Low expression of SHP-2 is associated with less favorable prostate cancer outcomes

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Abstract

Src homology 2 domain-containing tyrosine phosphatase-2 (SHP-2) is an important regulator of cell signaling because of its ability to dephosphorylate receptors of growth factors as well as the cytokines and tyrosine-phosphorylated proteins associated with these receptors. In the current study, we used four different prostate cancer cell lines: PC3, DU145, LNCaP and LNCaP-IL6+. Tumor specimens from 122 patients with prostate cancer were analyzed using a tissue microarray. Our data demonstrate that all four prostate cancer cell lines express the SHP-2 protein. Additionally, low staining intensity and SHP-2 expression in the cytoplasm of cancer cells in prostate tumor specimens was inversely correlated with prostate volume ($p=0.041$ and $p=0.042$, respectively) whereas nuclear staining was positively correlated with extracapsular extension (ece) ($p=0.039$). In our post-prostatectomy specimens, we found that patients with low SHP-2 expression had less-favorable outcomes with respect to biochemical recurrence (BCR) and clinical progression ($p=0.005$ and $p=0.018$ respectively). The loss of cytoplasmic SHP-2 expression is associated with increased growth and prostatic cancer progression.

Keywords: Src homology 2 domain-containing tyrosine phosphatase-2 (SHP-2), prostate cancer, tissue microarray (TMA)

Introduction

The growth of prostate cancer cells is regulated by several factors, including steroid hormones, growth factors and cytokines [1]. For patients with locally advanced and/or metastatic prostate cancer, hormone manipulation through androgen blockade is the main treatment modality [2]. However, the treatment response is often transient and a majority of patients become resistant to ablation treatment within 2–3 years. Once failure occurs, few therapeutic options remain. In therapy-resistant tumors, however, androgen receptors (ARs) may remain active, possibly because of cross talk between AR and multiple cell membrane receptor-initiated pathways. These activated intracellular signaling cascades include mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), phosphatidylinositol-3 kinase/AKT (PI3K/AKT) [3] and signal transducer and activator of transcription 3 (STAT3) [4]. Additionally, protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs) play a crucial role in the regulation of these pathways. In fact, if the balance between PTPs and PTKs is disrupted and tyrosine-phosphorylated proteins accumulate, abnormal proliferation and cell function may result [5].

We have recently shown that the down-regulation of SHP-1 in prostate cancer cells is associated with high rates of cell proliferation, increased risk of biochemical recurrence and clinical progression following radical prostatectomy [6]. SHP-2, a Src homology 2 (SH2) domain-containing PTP sharing homology with SHP-1 [7], is expressed widely in a variety of cells and tissues [7]. SHPs are intimately involved in many cellular activities,

including cytoskeleton maintenance, cell division and differentiation [8]. SHP-2 is believed to up-regulate a variety of signal transduction processes. In a number of studies, SHP-2 has also been shown to promote cell survival [8]. Indeed, SHP-2 promotes the activation of the Ras-MAPK signaling pathway through receptors for various growth factors and cytokines [9]. However, other investigations have demonstrated that SHP-2 inhibits PDGF signaling [10] and accelerates the induction of apoptosis [11] through STAT5 dephosphorylation [12].

Material and methods

Cell cultures. LNCaP cells derived from metastatic prostate cancer of the lymph nodes, DU145 cells derived from metastatic prostate cancer of the brain, and PC3 cells derived from metastatic prostate cancer of the bone were obtained from American Type Culture Collection (Manassas, VA). LNCaP cells were maintained in RPMI 1640 medium (Invitrogen, San Diego, CA) containing 10% fetal calf serum (FCS; Invitrogen). DU145 and PC3 were maintained in a humidified atmosphere of 5% CO_2 at 37°C in DMEM medium (Invitrogen) containing 10% FCS. The LNCaP-IL6+ sub cell line was provided by Zoran Culig [13] and was grown in the manner described above for LNCaP cells (but supplemented with 5ng/mL of IL6).

