

# Screening of Genetic and Expression Alterations of SRC1 Gene in Prostate Cancer

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**BACKGROUND.** Genetic alterations of the SRC1 gene have not been thoroughly studied in prostate cancer.

**MATERIALS AND METHODS.** Five prostate cancer cell lines and 32 xenografts were screened for mutations and gene copy number alterations. Subsequently, frequencies of detected sequence variations were further analyzed in 44 clinical prostate cancers, 6 benign prostate hyperplasias, and 48 normal controls. Finally, the protein expression of SRC1 in 254 clinical prostate tumors was investigated.

**RESULTS.** Three non-recurrent sequence variations, and one single nucleotide polymorphism in the coding region of SRC1, as well as one case of SRC1 gene amplification were found. The protein expression of SRC1 was higher in androgen ablation resistant than untreated prostate carcinomas, but the difference was not statistically significant ( $P = 0.0796$ ).

**CONCLUSIONS.** Genetic alterations of SRC1 are rare in prostate cancer. The nuclear protein accumulation of SRC1 seems to be mildly increased in androgen ablation resistant prostate cancers. *Prostate* 66: 1391–1398, 2006. © 2006 Wiley-Liss, Inc.

**KEY WORDS:** prostatic; neoplasia; NCOA1; mutation; amplification

## INTRODUCTION

The mechanisms of the progression of prostate cancer from androgen dependence to independence, that is, androgen ablation resistance are incompletely known. However, it has become evident that androgen receptor (AR) signaling is re-activated at the emergence of ablation resistant tumor clones [1]. In about 30% of the recurrent tumors, the resistance is most likely due to amplification of the AR gene [2]. In 10–30% of tumors treated with antiandrogens, such as flutamide or bicalutamide, the resistance could be explained by point mutations in the coding region of the AR gene [3,4]. However, in the majority of cases, the

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mechanisms for re-activation of AR signaling remain unknown.

Activation of AR is a complex process that involves a large number of proteins that act both as repressors and activators [5,6]. Such nuclear receptor co-regulators interact with receptors altering their transactivation properties by promoting several events, for example, chromatin remodeling, recruitment of pre-initiation complex and movement of RNA polymerase [5,6]. A large number of co-regulators has already been identified. Probably the most intensively investigated co-activator group is the p160/SRC protein family, consisting of three homologous 160 kDa proteins: nuclear receptor co-activator 1 (NCOA1), better known as steroid receptor co-activator-1 (SRC1), nuclear receptor co-activator 2 (NCOA2, alias TIF2, SRC2, GRIP1) and nuclear receptor co-activator 3 (NCOA3, alias AIB1, SRC3, RAC3, ACTR). p160 proteins recruit the CREB (cAMP responsive element binding protein 1) binding protein (CREBBP alias CBP) and p300 [7]. Both p160 and CBP/p300 possess histone acetyltransferase activity allowing these complexes to acetylate lysine residues of histone proteins. This will eventually lead to relaxing of the chromatin, which then allows basal transcription machinery and other factors to access the promoter of the target gene [7].

Although the effects of the co-regulators on the physiological function of AR are obviously important, little is known about their role during prostate cancer tumorigenesis. There is substantial evidence from *in vitro* studies that alterations in AR co-regulators could be involved in the progression of prostate cancer [5,6]. However, alterations of these co-regulators in clinical prostate cancers have not been thoroughly investigated. One of the most studied co-regulator in prostate cancer is SRC1. Immunohistochemical analyses of SRC1 have suggested that the expression is increased in ablation resistant prostate tumors [8] as well as in untreated tumors with aggressive feature, such as high Gleason score and advanced stage [9]. Data on mRNA expression are more controversial. Fujimori et al. [10] reported that the expression of SRC1 is higher in untreated tumors with high grade or poor response to endocrine therapy than in those with a lower grade or good response to endocrine therapy. We have previously shown that the expression of SRC1 mRNA is lower in ablation resistant than untreated prostate carcinomas [11].

