Aberrant Presentation of HPA-Reactive Carbohydrates Implies Selectin-Independent Metastasis Formation in Human Prostate Cancer

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Abstract

**Purpose:** To investigate the impact of prostate cancer cell surface glycosylation as part of the tumor cell–endothelial cell interaction in prostate cancer metastasis.

**Experimental Design:** Glycosyltransferase expression was profiled in metastasis-derived prostate cancer cell lines and compared with primary epithelium. Prostate cancer cells were examined for HPA- and selectin-binding and adhesion to endothelium. Spontaneous metastasis xenograft models were established to test the lectin HPA-binding sites as a marker of metastatic competence and to evaluate E-selectin-binding sites in vivo. The importance of selectins for metastasis formation was analyzed using Sele−/−/Selp−/− mice. The clinical relevance of HPA- and E-selectin-binding sites in prostate cancer was determined.

**Results:** Glycosyltransferases involved in the synthesis of common HPA-binding sites are downregulated in prostate cancer cells. An absence of HPA-reactive carbohydrates specifically indicates spontaneous metastatic spread of prostate cancer xenografts in vivo and a poor prognosis of patients with prostate cancer. HPA-binding sites decrease in lymph node metastases compared with corresponding primary tumors. Common selectin ligands are absent on prostate cancer cells, which do not adhere to recombinant selectins or endothelium under shear stress in vitro. Spontaneous metastasis formation is largely independent of selectins in vivo. E-selectin-binding sites are detectable in only 2% of patients with prostate cancer without prognostic significance.

**Conclusion:** Prostate cancer is characterized by an inverse functional and prognostic importance of HPA-binding sites compared with other adenocarcinomas. Accordingly, this study surprisingly shows that the selectin–selectin ligand axis, which is essential for extravasation and thus metastasis formation in several malignancies, can be circumvented in prostate cancer. Clin Cancer Res; 20(7); 1–12. ©2014 AACR.

Introduction

Prostate cancer is the predominant neoplasm in males and represents the second leading cause of cancer-related deaths in men (1). As with all other cancers, it is the development of distant metastases, which is responsible for the majority of prostate cancer–associated deaths. During the multistep process of metastatic spread, primary tumor cells are interacting with their microenvironment via their glyocalyx (2), which is commonly aberrantly composed in carcinoma cells compared with their normal counterparts (3). This altered cell surface glycosylation, which has widely been explained by an altered glycosyltransferase expression (4), is often associated with invasion and metastasis (5). In particular, increased cell surface presentation of terminal N-acetylgalactosamine- (GalNAc-) and N-acetylgalactosamine- (GlcNAc)-residues correlate with progression and metastasis in breast and colorectal cancer as determined by the specific binding of the lectin Helix pomatia agglutinin (HPA) toward these terminal glycoconjugates. Hence, HPA has been shown to be a marker of metastatic competence in human breast and colorectal xenograft primary tumors in severe combined immunodeficient (SCID) mice (2) and of a poor patient prognosis in these malignancies (6, 7) as well as in adenocarcinoma of the lung (8), gastric cancer (9), and malignant melanoma (10). HPA-reactive carbohydrates are typical for two of the most prominent O-glycans in cancer, namely Tn antigen and core 2 O-glycans (4, 11), which are synthesized through
of metastatic behavior in vivo antigen (PSA) values in patients (22). The impact of HPA-human breast, colorectal (HT29), and pancreatic cancer metastasis formation from xenograft primary tumors of migrate into host organs of distant metastases as well selectin-, integrin- and chemokine-mediated interactions to inflamed tissues (17). Our hypothesis is that circulating crucially involved in the adhesion cascade of leukocytes into organ invasion of human eosinophilic leukemia cells (16). Furthermore, the importance of HPA-reactive glyco- synthesis of sialylated Lewis X and A intermediates for the synthesis of sialylated Lewis X and A synthases (C2GNT1,2,3), respectively (Fig. 1A). Important- indicates a particular importance of adhesion partners other than selectins that accomplish the unique selectin-independent extravasation in this malignancy.

