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

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Running title: Cell surface glycosylation and PCa metastasis patterns

The authors disclose no potential conflicts of interest.

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Translational Relevance

This is the first study applying spontaneous metastasis xenograft models of human prostate cancer (PCa) to test the functional relevance of aberrant cell surface glycans and downstream molecules of the leukocyte adhesion cascade for PCa 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 PCa xenografts and decrease in clinical lymph node metastases. Corresponding to the fact that such carbohydrates are common intermediates for selectin ligand synthesis, PCa 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.
Abstract

Purpose: To investigate the impact of prostate cancer (PCa) 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 PCa cell lines and compared with primary epithelium. PCa 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 PCa was determined.

Results: Glycosyltransferases involved in the synthesis of common HPA-binding sites are down-regulated in PCa cells. An absence of HPA-reactive carbohydrates specifically indicates spontaneous metastatic spread of PCa xenografts in vivo and a poor prognosis of PCa patients. HPA-binding sites decrease in lymph node metastases compared with corresponding primary tumors. Common selectin ligands are absent on PCa 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 PCa patients without prognostic significance.

Conclusion: PCa is characterized by an inverse functional and prognostic importance of HPA-binding sites compared to 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 PCa.
Introduction

Prostate cancer (PCa) 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 PCa-associated deaths. During the multistep process of metastatic spread, primary tumor cells are interacting with their microenvironment via their glycocalyx (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-acetylglucosamine- (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) towards these terminal glycoconjugates. Hence, HPA has been shown to be a marker of metastatic competence in human breast and colorectal xenograft primary tumors in 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, 12), which are synthesized through the sequential activity of polypeptide GalNAc-transferases (pp-GalNac-T’s), core 1 synthase (C1GALT1) and core 2 synthases (C2GNT1,2,3), respectively (Fig. 1 A). Importantly, as summarized in Fig. 1 A, these glycans are common intermediates for the synthesis of sialylated Lewis X and A antigens (sLeX and sLeA) (13), which are presented at the surface of human breast and gastrointestinal adenocarcinoma (14-16) as well as leukemia cells (17). 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 (18). Our hypothesis is that circulating tumor cells (CTC) imitate this adhesion cascade involving selectin-, integrin- and chemokine-mediated interactions in order to migrate into host organs of distant metastases as well (19-21) (Fig. 1 A, lower panel). Accordingly, spontaneous metastasis formation from xenograft primary tumors of human breast, colorectal (HT29) and pancreatic cancer (PaCa5061) drastically decreases in Sel£^-/- Selp^-/- SCID and Pfp^-/-Rag2^-/- mice (14-16). Likewise, engraftment and organ invasion of human eosinophilic leukemia cells (EOL-1) are remarkably reduced in selectin deficient mice (17). Furthermore, the importance of HPA-reactive glycoconjugates as intermediates of selectin ligand synthesis has also been shown previously, as pre-incubation of human breast cancer cells with HPA inhibits their adhesion towards vascular endothelium (7, 22).

In PCa, 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 PSA-values in patients (23). The impact of HPA- and selectin-binding sites for metastasis formation and patient prognosis in PCa, however, has so far not been analyzed in detail. We therefore aimed to determine glycosylation patterns in PCa with a particular focus on HPA-reactive carbohydrates and selectin ligands (including their potential relevance for PCa adhesion to selectins/ endothelium); to test HPA as a marker of metastatic competence in PCa xenograft models and as a prognostic factor in clinical PCa; to analyze E-selectin binding sites in PCa xenograft tumors and in patients and to determine whether selectin binding is essential for metastasis formation in PCa.
Materials and Methods

