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Src family kinase activity is up-regulated in hormone refractory prostate cancer.

Tatarov O¹, Mitchell TJ¹, Seywright M², Leung HY³, Brunton VG⁴ and Edwards J¹.

¹ Division of cancer Sciences and Molecular Pathology, Faculty of Medicine,
Glasgow Royal Infirmary, Glasgow

² Dept of Pathology, Western Infirmary, Glasgow.

³ Cancer Research UK, Beatson Laboratories, Garscube Estate, Glasgow.

⁴ Edinburgh Cancer Research Centre, Western General Hospital, University of
Edinburgh, Edinburgh.

Clinical Cancer Research

Corresponding author

Dr Joanne Edwards

University Department of Surgery

Division of Cancer Sciences & Molecular Pathology

Level 2, Queen Elizabeth Building

Glasgow Royal Infirmary

Glasgow

G31 2ER

Tel 44 141 211 5441, Fax 44 141 552 3229

Email je10b@clinmed.gla.ac.uk

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Statement of Translational Relevance

Src kinase inhibitors are currently entering phase 3 clinical trials for treatment of prostate cancer, the current study demonstrates that only a subset of patients have increased Src activity and that these patients have a poor prognosis, relapsing quicker and have reduced shorter overall survival. These results suggest that potentially only this subset of patients would respond to this therapy and therefore it might be necessary to select patients prior to receiving Src inhibitors in a similar manner used with Herceptin and Tamoxifen in breast cancer.

Abstract

Purpose

Although 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 AIPC and if patients could be selected who were more likely to respond to therapy.

Experimental Design

A unique cohort of matched prostate tumour samples, taken prior to 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, these patients had significantly shorter time to relapse ($P=0.005$) and overall survival

($P < 0.0001$) and activated SRK expression correlated with 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.

Introduction

Prostate cancer is the most common cancer in men and is the second leading cause of cancer related deaths in men in the US and UK (1). Treatment options for locally advanced and metastatic prostate cancer are limited to androgen deprivation using anti-androgen drugs 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 non-receptor 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 tyrosine Y⁵²⁷, the molecule is locked in an inactive state, whereas autophosphorylation of tyrosine Y⁴¹⁹ allows the protein to unfold and assume a catalytically active conformation. Inactive Src is mostly located in the perinuclear region of the cell while the activated protein translocates to the cell periphery towards the inner surface of the cell membrane (4).

Apart from Src, other Src-family kinases (SFKs) have also been implicated in prostate cancer. CGH analysis of paired prostate cancer samples, taken from patients prior to hormone deprivation therapy and following hormone relapse, revealed 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 demonstrated effectiveness in prostate cancer cell line studies and animal models and a Phase II clinical trial is underway to assess its effectiveness in androgen-deprived prostate cancer (7). However, several important issues have not yet been resolved. The role of Src in tumour cell proliferation remains controversial and, therefore, the conventional estimation of treatment outcome by measurement of tumour 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 tumour profiling, for example, and develop a more targeted approach on the basis of this. Estimation of phospho SrcY⁴¹⁹ (activated form of SFKs) as a biomarker has previously been suggested for this purpose (8,9).

The major drawback of existing cell line studies, investigating the role of Src inhibitors in prostate cancer (10-12), 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 tumours in our study do express the AR it would be useful to also study AR