Translational Relevance

This is the first study applying spontaneous metastasis xenograft models of human prostate cancer to test the functional relevance of aberrant cell surface glycans and downstream molecules of the leukocyte adhesion cascade for prostate cancer metastasis. Carbohydrate residues recognized by the lectin *Helix pomatia* agglutinin (HPA), which predict metastasis formation and a poor prognosis in other malignancies, are surprisingly absent in highly metastatic prostate cancer xenografts and decrease in clinical lymph node metastases. Corresponding to the fact that such carbohydrates are common intermediates for selectin ligand synthesis, prostate cancer metastasis formation is largely independent of selectins. These findings are also reflected by a beneficial prognosis of patients with HPA-positive prostate cancers and by a particularly low incidence of E-selectin-binding sites in prostatectomy specimens underlining the translational relevance of our model. Moreover, this study indicates a particular importance of adhesion partners other than selectins that accomplish the unique selectin-independent extravasation in this malignancy.

the sequential activity of polypeptide GalNAc-transferases (pp-GalNac-T’s), core 1 synthase (C1GALT1) and core 2 synthases (C2GNT1,2,3), respectively (Fig 1A). Important- as summarized in Fig. 1A, these glycans are common intermediates for the synthesis of sialylated Lewis X and A antigens (sLeX and sLeA; ref. 12), which are presented at the surface of human breast and gastrointestinal adenocarcinoma (13–15) as well as leukemia cells (16). sLeX and sLeA are the main ligands for E- and P-selectin and therefore crucially involved in the adhesion cascade of leukocytes into inflamed tissues (17). Our hypothesis is that circulating tumor cells (CTC) imitate this adhesion cascade involving selectin-, integrin- and chemokine-mediated interactions to migrate into host organs of distant metastases as well (refs. 18–20; Fig 1A, bottom). Accordingly, spontaneous metastasis formation from xenograft primary tumors of human breast, colorectal (HT29), and pancreatic cancer (PaCa5061) drastically decreases in Sele−/−/Selp−/− SCID and Pfp−/−/Rag2−/− mice (13–15). Likewise, engraftment and organ invasion of human eosinophilic leukemia cells (EOL-1) are remarkably reduced in selectin-deficient mice (16). Furthermore, the importance of HPA-reactive glyco-conjugates as intermediates of selectin ligand synthesis has also been shown previously, as preincubation of human breast cancer cells with HPA inhibits their adhesion toward vascular endothelium (7, 21).

In prostate cancer, we recently established spontaneous metastasis xenograft models and identified abnormally presented β (1, 6) branched oligosaccharides as a marker of metastatic behavior in vivo and elevated prostate specific antigen (PSA) values in patients (22). The impact of HPA- and selectin-binding sites for metastasis formation and patient prognosis in prostate cancer, however, has so far not been analyzed in detail. We therefore aimed to determine glycosylation patterns in prostate cancer with a particular focus on HPA-reactive carbohydrates and selectin ligands (including their potential relevance for prostate cancer adhesion to selectins/endothelium), to test HPA as a marker of metastatic competence in prostate cancer xenograft models and as a prognostic factor in clinical prostate cancer, to analyze E-selectin binding sites in prostate cancer xenograft tumors, and in patients to determine whether selectin binding is essential for metastasis formation in prostate cancer.

Materials and Methods

Cell lines and culture conditions

PC-3, DU-145 (prostate cancer), PPEC (human primary, nonmalignant prostate epithelial cells), HT29 (colon cancer), EOL-1 (eosinophilic leukemia), and PaCa5061 (pancreatic adenocarcinoma) were used as described before (refs. 14, 22; see also Supplementary Table S1; refs. 16, 23). VCaP (prostate cancer) cells were obtained from American Type Culture Collection and cultured in RPMI-1640 supplemented with 2 mmol/L l-glutamine, 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (all Invitrogen) at 37 °C in a humidified atmosphere of 5% CO₂ (24). Human pulmonary microvascular endothelial cells (HPMEC, Supplementary Table S1; refs. 25–27) were from PromoCell and cultured in endothelial cell growth medium MV supplemented with the corresponding supplement mix (PromoCell). All experiments with primary cells were performed during the first six passages.

Quantitative real-time-PCR glycosylation array

RNA isolation and cDNA synthesis from cell culture grown PC-3, DU-145, VCaP, and PPEC was performed as described (22); expression of glycosyltransferases was assessed using the Human Glycosylation RT² Profiler PCR array including 84 glycosyltransferase and glycosidase genes (Qiagen). All arrays were repeated twice with independently isolated RNAs.