Cell Lines and Culture Conditions. PC-3, DU-145 (prostate cancer), PPEC (human primary, non-malignant prostate epithelial cells), HT29 (colon cancer), EOL-1 (eosinophilic leukemia) and PaCa5061 (pancreatic adenocarcinoma) were used as described before (15, 23) (see also Table S1 and refs. (17, 24)). VCaP (prostate cancer) were obtained from ATCC (Manassas, USA) and cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin (all Invitrogen, Karlsruhe, Germany) at 37°C in a humidified atmosphere of 5% CO₂ (25). Human pulmonary microvascular endothelial cells (HPMEC, Table S1) (26-28) were from PromoCell (Heidelberg, Germany) 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 (qRT)-PCR Glycosylation Array. RNA isolation and cDNA synthesis from cell culture grown PC-3, DU-145, VCaP and PPEC was performed as described (23); expression of glycosyltransferases was assessed using the Human Glycosylation RT² Profiler™ PCR array including 84 glycosyltransferase and glycosidase genes (Qiagen, Hamburg, Germany). All arrays were repeated twice with independently isolated RNAs.

HPA-binding flow cytometry and lectin histochemistry. Tumor cells were detached and incubated for 30 min at 4°C with FITC-conjugated HPA (Sigma) diluted 1:100 in lectin buffer (0.05 M TRIS-buffered saline, pH 7.6, added with 1 mM CaCl₂ and MgCl₂). Binding specificity was evaluated by inhibiting HPA with 100 mM D-GalNAc (Sigma) prior to 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, Münster, Germany). Data were analyzed using CyView™ software (Partec).

Xenograft primary tumors of all PCa 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, de-paraffinized and pre-treated with 0.1 % trypsin (obtained as trypsin powder from Biochrom (Berlin, Germany), substance activity 1512 USP U/mg) in lectin buffer for 15 min at 37 °C. Afterwards, sections were incubated with biotinylated HPA (Sigma) and stained as described before (23). Again, 100 mM D-GalNAc was used for inhibition of HPA on parallel sections.

**Subcutaneous Xenograft Mouse Models.** Male *Pfp/ Rag2*−/− double-knockout mice (8-12 weeks, 20-25g) from Taconic (Hudson, USA) were used as described (23). 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 (23). This study firstly describes the use of VCaP cells as a suitable model of metastatic PCa. For VCaP tumor growth it was necessary to mix 1 x 10^6 cells 1:2 with matrigel (BD, Heidelberg, Germany) immediately before injection. *Pfp/ Rag2*−/− mice were crossbred with E- and P-selectin deficient mice (Jackson Laboratory, Bar Harbor, ME, USA, stock 002916) and selectin-deficiency was verified as described (14).
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 histological analyses as described (29). Three representative lung sections from three 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 homogenised 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%. 200 μl 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 histological examinations.

**Quantification of disseminated tumor cells (DTC) and CTC by Alu-PCR.** DNA concentrations of all samples were quantified using a NanoDrop spectrophotometer (Peqlab, Erlangen, Germany). 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 normalised to 30 ng/μl using AE buffer (Qiagen). The concentrations of blood-DNA were quite similar in all samples (approx. 10 ng/μl) and were therefore not normalised. qPCR was performed with established human-specific Alu-primers (30). 2 μl 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 (23). The detection limit for specific human Alu-sequence signals was determined for each tissue type by testing DNA from five healthy (non-injected) Pfp/ Rrag2−/− 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 H.E.-stained lung sections per mouse (29). Human cancer cells were recognized by their characteristically large, basophilic, and polymorphic nuclei, which were clearly distinguishable from the smaller nuclei of mouse cells (Fig. 2 + 4). To evaluate potential differences between wildtype and E-/P-selectin deficient Pfp/ 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 S1P\(_1\) (polyclonal rabbit, SantaCruz#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 PCa 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, de-paraffinized tissue or microarray sections as described before (16). Human pancreatic adenocarcinoma grown in pfp/rag2 mice served as a positive control (16). 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 (TMA). 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 PCa patients, respectively. All patients underwent radical prostatectomy at the Department of Urology at our Medical Center (1992 - 2005). Prostate specific antigen (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 (31-34). HPA- and E-selectin-binding towards PCa epithelium was evaluated (negative vs. positive; positivity was considered when > 50 % of tumor cells were stained) and correlated with histopathological and clinical follow-up data. Next, PCa heterogeneity TMAs were analyzed to investigate whether HPA-binding is heterogeneous in PCa 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 from 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 down-regulated in PCa 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 down-regulated in PCa cells. Accordingly, all tested PCa 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. 1 B).

