Metastatic Progression of Prostate Cancer Is Mediated by Autonomous Binding of Galectin-4-O-Glycan to Cancer Cells

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Abstract

Metastatic prostate cancer continues to pose a difficult therapeutic challenge. Prostate cancer progression is associated with aberrant O-glycosylation of cancer cell surface receptors, but the functional impact of such events is uncertain. Here we report spontaneous metastasis of human prostate cancer xenografts that express high levels of galectin-4 along with genetic signatures of EGFR-HER2 signaling and O-glycosylation. Galectin-4 expression in clinical specimens of prostate cancer correlated with poor patient survival. Galectin-4 binding to multiple receptor tyrosine kinases stimulated their autophosphorylation, activated expression of pERK, pAkt, fibronectin, and Twist1, and lowered expression of E-cadherin, thereby facilitating epithelial–mesenchymal transition, invasion, and metastasis. In vivo investigations established that galectin-4 expression enabled prostate cancer cells to repopulate tumors in orthotopic and heterotopic tissues. Notably, these effects of galectin-4 relied upon O-glycosylation mediated by C1GALT1, a galactosyltransferase implicated in other cancers. Parallel changes in galectin-4 and O-glycosylation triggered aberrant receptor signaling and more aggressive invasive character in prostate cancer cells, which through better survival in the circulation also contributed to the bulk cell progeny of distal tumors. Our findings establish galectin-4 and C1GALT1-mediated glycosylation in a signaling axis that is activated during prostate cancer progression, with implications for therapeutic targeting of advanced metastatic disease. 

Introduction

Prostate cancer remains the most common malignancy in men worldwide. Although effective therapies are available for early-stage prostate cancer, there is still no cure for the metastatic disease. During metastasis, the malignant cancer cells migrate into the lymph and blood vessels via epithelial–mesenchymal transition (EMT), survive in the circulation system, and ultimately colonize distant organs and result in the development of life-threatening disease. Many transcription factors involved in the EMT program, such as Snail, Slug, and Twist also regulate the stem-like cell properties of carcinoma cells in cancer progression (1, 2). Furthermore, clinical evidence suggests that the expression level of Twist in clinical specimens from radical prostatectomy is highly correlated with biochemical recurrence of prostate cancer as indicated by a persistent increase in serum prostate-specific antigen (PSA) value (3). The expression of EMT-related transcription factors can be activated by signaling of specific growth factors and corresponding receptor tyrosine kinase (RTK) pathways, such as EGFR, PDGFR, HGF, and IGF1R (1, 2).

The aberrant glycosylation of surface proteins of malignant cells has been implicated in the various steps of tumor progression and can regulate intracellular and intercellular signaling to promote cell proliferation, metastasis, EMT, and angiogenesis (4, 5). The glycan-encoded information of the glycoconjugates is translated into cellular responses by the glycan-recognizing proteins, lectins. Here, we found that β-galactoside–binding protein galectin-4, encoded by the LGALS4 genes, is upregulated in highly metastatic prostate cancer cells. Galectin-4 knockdown from the metastatic prostate cancer cells suppressed tumorigenicity and metastasis, and conversely forced expression of galectin-4 in various prostate cancer cell lines boosted their tumorigenicity and metastasis. Galectins are a subfamily of animal lectins expressed in the extracellular environment, cell membrane, cytoplasm, and nucleus. In normal tissues, galectin-4 is primarily expressed in epithelial cells along gastrointestinal tissue (6). Galectins are synthesized as a cytosolic soluble protein and can...
be externalized via a nonclassic pathway to mediate cell–cell and cell–matrix interactions or as innate immune lectin to kill bacteria (7, 8). The function of galectin-4 in cancer is not well studied. However, the correlation between galectin-4 level and malignancy has been reported in many cancers. High levels of expression of galectin-4 have been shown in colon adenocarcinoma and breast carcinomas by immunohistochemical staining (6). Galectin-4 can enhance the adhesion of human colon cancer T84 cells to HUVEC.

