector controls, which do not}
express sFRP4. Furthermore, in characterizing PC3-sFRP4 clones, it became apparent that sFRP4 was expressed in the cytoplasm of cells and then secreted into the conditioned medium. Examination of the in vivo and in vitro data together suggests that functional sFRP4 is secreted (membranous expression by immunohistochemistry), where it may act in an autocrine/paracrine fashion to inhibit activation of the Wnt-signaling pathway. This in turn would lead to a decrease in the rate of PC cell proliferation which may account for the number of late relapses in patients whose tumors had >20% cells expressing membranous sFRP4.

sFRP4 has been implicated in apoptosis (34–36), proliferation (33, 37), organ differentiation (38), and carcinogenesis (7, 33, 39). DDC-4, the rat homologue of sFRP4, was identified in involuted mammary glands, ovarian corpus luteum, and prostate, suggesting it is associated with apoptosis (34). In human tissue, sFRP4 mRNA expression is not only up-regulated in failing myocardium, but correlates with the proapoptotic Fas/Fas-antagonist ratio (35). Similarly, James et al. (36) found sFRP4 protein expression in human osteoarthritic articular cartilage coincided with the presence of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive chondrocyte nuclei, suggesting a role for sFRP4 in chondrocyte apoptosis (36). On the other hand, sFRP4 mRNA expression was up-regulated in human endometrial carcinoma compared with normal endometrium (33), and, in the decidual cells of rats, sFRP4 expression correlates with the expression of proliferating cell nuclear antigen (37), suggesting a role in cellular proliferation. Despite abundant evidence for the role of Wnt signaling in development (8–10), only one study has demonstrated sFRP4 expression in this process, i.e., in the developing murine lung (38). In addition, sFRP4 mRNA expression has been demonstrated in endometrial (33), breast (33, 39), and prostate cancers (7). Wong et al. (39) found sFRP4 mRNA was down-regulated in breast carcinoma compared with normal breast tissue in contrast to Abu-Jawdeh et al. (33), who found sFRP4 mRNA up-regulation in breast cancer stroma without any epithelial expression.

Other sFRP family members, in particular sFRP1 and sFRP2, have been implicated in carcinogenesis, although their phenotypes are distinct from each other (40–43). Initial data demonstrated that MCF-7 cells overexpressing sFRP1 were more sensitive to apoptotic stimuli (40), whereas, in another study, sFRP1 was up-regulated in starved and apoptotic cells, again suggesting expression was associated with growth inhibition (41). In keeping with these findings, loss of sFRP1 mRNA expression was associated with progression from normal breast epithelium to breast cancer (41, 43), although there was no correlation between sFRP1 expression and markers of apoptosis (43). In glioma cell lines, sFRP1 and sFRP2 expression promoted tumor cell survival under nonsupportive conditions, but inhibited the migration of glioma cells, suggesting a different role in growth as opposed to metastasis (42). However, only sFRP2 promoted tumor growth in vivo (42), and, in contrast to sFRP1, sFRP2 expression conferred resistance to apoptotic stimuli in vitro (40). It is not clear whether the differences noted among the different sFRPs reflect different specificity for the various Wnt ligands and/or frizzled receptors, which, in turn, would affect which downstream pathway is modulated by their action. Certainly, the relationship between sFRP levels and downstream changes in β-catenin has been inconsistent, suggesting that more than one downstream pathway may be involved (39–43).

The proto-oncogenic effects of the Wnts have been apparent for many years (8, 9). Initially, expression of Wnt-1, Wnt-3, and Wnt-10B induced by the mouse mammary tumor virus was found to promote carcinogenesis specifically in the mouse mammary gland (8), whereas Wnt-1 (39) and Wnt-10B (44) were overexpressed in human breast cancers. More recently, aberrant expression of Wnts and frizzled receptors have been demonstrated in diverse cancers including endometrial carcinomas (45), head and neck squamous cell carcinomas (46) and colon cancer (47). Furthermore, Wnt-1 expression was important for cell proliferation and survival in an in vitro head and neck squamous cell carcinoma model, whereas inhibition of Wnt-1 by either an anti-Wnt-1 antibody or recombinant sFRP1 decreased the proliferation rate (46). This finding is in keeping with our data showing sFRP4, an inhibitor of Wnt signaling, diminished the rate of growth of the PC cell line, PC3.