**Metastatic PCa xenograft primary tumors and spontaneous lung metastases are HPA-negative; E-selectin-binding sites are absent in PCa 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 g, 1.68 g, and 2.32 g (Fig. 2 A) after a mean growth period of 39 ± 3.8 d, 136 ± 12 d, and 119 ± 26.6 d (p<0.0001, Fig. 2 B) for PC-3, VCaP, and DU-145, respectively. The rates and median numbers of detected DTC and CTC are depicted in Fig. 2 C-E. This is the first description of VCaP as a suitable spontaneous metastasis model of human PCa. Histology confirmed the presence of spontaneous lung metastases in the PC-3 model (Fig. 2). The 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. 2 C-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, HT29 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 unfavourable prognosis and HPA-binding decreases in lymph node metastases.** 696 of 1,285 PCa patients 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 to the HPA-positive cohort ($p=0.009$, Fig. 3 A). The adverse prognostic effect of HPA-negativity is more pronounced in the subset of R1-resected patients ($p=0.003$, Fig. 3 B). Accordingly, tumor stages ($p<0.0001$) and grades ($p=0.002$) are increased in the HPA-negative cohort (Fig. 3 C-D, Table 2). The percentage of overall biochemical relapses is increased in the HPA-negative patient group ($p=0.006$, Table 2, Fig. 3 E). 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).

Based on 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 PCa patients (Fig. 3 F). Only one patient is homogeneously HPA-positive, whereas seven patients (9 %) are homogeneously HPA-negative. An average of 26.3 % of tumor spots is HPA-positive per patient. 88 % 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 (Fig. 3 F, $p<0.0001$) indicating a decrease of HPA-reactive sugar residues during
PCa 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 (upper panel) and lymph node metastases (lower panel) are shown in Fig. 3.

**E-/P-selectin are not essential for metastasis formation and E-selectin-binding sites are seldomly presented in prostate cancer tumors.** After engraftment of highly metastatic PC-3 cells into E- and P-selectin deficient Pfpo/Rag2−/− mice, the number of DTC in the lungs (Fig. 4 A) and CTC in the blood (Fig. 4 B) remains unchanged. Since the numbers of DTC in the lungs detected by Alu-PCR might at least partially be caused by intravascular DTC 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 DTC from 88 ± 543.6 in wild type to 1305 ± 1645.5 in E-/P-selectin deficient mice (p=0.038, Fig. 4 C) suggesting a disturbed extravasation in selectin-deficiency. Nevertheless, the median number of intrastromal metastases is almost similar in both groups (Fig. 4 D). Immunohistochemical staining of vascular lung endothelium (S1P1) clearly demonstrates the presence of intrastromal PC-3 cells in selectin deficient mice (Fig. 4, middle panel). 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 PCa. Interestingly, this small subset of E-selectin-positive patients (as depicted in Fig. 4 E) tends to have a decreased biochemical relapse-free survival after surgery (63.1 vs. 101.5 months, p=0.15). The clinico-pathological features and outcomes of both groups, however, do not differ in a significant manner (Fig. 4 E).
Discussion

This study demonstrates for the first time that PCa progression is accompanied by decreased cell surface glycoconjugates terminating in GalNAc and GlcNAc (=HPA-reactive carbohydrates) as (I.) the corresponding glycosyltransferases are down-regulated in metastasis-derived PCa cell lines compared with non-malignant prostate epithelium, (II.) HPA-negativity is associated with metastasis formation in xenograft mouse models (III.) HPA-negativity indicates an unfavourable prognosis of PCa 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 PCa 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 (4, 11, 12) are common intermediates for the synthesis of selectin ligands (7, 22), extravasation of circulating tumor cells is not crucially dependent on selectin - selectin ligand interactions in PCa. Again, this is a peculiarity of PCa and contrary to different other human malignancies (14-17).

