

Src Family Kinase Activity Is Up-Regulated in Hormone-Refractory Prostate Cancer

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Abstract Purpose: Although Src family kinase (SFK) inhibitors are now in clinical trials for the treatment of androgen-independent prostate cancer (AIPC), there are no studies relating SFK activation to patient survival. This study was designed to determine if SFK activation was up-regulated with the development of AIPC and if patients could be selected who were more likely to respond to therapy.

Experimental Design: A unique cohort of matched prostate tumor samples, taken before hormone deprivation therapy and following hormone relapse, was used to determine by immunohistochemistry on an individual patient basis if SFK activity changed with progression to AIPC and whether this related to patient outcome measures. Using matched, hormone-sensitive and hormone-refractory cell lines, we determined if hormone status affected the way prostate cancer cells respond to suppression of SFK activity by the small-molecule inhibitor dasatinib.

Results: In the current study, 28% of patients with AIPC exhibited an increase in SFK activity in prostate cancer tissue, these patients had significantly shorter overall survival ($P < 0.0001$), and activated SFK expression correlated with the presence of distant metastases. Dasatinib inhibited phosphorylation of Src and Lyn and the downstream substrate FAK in hormone-sensitive and hormone-refractory cell lines. Although migration was reduced by dasatinib in both cell lines, proliferation of hormone-refractory cells only was inhibited.

Conclusion: Appropriate patient selection may allow better targeting of prostate cancer patients who are likely to respond to the treatment with SFK inhibitors at the same time improving the outcome of clinical trials.

Prostate cancer is the most common cancer in men and is the second leading cause of cancer-related deaths in men in the United States and United Kingdom (1). Treatment options for locally advanced and metastatic prostate cancer are limited to androgen deprivation therapy or surgical castration. Unfortunately, nearly all of these patients eventually develop androgen-independent prostate cancer (AIPC) for which currently there are no established effective therapies.

Our understanding of the mechanisms involved in the development of AIPC has considerably improved over the last decade. When deprived of androgen stimulation, androgen-sensitive prostate cancer (ASPC) cells develop the ability to survive and thrive by up-regulating oncogenic pathways where tyrosine kinases often play a crucial role (2). The nonreceptor tyrosine kinase Src is thought to facilitate the interaction between intracellular molecular cascades as well as form complexes with the androgen receptor (AR), which is expressed by the majority of AIPC cells. Tyrosine phosphorylation is an important factor in the regulation of AR activity resulting in translocation of the receptor into the nucleus and increase in DNA synthesis. Src activation by growth factors in prostate cancer cells has been shown to correlate with AR tyrosine phosphorylation, especially under androgen-depleted conditions. Thus, evidence exists of an important functional relationship between Src and AR in prostate cancer, which makes it an attractive target in the search of new treatment modalities for AIPC (3).

Deregulation of Src activity rather than its expression is thought to be an important factor in oncogenesis. When Src is phosphorylated on Y⁵²⁷, the molecule is locked in an inactive state, whereas autophosphorylation of Y⁴¹⁹ allows the protein to unfold and assume a catalytically active conformation. Inactive Src is mostly located in the perinuclear region of the cell, whereas the activated protein translocates to the cell periphery toward the inner surface of the cell membrane (4).

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Translational Relevance

Src kinase inhibitors are currently in phase III clinical trials for treatment of advanced prostate cancer. This study shows that, in a subset of patients, there is an increase in Src activity in transition of prostate cancer to hormone-independent state and that these patients have a poor prognosis, develop hormone relapse quicker, and have reduced overall survival. These results suggest that potentially therapy in these patients may be more effective; therefore, patient's selection might be necessary before receiving Src inhibitors in a similar manner used with Herceptin and tamoxifen in breast cancer.

Apart from Src, other Src family kinases (SFK) have also been implicated in prostate cancer. Comparative genomic hybridization analysis of paired prostate cancer samples, taken from patients before hormone deprivation therapy and following hormone relapse, revealed that Fgr was significantly amplified in samples from patients with AIPC (5). Another study showed that SFK member Lyn could play a role in the development of hormone resistance in prostate cancer. Inhibiting the interaction of Lyn with its substrates by sequence-based peptides induced extensive apoptosis of hormone-refractory DU-145 explants in nude mice (6).

The dual Src/Abl kinase inhibitor dasatinib has shown effectiveness in prostate cancer cell line studies and animal models and a phase II clinical trial is under way to assess its effectiveness in androgen-deprived prostate cancer (7). However, several important issues have not yet been resolved. The role of Src in tumor cell proliferation remains controversial; therefore, the conventional estimation of treatment outcome by measurement of tumor size may not be appropriate. Although evidence supporting the role of Src in cell motility and the development of metastatic disease exists, currently, there are no established methods to test this in the clinical setting. Furthermore, due to significant heterogeneity of prostate cancer, not every patient with the disease might be suitable for therapy with Src inhibitors. The challenge, therefore, is to identify a subset of patients who are likely to respond to the treatment using individual tumor profiling, for example, and develop a more targeted approach based on this. Estimation of phospho-Src Y⁴¹⁹ (activated form of SFKs) as a biomarker has previously been suggested for this purpose (8, 9).

The potential drawback of existing cell line studies, investigating the role of Src inhibitors in prostate cancer, is the use of DU-145 and PC-3 cell lines that, unless specifically introduced, conventionally do not express AR, and as the majority of tumors in our study do express the AR, it would be useful to also study AR-positive hormone-refractory cell line (10–12). Chang et al. recently investigated a series of prostate cancer cell lines and have shown that the cells expressing the highest levels of Src were those with the lowest activated/total Src ratios (13). In addition, they reported that Src mediates cell proliferation in DU-145 and PC-3 cells but show that the mechanism between both cell lines was different. It is therefore possible that hormone-refractory AR-positive cells would also employ a different mechanism. Park et al. used PC-3AR-A1, an AR-expressing variant of PC-3 cells,

and the highly tumorigenic PC-3MM2GL cell line in an animal model to compare the response of primary tumors and the incidence of lymph node metastases after treatment with dasatinib (14). There was no difference between the two cell lines and both types of primary tumors were reduced in size compared with controls as was the incidence of lymph node metastases, although AR-expressing implants tended to grow slower. In our *in vitro* experiments, we used LNCaP cells that depend on androgens for their growth and therefore respond to androgen stimulation and LNCaP-SDM cells, which were derived from LNCaP cells by gradual withdrawal of androgens from the culture medium (15). Although they retain the ability to respond to androgen stimulation, LNCaP-SDM cells are routinely cultured in dextran charcoal-stripped FCS, containing similarly low concentration of androgens as in the serum of patients undergoing hormone deprivation therapy. More importantly, LNCaP-SDM cells express AR as in the significant majority of prostate tissue specimens taken from patients with hormone-refractory prostate cancer and therefore represent a possible model of AIPC (16, 17).