positive hormone refractory cell line. However Chang et al recently investigated a series of prostate cancer cell lines and reported that the cells that expressed 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 DU15 and PC3 cells, but demonstrate that the mechanism between both cell lines was different (13). It is therefore possible that hormone refractory AR positive cells would also employ a different mechanism. Park et al. used PC-3AR-A1, a 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 tumours and the incidence of lymph node metastases after treatment with dasatinib (14). There was no difference between the two cell lines, both types of primary tumours were reduced in size compared to 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 which 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 foetal calf serum, 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 demonstrating 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 (IHC) in paired prostate tissue samples taken from patients prior to 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 to ASPC, as indicated by pSrcY⁴¹⁹ 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 while 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 (MREC) for Scotland. Application was subsequently submitted for the site-specific assessment and permission to recruit was granted by the Local Research Ethics Committees (LREC). No patients in this study had neoadjuvant radiotherapy or androgen deprivation therapy. Each patient in our cohort was required to have tissue sample taken prior to hormone deprivation therapy by means of trans-urethral resection of the prostate (TURP) or trans-urethral ultrasound (TRUS) biopsy of prostate, more than 50% fall in PSA as an indicator of response to androgen deprivation therapy, subsequent hormonal therapy was then administered to which the patient did not respond and PSA concentrations continued to rise above the PSA nadir to concentrations greater than 0.2ng/ml. The second tissue sample was taken after failure to respond to the second hormonal therapy and following at least 2 sequential rises in PSA level above 0.2ng/ml, indicating biochemical relapse. Clinical data, recorded for each patient included age (median 70, inter quartile range 66-73), PSA at diagnosis (median 110 ng/ml, inter quartile range 20-780), PSA at relapse (median 10 ng/ml, inter quartile range 4-11), Gleason grade at diagnosis (median 7, range 4-9) and Gleason grade at

relapse (median 8, range 6-10). All patients under went biochemical relapse (median time to relapse 2.53 years, inter quartile range 1.57-4.43 years). At last follow-up, 34 patients had died of their disease and 12 patients had died of other causes, median follow up was 8.3 years and inter quartile range was 5.2-9.1 years. Following diagnosis 10 % (5/50) patients received surgical orchidectomy and 90% (45/50) patients received LHRH analogue in combination with anti androgen therapy. Following biochemical relapse 64% (32/50) patients received radiotherapy, no patients in the current cohort received taxane therapy. Formalin fixed paraffin embedded tissue blocks were retrieved for each patient and 5 µm sections were cut using Leica RM 1235 microtome and applied onto silane-coated glass slides.

Immunohistochemistry. Tissue sections were dewaxed in xylene and rehydrated in graded alcohol. Antigen retrieval was performed 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 performed in humidified chamber overnight at 4⁰C (anti-phospho SrcY⁴¹⁹ 1:250 and anti-phospho SrcY⁵³⁰ 1:100, Cell Signalling Technologies, FGR 1:100, Abgent and Lyn 1:10, BD Biosciences) or for 1 hour at room temperature for total Src (1:1000, Cell Signalling Technologies). Tissue was then incubated in Envision solution (DAKO, UK) for 30 min and developed by application of 3,3'-diaminobenzidine (DAB) as a chromogen (DAKO, UK). Sections were counterstained with haematoxylin, dehydrated through graded alcohol and xylene and mounted in DPX. In each immunohistochemistry run LNCaP cell pellets and colon tissue is 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 2 independent observers, using a weighted histoscore method also known as the H-Score at magnification x 400 (18,-19). Each cellular location (membranes, cytoplasm and

nuclei) were 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 x % cells staining weakly positive) + (2 x % cells staining moderately positive) + (3 x % 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 (18-19).

Cell culture. Prostate cancer cell lines LNCaP and LNCaP-SDM were a kind gift from Professor C Robson (Northern Institute for Cancer Research, Newcastle). LNCaP cells were routinely maintained in RPMI 1640 (Invitrogen, UK) containing phenol red and supplemented with 10% foetal calf serum (Invitrogen, UK), 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 foetal calf serum (Cambrex, UK) known to contain negligible amount of androgens.

