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14. ABSTRACT

The purpose is to identify the role of mucin-type O-glycan associated glycotopes mediated metastasis of prostate cancer cells. Significant problem in the management of prostate cancer is emergence of hormone independent cancer progression and metastasis to vital organs such as lymph node, lung, bone and brain, finally leads to death. Currently the difficulty in finding solution for prostate cancer metastasis is unavailability of in vitro cell model system which mimics clinical progression of prostate cancer. Altered cell surface glycosylation, ie increased expression of sLex antigen, is characteristics of metastatic cancers, including prostate cancer. The main objective of current application to identify the role of mucin glycan associated epitopes involved in prostate cancer metastasis and role of extended core 1 structure in prostate cancer progression. Dr. Lin's laboratory has established a cell model system using the LNCaP cell line consisting of the parental C-33, the high passage C-81 cells and LNCaP cell derived C4-2B bone metastatic cells. The characteristics of this cell model system closely resemble the clinical progression of prostate cancer.

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MECA-79, L-selectin ligand, glycosyltransferases, sLex

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MUCIN GLYCAN: EXPRESSION AND POTENTIAL ROLE IN PROSTATE CANCER METASTASIS

ABSTRACT :

Purpose: The purpose of the current project is to identify the role of mucin-type O-glycan associated glycotopes mediated metastasis of prostate cancer cells.

Scope: Significant problem in the management of prostate cancer is emergence of hormone independent cancer progression and metastasis to vital organs such as lymph node, lung, bone and brain, finally leads to death. Currently the difficulty in finding solution for prostate cancer metastasis is unavailability of *in vitro* cell model system which mimics clinical progression of prostate cancer. Altered cell surface glycosylation, such as increased expression of sLe^x antigen, is characteristics of metastatic cancers, including prostate cancer. The main objective of current application to identify the role of mucin glycan associated epitopes involved in prostate cancer metastasis and role of extended core 1 structure in prostate cancer progression. Dr. Ming-Fong Lin's laboratory has established a cell model system using the LNCaP cell line consisting of the parental C-33, the high passage C-81 cells and LNCaP cell derived C4-2B bone metastatic cells. The characteristics of this cell model system closely resemble the clinical progression of prostate cancer.

Major findings: In the first year of DOD funding, low and high passages of LNCaP cells, mimic the clinical conditions of early and late-stage human prostate cancer, were employed for characterization of L-selectin ligand (glycotopes) and *in vitro* tumorigenicity. C-81 cells exhibit higher *in vitro* migratory and invasive properties as compared with C-33 cells. The L-selectin ligand and mucin glycan-associated MECA-79 epitope were elevated in C-81 cells. An increase of these glycotopes positively correlates with elevated tumorigenicity and expression of key glycosyl- and sulfotransferase genes. These results suggest modulated expression of selective glycogenes correlates with altered tumorigenicity of cancer cells. We also knock down the core 1 β3galT-1 enzyme.

Task 1: We observed C-81 cells showed a greater degree of *in vitro* migratory and invasive properties than the C-33 cells. Further, C-81 cells exhibit elevated expression of L-selectin ligand (MECA-79 epitope), and increased expression of key glycogenes involved in the synthesis of this ligand. These glycogenes and mucins were upregulated treatment with proinflammatory cytokines. Thus, modulation of selective glycogenes in cancer cells could modify the selectin ligands to alter the metastatic potential of these cells. This property resembles the known tumorigenic trend of these two cell clones.

Significance: The results obtained during the first 10 months (03/01/08-12/30/08) of DOD funding are significant towards identification of mucin glycan-associated glycotopes in the metastasis of Prostate cancer cells.

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1 INTRODUCTION:

Prostate cancer is the most common cancer in men in the United States and Western Europe, and the second most common cause of cancer-related death in men over age 55 (1). Progression of prostate cancer from hormone-dependent to hormone-independent stage renders these patients incurable. Current treatments, while effective in suppressing tumor growth and relieving tumor associated pain, provide only a short term relief. Eventually, mortality of these patients is resulted from metastasis of tumors to distant sites, including lymph nodes, lungs, bone and brain (2). However, the mechanism of prostate cancer metastasis is currently not known. Altered cell surface glycosylation, such as increased expression of sLe^x antigen, is characteristics of metastatic cancers, including prostate cancer (3-5). The goal of this application is to identify mucin glycans involved in prostate cancer metastasis.