![Figure 1](image-url)

**Figure 1.**
Identification of metastasis-associated genes from spontaneous metastases of orthotopic human prostate cancer xenograft in a murine model. **A,** flow chart for in vivo development of metastases and the derived tumor cell populations from 22Rv1 model. Details are described in Materials and Methods. **B,** Venn diagrams illustrate the distribution and overlap of differentially expressed genes. **C,** hierarchical clustering displays differential gene expression profiles for 22Rv1, 22Rv1-PT, and 22Rv1-M3 (22Rv1-M3-1 and 22Rv1-M3-2 are from two independent metastases). Two RNA extractions from each sample were hybridized in microarray analysis. **D,** the galectin-4 mRNA levels of prostate cancer cells were determined by qRT-PCR. **E,** protein expression of intracellular galectin-4 in different types of prostate cancer cells was visualized by Western blotting. **F,** cellular expression of galectin-4 (green) in prostate cancer cells was visualized by IFA. Nuclei (blue), DAPI staining. **G,** secretion of galectin-4 in the conditioned medium of different prostate cancer cells was measured by ELISA. Scale bar, 50 μm. Bars, SEM. **,** *P < 0.001 by unpaired Student t test.
endothelial cells, implying that galectin-4 might mediate in the initial attachment and/or spreading of neoplastic cells during metastasis (9). Galectin-4 was upregulated in highly metastatic gastric cancer cell lines and the level of galectin-4 significantly correlated with prognosis of gastric cancer (10). Recently, galectin-4 was also identified to be a novel predictor of lymph node metastasis of lung adenocarcinoma with galectin-4-negative patients showing survival advantage as compared with galectin-4-positive patients (11). However, the role of galectin-4 in hepatocellular cancer and colon cancer has been controversial.

Figure 2.
Validation of galectin-4 as a metastasis driver gene. A, $5 \times 10^4$ cells of 22Rv1-Luc2 cells, 22Rv1-M3, M3-shCtrl, and M3-shGal4 were inoculated into mouse prostate and the BLI was measured weekly. Left, growth curves of primary prostate cancer were determined by BLI, n = 8–12. Right, the BLI of representative mice are shown as a serial time course. Eight over 12 mice in the shGal4 group exhibited undetectable tumor BLI. B, quantification of lung metastasis from orthotopic prostate cancer xenograft. Left, endpoint lung metastases were quantified by ex vivo BLI. Right, representative histologic sections of lungs. E, endpoint tumor mass of 22Rv1, 22Rv1-M3, M3-shCtrl, and M3-shGal4 orthotopic xenografts. Right, representative histologic sections of lungs. D, $1 \times 10^5$ cells of 22Rv1-M3, M3-shCtrl, or M3-shGal4 were inoculated into the tail vein of nude mice and the systemic metastasis were monitored weekly for 8 weeks. Left, the longitudinal BLI intensity was analyzed for tumor colonization; n = 6–8. Right, whole body BLI images of representative mice exhibited systemic metastases. E, mouse lungs from D were removed at endpoint and metastasis was estimated by quantification of BLI intensity. F, and G, ELISA determined the plasma concentration of galectin-4 in mice bearing orthotopic prostate cancer in A or metastatic prostate cancer in C, respectively. Scale bar, 100 μm. Bars, SEM. ***, P < 0.001 by unpaired Student t test.
Despite the disagreement across cancer types, we identified galectin-4 as a major driver gene governing prostate cancer metastasis in a murine model. In this study, we dissected the molecular mechanisms and signaling pathways caused by galectin-4 overexpression that drive certain EMT-like events in prostate cancer cells and thus the metastatic progression and provide evidence for the clinical correlation of galectin-4 with poor survival in prostate cancer patients.

Figure 3.
Galectin-4 upregulation in clinical prostate cancer correlated with clinical malignancy, advancement, and poor survival in prostate cancer patients. A and B, IHC analysis for galectin-4 expression in clinical prostate cancer specimens of different stages. Scale bar, 50 μm. B, the statistical significance of galectin-4 level and stage (left, \( P < 0.001 \)) or Gleason score (right, \( P < 0.001 \)) was examined in \( \chi^2 \) test. C, cumulative survival probabilities (Kaplan–Meier) for prostate cancer subgroups. The statistical significance was determined by log-rank test. D, correlation of galectin-4 expression score in prostate cancer specimens with the overall survival of prostate cancer patients. ***, \( P < 0.001 \) by Fisher exact test. E, correlation of galectin-4 score in prostate cancer specimens with the PSA recurrence in prostate cancer patients. ***, \( P < 0.001 \) by Fisher exact test. F, galectin-4 mRNA expression in clinical samples of normal and cancerous human prostate tissues from commercial cDNA array were analyzed by qRT-PCR. Box plot represents the median with maximum and minimum, *, \( P < 0.05 \) by unpaired Student \( t \) test. G, the levels of galectin-4 transcripts in normal and cancerous human prostate carcinoma tissues. Raw data were acquired from the Oncomine, Tomlins Prostate Dataset. Transcript level of galectin-4 in prostate cancer samples \( (n = 25) \), was compared with samples of PIN and BPH \( (n = 10) \), \( n = 10 \). Box plot represents the median with 90th and 10th percentiles, and statistical significance was analyzed by Student \( t \) test.
Materials and Methods