HPA-binding flow cytometry and lectin histochemistry

Tumor cells were detached and incubated for 30 minutes at 4°C with fluorescein isothiocyanate (FITC)-conjugated HPA (Sigma) diluted 1:100 in lectin buffer (0.05 mol/L TRIS-buffered saline, pH 7.6, added with 1 mmol/L CaCl₂ and MgCl₂). Binding specificity was evaluated by inhibiting HPA with 100 mmol/L D-GalNAc (Sigma) before incubation. All samples were washed once, marked dead or alive by propidium iodide staining (Sigma), and subjected to flow cytometry (FACS) using a CyFlow Cube cytometer (Partec). Data were analyzed using CyView software (Partec).

Xenograft primary tumors of all prostate cancer cell lines, HT 29 and PaCa5061 as well as prostate cancer prognosis and heterogeneity tissue microarrays (see below) were evaluated for HPA-binding sites by a standard lectin histochemistry in accordance with several previous studies in
different human adenocarcinomas (2, 6–10). Briefly, tissue sections were treated overnight with xylol, deparaffinized, and pretreated with 0.1% trypsin (obtained as trypsin powder from Biochrom, substance activity 1512 USP U/mg) in lectin buffer for 15 minutes at 37°C. Afterward, sections were incubated with biotinylated HPA (Sigma) and stained as described before (22). Again, 100 mmol/L D-GalNAc was used for inhibition of HPA on parallel sections.

Subcutaneous xenograft mouse models

Male Pfp/Rag2<sup>−/−</sup> double-knockout mice (8–12 weeks, 20–25 g) from Taconic were used as described (22).
Animals were maintained under pathogen-free conditions in individually ventilated cages and fed with sterile standard food and water ad libitum. All animal experiments were approved by the local animal experiment approval committee (project No. G08/75). PC-3 and DU-145 cells were xenotransplanted as described (22). This study firstly describes the use of VCaP cells as a suitable model of metastatic prostate cancer. For VCaP tumor growth, it was necessary to mix 1 × 10⁶ cells 1.2 with Matrigel (BD Biosciences) immediately before injection. Pfp/Rag2⁻/⁻ mice were crossbred with E- and P-selectin-deficient mice (Jackson Laboratory, stock 002916) and selectin deficiency was verified as described (13).

When primary tumors exceeded 2 cm³ or ulcerated the mouse skin, the mice were terminally narcotized and sacrificed by cardiocentesis. Right lungs were excised en bloc and prepared for histologic analyses as described (28). Three representative lung sections from 3 animals of the PC-3 group were subjected to HPA lectin histochemistry to determine the presence of HPA-reactive carbohydrates in spontaneous lung metastases. The left lungs were homogenized in a sample disruptor (TissueLyser II, Qiagen) and subjected to DNA-isolation (QIAamp DNA Mini Kit, Qiagen). Bone marrow was collected by flushing the left femora with 1 mL NaCl 0.9%. Two hundred microliters of blood and the bone marrow suspensions were subjected to DNA isolation using the QIAamp DNA Blood Mini Kit. Finally, primary tumors were removed, weighed, and processed for histologic examinations.

Quantification of disseminated tumor cells and CTC by Alu-PCR

DNA concentrations of all samples were quantified using a NanoDrop spectrophotometer (Peqlab). As the content of detectable Alu-sequences in the following qPCR would have been affected simply by varying DNA concentrations, all lung and bone marrow DNA samples were normalized to 30 ng/µL using AE buffer (Qiagen). The concentrations of blood-DNA were quite similar in all samples (~10 ng/µL) and therefore not normalized. Quantitative PCR (qPCR) was performed with established human-specific Alu primers (29). Two microliters total DNA (i.e., 60 ng lung/bone marrow-DNA, 20 ng blood-DNA) were used for each qPCR. Numerical data were determined against a standard curve as described (22). The detection limit for specific human Alu-sequence signals was determined for each tissue type by testing DNA from 5 healthy (noninjected) Ppf/Rag2⁻/⁻ mice of similar sex and age. For each sample, analyses were performed in duplicates and as independent experiments at least twice.

