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Evaluation of Somatostatin and CXCR4 Receptor Expression in a Large Set of Prostate Cancer Samples Using Tissue Microarrays and Well-Characterized Monoclonal Antibodies

Christoph Werner [a,b], Olaf Dirsch [c,d], Uta Dahmen [e], Marc-Oliver Grimm [f], Stefan Schulz [b], Amelie Lupp [b,*]

[a] Department of Internal Medicine III, Jena University Hospital, Jena, Germany
[b] Institute of Pharmacology and Toxicology, Jena University Hospital, Jena, Germany
[c] Institute of Pathology, Jena University Hospital, Jena, Germany
[d] Institute of Pathology, Klinikum Chemnitz, Chemnitz, Germany
[e] Department of General, Visceral and Vascular Surgery, Jena University Hospital, Jena, Germany
[f] Department of Urology, Jena University Hospital, Jena, Germany

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ABSTRACT

BACKGROUND: Prostate cancer (PCa) is the most common type of cancer among men in Western countries. Despite numerous therapeutic options, few treatments are available for patients with end-stage disease. In the present study, different somatostatin receptors (SSTs) and the chemokine receptor CXCR4 were evaluated for their suitability as novel therapeutic targets in PCa. **MATERIALS AND METHODS:** The expression of SST subtypes 1, 2A, 3, and 5 and of CXCR4 was evaluated in 276 PCa tumor samples on a tissue microarray (TMA) in 23 whole-block tumor samples and in 3 PCa cell lines by immunohistochemistry using well-characterized monoclonal antibodies. **RESULTS:** Overall, the frequency and intensity of expression of SSTs and CXCR4 were very low among the PCa samples investigated. Specifically, SST5, SST2A, and SST3 were expressed, albeit at low intensity, in 10.5%, 9.1%, and 0.7% of the TMA samples, respectively. None of the TMA samples showed SST1 or CXCR4 expression. Only a single small-cell-type neuroendocrine carcinoma that was coincidentally included among the whole-block samples exhibited strong SST2A, SST5, and CXCR4 and moderate SST3 expression. Independent of the tumor cells, the tumor capillaries in many of the PCa samples were strongly positive for SST2A, SST3, SST5, or CXCR4 expression. SST expression in the tumor cells was associated with advanced tumor grade and stage. **CONCLUSION:** Overall, SST and CXCR4 expression levels are clearly of no therapeutic relevance in PCa. SST- or CXCR4-based therapy might be feasible, however, in rare cases of small-cell-type neuroendocrine carcinoma of the prostate.

Introduction

Prostate cancer (PCa) is the most common type of cancer among men in Germany and the majority of Western countries [1]. Overall, cancer-specific mortality due to PCa is quite low because of low progression rates and early detection [1,2]. In recent years, the rate of early detection of PCa has increased because of prostate-specific antigen (PSA) screening, but no corresponding reduction in mortality rate has been noted [1–3]. Many therapeutic strategies for PCa treatment are available, including active surveillance, pharmaceutical castration, radiation, brachytherapy, and radical prostatectomy, which is the most often used modality in Germany [4–6]. Despite the numerous therapeutic options, some patients with end-stage PCa are still encountered in clinical practice. For those patients, nuclear-medicinal treatment options are still possible, such as radium-223 treatment [7–9] or prostate-specific membrane antigen radiopeptide therapy [8,10–14]. However, other therapeutic options for patients with end-stage PCa are still needed.

Peptide receptor radionuclide therapy (PRRT) with radiolabeled somatostatin analogues is routinely used to treat well-differentiated gastroenteropancreatic neuroendocrine neoplasms, which are well known for their overexpression of somatostatin receptors (SSTs), especially SST2 [15–18]. In contrast, PRRT based on the chemokine receptor CXCR4 was only recently shown to be a feasible treatment option for patients with highly aggressive cancers such as small-cell lung cancers, leukemias, and lymphomas [19,20]. In PCa, however, the available data on SST and CXCR4 expression are scarce and highly contradictory, in part because a wide variety of mostly polyclonal antibodies has been used for the immunohistochemical investigations. In addition, many studies were limited by

* Address all correspondence to: Amelie Lupp, Institute of Pharmacology and Toxicology, Jena University Hospital, Friedrich Schiller University Jena, Drackendorfer Str. 1, D-07747 Jena, Germany.
E-mail address: Amelie.Lupp@med.uni-jena.de. (A. Lupp).

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high rates of background staining and only cytoplasmic, or even nuclear, staining for these membrane-bound receptors (for an overview of the studies of SST and CXCR4 expression in PCa from the past 17 years, see Tables 1 and 2).

In the present study, we aimed to determine if SST- or CXCR4-based PRRT would be a feasible treatment option for patients with end-stage PCa. We measured SST and CXCR4 expression in a large set of PCa samples by immunohistochemistry using well-characterized rabbit monoclonal antibodies. These monoclonal antibodies, which have several advantages over polyclonal ones, were previously generated and extensively characterized by our group [42–46] and have also been validated by other researchers (e.g., [47–49]).

Materials and Methods

Patients and Clinical Data

Formalin-fixed and paraffin-embedded specimens of prostate tissue were retrospectively collected from routine diagnostic samples taken at the Institute of Pathology, Jena University Hospital, Jena, Germany. Specimens from 306 radical prostatectomies performed between January 2010 and March 2013 at the Department of Urology, Jena University Hospital, Jena, Germany, were used to construct a tissue microarray (TMA). Another 23 PCa samples were evaluated as conventional complete blocks. Clinical data recorded at the respective institutions were collected and anonymized for further evaluation. All procedures performed in this study were in accordance with the 1964 Helsinki Declaration and its later amendments. The ethics committee of the Jena University Hospital, Jena, Germany, granted permission for the study to be undertaken. The patients included in the study gave informed consent by responding to a request sent by mail.

The UICC-TNM Classification (ninth edition) system was used to assign the stage of the tumors used in the study. The Gleason score was used to assign tumor grade. Preoperative serum levels of PSA were available for 259 patients. Postoperative PSA values measured within 3 months after surgery were available for only 121 patients. The number of patients with available PSA measurements decreased further as the length of follow-up increased.

Cytoblocks

HEK-293 cells and the prostate cancer cell lines LNCaP, DU145, and PC-3 (DMSZ, Braunschweig, Germany) were grown in 75-cm^2 culture flasks to 80% confluency. The cells were then washed once with phosphate-buffered saline and transferred into 10% buffered formalin (J.T. Baker, Deventer, The Netherlands) for 2 hours. After centrifugation for 10 minutes at 3500 $\times g$, the supernatant was removed, and 1 ml pooled human plasma was added to the samples. The samples were then vortexed briefly, supplemented with 100 µl fibrinogen (50%-70% protein; ≥80% clottable; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and vortexed again. The resulting clots were incubated for another 24 hours in 10% buffered formalin and then embedded in paraffin blocks.