Western blot analysis. Cells were lysed in RIPA buffer (50 mM Tris pH7.6, 150 mM sodium chloride, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mM sodium fluoride, 1 mM sodium ortho-vanadate and 1:100 Calbiochem protease inhibitor cocktail set 1) and centrifuged at 12 000 rpm for 10 min, the supernatant removed and protein concentration determined using BCA/CuSO₄ assay. 40 µg of protein per well was resolved by 4-12% gradient Bis-Tris gel electrophoresis (Invitrogen, UK); proteins were transferred to nitrocellulose membranes (Millipore, UK), which were blocked for 1 hour in 5% BSA and probed with primary antibodies: anti-phospho SrcY⁴¹⁹ (1:10000), anti-total Src (1:10000 Cell Signaling Technologies,

UK), anti-phospho FAKY³⁹⁷ (1:10000), anti-phospho FAK⁸⁶¹ (1:10000 Biosource, Belgium), anti-total FAK (1:10000 BD Biosciences, UK) at 4⁰C overnight. Membranes were then incubated with secondary antibodies (anti-rabbit 1:5000 or anti-mouse 1:5000, Cell Signalling Technologies) and visualized with ECL kit (Amersham, UK). Where necessary, the membranes were stripped by incubating with Re-Blot Plus stripping buffer (Chemicon, UK) before re-probing with other antibodies including anti- α Tubulin (1:8000 Santa Cruz, USA) to confirm equal protein loading.

Steroid exposure and withdrawal experiment. LNCaP and LNCaP-SDM cells were seeded in standard culture media at a density of 1×10^6 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 media: serum-free medium, steroid depleted medium containing 10% charcoal-stripped serum and medium containing 10% full foetal calf serum. After 60 hours, which allows for the metabolism of intracellular steroid hormones, cell lysates were prepared and western blot analysis performed as described above.

Src inhibitor exposure experiment. LNCaP and LNCaP-SDM cells were seeded at a density of 1×10^6 per 9 cm Petri dish and cultured using standard culture media until 80% confluent and then treated overnight with a range of dasatinib concentrations. Cell lysates were prepared and western blot analysis performed as described above. Dasatinib was provided by Bristol Myers Squibb and made up as 10 mM stock in DMSO.

Immunoprecipitation. Cell lysates were prepared as described in previous sections and samples containing 500 μ g of protein in 500 μ l incubated with anti-total Lyn (1:50 BD Biosciences) or anti-total Src (1:100 Cancer Research UK) at 4⁰C overnight. Then, anti-mouse IgG agarose beads (Sigma, UK) were added to the samples (20 μ l per sample) and further incubated for 1 hour at 4⁰C. Immune

complexes were analyzed as per western blot protocol with anti-phospho SrcY⁴¹⁹ antibody (1:10000 Cell Signaling Technologies).

Cell migration. To follow random migration, cells were plated at a low density (5×10^4) on glass-bottomed 6-well plates (Iwaki, Japan), allowed to attach overnight and then treated with increasing concentrations of dasatinib, diluted in standard culture media. Migration was monitored by video time-lapse microscopy for 24 hours using a Zeiss Axiovert S100 microscope with x20 objective powered by AQM Advance software (Kinetic Imaging, UK).

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 cells per well of a glass-bottomed six-well plate (Iwaki, Japan). The following day, the wounds through the confluent cells monolayer were made using a fine pipette tip, three wound per each well. The medium was then replaced with standard for each cell line culture medium containing increasing concentrations of dasatinib. The assay was performed over 24 hours with images taken from 3 fields per each well every 30 minutes using a Zeiss Axiovert S100 microscope at x20 magnification. Cell migration was measured by subtraction of the distance between the edge of the wound at time 0 and 24 hours using ImageJ software.

Cell proliferation. Proliferation was assessed using the WST-1 assay as per manufactures instructions. Cells were seeded in 96 well plates at a density 5×10^3 cells per well in standard culture media, allowed to attach overnight and the following day treated with increasing concentrations of dasatinib, DMSO was added to the control well. The assay was performed at 48, 72 and 96 hours by adding 10 μ L of WST-1 reagent prior diluted in Electro Coupling Solution (ECS) to each well. The optical

absorbance level was measured after 2 hours incubation at 37⁰C 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 media for 24 hours after which LNCaP cells were placed in the steroid depleted medium containing 10% charcoal stripped fetal calf serum and LNCaP-SDM cells in RPMI1640 supplemented with 10% full fetal calf serum. WST-1 assay was performed at 48, 72 and 96 hours as described above.

