A unique galectin signature in human prostate cancer progression suggests galectin-1 as a key target for treatment of advanced disease

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The authors disclose no potential conflicts of interest
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Key words: Tumor microenvironment, Prostate Cancer, Galectins, Angiogenesis.

Running Title: Galectin signature of human prostate cancer
ABSTRACT

Galectins, a family of glycan-binding proteins, influence tumor progression by modulating interactions between tumor, endothelial, stromal and immune cells. Despite considerable progress in identifying the roles of individual galectins in tumor biology, an integrated portrait of the galectin network in different tumor microenvironments is still missing. We undertook this study to analyze the 'galectin signature' of the human prostate cancer (PCa) microenvironment with the overarching goal of selecting novel molecular targets for prognostic and therapeutic purposes. In examining androgen-responsive and castration-resistant PCa cells and primary tumors representing different stages of the disease, we found that galectin-1 (Gal-1) was the most abundantly expressed galectin in PCa tissue and was markedly upregulated during disease progression. In contrast, all other galectins were expressed at lower levels: Gal-3, -4, -9 and -12 were downregulated during disease evolution, while expression of Gal-8 was unchanged. Given the prominent regulation of Gal-1 during PCa progression and its predominant localization at the tumor-vascular interface, we analyzed the potential role of this endogenous lectin in PCa angiogenesis. In human PCa tissue arrays, Gal-1 expression correlated with the presence of blood vessels, particularly in advanced stages of the disease. Silencing Gal-1 in PCa cells reduced tumor vascularization without altering expression of other angiogenesis-related genes. Collectively, our findings identify a dynamically regulated 'galectin-specific signature' that accompanies disease evolution in PCa, and they highlight a major role for Gal-1 as a tractable target for anti-angiogenic therapy in advanced stages of the disease.
PRECIS

The dynamically regulated expression signature for an important class of cell surface glycan-binding molecules in prostate cancer suggests a tractable target for anti-angiogenic therapy in advanced disease.
INTRODUCTION

Prostate cancer (PCa) is the second most common cancer in men, and represents a significant cause of mortality worldwide (1). Localized PCa is efficiently treated by association of surgery with radiation therapy and androgen ablation. However, PCa evolves towards stages in which tumor cells acquire properties allowing their distant dissemination (2) and castration-resistant growth (3). No current treatments are applicable to these situations and the prospect for cure decreases radically. These particular features urge the search of novel prognosis strategies that could delineate the transition from hormone-sensitive toward hormone-resistant tumor growth and innovative therapeutic approaches suitable for castration-refractory stages of the disease.

Effective cancer therapies typically capitalize on molecular differences between healthy and neoplastic tissues. In the post-genomic era, the study of the glycome has enabled the association of specific glycan structures with the transition from normal to neoplastic tissue (4, 5). The function of deciphering the biological information encoded by the glycome is assigned to endogenous glycan-binding proteins or lectins, whose expression and function are regulated during tumor progression (5). Galectins, a family of glycan-binding proteins, play pivotal roles as regulators of tumor biology by directly influencing tumor transformation, invasiveness, angiogenesis and tumor-immune escape (6,7). These lectins are defined by a common structural fold and a conserved carbohydrate recognition domain (CRD) that recognizes N- and O-glycans expressing the disaccharide N-acetyllactosamine (Galβ(1-4)-GlcNAc), although differences in glycan-binding preferences of individual members of the family have been reported (7). Galectins that are traditionally classified as ‘proto-type’ (Gal-1, -2, -5, -7, -10, -11, -13, -14, -15) have one CRD that can dimerize, whereas ‘tandem-repeat’ galectins (Gal-4, -6, -8, -9 and -12) contain two homologous CRDs in tandem in a single polypeptide chain. Gal-3 is unique in that it contains a CRD
connected to a non-lectin N-terminal region that is responsible for oligomerization (7). Extracellularly, galectins interact with cell surface glycoconjugates and trigger cellular signaling to control migration, immunity and angiogenesis. Intracellularly, galectins can control tumor transformation, proliferation and survival (7, 8).

Previous studies have identified galectins as key components of the prostate cancer microenvironment (9-11). Expression of Gal-1 controls the differentiation and survival of PCa cells (9, 12) and inhibits T-cell transmigration (13). On the other hand, Gal-3 controls homotypic and heterotypic aggregation of PCa cells (14-16) and controls their viability (17). Tumor cell expression of Gal-3 has been proposed to delineate the transition from benign stages to castration-resistant malignant disease (18) and its regulated expression is associated with promoter methylation (19). Silencing Gal-3 results in decreased migration, invasion and proliferation of PCa cells (20). Moreover, Gal-8, which was originally identified as prostate cancer tumor antigen 1 (PCTA1) (21), can modulate integrin-mediated cell-extracellular matrix interactions (22). However, in spite of considerable progress in dissecting the functions of individual members of the galectin family, there is still no integrated portrait of the ‘galectin signature’ of the human prostate cancer microenvironment.