Interestingly, one recent study on the glycosylation potential of human PCa also demonstrates a low mRNA expression of different polypeptide-GalNAc-transferases (pp-GalNAc-T's) in PCa (35) suggesting a minor relevance of O-glycosylation initiation in PCa in general. This observation is also reflected by the particularly low incidence of Tn antigen found in PCa (4 to 26 %) (36), even though it’s typically highly presented in several other malignancies (11). In contrast, Gao et al. and one of our previous studies rather demonstrated a remarkable increase of N-
acetylglucosaminlytransferase V (GnT-V) and GnT-Vb, respectively, indicating a particular relevance of β1,6-branched complex-type N-glycans in PCa (23, 35). However, approximately 45 % of PCa patients were classified 'HPA-positive' in our study. This might be possibly due to the abundant presentation of core 2 O-glycans by mucin-1 (37), which is an oncoprotein that has been shown to be over-expressed in up to 60 % of PCa patients (38) and contains numerous carbohydrate chains with terminal GlcNAc-residues. Interestingly, ectopic over-expression of highly core 2-glycosylated mucin-1 in C4-2B PCa cells leads to a decreased PCa xenograft tumor growth in vivo (37), 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 PCa cells is associated with an absence of sLe^A^ and sLe^X^ on their surface. Due to 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 PCa. We corroborated this conclusion by the low incidence of E-selectin-binding sites in clinical PCa 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 PCa.

Following the steps of the leukocyte adhesion cascade, different integrins and chemokines have actually been proven to be relevant for adhesion and transmigration in PCa. For instance, CXCL13/CXCR5-mediated clustering of α,β3-integrin drives adhesion of PCa cells towards human bone marrow endothelium, with CXCL13 serum levels being positively correlated with PCa progression (39). Furthermore, α3β1-integrin expression in prostatectomy specimens is significantly associated with a poor prognosis (40) and β4-integrin expression is remarkably up-
regulated in PCa bone metastases (41). In contrast, another study already pointed out that PCa cells adhere to and traverse bone marrow endothelium via sequential dependence on E-selectin, β1- and αvβ3-integrin (42). In that and a previous study of the same group (43), however, it was obviously necessary to over-express α-1,3-fucosyltransferases (FT 3, 6, and 7) in PCa cell lines in order to observe any adhesive events in vitro at all (presumably due to the subsequent elevation of sLeX on FT-transfected cells). This strongly supports our findings that sLeA/sLeX presentation and shear stress-resistant adhesion towards HPMEC and P-selectin are not detectable using native PCa cells in vitro. Using the same transfectants for in vivo homing studies, the authors showed an increased retention of FT-over-expressing tumor cells within the bone marrow compared with native PCa cells (42). The genetically engineered over-expression of E-selectin ligands on PCa 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 et al. interestingly found that bone retention still occurred in up to 50% of mice after pre-treating mice with an E-selectin blocking antibody. In contrast, blockade of PCa cells with a β1-integrin antibody reduced cell retention by 88% (42).

Taken together, these and our own findings indicate selectin-independent, presumably integrin-driven metastasis patterns as a characteristic of PCa. Interestingly, this unusual biological behavior is obviously associated with unusual metastasis patterns in clinical PCa. As initially shown by Oscar Batson in 1940, metastases to the vertebrae of the lumbar spine are the most frequent ones in PCa and typically occur via a valveless prevertebral vein plexus (Batson’s plexus (44)). 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 PCa (45). As the blood flow in prevertebral
plexus is normally directed towards the lower vena cava and by this away from the spine (46), vertebrae 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 PCa independent of selectins still remain to be determined.