To identify the role of mucin glycan-associated glycotopes in the metastasis of prostate cancer cells, we have utilized the prostate cancer (LNCaP) model system developed by Dr. Ming Fong Lin laboratory. LNCaP cells of low-passage (C-33) and high-passage (C- 81) cells were used in this study. The properties exhibited by these two cell clones mimic the early and the late stages of human prostate cancer in the clinic. Thus, this is a useful *in vitro* cell model for studying the mechanisms of clinical progression of prostate cancer.

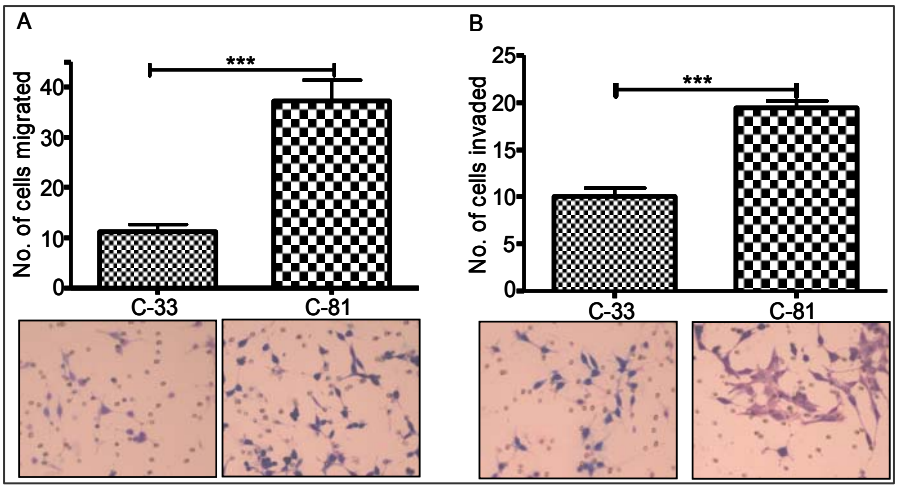
Cancer metastasis is associated with expression of altered glycoconjugates in both structure and quantity. Most tumor antigens are associated with mucins. In prostatic carcinoma, nearly total absence of normally occurring ABO and type 1-based Lewis antigens, Le^a and Le^b, and over expression of sialylated and fucosylated epitope such as sialyl $Lewis^x$ (sLe^x) on mucin glycans has been reported. These epitopes serve as the ligands for carbohydrate-binding, calcium-dependent lectins/selectins. These selectin-ligand interactions are thought to play significant roles in blood-borne metastasis of cancer cells. Recent studies show that introduction of core 2 branching enzyme (Core 2 β6 N-acetylglucosaminyltransferase, C2GnT) to prostate cancer cells enhanced their attachment and metastasis to other parts of the body in an *in vivo* animal model system (6). Bone marrow epithelial cells (BMEC) containing E-selectin helps initiate adhesion of prostate tumor cells, which express E-selectin ligand-1 (ESL-1) bearing sLe^x epitope. Therefore, expression of sLe^x antigen is associated with bone metastasis of prostate cancer (7). One goal of this application is to test sLe^x as a progression and metastatic biomarker of LNCaP cells. This hypothesis is well supported by the clinical observation that overexpression of sLe^x was associated with hormone-resistant, highly metastatic prostate cancer (5). The results of this study could develop specific inhibitors to block the expression of specific glycosyltransferase involved in production of sLe^x on mucin glycans and thereby prevent prostate cancer growth and metastasis.

2 BODY:

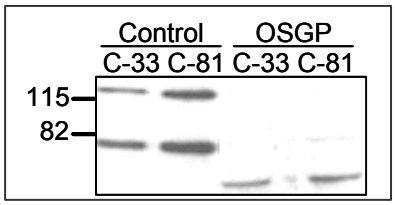
2.1 *Task 1:* To identify the involvement of mucin glycan-associated glycotopes in the metastasis of prostate cancer cells.