Cell lines
PC-3, 22Rv1, LNCaP, and DU-145 cell lines were obtained from the ATCC from 2002 to 2003. All cell lines and their derivatives were authenticated by comparing in ATCC database of short tandem repeat DNA profiles within 6 months of the last experiment. For in vivo measurement of tumor growth and metastasis, these cell lines were stably transfected with firefly luciferase luc2 of pGL4 (Promega) driven by a hybrid EF1α/eIF4g promoter (InvivoGen) through lentiviral infection. All cell lines were routinely cultured in RPMI1640 supplemented with 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, and 10% FBS.

Orthotopic xenografting and establishment of the metastatic sublines
BALB/c nude mice (6–8 weeks old) were obtained from the National Laboratory Animal Center and all animal work was conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee, Academia Sinica (Taipei, Taiwan). To derive highly malignant cells, 2 × 10^5 of luciferase-expressing 22Rv1 cells (22Rv1-Luc2) were implanted in the anterior prostate of nude mice as described previously (14). After 12–14 weeks, the host mice were necropsied and the metastases from lumbar lymph nodes and primary tumors were dissected under laminar flow. Tumor tissues were minced using sterile scalpels and further digested with Collagenase D (Roche) for 1 h. The lymph node metastatic cancer cells, named 22Rv1-M1, were orthotopically reimplanted in nude mice. At 12 weeks, the secondary metastases were isolated in the lumbar lymph nodes and designated as 22Rv1-M2 cells. Suspension of 1 × 10^7 22Rv1-M2 cells in DPBS was injected into nude mice through the tail vein, and mice developed metastases (22Rv1-M3) after 6 weeks. This procedure was repeated once to mice through the tail vein, and mice developed metastases growth, mice received D-luciferin at 150 mg/kg by intraperitoneal injection. The correlation analysis of galectin-4 expression and patient survival curve was determined using the log-rank test. Clinical relevance of variable galectin-4 expression was assessed using Fisher exact test (two-tailed).

Results

Metastatic prostate cancer cells express a high level of galectin-4
To study prostate cancer progression, we tagged 22Rv1 castration-resistant prostate cancer cell line by expression of a luciferase reporter gene and orthotopically implanted the heterogeneous cell populations into nude mice prostates. Mice carrying 22Rv1 tumors consistently developed spontaneous metastases in lymph nodes (Supplementary Fig. S1A). After recovering the prostate cancer cells from primary culture of metastatic lesions and serial in vivo propagation, metastases-derived sublines (22Rv1-M1, M2, M3, and M4) were established (Fig. 1A). These 22Rv1-derived cells exhibited increased invasion activity along with the progression of primary tumor-derived 22Rv1-PT, metastatic 22Rv1-M3 (two independent isolates, 22Rv1-M3-1 and 22Rv1-M3-2), and 22Rv1-M4 in comparison with the parental 22Rv1-Luc2 cells (Supplementary Fig. S1B). These aggressive cancer cells acquired increased capacity for colonization as demonstrated by experimental metastasis through tail vein injection, whereas the 22Rv1-Luc2 cells were unable to form macrometastasis lesions in the lung at 6 weeks after inoculation as shown by bioluminescence imaging (BLI) and histologic examination (Supplementary Fig. S1C and S1D). To identify genes associated with the metastatic phenotype, we conducted a transcription microarray analysis (GEO accession number: GSE76034) by comparing the 22Rv1 cells with the localized 22Rv1-PT cells or the metastatic sublines, 22Rv1-M3-1 and -2. Genes with a 3-fold or higher change of expression were selected. The metastasis-associated genes were deduced from comparisons by including the differential genes shared by 22Rv1-M3-1 and 22Rv1-M3-2, but excluding those from 22Rv1-PT versus 22Rv1. Fifty-six genes that were differentially expressed in metastatic subpopulations (19 upregulated and 37 downregulated genes), but not in 22Rv1-PT, were identified (Fig. 1B and C). We knocked down eight of these upregulated genes one-by-one and found that the LGALS4 (galectin-4) was required for the metastatic prostate cancer cells to colonize various tissues (Supplementary Fig. S1E and S1F). The 22Rv1-M3-2 cells, hereafter named 22Rv1-M3, were used to characterize the role of galectin-4 in prostate cancer progression. The expression levels of galectin-4 at the mRNA and protein levels in different cell isolates exhibited a prominent surge as 22Rv1 progressed into 22Rv1-M3 and 22Rv1-M4 (Fig. 1D and E). The strong expression of galectin-4 was heterogeneous and due to increased proportions of galectin-4–positive cells, as demonstrated by indirect fluorescent antibody (IFA; Fig. 1F). The extracellular galectin-4 was heightened in 22Rv1-M3 and further in 22Rv1-M4, as measured by ELISA (Fig. 1G). The galectin-4 levels in conditioned medium from 22Rv1, 22Rv1-PT, and 22Rv1-M4 cells corresponded with their relative expression of mRNA and protein, whereas knockdown of galectin-4 in 22Rv1-M3 cells stripped off galectin-4 in its conditioned