Prostate cancer tissue microarray analysis and cell line arrays. The prostate cancer tissue microarray (TMA) included tumor specimens obtained from 122 patients treated between 1998 and 2003 at Skåne University Hospital, Malmö, Sweden by open radical prostatectomy for localized prostate cancer. The patients provided informed consent, and the study was approved by the Regional Ethical Review Board at Lund University, Sweden (approval number DN. 445-07). Patient and tumor characteristics are detailed in (Table 1). After a mean follow-up period of 58.8 months, two patients had died from prostate cancer, two patients had died from other causes, and 118 patients remained alive.

Tissue microarrays were constructed as previously described [14]. In brief, areas representing cancer and normal prostate tissue were marked on hematoxylin/eosin-stained sections. Two 1.00 mm tissue cores were then

extracted and mounted using a manual array device (MTA-1 Beecher Instruments Inc., Sun Prairie, WI). Immunohistochemical (IHC) staining for SHP-2 was performed using an automated IHC staining procedure (Techmate 500 Dako, Copenhagen, Denmark) and a polyclonal anti-human SHP-2 antibody (C-18) (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, 4-µm-thick sections were dried, de-paraffinized, rehydrated and incubated with Target Retrieval Solution (pH 9.9; Dako) in a microwave oven for two minutes each at 900, 750, 650 and 300 W. The sections were incubated at a primary antibody concentration of 0.2 µg/ml. Dako real envision detection system peroxidase/DAB (Dako) was used for secondary antibody incubation and visualization. Hematoxylin was used to counter-stain cell nuclei. Evaluation of the stained sections was completed by three of the authors (HT, LB and PH). We were able to evaluate SHP-2 expression in 105 cases. The remaining samples were lost or damaged during sectioning or staining. Cytoplasmic staining intensity was graded from 0 to 3; 0 corresponding to ´no staining,` 1 to ´weak staining,` 2 to ´moderate staining,` and 3 to ´strong staining.' Nuclear staining was graded as either positive or negative. The percentage of stained cells was categorized as 0, 30, 50, 70 or 100%. For survival analysis, SHP-2 staining intensity was classified into high (scores of 2 and 3) and low (scores of 0 and 1). Similarly, the percentage of stained cells was classified into ≤50% and >50%. LNCaP, LNCaP-IL6+, DU145 and PC3 cell lines were pelleted and fixed in 4 % paraformaldehyde, embedded in paraffin and IHC stained as described above.

SHP-2 tyrosine phosphatase activity assay. In order to measure the activity of SHP-2 in the cytoplasm and nucleus, sub-cellular fractionation of LNCaP, LNCaP-IL6+, DU145 and PC3 was perfomed using lysis buffer; 10Mm HEPES pH 7.9, 1.5 mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 1 % Triton X-100, 15 % Complete protease inhibitor, 1mM PMSF for cytoplasmic extraction and RIPA buffer for nuclei extraction. To confirm the fractionation, 30 µg of protein was subjected to a 10 % SDS-PAGE and transferred to a PVDF membrane. The membrane were blocked in Odyssey´s blocking buffer (Li-Cor Biotechnology, Nebraska, USA) and probed with polyclonal goat anti-Lamin B diluted 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA). Thousand micrograms of fractionated lysates from cytoplasm and nuclei, respectively were then incubated with a rabbit anti-human SHP-2 antibody for 2 hours at 4°C and immunoprecipitated with protein G-beads (Amersham Biosciences, Amersham, UK) for 1 hour at 4°C. A PTP-activity assay was then performed, where 20 µl of bead solution was incubated with 20 µl of buffer (100 mM Bis-Tris pH 6.0, 150 mM NaCl, DTT 5 mM) and 60 µl of *p*NPP-solution for 45 minutes at 37°C. To determine the background activity, one sample was immunoprecipitated with rabbit anti-human IgG-antibody (DAKO, Copenhagen, Denmark**).** After 45 minutes, the OD was measured at 405 nm. The OD correlates with the amount of free phosphate-groups that is representative of the SHP-2-specific PTP activity in the analyzed cells.