TMA Construction

A TMA was constructed according to the recommendations of Parsons and Grabsch [50] using the Manual Tissue Arrayer MTA1 (Beecher Instruments, Inc., Sun Prairie, WI). Three tissue cylinders of 0.6 mm in diameter were taken from each paraffin-embedded tumor block after an area of interest was identified by haemotoxylin and eosin (H&E) staining. The tissue cylinders were then transferred to the cores of two different TMA recipient blocks. Two cylinders were placed on one block and the third on the other block such that they lied differently in the inner and outer areas of the array to avoid technical bias in staining. Each of the 16 TMA blocks produced (consisting of 10 × 8 tissue cylinders) was marked by specific asymmetry of the tissue cylinders to ensure unambiguous orientation within the TMA section, as well as unambiguous identification of the TMA block itself. Each TMA block included negative and positive controls consisting of cylinders from cytoblocks of wild-type HEK-293 cells (DSMZ, Braunschweig, Germany) and HEK-293 cells stably transfected with one of the SSTs, human pancreatic islets (for SST1, SST2A, SST3, and SST5), germinal centers of human lymph nodes (for SST2, SST5, and CXCR4), and nontumorous prostatic tissue (see Figure 1 for representative images of the positive controls).

Immunohistochemistry

For immunohistochemical staining, 2.5-µm sections were prepared from the TMA blocks and 23 additional tumor blocks and floated onto positively charged slides. The slides were dewaxed, microwaved in 10 mM citric acid (pH 6.0) for 16 minutes at 600 W, and then incubated with the respective primary antibodies overnight at 4°C (for detailed information about the sources, clones, and dilutions of the antibodies, see Table 3). Detection of the primary antibody was performed using a biotinylated anti-rabbit or anti-mouse IgG followed by incubation with peroxidase-conjugated avidin (Vector ABC Elite kit, Vector Laboratories, Burlingame, CA; dilution 1:20). Binding of the primary antibody was visualized using 3-amino-9-ethylcarbazole in acetate buffer (BioGenex, San Ramon, CA; dilution 1:5). The sections were then rinsed, counterstained with Mayer's hematoxylin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and mounted in Vectamount mounting medium (Vector Laboratories, Burlingame, CA). The stained sections were digitalized using an automated slide scanner (NanoZoomer 2.0 HT; Hamamatsu Photonics, Hamamatsu, Japan).

Table 1
Immunohistochemical Studies of SST Expression in Prostate Carcinomas

Study	Samples (*n*)	SST Subtypes	Type of Antibody	Positivity (%)	Type of Staining	Correlations with Clinical Data
Dizeyi et al. 2002 [21], Hansson et al. 2002 [22]	27	1, 2, 3, 4, 5	Rabbit polyclonal (Dr. Helboe)	0% (SST5)-93% (SST4)	Cytoplasmic	No correlations with Gleason score
Cariaga-Martinez et al. 2009 [23]	45	2	Rabbit polyclonal (Santa Cruz)	95% (lost in 2 cases)	Cytoplasmic	Inverse correlation with Gleason score
Morichetti et al. 2010a [24]	40	1, 2, 3, 4, 5	Rabbit polyclonal (Chemicon International)	79.6%-86.7%, strong staining: 5.5%-13.9%	Membrane (SST3, SST4), cytoplasmic (SST1-SST5), nuclear (SST4, SST5)	Strong staining, increasing from normal to HGPIN to PCa
Morichetti et al. 2010b [25]	60 + 20 controls	1, 2, 3, 4, 5	Rabbit polyclonal (Chemicon International)	79.5%-96.1%, strong staining: 6.1%-15.2%	Same as Morichetti et al. 2010a	Strong staining, increasing from normal to HGPIN to PCa
Mazzucchelli et al. 2010 [26], Montironi et al. 2013 [27]	40	1, 2, 3, 4, 5	Rabbit polyclonal (Chemicon International)	53.8%-86.7%, strong staining: 3.3%-13.9%	Same as Morichetti et al. 2010a	Decreased in PCa compared with normal tissue
Hennigs et al. 2014 [28]	2195 (TMA)	2	Rabbit polyclonal (Atlas Antibodies)	44%	Membrane and cytoplasmic	Inverse correlation with Gleason score, pT, Ki-67, preoperative PSA, positive surgical margins; lower SST2 expression in recurrent tumor and MTS

HGPIN, high-grade prostatic intraepithelial neoplasia; *MTS*, metastasis/metastases; *n/s*, not specified.

Table 2
Immunohistochemical Studies of CXCR4 Chemokine Receptor Expression in Prostate Carcinomas

Study	Samples (*n*)	Type of Antibody	Positivity (%)	Type of Staining	Correlations with Clinical Data
Sun et al. 2003 [29]	>600 (TMA)	Mouse monoclonal (R&D Systems)	100%	Nuclear and cytoplasmic	Higher in PCa than in benign tissue, increasing with aggressiveness
Darash-Yahana et al. 2004 [30]	43 (tumor tissue panel) + 75 PT + 11 LN/B MTS	Mouse monoclonal (two different; R&D Systems)	100%, strong staining: 28% tumor tissue panel, 19% PT, 81% LN/B-MTS	Cytoplasmic and membrane	No expression in normal tissue, expression higher in tumor with MTS and in MTS
Mochizuki et al. 2004 [31]	35	Mouse monoclonal (R&D Systems)	57.1%	Nuclear and cytoplasmic	Negative in normal tissue, higher in patients with B-MTS, no correlation with T-stage, Gleason score, presence of LN or lung MTS
Hirata et al. 2007 [32]	50	Mouse monoclonal (R&D Systems)	45%-100%	Nuclear and cytoplasmic	Association with CXCL12 G801A genotype; highest with A/A genotype (100%), followed by G/A genotype (75%) and G/G genotype (45%)
Akashi et al. 2008 [33]	52	Goat polyclonal (Santa Cruz)	94.2%	Cytoplasmic	High expression associated with poor cancer-specific survival. No correlation with grading, extent of B-MTS, clinical response to hormone therapy or PSA level
Xing et al. 2008 [34]	40 + 10 controls	Mouse polyclonal (R&D Systems)	82.5%	Cytoplasmic	No expression in normal tissue; correlation with staging, higher in metastatic disease; no association with Gleason score, PSA level
Jung et al. 2011 [35]	57 (TMA)	Goat polyclonal (Santa Cruz)	93%	Cytoplasmic	Associated with local recurrence, distant MTS, cancer-specific survival; no correlation with age, Gleason score, T-stage, PSA level biochemical recurrence
Okera et al. 2011 [36]	63	Goat polyclonal (Santa Cruz)	56%	Cytoplasmic	No association with patient outcomes
Domanska et al. 2012 [37]	45 (15 PT, 15 LN-MTS, 15 B-MTS)	Rabbit polyclonal (Abcam)	PT negative, LN-MTS 13% positive, B-MTS 67% positive	Nuclear and cytoplasmic	Higher expression in B-MTS than in LN-MTS and PT
Delongchamps et al. 2015 [38]	40	Mouse monoclonal (Abnova)	25% in center of tumor, 85% at tumor front	Nuclear and cytoplasmic	High expression at tumor front associated with high Gleason score and locally advanced disease
Gravina et al. 2015 [39]	78 localized, 12 LN-MTS, 4 B-MTS + array (46 PT, 8 B-MTS)	Rabbit polyclonal (GenScript)	100%; strong staining: PT 35.5%, LN-MTS 50%, B-MTS 100%	Nuclear and cytoplasmic	No expression in normal tissue, higher expression in B-MTS than in LN-MTS and PT
Diao et al. 2016 [40]	148	Mouse monoclonal (R&D Systems)	100%	Cytoplasmic and membrane	Higher in PCa than in normal tissue; correlation with microvessel density, young age, grade, presence of MTS, androgen receptor negativity
Mushtaq et al. 2018 [41]	12 + 11 controls	Mouse monoclonal (R&D Systems)	No information provided	Cytoplasmic	No expression in benign lesions, in contrast to PCa