To our knowledge, up until now, there have been no publications showing correlation of SFK expression and activation with clinical parameters including survival in prostate cancer. To address this issue, we investigated SFK expression and activation by immunohistochemistry in paired prostate tissue samples taken from patients before androgen deprivation therapy and following hormone relapse. Here, for the first time, we show that, in a subset of prostate cancer patients, an increase in SFK activity in AIPC samples compared with ASPC, as indicated by phospho-Src Y⁴¹⁹ immunostaining, is associated with a significant decrease in survival. Our tissue culture experiments have shown that treatment with dasatinib resulted in inhibition of proliferation and migration of LNCaP-SDM cells, whereas only migration was suppressed in the parental LNCaP cell line, suggesting that inhibition of SFKs may provide therapeutic benefit in AIPC.

Materials and Methods

Patient cohort characteristics. Ethical approval was obtained for the patients' recruitment from multiple research ethics committee for Scotland. Application was subsequently submitted for the site-specific assessment and permission to recruit was granted by the local research ethics committees. No patients in this study had neoadjuvant radiotherapy or neoadjuvant androgen deprivation therapy. Each patient in our cohort was required to have prostate tissue samples taken before hormone deprivation therapy by means of transurethral resection of the prostate or transrectal ultrasound biopsy of prostate and >50% fall in PSA as an indicator of response to androgen deprivation therapy. Transrectal ultrasound biopsies consisted of minimum 12 cores from both left and right prostatic lobes with minimum of four cores taken from the transition zone. Subsequent, second-line hormonal therapy was then administered to which the patient did not respond and PSA concentrations continued to increase above the PSA nadir to concentrations >0.2 ng/mL. The second prostate tissue sample was taken during "channel" transurethral resection of the prostate after failure to respond to the second-line hormonal therapy and following at least two sequential increases in PSA level >0.2 ng/mL, indicating biochemical relapse. Clinical data recorded for each patient included age [median, 70; interquartile range (IQR), 66-73], PSA at diagnosis (median, 23.2 ng/mL; IQR, 4.6-35.6), PSA at relapse (median, 10 ng/mL; IQR, 4-11), Gleason grade at diagnosis (median, 7; range, 4-9), and Gleason grade at relapse (median, 8; range, 6-10). All patients developed biochemical relapse (median time to relapse, 2.53 years; IQR, 1.57-4.43 years). At last

follow-up, 34 patients had died of prostate cancer and 12 patients had died of other causes, median follow-up was 8.3 years, and IQR was 5.2 to 9.1 years. Following diagnosis, 10% (5 of 50) of patients underwent surgical orchidectomy and 90% (45 of 50) received LHRH analogue initially and then antiandrogen therapy subsequently. Following biochemical relapse, 64% (32 of 50) patients received radiotherapy; no patients in the current cohort received taxane therapy. Formalin-fixed paraffin-embedded prostate tissue blocks were retrieved for each patient and 5 μ m sections were cut using Leica RM 1235 microtome and applied onto silane-coated glass slides.

Immunohistochemistry. Prostate tissue sections were dewaxed in xylene and rehydrated in graded alcohol. Antigen retrieval was done under pressure for 5 min in citrate buffer (pH adjusted to 6.0). Endogenous peroxidase was blocked in 0.3% hydrogen peroxide for 10 min and blocked using 1.5% horse serum. Incubation with primary antibody was done in humidified chamber overnight at 4°C (anti-phospho-Src Y⁴¹⁹ 1:250 and anti-phospho-SrcY⁵³⁰ 1:100, Cell Signaling Technology; Fgr 1:100, Abgent; and Lyn 1:10, BD Biosciences) or for 1 h at room temperature for total Src (1:1,000, Cell Signaling Technology). Tissue was then incubated in Envision solution (DAKO UK) for 30 min and developed by application of 3,3'-diaminobenzidine as a chromogen (DAKO UK). Sections were counterstained with hematoxylin, dehydrated through graded alcohol and xylene, and mounted in DPX. In each immunohistochemistry run, LNCaP cell pellets and colon tissue are included as positive controls and an isotype-matched antibody is used on colon samples to provide a negative control.

Scoring method. Protein expression levels were assessed blindly by two independent observers using a weighted histoscore method also known as the H-score at magnification \times 400. Each cellular location (membranes, cytoplasm, and nuclei) was scored separately. The weighted histoscore method assesses the staining intensity and the percentage of cells stained with that intensity for the full slide. It is calculated by (1 \times % cells staining weakly positive) + (2 \times % cells staining moderately positive) + (3 \times % cells staining strongly positive). This provides a semi-quantitative classification of staining intensity, with the maximum score being 300 (if 100% of cells stain strongly positive) and minimum score being 0 (if 100% or cells are negative). The weighted histoscore method is a well-established method for scoring tissue that has heterogeneous staining (18).

Cell culture. Prostate cancer cell lines LNCaP and LNCaP-SDM were kind gifts from Prof. C. Robson (Northern Institute for Cancer Res-

earch). LNCaP cells were routinely maintained in RPMI 1640 (Invitrogen) containing phenol red and supplemented with 10% FCS (Invitrogen) and 1% glutamine. LNCaP-SDM cells have been developed using parental LNCaP cells as a model of hormone-resistant prostate cancer by gradual withdrawal of androgens from the medium. These cells were routinely cultured in RPMI 1640 supplemented with 1% glutamine and 10% charcoal-stripped FCS (Cambrex) known to contain negligible amount of androgens.

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.6), 150 mmol/L sodium chloride, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and 1:100 Calbiochem protease inhibitor cocktail set 1] and centrifuged at 12,000 rpm for 10 min, the supernatant was removed, and protein concentration was determined using BCA/CuSO₄ assay. Protein (40 μ g/well) was resolved by 4% to 12% gradient bis-Tris gel electrophoresis (Invitrogen); proteins were transferred to nitrocellulose membranes (Millipore), which were blocked for 1 h in 5% bovine serum albumin and probed with primary antibodies: anti-phospho-Src Y⁴¹⁹ (1:10,000), anti-total Src (1:10,000; Cell Signaling Technology), anti-phospho-FAK Y³⁹⁷ (1:10,000), anti-phospho-FAK Y⁸⁶¹ (1:10,000; Biosource), and anti-total FAK (1:10,000; BD Biosciences) at 4°C overnight. Membranes were then incubated with secondary antibodies (anti-rabbit 1:5,000 or anti-mouse 1:5,000; Cell Signaling Technology) and visualized with ECL kit (Amersham). Where necessary, the membranes were stripped by incubating with Re-Blot Plus stripping buffer (Chemicon) before reprobing with other antibodies including anti- α -tubulin (1:8,000; Santa Cruz Biotechnology) to confirm equal protein loading.