Morphological and immunohistochemical analysis of spontaneous lung metastases

Pulmonary metastases were examined histologically in 10 standardized hematoxylin and eosin (H&E)-stained lung sections per mouse (28). Human cancer cells were recognized by their characteristically large, basophilic, and polymorphic nuclei, which were clearly distinguishable from the smaller nuclei of mouse cells (Figs. 2 and 4). To evaluate potential differences between wild-type and E-/P-selectin-deficient Ppf/Rag2⁻/⁻ mice, the lungs of 10 mice per group were analyzed by two blinded investigators with a particular focus on the differentiation between intrastromal metastases and intravascular tumor cells. Tumor cell location was considered as to be intravascular, when erythrocytes or blood plasma were adjacent to cancer cells or a surrounding layer of vascular endothelium was morphologically present. In addition, immunostainings for S1P1 (polyclonal rabbit, Santa Cruz #25489) were performed on consecutive lung tissue slides to ascertain the presence of intrastromal PC-3 cells in selectin-deficient mice.

Detection of E-selectin-binding sites on tissue sections by immunofluorescence

Cell surface E-selectin-binding sites were assessed in prostate cancer xenograft tumors and prostatectomy cancer epithelium using a rh-E-selectin/IgG1-Fc chimera or IgG-Fc (isotype control, both from R&D Systems) on xylol-treated, deparaffinized tissue, or microarray sections as described before (15). Human pancreatic adenocarcinoma grown in Ppf/Rag2⁻/⁻ mice served as a positive control (15). The use of anonymized human tissue microarrays and clinical follow-up data was permitted by the local ethical review committee (Project No.WF-060/12).

Prostate cancer prognosis and heterogeneity tissue microarrays

The clinical impact of glycoconjugates terminating in GalNAc and/or GlcNAc and of E-selectin-binding sites was analyzed using HPA lectin histochemistry and E-selectin immunofluorescence on TMA slides containing primary tumor samples from 1,285 or 1,600 patients with prostate cancer, respectively. All patients underwent radical prostatectomy at the Department of Urology at our Medical Center (1992–2005). PSA values were measured quarterly in the first year, followed by biannual measurements in the second and annual measurements after the third year following surgery. Biochemical relapse (BCR) was defined as a postoperative PSA of 0.2 ng/mL and rising thereafter; patients without evidence of recurrence were censored at last follow-up. Prostatectomy specimens were transferred onto a TMA format as described before (30–33). HPA- and E-selectin–binding toward prostate cancer epithelium was evaluated (negative vs. positive; positivity was considered when >50% of tumor cells were stained) and correlated with histopathologic and clinical follow-up data. Next, prostate cancer heterogeneity TMAs were analyzed to investigate whether HPA binding is heterogeneous in prostate cancer and whether the binding status differs between primary tumors and lymph node metastases. This additional microarray included a total of 1,727 tissue punches, taken in 20 different remote areas of each primary tumor and one tissue punch each from 1–8 matched lymph node metastases (n = 76).
These clinical studies were approved by the local ethics committee (WF-049/09).

**Results**

**Glycosyltransferases involved in the biosynthesis of HPA-reactive sugar residues are downregulated in prostate cancer cells**

Significant changes of glycosyltransferase expression involved in the synthesis of HPA-reactive carbohydrates are summarized in Table 1. Note that several polypeptide GalNAc-transferases, core 1 and 2 synthases are downregulated in prostate cancer cells. Accordingly, all tested prostate cancer cell lines bind GalNAc/GlcNAc-specific HPA at a low (VCaP, DU-145) to moderate (PC-3) level compared with HT29 colon cancer cells (Fig. 1B).

Metastatic prostate cancer xenograft primary tumors and spontaneous lung metastases are HPA-negative; E-selectin-binding sites are absent in prostate cancer xenograft tumors

Xenograft tumors developed in 5 of 5 PC-3- and DU-145-bearing mice and 7 of 9 mice injected with VCaP in Matrigel. The median tumor weights are 1.85, 1.68, and 2.32 grams (Fig. 2A) after a mean growth period of 39 ± 3.8, 136 ± 12, and 119 ± 26.6 days (P < 0.0001; Fig. 2B) for PC-3, VCaP, and DU-145, respectively. The rates and median numbers of detected disseminated tumor cells (DTC) and CTC are depicted in Fig. 2C–E. This is the first description of VCaP as a suitable spontaneous metastasis model of human prostate cancer. Histology confirmed the presence of spontaneous lung metastases in the PC-3 model (Fig. 2).
Table 1. Several glycosyltransferases involved in the synthesis of HPA-reactive glycoconjugates are downregulated in metastasis-derived prostate cancer cells compared with primary nonmalignant prostate epithelium (PPEC)