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Table 1  Cox proportional hazard analysis of membranous secreted frizzled-related protein 4 (sFRP4) expression and clinicopathological features of localized prostate cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio (95% CI)</th>
<th>P</th>
<th>Hazard Ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gleason score</td>
<td>1.6</td>
<td>&lt;0.0001</td>
<td>1.4</td>
<td>0.002</td>
</tr>
<tr>
<td>Continuous variable</td>
<td>(1.3–1.9)</td>
<td></td>
<td>(1.1–1.7)</td>
<td></td>
</tr>
<tr>
<td>Pathological stage</td>
<td>3.4</td>
<td>&lt;0.0001</td>
<td>2.5</td>
<td>0.001</td>
</tr>
<tr>
<td>gT3/T4 vs. gT2</td>
<td>(2.0–5.9)</td>
<td></td>
<td>(1.5–4.4)</td>
<td></td>
</tr>
<tr>
<td>Preoperative PSA</td>
<td>2.5</td>
<td>0.0004</td>
<td>2.1</td>
<td>0.005</td>
</tr>
<tr>
<td>≥10 vs. &lt;10 ng/ml</td>
<td>(1.5–4.1)</td>
<td></td>
<td>(1.3–3.4)</td>
<td></td>
</tr>
<tr>
<td>Surgical margins</td>
<td>1.7</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive vs. negative Adjuvant treatment</td>
<td>(1.1–2.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any vs. nil</td>
<td>2.4</td>
<td>0.0006</td>
<td>1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Seminal vesicle involvement</td>
<td>(1.5–3.9)</td>
<td></td>
<td>(0.6–1.9)</td>
<td></td>
</tr>
<tr>
<td>Positive vs. negative Membranous sFRP4 expression</td>
<td>3.8</td>
<td>&lt;0.0001</td>
<td>1.7</td>
<td>0.03</td>
</tr>
<tr>
<td>≤20% vs. &gt;20%</td>
<td>(1.3–3.4)</td>
<td></td>
<td>(1.1–2.8)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 provides a Cox proportional hazard analysis of membranous secreted frizzled-related protein 4 (sFRP4) expression and clinicopathological features of localized prostate cancer.
Our data suggest that the Wnt-signaling pathway is important in PC specifically through its effects on cellular proliferation rather than apoptosis. It appears reasonable to assume sFRP4 exerts its effect by binding to one of the Wnt ligands but apart from one study which showed an interaction between sFRP4 and Wnt-8 in a Xenopus model, there is no evidence as to which of the Wnt ligands sFRP4 might bind in mammalian systems. The decrease in phosphorylated GSK3\(^{-}\) in PC3-sFRP4 clones compared with PC3-empty vector suggests that the upstream Wnt pathway activity in PC is connected to the downstream GSK3\(^{-}\)-catenin. However, it is not clear why some PCs express sFRP4 at the membrane and some do not, although both have cytoplasmic sFRP4 expression. A possible explanation is that sFRP4 may be mutated in some tumors preventing secretion, or there may be deregulation of sFRP4 secretion through other unknown effects on protein trafficking.

The downstream targets of Wnt signaling, in particular the Wnt/\(\beta\)-catenin pathway, are important in a number of cancers including PC. Aberrations in this pathway in colon cancer are well-described with 80% of sporadic colon cancers having a mutation of the adenomatous polyposis coli gene and the remaining 20% having a high rate of \(\beta\)-catenin mutations (48). Mutations in \(\beta\)-catenin have also been found in hepatocellular carcinomas (9, 48), endometrioid ovarian cancers (9) and endometrial cancers (9), but are rare in PC (49, 50). In breast cancer, expression of nuclear wild-type \(\beta\)-catenin is an independent predictor of outcome (51). In PC, loss of \(\beta\)-catenin expression at the membrane in conjunction with E-cadherin has long been known to be associated with a poor prognosis (52), but, more recently, \(\beta\)-catenin was found to have a role in modulating the androgen receptor (20–24). Interestingly, one study found that 20% of metastatic PCs lesions examined expressed \(\beta\)-catenin in the nucleus (22).

Despite the in vitro evidence suggesting that the sFRP4 phenotype in PC is mediated by the Wnt/\(\beta\)-catenin pathway, there was no correlation between membranous sFRP4 expression and nuclear \(\beta\)-catenin expression in the localized PC cohort (data not shown). This may be related to the competing influence of both the Wnt pathway and the phosphatidylinositol 3-kinase/Akt pathway on \(\beta\)-catenin through GSK3\(^{-}\). In addition, it may be attributable to sFRP4 signaling through more than one pathway. The sFRP4-Wnt interaction may mediate signaling through both the Wnt/\(\beta\)-catenin and Wnt/Ca\(^{2+}\) pathways, as well as the ErbB1 pathway, which has recently been described in mammary epithelial cell lines (53).

Fig. 5 Changes in cellular proliferation in PC3-sFRP4 4 cells compared with parental PC3 cells and PC3-empty vector control cells. A, growth curves demonstrating different rates of exponential growth for PC3 cells/PC3-empty vector (clone and pooled transfectants) and PC3-secreted frizzled-related protein 4 (sFRP4 (PC3-sFRP4); three stable clones) as assessed by manual counting of cell numbers. The data points are representative of the mean ± SE of three experiments. Where error bars are not shown, the SE was smaller than the size of the symbol. PC3 (○), PC3-empty vector clone (□); PC3-empty vector pool (○); PC3-sFRP4 clone 13 (△); PC3-sFRP4 clone 19 (●); and PC3-sFRP4 clone 26 (●). B, frequency histogram demonstrating a decrease in the percentage of cells proliferating in the exponential growth phase for the PC3-sFRP4 clones (□) compared with PC3/PC3-empty vector (●), as assessed by BrdUrd incorporation. C, histogram demonstrating the increase in mean doubling time of the PC3-sFRP4 clones compared with PC3 and PC3-empty vector clones, as assessed by both manual counting and Cell Titer 96 proliferation assays.
because of its inhibitory effect on cellular growth, which is likely mediated, at least in part, via the Wnt/β-catenin pathway. This study provides new evidence for a potential role of Wnt signaling in prostate carcinogenesis, with consequent application as both a prognostic marker and potential therapeutic target.

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Membranous Expression of Secreted Frizzled-Related Protein 4 Predicts for Good Prognosis in Localized Prostate Cancer and Inhibits PC3 Cellular Proliferation in Vitro

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