Figure 4.
Galectin-4 mediates autocrine activation of multiple RTKs and the downstream signaling. A, microarray data were analyzed using the GSEA program and identified the HER2 (ERBB2) gene set (P < 0.001, FDR < 0.25) and EGFR gene set (P < 0.001, FDR < 0.25) are significantly enriched in 22Rv1-M3 cells. B, immunoblot of phosphorylated and total EGFR, HER2, HER3, IGFIR, Akt1, ERK, and GSK3β in 22Rv1 and 22Rv1-M3. C, time course of galectin-4–mediated phosphorylation of RTKs and downstream effectors were analyzed. M3-shGa4 cells were starved for 24 hours, treated with 0.2 µg/mL rhGalectin-4 for the indicated time, and analyzed by Western blotting. D, IHC analysis for the expression of galectin-4, pEGFR, pHER2, pHER3, and E-cadherin in tumors derived from orthotopic xenografts of different cells. Scale bar, 50 µm. E, the effect of EGFR/HER2 inhibitor on galectin-4–mediated response. M3-shGa4 cells were cotreated with 0.2 µg/mL rhGalectin-4 and lapatinib (10 µmol/L) for 16 hours. Phosphorylation of RTKs and the downstream effectors were examined by Western blotting. F, the carbohydrate dependence of galectin-4–mediated RTK phosphorylation. Prostate cancer cells were treated with 0.2 µg/mL rhGalectin-4 in the presence or absence of 10 mmol/L lactose or sucrose for 16 hours, and the levels of RTKs phosphorylation and the downstream Twist1 were determined in Western blotting.
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medium (Supplementary Fig. S1G and S1H). Furthermore, implantation of 22Rv1-M3 cells in mice caused a faster tumor growth rate with more lung metastases compared with 22Rv1 cells, while galectin-4 deprivation (M3-shGal4) significantly inhibited the tumor growth and lung metastasis compared with the scramble control, M3-shCtrl (Fig. 2A–C). As determined by intravenous inoculation of prostate cancer cells, metastatic colonization by prostate cancer in various tissues and the lung required high levels of galectin-4 expression, and galectin-4 deprivation resulted in no detectable metastatic lesions in this assay (Fig. 2D and E). To determine whether galectin-4 was released into the blood circulation, plasma samples of tumor-bearing mice were analyzed by ELISA. Mice bearing 22Rv1-M3 tumors or M3-shCtrl tumors had significantly higher galectin-4 levels than those with 22Rv1 tumor, and the circulating galectin-4 was hardly detectable in mice bearing M3-shGal4 tumors (Fig. 2F). The metastatic tumors inoculated intravenously also released a high-level of galectin-4 in the host circulation (Fig. 2G). These data demonstrate that overexpression of galectin-4 in prostate cancer cells was required to promote tumorigenicity and metastasis.

Clinical significance of galectin-4 expression in prostate cancer

The pathologic role of galectin-4 in clinical prostate cancer is not known. To address whether dysregulation of galectin-4 is associated with clinical prostate cancer progression, the galectin-4 expression in prostate cancer specimens was examined using commercial tissue microarrays (n = 192). High-level expression of galectin-4 correlated with the high pathologic stage and high Gleason score of prostate cancer specimens (Fig. 3A and B). To evaluate the prognostic value of galectin-4 in clinical prostate cancer, samples from another cohort of prostate cancer patients (n = 253) were analyzed for the correlation between the galectin-4 level and patient survival. Prostate cancer specimens with higher levels of expression of galectin-4 significantly correlated with poorer overall survival and a higher rate of prostate cancer biochemical recurrence (Fig. 3C–E). In addition, the mRNA level of galectin-4 in a third patient cohort (commercial cDNA array) was detected as significantly higher in cancerous prostate than normal prostate tissues (Fig. 3F). Consistent with these qPCR results, mining the cancer profiling database Oncomine, Tomlins Prostate Dataset (15), showed significantly higher galectin-4 mRNA in prostate cancer patients (n = 25) compared with benign prostatic hyperplasia (BPH) or prostatic intraepithelial neoplasia (PIN) patients (n = 10; Fig. 3G).