Steroid exposure and withdrawal experiment. LNCaP and LNCaP-SDM cells were seeded in standard culture medium at a density of 1 \times 10⁶ per 9 cm dish and allowed to attach overnight. The following day, the cells were washed twice in PBS and the medium was changed. Each cell line was exposed to three types of medium: serum-free medium, steroid-depleted medium containing 10% charcoal-stripped serum, and medium containing 10% full FCS. After 60 h, which allows for the metabolism of intracellular steroid hormones, cell lysates were prepared and Western blot analysis was done as described above.

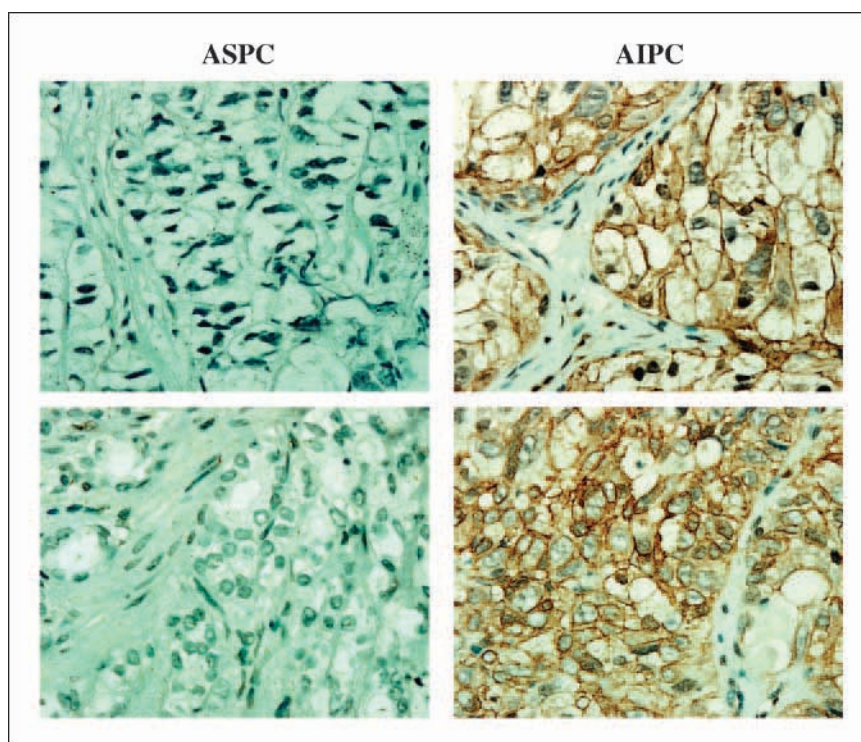
Src inhibitor exposure experiment. LNCaP and LNCaP-SDM cells were seeded at a density of 1 \times 10⁶ per 9 cm Petri dish and cultured using standard culture medium until 80% confluent and then treated

Table 1. Changes in expression in the transition from hormone-sensitive to hormone-refractory disease

Antibody	Histoscores		No. (%) patients			P
	ASPC	AIPC	Increase	Fall	No change	
Phospho-Src Y ⁴¹⁹						
Cytoplasm	45 (18.75-80)	70 (40-90)	13 (26)	3 (6)	34 (68)	0.035
Membrane	20 (0-60)	50 (0-90)	14 (28)	3 (6)	33 (66)	0.017
Phospho-Src Y ⁵²⁷						
Cytoplasm	80 (50-112.5)	50 (20-90)	6 (12)	18 (36)	26 (52)	0.002
Membrane	50 (20-80)	20 (0-70)	13 (26)	21 (42)	16 (32)	0.101
Total Src						
Cytoplasm	130 (100-180)	100 (80-126.25)	4 (8)	16 (32)	30 (60)	0.009
Membrane	55 (20-116.25)	55 (30-110)	10 (20)	13 (26)	27 (54)	0.926
Total Lyn						
Cytoplasm	28 (5-70)	50 (20-80)	17 (34)	8 (16)	25 (50)	0.094
Membrane	38 (10-80)	70 (35-95)	25 (50)	6 (12)	19 (38)	0.005
Total FGR						
Cytoplasm	65 (39-100)	50 (30-78)	8 (16)	15 (30)	27 (54)	0.104
Membrane	95 (69-112)	90 (63-108)	8 (16)	14 (28)	28 (56)	0.567

NOTE: Protein expression for each antibody including median histoscores and IQRs. Membrane and cytoplasm staining was scored separately and the patients were subdivided into those who had an increase in expression from ASPC to AIPC, decrease, and no change. The difference in staining between ASPC and AIPC samples was assessed by Wilcoxon signed-rank test.

Fig. 1. Immunohistochemistry analysis of paired prostate cancer samples taken before the initiation of hormone deprivation therapy (ASPC samples) and following hormone relapse (AIPC samples). Expression of phospho-Src Y⁴¹⁹. Magnification, $\times 40$.



overnight with a range of dasatinib concentrations. Cell lysates were prepared and Western blot analysis was done as described above. Dasatinib was provided by Bristol-Myers Squibb and made up as 10 mmol/L stock in DMSO.

Immunoprecipitation. Cell lysates were prepared as described in previous sections and samples containing 500 μg protein in 500 μL were incubated with anti-total Lyn (1:50; BD Biosciences) or anti-total Src (1:100; Cancer Research UK) at 4°C overnight. Then, anti-mouse IgG agarose beads (Sigma) were added to the samples (20 μL /sample) and further incubated for 1 h at 4°C. Immune complexes were analyzed as per Western blot protocol with anti-phospho-Src Y⁴¹⁹ antibody (1:10,000; Cell Signaling Technology).

Cell migration. To follow random migration, cells were plated at a low density (5×10^4) on glass-bottomed 6-well plates (Iwaki), allowed to attach overnight, and then treated with increasing concentrations of dasatinib, diluted in standard culture medium. Migration was monitored by video time-lapse microscopy for 24 h using a Zeiss Axiovert S100 microscope with $\times 20$ objective powered by AQM Advance software (Kinetic Imaging).

Wound-healing assay was carried out to investigate the ability of prostate cancer cells to migrate into a denuded area in the presence or absence of dasatinib. LNCaP and LNCaP-SDM cells were plated at a density of 1×10^6 per well of a glass-bottomed 6-well plate (Iwaki). The following day, the wounds through the confluent cell monolayer were made using a fine pipette tip, three wounds per each well. The medium was then replaced with standard for each cell line culture medium containing increasing concentrations of dasatinib. The assay was done over 24 h with images taken from three fields per each well every 30 min using a Zeiss Axiovert S100 microscope at $\times 20$ magnification. Cell migration was measured by subtraction of the distance between the edge of the wound at time 0 and 24 h using ImageJ software.