Characteristic		***Intensity***	***Percentage***	***Nuclear***
Median (range) age at surgery (Years)	63 (48-74)	p = 0.525	p = 0.190	p = 0.948
Mean (range) preoperative PSA (ng/ml)	9.6 (2.6-36.5)	p = 0.745	p = 0.022	p = 0.804
Mean prostate volume (range) (ml)	40 (16-166)	*p = 0.041* *cc = -0.2*	*p = 0.042* *cc = -0.2*	p =0.142
Mean tumor volume (range) (ml)	4.4 (0-15)	p = 0.934	p = 0.339	p = 0.174
	n			
Clinical stage		n.d	n.d	n.d
T1	57			
T2	64			
T3	1			
Gleason sum		p = 0.747	p = 0.313	p = 0.887
≤ 6	68			
7	46			
≥ 8	8			
Extracapsular extension*		p = 0.319	p = 0.738	*p = 0.039* *cc = 0.21*
Yes	70			
No	44			
Seminal vesicle invasion*		p = 0.975	p = 0.528	p = 0.678
Yes	15			
No	106			
Positive surgical margins*		p = 0.434	p = 0.725	p = 0.505
Yes	69			
No	52			
Lymph node involvement*		p = 0.443	p = 0.522	p = 0.552
Yes	2			
No	43			

***** Histopathological data was not available for every patient

Table 1
Clinical and pathological characteristics of the patient cohort, and statistical analysis of SHP-2 intensity, percentage and nuclear staining.

Statistical Analysis. Spearman's correlation was used to correlate SHP-2 expression with clinical parameters. A Cox's proportional hazard regression model was used for univariate and multivariable analyses. Low staining intensity and the percentage of positive cells were used for the reference categories, and negative nuclear staining was used for calculating the

hazard ratios. Biochemical recurrence (BCR) was defined as two PSA tests >0.2 ng/ml. Clinical progression included BCR, death from prostate cancer or adjuvant radiation therapy. All statistical analyses were performed using SPSS version 16.0 (SPSS Inc.). Diagrams representing phosphatase activity are presented as mean ± SEM. Data in the diagrams were analyzed using Student's *t*-test. All statistical tests were two-sided. *P*-values <0.05 were considered significant.

Results

Analysis of SHP-2 protein expression and function in LNCaP, LNCaP-IL6+, DU145 and PC3 cells. SHP-2 immunoreactivity was detected in the cytoplasm and nuclei in all four cell lines, however, DU145 and LNCaP-IL-6+ showed higher expression of SHP-2 and more cells that expressed SHP-2 in the nuclei (Fig. 1A). Tyrosine phosphatase analysis revealed that the SHP-2 activity was overall higher in the LNCaP-IL6+ cell line compared with the LNCaP, DU145 and PC3 cell lines. All four cell lines showed high SHP-2 activity in the nucleus, which was similar to or higher (LNCaP-IL6+, $p<0.03$) than that in the cytoplasm. (Fig 1B).

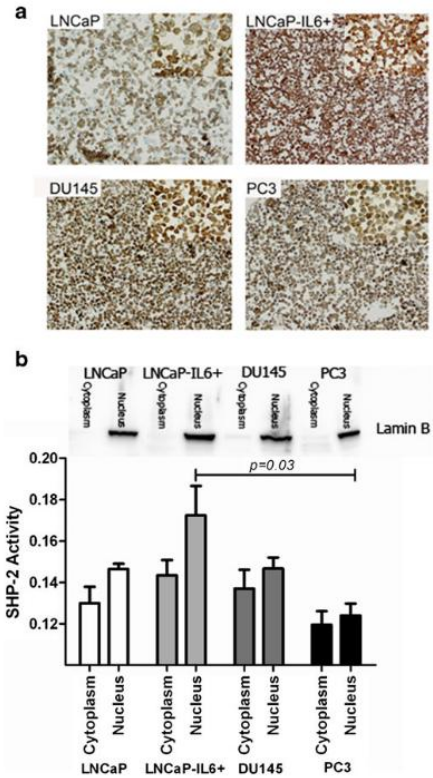

Figure 1.
SHP-2 expression and phosphatase activity in prostate cancer cell lines. **A.** SHP-2 expression in LNCaP, LNCaP-IL6+, DU145 and PC3 cell lines analyzed with immunohistochemical staining. **B.** Phosphatase activity of SHP-2 in cytoplasmic and nuclear fractions analyzed with pNPP phosphatase assay. Lamin B was used as a control of the fractionation.