Galectin-4 mediated activation of RTKs, ERK, and Akt signaling

To reveal the oncogenic function that is acquired in 22Rv1-M3 cells, microarray data were analyzed using gene-set enrichment analysis (GSEA) to identify statistically significant functionally related gene sets, using the oncogenic categories of the Molecular Signatures Database (MSigDB). This GSEA algorithm revealed that EGFR-HER2, as well as the downstream RAF, MEK, Akt, and β-catenin signatures were enriched in 22Rv1-M3 cells compared with 22Rv1 cells (Fig. 4A and Supplementary Fig. S2A). Next, expression of RTK genes was assessed by qRT-PCR. EGFR family receptors were prominently expressed in 22Rv1 cells, and the transcripts of several RTKs were upregulated in 22Rv1-M3 cells compared with 22Rv1 cells, including HER2, HER3, and IGF1R (Supplementary Fig. S2B). The role of galectin-4 in the activation of these receptors and their downstream signaling was then examined by comparing 22Rv1, 22Rv1-M3 cells, and galectin-4-deprived cells using Western blot analysis. The phosphorylation levels of EGFR, HER2, HER3, and IGF1R were higher in 22Rv1-M3 than 22Rv1, whereas galectin-4 knockdown inhibited the phosphorylation of all these RTKs and also hampered the downstream signaling including phosphorylation of ERK1/2, Akt (T308 and S473), and GSK3β (S9; Fig. 4B).

Because ligand-mediated RTK activation usually induces rapid phosphorylation of RTKs and downstream signaling molecules, the kinetics of galectin-4-mediated RTK activation was analyzed by adding exogenous galectin-4 to a culture medium of M3-shGal4 cells under serum starvation. The Western blot data suggested that galectin-4–mediated RTK activation was initiated within 5 minutes and peaked at 15 minutes (Fig. 4C). The in vivo activation of RTK signaling by galectin-4 was further evaluated by IHC with the orthotopic tumors derived from different prostate cancer cells. Phosphorylation levels of HER2 and HER3 in high galectin-4–expressed 22Rv1-M3 tumors were much higher than the levels in 22Rv1 tumors, which were inhibited in galectin-4–deprived tumors (Fig. 4D). As the RTK-mediated response is tyrosine kinase-dependent, specific inhibition of EGFR and HER2 with lapatinib effectively blocked the phosphorylation of EGFR, HER2, and HER3, but not IGF1R, upon galectin-4 stimulation (Fig. 4E). However, Akt and ERK, common signaling nodes downstream of most RTKs, were not suppressed following lapatinib treatment, suggesting the involvement of other RTKs in galectin-4 signaling and the ineffectiveness of lapatinib monotherapy for aggressive prostate cancer (Fig. 4E). To further determine whether galectin-4–mediated RTK activation is dependent on the galectin–carbohydrate interaction, lactose was used to compete the galectin-4 binding. In four prostate cancer cell lines and 22Rv1–derived 22Rv1-M3 cells, lactose but not sucrose was able to compete the effect of galectin-4 in mediated RTKs activation and the downstream cellular responses of Twist1 expression (Fig. 4F).

Figure 5.

O-glycosylation–dependent binding of galectin-4 to RTKs. A, galectin-4 or RTKs complexes were immunoprecipitated from the membrane fraction of 22Rv1-M4 cells with respective specific antibodies. Individual RTKs and galectin-4 were detected in the galectin-4 or each RTK immuno-complex by Western blotting. B, proximity ligation assay for interactions between galectin-4 and EGFR, HER2, or HER3 on the cell surface was conducted in 22Rv1-M4 cells and imaged by confocal microscopy. Red, PLA signals; green, counterstain of F-actin. Bar, 10 μm. C, specific binding of fluorophore-conjugated 488–galectin-4 to the cell surface of 22Rv1 cells. Cells were incubated with 1 μg/ml 488–galectin-4 in the presence of 10 mmol/L Lactose (competitor) or sucrose (control). Right, the mean fluorescent intensity (MFI) values from triplicates are represented as means ± SEM. D, GSEA enrichment score curves of the O-link glycosylation gene set in KEGG was identified. E, C1GALT1 mRNA level in different prostate cancer cells and their differential patterns of cell surface glycans were determined by qRT-PCR (left) and VVL lectin staining (right), respectively. F, the binding of 488–galectin-4 or VVL to 22Rv1 or PC-3 cells transfected with control shRNA or shC1GALT1 were analyzed using flow cytometry. G, the role of C1GALT1 in RTKs activation by ligands. C1GALT1 were knocked down in PC-3-tetO-Gal4 cells with or without inducing the galectin-4 expression. Phosphorylation of RTKs was examined by Western blotting. H, C1GALT1 knockdown PC-3 cells were treated with 10 ng/ml EGF or 50 ng/ml heregulin for 30 minutes and analyzed by Western blotting. Data are expressed as mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001 by unpaired Student t test.

