Immunohistochemical detection of tyrosine phosphatase SHP-1 predicts outcome after radical prostatectomy for localized prostate cancer

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Abstract

The protein tyrosine kinase (PTK) receptors and cytosolic signaling proteins as well as the protein tyrosine phosphatases (PTPs) have important roles in regulation of growth of the benign and malignant prostate gland. Here we studied expression of the protein tyrosine phosphatase SHP-1 in prostate cancer cell lines and in human prostatic tissues. SHP-1 is expressed at a high level in LNCaP prostate cancer cells compared with PC3 cells. Silencing of SHP-1 expression with siRNA in LNCaP cells led to an increased rate of proliferation, whereas overexpression of SHP-1 by means of transient transfection in PC3 cells led to a decrease in proliferation. Corresponding changes were observed in cyclin D1 expression. We further demonstrate that LNCaP and PC3 cells respond differently to IL-6 stimulation. SHP-1 overexpression in PC3 cells reversed IL-6 stimulation of proliferation whereas in SHP-1-silenced LNCaP cells IL-6 inhibition of proliferation was not affected. In addition, IL-6 treatment led to higher levels of phosphorylated STAT3 in SHP-1-silenced LNCaP cells than in control cells. Next, SHP-1 expression in human prostate cancer was analyzed by immunohistochemical staining of tissue microarrays comprising tumor specimens from 100 prostate cancer patients. We found an inverse correlation between the tumor level of SHP-1 expression and time to biochemical recurrence and clinical progression among prostate cancer patients. In conclusion, our results suggest that a decreased level of SHP-1 expression in prostate cancer cells is associated with a high proliferation rate and an increased risk of recurrence or clinical progression after radical prostatectomy for localized prostate cancer.
Introduction

Prostate cancer is one of the most frequent cancers in males in Western countries\(^1\). Prostate-specific antigen (PSA) is used widely as a marker in initial diagnosis of prostate cancer and for monitoring the response to treatment. Radical prostatectomy (RP) serves as the first line of treatment for patients with localized prostate cancer\(^2\). However, in many cases the cancer will recur, as evidenced by rising levels of serum PSA, a phenomenon known as biochemical recurrence (BCR). This is an important concern and it is currently difficult to identify those patients at a high risk of tumor recurrence\(^3\). There are very few biomarkers that can be used to differentiate aggressive from non-aggressive tumors after diagnosis. Thus, molecular markers associated with and predicting disease recurrence are needed to help identify patients with an increased risk of developing tumor recurrence despite efficient local therapy.

Phosphorylation of proteins at specific tyrosine residues has an important role in regulating cell proliferation in multiple aspects of oncogenesis. The level of tyrosine phosphorylation of cellular proteins is controlled by protein tyrosine kinases (PTKs) and the level of dephosphorylation by protein tyrosine phosphatases (PTPs)\(^4\). Extensive studies have been performed to determine the role of PTKs in carcinogenesis and growth regulation. For example, Src-related kinases have been reported to have increased activity or expression levels in several malignancies including prostate cancer\(^5,6\). It has been shown that inhibition of Src activation causes cell cycle arrest and reduces cell migration and cell growth in prostate cancer\(^7\). The epidermal growth factor receptor (EGFR) has also been shown to be upregulated in prostate cancer\(^8\). This receptor can be targeted with
monoclonal antibodies or with tyrosine kinase inhibitors, suppressing the growth and invasion of androgen-dependent and -independent prostate cancer cells in vitro. The functional roles of PTPs in human cancer are, however, much less well understood.

The Src-homology PTP SHP-1 is a non-receptor phosphatase that contains two SH2 domains at its N-terminus, a single catalytic domain, and a C-terminal tail that contains tyrosine residues. SHP-1 is expressed in normal hematopoietic and epithelial cells. Decreased or abolished expression in leukemia and lymphoma has been related to both malignant transformation and tumor cell invasiveness. Moreover, SHP-1 expression in leukemic cells can lead to decreased proliferation and induction of morphological changes. Transient transfection of SHP-1 in the breast cancer cell line MDA-MB-231, with undetectable endogenous SHP-1, reduced cell proliferation 2–3 fold. Zapata et al have detected SHP-1 expression in the normal human prostate as well as in prostate cancer and they showed evidence of decreased SHP-1 expression in prostate cancer. SHP-1 has been shown to be involved in regulating signals of cytokines such as IL-3R in leukemic cells, PDGF- and EGF receptor and other tyrosine kinase receptors in non-hematopoietic cells.