We have also demonstrated that a prostate cancer xenograft LuCaP70 contains amplification of the SRC1 gene together with increased expression of the gene [11]. Subsequently, we screened 47 ablation resistant tumors for SRC1 gene amplification using fluorescence *in situ* hybridization (FISH). No amplifications were found suggesting the amplification is a

rare event in prostate cancer. The fact, that a xenograft containing this particular gene amplification was found, suggests that SRC1 may be a target for genetic aberration in prostate cancer. Therefore, we decided to screen prostate tumors for SRC1 mutations using denaturing high-performance liquid chromatography (DHPLC) and sequencing. In addition, 13 prostate cancer xenografts were studied for SRC1 gene amplification by FISH. Finally, we measured the expression of SRC1 in 254 clinical samples of prostate tumors using tissue microarrays (TMA) and immunohistochemistry.

## MATERIALS AND METHODS

### Cancer Cell Lines and Xenografts

The prostate cancer cell lines LNCaP, DU-145, PC-3 and 22Rv1 were obtained from the American Type Culture Collection (Rockville, MD), whereas LAPC4 was kindly provided by Dr. C. Sawyers (UCLA, Los Angeles, CA). 19 LuCaP (LuCaP 23.1, 23.8, 23.12, 35, 35V, 49, 58, 69, 70, 73, 77, 78, 81, 86.2, 92.1, 93, 96, 105, 115) and 13 PC (PC-82, 133, 135, 295, 310, 324, 329, 339, 346, 346I, 346B, 346BI and 347) human prostate cancer xenografts were made available by two of the authors (R.V. and W.W., respectively). Total RNA from the cell lines and xenografts was isolated using Trizol reagent (Invitrogen Corporation, Carlsbad, CA) or with Qiagen RNeasy MiniKit (Qiagen, Inc., Valencia, CA) according to the manufacturers' instructions. The first strand cDNA was synthesized with a Superscript<sup>TM</sup> II reverse transcriptase and oligo d(T) primer according to the manufacturer's instructions (Invitrogen Corp.). As a control, human prostate total RNA (Clontech Laboratories, Inc., Palo Alto, CA) was used and reverse transcribed using AMV Reverse Transcriptase (Finnzymes, Espoo, Finland) according to the manufacturers instructions.

Freshly frozen clinical samples (6 benign prostate hyperplasia, 30 untreated, and 14 ablation-resistant prostate cancers) were obtained from the Tampere University Hospital (Tampere, Finland). The specimens were histologically examined for the presence of tumor tissue (>50% of cells) using hematoxylin and eosin-stained slides. The clinicopathological variables of the cases are shown in Table I. In addition, 48 normal samples were obtained from healthy Finnish female blood donors. DNA was extracted from these samples with routine techniques, and were first amplified with GenomiPhi<sup>TM</sup>-DNA amplification kit (Amer-sham, GE Healthcare, UK) according to the manufacturer's instructions and diluted 1:5 for the subsequent PCR reactions used in DHPLC.

For construction of tissue microarrays (TMA), retrospective material of formalin-fixed, paraffin-embedded

**TABLE I. Clinicopathological Variables of the Tumor Materials**

	Material for DHPLC N (%)		Material for immunohistochemistry N (%)	
	Untreated tumors (n = 30)	Ablation resistant tumors (n = 14)	Untreated tumors (n = 205)	Ablation resistant tumors (n = 49)
WHO-grade:				
I	9 (30)			
II	14 (47)			
III	7 (23)			
Gleason-score:				
<7			78 (38)	
7			99 (48)	
>7			27 (13)	
unknown			1 (1)	
Clinical TMN-stage:				
T2N0XM0	14 (47)			
T3N0M0	10 (34)			
T3NXM0	1 (3)			
T4NXM0	1 (3)			
T2N1M0	1 (3)			
T3-4NXM1	2 (7)			
TXN0M0	1 (3)			
pT-stage:				
pT1			1 (1)	
pT2			135 (65)	
pT3			68 (33)	
pTX			1 (1)	
Therapy:				
Orchiectomy		4 (29)		24 (49)
LHRH analog		3 (22)		13 (27)
Estrogen		2 (14)		
Orchiectomy and estrogen		2 (14)		
Orchiectomy and antiandrogen		1 (7)		6 (12)
LHRH analog and antiandrogen		1 (7)		4 (8)
Bicalutamide				1 (2)
LHRH analog and bicalutamide and estramustine				1 (2)
Unknown		1 (7)		
The mean (range) time from the onset of androgen ablation to the progression (months)		38 (15–68)		31 (1–112)

samples of 205 untreated prostate cancers from consecutive prostatectomy treated patients as well as 49 androgen ablation resistant tumors were obtained from the Tampere University Hospital. The clinicopathological variables of the cases are given in Table I. The median follow-up time of the prostatectomy treated patients was 103.3 months. A rise in PSA levels or the emergence of metastases was considered as progression.