SHP-2 expression in prostate cancer tissue. SHP-2 expression was observed in epithelial and cancer cells as described below. In some cases, staining of stromal cells was also observed. SHP-2 expression was evaluated in 105 samples. The remaining cases were not analyzed because of the core loss during sectioning or staining. Representative photomicrographs are shown in Fig. 2. Three cores showed no cytoplasmic staining, 15 cores showed weak staining, 58 showed moderate staining, and

29 showed strong staining. In 68 cores, nuclear staining was absent, and in 37 cores, the nuclei were positively stained.

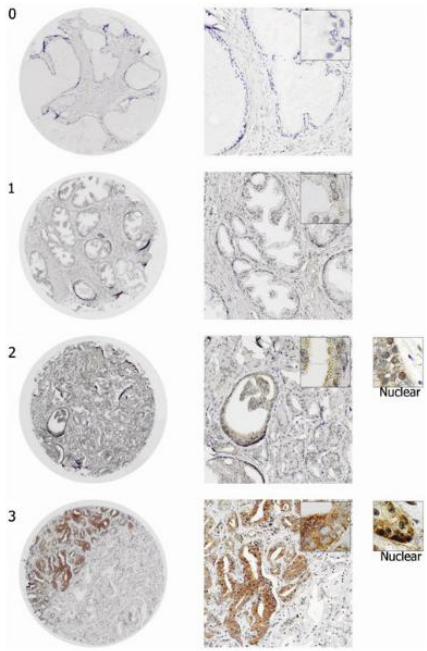

Figure 2.
Representative photomicrographs of SHP-2 immunohistochemistry in prostate cancer cores. SHP-2 expression was scored on the basis of staining intensity from 0–3. Zero corresponds to ´no staining,` 1 to ´weak staining,` 2 to ´moderate staining` and 3 to ´strong staining.` Nuclear staining was scored qualitatively as either positive or negative (insets in 2 and 3).

Correlation of SHP-2 with clinicopathological parameters and survival analysis. We used statistical analyses to assess the potential association of the intensity of cytoplasmic SHP-2 expression, the percentage of stained cells and nuclear staining with tumor characteristics (Table 1). SHP-2

staining did not correlate with preoperative serum PSA levels, Gleason grades or other tumor characteristics. The intensity of SHP-2 immunostaining was classified into low (scores 0 and 1) *versus* high (scores 2 and 3) groups. The percentage of positive cells was divided into ≤50% *versus* >50% groups. Using a Spearman's analysis, we found that both the SHP-2 intensity and the percentage of positive cells were inversely correlated with prostate volume (p=0.04 and p=0.042 respectively) whereas nuclear SHP-2 staining was positively correlated with extra-capsular extension (p=0.039).

Survival analysis. BCR, defined as two post-operative serum PSA analyses >0.2 ng/ml, is commonly used to monitor outcomes following radical prostatectomy. We investigated whether the SHP-2 intensity, percentage of positive cells, and presence of nuclear staining was correlated with clinical outcomes, using BCR-free time and clinical progression-free time as our primary endpoints. Twenty-three of the 105 (22%) patients experienced BCR during the follow-up period. Thirty-one (30%) patients demonstrated disease progression.

A univariate analysis showed that the percentage of SHP-2 positive cells was a prognostic factor of BCR (hazard ratio (HR) 0.264, 95% CI 0.130–0.673, p=0.005) and clinical progression (HR 0.359, 95% CI 0.153–0.841, p=0.018). A multivariable analysis adjusted for preoperative PSA level, Gleason score, extracapsular extension, seminal vesicle invasion and positive surgical margins demonstrated that SHP-2 remained an independent prognostic factor of BCR-free time (HR 0.246, 95% CI 0.066–0.920, p=0.037) but not clinical progression (Table 2).