Cell proliferation. Proliferation was assessed using the WST-1 assay as per manufacturer's instructions. Cells were seeded in 96-well plates at a density of 5×10^3 per well in standard culture medium, allowed to attach overnight, and the following day treated with increasing concentrations of dasatinib; DMSO was added to the control well. The assay was done at 48, 72, and 96 h by adding 10 μL WST-1 reagent before

dilution in Electro Coupling Solution to each well. The absorbance level was measured after 2 h incubation at 37°C using a 96-well microplate reader at 450 nm with reference wavelength 600 nm.

To study the effect of androgens on proliferation in the presence of dasatinib, LNCaP and LNCaP-SDM cells were plated in standard culture medium for 24 h after which LNCaP cells were placed in the steroid-depleted medium containing 10% charcoal-stripped FCS and LNCaP-SDM cells were in RPMI 1640 supplemented with 10% full FCS. WST-1 assay was done at 48, 72, and 96 h as described above.

Statistical analysis. Statistical analysis was done using Microsoft Excel and the SPSS statistical package (version 9.0). Small changes in protein expression between paired ASPC and AIPC tumors could be due to random errors in the assessment of histoscores. To identify individual patients in whom there was strong evidence of a genuine increase or decrease in protein expression, it was required that the change in expression exceeded a threshold equal to 2 SDs of the interobserver difference for that protein. This threshold was chosen because, if there was in reality no difference in protein expression between ASPC and AIPC tumors in a given patient, there would be only a 5% probability of an apparent difference being observed that exceeded the threshold due to random variation. This assumes that the random variation between two different observers assessing the same tumor is of a similar magnitude to the random variation that would affect a single observer assessing two different tumors with the same level of protein expression. Changes in protein expression in individual patients that exceeded this threshold were termed significant. Wilcoxon signed-rank tests were used to compare expression between ASPC and AIPC tumors. Survival analysis including time to relapse, time to death from relapse, and overall survival was conducted using the Kaplan-Meier method and curves were compared with the log-rank test.

Results

SFK protein expression in ASPC and AIPC. To determine the level of Src, Lyn, and Fgr expression and activation in the

transition from ASPC to AIPC, we used 100 paraffin-embedded prostate tissue sections taken from 50 patients with prostate cancer who underwent hormone deprivation therapy and subsequently developed hormone relapse. Due to tissue heterogeneity, full prostate tissue sections were stained using antibodies against total Src, Lyn, Fgr, and phospho-Src Y⁴¹⁹ representing an active form of SFKs and phospho-SrcY⁵²⁷, a marker suggestive of inactive Src (Table 1). The sequences surrounding Src autophosphorylation site Y⁴¹⁹ are highly conserved in the majority of SFK and positive staining using this antibody represents autophosphorylation not only of Src but also of other Src family members. In our cohort, there was a significant increase in overall phospho-Src Y⁴¹⁹ immunostaining in the transition from ASPC to AIPC; more intense cytoplasm and membrane staining was observed in AIPC samples compared with ASPC (Fig. 1). In addition, although the majority of matched specimens on an individual patient basis showed no change in expression of phospho-Src Y⁴¹⁹ in the transition from ASPC to AIPC, a subgroup of patients did have tumors that exhibited an increase in expression as determined by the weighted histoscore technique (Table 1). Small changes in protein expression between paired ASPC and AIPC tumors could be due to random errors in the assessment of histoscores; therefore, the mathematical method as described in Statistical Analysis was employed for selection of patients with genuine increases in expression. Changes in protein expression in individual patients that exceeded this threshold were termed an increase in expression. Using this method, 13 (26%) and 14 (28%) patients were noted to have an increase in expression of activated SFKs (phospho-Src Y⁴¹⁹) in the cytoplasm and membrane, respectively (Table 1). In addition, there was an increase in overall Lyn membrane immunostaining in the transition from ASPC to AIPC; more intense cytoplasm and membrane staining was observed in AIPC samples compared with ASPC (although only membrane staining reached significance). No change in expression was observed for Fgr; interestingly, expression of total Src and phospho-SrcY⁵²⁷ in the cytoplasm fell significantly after the patients developed hormone relapse, whereas the membrane staining did not change (Table 1).

Correlation of SFK expression with clinical parameters. Development of bone metastases (as confirmed by bone scans) in patients with advanced prostate cancer is the cause for significant morbidity and is considered to be an important negative prognostic sign. In our cohort, following the diagnosis of prostate cancer, the majority of the patients (46 or 92%) had bone scans; in 8 (16%) patients, it was positive, implying the presence of bone metastases; and in 38 (76%) patients, it was negative. After the development of AIPC, the patients who had positive bone scans at diagnosis and no bone scan after relapse were considered as having bone metastases as well as those who had negative bone scans at diagnosis and positive scans at relapse (26 or 52%). The membranes in the tumor samples taken from patients with AIPC stained more intensely with the anti-phospho-Src Y⁴¹⁹ antibody if there was evidence of bone metastases ($P = 0.011$; Supplementary Material). No significant correlation was observed between presence of bone metastases and cytoplasmic staining with antibodies against phospho-Src Y⁴¹⁹, phospho-SrcY⁵²⁷, total Src, Fgr, or Lyn.

The cohort was then further subdivided into groups according to Gleason grade at diagnosis and PSA value at relapse, important prognostic criteria in patients with prostate cancer.

High or low PSA at relapse were not associated with immunohistochemistry histoscores and only expression of cytoplasmic Lyn in the hormone-refractory tumors was associated with Gleason grade ($P = 0.029$; Supplementary Material).