**DHPLC**

For the DHPLC analysis, fragments covering the coding area as well as part of 5'- and 3'-UTRs (untranslated region) of the SRC1 (nucleotides 55-

4606 from the beginning of transcription according to GenBank Acc.no. NM\_003743) gene were PCR amplified from cDNA or DNA using Phusion™ High-Fidelity DNA polymerase (Finnzymes). The primer sequences used are shown in Table II. PCR cycling was performed as follows: 98°C for 30 sec followed by 35 sets of cycles of 98°C for 7 sec, 57–64°C for 20 sec 72°C for 25 sec and a final extension for 5 min with the final volume of 20 µl. PCR products were then mixed with the control DNA and subjected to a denaturation at +95°C for 5 min followed by annealing by cooling to 65°C during 30 min.

DHPLC was performed using Agilent 1100 LC HPLC instrumentation (Agilent Technologies, Palo

**TABLE II. Primers and Annealing Temperatures for PCR, as well as Temperatures and Gradients used in DHPLC**

	Name	Sequence	PCR annealing temperature	DHPLC run temperature	Gradient
1	SRC1s1	TTGGCCTTAAATGAGGTGAGA	57	59	56–68
	SRC1as1	ATCCAAAGCCTCCAAAAGAA		60	55–67
2	SRC1s2	TGCAAGATTTTGAAGAAAACAGT	57	59	57–69
	SRC1as2	TTCTACACCCGTAATAGCTGGA		60	57–69
3	SRC1s3	GCTTGCCAGCGTTATGAAG	58	59	58–70
	SRC1as3	CATGTTGCTGTTGGATGGTG		60	58–70
4	SRC1s4	CATGCAACCTTTCATCATGG	57	60	56–68
	SRC1as4	AGGAATTGTTTGGCATCCTG		61	55–67
5	SRC1s5	GCACAGTTCATGTCTCCAAGG	57	59	59–71
	SRC1as5	GCAGAGGCAGAGTTGGAAGT		60	59–71
6	SRC1s6	CTTCCAACCTCTGCCTCTGCT	57	59	59–71
	SRC1as6	TTTCTCAGGAGTGGGTTTG		60	56–68
7	SRC1s7	AAACCTGAGCCTGGATGATG	57	60	56–68
	SRC1as7	TGTTGTGGGTGGACAGAGAA		61	56–68
8	SRC1s8	CCAATTTGGACAACCAGGAA	57	60	56–68
	SRC1as8	GCTTGCCGATTTTGGTGTAT		61	56–68
9	SRC1s9	GCCCAGGCAAACCTCTAAACA	57	60	58–70
	SRC1as9	TGCTTCAGGATTTGCTGCTA		61	57–69
10	SRC1s10	GAACCCACCTGCTTCTACC	57	61	58–70
	SRC1as10	TGCTTCAGGATTTGCTGCTA		62	58–70
11	SRC1s11	GCAAGGAGCGATAGGAAACA	60	Direct	
	SRC1as11	TGAAGAATGGCTGCAGATTG		Sequencing	
12	DNAmut2s	TCTTTCAACCTCCAAACCAT	52	56	50–62
	DNAmut2as	CCACGTGCAGTATGCTGTAGA		57	47–59

Alto, CA) equipped with a Varian CP28353 Helix DNA Column (50 × 3.0 mm) (Varian, Inc., Palo Alto, CA). The melt temperatures, determined by the Stanford melt algorithm (<http://insertion.stanford.edu/melt.html>), and temperatures of one degree above the melt temperatures were used in the DHPLC runs (Table II). The gradients used (Table II) were obtained from the Stanford melt algorithm and further adjusted by empirical testing. Samples that varied in retention time and/or peak shape were re-amplified and purified using either Qiagen QIAquick PCR purification Kit (Qiagen, Inc., Valencia, CA) or Montage filtration system (Millipore Corporation, Bedford, MA).