<table>
<thead>
<tr>
<th>Glycosyltransferases</th>
<th>Gene</th>
<th>Ct-value PPEC</th>
<th>Fold up-/downregulation vs. PPEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppGalNAc-T’s (polypeptide)</td>
<td>GALNT3</td>
<td>23.76</td>
<td>PC-3: −2.95, VCaP: −1.34, DU-145: −31.25b</td>
</tr>
<tr>
<td></td>
<td>GALNT6</td>
<td>27.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(GALNT8)</td>
<td>34.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GALNT12</td>
<td>29.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GALNT14</td>
<td>27.63</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-galactosaminyltransferases</td>
<td>GALNT3</td>
<td>23.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GALNT6</td>
<td>27.03</td>
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<td></td>
<td>(GALNT8)</td>
<td>34.1</td>
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<td></td>
<td>GALNT12</td>
<td>29.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GALNT14</td>
<td>27.63</td>
<td></td>
</tr>
<tr>
<td>O-glycan core structure glycosyltransferases</td>
<td>C1GALT1</td>
<td>23.75</td>
<td>PC-3: −2.87b, VCaP: −6.43c, DU-145: −1.32</td>
</tr>
<tr>
<td></td>
<td>C2GNT1</td>
<td>28.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C2GNT2</td>
<td>26.74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C2GNT3</td>
<td>27.97</td>
<td>PC-3: −29.96b, VCaP: −89.63b, DU-145: −2.27a</td>
</tr>
</tbody>
</table>

aP < 0.05.
bP < 0.001.
cP < 0.0001.

detection limits for specific Alu sequences were 20, 5, and 1 tumor cells per used DNA in the lung, bone marrow, and blood, respectively (red dotted lines shown in Fig. 2C–E).

HPA binding is completely absent in VCaP primary tumors and in more than 80% of PC-3 primary tumors, whereas DU-145 tumors show a weak, homogeneous staining pattern throughout the samples. An average of 57 of 68 (83.8%) lung metastases per mouse is HPA-negative (P < 0.05). E-selectin-binding sites are only marginally detectable in PC-3 tumors and are absent in VCaP and DU-145 xenografts. In contrast, IT29 colon and PaCa5061 pancreatic adenocarcinoma xenograft primary tumors present increased levels of HPA- and E-selectin-binding sites in vivo (Fig. 2).

HPA-negative patients have an unfavorable prognosis and HPA-binding decreases in lymph node metastases

Six hundred and ninety-six of 1,285 patients with prostate cancer show no detectable HPA binding, when one representative tissue spot is analyzed per patient, indicating the absence of carbohydrates terminating in GalNAc or GlcNAc at the cancer epithelium cell surface in the majority of cases. Importantly, HPA-negative patients have a decreased biochemical relapse (BCR)-free survival in comparison with the HPA-positive cohort (P = 0.009; Fig. 3A). The adverse prognostic effect of HPA negativity is more pronounced in the subset of R1-resected patients (P = 0.003; Fig. 3B). Accordingly, tumor stages (P<0.0001) and grades (P = 0.002) are increased in the HPA-negative cohort (Fig. 3C and D; Table 2). The percentage of overall biochemical relapses is increased in the HPA-negative patient group (P = 0.006; Table 2; Fig. 3E). In addition, HPA-negative patients have elevated PSA values (P = 0.02; Table 2). However, loss of HPA-binding sites is not an independent predictive biomarker (P = 0.562, multivariate Cox analysis including Gleason score, pT stage, pN and R status).

On the basis of our analysis of up to 20 different primary tumor spots taken from different remote areas of each primary tumor (n = 76), we report a heterogeneous HPA-binding pattern in 89.5% of all patients with prostate cancer (Fig. 3F). Only one patient is homogeneously HPA-positive, whereas 7 patients (9%) are homogeneously HPA-negative. An average of 26.3% of tumor spots is HPA-positive per patient. Eighty-eight percent of all patients have at least one HPA-positive primary tumor spot. Interestingly, this number decreases to 50% in the corresponding lymph node metastases of the same patients (P <0.0001; Fig. 3F), indicating a decrease of HPA-reactive sugar residues during prostate cancer progression and metastatic spread. Lymph node spots were lost in 6 cases during sample processing. Representative pictures of positive and negative HPA binding on primary tumors (top) and lymph node metastases (bottom) are shown in Fig. 3.