Statistical analysis.

Statistical analysis was performed using Microsoft Excel and the SPSS statistical package (version 9.0). Small changes in protein expression between paired ASPC and AIPC tumours could be due to random errors in the assessment of histoscores. To identify individual patients in whom there was strong evidence of a genuine rise or fall in protein expression, it was required that the change in expression exceed a threshold equal to two standard deviations of the inter-observer difference for that protein. This threshold was chosen because, if there was in reality no difference in protein expression between ASPC and AIPC tumours 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 tumour is of a similar magnitude to the random variation that would affect a single observer assessing two different tumours 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 tumours. 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. In order to determine the level of Src, Lyn and Fgr expression and activation in the transition from ASPC to AIPC we used one hundred paraffin embedded tissue sections taken from fifty patients with prostate cancer who underwent hormone-deprivation therapy and subsequently developed hormone relapse. Due to tissue heterogeneity, full tissue sections were stained using antibodies against total Src, Lyn, Fgr and phospho SrcY⁴¹⁹ representing an active form of SFKs and phospho SrcY⁵²⁷, a marker suggestive of inactive Src (Table 1). The sequences surrounding Src autophosphorylation site tyrosine Y⁴¹⁹ are highly conserved in the majority of SFK and positive staining using this antibody represents autophosphorylation not only of Src but also other Src family members. In our cohort, there was a significant increase in overall pSrcY⁴¹⁹ immunostaining in the transition from ASPC to AIPC, more intense cytoplasm and membrane staining was observed in AIPC samples compared to ASPC (Fig. 1). In addition, although the majority of matched specimens on an individual patient basis showed no change in expression of pSrcY⁴¹⁹ as determined by the weight histoscore in the transition from ASPC to AIPC, a subgroup of patients did have tumours that exhibited a rise in expression as determined by the weighted histoscore technique in the transition from ASPC to AIPC (table 1). Small changes in protein expression between paired ASPC and AIPC tumours could be due to random errors in the assessment of histoscores, therefore the mathematic method as described in the methods statistical analysis section was employed for selection of patients with genuine rises 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 (pSrcY⁴¹⁹) 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 to ASPC (although only membrane staining reached significance). No change in expression was observed for Fgr and, interestingly, expression of total and inactive forms of Src 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 patients (16%) it was positive, implying the presence of bone metastases, and in 38(76%) 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(52%)). The membranes in the tumour samples taken from patients with AIPC stained more intensely with the phospho-SrcY⁴¹⁹ antibody if there was evidence of bone metastases (P=0.011)(Fig 2A). No significant correlation was observed between the presence of bone metastases and cytoplasmic staining with antibodies against phospho-SrcY⁴¹⁹, 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 IHC histoscores

and only expression of cytoplasmic Lyn in the hormone refractory tumours was associated with Gleason grade ($p=0.029$)(Fig. 2A).

Survival analysis. Use of paired prostate cancer specimens taken from patients prior to commencement of hormone manipulation therapy and after androgen escape, made it possible to correlate IHC 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 (pSrcY⁴¹⁹ expression) at the membrane in the transition from ASPC to AIPC, had a 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 to 2.98(1.93-4.63) years for those who had decreased membrane signal or no change ($P=0.005$, Fig. 2B). Furthermore, the time from biochemical relapse to death was also reduced: 1.14 (0.58-2.15) years versus 1.87 (1.1-1.86) years ($P=0.011$, Fig. 2C). 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 pSrcY⁴¹⁹ expression was 2.91(Inter Quartile Range 2.42-4.92) years) compared to 6.33(IQR 4.15-7.27) years for those patients with tumours that had a decrease or no change in pSrcY⁴¹⁹ expression ($P<0.0001$, Fig. 2D). No association with the time to relapse or survival was observed for Fgr, total or inactive forms of Src, however an increase in membrane Lyn expression was associated with a shorter time to relapse ($P=0.022$). Median time from diagnosis to hormone escape in this subgroup of patients was 2.29(1.78-2.79) years compared to 3.2(2.45-3.99) years in those who had a decreased membrane signal or no change ($P=0.022$). 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 to 3.2(1.98-4.45) years in those who had decreased membrane signal or no change

($P=0.040$). However, no association with time from biochemical relapse to death or overall survival were observed.