Our findings identify a unique galectin expression profile which delineates different stages of PCa progression. From all galectins analyzed, Gal-1 is uniquely expressed at high levels in human PCa and contributes to tumor progression by promoting neovascularization. These results underscore the importance of Gal-1 as an attractive therapeutic target in advanced stages of PCa.
MATERIALS AND METHODS

Human samples

Radical prostatectomies were obtained from the archival tissue bank of the Department of Pathology, Hospital Alemán (Buenos Aires, Argentina). Samples were classified according to TNM classification (UICC, 2002) by two independent pathologists (G.C., O.M.). Specimens (n=61) covered all stages of PCa evolution (T1, T2, T3 and T4) in addition to benign cases (BHP) (Table 1). None of these patients received pre-operative therapy. Protocols were approved by the Local Ethics Committee (Hospital de Clínicas ‘José de San Martín’).

Cells and animals

Human prostate cancer cell lines used included: the hormone-responsive LNCaP cell line and the castration-resistant cell lines 22Rv1 and PC-3 with or without androgen receptor (AR) expression respectively. The LNCaP and 22Rv1 cell lines were provided by Anne Chauchereau (Institute Gustave Roussy, France). LNCaP cells were also provided by Elba Vazquez (University of Buenos Aires, Argentina). These cell lines were originally obtained from the American Type Culture Collection (ATCC). Cell morphology was evaluated by microscopic examination on a daily basis. Growth properties of LNCaP cells were regularly tested through their responsiveness to androgens using MTT assay. Cells were incubated for 24 h in red phenol-free RPMI, 10% Charcoal-treated serum and medium was supplemented with 10⁻¹⁰M R1881 (AR agonist) for 3 days before analyzing growth and gene expression. PSA induction was evaluated by real time RT-PCR. Routine tests for 22Rv1 cells included examination of androgen-insensitive growth (MTT method) and PSA induction by R1881 (real time RT-PCR). The PC-3 cell line was provided by Elba Vazquez (University of Buenos Aires, Argentina). Growth of these cells was routinely tested for androgen sensitivity and the AR and PSA phenotypes by real time RT-PCR. Bovine aortic endothelial cells (BAEC) were provided by
Maria T. Elola (University of Buenos Aires, Argentina). BAEC were tested for their ability to form tubular structures in the presence of vascular endothelial growth factor (VEGF). Each cell line was routinely tested for Mycoplasma contamination by genomic PCR. LNCaP, 22Rv1 and PC-3 cells were cultured in RPMI and BAEC were cultured in DMEM. Medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (PAA, Cell Culture, Austria), 2mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. BAEC were used at passage 14 or less. For some experiments, 22Rv1 cells (50,000) were plated into 12-well plates, cultured for 2 days under normal oxygen supply, and then exposed to hypoxic (1% O₂) or normoxic conditions for an additional 15 h. Nude mice were obtained from The National University of La Plata (La Plata, Argentina) and maintained in accordance with the Institutional Animal Care and Use Committee guidelines (IBYME, Buenos Aires, Argentina).

**Reagents**

The following anti-galectin antibodies (Santa Cruz Biotechnology, Inc., USA) were used: rabbit anti-Gal-1 (H-45), anti-Gal-8 (H-80), anti-Gal-3 (H-160), anti-Gal-4 (T-20), anti-Gal-12 (H-166) and goat anti-Gal-9 (C-20) antibodies. A purified anti-Gal-1 polyclonal rabbit IgG generated in G.A.R’s laboratory was used (23,24). Anti-human carbonic anhydrase IX polyclonal antibody (H-120) was obtained from Santa Cruz. Media and trypsin/EDTA were obtained from Gibco-Invitrogen (Life Technologies). Blocking anti-Gal-1 monoclonal antibody (mAb; F8.G7) was generated and validated as described (25, 26). Growth factor-reduced Matrigel was obtained from BD Biosciences.

**Immunohistochemistry**

Immunohistochemistry was performed on paraffin-embedded tissue samples. Samples were deparaffinized by 5 min incubation in xylene, 100%, 95% and 75% ethanol. Endogenous peroxidase
activity was quenched by 10 min incubation with 1% H₂O₂. Non-specific binding was blocked using normal horse serum in 0.05% saponin. Samples were incubated with the appropriate antibodies at the optimal dilutions for 1 h at room temperature. The following antibodies were used: polyclonal anti-Gal antibodies and preimmune sera from Santa Cruz (1:200 dilution) and purified anti-Gal-1 rabbit IgG (1:1,500). Immunoreactions were developed using the avidin-biotin-peroxidase Vectastain ABC Kit (Vector). Galectin expression was graded as follows: 0 (negative); 1+ (poor intensity); 2+ (moderate intensity); 3+ (high intensity) and 4+ (very high intensity). PCa cell lines were adhered to poly-L-lysine (Sigma)-coated coverslips for 2 h at 37°C (50,000 cells per coverslip), fixed with 4% paraformaldehyde for 5 min and processed for immunocytochemistry as described for tissues.