B, bone; *LN*, lymph node; *MTS*, metastasis/metastases; n/s, not specified; *PT*, primary tumors.

Staining in all sections was scored using the semiquantitative Immunoreactivity Score (IRS) according to Remmele and Stegner [51]. To determine the IRS, the percentage of positively stained tumor cells in each section was stratified into five gradations [no positive cells (0), <10% positive cells (1), 10%-50% positive cells (2), 51%-80% positive cells (3), and >80% positive cells (4)]. The grade of positive staining was then multiplied by the staining intensity, which was quantified in four gradations [no staining (0), weak staining (1), moderate staining (2), and strong staining (3)]. Thus, the final IRS values ranged from 0 to 12. All initial scoring was performed by the same investigator (C.W.).

The presence of tumor in each of the tissue cylinders on the TMA was evaluated in H&E-stained sections on the basis of cell morphology. In addition, alpha-methylacyl-CoA racemase (AMACR) was used as nonspecific tumor marker, chromogranin A (CgA) as a marker for neuroendocrine differentiation, and Ki-67 as a proliferation marker. Furthermore, expression of extracellular signal–regulated kinase (ERK) 1/2 and phosphorylated (activated) ERK (pERK) was evaluated in order to include a downstream regulator of cell proliferation.

All initial assessments of histological staining were reevaluated by an experienced pathologist (O.D.). Both investigators were blinded to the clinical diagnoses of the sample donors. In case of discrepant scores, final decision was achieved by consensus.

TMA samples of a given patient were only included in the final analysis if tumor tissue was present in at least two of the three cylinders of this patient. Then, for each patient, the arithmetic mean of the IRS scores of all tumor-carrying cylinders (i.e., of two or three cylinders) was calculated. Only tumors with an IRS value ≥ 2 for a given receptor or marker were considered positive for that receptor or marker.

Statistics

The IBM SPSS statistics program version 25.0 (Armonk, NY) was used for graphical data processing and statistical analysis. Because the data were not normally distributed (Kolmogorov-Smirnov test), Mann-Whitney *U* test, χ^2 test, Kendall's τ-*b* test, and Spearman's rank correlation were performed. For survival analysis, the Kaplan-Meier method with log-rank test was used. *P* values $\leq$ .05 were considered statistically significant.

Results

Patient Characteristics

Two hundred seventy-six of the 306 tumor samples on the TMA with tumor present in at least one of the three tissue cylinders were included in the final analysis. The mean age at the time of surgery of the 276 patients included in the final analysis was 65.5 years (median: 66.0 years; range: 47-79 years). Slightly more than half (51.8%) were between 61 and 70 years of age. The mean body mass index of the included patients was 27.3 kg/m^2 (median: 27.0 kg/m^2, range: 19.3-38.1 kg/m^2).

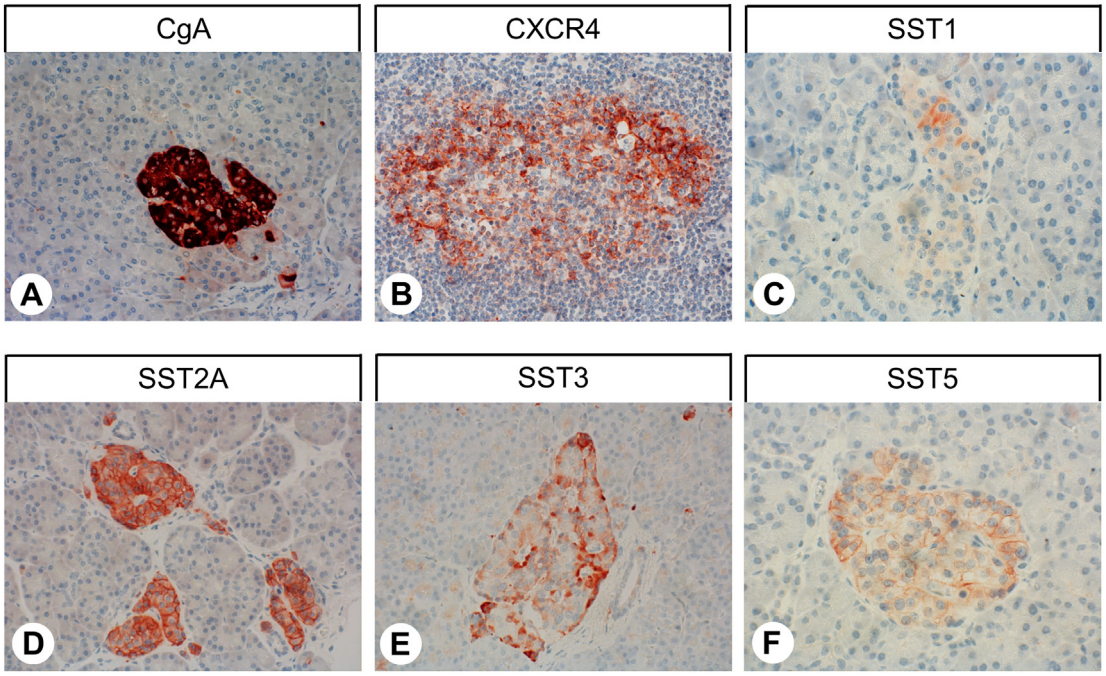

Figure 1. Positive controls for immunostaining. Typical examples of positive controls for (A) CgA; (B) the chemokine receptor CXCR4; and (C-F) the somatostatin receptors SST1, SST2A, SST3, and SST5. CgA, SST1, SST2, SST3, and SST5: pancreatic islets; CXCR4: germinal center of a lymph node. Immunohistochemistry (red-brown color), counterstaining with hematoxylin. Original magnification: 400× in A, B, D, and E; 630× in C and F.