SFK activity in prostate cancer cell lines. Having demonstrated that SFK activation may affect survival of patients with AIPC, we examined SFK activity in hormone sensitive LNCaP and hormone refractory LNCaP-SDM prostate cancer cell lines. In order to assess the effect of androgen stimulation and withdrawal on Src kinase activity, both cell lines were exposed to various culture media including serum-free medium, medium containing dextran charcoal-stripped foetal calf serum (steroid depleted medium) and full foetal calf serum. SFK activity in LNCaP cells experiencing acute androgen 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 to 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 foetal calf serum containing physiological 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 reduction of SFK activity using the autophosphorylation of Src at tyrosine Y⁴¹⁹ as a marker of activation. Complete inhibition of SFK activity in LNCaP cells was seen at 50 nM (Fig. 3B). SFK activity was also suppressed in LNCaP-SDM cells (Fig. 3C), although complete inhibition was achieved at slightly higher concentrations (75 nM). Interestingly, in LNCaP-SDM cells increasing the dose of dasatinib resulted in an increase in total Src. FAK phosphorylation on tyrosine Y⁸⁶¹ (Src-dependent

phosphorylation site) was also inhibited by treatment with dasatinib in both cell lines in a dose-dependent manner while the levels of phospho FAKY³⁹⁷ (Src-independent autophosphorylation site) and total FAK remained unchanged (Fig. 3B and C).

Due to cross-reactivity of the 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 (data not shown) and LNCaP-SDM (Fig. 4A and B) cells exhibited a similar dose-dependent reduction in migration when treated with increasing concentrations of dasatinib for 48 hours. LNCaP cells cultured in full medium, containing foetal calf serum 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 physiological concentrations of androgens (foetal calf serum), 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. 4C 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 while 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 nM) (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 M (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).

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 (2,20). 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 tumour profiling and combination treatments could be used to overcome drug resistance (21).

Numerous in vitro studies have confirmed the importance of Src in the development of prostate cancer and its progression to a hormone independent state (19-27). 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 upregulated 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 a 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 (26), although our data suggests that its expression does not seem to change with hormone escape. Goldenberg-Furmanov et al. reported that expression of Lyn, another SFK member, tends to be higher in poorly differentiated regions of prostatic tumours and Lyn deficient mice have underdeveloped prostate gland (28). In this study, we demonstrate that expression of Lyn does correlate with Gleason grade and, furthermore, this is a feature of hormone refractory prostate cancer. It has been suggested that various SFK member may play different role in prostatic oncogenesis (29). Experiments using siRNA 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 (28).

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 (25). 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 (20). In animal models, dasatinib treatment was demonstrated to affect primary tumour growth of prostate cancer cells implants as well as reduce the rate of lymph node metastases (29). 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 (30). This is a significant drawback given that there is evidence of AR and Src interactions, especially in a low androgen environments (31). 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 and should not be ignored. LNCaP-SDM cells were developed by gradual withdrawal of androgens from the culture medium mimicking hormone deprivation therapy; they retain functional AR and, therefore, provide a good 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 while cell-cell junctions become weaker and focal adhesions at the rear of the moving cell disassemble (32). 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 as well as hormone refractory prostate cancer in order to 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 and, 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 PDGFR β , Abl, c-Kit and EphA2 (33-34). 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 (34-35). Our experiments showed that dasatinib inhibited proliferation of hormone-refractory LNCaP-SDM cells whereas proliferation of the hormone sensitive LNCaP cells was not affected. However, in a recent publication, it has been demonstrated that dasatinib inhibited proliferation of both LNCaP and PC3-MM2GL cell line with dasatinib concentrations above 100 nM (30). Thus, proliferation of hormone resistant prostate cancer cells is more likely to be affected by SFK inhibitors probably 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 anti-androgens, chemotherapy or other small molecule inhibitors in patients with prostate cancer should be considered (35). As Src inhibitors could potentially be used to prevent the development of metastatic disease, methods to assess this in clinical trials are urgently required.