Immunohistochemistry of tissue microarrays was performed using 4 µm-thick formalin-fixed, paraffin-embedded sections of tissue microarray slides containing 29 paired cores (2 different areas of each single tumor from 29 tumors analyzed) of invasive PCa (BC19013; US Biomax) (Table 1B). Slides were soaked in xylene and passed through graded alcohols and distilled water prior to use. Slides were then pre-treated with citrate buffer pH 6.0 (Invitrogen) in a steam pressure cooker (Decloaking Chamber CD2008US, Biocare Biomedical) according to manufacturers’ recommended settings (127°C for 30 sec, followed by 90°C). Slides were blocked for peroxidase activity using a specific blocker (DAKO) and washed for 5 min in buffer. Individual slides were incubated with a mouse anti-human CD34 mAb (clone QBEND-10, RTU, Immunotech) and a rabbit anti-human Gal-1 polyclonal antibody (1:10,000) generated and used as described (23,24). After 1 h incubation, slides were washed and processed by the appropriate Envision+ kit (DAKO) as per manufacturer’s instructions, developed using a DAB chromogen (DAKO) and counterstained with hematoxylin. Stained slides were digitally scanned using Aperio ScanScope XT workstation at the 20X setting (Aperio Technology, Inc.). Core images were then analyzed using
ImageScope software (version 10.0.35.1800, Aperio Technology). Briefly, pathologists (S.J.R., J.L.K.) identified areas of tumors as regions of interest (ROI) and excluded areas without significant tumor using standard ImageScope software functions. The ROIs were then analyzed using a standard analysis algorithm (color deconvolution v9.0, Aperio Technology) to quantify the average optical density of Gal-1 staining and the percentage of positive pixels in the annotated tumor area.

**Real time RT-PCR**

Transcriptional profile of galectins was analyzed in human PCa cell lines (log phase of growth) that are representative of different stages of tumour progression. Transcriptional profile of angiogenesis-related genes was analyzed in plugs generated by injection of Gal-1-sufficient or Gal-1-silenced human 22Rv1 cells into nude mice. In all cases, RNA was purified using TRIzol reagent (Invitrogen) coupled to DNAse (RQ1, Promega) treatment. Four hundred ng of total RNA was used for the reverse transcription reaction by using SuperScript III Reverse Transcriptase, random hexamers (2.5 µg/ml) and dNTPs (500 nM) according to manufacturer’s instructions (Invitrogen) during 50 min at 45°C, following by RNAse H treatment for 30 min at 37°C. One µl of a 1:25 dilution of cDNA was used as template in real time PCR. Relative gene expression was analyzed using SYBR Green PCR kit (Applied Biosystem, Life Technologies). PCR conditions were as follows: 5 min 95°C, 40 cycles 30 sec at 95°C, 32 sec at 59°C, 45 sec at 72°C. Amplification fragments were analyzed by electrophoresis on a 2% agarose TAE gel and by thermal dissociation curves (Tm) to characterize the amplicon corresponding to each primer couple. Primers used are listed in Tables S1 and S2. Cyclophilin A was used as an internal reference gene (27). Equivalent amounts of RNA were tested to rule out genomic DNA contamination.
**Immunoblotting**

Specificity of anti-galectin antibodies was evaluated by immunoblotting (Figure S2). Cells were lysed in RIPA buffer (50 mM Tris-HCl-pH 8, 150 mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, 1 mM sodium vanadate and Protease Inhibitor Cocktail Set III (Calbiochem). Equal amounts of protein (10-30 µg) were resolved by 15% SDS-PAGE, blotted onto PVDF membranes (GE Healthcare), blocked with 5% BSA and probed with anti-galectin or anti-β-tubulin (H-235 1:200, Santa Cruz) antibodies or preimmune rabbit antiserum. The following dilutions of antibodies were used: anti-Gal-1 (1:500), Gal-3 (1:400), Gal-4 (1:100), Gal-8 (1:400), Gal-9 (1:100), Gal-12 (1:100) and rabbit IgG anti-Gal-1 at 1:1,500). Bound antibodies were detected with peroxidase-labeled anti-rabbit total immunoglobulins (1:3,000; Sigma) or by peroxidase-labeled rabbit anti-goat IgG (1:2,000; Sigma). Peroxidase activity was detected using a luminol-based method and chemiluminescence was determined using a Fuji Photo Film Analyzer.