Interleukin-6 (IL-6) is a cytokine that has been implicated in the development and progression of several types of tumors including those in the prostate. The expression of IL-6 and its receptor has been shown in human prostate cancer cell lines and in clinical specimens of prostate cancer and benign prostate hyperplasia. High levels of IL-6 are observed in the plasma of patients with prostate cancer, which correlate with more
advanced stages of the disease, therapy resistance, and poor prognosis. In *in vitro* experiments IL-6 has been reported to affect cell proliferation and to induce neuroendocrine (NE) differentiation of prostate cancer cells in a complicated manner depending on autocrine or paracrine cytokine action. IL-6 binds to its receptor and mainly transduces signals through MAP kinase/ERK, PI3K/AKT and STAT3 pathways. It has been shown in oligodendrocytes from the motheaten mice, which have a genetic defect in the SHP-1 gene, that SHP-1 expression controls the activation of STAT3 by IL-6 signaling.

In this study we examined SHP-1 in prostate cancer cell lines and in clinically well-annotated tumor tissue samples. We demonstrate that the prostatic cancer cell lines LNCaP and PC3 express SHP-1 mRNA and protein at different levels. To investigate the functional role of SHP-1 in the prostate cancer cell lines, SHP-1 expression was inhibited with siRNA (small interfering RNA) or overexpressed by means of transient and stable transfection. In LNCaP cells, with a high endogenous level of SHP-1 expression, inhibition of SHP-1 resulted in an increase in cellular proliferation and cyclin D1 expression. On the other hand, PC3 cells, which express low amounts of endogenous SHP-1 protein, exhibited a decrease in proliferation and cyclin D1 when SHP-1 was overexpressed. We further demonstrate that LNCaP and PC3 cells respond differently to IL-6 stimulation. IL-6 inhibits LNCaP cell growth, whereas it increases proliferation in PC3 cells. After inhibiting SHP-1 expression in IL-6 stimulated LNCaP cells there was no difference in response compared to control cells, whereas overexpression of SHP-1 in PC3
cells abolished the proliferative effect of IL-6. We also show that levels of phosphorylated STAT3 are increased in SHP-1 silenced LNCaP cells after IL-6 treatment.

In tumor samples obtained from 100 patients surgically treated by means of open radical prostatectomy, the level of SHP-1 expression varied from high (as in normal prostate) to undetectable. Expression of SHP-1 did not correlate with serum preoperative PSA levels, Gleason score, tumor volume or prostate volume. Interestingly though, survival analysis revealed that low tumor-specific SHP-1 expression was associated with significantly shorter biochemical recurrence (BCR)-free and clinical progression-free times. The association remained significant in multivariate analysis, adjusted for established prognostic parameters.
Material and Methods

Cell cultures

The lymph node metastasis-derived human prostate cancer cell line LNCaP and the bone metastasis-derived prostate cancer cell line PC3 were obtained from the American Type Culture Collection (Manassas, VA). LNCaP cells were maintained in RPM1 1640 and PC3 cells in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen, San Diego, CA) containing 10% fetal calf serum (FCS; Invitrogen) in a humidified atmosphere of 5% CO₂ at 37 °C.

Quantitative real-time PCR (qPCR)

Total RNA was isolated from cell lines PC3 and LNCaP with TRIzol (Invitrogen, San Diego, CA, USA) and cDNA was prepared from two µg of RNA using a First Strand cDNA Synthesis Kit according to the manufacturer’s instructions (Amersham Biosciences, Amersham, UK). Quantitative PCR was performed using Finnzymes DyNAmo SYBR Green qPCR Kits (Finnzymes Oy, Finland) according to the manufacturer’s instructions. Oligonucleotide sequences were as follows: SHP-1, 5’-GCC CAG TTC ATT GAA ACC AC-3’ (sense) and 5’-GAG GGA ACC CTT GCT CTT CT-3’ (antisense); GAPDH, 5’-CGA CCA CTT TGT CAA GCT CA-3’ (sense) and 5’-AGG GGT CTA CAT GGC AAC TG-3’ (antisense). The following PCR conditions were used: denaturation at 95 °C for 10 min followed by 35 cycles of 94 °C for 1 min, annealing for 1 min at 57 °C and elongation for 1 min at 72 °C, and a final elongation step at 72 °C for 10 min.

cDNA microarray

Gene expression levels in PC3 and LNCaP cells were analyzed by using an Illumina Beadchip cDNA microarray. In brief, total RNA from cells was extracted, utilizing a two-
step procedure. A first extraction with TRIzol reagent (Invitrogen) was followed by an RNeasy (Qiagen, Valencia, CA) clean-up. One hundred and fifty ng of RNA from each sample was amplified and Cy3-labeled by utilizing a TotalPrep RNA amplification kit (Ambion, Austin, TX) and hybridized to a HumanRef-8 v2 expression BeadChip (Illumina, San Diego, CA). The chip was analyzed in a Bead Station 500, using BeadStudio software (Illumina).