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Table 1.

| Antibody | Histoscores | | Number of Patients | | | P value |
|----------------------------|---------------|----------------|--------------------|----------|-----------|---------|
| | ASPC | AIPC | Increase | Fall | No change | |
| pSrcY⁴¹⁹ | | | | | | |
| 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 |
| pSrcY⁵²⁷ | | | | | | |
| 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 |

Protein expression for each antibody including median histoscores and inter-quartile ranges. 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 prior to the initiation of hormone deprivation therapy and following hormone relapse. Expression of (A) pSrcY⁴¹⁹, (B) pSrcY⁵²⁷ (C) total Src and (D) Lyn (E) Fgr. Magnification x40.

Fig. 2.

(A) Relationship between membrane pSrcY⁴¹⁹ and presence of bone metastasis at relapse and cytoplasmic Lyn expression in hormone refractory tumours and Gleason grade.

Survival analysis of membrane immunostaining with anti-phospho-SrcY⁴¹⁹ antibody in transition from ASPC to AIPC. Kaplan-Meier survival curves plotting time (B) from prostate cancer from diagnosis to hormone relapse, (C) from hormone relapse to death and (D) from prostate cancer diagnosis to death. Dashed lines represent patients who had an increase in immunohistoscopes in the transition from hormone sensitive to hormone resistant prostate cancer whereas solid lines reflect the 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 media containing no serum (serum-starved, SS), dextran-charcoal stripped fetal calf serum (steroid depleted medium, SDM) and fetal calf serum (full medium, FM). Cell lysates were immunoblotted with anti-phospho-SrcY⁴¹⁹, anti-Src and anti-tubulin antibodies. (B) LNCaP cells and (C) LNCaP-SDM cells were exposed to increasing concentrations of dasatinib in standard corresponding culture media for 16 hours. Cell lysates were immunoblotted with anti-phospho-SrcY⁴¹⁹, 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 media for 16 hours.

Cell lysates were then immunoprecipitated with either Src or Lyn specific antibodies and probed with anti-phospho-SrcY⁴¹⁹ antibody.

Fig. 4. Dasatinib inhibits motility of LNCaP-SDM cells. (A) A wound healing assay performed using LNCaP-SDM cells exposed to a range of dasatinib concentrations. Images were taken after 48 hours and show the relative closure of the wound. (B) The difference in distance (expressed in nm) between the edge of wounds at the beginning of the experiment and after 48 hours observed by time lapse microscopy were plotted against inhibitor concentration. Each value represents the mean \pm S.D. of three separate measurements (three wounds per well). (C) LNCaP-SDM cells were plated at low density and exposed to increasing concentrations of dasatinib for 24 hours. Motility was observed by time lapse microscopy and separate pictures were taken from the stacks at 4 hourly intervals. (D) The number of protrusions produced by the cells over 24 hours in each concentration of inhibitor was calculated. Values are expressed as the mean + S.D. of three individual fields, each field containing around 60 cells. Experiments were repeated three times.

Fig. 5. Dasatinib inhibits proliferation of LNCaP-SDM cells. Cells were plated on 96 well plates and after 24 hours were treated with a range of dasatinib concentrations. After 96 hours a WST-1 assay was performed. The values reflect the mean \pm S.D. of 6 wells, presented as percentages of the untreated control. Experiments were repeated three times.