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Legend to Figures

**Fig. 1.** O-glycan biosynthesis, HPA-reactive sugar residues and synthesis of selectin ligands. **A,** Representation of O-glycosylation pathways relevant to the study (35). Tn-antigen and core 2 O-glycans present terminal GalNAc or GlcNAc and are specifically recognized by the lectin *Helix pomatia* agglutinin (HPA) (12, 47). The core 2 structure is a critical scaffold for the production of the main selectin ligands sLeA and sLeX (13) (upper panel). Selectin ligands are involved in the initiation of flow adhesion of leukocytes to vascular endothelium during inflammation. There is a rising body of evidence indicating that these pathways are mimicked by circulating tumor cells (CTC) for extravasation at a distant site during metastasis formation (18). Several molecules downstream of selectins in this cascade such as integrins and chemokines (not illustrated) are relevant as well (lower panel). **B,** In accordance with the glycosyltransferase expression changes summarized in Table 1, PCa cells show a moderate (PC-3) to weak (VCaP, DU-145) HPA-binding compared with HT29 colon cancer cells (2). Black histograms represent HPA-binding after inhibition with D-GalNAc. GalNAc: N-acetylgalactosamine; GlcNAc: N-acetylglucosamine, (s)Le^AX^: (sialylated) Lewis^AX^-antigen.

**Fig. 2.** HPA-binding is absent in metastatic PCa xenograft tumors and lung metastases; E-selectin binding sites are not detectable in PCa xenografts. **A-E,** Tumor growth, growth period and spontaneous metastasis formation after s.c. xenotransplantation of PC-3, VCaP and DU-145 into *Pfp/Rag2^-/-* mice. Disseminated tumor cells (DTC) in lungs (C) and bone marrow (D) as well as circulating tumor cells (CTC) in the blood (E) were quantified by *Alu*-PCR (detection limits are indicated by red lines). Photomicrographs show representative samples of HPA-binding histochemistry and E-selectin-binding immunofluorescence on xenograft tumors.
HT29 colon cancer and PaCa5061 pancreatic adenocarcinoma xenografts served as positive controls (2, 6, 15, 16, see also Table S1). Inserts demonstrate HPA-binding after inhibition with D-GalNAc. F, HPA-reactive carbohydrates are detectable in only 16.2% of spontaneous PCa lung metastases (bars represent means ± SD of three mice (10 lung sections each)). *p < 0.05 vs. DU-145; ***p < 0.0001 vs. VCaP and DU-145; #p < 0.05.

**Fig. 3. Adverse prognosis and lymph node metastases are accompanied by decreased HPA-reactive glycoconjugates.** A-B, Reduced biochemical relapse-free survival in HPA-negative PCa patients. C-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 (upper panel) and LN (lower panel).

**Fig. 4. PCa 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−/− Pfp/Rag2−/− mice. C-D, Morphological analyses reveal an increased number of DTC still present in lung vessels in E-/P-selectin−/− 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−/− mice strongly indicating additional, selectin-independent mechanisms for PCa extravasation. Representative
S1P$_1$-immunostainings (middle panel) taken from E-/P-selectin$^{-/-}$ mice illustrate intravascular (left picture) vs. intrastromal (right picture) cancer cells (black arrows) by labelling 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; * p < 0.05.


**Figure 1**

A. Simplified schematic representation of core 1 and core 2 carbohydrate structures associated with Tn- and T-antigens.

B. Flow cytometry plots showing HPA-reactive staining in various cell lines.

Legend:
- HPA-reactive residues
- E-/P-Selectin ligands
- GalNAc
- galactose
- GlcNAc
- fucose
- sialic acid

**Notes:**
- The figure illustrates the synthesis and expression of specific carbohydrate structures (GalNAcT, C1GalT1, C2GnT1,2,3) and the subsequent modifications by enzymes (β3GalT5, β4GalT1-6, α4FUT, α3FUT, ST3Gal).
- The figure also highlights the role of these structures in cancer cell metastasis, showing interactions with blood flow, vascular endothelium, vessel lumen, and metastatic organ's connective tissue.