E-/P-selectin are not essential for metastasis formation and E-selectin-binding sites are seldom presented in prostate cancer tumors

After engraftment of highly metastatic PC-3 cells into E- and P-selectin–deficient Pfp/Rag2−/− mice, the number of DTCs in the lungs (Fig. 4A) and CTCs in the blood (Fig. 4B) remains unchanged. Because the numbers of DTC in the lungs detected by Alu-PCR might at least partially be caused by intravascular DTCs and may not necessarily represent true metastases, the contralateral lungs were examined morphologically. By this approach, we demonstrate an increase of the median number of intravascular DTCs from 88 ± 543.6 in wild-type to 1,305 ± 1,645.5 in E-/P-selectin–deficient mice (P = 0.038; Fig. 4C), suggesting a disturbed extravasation in selectin deficiency. Nevertheless, the median number of intrastromal metastases is almost similar in both groups (Fig. 4D). Immunohistochemical staining of vascular lung endothelium (S1P1) clearly demonstrates the
Figure 3. Adverse prognosis and lymph node metastases are accompanied by decreased HPA-reactive glycoconjugates. A and B, reduced BCR-free survival in HPA-negative prostate cancer patients. C and D, HPA-negativity correlates with higher tumor stages and increased Gleason scores. E, the percentage of patients suffering from BCRs is increased in the HPA-negative cohort. F, the number of cases with at least one HPA-reactive specimen decreases from 88% in primary tumors (PT) to 50% in lymph node metastases (LN). Photomicrographs show representative samples of HPA-positive and -negative PT (top) and LN (bottom).
presence of intrastromal PC-3 cells in selectin-deficient mice (Fig. 4, middle). The growth period and tumor weight at necropsy are not affected by selectin deficiency (not shown).

E-selectin binding is detectable in only 32 of 1,600 prostatectomy samples demonstrating an incidence of E-selectin ligands of 1:50 in clinical prostate cancer. Interestingly, this small subset of E-selectin–positive patients (as depicted in Fig. 4E) tends to have a decreased BCR-free survival after surgery (63.1 vs. 101.5 months; \( P = 0.15 \)). The clinicopathologic features and outcomes of both groups, however, do not differ in a significant manner (Fig. 4E).

**Discussion**

This study demonstrates for the first time that prostate cancer progression is accompanied by decreased cell surface glycoconjugates terminating in GalNAc and GlcNAc (= HPA-reactive carbohydrates) as (i) the corresponding glycosyltransferases are downregulated in metastasis-derived prostate cancer cell lines compared with nonmalignant prostate epithelium; (ii) HPA-negativity is associated with metastasis formation in xenograft mouse models; (iii) HPA-negativity indicates an unfavourable prognosis of prostate cancer patients; and (iv) the incidence of HPA-reactive carbohydrates decreases in lymph node metastases compared with primary tumors; (v) E- and P-selectin are not essential for spontaneous pulmonary metastasis formation in vivo; and (vi) selectin-binding sites are only rarely present in prostatectomy specimens (incidence 1:50).

Taken together, we demonstrate an inverse functional and prognostic relevance of HPA-binding sites in prostate cancer when compared with several other human adenocarcinomas as outlined in the Introduction (2, 6–10). Likewise, in accordance with our hypothesis that such glycoconjugates (e.g., Tn antigen and core 2 O-glycans;...}