Cell-cell interactions involving selectins and their carbohydrate ligands present on opposite cells play a key role in a variety of biological events, such as trafficking and homing of lymphocytes to specific tissues or lymphoid organs in the body [13]. For example, the L-selectin ligand in the high endothelial venues of the lymphoid organs is responsible for the arrest of the L-selectin-carrying leukocytes that traffick through these organs. Current study demonstrates that LNCaP cells express L-selectin ligand, which may facilitate metastasis of these cells to the regional lymph nodes by hitching on the circulating leukocytes. This finding is consistent with the fact that these cells are originally derived from the lymph nodes of a patient with metastatic prostate cancer [14].

a. Elevated *in vitro* migration and invasion properties in C-81 LNCaP cells. The metastatic potential of human prostate cancer cells was analyzed by *in vitro* Matrigel Boyden chamber assay. As compared to C-33 LNCaP cells, the C-81 cells showed a significantly higher level of motility through polyethylene terapthalate (Fig. 1A) and a higher degree of invasion through matrigel (Fig. 1B).

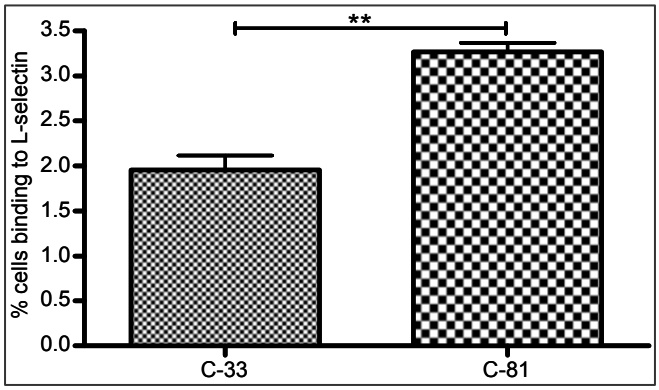


b. MECA-79 epitope is located in mucin-type glycans. The MECA-79 epitope was detected in two protein bands at 77 and 131 kDa from lysates of both C-33 and C-81 cells. The high passage LNCaP cells showed greater intensity (2.6 fold of 131 kDa and 1.8 fold of 77 kDa) than low passage LNCaP cells. The biochemical properties of MECA-79 epitopes present in C-33 and C-81 cells were characterized by digestion of cell lysates with N-glycanase, KS-II, Ch-ABC and OSGP. The MECA-79 epitope was resistant to N-glycanase, KS-II and Ch-ABC (Data not shown). However, the two protein bands carrying MECA-79 epitope



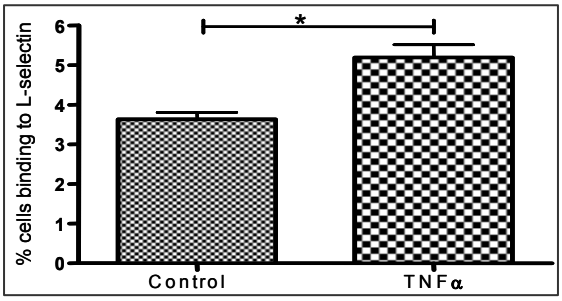
were digested by OSGP, which specifically cleaves the peptide backbone of glycoproteins carrying sialylated mucin type glycans (Fig. 2).

c. As per approved statement of work (SOW), we have identified *in vitro* binding of L-selectin to low and high passage LNCaP cells with different affinity, the C-81 clone showed higher binding (70%) than C-33 cells (Fig 3).