**Cell Lines:**
- PC-3
- VCaP
- DU-145
- HT29

**Data Representation:**
- Counts are presented on a log scale, with counts per 10,000.

**Context:**
- The figure is part of a scientific manuscript discussing the role of specific carbohydrate structures in cancer cell behavior.
- The data is derived from experiments conducted on various cell lines.

**References:**
- The figure includes references to scientific literature discussing the synthesis and function of these carbohydrate structures in cancer research.

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Figure 2

A. Tumor weight [g] of HPA-binding sites and E-Selectin-binding sites.


C. Number of DTCs (60 ng DNA) in the left lung.

D. Number of DTCs (60 ng DNA) in the bone marrow.

E. Number of CTCs (20 ng DNA) in blood.

F. HPA-binding in PC-3 lung metastases.
Figure 3

A. Cumulative survival of all patients (n = 1,285)

- HPA-pos: n = 589
- HPA-neg: n = 696

B. Cumulative survival of R1-resected patients (n = 277)

- HPA-pos: n = 126
- HPA-neg: n = 151

C. No. of patients [% of all stages]

- pT2
- pT3a
- pT3b
- pT4

D. No. of patients [% of all grades]

- Gleason Score ≤ 6
- 3+4
- 4+3
- ≥ 8

E. No. of all patients [%]

F. Pat. # HPA-positive HPA-negative

- Rate of HPA+ cases
  - PT spots
  - LN spots

*p < 0.0001
*p = 0.006
*p = 0.002
*p = 0.003
*p = 0.009
*p = 0.009
*p = 0.003
*p = 0.003
*p = 0.003
Figure 4

A. No. of DTC/60 ng DNA (lung)

B. No. of CTC/20 ng DNA (blood)

C. No. of intravascular lung metastases

D. No. of intrastromal lung metastases

E. Cumulative survival

Prostatectomy specimen (TMA)

pfp/rag2−/− x E−/P-Selectin−/−

anti-S1P1

wt E−/P-Selectin−/−

Image 402x636 to 562x754
Image 402x504 to 562x623
Image 318x342 to 499x479
Image 112x341 to 294x479
Image 336x197 to 499x326
Image 171x220 to 313x318

<table>
<thead>
<tr>
<th>Glycosyl-</th>
<th>gene</th>
<th>Ct-value</th>
<th>fold up-/down-regulation vs. PPEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>transferases</td>
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<td>PPEC</td>
<td>PC-3</td>
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<tr>
<td>GALNT3</td>
<td>23.76</td>
<td>-2.95</td>
<td>-1.34</td>
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<tr>
<td>GALNT6</td>
<td>27.03</td>
<td>1.45</td>
<td>*<em>-119.91</em></td>
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<tr>
<td>GALNT8</td>
<td>34.1</td>
<td>*<em>-3.1</em></td>
<td>-2.8*</td>
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<td>GALNT12</td>
<td>29.92</td>
<td>7.47*</td>
<td>-1.51</td>
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<td>GALNT14</td>
<td>27.63</td>
<td>*<em>-56.95</em></td>
<td>-2.26</td>
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<tr>
<td>C1GALT1</td>
<td>23.75</td>
<td><strong>-2.87</strong></td>
<td><strong>-6.43</strong>*</td>
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<tr>
<td>C2GNT1</td>
<td>28.05</td>
<td><strong>-5.45</strong></td>
<td>6.04*</td>
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<tr>
<td>C2GNT2</td>
<td>27.64</td>
<td><strong>-15.47</strong></td>
<td><strong>-22.51</strong></td>
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<tr>
<td>C2GNT3</td>
<td>27.97</td>
<td><strong>-29.96</strong></td>
<td><strong>-89.63</strong></td>
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</table>