**Western Blotting**

Cells were lysed with ice-cold lysis buffer: 9 mM Hepes, 0.08 mM EDTA, 0.08 mM EGTA, 9 mM KCL, 5% NP-40 and complete protease inhibitor (Roche, Mannheim, Germany) at 4 °C for 10 min. The samples were centrifuged for 15 min at 16 000 × g at 4 °C and the supernatants were collected. Protein concentrations were determined by using a BCA protein assay (Pierce Biotechnology, Inc., Rockford USA). Thirty µg of each protein sample was subjected to 10% SDS-PAGE and transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, USA). The membranes were blocked in Odyssey’s blocking buffer (Li-Cor Biotechnology, Nebraska, USA) and probed with antibodies against polyclonal rabbit anti-SHP-1 diluted 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal mouse anti-Actin diluted 1:5000 (Sigma-Adrich, St.Louis, MO), monoclonal rabbit anti-cyclin D1 diluted 1:1000 (Abcam, Cambridge, MA) or monoclonal rabbit anti-phosphotyrosine STAT3 diluted 1:1000 (Cell Signaling Technologies, MA) in blocking buffer diluted 1:3 in PBS for 2 h at room temperature (RT) or overnight at 4 °C, followed by IRDye goat anti-mouse IgG or IRDye goat anti-rabbit IgG secondary antibodies diluted 1:3000 and 1:1000, respectively, for 45 min at RT and visualized using the Odyssey detection system.
Transfection, proliferation and IL-6 stimulation of prostate cancer cells

For transient transfection studies, PC3 and LNCaP cells were plated in 96-well plates, serum-starved overnight and transfected with pEGFP or pEGFP-SHP1-WT plasmids (kind gifts from Prof. Frank Böhmer, Jena University, Germany), SHP-1 siRNA or scrambled control siRNA (Dharmacon, Thermo Fisher Scientific, Lafayette, CO) using lipofectamin 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 h the medium was changed to medium containing 10% FCS and the cells were cultured for another 72 h. Stable transfection of PC3 cells was performed by selection with G418 (500 µg/ml) for 8 weeks. After selection the cells were plated in 96-well plates, serum-starved overnight and cultured in medium containing FCS for 48 h. Stimulation studies were performed by plating transfected cells, which had been serum-starved overnight, in 96-well plates and incubating in medium supplemented with 1% dextran-charcoal treated FCS (DC-FCS) in the presence or absence of recombinant IL-6 (Peprotech, London, UK) for 72 h. [³H]-thymidine (0.5 µCi/well) was added 6 h prior to termination of the experiment and thymidine incorporation was measured by scintillation counting in a Wallac MicroBeta TriLux (Wallac Oy, Turku, Finland). For analysis of cyclin D1 expression transiently-transfected LNCaP cells and stably-transfected PC3 cells were plated in 6-well plates (Sarstedt, Germany), serum-starved overnight and cultured in medium containing 10% FCS for 6 and 24 h. STAT3 protein expression was analyzed in transfected LNCaP cells treated with or without 50 ng/ml IL-6 for 20 min.

Cell cycle analysis by flow cytometry

The effect of SHP-1 on cells entering the S-phase of the cell cycle was determined by flow cytometry. PC3 and LNCaP cells were plated in 6 cm plates (Sarstedt, Germany), serum-
starved overnight and then transfected with either pEGFP plasmids, pEGFP-SHP1-WT plasmids or SHP-1 siRNA (Dharmacon, Thermo Fisher Scientific) using lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 h, FCS was added to the medium (10%). The cells were washed with PBS and fixed in ice-cold 70% ethanol for 15 min. After treatment with RNase A (100 µg/ml), propidium iodide (50 µg/ml) and 0.05% Triton X-100 for 30 min, the number of cells in the S-phase was analyzed by using a FACS Calibur flow cytometer (BD Bioscience, New Jersey, USA).