### Sequencing

All samples showing more than one peak (or an abnormal peak) in DHPLC were sequenced. In addition, SRCa- and SRCe splice variants at the 3' end of the gene were amplified with primers SRC1s11 and SRC1as11 (Table II), purified from the gel using QIAquick Gel Extraction Kit (Qiagen, Inc.) according to the manufacturer's instructions and sequenced directly. The region of SRC1 interacting with AR was amplified with primers CATGGTCAGGCAAACCTT and CTGTCGGTGTCTGTTGACTGT, and sequenced directly. Sequencing was performed using the Big-

Dye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the ABI PRISM<sup>®</sup> 3100 sequencer (Applied Biosystems) according to the manufacturer's instructions.

### Q-RT-PCR

Expression of SRC1 and house keeping gene TATA-box Binding Protein (TBP) in PC xenograft samples was analyzed using Q-RT-PCR as previously described [11]. Briefly, PCR reactions were performed using the LightCycler<sup>™</sup> apparatus (Roche Diagnostics, Mannheim, Germany) with either the LC Fast Start DNA SYBR Green I Kit (SRC1) or the LC DNA Master Hybridization Probes (TBP) (Roche Diagnostics). To ensure the specificity of the PCR product, a melting curve analysis was performed, and all PCR products were analyzed in a 1.5% agarose gel. For preparing standard curves, the total cDNA from LNCaP cells were used at several dilutions. The SRC1 expression values were normalized to the expression values of TBP.

### FISH

For the gene copy number analysis, human genomic BAC clones for SRC1 (GenBank Acc.no. AC013459) and

for STEAP3 alias pHyde (GenBank Acc.no. AC016673) were labeled by nick translation with digoxigenin-dUTP (Roche Diagnostics) and FITC-dUTP (NEN, Boston, MA), respectively, and the dual-color hybridization was performed as previously described [11]. Briefly, frozen tissue sections of the xenografts were fixed with a series of 50%, 75%, and 100% Carnoy's fixative (3:1 methanol:acetic acid), followed by denaturation in 70% formamide/2 × SSC solution (pH 7) at 70°C for 3 min and dehydration. Hybridization was carried out at 37°C in a humid chamber. After washes, slides were stained with 1:300 anti-digoxigenin-rhodamine (Roche Diagnostics), washed, and counterstained with an antifade solution (Vectashield, Vector laboratories, Burlingame, CA) containing 4,6-diamidino 2-phenylindole (DAPI). FISH signals were scored from non-overlapping and intact nuclei using Olympus BX50 epifluorescence microscope (Tokyo, Japan). Amplification of the gene was defined as a >twofold higher copy number for SRC1 than for the reference probe (STEAP3), or more than five copies of SRC1, or tight clusters of SRC1 signals.

**Immunohistochemistry**

Protein expression was analyzed using polyclonal rabbit antibody against SRC1 (PA1-840 Affinity Bio-Reagents, Golden, CO). TMA sections were deparaffinized, followed by antigen retrieval (autoclave cooking in 10 mM of sodium citrate buffer, pH 6.0, at 121°C for 2 min). The antibody was diluted 1:200 in PowerVision blocking solution ImmunoVision Technologies Corporation, Brisbane, CA) and incubated overnight at +4°C. Bound antibody was visualized with PowerVision+™ Poly-HRP IHC Detection Kit (ImmunoVision Technologies Corp.). Sections were counterstained with hematoxylin. Nuclear staining was scored from 0 to 3 (0 no staining, 1 weak, 2 moderate, and 3 high intensity staining).