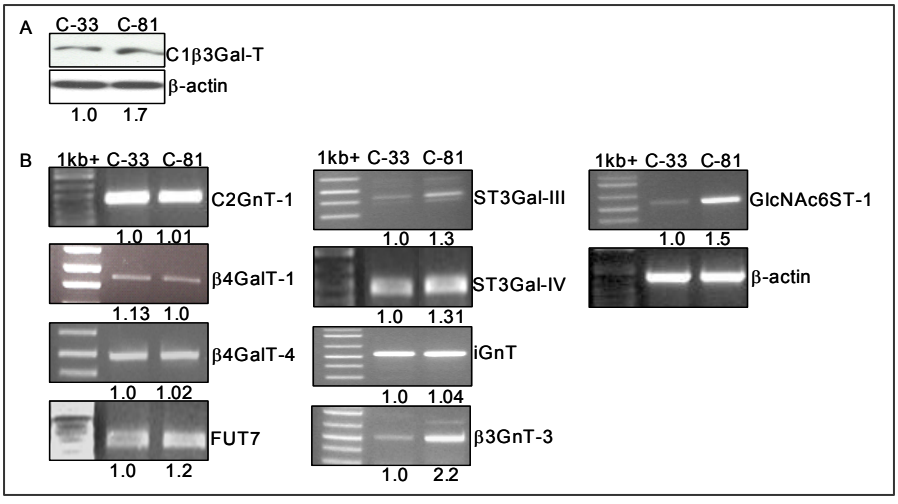


The C-33 and C-81 LNCaP cell model has been shown to mimic the clinical progression of prostate cancer metastasis [10, 11] and has been widely used for studying the mechanisms of prostate cancer progression [15-19]. We find that C-81 cells express more L-selectin ligand and exhibit higher levels of *in vitro* migratory and invasive properties as compared with C-33 cells. The results indicate that increased expression of L-selectin ligand correlates with elevated metastatic potential of LNCaP cells.

d. Treatment of C-81 LNCaP cells with TNFα (20ng/ml) for 36hr showed 1.4 fold higher binding of L-selectins than untreated control cells (Fig 4).



e. Analysis of glycogenes involved in the synthesis of L-selectin ligand in LNCaP cells. To understand the difference in metastatic potential as related to L-selectin ligand expression between C-33 and C-81 LNCaP cells, a combination of western blotting and RT-PCR analysis of key glycogenes involved in the synthesis of L-selectin ligand was performed. Western blot analysis showed a 70% increase of the C1β3GalT-1 enzyme (core 1 synthase)

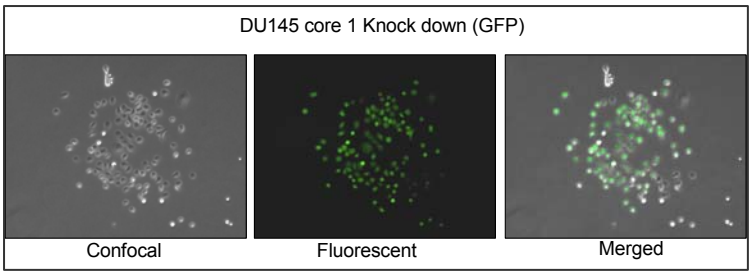


in C-81 cells as compared to C-33 cells (Fig. 5A). RT-PCR analysis of the expression of glycosyltransferase and sulfotransferase genes involved in L-selectin ligand synthesis showed a significantly higher expression of the following glycogenes in C-81 LNCaP cells as compared to those in C-33 cells: β3GnT-3 (120%), FUT7 (20%), GlcNAc6ST-1 (50%), ST3Gal-III (30%) and ST3Gal-IV (30%) (Fig.5B). There was no difference in the expression of C2GnT-1, β4GalT-1, β4GalT-4 and iGnT genes between these two LNCaP cells although expression levels were relatively high in both cells

6-sulfo-sLe^x is the L-selectin ligand, which also can be recognized by MECA-79 antibody. This antibody is specific for mucin core 1-associated 6-sulfo-N-acetyllactosamine [Galβ1-4(6-sulfo)GlcNAcβ1-3Galβ1-3GalNAcα] [20] with or without further modifications, which include α2-3 sialylation of Gal, α1-3 fucosylation of GlcNAc, and/or C6 sulfation of Gal [8, 9]. MECA-79 specifically binds to luminal surface of HEV and inhibits binding of lymphocyte to HEVs *in vivo* and *in vitro*. MECA-79 epitope can be found in mucin-type [12] and N-linked [21] glycans. But, they are present only in mucin-type glycans in LNCaP cells (Fig. 2).