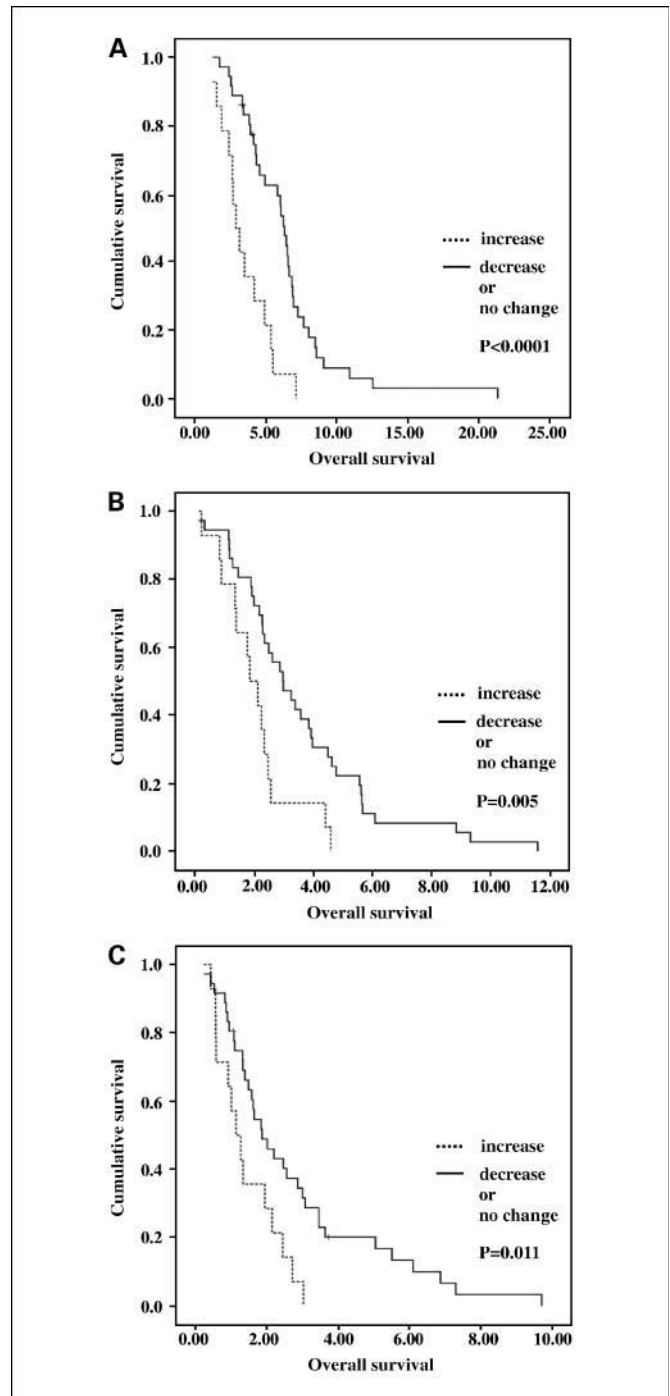


Fig. 2. Survival analysis of membrane immunostaining with anti-phospho-Src Y⁴¹⁹ antibody in transition from ASPC to AIPC. Kaplan-Meier survival curves plotting time from prostate cancer diagnosis to hormone relapse (A), from hormone relapse to death (B), and from prostate cancer diagnosis to death (C). Dashed lines, patients who had an increase in immunohistochemistry in the transition from hormone-sensitive to hormone-resistant prostate cancer; solid lines, patients who had decrease or no change.

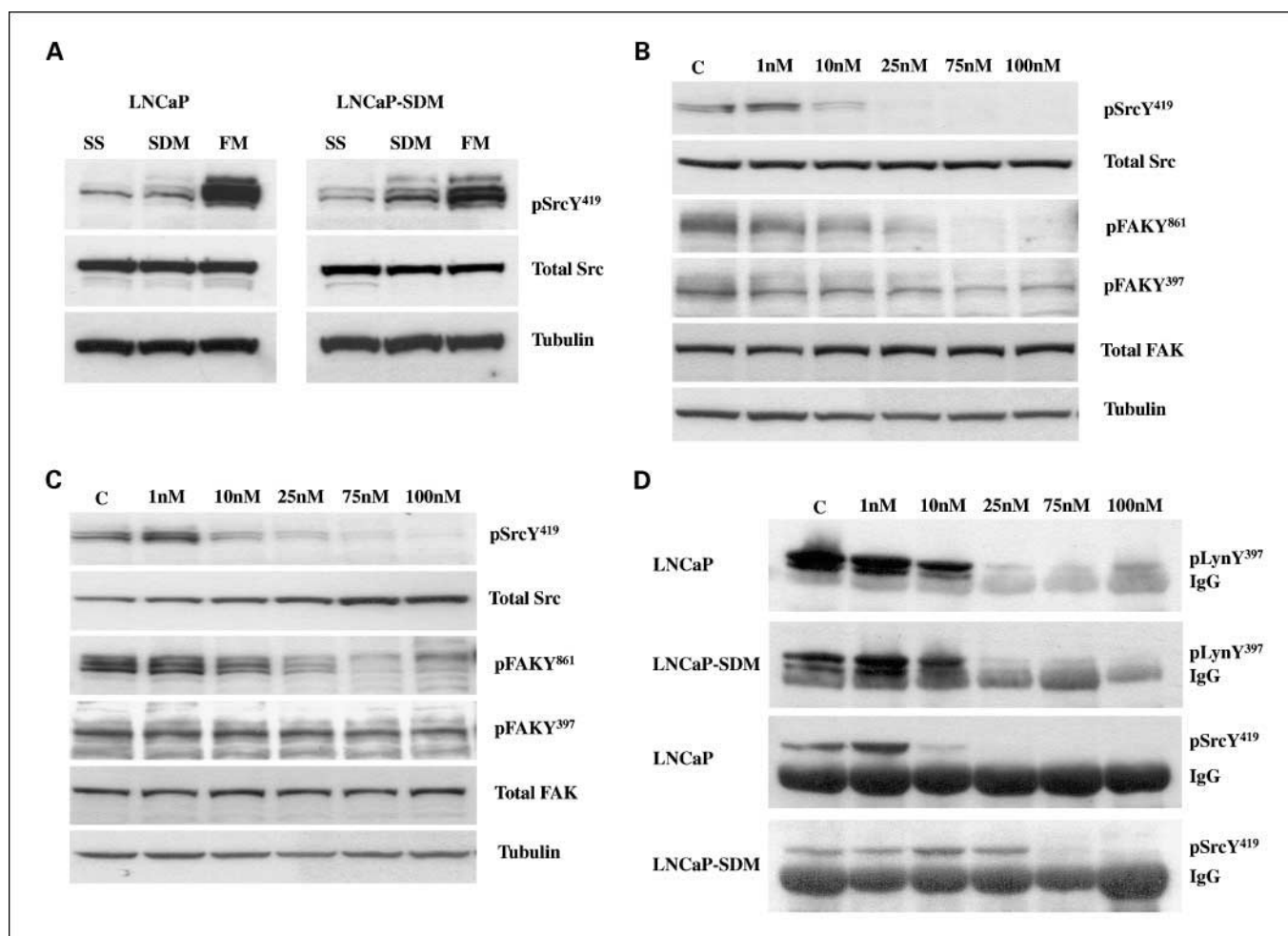


Fig. 3. Dasatinib inhibits phosphorylation SFKs and downstream marker FAK. **A**, LNCaP and LNCaP-SDM cells were cultured in medium containing no serum (serum-starved; *SS*), dextran/charcoal-stripped FCS (steroid-depleted medium; *SDM*), and FCS (full medium; *FM*). Cell lysates were immunoblotted with anti-phospho-Src Y⁴¹⁹, anti-Src, and anti-tubulin antibodies. LNCaP (**B**) and LNCaP-SDM (**C**) cells were exposed to increasing concentrations of dasatinib in standard corresponding culture medium for 16 h. Cell lysates were immunoblotted with anti-phospho-Src Y⁴¹⁹, anti-phospho-FAK, anti-FAK, anti-Src, and anti-tubulin antibodies. **D**, LNCaP and LNCaP-SDM cells were exposed to increasing concentrations of dasatinib in standard corresponding culture medium for 16 h. Cell lysates were then immunoprecipitated with either Src- or Lyn-specific antibodies and probed with anti-phospho-Src Y⁴¹⁹ antibody.