**Statistical Analysis**

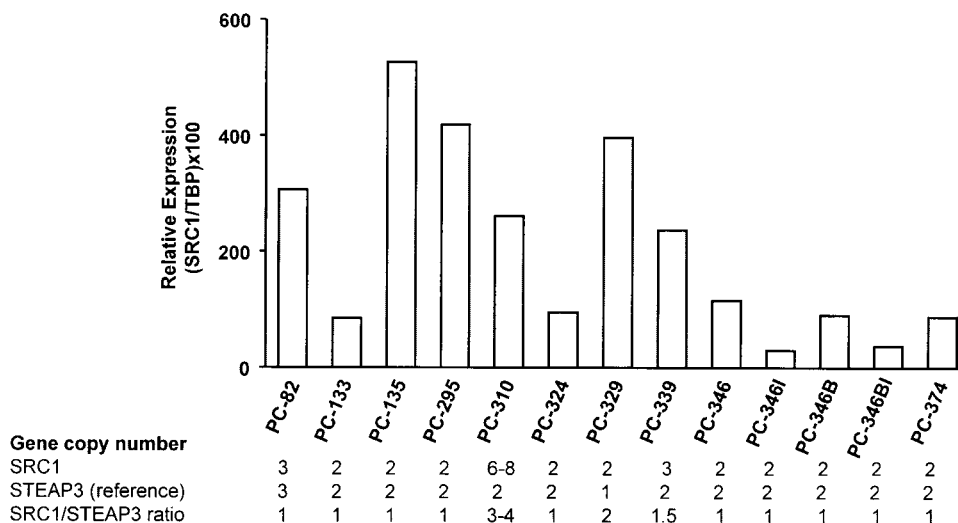
Fisher's exact and  $\chi^2$ -tests were used to evaluate the association of SRC1 protein expression with different clinicopathological variables. Progression-free survival analysis was done with Kaplan–Meier method together with Wilcoxon rank test.

**RESULTS AND DISCUSSION**

Although SRC1 has been implicated in the progression of prostate cancer [7–11], no mutation analysis of the gene in prostate cancer has been reported so far. Therefore, we decided to screen five prostate cancer cell lines and 32 xenografts samples for mutations. Many of these models have been established from patients with androgen ablation-resistant disease and they contain large number of genetic alterations [12,13]. Thus, if mutations in SRC1 are common in prostate cancer, one would expect to find them in these specimens. Screening of the cell lines and xenografts by DHPLC and following sequencing revealed three sequence variations in four independent xenografts (Table III). In LAPC4, a heterozygous silent (Cys129Cys) mutation T → T/C at base 378 (starting from the initiation of translation according to GenBank Acc.no. NM\_003743) was found. In addition, a silent (Thr154Thr) base substitution C → C/G at base 462 was found in both LuCaP96 and PC82. Finally, in 22Rv1, a heterozygous base substitution G → G/C at 466, leading to missense Val156Ile mutation, was detected. To further investigate the frequency of these changes in prostate cancer, 30 untreated, 14 ablation resistant tumors and 6 BPH specimens as well as 48 (96 chromosomes) blood samples from healthy donors were screened using DHPLC and subsequent sequencing. The mutations Cys129Cys, and Val156Ile were not found in any of the samples suggesting that they are rare events in prostate cancer. On the other hand, the C462G (Thr154Thr) variation was found in 15.9% of the

**TABLE III. Summary of the Sequence Alterations of SRC1 in Prostate Cancer**

Samples	Alteration			
	T378C (Cys129Cys)	C462G (Thr154Thr)	C462T (Thr154Thr)	G466C (Val156Ile)
22Rv1				X
LAPC4	X			
LuCaP96		X		
PC82		X		
Frequency:				
Cell lines and xenografts (n = 37)	1 (2.7%)	2 (5.4%)	0 (0.0%)	1 (2.7%)
Clinical cancer specimens: untreated (n = 30)	0 (0.0%)	4 (13.3%)	0 (0.0%)	0 (0.0%)
ablation resistant (n = 14)	0 (0.0%)	3 (21.0%)	1 (7.1%)	0 (0.0%)
Population control samples (n = 48)	0 (0.0%)	7 (14.6%)	0 (0.0%)	0 (0.0%)