Mucin glycan-associated 6-sulfo-sLe^x can be found on extended core 1 and core 2 branch as shown in Figure 5. Synthesis of this epitope involves several glycosyl- and sulfo-transferases working in a sequential ordered manner [20]. Mucin glycan synthesis is initiated by the formation of GalNAcαSer/Thr as catalyzed by peptidyl GalNAc transferases, which is followed by the formation of core 1 [Galβ 1-3GalNAc] as catalyzed by core 1 synthase (C1 β3GalT-1). After the formation of core 2 [GlcNAcβ6(Galβ1-3)GalNAcα1R] and/or extended core 1 [GlcNAcβ1-3Galβ1-3GalNAcα1R], GlcNAc 6-sulfate is generated before β1-4 galactosylation of GlcNAc. This enzymatic step is followed by sialylation and then fucosylation to complete the synthesis of the L-selectin ligand. L-selectin ligand can be located at core 1 and core 2, but only core 1-associated L-selectin ligand can be recognized by MECA-79 antibody [20]. The increase in MECA-79 epitope and L-selectin ligand in C-81 LNCaP cells as compared to C-33 cells can be explained by upregulation of the expression of several glycogenes involved in the synthesis of these glycotopes. C-81 cells express 70% more C1 β3GalT-1 than C-33 cells. Since core 1 is the obligatory precursor for core 2 [22], more core 1 can lead to the synthesis of more core 2 branch. In addition, C81 cells express 120% more core 1 extension enzyme (β3GnT-3), which can lead to production of more extended core 1 structure. Furthermore, C-81 cells express more sulfotransferase (GlcNAc6ST-1), sialyltransferases (ST3Gal-III and -IV) and fucosyltransferase (FUT7). The expression pattern of these glycosyltransferases positively correlated with higher level expression of MECA-79 epitope and L-selectin ligand on C-81 cells than in C-33 cells. Furthermore, it is of interest to note that alteration of the expression of selective glycosyltransferase and sulfotransferase genes is correlated with the metastatic potential of LNCaP cells, suggesting the important role played by these glycogenes in carcinogenesis.

f. We have knock down the expression of core 1β3Gal-T enzyme from highly metastatic prostate cancer cells, DU145 and C-81 cells. The core 1β3Gal-T siRNA transfected cell contains both mixtures of transfected and untransfected cell populations (Fig 6). The Knock down (GFP expressing) cells can be easily isolated from non-transfected cells by FACS.



g. We are in processing of isolating green fluorescent protein expressing core 1β3Gal-T knock downed (siRNA transfected) cells only by FACS system, then expand these single cell population into mass volume for studying its effect on metastatic potential in an animal model system. Further, we will analyse tumor cell metastasis by luciferase and immunohistochemical assays.

2.2 ***Task 2:*** **Role of core 1 extension glycosyltransferase (β3GnT-3) in prostate cancer progression and metastasis.**

2.3 ***Task 3:*** **To validate the results obtained in LNCaP cell model system in human prostate cancer tissue samples**.

As per statement of work, works mentioned in task 2 and 3, we have initiated all the preliminary work necessary for our proposed work in task 2 and 3.

3 KEY RESEARCH ACCOMPLISHMENTS:

a. In the first year of funding, we have observed C-81 cells showed a greater degree of *in vitro* migratory and invasive properties than the C-33 cells. Further, C-81 cells exhibit elevated expression of L-selectin ligand (MECA-79 epitope), and increased expression of key glycogenes involved in the synthesis of this ligand. These glycogenes and mucins were upregulated treatment with proinflammatory cytokines. Thus, modulation of selective glycogenes in cancer cells could modify the selectin ligands to alter the metastatic potential of these cells. This property resembles the known tumorigenic trend of these two cell clones.

b. we **presented a poster** on the role of mucin-glycans on prostate cancer metastasis at the AACR annual meeting 2008, held at San Diego, CA (Appendix 1). In this meeting we have presented role of glycoslytransferases which regulating the expression of mucin-glycan synthesis under normal and proinflammatory cytokine treatment conditions and metastatic potential of prostate cancer.

c. We have also **published a paper** (Appendix 2) on the role of L-selectin ligand on LNCaP cells *in vitro* migration and invasion. In this manuscript, we communicated the data on the mucin glycan-associated glycotopes in the metastasis of prostate cancer cells (Task 1).

Our project experiments are being conducted as per the approved SOW and in the speculated time-periods as mentioned in the SOW.

4 REPORTABLE OUTCOMES:

a. Presented a poster in AACR Annual meeting, 2008, held at San Diego, CA (Appendix 1).

b. Published a paper on Elevated expression of L-selectin ligand in lymph node-derived human prostate cancer cells correlates with increased tumorigenicity (Appendix 2).

5 CONCLUSIONS:

The experimental data so far obtained during the first 10 months of the DOD project address the key problem in the prostate cancer metastasis, i.e., to identify the role involvement of mucin-glycans in hormone-refractory prostate cancer metastasis.