**Prostate cancer tissue microarray analysis**

The prostate cancer tissue microarray (TMA) included tumor specimens obtained from 122 patients treated by means of radical prostatectomy for localized prostate cancer. The patients gave written consent and the study was approved by the local ethics committee (ref. 445-07). Patient and tumor characteristics are given in Table 1 and include age at surgery, preoperative concentration of serum PSA, prostate volume, tumor volume, clinical stage, Gleason score, extra capsular extension, seminal vesicle invasion, positive surgical margins and lymph node involvement. After a mean follow-up period of 58.8 month, two patients had died from prostate cancer, two from other causes and 118 patients were alive. Twenty of the 100 patients in whom SHP-1 could be evaluated had a BCR during the follow-up period and 25 showed progression of the disease.

Tissue microarrays were constructed as described previously. In brief, areas representing cancer as well as normal prostate tissue were marked on hematoxylin/eosin-stained sections and two 1.00 mm tissue cores were extracted and mounted using a manual arraying device (MTA-1 Beecher Instruments Inc., Sun Prairie, WI).

Immunohistochemical (IHC) staining of SHP-1 was performed with a polyclonal anti-
human SHP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using an automated
IHC staining procedure (Techmate 500 Dako, Copenhagen, Denmark). Briefly, 4-µm-
thick sections were dried, deparaffinized, rehydrated and incubated with Target Retrieval
Solution (pH 9.9; Dako) in a microwave oven at 900, 750, 650 and 300 W for 2 min at
each energy level. The sections were incubated with primary antibody at a final
concentration of 0.2 µg/ml. Dako real envision detection system peroxidase/DAB (Dako)
was used for incubation with secondary antibodies and for visualization. Hematoxylin was
used to counter-stain cell nuclei. The stained sections were evaluated by two experienced
clinical pathologists (KJ and RE) and by three of the other authors (HT, LB and PH).

Cytoplasmic staining intensity was graded from 0 to 3, with 0 corresponding to ‘no
staining’, 1 to ‘weak staining’, 2 to ‘moderate staining’ and 3 to ‘strong staining’. For
survival analysis, SHP-1 expression was dichotomized into high (scores 2 & 3) and low
(scores 0 & 1). PC3 and LNCaP cell pellets were fixed in 4% paraformaldehyde,
embedded in paraffin and IHC staining was performed as described above.

**Statistical Analysis**

Spearman’s rank correlation test was used to analyze the correlation between SHP-1
expression and clinical parameters. Kaplan–Meier analysis and the log rank test were used
to analyze differences in BCR-free time according to SHP-1 expression. Biochemical
recurrence was defined as PSA >0.2 ng/ml with a confirmatory level and clinical
progression included BCR, death from prostate cancer and adjuvant radiation therapy.

Cox’s proportional hazard regression model was used for univariate and multivariate
analysis. Low staining intensity was used as the referent category when calculating hazard
ratios. Diagrams representing cell proliferation are presented as mean ± SE and the data
was analyzed using Student’s t-test. Data presented are representative of three independent experiments. All calculations were performed using SPSS version 15.0 (SPSS Inc.) All statistical tests were two-sided, and $p$ values less than 0.05 were considered to be statistically significant.
Results

SHP-1 expression in prostate cancer cell lines

Examination of SHP-1 mRNA expression by means of cDNA microarray and real-time quantitative PCR in LNCaP cells and PC3 cells revealed almost five-fold greater SHP-1 expression in LNCaP cells compared with PC3 cells (Fig. 1a). The difference in expression was confirmed at the protein level, using both immunocytochemistry and Western blot analysis (Fig. 1b).

Functional analysis of SHP-1 in prostate cancer cell lines

To investigate whether SHP-1 affects proliferation in prostate cancer cells, we inhibited expression of SHP-1 with siRNA in LNCaP cells, which express high levels of endogenous SHP-1, and overexpressed SHP-1 in PC3 cells, with low endogenous SHP-1 expression. The transfected cells were analyzed for [3H]-thymidine uptake after 72 h or 48 h in case of stably-transfected PC3 cells (Fig. 2a). The SHP-1 siRNA-treated LNCaP cells showed a higher proliferation rate than the controls that were transfected with a scrambled control siRNA. Interestingly, in both the transient as well as the stably-transfected PC3 cells, the proliferation rate was decreased in comparison with control cells. Western blot analysis was performed to confirm successful overexpression or knock-down of SHP-1 (Fig. 2b). Indeed, SHP-1 siRNA treatment of LNCaP cells reduced SHP-1 expression, whereas overexpression of SHP-1 was demonstrated in PC3 cells. To confirm the proliferation data we stained the cells with propidium iodide to perform cell cycle analysis using flow cytometry (Fig. 2c). The analysis showed that more cells entered the S-phase in siRNA-treated LNCaP cells than in parental or siControl RNA-treated cells, whereas upon overexpression of SHP-1 in PC3 cells the number of cells that entered the S-phase was
reduced. To understand the mechanism underlying the growth arrest and proliferation in siRNA-treated LNCaP and SHP-1-overexpressing PC3 cells we analysed the level of cyclin D1 protein that regulates G1 to S phase progression of the cell cycle (Fig. 2d). The cells were treated with serum for 6 and 24 h, and indeed cyclin D1 expression was stronger in siRNA-treated LNCaP cells than in control cells, whereas in SHP-1-overexpressing PC3 cells the cyclin D1 levels were decreased when compared to the control.