**Lentivirus vector production and transduction**

pLv-HTM plasmid (provided by Trono Didier, Geneva University) is a self-inactivation third generation HIV-1-derived vector (28). Annealed oligonucleotides coding for shRNA were ligated into ClaI and MluI double-restricted plasmids by standard cloning. Restriction enzymes and T4 DNA ligase were from New England BioLabs. Production of siRNA was under the control of H1 (RNA polymerase type II) promoter. As reporter gene, green fluorescent protein (GFP) was expressed under the control of eukaryotic EF-1α promoter. Plasmids were verified by sequence analysis. Lentiviral particles were produced by transient transfection of 293T cells. Briefly, subconfluent 293T cells were co-transfected with 20 µg plasmid vector, 15 µg pCMVR8.74 and 5 µg pMD.G (pseudotyped VSVG) using calcium phosphate. Supernatants were harvested at 48 h and 72 h and stored at -80°C until use. Viral titers expressed as TU/ml were determined by assessing
transduction of 22Rv1 cells with serial dilutions of virion preparations. 22Rv1 PCa cells were transduced with virus at MOI=5 in the presence of 5 µg/ml protamine sulfate (Sigma). After one week, transduced cells (GFP+) were purified using a FACSria II cell sorter (BD Bioscience).

In vitro capillary-like tube formation and in vivo Matrigel plug assay

Matrigel (150 µl) (BD Biosciences) was added to 24-well plates and allowed to polymerize for 2 h at 37°C. Conditioned media were added to wells and 2.5 x 10^4 BAEC were seeded on each well. Tube formation was evaluated in 5 different fields of each well and photographed at 18 h using an inverted microscope. For in vivo assays, cold Matrigel was mixed with 5 x 10^6 22Rv1 cells in the absence or presence of a Gal-1 blocking or isotype control mAb (7.5 mg/kg). The mixture (500 µl) was subcutaneously injected into six week-old male nude mice. Five days later, Matrigel plugs were harvested and photographed. Matrigel plugs were homogenized in 500 µl H_2O on ice and cleared by centrifugation at 200 x g for 6 min at 4°C. Hemoglobin content was determined using the Drabkin’s reagent (Wiener Lab, Buenos Aires, Argentina).

Statistical analysis

Data are presented as mean ± standard deviation (S.D.) of at least three independent experiments in triplicate. Comparisons between two groups were performed by using paired Student’s t-test or Spearman correlation test as indicated. Differences were considered significant when P values were less than 0.05.
RESULTS

Identification of ‘the galectin signature’ of human prostate cancer cells

To delineate the galectin expression profile during PCa progression, we first examined the galectin transcriptional pattern of several human PCa cell lines, which are representative of different stages of the disease. These include the hormone-responsive cell line LNCaP and the castration-resistant cell lines 22Rv1 and PC-3, which are androgen receptor (AR) positive (22Rv1) or negative (PC-3) respectively. Total RNA was extracted in the log phase of growth and analyzed by quantitative RT-PCR (Figure 1A). Gal-1 was found to be the most abundantly expressed galectin in all cells analyzed and its expression was higher in PCa cells exhibiting more aggressive behavior in vivo (22Rv1 and PC-3) (27). Transcripts for Gal-8, which has been postulated as PCa marker (21, 29), was expressed at moderate levels in all PCa cell lines tested. Gal-3 mRNA was only detected in castration-resistant, AR negative PC-3 cells. Transcripts for all other galectin family members (Gal-2, -4, -7, -9, -10, -12 and -13) were expressed at very low levels.

To further delineate the ‘galectin-specific PCa signature’, we assessed the expression of galectin family members at the protein level (focusing on galectins with higher transcript abundance). Immunocytochemical analysis confirmed that Gal-1 was the most abundantly expressed galectin in the PCa cell lines analyzed, showing up-regulated expression in the most aggressive cell lines (Figure 1B). On the other hand, Gal-3 was selectively expressed in the PC-3 cell line, Gal-8 was detected in all the three cell lines and Gal-9 and Gal-12 showed a modest expression in all cell lines analyzed. These results indicate a fine regulation of galectin expression in PCa cell lines characterized by differences in phenotype, hormone-dependency and aggressiveness.
Analysis of the galectin expression profile of human primary prostate tumors

The differential expression of galectins in PCa cell lines prompted us to investigate the galectin profile in prostatectomies obtained from 61 patients with newly diagnosed untreated disease. Samples included a large spectrum of PCa stages (T1, T2, T3 and T4 according to TNM classification; UICC, 2002) in addition to a benign stage (BHP) (Table 1). Similar to PCa cell lines, Gal-1 was highly expressed in primary tumors and its expression was up-regulated in more advanced lesions (Figure 2). On the other hand, although typically expressed at lower levels, Gal-3, -4, -9 and -12 decreased gradually as the disease progressed toward more aggressive stages. Conversely, Gal-8 was expressed at moderate levels in lesions corresponding to all stages. These data delineate a ‘galectin-specific signature’ characterized by selective up- or down-regulation of galectins during PCa progression and highlight a potential role for Gal-1 as a sensitive biomarker in advanced stages of the disease.