The pathological staging of the included tumor samples ranged from pT2a to pT4: 21 (7.6%) pT2a, 11 (4.0%) pT2b, 147 (53.3%) pT2c, 44 (15.9%) pT3a, 48 (17.1%) pT3b, and 3 (1.1%) pT4. The T status was unknown for two (1.0%) of the samples. At the time of surgery, 203 of the TMA donors (73.5%) had no lymph node metastases, whereas in 43 of the patients (15.6%), lymph node metastases were already present. The lymph node status was not known at the time of surgery for 30 (10.9%) of the TMA donors. Four (1.4%) of the patients had distant metastases.

The Gleason score of the included TMA samples ranged from 5 to 10: 3 (1.1%) Gleason score 5, 55 (19.9%) Gleason score 6, 110 (39.8%) Gleason score 7a, 51 (18.5%) Gleason score 7b, 17 (6.2%) Gleason score 8, 39 (14.1%) Gleason score 9, and 1 (0.4%) Gleason score 10. According to the UICC grading, 22 tumors (8.0%) were G1, 148 (53.6%) G2, 104 (37.7%) G3, and 2 (0.7%) G4. The weights of the prostates at the time of surgery ranged from 15 g to 150 g with a mean of 50.9 g and a median of 49.0 g. Twenty (7.2%) of the TMA donors received antiandrogen treatment before surgery. Serum PSA levels were available for 259 of the patients. The mean serum PSA value was 11.2 ng/ml (median: 7.0; range: 0.3-171.0 ng/ml). One hundred fifty (54.3%) of the PSA values were between 4 ng/ml and 10 ng/ml, and 31 (11.2%) were below 4 ng/ml. Follow-up PSA levels were available for some donors up to 24 months after surgery. Thirty-eight of the TMA donors experienced biochemical relapse after surgery.

Receptor Expression Patterns

Representative examples of immunostaining for the SSTs, CXCR4, and CgA in the TMA samples are depicted in Figure 2. Overall, the antibodies against the SSTs as well as those against CXCR4 produced distinct immunostaining of the plasma membrane but also of the cytoplasm of the tumor cells. Apart from (and independent of) the staining in the tumor cells, in 30% of the cases, the SST5 and, in about 10% of the tumors, the SST2A, SST3, and CXCR4 were found to be strongly expressed on the tumor capillaries (see Figure 2, *D* and *F*). SST1 and SST3 were also strongly expressed on single cells scattered throughout the tumor tissues, most probably representing immune cells (see Figure 2, *E* and *G*).

One poorly differentiated small-cell-type neuroendocrine carcinoma of the prostate was included among the 23 conventional tumor blocks. The staining results for that tumor are shown in Figure 3. In contrast to the other tumor samples, with this small-cell-type neuroendocrine tumor, a strong CgA, a high Ki-67, as well as a strong SST2A, SST5, and CXCR4 expression and a moderate SST3 expression were noted.

Figures 4 and 5 show the IRS values of all samples (taking into account also the negative ones) and the numbers of samples that were positive (IRS $\geq$ 2) for the different SSTs and the CXCR4 on the TMA and among the 23 conventional tumor block samples, respectively. For all receptors, but

Table 3
Antibodies Used for Immunohistochemical Staining

Antibody	Clone	Type	Epitope	Supplier	Dilution
SST1	UMB-7	Rabbit monoclonal	ENLESGGVFRNGTCTSRITTL (residues 377-391)	Epitomics, Burlingame, CA	1:25
SST2A	UMB-1	Rabbit monoclonal	ETQRTLLNGDLQTSI (residues 335-369)	Epitomics, Burlingame, CA	1:10
SST3	UMB-5	Rabbit monoclonal	QLLPQEASTGEKSSTMRISYL (residues 398-418)	Epitomics, Burlingame, CA	1:20
SST5	UMB-4	Rabbit monoclonal	QEATPPAHRAAANGLMQTSKL (residues 344-364)	Epitomics, Burlingame, CA	1:10
CXCR4	UMB-2	Rabbit monoclonal	KGKRGGHSSVSTESESSSFHSS (residues 338-359)	Epitomics, Burlingame, CA	1:2
AMACR	13H4	Rabbit monoclonal	Full-length recombinant AMACR	DAKO, Carpinteria, CA	Solution ready to use
CgA	LK2H10	Mouse monoclonal		BioLogo, Kronshagen, Germany	1:50
Ki-67	MIB-1	Mouse monoclonal		DAKO, Carpinteria, CA	
ERK1/2	137F5	Rabbit monoclonal		Cell Signaling Technology, Danvers, MA	1:200
pERK1/2	D13.14.4E	Rabbit monoclonal		Cell Signaling Technology, Danvers, MA	1:400

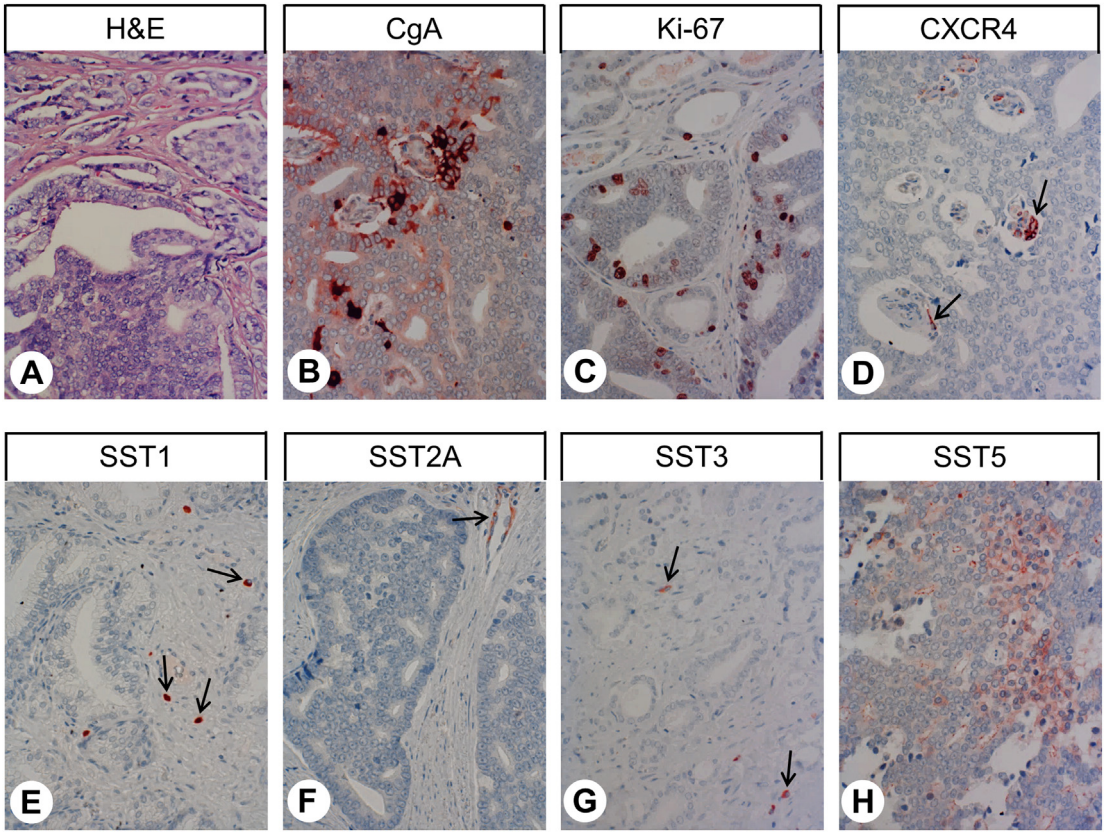

Figure 2. (A) H&E staining and (B-H) immunohistochemical staining (red-brown color) showing typical expression patterns of CgA; Ki-67; chemokine receptor CXCR4; and somatostatin receptors SST1, SST2A, SST3, and SST5 in prostate cancer tissues on the tissue microarray. Counterstaining with hematoxylin. Original magnification: 400×.