Survival analysis. Use of paired prostate cancer specimens taken from patients before commencement of hormone manipulation therapy and after androgen escape made it possible to correlate immunohistochemistry scores of each individual patient with various survival parameters including time from diagnosis to biochemical relapse, time from relapse to death, and overall survival. Statistical analysis revealed that a subset of patients who had an increase in SFK activity (phospho-Src Y⁴¹⁹ expression) at the membrane in the transition from ASPC to AIPC had significantly shorter time to relapse; median time from diagnosis to hormone escape in this subgroup of patients was 1.86 (1.36-2.47) years compared with 2.98 (1.93-4.63) years for those who had decreased membrane signal or no change ($P = 0.005$; Fig. 2A). Furthermore, time from biochemical relapse to death was also reduced: 1.14 (0.58-2.15) versus 1.87 (1.1-1.86) years ($P = 0.011$; Fig. 2B). Therefore, the combination of reduced time to relapse combined with reduced time to death following relapse resulted in a significant reduction in overall survival; median survival for those with an increase in membrane phospho-Src Y⁴¹⁹ expression was 2.91

(IQR, 2.42-4.92) years compared with 6.33 (IQR, 4.15-7.27) years for those patients with tumors that had a decrease or no change in phospho-Src Y⁴¹⁹ expression ($P < 0.0001$; Fig. 2C). However, when multivariate Cox regression analysis was done for the patients with an increase in SFK activity taking into account the presence of metastases at relapse and Gleason score, SFK activity was noted to be independently significant only for the time from relapse to death ($P = 0.018$) and overall survival ($P = 0.01$).

No association with the time to relapse or survival was observed for Fgr, total Src, and phospho-SrcY⁵²⁷; however, an increase in membrane Lyn expression was associated with a shorter time to relapse ($P = 0.022$; data not shown). Median time from diagnosis to hormone escape in this subgroup of patients was 2.29 (1.78-2.79) years compared with 3.2 (2.45-3.99) years in those who had a decreased membrane signal or no change. This was also observed for an increase in cytoplasmic Lyn expression; median time from diagnosis to hormone escape in this subgroup of patients was 2.41 (2.01-2.73) years compared with 3.2 (1.98-4.45) years in

those who had decreased membrane signal or no change ($P = 0.040$). However, multivariate Cox regression analysis showed that increase in Lyn expression was not independently significant when the presence of metastases at relapse and Gleason score were considered. No association with time from biochemical relapse to death or overall survival were observed.

SFK activity in prostate cancer cell lines. Having observed an association of SFK activation and decreased survival of AIPC patients, we examined SFK activity in hormone-sensitive LNCaP and hormone-refractory LNCaP-SDM prostate cancer cell lines. To assess the effect of androgen stimulation and withdrawal on Src kinase activity, both cell lines were exposed to various culture medium including serum-free medium, medium containing dextran charcoal-stripped FCS (steroid-depleted medium), and full FCS. SFK activity in LNCaP cells experiencing acute an-

drogen withdrawal in steroid-depleted medium was reduced to the same degree as in the cells that were serum starved (Fig. 3A). In LNCaP-SDM cells routinely cultured in charcoal-stripped serum, long-term androgen deprivation resulted in higher basal level of SFK activity compared with LNCaP cells in equivalent culture conditions. When deprived of growth factors in serum-free medium, LNCaP-SDM cells displayed a further reduction in SFK activity, whereas treatment of these cells with full FCS containing physiologic amount of androgens increased SFK activity, although it did not reach the level observed in parental LNCaP cells (Fig. 3A).

Dasatinib inhibits SFK activity in LNCaP and LNCaP-SDM cells. Dasatinib was used to investigate the effects of Src inhibition on LNCaP and LNCaP-SDM cells. Treatment of LNCaP and LNCaP-SDM cells with dasatinib resulted in a dose-dependent