Galectin-1 is a novel target for anti-angiogenic therapies in advanced human PCa

Because Gal-1 expression is associated with PCa aggressiveness and has emerged as a novel pro-angiogenic factor in other tumor types (30, 31), we asked whether this lectin was differentially expressed in tumor areas associated to blood vessels in human prostate cancer. To address this issue, we investigated whether a correlation exists between Gal-1 and CD34 expression using a human tissue array comprised of 29 paired cores of invasive PCa classified according to proliferation rates and cell morphology. A positive correlation was found between Gal-1 and CD34 expression in arrays representing advanced stages of human PCa (Figure 3A). This correlation was not observed in arrays of human breast cancer (Figure 3A), suggesting tissue-specific pro-angiogenic effects of this lectin.

Given the promising value of anti-angiogenic therapies in castration-resistant advanced PCa (32), we examined the role of Gal-1 in PCa angiogenesis. We evaluated the effect of conditioned
medium (CM) obtained from a Gal-1-positive PCa cell line (22Rv1 CM) on in vitro BAEC tubulogenesis. As shown in Figure 4, CM from 22Rv1 cells (Gal-1 concentration: 10.7 ng/ml) induced the formation of tubular structures reflective of EC morphogenesis. To evaluate the involvement of Gal-1, we exposed BAEC to CM from 22Rv1 PCa cells in the presence of an anti-Gal-1 neutralizing mAb (25,26). Neutralization of soluble Gal-1 considerably reduced tube formation (3.76 ± 1.50 fold; n=10) compared to BAEC exposed to PCa CM in the presence of a control isotype Ab (Figure 4A and B).

The in vitro effects of Gal-1-expressing PCa cells on EC morphogenesis prompted us to investigate the role of this endogenous lectin in angiogenesis in vivo using two different approaches in order to differentiate the source of Gal-1 (tumor and microenvironment vs. tumor alone). First, we injected a Matrigel mixture containing 22Rv1 PCa cells and a blocking anti-Gal-1 mAb (or its isotype control) into nude mice. Second, we used 22Rv1 tumor cells that were transduced with a human specific Gal-1 shRNA-coding lentivirus (Gal-1-shRNA-LV) purified to homogeneity by cell sorting. Non-sorted bulk transduced 22Rv1 cells, with partial down-regulation of Gal-1, were also tested (Figure 5A). A marked reduction of microvessel density was observed using both experimental approaches (anti-Gal-1 mAb and Gal-1 shRNA-LV), indicating that tumor cells were the main source of Gal-1, at least at early time points of tumor implantation (Figure 5B and C). Intermediate effects were observed when Gal-1 was partially down-regulated in PCa cells (Figure 5B and C). Altogether, our results reveal a key role for Gal-1 as a mediator of PCa-induced angiogenesis.

As angiogenesis relies on the expression of hypoxia-regulated genes and Gal-1 is regulated by hypoxia in different tumor types (26,33,34), we then examined the effects of hypoxia on the galectin expression profile of human PCa. For this purpose, 22Rv1 PCa cells were cultured under hypoxic or normoxic conditions and the galectin transcriptional profile was evaluated. We could observe no significant modification of the galectin expression profile except for Gal-1 that was modestly up-regulated in response to hypoxia (1.3-fold, p=0.015) (data not shown). To further understand the
molecular mechanisms underlying Gal-1-mediating angiogenesis, we screened different molecules classically associated with angiogenesis (bFGF, VEGF-A, CD142, uPA, CXCR4, PDGF-AA and MMP-9 as activators of angiogenesis; TSP-1, TIMP-1, CXCL10 and SPP1 as inhibitors of angiogenesis; and CA-IX as a marker of hypoxia) in Gal-1-silenced human PCa tumors. RNA was purified from in vivo plugs generated by injection of Gal-1-sufficient and Gal-1-silenced human 22Rv1 cells into nude mice and angiogenesis-related genes were determined by real time RT-PCR. Silencing Gal-1 in PCa cells growing in Matrigel plugs in vivo did not alter expression of angiogenesis-related genes neither in the tumor itself (human) nor in the tumor microenvironment (mouse) (Figure 6). These results suggest that Gal-1-induced PCa angiogenesis is independent of the up-regulation or down-regulation of classical pro-angiogenic or anti-angiogenic factors and places Gal-1 as a critical mediator of tumor angiogenesis.
DISCUSSION

Prostate cancer is no longer viewed as a disease of abnormally proliferating epithelial cells, but rather as a disease involving complex interactions between prostate cancer epithelial cells and the tumor microenvironment. Multiple signaling pathways and biological events mediate tumor growth, including androgen receptor signaling, tyrosine kinase receptor signaling, angiogenesis and tumor-immune escape (35). Interactions between multivalent lectins and glycans participate in this complex network by modulating stromal, endothelial and immune cell compartments (6, 7). Although original assumptions based on conserved carbohydrate specificity and structural homology suggested that galectins may have redundant functions, recent information challenged this view demonstrating specific roles for each member of the galectin family in the regulation of tumor cell invasiveness, inflammation and angiogenesis (7). In search for novel biomarkers and therapeutic targets, here we identified a ‘galectin-specific signature’ associated with PCa progression. Galectin expression was profiled in PCa cell lines with diverse androgen-dependence properties and AR expression and in human primary tumors obtained from treatment-free patients at different stages of the disease.