especially SST2A and SST5, expression levels varied considerably among individual patients, which are mirrored by the high number of outliers depicted in Figure 4*B* and the length of the whiskers in Figure 5*B*. Generally, the SST and CXCR4 expression levels were very low in the PCa samples investigated. On the TMA, SST5 was the most prominently expressed receptor, followed by SST2A and SST3. By contrast, among the conventional whole-block tumor samples, CXCR4 was the most highly expressed receptor, followed by SST5, SST2A, and SST3. Additionally, both the intensity of expression and the number of positive samples were distinctly higher among the whole-block tumor samples than on the TMA. Of the TMA samples, 29 (10.5%) were positive for SST5 expression, 25 (9.1%) for SST2A expression, and 2 (0.7%) for SST3 expression, although the median IRS was 0 for each of the receptors across all of the TMA samples. Of the positive TMA samples (IRS $\geq$ 2), the median IRS values for the SST2A, SST3, and SST5 were 2.67, 3.17, and 2.33, respectively. None of the TMA samples was positive for SST1 or CXCR4. Of the whole-block samples, 13 (56.5%) were positive for SST5 expression, 8 (34.8%) for SST2A expression, 3 (13.0%) for SST3 expression, and 12 (52.2%) for CXCR4 expression, with median IRS values of 1, 0, 0, and 2, respectively, across all of the whole-block samples. Of the positive whole-block samples (IRS $\geq$ 2), the median IRS values of the SST2A, SST3, SST5, and CXCR4 were 2.00, 3.00, 4.00, and 2.00, respectively. Similar to the TMA samples, none of the whole-block samples was positive for SST1 expression.

The TMA samples were additionally stained for the tumor markers AMACR and CgA, as well as for ERK and pERK. Two hundred fifty-one (90.9%) of the TMA samples were positive for AMACR staining, and 119 (43.1%) were positive for CgA expression. ERK and pERK staining was positive in 257 (93.1%) and 111 (40.2%) of the TMA samples, respectively. The median IRS values across all the TMA samples for AMACR, CgA, ERK, and pERK were 2 (range: 0-3), 1 (range: 0-11), 2.7 (range: 0-8), and 0.3 (range: 0-5), respectively, and thus were also quite low.

Among the TMA samples, SST2A immunostaining was positively correlated with SST3, SST5, CXCR4, CgA, and AMACR expression, as well as with Gleason score (Table 4). In addition, SST3 immunostaining was correlated with SST5 and CgA expression and with Gleason score; SST5 immunostaining with CXCR4, CgA, and AMACR expression; and CXCR4 immunostaining with CgA and AMACR expression (Table 4). A positive association was also noted between CgA and AMACR expression (Table 4). Additional positive correlations were observed between the expression intensities of SST2A and ERK (r_{sp} = 0.185, P = .002), SST3 and ERK (r_{sp} = 0.142, P = .018), SST5 and ERK (r_{sp} = 0.221, P < .001), SST5 and pERK (r_{sp} = 0.330, P < .001), CXCR4 and pERK (r_{sp} = 0.182, P = .002), CgA and ERK (r_{sp} = 0.290, P < .001), CgA and pERK (r_{sp} = 0.365, P < .001), and AMACR and ERK (r_{sp} = 0.186, P = .002). A positive correlation was also seen between ERK and pERK expression (r_{sp} = 0.255, P < .001).

Correlations with Clinical Data

No associations between patient age, preoperative PSA values, or prostate weight and SST, CXCR4, CgA AMACR, ERK, or pERK expression were found. Gleason score was, however, positively correlated with SST2A and SST3 expression (Table 4), as well as with ERK expression (τ = 0.149, P = .001) and PSA level (τ = 0.222, P < .001). A positive association was also noted between patient age and prostate weight (r_{sp} = 0.277, P < .001).

T stage was positively correlated with ERK expression (τ = 0.135, P = .004), patient age (τ = 0.098, P = .036), preoperative PSA value (τ = 0.217, P < .001), and Gleason score (τ = 0.474, P < .001). The presence of lymph node metastases was associated with significantly higher expression of SST2A (mean IRS: N0: 0.413, N1: 0.806; P = .010), SST3 (mean IRS: N0: 0.051, N1: 0.194; P = .018), and ERK (mean IRS: N0: 2.825, N1: 4.050; P = 0,001), as well as with higher preoperative PSA value (N0: 9.44 ng/ml, N1: 23.25 ng/ml; P < .001), higher T stage (P < .001), and higher Gleason score (P < .001).

SST2A expression was positively correlated with biochemical relapse after 6 months (P = .019). Furthermore, SST5 expression and CgA