**IL-6 stimulation of prostate cancer cell lines**

Since IL-6 has been implicated to be involved in the development and progression of prostate cancer, we studied whether SHP-1 is involved in the response of LNCaP and PC3 cells to IL-6 stimulation. As shown previously, LNCaP cells showed a growth inhibitory response to IL-6, whereas in PC3 cells IL-6 had a growth stimulatory response (Fig. 3a). Because SHP-1 is a phosphatase that is involved in modulation of receptor signaling, we also analyzed the IL-6 response in SHP-1 siRNA-treated LNCaP cells, and SHP-1 overexpressing PC3 cells. In LNCaP cells, silencing SHP-1 expression did not affect the growth inhibitory response to IL-6, whereas in PC3 cells, overexpressing SHP-1 abolished the growth stimulatory response to IL-6 (Fig. 3b). Since SHP-1 has been implicated to have a role in activation of STAT3 by IL-6 we analyzed the phosphorylation status of STAT3 after IL-6 treatment in LNCaP cells. Indeed, the siRNA-treated LNCaP cells showed increased levels of phosphorylated STAT3 compared to siControl-treated cells (Fig 3c). Because PC3 cells do not express endogenous STAT3 protein (data not shown) they were not analyzed for phosphorylated STAT3 levels.

**SHP-1 expression in prostate cancer**
Analysis of SHP-1 expression in prostate cancer tissue revealed that SHP-1 immune reactivity was localized to the cytoplasm of prostate epithelial cells and the tumor cells that were stained for SHP-1. Expression of SHP-1 could be evaluated in 100 cases – the remaining cases were not available for analysis owing to core loss during sectioning and staining. Staining was strong in normal epithelial cells, whereas in tumor cells staining intensity was heterogeneous, varying from absent to strong. Representative photomicrographs are shown in Figure 4. Nine of the cores showed no staining, thirty-three had weak staining, fifty had a moderate staining and eight showed strong staining. No staining was seen in stromal cells but occasionally we found infiltrating cells of hematopoietic origin in the stromal compartment, with strong immunoreactivity towards SHP-1.

**Correlation of SHP-1 with PSA and clinicopathological parameters**

We used statistical analyses to assess the potential association of the intensity of SHP-1 expression with preoperative levels of serum PSA, Gleason grades and other tumor characteristics. Expression of SHP-1 was dichotomized into high (scores 2 & 3) and low (scores 0 & 1). In Spearman’s rank analysis we found no statistically significant correlations between SHP-1 expression and preoperative PSA levels ($p=0.835$; Fig. 5a), Gleason scores ($p=0.857$; Fig. 5b), tumor volume ($p=0.082$; Fig. 5c) or prostate volume ($p=0.243$; Fig. 5d).

**Survival analysis**

A postoperative rise in serum PSA levels (biochemical recurrence, BCR), defined as a PSA concentration $>0.2$ ng/ml in blood, with a confirmatory value, is widely used to monitor the outcome of radical prostatectomy. We investigated whether or not SHP-1
expression was correlated to clinical outcome, using BCR-free time and clinical progression-free time as primary endpoints. Twenty of the 100 (20%) patients in whom SHP-1 could be evaluated experienced BCR during the follow-up period and twenty-five (25%) showed disease progression.

As shown in Figure 6, patients with low levels of tumor-specific SHP-1 expression in prostatectomy specimens had a significantly poorer outcome with respect to BCR and clinical progression compared with patients with high SHP-1 expression ($p=0.021$ and $p=0.004$ respectively). Univariate analysis showed that SHP-1 expression was a significant prognostic factor as regards BCR (hazard ratio (HR) 0.591, 95% CI 0.370–0.942, $p=0.027$) and clinical progression (HR 0.552, 95% CI 0.360–0.846, $p=0.006$). In multivariate analysis including adjustment for preoperative PSA level, Gleason score, and presence or absence of extra capsular extension, seminal vesicle invasion and positive surgical margins, SHP-1 remained an independent prognostic factor as regards BCR-free time (HR 0.488, 95% CI 0.275–0.867, $p=0.014$) and clinical progression (HR 0.468, 95% CI 0.283–0.774, $p=0.003$) (Table 2).
Discussion

Here we have shown that the PTP SHP-1 is expressed at a high level in normal prostate tissue and is commonly decreased to a low or non-detectable level in prostate cancer. Most importantly, decreased levels of SHP-1 were associated with a significantly shorter time to biochemical recurrence and clinical progression after radical prostatectomy for localized prostate cancer.