Table 1: Several glycosyltransferases involved in the synthesis of HPA-reactive glycoconjugates are down-regulated in metastasis-derived PCa cells compared with primary non-malignant prostate epithelium (PPEC). *p < 0.05, **p < 0.001, ***p < 0.0001.
Table 2. Clinicopathological features of the study populations (completed follow-up ≥ 1 month)

<table>
<thead>
<tr>
<th></th>
<th>HPA positive patients (n=589)</th>
<th>HPA negative patients (n=696)</th>
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<tbody>
<tr>
<td><strong>Patient age [years]</strong></td>
<td></td>
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<tr>
<td>mean (median)</td>
<td>62.5 (62.7)</td>
<td>62.24 (62.6)</td>
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<tr>
<td>range</td>
<td>43.2-76.1</td>
<td>40.6-76.3</td>
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<tr>
<td><strong>Follow-up [months]</strong></td>
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<tr>
<td>mean (median)</td>
<td>96.9 (120.4)</td>
<td>93.1 (105.3)</td>
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<tr>
<td>range</td>
<td>1.6-219.2</td>
<td>1.2-228.7</td>
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<tr>
<td><strong>preoperative PSA [ng/mL]</strong></td>
<td></td>
<td>P</td>
</tr>
<tr>
<td>mean (median)</td>
<td>10.5 (7.6)</td>
<td>11.9 (8.0)</td>
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<tr>
<td>range</td>
<td>0.0-74.6</td>
<td>0.0-102.8</td>
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<tr>
<td>missing data</td>
<td>13 pat. (2.2 %)</td>
<td>21 pat. (3 %)</td>
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<tr>
<td><strong>pT stage</strong></td>
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<tr>
<td>pT2</td>
<td>367 (62.3%)</td>
<td>345 (49.6%)</td>
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<tr>
<td>pT3a</td>
<td>144 (24.4%)</td>
<td>186 (26.7%)</td>
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<tr>
<td>pT3b</td>
<td>66 (11.2%)</td>
<td>139 (20%)</td>
</tr>
<tr>
<td>pT4</td>
<td>12 (2%)</td>
<td>26 (3.7%)</td>
</tr>
<tr>
<td><strong>Prostatectomy Gleason Score</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 6</td>
<td>262 (44.4%)</td>
<td>235 (33.8%)</td>
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<tr>
<td>3+4</td>
<td>259 (44%)</td>
<td>333 (47.8%)</td>
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<tr>
<td>4+3</td>
<td>57 (9.7%)</td>
<td>109 (15.7%)</td>
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<tr>
<td>≥ 8</td>
<td>11 (1.9%)</td>
<td>19 (2.7%)</td>
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<tr>
<td><strong>pN stage</strong></td>
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<td></td>
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<tr>
<td>pN0</td>
<td>495 (84%)</td>
<td>590 (84.8%)</td>
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<tr>
<td>pN1-3</td>
<td>23 (3.9%)</td>
<td>40 (5.7%)</td>
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<td>pNx</td>
<td>71 (12.1%)</td>
<td>66 (9.5%)</td>
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<tr>
<td><strong>Surgical margin status</strong></td>
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<tr>
<td>R0</td>
<td>463 (78.6%)</td>
<td>544 (78.2%)</td>
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<tr>
<td>R1</td>
<td>126 (21.4%)</td>
<td>151 (21.7%)</td>
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<tr>
<td>Rx</td>
<td>0 (0%)</td>
<td>1 (0.1%)</td>
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<tr>
<td><strong>Overall biochemical recurrences</strong></td>
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<tr>
<td></td>
<td>182 (30.9%)</td>
<td>266 (38.2%)</td>
</tr>
</tbody>
</table>

P < 0.0001

P < 0.002

P = 0.006
Aberrant Presentation of HPA-reactive Carbohydrates Implies Selectin-Independent Metastasis Formation in Human Prostate Cancer

Tobias Lange, Mareike Kupfernagel, Daniel Wicklein, et al.

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