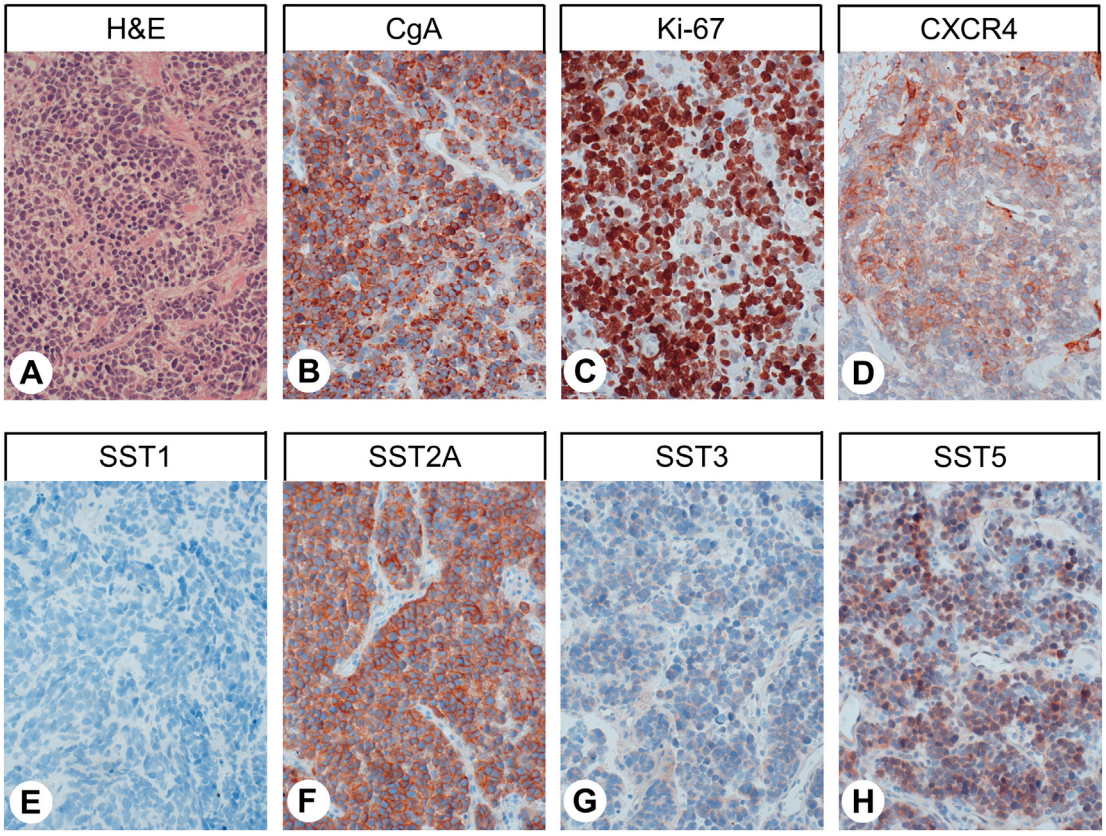

Figure 3. (A) H&E staining and (B-H) immunohistochemical staining (red-brown color) showing expression patterns of CgA; Ki-67; chemokine receptor CXCR4; and somatostatin receptors SST1, SST2A, SST3, and SST5 in a poorly differentiated small-cell-type neuroendocrine carcinoma of the prostate. Counterstaining with hematoxylin. Original magnification: 400×.

expression were correlated with relapse after 24 months ($P = .019$ and $P = .048$, respectively).

Patients who received preoperative antiandrogen treatment had higher T stage ($P = .014$) and Gleason score ($P = .030$) compared with those who did not receive antiandrogen therapy, but there was no association between antiandrogen therapy and the levels of SST or CXCR4 receptor expression.

Regarding tumor vessels, CXCR4 positivity of the tumor microvessels was positively associated with Gleason score ($P = .017$) as well as the presence of distant metastases ($P = .012$).

Receptor Expression in Human Prostate Carcinoma Cell Lines

The staining results obtained for the prostate cancer cell lines LNCaP, DU145, and PC-3 are depicted in Figure 6. All three cell lines were devoid of any SST1, SST2, or SST3 expression but showed noticeable SST5 positivity, which was most evident in the LNCaP and PC-3 cells. The LNCaP cells—and to a much lesser extent the DU145 and PC-3 cells—were also positive for CXCR4 expression. As expected, the PC-3 cells showed strong CgA expression.

Discussion

Receptor Expression Patterns

In contrast to the existing data for both SSTs [21–28] and CXCR4 [29–41], our investigations revealed only negligible SST and CXCR4 expression in the PCa samples overall. One potential reason for these discrepancies is that we used well-characterized monoclonal antibodies to measure the expression levels, whereas most of the previous investigations used polyclonal antibodies from various commercial and noncommercial sources, which might also explain the great variability in the SST and CXCR4 expression levels among the previous studies. We used the IRS to represent the expression levels of the receptors and markers in the cancer tissues, taking both the frequency and the intensity of expression into account. Only samples displaying an IRS ≥ 2 were considered positive for expression of a given receptor or marker. Some previous studies provided no information as to whether the staining frequency and intensity were both measured and which method was used to perform the measurements. Because a given receptor must display at least moderately strong expression intensity (i.e., IRS ≥ 6) for therapies targeting it to be clinically useful, our results clearly indicate that only very few patients with PCa would likely benefit from SST- or CXCR4-based diagnostics or therapy. Of the 276 evaluable tumors on the TMA, only 3 (1.1%) displayed an IRS of 6 for SST5, and only 1 (0.4%) displayed an IRS of 6 for SST2A. All other TMA samples had IRS values below the threshold for the SSTs and CXCR4.

Neuroendocrine differentiation in PCa occurs during tumor progression and is typically observed in tumors with metastases and in castration-resistant tumors as a result of antiandrogenic therapy [52]. For the neuroendocrine marker CgA, 43.1% of the TMA samples had an IRS ≥ 2, and only 17.8% (49) had an IRS ≥ 6 (with maximum IRS values of 11). However, of the 49 samples with a CgA IRS ≥ 6, only 1 had an IRS of 6 for an SST (SST5), whereas 14 were completely devoid of any SST expression. Thus, although there was a significant correlation between SST and CgA expression, many of the tumors, despite exhibiting substantial neuroendocrine differentiation, did not express any SST (or expressed them at only a negligible level). In contrast, tumors with high SST expression were not necessarily highly CgA positive. Notably, the overall median CgA IRS in our investigation was only 1, indicating a generally low percentage of neuroendocrine differentiation among the tumor samples evaluated.

Most of the patients in our study were likely still in an early stage of PCa because only a few patients had known lymph node metastases and very few had distant metastases. Additionally, only a small number of patients

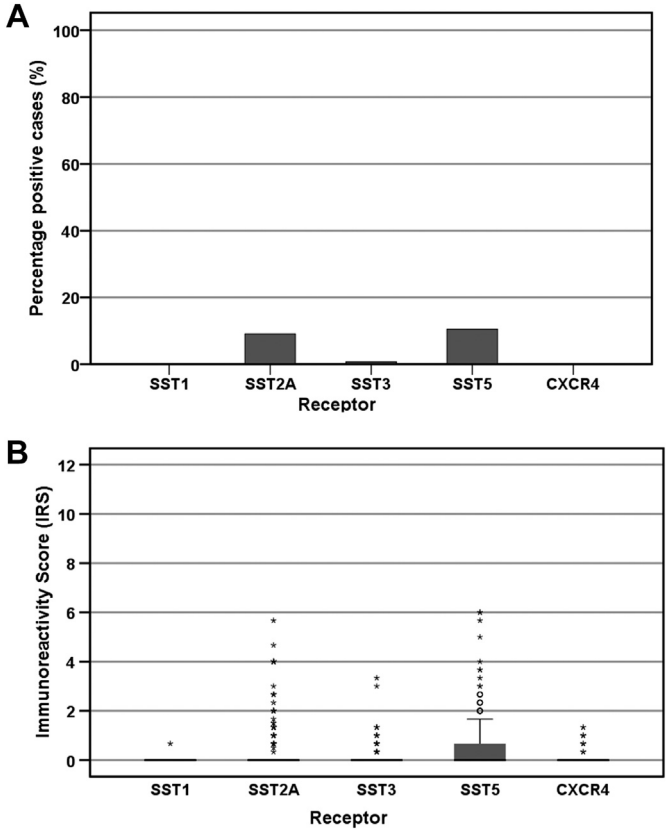

Figure 4. Expression profiles of the SST subtypes SST1, SST2A, SST3, and SST5 and the chemokine receptor CXCR4 in prostate cancer tissues on the tissue microarray. (A) Percentage of tumors positive for the different SSTs and CXCR4. Tumors with an IRS ≥ 2 were considered positive. (B) Box plots of the expression levels (IRS values) of the SSTs and CXCR4 of all samples (taking into account also the negative ones). Median values, upper and lower quartiles, minimum and maximum values, and outliers are depicted. The outliers are defined as follows: circles: mild outliers, 1.5 to 3 times more extreme than the upper or lower quartiles; asterisks: extreme outliers, more than 3 times as extreme as the upper or lower quartiles.