In an attempt to define the functional role of SHP-1 in prostate cancer, we used the prostatic cancer cell lines LNCaP and PC3, which express SHP-1 mRNA and protein at relatively high and low levels, respectively. We showed that inhibition of SHP-1 expression with siRNA resulted in increased cellular proliferation, whereas overexpression decreased the proliferation rate. These results suggest that SHP-1 has an important role in maintaining normal prostate function and in regulation of prostate cancer cell growth. Our results are in agreement with those of Zapata et al., who reported decreased SHP-1 expression in prostate cancer as well as inhibition of growth of PC3 cells overexpressing SHP-1. Corresponding results have also been shown in breast cancer cells. Wu et al. have reported that introduction of SHP-1 to breast cancer cells with low endogenous expression levels results in decreased cell proliferation. These observations suggest that down regulation of SHP-1 is associated with increased proliferation of malignant cells. A large number of studies have been focused on the role of SHP-1 in hematopoietic cells, whereas the role of SHP-1 in epithelial cells and epithelium-derived carcinomas is less well characterized. In hematopoietic cells SHP-1 has been suggested to contribute to the termination of mitogenic signals of growth factors by dephosphorylating critical phosphorylated molecules. SHP-1 has been shown to be associated with and to
dephosphorylate cell membrane receptors such as INFα/β receptors and the Epo receptor, and/or other substrates such as JAK\textsuperscript{38,39}. It is possible that SHP-1 regulates the JAK/STAT signaling transduction pathway via this mechanism, leading to a decrease in proliferative signals\textsuperscript{38-40}. SHP-1 has also been shown to be associated with epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and interleukin-3 receptor (IL-3R) in epithelial cells\textsuperscript{18-20,40}, thereby possibly modulating cell signaling. EGFR is involved in the pathogenesis of several types of tumor\textsuperscript{41} and both EGF and EGFR are frequently upregulated in advanced stages of prostate cancer\textsuperscript{8}. Activated EGFR pathways are considered to belong to mechanisms that take over upon development of androgen resistance\textsuperscript{42-44}. Similarly, the IL-6 pathway has been suggested to play an important role in the development and growth of androgen-independent prostate cancer\textsuperscript{45}.

As shown before by others\textsuperscript{46-48} we also found that a short term treatment with IL-6 inhibits growth of androgen-responsive LNCaP cells, whereas in androgen-unresponsive PC3 cells it evokes a growth stimulatory effect. We then analyzed how SHP-1 expression affects the IL-6 response in these cell lines. We show that the response to IL-6 was not affected in SHP-1 silenced LNCaP cells, whereas in SHP-1 overexpressing PC3 cells the growth stimulatory response to IL-6 was abolished. These results suggest that SHP-1 is involved in IL-6 signaling as a negative regulator. An LNCaP subline which has been generated through continuous treatment with IL-6 (LNCaP-IL6+), exhibits an increased basal proliferation rate and does not show the growth inhibitory response to IL-6\textsuperscript{47} as parental LNCaP cells do. It is obvious that LNCaP-IL6+ cells utilize different signaling pathways than the parental LNCaP cells and therefore, it would be interesting to study the
role of SHP-1 in regulation of proliferation and neuroendocrine differentiation in these cells.

The action of IL-6 may involve the use of multiple pathways such as MAP kinase/ERK, PI3K/AKT and STAT3 to differentially regulate androgen receptor (AR) transactivation and/or AR-mediated cell growth in prostate cancer cells. SHP-1 is known to reduce activation of ERK, AKT and STAT3. We demonstrate that IL-6 activates STAT3 in LNCaP cells, and these results are in agreement with Spiotto et al. who have shown that the growth inhibitory response to IL-6 in LNCaP cells correlates with activated STAT3. We also show that silencing of SHP-1 expression increases the levels of phosphorylated STAT3 in LNCaP cells. These results suggest that SHP-1 is involved in the IL-6/STAT3 pathway in LNCaP cells. In PC3 cells, lacking STAT3 expression (data not shown), it could be the MAP kinase/ERK pathway that is involved in IL-6 stimulation. On the other hand, constitutively activated STAT3 levels have been shown to be elevated in prostate cancer. These elevated levels are associated with more aggressive and poorly-differentiated tumors, a significantly greater rate of BCR and induction of metastatic behavior of human prostate cancer. In prostate cancer cell lines STAT3 induces motility and invasiveness.