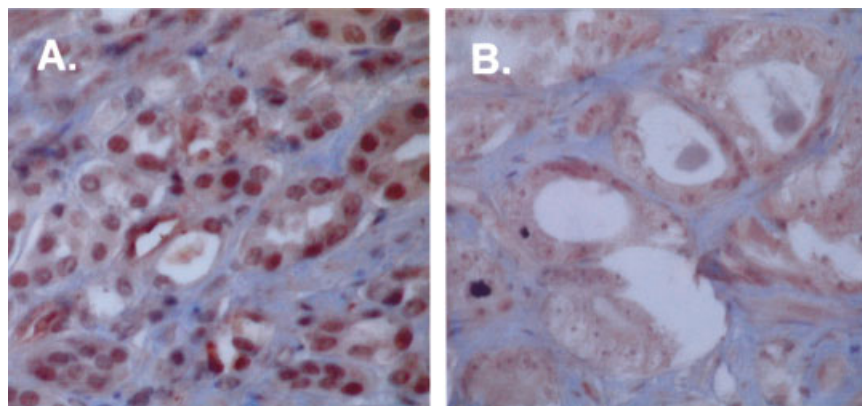
**Fig. 1.** The relative expression of SRC1 in PC-xenografts determined by real-time quantitative RT-PCR. The gene copy number of SRC1 and STEAP3 (reference) as well as their ratio is shown below.

prostate carcinomas, and in 14.6% of normal samples, indicating that it is a common polymorphism. Indeed, this sequence variation has been reported to be a single nucleotide polymorphism (NCBI-dbSNP rs# cluster id: rs11125744). In addition, we found another synonymous sequence variation in the same base pair, C462T (Thr154Thr), in one hormone-refractory patient tumor. In summary, only one putatively significant mutation (Val156Ile) was found in 81 (cell lines, xenografts, and clinical tumors) cancer specimens indicating that mutations in SRC1 are very rare in prostate cancer.

The first round mutation screening of the 5 cell lines and 32 xenografts were made from cDNA instead of DNA due to the high number of small exons (altogether 20 exons) encoding only less than 5 kb transcript. Thus, we were able to screen the whole coding region of the gene with 11 primer pairs. The sequence variations identified in the cell lines and xenografts, were further analyzed in DNA from clinical tumors (with primers

DNAmut2s, DNAmut2as, Table I). SRC1 gene has two splice variants, SRC1e and SRC1a [14], which are equally expressed in cell lines and tissues. SRC1e has been reported to have more potential for the activation of androgen receptor (AR) [15,16]. Both splice variants were screened for mutations. We utilized heteroduplex analysis with DHPLC to screen for mutations, because the sensitivity and specificity of DHPLC has been reported to be very high [17]. However, since only a few mutations were found by DHPLC we wished to ensure that no sequence variations were missed. Therefore, we also directly sequenced the region of SRC1 responsible for binding to the AR. No alterations in this region were found either by DHPLC or by direct sequencing.

We have recently shown that LuCaP70 contains high-level amplification of the SRC1 gene [11]. Therefore, the PC-series xenografts were analyzed for gene copy number alterations by FISH. Most of the xenografts showed two copies of SRC1 at locus 2p23 and the



**Fig. 2.** An example of nuclear SRC1 staining in (A) immunopositive, and (B) negative prostate cancer.

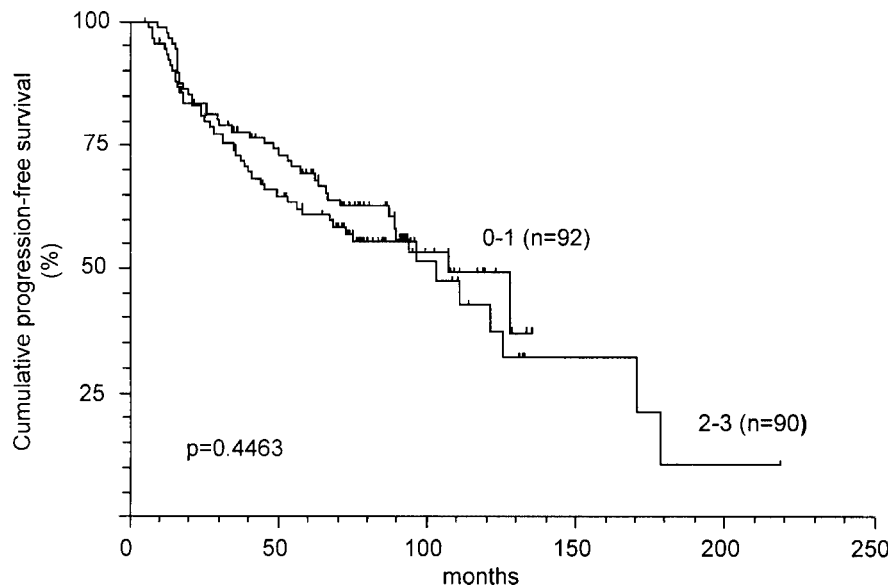
reference gene STEAP3, located at 2q14, (Fig. 1). Whereas, PC-310 showed six to eight SRC1 signals and only two control signals indicating three- to fourfold amplification of the SRC1. Taken together with our earlier findings [11], this is a second tumor that contains the SRC1 amplification. However, the frequency of amplification seems to be low, as we have found only two cases of amplification out of 70 (23 xenografts, 47 ablation-resistant tumors) samples analyzed. We analyzed also the 13 PC-xenografts for SRC1 mRNA expression using Q-RT-PCR (Fig. 1). The expression level was highest in PC-135, PC-295, and PC-329, whereas PC-310 showed somewhat over median level of expression. Although we have previously shown that LuCaP70, which contains the gene amplification, shows high expression of SRC1 mRNA [11], the data here suggest that SRC1 might not be the primary target gene for the amplification at the SRC1 locus.