| Table 2. Clinicopathologic features of the study populations (completed follow-up ≥ 1 month) |
|---------------------------------------------|---------------------------------------------|------------------|
|                                             | HPA-positive patients (\( n = 589 \)) | HPA-negative patients (\( n = 696 \)) | \( P \)          |
| Patient age, y                              |                                      |                                |                 |
| Mean (median)                               | 62.5 (62.7)                          | 62.24 (62.6)                 |                 |
| Range                                       | 43.2–76.1                            | 40.6–76.3                    |                 |
| Follow-up, months                           |                                      |                                |                 |
| Mean (median)                               | 96.9 (120.4)                         | 93.1 (105.3)                 |                 |
| Range                                       | 1.6–219.2                            | 1.2–228.7                    |                 |
| Preoperative PSA, ng/mL                     |                                      |                                |                 |
| Mean (median)                               | 10.5 (7.6)                           | 11.9 (8.0)                   | 0.023           |
| Range                                       | 0.0–74.6                             | 0.0–102.8                    |                 |
| Missing data                                | 13 pat. (2.2%)                       | 21 pat. (3%)                 |                 |
| \( \text{No. of patients (\%)} \)          |                                      |                                |                 |
| pT Stage                                    |                                      |                                |                 |
| \( \text{pT2} \)                             | 367 (62.3\%)                         | 345 (49.6\%)                 |                 |
| \( \text{pT3a} \)                            | 144 (24.4\%)                         | 186 (26.7\%)                 |                 |
| \( \text{pT3b} \)                            | 66 (11.2\%)                          | 139 (20\%)                   |                 |
| \( \text{pT4} \)                             | 12 (2\%)                             | 26 (3.7\%)                   |                 |
| Prostatectomy Gleason score                 |                                      |                                | \(<0.0001\)     |
| \( \leq 6 \)                                 | 262 (44.4\%)                         | 235 (33.8\%)                 |                 |
| \( 3+4 \)                                   | 259 (44%)                            | 333 (47.8\%)                 |                 |
| \( 4+3 \)                                   | 57 (9.7\%)                           | 109 (15.7\%)                 |                 |
| \( \geq 8 \)                                | 11 (1.9\%)                           | 19 (2.7\%)                   |                 |
| pN Stage                                    |                                      |                                | 0.002           |
| \( \text{pN0} \)                             | 495 (84\%)                           | 590 (84.8\%)                 |                 |
| \( \text{pN1–3} \)                           | 23 (3.9\%)                           | 40 (5.7\%)                   |                 |
| \( \text{pNx} \)                             | 71 (12.1\%)                          | 66 (9.5\%)                   |                 |
| Surgical margin status                      |                                      |                                |                 |
| \( \text{R0} \)                              | 463 (78.6\%)                         | 544 (78.2\%)                 |                 |
| \( \text{R1} \)                              | 126 (21.4\%)                         | 151 (21.7\%)                 |                 |
| \( \text{Rx} \)                              | 0 (0\%)                              | 1 (0.1\%)                    |                 |
| Overall biochemical recurrences             | 182 (30.9\%)                         | 266 (38.2\%)                 | 0.006           |
Figure 4. Prostate cancer metastasis formation is largely independent of E- and P-selectin. A-B, The numbers of DTC in the lungs and CTC in the blood remain unchanged (Alu-PCR) after s.c. engraftment of PC-3 into E-/P-selectin<sup>−/−</sup> Pfp/Rag2<sup>−/−</sup> mice. C-D, Morphological analyses reveal an increased number of DTC still present in lung vessels in E-/P-selectin<sup>−/−</sup> mice. The number of intrastromal metastases, however, is similar in both groups. H.E.-stained photomicrographs show examples of transmigrating PC-3 cells (upper panel) and a single cell metastasis present in the alveolar septum (lower panel, black arrow) in E-/P-selectin<sup>−/−</sup> mice strongly indicating additional, selectin-independent mechanisms for prostate cancer extravasation. Representative S1P<sub>1</sub>-immunostainings (middle panel) taken from E-/P-selectin<sup>−/−</sup> mice illustrate intravascular (left picture) vs. intrastromal (right picture) cancer cells (black arrows) by labeling murine vascular endothelium (red arrows). Intravascular erythrocyte (∗)/leukocyte (†). E, Prostatectomy specimens represent E-selectin-binding sites in only 2% of patients without prognostic significance for this small subset. TMA: tissue microarray; <sup>∗</sup> P < 0.05.
of nonselectin ligands (7, 21), extravasation of CTCs is not
ruled out for selectin ligands. Again, this is a peculiarity of prostate cancer and contrary to other human malignancies (13–16).

Interestingly, one recent study on the glycosylation potential of human prostate cancer also demonstrates a low mRNA expression of different polypeptide-GalNAc-transferases (pp-GalNAc-Ts) in prostate cancer (34), suggesting a minor relevance of O-glycosylation initiation in prostate cancer in general. This observation is also reflected by the particularly low incidence of Tn antigen found in prostate cancer (4%–26%; refs. 35), even though it is typically highly presented in several other malignancies (4). In contrast, Gao and colleagues and one of our previous studies rather demonstrated a remarkable increase of N-acetylgalcosaminyltransferase V (GnT-V) and GnT-Vb, respectively, indicating a particular relevance of β1,6-branched complex-type N-glycans in prostate cancer (22, 34). However, approximately 45% of patients with prostate cancer were classified "HPA-positive" in our study. This might be possible due to the abundant presentation of core 2 O-glycans by mucin-1 (36), which is an oncoprotein that has been shown to be overexpressed in up to 60% of patients with prostate cancer (37) and contains numerous carbohydrate chains with terminal GlcNAc-residues. Interestingly, ectopic overexpression of highly core 2-glycosylated mucin-1 in C4-2B prostate cancer cells leads to a decreased prostate cancer xenograft tumor growth in vivo (36), which is now supported by the beneficial clinical course of our HPA-positive patient cohort and by the loss of HPA-binding sites in lymph node metastases as well as metastatic xenograft tumors.