I am greatly thankful to DOD funding resources and extremely sorry for not able to complete the proposed work. I have resigned my position from Dr. Cheng's lab on 31st December 2008, after that I am unable to continue this project further. I also requested my mentor, to take this project with me in my future lab to complete the proposed work. I am not allowed to take with me this project; beyond this I am unable to do anything further. I would like to thank the DOD once again for giving me a wonderful opportunity to carryout my prostate cancer research work.

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Appendix – 1

AACR Poster Abstract:

TNFα enhancement of Glycosyltransferases involved in the biosynthesis of sialyl Lewis X (sLe^x), and metastatic potential of human prostate cancer cells

Prakash Radhakrishnan[1*], Ming-Fong Lin[1,2], Rakesh Singh[1,2,3] and Pi-Wan Cheng[1,2]

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The objective of current study was to examine the effects of pro-inflammatory cytokines on the expression of glycosyltransferases and sulfotransferases responsible for the synthesis of the carbohydrate epitopes involved in the metastasis of prostate cancer cells. Androgen-independent high-passage human lymph node-derived metastatic prostate cancer cells (C-81 LNCaP), which expressed functional androgen receptor and mimicked the high metastatic potential of advanced prostate cancer, were used. These cells exhibited migratory and invasive property reflective of high metastatic potential under *in vitro* Boyden chamber assay, and metastasis in a *s.c.* tumor xenograft model. These cells bind to P-, L-, and E-selectins, which recognize sLe^x-containing glycans. Such interactions can facilitate cancer metastasis by promoting binding of cancer cells to platelets and inflamed endothelial cells at distant sites, leukocytes and high endothelial venue in lymphoid tissues, and inflamed endothelial cells at distant sites, respectively. Binding of these cells to P- and E-selectins, and anti-sLe^x antibody was increased by 12, 240, and 248% following treatment with a pro-inflammatory cytokine (TNFα), respectively. Treatment of TNFα-treated cells with either anti-sLe^x antibody or E-selectin significantly suppresses migration (81 and 52%, respectively) and invasion (45 and 56%, respectively), suggesting involvement of sLe^x in these processes. RT-PCR analysis of glycosyltransferase and sulfotransferase genes revealed increased expression of core 2 N-acetylglucosaminyltransferase 2/M (C2GnT-2/M) (60%), fucosyltransferase-VII (FUT7) (50%), α2-3sialyltransferase (ST3Gal-III) (20%), and sulfotransferase (GlcNAc6ST-1)(60%), but not FUT4, ST3Gal-VI, C2GnT-1/L, and GlcNAc6ST-3 in TNFα-treated cells. We conclude that TNFα treatment stimulates the expression of selective glycosyltransferase and sulfotransferase genes in metastatic human prostate cancer cells to generate more sLe^x, which leads to increased binding of these cells to selectins and thus promotes the metastatic potential. These results could explain how inflammation potentiates the metastatic potential of advanced prostate cancer and support the well-accepted hypothesis that inflammation contributes to cancer metastasis. (Supported by NRI-Cancer Glycobiology Program, Eppley Cancer Center pilot project, and DOD Post-Doctoral fellowship)

Key words: LNCaP cells, sLe^x, TNFα, Lymph node, Metastasis

Appendix – 2

Manuscript Abstract: Glycoconj J. 2009 Jan;26(1):75-81. Epub 2008 Aug 1.

Elevated expression of L-selectin ligand in lymph node-derived human prostate cancer cells correlates with increased tumorigenicity

Prakash Radhakrishnan · Ming-Fong Lin · Pi-Wan Cheng

Abstract: Human prostate cancer LNCaP cells including C-33 and C-81 cells were originally derived from the lymph nodes of a patient with metastatic prostate cancer. These two cells, mimic the clinical conditions of early and late-stage human prostate cancer, were employed for characterization of L-selectin ligand and *in vitro* tumorigenicity. C-81 cells exhibit higher *in vitro* migratory and invasive properties as compared with C-33 cells. The L-selectin ligand and mucin glycan-associated MECA-79 epitope were elevated in C-81 cells. An increase of these glycotopes positively correlates with elevated tumorigenicity and expression of key glycosyl- and sulfotransferase genes. These results suggest modulated expression of selective glycogenes correlates with altered tumorigenicity of cancer cells.

Key words: LNCaP cells · MECA-79 · L-selectin ligand · glycosyltransferase