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C1GALT1 expression determined the galectin-4–mediated RTK response

To find out whether galectin-4 binds directly to these cell-surface receptors and triggers the downstream signaling, we immunoprecipitated the galectin-4 or individual RTKs from 22Rv1-M3 and 22Rv1-M4 cells with specific antibodies and detected whether galectin-4 and RTKs coexist in the same immunocomplexes. As detected by Western blotting, galectin-4 was reciprocally coimmunoprecipitated with each RTK, suggesting its binding to RTKs (Fig. 5A and Supplementary Fig. S2C). In agreement, proximity ligation assay of extracellular EGFR, HER2, or HER3 with galectin-4 also represented their colocalization on the cell surface (Fig. 5B). Next, we examined whether the galectin-4–mediated response is dependent on carbohydrate recognition. Direct binding of galectin-4 to the cell surface was detected positive by flow cytometry, which was selectively competed out by lactose at high concentration, rather than sucrose (Fig. 5C). Most membrane proteins are glycosylated and bear N-linked and O-linked glycans. Analysis for the differential genes using GSEA identified that the O-glycosylation pathway was upregulated in 22Rv1-M3 cells relative to 22Rv1 (Fig. 5D). The mRNA quantification of glycosylation enzymes confirmed that core 1 O-glycosylation enzymes (C1GALT1, ST3GAL1, and ST6GALNAC4) were upregulated in 22Rv1-M3; however, the core 3 synthase (B3GNT6) and N-glycosylation enzyme (MGAT5) were expressed at low levels in 22Rv1 and 22Rv1-M3 cells (Supplementary Fig. S2D). As C1GALT1 is the only known enzyme in humans that catalyzes the initiation of biosynthesis of core 1 O-glycan structure, we knocked down C1GALT1 expression in prostate cancer cells to dissect the role of O-glycans in the RTK activation by galectin-4 (Supplementary Fig. S2E). The C1GALT1 mRNA gradually increased along with 22Rv1, 22Rv1-M3, and 22Rv1-M4, which accompanied the decrease of Tn antigen on the cell surface as determined by VVI lectin binding (Fig. 5E). Furthermore, knockdown of C1GALT1 in 22Rv1 and PC-3 decreased the galectin-4 binding, but increased the VVI binding (Fig. 5F). Targeted ablation of C1GALT1 in PC-3 and 22Rv1-M3 cells resulted in loss of the galectin-4–mediated RTK activation, indicating that the galectin-4 effect was dependent on binding to surface core 1 O-glycans (Fig. 5G and Supplementary Fig. S3A). In contrast, C1GALT1-depleted cells remained responsive to the physiologic ligands of ERBB receptors, EGFR, and heregulin (Fig. 5H and Supplementary Fig. S3B).

Galectin-4 regulated invasion activity and EMT

Growth factor–mediated activation of RTKs transduces signals to downstream signaling effectors, such as ERK and Akt, and was found to promote EMT and cancer metastasis. The galectin-4 effect on prostate cancer cells was then analyzed by the expression of EMT-related molecules using Western blotting. 22Rv1-M3 cells expressed higher levels of fibronectin, vimentin, β-catenin, and Twist1, but lower levels of E-cadherin than 22Rv1, indicating EMT-like phenotypes (Fig. 6A). Knockdown of galectin-4 in 22Rv1-M3 reverted the expression of the EMT molecules (Fig. 6A). Transcript levels of fibronectin, vimentin, Twist1, and E-cadherin were correlated with their respective protein levels (Supplementary Fig. S4A). Transwell assays were performed to study the cell invasion activity of these prostate cancer cells and the role of galectin-4. 22Rv1-M3 cells exhibited stronger invasion activity than 22Rv1, and galectin-4 deprivation in 22Rv1-M3 cells significantly diminished the cell invasion (Fig. 6B). To reflect the EMT in these cells, E-cadherin expression detected by immunostaining showed a lower level of E-cadherin in 22Rv1-M3 cells than 22Rv1 cells, and the E-cadherin level was increased in 22Rv1-M3 following the knockdown of galectin-4 (Figs. 4D and 6C). The treatment with ectopic galectin-4 protein (recombinant galectin-4 or 22Rv1-M3 conditioned medium) also rescued the EMT phenotype in M3-shGal4 cells, as demonstrated by the RTKs activation and increased Twist1 expression as well as cell invasion activity (Supplementary Fig. S4B and S4C). In addition, carbohydrate dependency in galectin-4–mediated invasion was demonstrated by using lactose competition (Supplementary Fig. S4C). Overexpression of galectin-4 in various prostate cancer cells increased the cell invasion activity, RTK phosphorylation, and EMT markers (Figs. 6D and E and Supplementary Fig. S5A–S5C). The EMT induction by galectin-4 overexpression also enhanced the invasive growth, metastasis, EGFR-HER2 activation, and decreased E-cadherin in 22Rv1 and PC-3 tumors (Fig. 6F and G and Supplementary Fig. S5A–S5F). Interestingly, knocking down C1GALT1 in 22Rv1-M4 cells resulted in loss of the RTK activation by galectin-4 and significantly hampered the cell invasion activity presumably due to the loss of core 1 O-glycans conjugated to RTKs for its binding (Fig. 6H). Of note, depriving C1GALT1 in 22Rv1-derived cells even decreased the protein expression of EGFR and IGF1R, not HER2 or HER3.