To study the expression of SRC1 protein in prostate cancer, we stained 254 clinical tumor samples representing untreated (n=205) and ablation resistant (n=49) prostate carcinomas (Fig. 2). One hundred six (52%) of the prostatectomy specimens were scored as 0 or 1 (negative or weak), and 99 (48%) as 2 or 3 (moderate to strong). Of the ablation resistant tumors, 18 (37%) were scored as 0 or 1, and 31 (63%) as 2 or 3. Statistical analysis showed that this difference was not a significant ( $P=0.0796$ ) increase in the expression of SRC1 in ablation-resistant tumors (Table IV). The finding is in line with a previous study by Gregory et al. [8] who showed increased expression of SRC1 protein in a small number of ablation-resistant prostate

**TABLE IV. Association of Clinicopathological Variables with the Expression of SRC1 in Prostate Cancer**

	SRC1 staining intensity		P-value
	0-1	2-3	
Untreated	106 (52)	99 (48)	0.0796
Ablation resistant	18 (37)	31 (63)	
Untreated:			
Gleason score:			0.8015
<7	43 (55)	35 (45)	
=7	49 (49)	50 (51)	
>7	14 (52)	13 (48)	
pT-stage:			0.0239
pT1 + pT2	63 (58)	45 (42)	
pT3	39 (42)	54 (58)	

tumors. Clearly, the present finding illustrates that the conclusion drawn in our previous study showing decreased mRNA expression of SRC1 in ablation-resistant tumors, does not hold for SRC1 protein expression levels [11]. This could be explained by the fact that mRNA expression level is not always directly associated with protein expression level. Alternatively, it may be that in the ablation-resistant tumors SRC1 protein is accumulated into nuclei, possibly due to high nuclear content of AR. In prostatectomy treated patients, SRC1 staining was shown to be associated with pT-stage ( $P=0.0239$ ), but not with Gleason score ( $P=0.8015$ ) (Table IV). Recently, Agoulnik et al. [9] showed that increased SRC1 protein expression, as



**Fig. 3.** Kaplan–Meier curves of progression-free survival in prostatectomy treated patients categorized into SRC1 negative (staining intensity 0–1) and positive (2–3) cases.

determined by immunohistochemistry, was associated with both clinical stage and Gleason grade in their material of prostatectomy treated patients. This discrepancy could be explained, by the different antibodies used in these studies. The antibody (PA1-840 Affinity BioReagents, Golden, CO) used in the present study recognizes the aminoterminal part of the protein (amino acids 8–24), whereas the antibody used by Agoulnik et al. (MAI-840, Affinity BioReagents) is raised against a peptide corresponding to amino acid residues 477–947. Similarly to the findings by Agoulnik et al. [9], we found no association ( $P = 0.4463$ ) between progression-free survival and SRC1 expression (Fig. 3).

In conclusion, the nuclear protein expression of SRC1 seems to be mildly increased in androgen ablation resistant prostate cancers. In the prostatectomy treated patients, the immunostaining was associated with clinical stage, but not with Gleason score or progression-free survival. Although two cases of SRC1 gene amplifications and one case of missense mutation indicate that the gene may be targeted by genetic alterations, the low frequency of the aberrations suggests that genetic alterations in SRC1 are not commonly involved in the progression of prostate cancer.

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