	BCR free time		Progression free time	
SHP-2 intensity	RR (95% CI)	*p-value*	RR (95% CI)	*p-value*
Univariate				
Low (score 0-1)	1.000		1.000	
High (score 2-3)	0.461 (0.166-1.280)	0.137	0.577 (0.229-1.454)	0.253
Multivariate				
Low (score 0-1)	1.000		1.000	
High (score 2-3)	1.150 (0.283-4.676)	0.845	1.346 (0.387-4.678)	0.640
SHP-2 percentage				
Univariate				
Low (score 0-1)	1.000		1.000	
High (score 2-3)	0.264 (0.130-0.673)	*0.005*	0.359 (0.153-0.841)	*0.018*
Multivariate				
Low (score 0-1)	1.000		1.000	
High (score 2-3)	0.246 (0.066-0.920)	*0.037*	0.362 (0.121-1.085)	0.070
SHP-2 Nuclear				
Univariate				
Low (score 0-1)	1.000		1.000	
High (score 2-3)	0.693 (0.252-1.910)	0.479	0.670 (0.267-1.681)	0.394
Multivariate				
Low (score 0-1)	1.000		1.000	
High (score 2-3)	0.834 (0.261-2.667)	0.759	0.849 (0.314-2.294)	0.746

Table 2. Cox's uni- and multivariate analysis of biochemical recurrence-free and progression-free time according to SHP-2 staining intensity, percentage of positive cells and absence or presence of nuclear staining. Multivariate analysis included adjustment for preoperative PSA level, Gleason score, and presence or absence of extracapsular extension, seminal vesicle invasion and positive surgical margin

Discussion

Recently, we demonstrated that decreased SHP-1 expression in prostate cancer cell lines was associated with high proliferation rates. Moreover, in biopsy cores obtained clinically, decreased SHP-1 expression was associated with an increased risk of recurrence and clinical progression following radical prostatectomy [6]. Here, we show that decreased SHP-2 expression or a lack of such expression is correlated with clinical progression and shorter time to BCR. Furthermore, the multivariable

analysis showed that SHP-2 was an independent predictor of BCR after controlling for preoperative PSA level, Gleason score, extra capsular extension, seminal vesicle invasion and positive surgical margins. In addition, low SHP-2 staining intensity and low SHP-2 positive cell staining percentage of SHP-2-positive cells correlated with increased prostate volume.

The catalytic activity of SHP-2 is required for the modulation of ERK/MAPK and PI3K/AKT pathways downstream of receptor tyrosine kinases [7]. Moreover, SHP-2 is required for the full activation of Ras since it is thought to regulate an upstream element necessary for Ras activation [9]. In MCF10A non-malignant mammary cells, Gab2 over-expression increases the proliferative potential in a SHP-2-dependent manner [15]. However, SHP-2 has also been reported to promote cell death and induce apoptosis [10-12].

In this study we analysed the expression and activity of SHP-2 in the prostate cancer cell lines LNCaP, LNCaP-IL6+, DU145 and PC3. In all cell lines SHP-2 was detected in both cytoplasm and nuclei. Similarly, SHP-2 activity was also demonstrated in both cytoplasm and nuclei. The level of nuclear SHP-2 activity in LNCaP-IL6+ was higher than in other prostate cancer cell lines. The function of nuclear SHP-2 in prostate cancer could be involved in migration and invasion since nuclear expression correlated with extracapsular extension of the cancer. Prior studies have also reported SHP-2 in the nucleus [16-19]. Since SHP-2 lacks a typical nuclear localization signal sequence, it could be transported to the nucleus in association with