Discussion

The aberrant interactions of tumor cell surface glycans-lectin influence the adhesion of cancer cells to the ECM or endothelium, favoring tumor dissemination (16). During malignancy progression, cancer cells prevalently acquire expression of O-glycans that are normally present on embryonic tissues, but not on well-differentiated adult tissues. Cancerous epithelial cells express extraordinary core 1 (T antigen and sialyl-T antigen) or core 2 O-glycans in association with metastasis, whereas normal cells primarily express core 3 or core 4 (5, 17). Indeed, restoring the expression of core 3 synthase in prostate cancer cell lines

![Figure 6](cancerres.aacrjournals.org)
interrupted the integrin α2β1 complex and inhibited invasion activity, tumor growth, and metastasis (18). The expression of core 1- or core 2-derived structures is initiated with C1GALT1 enzyme catalysis by adding a galactose to GalNAc-O-Ser/Thr linkage. In fact, C1GALT1 is involved in carcinogenesis of breast, colon, and hepatocellular cancers, in which C1GALT1 overexpression promotes cancer invasion by activating β1 integrin, FGFR2, and MET signaling (19, 20). In the 22Rv1-derived sublines, the elevated expression of C1GALT1, ST3GAL1, and ST6GALNAC4 along with lack of expression of B3GNT6 (the only core 3 synthase) and GCNT3 indicated that their O-glycans are mainly in core 1 structure, not core 2 or core 3. The disaccharide structure of T antigen conjugated to surface proteins or lipids may serve as binding receptors for galectin-4 to exert a signaling function. As a result, targeted ablation of C1GALT1 disrupted the galectin-4 binding to its cell surface receptors (Fig. 5F). Given the dominant expression of truncated core 1 O-glycans in cancers including metastatic prostate cancer, it is interesting to either inhibit the O-glycan binding to galectins or inhibit the enzymes synthesizing the O-glycans. Likewise, high level of core 1 O-glycan and low level of core 2 O-glycan were shown in malignant prostatic tumor cells and aggressive cell lines, PC-3 and DU145, which provided prostate cancer cell resistance to the proapoptotic effect mediated by the galectin-1–core 2 glycan interaction (21). In metastatic lung cancer, deficiency in core 2 formation due to increase of ST6GALNAC4 and decrease of GCNT3, displayed T antigens on the cell surface, and the disaccharide of T antigen mediated the interaction with galectin-3–carrying myeloid cells to promote metastasis (22). These findings connect the oncocalcit glands with EMT and RTK activation of tumor cells via galectin-4 regulation in metastasis and tumorigenesis.

The circulating galectin-2, -4, and -8 in blood is increased in patients with colon and breast cancers, especially those with metastasis, as compared with healthy individuals, and these extracellular galectins, through binding to T antigen–conjugated MUC1 on the cancer-cell surface enhanced the cancer–endothelium adhesion (23). In this study, we revealed that concomitant changes in O-glycosylation and galectin-4 overexpression in prostate cancer cells enable oncogenic signals to impart cancer metastasis by inducing EMT-like phenotypes. These prostate cancer cell lines expressed low levels or no ligands for EGF, HER3, or IGF1R (Supplementary Fig. S7A). The galectin-4–mediated RTK phosphorylation in the absence of natural ligands favored the model of a direct binding between galectin-4 and the O-glycans conjugated to RTKs. However, the potential involvement of intracellular galectin-4 and MUC1 in the galectin-4–RTK interaction or signal amplification has not been excluded (24).