Originally described as Prostate Carcinoma Tumor Antigen-1 (PCTA-1), Gal-8 has been reported to be ubiquitously expressed in several tissues, but is up-regulated in PCa (29). Our results confirm that this ‘tandem-repeat’ galectin is expressed by PCa cell lines and primary tumors, but indicate that the degree of Gal-8 expression is comparable in all tumor stages. Given the complexity of Gal-8 isoforms, the variability of its intracellular localization and its ubiquitous expression pattern, this galectin is likely to be an important component of PCa biology (29), including modulation of cell proliferation, adhesion and angiogenesis (22, 36).

Interestingly, our results reveal that Gal-3, -4, -9 and -12 are down-modulated in advanced stage primary tumors. These data are consistent with earlier reports showing that Gal-3
expression decreases during tumor growth (10, 18) through mechanisms including promoter methylation (19) and metalloproteinase-mediated protein cleavage (20). Depending on its selective intracellular or extracellular localization, different biological properties have been assigned to Gal-3, resulting in a dual pro- or anti-tumorigenic effect (10, 37). Our results suggest that down-regulation of Gal-3, combined with the expression of other galectin members, is a hallmark of PCa progression. More importantly, our findings demonstrate that Gal-1 is the most abundantly expressed galectin in PCa and its expression correlates with disease severity, underscoring the relevance of this endogenous lectin as a possible biomarker and therapeutic target in high grade castration-refractory PCa.

Previous studies aimed at delineating the galectin transcriptional profile of a panel of human tumor cell lines revealed that all PCa cell lines analyzed (DU145, PC-3 and LNCaP) were negative for Gal-2, Gal-4, Gal-7 and Gal-9, but expressed considerable amounts of Gal-8 (38). Moreover, Gal-1 and Gal-3 were expressed in DU.145 and PC-3, but not in the LNCaP cell line (38). In contrast to these findings, we detected significant expression of Gal-1 in LNCaP cells both at the mRNA and protein levels, although at 20-fold lower levels than the androgen unresponsive 22Rv1 and PC-3 tumor cells. Moreover, Gal-1 expression augmented when LNCaP cells were cultured for several weeks in the absence of hormones (LNCaP-CR) (Figure S1A). As castration-sensitive or resistant LNCaP cells were both PSA-positive and responsive to an androgen receptor agonist (R1881) (Figure S2B), the discrepancies in Gal-1 expression among different studies might reflect different culture conditions, cell line sources or selection protocols. In this regard, our studies were performed on PCa cells isolated during the log phase of growth and the results were substantiated using primary tumors isolated at different stages of the disease.

Given the pleiotropic functions of Gal-1 in the tumor microenvironment, including its role in angiogenesis (26,31,39,40), cell adhesion and invasiveness (16,30) and immunosuppression (23-
up-regulation of Gal-1 may dramatically influence PCa progression. In this regard, Gal-1 is expressed in ECs (42-44) and is up-regulated in various cancer types (6). Here, we demonstrate that Gal-1 is the most highly expressed and regulated galectin in the PCa microenvironment and plays essential roles in PCa angiogenesis. The role of Gal-1 in angiogenesis seems to be tissue-specific as Gal-1 expression correlates with EC markers in advanced PCa but not in human breast cancer. These findings are consistent with the ability of Gal-1 to induce angiogenesis in oligodendroglioma (30), B16 melanoma (31), and Kaposi’s sarcoma (26), but not in other tumor types such as Lewis lung carcinoma (41).

Selective silencing strategies in tumors clearly demonstrated that the main cellular source of Gal-1 is represented by tumor cells. However, mechanisms by which ECs capture Gal-1 from the tumor microenvironment or tumor-induced EC activation up-regulates Gal-1 expression have also been described (31,39). Moreover, as Gal-3 and Gal-8 also contribute to angiogenesis in other tumor types, the spatiotemporal regulation of distinct members of the galectin family might ultimately dictate the vascularization phenotype (36,45, 46,47). Finally, Gal-1 silencing in PCa cells did not alter the expression of classical pro-angiogenic or anti-angiogenic mediators neither in tumor cells nor in the tumor microenvironment, highlighting a direct and critical role of this lectin in PCa angiogenesis.