proteins such as Gab1 [19]. Although it remains unclear, differential compartmentalization may alter SHP-2 function. For example, SHP-2 has been associated with the regulation of STAT5a as well as telomerase reverse transcriptase in the nucleus [17, 19]. However, further studies are required to determine whether this PTP functions similarly in prostate cancer.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Culig Z, Bartsch G, Hobisch A (2002) Interleukin-6 regulates androgen receptor activity and prostate cancer cell growth. Mol Cell Endocrinol 197:231-238.
2. Pilat MJ, Kamradt JM, Pienta KJ (1998) Hormone resistance in prostate cancer. Cancer Metastasis Rev 17:373-381.
3. Chatterjee B (2003) The role of the androgen receptor in the development of prostatic hyperplasia and prostate cancer. Mol Cell Biochem 253:89-101.
4. Matsuda T, Junicho A, Yamamoto T, et al (2001) Cross-talk between signal transducer and activator of transcription 3 and androgen receptor signaling in prostate carcinoma cells. Biochem Biophys Res Commun 283:179-187.
5. Mustelin T, Vang T, Bottini N. (2005) Protein tyrosine phosphatases and the immune response. Nat Rev Immunol 5:43-57.
6. Tassidis H, Brokken LJ, Jirstrom K, et al (2010) Immunohistochemical detection of tyrosine phosphatase SHP-1 predicts outcome after radical prostatectomy for localized prostate cancer. Int J Cancer 126: 2296-2307.
7. Neel BG, Gu H, Pao L (2003) The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. Trends Biochem Sci 28:284-293.
8. Chong ZZ, Maiese K (2007) The Src homology 2 domain tyrosine phosphatases SHP-1 and SHP-2: diversified control of cell growth, inflammation, and injury. Histol Histopathol 22: 1251-1267.

9. Matozaki T, Murata Y, Saito Y, Okazawa H, Ohnishi H (2009) Protein tyrosine phosphatase SHP-2: a proto-oncogene product that promotes Ras activation. Cancer Sci 100:1786-1793.
10. Vantler M, Huntgeburth M, Caglayan E, Ten Freyhaus H, Schnabel P, Rosenkranz S (2006) PI3-kinase/Akt-dependent antiapoptotic signaling by the PDGF alpha receptor is negatively regulated by Src family kinases. FEBS Lett 580:6769-6776.
11. Yuan L, Yu WM, Xu M, Qu CK (2005) SHP-2 phosphatase regulates DNA damage-induced apoptosis and G2/M arrest in catalytically dependent and independent manners, respectively. J Biol Chem 280:42701-42706.
12. Chen J, Yu WM, Bunting KD, Qu CK (2004) A negative role of SHP-2 tyrosine phosphatase in growth factor-dependent hematopoietic cell survival. Oncogene 23:3659-3669.
13. Hobisch A, Ramoner R, Fuchs D, et al (2001) Prostate cancer cells (LNCaP) generated after long-term interleukin 6 (IL-6) treatment express IL-6 and acquire an IL-6 partially resistant phenotype. Clin Cancer Res 7:2941-2948.
14. Kononen J, Bubendorf L, Kallioniemi A, et al (1998)Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 4:844-847.
15. Chan G, Kalaitzidis D, Neel BG (2008) The tyrosine phosphatase Shp2 (PTPN11) in cancer. Cancer Metastasis Rev 27:179-192.
16. Xu R, Yu Y, Zheng S, et al (2005) Overexpression of Shp2 tyrosine phosphatase is implicated in leukemogenesis in adult human leukemia. Blood 106:3142-3149.

17. Chughtai N, Schimchowitsch S, Lebrun JJ, Ali S (2002) Prolactin induces SHP-2 association with Stat5, nuclear translocation, and binding to the beta-casein gene promoter in mammary cells. The Journal of biological chemistry 277:31107-31114.
18. Fridberg M, Kjellstrom S, Anagnostaki L, et al (2008) Immunohistochemical:analyses of phosphatases in childhood B-cell lymphoma lower expression of PTEN and HePTP and higher number of positive cells for nuclear SHP2 in B-cell lymphoma cases compared to controls. Pediatr Hematol Oncol 25:528-540.
19. Jakob S, Schroeder P, Lukosz M, et al (2008) Nuclear protein tyrosine phosphatase Shp-2 is one important negative regulator of nuclear export of telomerase reverse transcriptase. J Biol Chem 28**:**33155-33161.