The suppressors of cytokine signaling-1 (SOCS-1) and SOCS-3 are regulatory proteins involved in terminating IL-6 signaling by inhibiting the JAK/STAT pathway. SOCS-1 expression is increased by IL-6 in prostate cancer cell lines and it has been shown that SOCS-1 has a growth-inhibitory effect in these cells. SOCS-1 down-regulation did not,
however, prevent IL-6 induced growth inhibition of LNCaP cells. Interestingly, we observed the same for SHP-1 down-regulation in LNCaP cells. Moreover, SOCS-3 is differently expressed in LNCaP and LNCaP-IL6+ cell lines. LNCaP cells are SOCS-3 negative and express increased levels of phosphorylated STAT3 in response to IL-6, whereas the SOCS-3 positive LNCaP-IL6+ cell line showed minimal response of STAT3 phosphorylation by IL-6. The relationship of SHP-1 and SOCS-1/3 in IL-6 signaling is presently not known but they can be expected to be related to each other. In any case, our results suggest that, similar to SOCS-1, the negative growth regulatory effect of SHP-1 (as in PC3 cells) is independent of STAT3. It is possible that various IL-6 responses such as those in proliferation and neuroendocrine differentiation are mediated by different signaling pathways and in a cell-specific manner.

In prostate cancer, cross-talk between AR and growth factor receptor signaling has an important role in mediation of androgen-regulated homeostasis, and disruption of this interaction is involved in the development of androgen-independent growth. Therefore, SHP-1 could have an important role in modulation of the signaling pathways initiated by growth factors and/or cytokines in the prostate. Further studies are needed to determine the exact role and the pathways involved in SHP-1 signaling in prostate cancer.

Serum PSA concentrations decrease to an undetectable level in most patients following radical prostatectomy. Reflecting successful eradication of tumor tissue, a subsequent rise in PSA levels is a marker of tumor recurrence. Unfortunately, current diagnostic and prognostic markers, including preoperative PSA levels and histological grade of the tumor,
do not sufficiently distinguish those patients at risk of disease recurrence. Therefore, it is of importance to find new molecular markers for better prognostic stratification of prostate cancer patients. Here we show that a decrease or loss of SHP-1 expression correlates with a significantly decreased time to BCR and clinical progression, and multivariate analysis showed that SHP-1 remained an independent predictor when adjusted for preoperative PSA level, Gleason score, and presence or absence of extra capsular extension, seminal vesicle invasion and positive surgical margins. We conclude that SHP-1 probably suppresses growth and invasive behavior of prostate cancer cells through its function as a negative regulator of signal transduction pathways initiated by various external stimuli. Together, our findings suggest that low SHP-1 expression might serve as a marker to identify prostate cancer patients at an increased risk of tumor recurrence.
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Figure legends

Figure 1. SHP-1 expression in prostate cancer cell lines. Relative mRNA expression levels in LNCaP and PC3 cells analyzed by means of cDNA microarray and confirmed with quantitative RT-PCR (qPCR) (a). Immunocytochemical and Western blot (b) analysis showing the protein expression of SHP-1 in the cancer cell lines LNCaP and PC3. Actin served as a loading control.

Figure 2. Effects of SHP-1 expression levels on cell proliferation. LNCaP cells were transfected with SHP-1 siRNA or siControl and PC3 cells transfected transiently or stably with pEGFP-SHP1-WT or the empty vector pEGFP. (a) Cells were grown in serum-free medium for 24 h prior to transfection. For stable transfection the cells were selected with G418 (500 µg/ml) for 8 weeks. Transiently and stably-transfected cells were incubated with serum (10%) for 72 h and 48 h, respectively, and [3H]-thymidine (0.5 µCi/well) was added 6 h prior to harvesting the cultures. (b) Western blot analysis of SHP-1 expression in transfected LNCaP, PC3 and stably-transfected PC3 cells as a control of transfection efficiency. Actin served as a loading control. (c) Cell cycle analysis of percentage of cells entering the S-phase in transfected LNCaP and PC3 cells. (d) Cyclin D1 expression in LNCaP cells and stably-transfected PC3 cells treated with serum (10%). Data in (a) is expressed as mean ± SE (n=3). *, p<0.05; ***, p<0.001.