In summary, our findings identify a distinctive ‘galectin signature’, which delineates tumor progression in human PCa and highlight a major role of Gal-1 as a novel target of anti-angiogenic therapies in advanced castration-resistant stages of PCa, where effective treatments are still lacking.
ACKNOWLEDGEMENTS

We thank Drs. Karim Fizazi and Catherine Gaudin (INSERM U981; IGR-France), Geraldine Gueron (University of Buenos Aires) and Carla Saleh (Pasteur Institute, France) for help and advice.

GRANT SUPPORT

Supported by grants from Prostate Action (UK) to G.A.R, D.J.L. and D.C, Agencia Nacional de Promoción Científica y Técnica Argentina (ANPCyT; PICT 2008-134 to D.J.L.; PICT 2010-870 to G.A.R.), Programa de Cooperación Franco-Argentino ECOS-Sud (A10S03 to G.A.R., A.C., D.J.L and D.C.), Fundación Sales to G.A.R, University of Buenos Aires to G.A.R., and Association pour la Recherche sur les Tumeurs de la Prostate -ARTP, France- to D.C.

AUTHORS’ CONTRIBUTIONS

Acquisition, analysis and interpretation of data: D.J.L.; L.D.G; L.G.; V.C.D.; L.N.; N.A.N.; J.L.K; S.J.K.; M.T.E and D.C.

Material support: D.C.; P.S.; G.C.; O.M.; M.S.; E.V. and A.C.

Development of methodology: D.C.; D.O.C.

Conception; design and supervision of the study: D.J.L; D.C.; G.A.R.

Writing of the paper: D.J.L.; D.C.; G.A.R.
REFERENCES


### Table 1: Description of human primary tumors analyzed

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<th>GRADE</th>
<th>AVERAGE AGE (years)</th>
<th>Number of patients</th>
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<td>A.</td>
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<tr>
<td>Hyperplasia</td>
<td>65 +/- 10</td>
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<tr>
<td>T1</td>
<td>69 +/- 10</td>
<td>10</td>
</tr>
<tr>
<td>T2</td>
<td>63 +/- 5</td>
<td>19</td>
</tr>
<tr>
<td>T3</td>
<td>60 +/- 5</td>
<td>18</td>
</tr>
<tr>
<td>T4</td>
<td>56 +/- 10</td>
<td>3</td>
</tr>
<tr>
<td>B.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>69 +/- 6</td>
<td>5</td>
</tr>
<tr>
<td>Grade 1</td>
<td>74 +/- 7</td>
<td>8</td>
</tr>
<tr>
<td>Grade 2</td>
<td>73 +/- 8</td>
<td>60</td>
</tr>
<tr>
<td>Grade 3</td>
<td>73 +/- 8</td>
<td>58</td>
</tr>
</tbody>
</table>

**A.** Radical prostatectomies were classified according to TNM scale. Specimens (n=61) covered all stages of PCa evolution, including T1 (tumor detected in less or 5% of the tissue), T2 (tumor confined to the prostate), T3 (tumor extends beyond the prostatic capsule) and T4 (tumor invades structures other than seminal vesicles), in addition to benign hyperplasia (BHP). Average ages and Gleason indexes from patients are shown (Gleason ≤ 6: tumor well differentiated, low grade; Gleason = 7: intermediate grade and Gleason ≥ 8: tumor poorly differentiated, high grade). **B.** Tissue arrays of radical prostatectomies obtained from US Biomax (TMA-BC19013) are classified according to: Grade 1 or well differentiated (cells appear normal and do not grow rapidly); Grade 2 or moderately-differentiated (cells appear slightly different from normal) Grade 3 or poorly differentiated (cells appear abnormal and tend to grow and spread more aggressively).
FIGURE LEGENDS

Figure 1: Galectin expression profile in human PCa cell lines. A. Transcriptional profile of galectins by real-time RT-PCR. Results are expressed as galectin mRNA relative to Cyclophilin A. Cell lines are presented according to androgen sensitivity and androgen receptor (AR) expression. Left, LNCaP cells (hormone responsive HR, AR+); Middle, 22Rv1 cells (castration resistant CR, AR+); Right, the more aggressive PC-3 cells (CR and AR-). Data are expressed as mean ± SD of four independent experiments. B. Immunocytochemical analysis of galectins in PCa cells adhered onto poly-L-Lysine-coated glasses (Magnification X100).

Figure 2: Galectin expression profile of human primary prostate tumors. A. Radical prostatectomies from naive patients (n=50 carcinomas and n=11 benign hyperplasia) were arranged according to T grade (TNM classification; UICC). Galectin expression was analyzed by immunohistochemistry in paraffin-embedded tissue sections from patients (Table 1). Magnification: 400 X. B. Galectin expression in patient samples was graded as follows: 0= negative; 1=low intensity; 2=moderate intensity; 3=high intensity and 4=very high intensity. Results are representative (A) or are the mean ± S.D. (B) of four independent experiments. * P <0.05 (Student’s t test), n.s = non-statistically different.