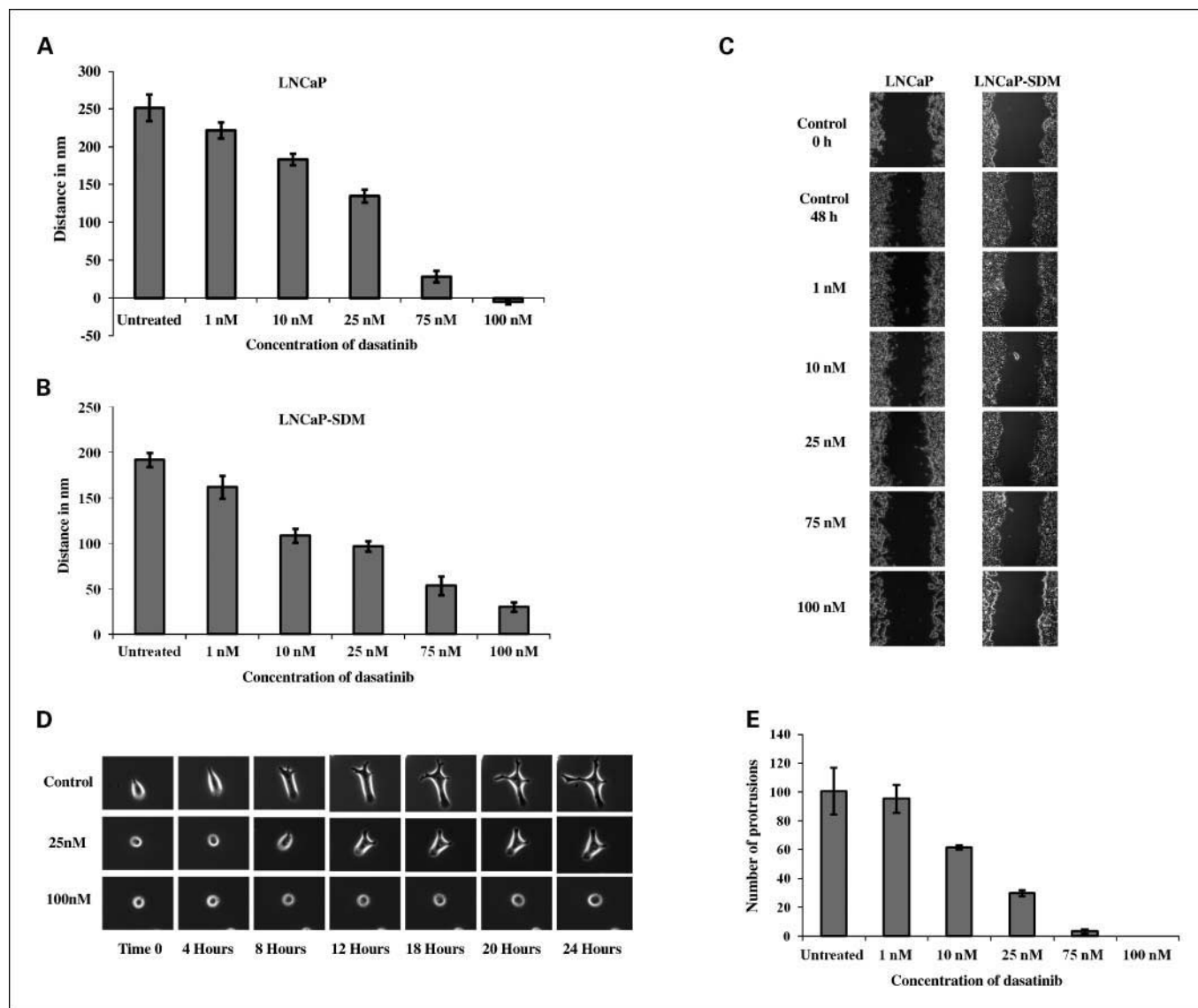


Fig. 4. Dasatinib inhibits motility of LNCaP-SDM cells. **A** wound-healing assay done using LNCaP (**A** and **C**) and LNCaP-SDM (**B** and **C**) cells following exposure to a range of dasatinib concentrations. **C**, images were taken after 48 h and show the relative closure of the wound. The difference in distance (in nm) between the edge of wounds at the beginning of the experiment and after 48 h observed by time-lapse microscopy were plotted against inhibitor concentration. Mean \pm SD of three separate measurements (three wounds per well). **D**, LNCaP-SDM cells were plated at low density and exposed to increasing concentrations of dasatinib for 24 h. Motility was observed by time-lapse microscopy and separate pictures were taken from the stacks at four hourly intervals. The number of protrusions produced by the cells over 24 h in each concentration of inhibitor was calculated. Mean \pm SD of three individual fields, each field containing \sim 60 cells. Each experiment was repeated three times.

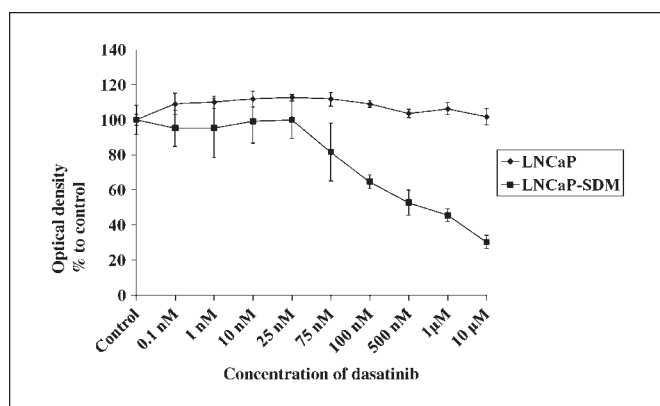


Fig. 5. Dasatinib inhibits proliferation of LNCaP-SDM cells. Cells were plated on 96-well plates and after 24 h treated with a range of dasatinib concentrations. After 96 h, a WST-1 assay was done. Mean \pm SD of six wells presented as percentages of the untreated control. Experiments were repeated three times.

reduction of SFK activity using the autophosphorylation of Src at Y⁴¹⁹ as a marker of activation. Complete inhibition of SFK activity in LNCaP cells was seen at 50 nmol/L (Fig. 3B). SFK activity was also suppressed in LNCaP-SDM cells (Fig. 3C), although complete inhibition was achieved at slightly higher concentrations (75 nmol/L). Interestingly, in LNCaP-SDM cells, increasing the dose of dasatinib resulted in an increase in total Src. FAK phosphorylation on Y⁸⁶¹ (Src-dependent phosphorylation site) was also inhibited by treatment with dasatinib in both cell lines in a dose-dependent manner, whereas the levels of phospho-FAK Y³⁹⁷ (Src-independent autophosphorylation site) and total FAK remained unchanged (Fig. 3B and C).

Due to cross-reactivity of anti-phospho-Src Y⁴¹⁹ antibody, it was necessary to immunoprecipitate Src and Lyn to study the effect of dasatinib on individual family members. Dasatinib inhibited Lyn autophosphorylation in both LNCaP and LNCaP-SDM cells at equivalent concentrations (Fig. 3D). Higher concentrations were necessary to inhibit Src autophosphorylation in the LNCaP-SDM cells (Fig. 3D).

Dasatinib inhibits prostate cancer cell migration. Time-lapse video microscopy was employed to investigate the effect of dasatinib on LNCaP and LNCaP-SDM cell migration into a denuded area using a wound-healing assay. Both LNCaP (Fig. 4A and C) and LNCaP-SDM (Fig. 4B and C) cells exhibited a similar dose-dependent reduction in migration when treated with increasing concentrations of dasatinib for 48 h. LNCaP cells cultured in full medium containing FCS were noted to have long protrusions, whereas the protrusions, produced by LNCaP-SDM cells when kept in androgen-depleted medium, rarely exceeded the length of the body of the cell. Interestingly, when cultured in the medium containing physiologic concentrations of androgens (FCS), the number of LNCaP-SDM cells forming long protrusions significantly increased (data not shown).

We then studied the effect of dasatinib on protrusion dynamics in LNCaP-SDM cells. Cells treated with low inhibitor concentrations formed protrusions at significantly lower rates than control cells, whereas cells exposed to high concentrations of dasatinib failed to produce protrusions. Images of representative cells are shown in Fig. 4D and a similar effect was observed in LNCaP cells (data not shown). Quantitative analysis revealed that as a result of dasatinib treatment there was a dose-

dependent reduction in the number of protrusions per cell (Fig. 4D).