Figure 3. Effect of IL-6 stimulation on proliferation of LNCaP and PC3 cells. Parental cells were stimulated with IL-6 for 72h in the presence of 1% DC-FCS (a). SHP-1 SiRNA-transfected LNCaP cells and PC3 cells stably-transfected with SHP-1 were
stimulated as described above (b). Levels of phosphorylated STAT3 in siRNA-treated LNCaP cells stimulated with 50 ng/ml IL-6 for 20 min (c). Data is expressed as mean ± SE (n=3). *, p<0.05; **, p<0.01.

**Figure 4.** Representative photomicrographs of SHP-1 immunohistochemistry in benign prostate tissue and in prostate cancer. Cytoplasmic SHP-1 expression in epithelial cells was scored on the basis of intensity of staining from 0–3, with 0 corresponding to ‘no staining’, 1 to ‘weak staining’, 2 to ‘moderate staining’ and 3 to ‘strong staining’.

**Figure 5.** Spearman’s rank analysis of SHP-1 staining intensity with preoperative serum PSA levels (a), Gleason score (b), tumor volume (c) and prostate volume (d). SHP-1 expression was dichotomized into high (scores 2 & 3) and low (scores 0 & 1).

**Figure 6.** Kaplan–Meier analysis of time to biochemical recurrence, (BCR-free time, months) (a) and clinical progression (progression-free time, months) (b) dichotomized into high (scoring 2 & 3) and low (scoring 0 & 1) SHP-1 expression. The log-rank test for significance yielded p values of 0.021 and 0.004, respectively.
Table 1
Clinical and pathological characteristics of the patient cohort.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median (range)</th>
<th>n</th>
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<tbody>
<tr>
<td>Median (range) age at surgery (Years)</td>
<td>63 (48-74)</td>
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<tr>
<td>Mean (range) preoperative PSA (ng/ml)</td>
<td>9.6 (2.6-36.5)</td>
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<tr>
<td>Mean prostate volume (range) (ml)</td>
<td>40 (16-166)</td>
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</tr>
<tr>
<td>Mean tumor volume (range) (ml)</td>
<td>4.4 (0-15)</td>
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<table>
<thead>
<tr>
<th>Clinical Stage</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>n</th>
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<td>64</td>
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<table>
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<tr>
<th>Gleason sum</th>
<th>≤ 6</th>
<th>7</th>
<th>≥ 8</th>
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<td>68</td>
<td>46</td>
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<td>122</td>
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<th>Seminal vesicle invasion*</th>
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<th>No</th>
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<td></td>
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<td>106</td>
<td>121</td>
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<table>
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<th>Positive surgical margins*</th>
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<th>n</th>
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<td></td>
<td>69</td>
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<table>
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<th>Lymph node involvement*</th>
<th>Yes</th>
<th>No</th>
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* Histopathological data was not available for every patient
Table 2
Cox’s uni- and multivariate analysis of biochemical recurrence-free and progression-free time according to low and high expression of SHP-1. Multivariate analysis included adjustment for preoperative PSA level, Gleason score, and presence or absence of extracapsular extension, seminal vesicle invasion and positive surgical margins.

<table>
<thead>
<tr>
<th>SHP-1 staining intensity</th>
<th>BCR free time</th>
<th>Progression free time</th>
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<tr>
<td></td>
<td>RR (95% CI)</td>
<td>p-value</td>
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<td><strong>Univariate</strong></td>
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<td>Low (score 0-1)</td>
<td>1.000</td>
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<tr>
<td>High (score 2-3)</td>
<td>0.591 (0.370-0.942)</td>
<td>0.027</td>
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<tr>
<td><strong>Multivariate</strong></td>
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<tr>
<td>Low (score 0-1)</td>
<td>1.000</td>
<td></td>
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<tr>
<td>High (score 2-3)</td>
<td>0.488 (0.275-0.867)</td>
<td>0.014</td>
</tr>
</tbody>
</table>
Figure 1

(a) cDNA

(b) LNCaP and PC3

Fold change

LNCaP  PC3

qPCR

LNCaP  PC3

Figure 1
138x84mm (300 x 300 DPI)
Figure 2
209x270mm (300 x 300 DPI)
Figure 3

137x150mm (300 x 300 DPI)
Figure 4
50x281mm (300 x 300 DPI)
Figure 5
152x108mm (300 x 300 DPI)
Figure 6
61x123mm (300 x 300 DPI)