In addition to overexpression of galectin-4 in metastatic prostate cancer, we further identified that upregulation of C1GALT1-mediated glycan synthesis is required to mediate galectin-4 binding and RTK activation, which suggests that C1GALT1 may be a feasible target for cancer therapy. O-glycosylation in RTKs has been demonstrated, including EGFR and IGF1R (25, 26). The elevated expression of all these RTKs in prostate cancer is correlated with clinical stage and poor prognosis. Unfortunately, latanib treatment was ineffective or exhibited a minor response in both hormone-therapy naïve and castration-resistant patients (27). Our data suggested that narrow breadth design for targeted therapy drugs may be insufficient and defective due to ample RTK activation in metastatic cancer. Likewise, blocking the interaction between inherent ligand and receptors would not target a population of prostate cancer overexpressing galectins as atypical ligands. A recent study proposed that using galectin-1–specific antibody can inhibit the association of galectin-1 with N-glycan on VEGFR thus suppressing the tumor growth in anti-VEGF–refractory tumors (28). Of note, C1GALT1 deficiency seems to down-regulate the protein levels of certain RTKs, such as EGFR and IGF1R. Further study of this effect found that the C1GALT1 knockdown did not regulate the IGF1R mRNA level, suggesting the C1GALT1–mediated IGF1R expression may be through translational or posttranslational control (Supplementary Fig. S7B). Currently the detailed mechanism is unavailable; however, depriving C1GALT1 indeed inhibited the phosphorylation of HER2 and HER3 by either endogenous or ectopic galectin-4 in prostate cancer cells without loss of protein levels in HER2 and HER3 (Fig. 5G and 6H and Supplementary Fig. S3A). Glycan modification of particular proteins can contribute to quality-control in protein-folding, stability and oligomerization (29, 30). For example, core 1 O-glycan–deficient podoplanin, a type 1 transmembrane mucin-type O-glycoprotein, was highly susceptible to proteolytic degradation by various proteases, such as MMP-2/9 (31). Therefore, deglycosylated receptors possibly results in protein instability or degradation.

An earlier study of the galectin profile in a cohort of therapy-naïve prostatectomies indicated that galectin-1 is the most abundant galectin in this setting and is substantially upregulated during progression, while galectin-3, -4, -9, and 12 are downregulated over the course of the disease. However, our survey indeed showed that galectin-4 correlated with clinical tumor progression, poor survival, and cancer recurrence. Whether elevation of galectin-4 expression is regulated by any stress conditions inherent in the tumor microenvironment or induced by castration and other therapies require further studies. To determine the effect of other galectins in our progression model, our further analysis for the expression of galectin-1, galectin-2 (prototype), galectin-3 (chimera), galectin-4, galectin-9, and galectin-12 (tandem-repeat) shows that 22Rv1-M3 cells expressed only galectin-4 and galectin-1 with little or undetectable levels of galectin-2, 3, 9, and 12 (Supplementary Fig. S7C). Although 22Rv1 cells and its derivatives, including galectin-4 knockdown cells, express comparable galectin-1, galectin-4 essentially and sufficiently regulated the RTKs phosphorylation, EMT, and invasion activity as shown by knockdown and overexpression experiments (Figs. 4B–F and 6A–G). In addition, PC-3 and DU145 cells are deficient in core 2 glycan and resistant to galectin-1–induced cell apoptosis (21). Collectively, these data suggest that galectin-4 plays a dominant role in regulating RTKs activity in prostate cancer cells. By acting as an atypical and promiscuous ligand, galectin-4 can mediate the activation of multiple RTKs to promote cancer progression. On the other hand, blockade of the galectin–glycan interaction may interrupt the RTK-mediated tumor metastasis.

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

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References


Correction: Metastatic Progression of Prostate Cancer Is Mediated by Autonomous Binding of Galectin-4-O-Glycan to Cancer Cells

In this article (Cancer Res 2016;76:5756–67), which appeared in the October 1, 2016, issue of Cancer Research (1), there are errors in Figs. 2A and 6E. The authors used one trimmed image/blot to fit the size of the others. In these two panels, they accidentally kept the “ruler image/blot” and deleted the “subject image/blot.” These errors do not affect the conclusions of the article.

The online version of the article has been corrected and no longer matches the print. The authors regret these errors.

Reference

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Metastatic Progression of Prostate Cancer Is Mediated by Autonomous Binding of Galectin-4-O-Glycan to Cancer Cells

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