Figure 3: Gal-1 expression positively correlates with the number of CD34+ blood vessels in advanced human prostate cancer. Expression of Gal-1 and CD34 was evaluated in an invasive PCA tissue-microarray (slides containing 29 paired cores: BC19013, US Biomax) by immunohistochemistry. Stained slides were digitally scanned using Aperio ScanScope XT
workstation and evaluated by ImageScope software. **A.** Correlation between Gal-1 and CD34 expression in tumor areas of grade III PCa (classification based on grade of proliferation and cell morphology as described in Materials and methods). Breast cancer tissue was analyzed for comparison purposes. **B.** Examples of PCa samples with intense or low Gal-1 and CD34 expression. Results are representative of three independent experiments. * P <0.05 (Spearman correlation test).

**Figure 4: Prostate cancer-derived Gal-1 promotes EC morphogenesis.** **A.** *In vitro* formation of tubular structures by BAEC cultured in Matrigel with 22Rv1-conditioned media (22Rv1 CM) in the absence or presence of an anti-Gal-1 mAb (F8.G7) or isotype control mAb. Recombinant VEGF was used as a positive control. **B.** Quantification of the number of tubular structures per field. Results are representative (A) or are the mean ± S.D. (B) of four independent experiments. * P <0.001 (Student’s t test).

**Figure 5: Prostate cancer-derived Gal-1 promotes angiogenesis in vivo.** Nude mice were injected with 5 x 10^6 22Rv1 PCa cells incorporated in Matrigel plugs. The role of Gal-1 was assessed by two different strategies: a) using 22Rv1 cells transduced with Gal-1 shRNA-coding lentivirus (bulk; n=3 or sorted cells; n=8 with ~40 and 80% Gal-1 down-regulation respectively) compared to control shRNA-LV cells (n=8); or b) by adding an anti-Gal-1 mAb (n=3) or isotype control (n=3). **A.** Immunoblot of Gal-1 in 22Rv1 cells. First lane: cells transduced with a control shRNA-LV and sorted according to GFP expression; second lane: Gal-1 shRNA-LV (bulk); third lane: cells transduced with a Gal-1 shRNA-LV and sorted. **B.** Representative photographs of *in vivo* plugs at day 5. **C.** Hemoglobin content in plugs at day 5, normalized to µg of protein. Results are representative (A,B) or are the mean ± S.D. (C) of four independent experiments. **P** <0.005 (Student’s t test).
Figure 6: Gal-1 silencing does not alter expression of angiogenesis-related genes in PCa. Total RNA was extracted from plugs generated in vivo by injection of Gal-1-sufficient (n=5) and Gal-1-silenced (n=5) human 22Rv1 cells into nude mice. Angiogenesis-related genes, including activators of angiogenesis (bFGF, VEGF-A, CD142, uPA, CXCR4, PDGF-AA and MMP-9), inhibitors of angiogenesis (TSP-1, TIMP-1, CXCL10 and SPP1) and a marker of hypoxia (CA-IX) were screened by real time RT-PCR adapted to a gene array platform. This screening allows the distinction of human genes (derived from the tumor) and mouse genes (reflecting the mouse microenvironment). Lower panel includes genes derived from both tumor and microenvironment, as these primers react with both human and mouse genes. Data show individual samples analyzed and the mean of individual plugs. *P<0.05 (Student’s t test).
A

LNCaP

22Rv1

PC-3

Relative Gal-1 mRNA vs Cyclo A

B

Pre-immune sera

Gal-1

Gal-3

Gal-8

Gal-9

Gal-12

LNCaP HR, AR+

22Rv1 CR, AR+

PC-3 CR, AR−
B

Galectin Expression (AU)

Tumor Progression (TNM Classification)

Laderach et al., Figure 2
A Prostate cancer
Grade III

Gal-1(OD)

CD34 (%)

Breast cancer

Gal-1                             CD34

Gal-1                             CD34

p=0.04

Laderach et al., Figure 3
A

100 μm

VEGF

22Rv1 CM

22Rv1 CM Isotype Control

22Rv1 CM Gal-1 mAb

B

Number of tubules per field

VEGF  
+ Isotype Control  
+ Gal-1 mAb  
22Rv1 CM

Laderach et al., Figure 4
A

Gal-1

β-tubulin

B

Isotype mAb
control shRNA-LV

Gal-1 shRNA-LV
(bulk)

Gal-1 shRNA-LV
(sorted)

Gal-1 mAb

C

Hemoglobin (g/µg protein)

Laderach et al., Figure 5
Laderach et al., Figure 6
A unique galectin signature in human prostate cancer progression suggests galectin-1 as a key target for treatment of advanced disease

Diego J Laderach, Lucas Gentilini, Laura Giribaldi, et al.

Cancer Res  Published OnlineFirst October 29, 2012.