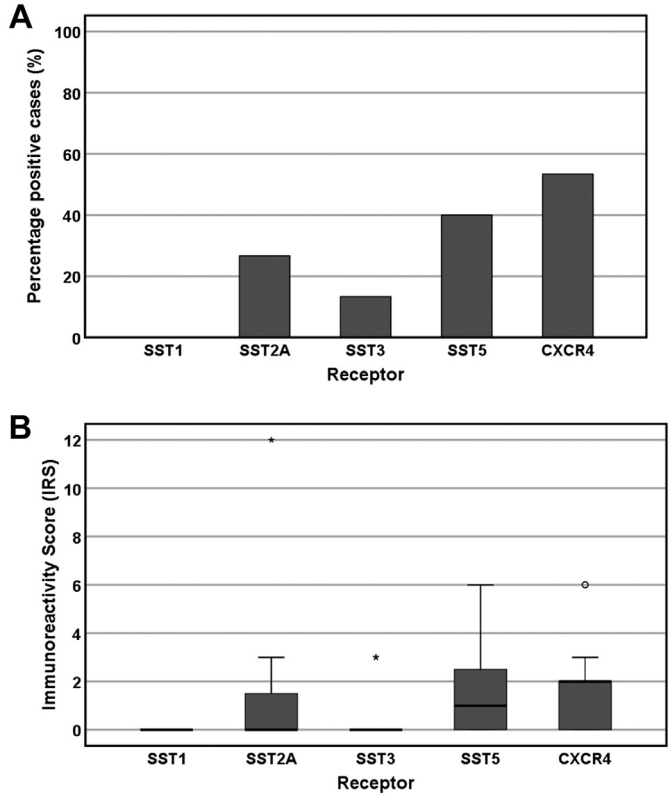

Figure 5. Expression profiles of the SST subtypes SST1, SST2A, SST3, and SST5 and the chemokine receptor CXCR4 in whole-block prostate cancer tissues. (A) Percentage of tumors positive for the different SSTs and CXCR4. Tumors with an IRS ≥ 2 were considered positive. (B) Box plots of the expression levels (IRS values) of the SSTs and CXCR4 of all samples (taking into account also the negative ones). Median values, upper and lower quartiles, minimum and maximum values, and outliers are depicted. The outliers are defined as follows: circles: mild outliers, 1.5 to 3 times more extreme than the upper or lower quartiles; asterisks: extreme outliers, more than 3 times as extreme as the upper or lower quartiles.

had received antiandrogenic therapy. Overall, our findings are in line with SST-PET/CT data from the literature in which positive lesions were observed only in select cases of patients with castration-resistant tumors, biochemical relapse, or multiple metastases [53–56]. That our patients were still in an early stage of PCa might also be the reason for the low rate of CXCR4 expression, as CXCR4 expression is primarily present in highly aggressive tumors, such as G3 gastroenteropancreatic neuroendocrine carcinomas, small-cell lung cancers, and lymphomas [57–60]. This finding also corroborates the results of two meta-analyses demonstrating that CXCR4 is primarily expressed in advanced PCa [61,62].

Of all the tumors evaluated in the present investigation, only the small-cell-type neuroendocrine carcinoma that was coincidentally included among the whole-block samples exhibited strong CgA, SST, and CXCR4 expression. The IRS values of that tumor were 12, 0, 12, 3, 6, and 6 for CgA, SST1, SST2A, SST3, SST5, and CXCR4, respectively, with a Ki-67 index of 97%. Small-cell-type neuroendocrine carcinomas of the prostate are very rare (<1% of cases), are highly aggressive, exhibit high Ki-67 levels, frequently show multiple metastases, and have exceptionally poor prognosis [63]. Therapeutic options for patients with small-cell-type neuroendocrine carcinomas are very limited. Our results suggest that SST- or CXCR4-based therapy might be effective in those patients, as recently demonstrated in a case report on a combination therapy of docetaxel and octreotide [64].

When comparing the results obtained with the TMA with those achieved with the whole-block tumor samples, generally higher SST and CXCR4 expression rates were observed with the complete blocks and therefore a much higher percentage of positive cases. That discrepancy might be due to the high heterogeneity of SST and CXCR4 expression within the individual tumors. This heterogeneity was clearly visible in the whole-block tumor samples and was most pronounced for CXCR4 expression, which was often confined to the cells surrounding necrotic areas and to the proliferation fronts of the tumors. Hence, with this receptor also the highest discrepancy between the results obtained with the TMA and the whole-block tumor samples was observed. Pronounced intraindividual variability in SST and CXCR4 expression is well documented in the literature on neuroendocrine tumors [60,65], as well as other tumor entities [66–68] and prostate cancer [38]. This variability might have led to an underestimation of SST and CXCR4 expression in the TMA samples in our investigation, although three tissue cylinders were taken per tumor block. Therefore, from our results, it has to be concluded that TMAs are not appropriate to determine the presence of SST and CXCR4 in PCa.

However, even among the whole-block samples, only very low median IRS values were observed (below 2 for all receptors), leading to similar results as those of the TMA: in PCa tumor cells, with the exception of very few select cases, SSTs and CXCR4 are expressed at negligible levels and therefore have no obvious diagnostic or therapeutic relevance.

In addition to the PCa tumor samples, also cytoblocks of three PCa cell lines were evaluated for their SST and CXCR4 expression profile: LNCaP, which is androgen sensitive and displays low metastatic potential; DU-145, which is hormone insensitive and shows moderate metastatic capacity; and PC-3, which represents a model for small-cell-type neuroendocrine

Table 4
Correlations Between Expression Intensities of Different SSTs, CXCR4, CgA, AMACR, Preoperative PSA Values, and Gleason Score in the TMA samples

		SST2A	SST3	SST5	CXCR4	CgA	AMACR	PSA	Gleason
SST1	*r*	−0.034	−0.017	0.085	−0.013	0.102	−0.030	0.043	−0.018
	P	.573	.775	.161	.825	.091	.618	.396	.743
SST2A	*r*		**0.245**	**0.468**	**0.215**	**0.324**	**0.384**	0.027	**0.154**
	P		**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	.580	**.003**
SST3	*r*			**0.195**	0.066	**0.236**	0.049	−0.047	**0.139**
	P			**.001**	.277	**<.001**	.415	.348	**.010**
SST5	*r*				**0.219**	**0.518**	**0.259**	0.036	0.056
	P				**<.001**	**<.001**	**<.001**	.458	.280
CXCR4	*r*					**0.269**	**0.128**	0.013	−0.055
	P					**<.001**	**.034**	.790	.308
CgA	*r*						**0.203**	−0.007	0.006
	P						**.001**	.913	.922
AMACR	*r*								0.107
	P								.077