The low expression of ppGalNAc-T’s, core 1, and 2 synthases especially in metastatic prostate cancer cells is associated with an absence of sLe^A and sLe^X on their surface. Because of the presence of intrastromal lung metastases in E/P-selectin−/− mice, we concluded that the selectin–selectin ligand axis is not essential for metastasis formation in prostate cancer. We corroborated this conclusion by the low incidence of E-selectin-binding sites in clinical prostate cancer tumors, which, in addition, did not show any significant prognostic importance. These observations strongly suggest selectin-compensating or -independent mechanisms that accomplish transendothelial migration in prostate cancer. Following the steps of the leukocyte adhesion cascade, different integrins and chemokines have actually been proven to be relevant for adhesion and transmigration in prostate cancer. For instance, CXCL13/CXCR5-mediated clustering of α5β1-integrin drives adhesion of prostate cancer cells toward human bone marrow endothelium, with CXCL13 serum levels being positively correlated with prostate cancer progression (38). Furthermore, α5β1-integrin expression in prostatectomy specimens is significantly associated with a poor prognosis (39) and β3-integrin expression is remarkably upregulated in prostate cancer bone metastases (40). In contrast, another study already pointed out that prostate cancer cells adhere to and traverse bone marrow endothelium via sequential dependence on E-selectin, β1-integrin, and αvβ3-integrin (41). In that and a previous study of the same group (42), however, it was obviously necessary to overexpress α1,3-fucosyltransferases (FT 3, 6, and 7) in prostate cancer cell lines to observe any adhesive events in vitro at all (presumably due to the subsequent elevation of sLe^A on FT-transfected cells). This strongly supports our findings that sLe^A/sLe^X presentation and shear stress-resistant adhesion toward HPMEC and P-selectin are not detectable using native prostate cancer cells in vitro. Using the same transfectants for in vivo homing studies, the authors showed an increased retention of FT-overexpressing tumor cells within the bone marrow compared with native prostate cancer cells (41). The genetically engineered overexpression of E-selectin ligands on prostate cancer cells, however, does not represent the clinical situation with respect to the low incidence of E-selectin-binding sites elucidated by our study. Nevertheless, Barthel and colleagues interestingly found that bone retention still occurred in up to 50% of mice after pretreating mice with an E-selectin blocking antibody. In contrast, blockade of prostate cancer cells with a β3-integrin antibody reduced cell retention by 88% (41).

Taken together, these and our own findings indicate selectin-independent, presumably integrin-driven metastasis patterns as a characteristic of prostate cancer. Interestingly, this unusual biologic behavior is obviously associated with unusual metastasis patterns in clinical prostate cancer. As initially shown by Oscar Batson in 1940, metastases to the vertebral spine of the bones of the body are the most frequent ones in prostate cancer and typically occur via a valveless prevertebral veinplexus (Batson’s plexus; ref. 43). These metastatic lesions occur independently of systemic dissemination and are predominant in patients with smaller primary tumors, suggesting backward venous spread as an early metastasis route in prostate cancer (44). As the blood flow in prevertebral plexus is normally directed toward the lower vena cava and by this away from the spine (45), vertebral metastases might rather appear through a kind of growth per continuitatem. The patterns of dynamic flow adhesion and thus selectin interactions as recognized to be necessary for systemic dissemination might be less relevant here. However, the precise mechanisms of how integrins or chemokines accomplish extravasation in prostate cancer independent of selectins still remain to be determined.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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by high-throughput fluorescence in situ hybridization on tissue micro-
525–37.
36. Premaratne P, Welen K, Damber JE, Hansson GC, Backstrom M. O-
Aberrant Presentation of HPA- Reactive Carbohydrates Implies Selectin-Independent Metastasis Formation in Human Prostate Cancer

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