Effect of dasatinib on prostate cancer cell proliferation. Growth of hormone-sensitive LNCaP cells is driven mostly by androgens, whereas hormone-refractory LNCaP-SDM cells are thought to depend on growth factors transmitting oncogenic signals via tyrosine kinases. Treatment with dasatinib inhibited proliferation of hormone-refractory LNCaP-SDM cells in a dose-dependent manner (IC₅₀, 500 nmol/L; Fig. 5) at concentrations corresponding to inhibition of SFK activity (Fig. 3C). Proliferation of hormone-sensitive LNCaP cells was not significantly inhibited by dasatinib even at concentrations up to 10 μ mol/L (Fig. 5). Interestingly, introduction of androgens to the steroid-depleted medium, used for routine culture of LNCaP-SDM cells, did not rescue proliferation suppressed by dasatinib (data not shown). In addition, treatment of LNCaP cells experiencing acute androgen withdrawal in steroid-depleted medium did result in further inhibition of proliferation, although this was observed at concentrations of dasatinib higher than 10 μ mol/L; therefore, this effect was thought to be nonspecific (data not shown).

Discussion

Hormone-sensitive prostate cancer cells proliferate and migrate as a result of androgen stimulation. Androgen deprivation therapy leads to the changes in the way prostate cancer cells operate by increasing their sensitivity to the low levels of androgens, up-regulating various oncogenic molecular pathways where the AR is activated by growth factors and antagonists or bypassing AR altogether (2, 19). These pathways frequently interact or crosstalk, which is why the strategy of inhibiting individual proteins or even pathways has not resulted in the development of effective therapies. Targeting key tyrosine kinases involved in the interaction between various cascades together with individual tumor profiling and combination treatments could be used to overcome drug resistance (20).

Numerous *in vitro* studies have shown the significance of Src in the development of prostate cancer and its progression to a hormone-independent state (11, 12, 21–25). Here, for the first time, we report the association between SFK activity and clinical data in prostate cancer patients. Our findings suggest that SFK activity is up-regulated in a subgroup of patients with AIPC, which ultimately affects their survival. Patients with the increase in SFK activity in the transition from ASPC to AIPC live, on average, 3.42 years shorter after the diagnosis of prostate cancer is made than the patients with the decrease or no change. This difference is dramatic given the relatively short overall lifespan of patients with hormone-refractory disease. Furthermore, an increase in SFK activity in patients with AIPC may result in higher likelihood of metastatic disease, which could potentially contribute to the reduction in survival.

It is not entirely clear at the moment which SFK member plays dominant role in the transition of prostate cancer to hormone independence. Fgr has previously been found amplified in hormone-refractory prostate cancer, although our data suggest that its expression does not seem to change with hormone escape (19). Goldenberg-Furmanov et al. reported that expression of Lyn, another SFK member, tends to be higher in poorly differentiated regions of prostatic tumors and Lyn-deficient mice have underdeveloped prostate gland (6). In this study, we

show that expression of Lyn does correlate with Gleason grade; furthermore, this is a feature of hormone-refractory prostate cancer. It has been suggested that various SFK members may play different role in prostatic oncogenesis. Experiments using small interfering RNA approach to silence Src or Lyn revealed that although Src knockout had only minimal effect on proliferation rate, cell migration was significantly reduced. In contrast, silencing Lyn resulted in reduced proliferation, although no migration experiment was conducted (14).

Use of Src kinase inhibitors in prostate cancer cell lines has been the subject of several publications. Recchia et al. reported a reduction in PC-3 cells adhesion, migration, and proliferation when treated with pyrrolopyrimidine class compounds (12). Dasatinib (BMS-354825), a selective inhibitor of Src and related kinase Abl, has been reported to suppress Src kinase activity at low nanomolar concentrations, reduce the phosphorylation of downstream proteins FAK and p130^{cas}, and as a result inhibit DU-145 cell adhesion, migration, and invasion (11). In animal models, dasatinib treatment was shown to affect primary tumor growth of prostate cancer cell implants as well as reduce the rate of lymph node metastases (14). However, the cell lines used in these studies do not express AR, which is found in the majority of prostate cancer specimens taken from patients with AIPC. This represents a potential drawback given that there is evidence of AR and Src interactions, especially in androgen deprivation conditions (26). Park et al. reported the use of a PC-3 cell line transfected with functional AR in an animal model, although no *in vitro* experiment was conducted (14). In this study, simulating acute androgen withdrawal by placing LNCaP cells in the steroid-depleted medium produced a dramatic reduction in Src kinase activity, confirming that Src interaction with AR is important in prostate cancer. In our experiments, we used LNCaP-SDM cells that were developed by gradual withdrawal of androgens from the culture medium mimicking hormone deprivation therapy; they retain functional AR and therefore provide a possible model of AIPC.

Src kinase plays a key role in cell migration by regulating the turnover of focal adhesions and adherence junctions. Cell movement is initiated by the focal adhesion assembly at the leading edge of the moving cell followed by formation of dynamic protrusions, allowing the cell to propagate in the direction of movement, whereas cell-cell junctions become weaker and focal adhesions at the rear of the moving cell disassemble (27). When treated with dasatinib, both hormone-sensitive and hormone-refractory cell lines exhibited reduced migration, which was dose-dependent. The cells were not able to produce protrusions or, when exposed to lower concentrations of dasatinib, produced protrusions at much lower rate than control

cells. This finding has certain clinical implications: Src inhibitors could be used in both hormone-sensitive and hormone-refractory prostate cancer to reduce or prevent the spread of malignant cells and the development of metastatic disease. However, at the moment, there are no reliable methods to evaluate this in clinical setting; therefore, we need to develop the necessary tools as a matter of urgency.

Controversy surrounding the role of Src in cancer cell proliferation is partly due to the lack of specificity many small-molecule inhibitors often display. Dasatinib, for instance, is known to inhibit platelet-derived growth factor receptor β , Abl, c-Kit and EphA2 (28). Although previous studies have shown that Src inhibitors may affect proliferation of prostate cancer cells, the concentrations required to elicit this effect considerably exceeded those needed to suppress adhesion and migration (10, 12). In a recent publication, it has been shown that dasatinib inhibited proliferation of both LNCaP and PC-3MM2GL cell line with dasatinib concentrations >100 nmol/L (14). However, our experiments have shown that dasatinib inhibited proliferation of hormone-refractory LNCaP-SDM cells at concentrations that suppressed SFK activity, whereas proliferation of the hormone-sensitive LNCaP cells was not affected. We concluded that proliferation of hormone-resistant prostate cancer cells is probably more likely to be affected by SFK inhibitors due to their dependence on growth factors rather than androgens.

Although SFK activity could be a contributing factor in the development of prostate cancer and its progression to the hormone-independent state, only a subgroup of patient with AIPC might be suitable for the treatment with Src inhibitors. Identifying these patients would provide more targeted approach resulting in the treatment being delivered more effectively. Therapeutic strategies employing Src inhibitors in combination with conventional antiandrogens, chemotherapy, or other small-molecule inhibitors in patients with prostate cancer should be considered (7). As Src inhibitors could potentially be used to prevent the development of metastatic disease, methods to assess this in clinical trials are urgently required.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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