Significant correlations ($P < .05$) are marked in bold. *r*, correlation coefficient [Spearman; Kendall τb (correlations with Gleason score)]; *P*, *P* value.

carcinoma, is androgen insensitive, and displays high metastatic ability [69,70]. All three cell lines showed expression of SST5, which was also the most commonly expressed receptor in the PCa tumor samples. Surprisingly, and in contrast to the results obtained for the small-cell-type neuroendocrine carcinoma, PC-3 cells were devoid of expression of any other SST subtype and also had lower CXCR4 expression than expected for such a highly proliferative cell line. In contrast, LNCaP cells showed higher CXCR4 expression than expected for a cell line with low proliferative potential. Previous studies have reported similar observations of higher CXCR4 expression in LNCaP compared with that in PC-3 cells [29,71]. Thus, regarding SST and CXCR4 expression patterns, none of the three cell lines can be regarded as representative of PCa tumors.

Correlations with Clinical Data

In contrast to observations in the literature [23,28], our results clearly indicate a positive association between SST expression and tumor grade and stage. There were positive correlations between SST2A and SST3 expression and Gleason score and preoperative PSA values, between SST2A and SST3 expression and lymph node metastases, and between SST2A

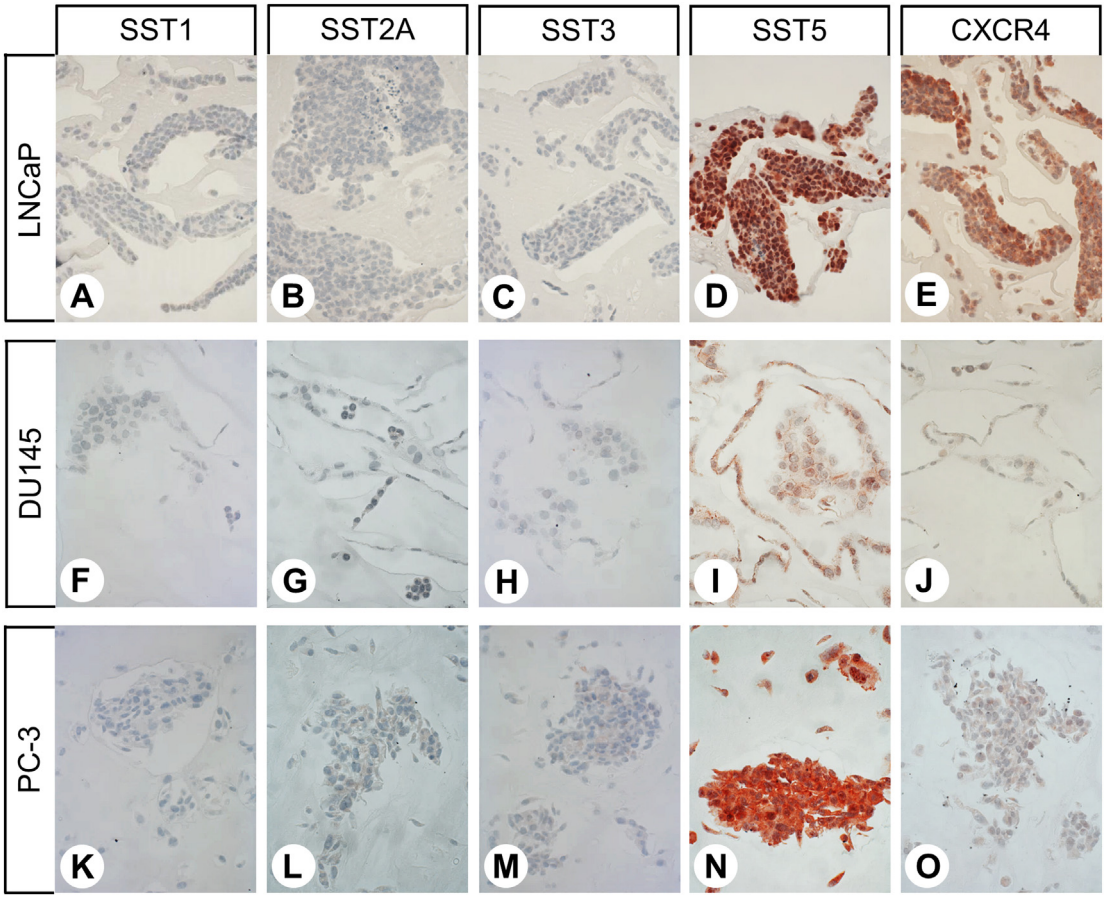

Figure 6. Immunohistochemical staining (red-brown color) showing expression patterns of the SST subtypes SST1, SST2A, SST3, and SST5 and the chemokine receptor CXCR4 in the prostate cancer cell lines (A-E) LNCaP, (F-G) DU145, and (H-O) PC-3. Counterstaining with hematoxylin. Original magnification: 400×.

and SST5 expression and biochemical relapse. These results are in agreement with the observation that SST2A, SST3, and SST5 expression was positively correlated with ERK and pERK expression. We also found correlations between CgA and CXCR4 expression and ERK and pERK expression. ERK overexpression has been demonstrated in many tumors and has been shown to play a role in tumor progression [72], which seems to apply also in PCa [73]. Fittingly, in our study, higher ERK expression was associated with elevated preoperative PSA values, advanced T stage, increased Gleason score, and higher AMACR expression.

Independent of SST and CXCR4 expression in the tumor cells, SST2A, SST3, SST5, and CXCR4 were often strongly expressed on the tumor capillaries. Similar observations were described previously for SSTs in PCa [21–27] and for CXCR4 in other tumor entities [66,68,74,75]. Additionally, in the present study, an association between CXCR4 positivity of tumor microvessels and the presence of distant metastases ($P = .012$) and higher Gleason score ($P = .017$) was observed. Neoangiogenesis plays an important role in the development, progression, and metastasis of many types of tumors, including PCa [76]. Therefore, targeting of tumor microvessels using anti-SST or anti-CXCR4 therapies might represent a promising (additional) therapeutic strategy for PCa.

Conclusions

Although SST and CXCR4 expression levels are associated with higher tumor grade and stage, they are generally low in PCa and therefore are of no obvious diagnostic or therapeutic relevance in that disease. SST-based or CXCR4-based therapy might be justified only in rare cases of small-cell-type neuroendocrine carcinoma of the prostate. Further studies on more advanced tumor stages specifically focusing on this rare tumor entity are necessary to validate the results obtained from the single case presented here. Because of the high intraindividual variability in SST and CXCR4 expression in PCa, whole-tumor samples should preferably be analyzed.

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Declaration of Competing Interest

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