ROLE OF BETA 1,6 BRANCHED N-LINKED OLIGOSACCHARIDES IN REGULATING KEY CELLULAR PROCESSES INVOLVED IN CANCER CELL INVASION

By

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As members of the Viva Voce Board, we recommend that the dissertation prepared by Amit Ranjan titled 'Role of beta1,6 branched N-linked oligosaccharides in regulating key cellular processes involved in cancer cell invasion' be accepted as fulfilling the requirements for the Degree of Doctor of Philosophy.

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DECLARATION

I declare that the thesis titled 'Role of β1,6 branched N-linked oligosaccharides in regulating key cellular processes involved in cancer cell invasion' is a record of the work carried out by me during the period September 2006 to July 2013 under the supervision of Dr. Rajiv D. Kalraiya. This work is original and it has not been submitted earlier as a whole or in part for a degree/diploma at this or any other institute or university.

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CERTIFICATE

I certify that the thesis titled 'Role of β1,6 branched N-linked oligosaccharides in regulating key cellular processes involved in cancer cell invasion' submitted for the degree of Doctor of Philosophy by Amit Ranjan is a record of the research carried out by him during the period September 2006 to July 2013 under my supervision. This work has not formed the basis for the award of any degree, diploma, associate ship or fellowship at this or any other institute or university.

Navi Mumbai,

March 2014

Dr. Rajiv D. Kalraiya

I DEDICATE THIS THESIS TO MY LATE GRANDMOTHER,
A CANCER VICTIM, WHO INSPIRED ME TO WORK IN THE FIELD OF CANCER BIOLOGY

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CONTENTS

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SYNOPSIS

Introduction:

Cancer is the second major cause of mortality worldwide including, India. The development of cancer involves complex, dynamic changes in the genome that initially leads to the generation of pre-neoplastic lesions and eventually gives rise to clonal variants that proliferate into tumors, usually over many decades. Estimates predict that during the process of tumor genesis, cells must acquire a minimum of six mutations to become malignant. These mutations are included in those genes that make malignant cells self-sufficient in growth signals, insensitive to growth – inhibitory signals, to replicate extensively, avoid apoptosis or programmed cell death, have sustained angiogenesis and competent to invade and produce distant metastasis, which form the hallmarks of cancer. Recently, two additional hallmarks; evasion of immune response and reprogramming of energy metabolism have been included to pre-existing lists [1]. Metastasis, a multistep, complex process is the major cause of cancer related mortality [2]. Invasion is the key event involved in majority of the steps of the metastatic cascade. Thus, cancer cell invasion is the hallmark of metastasis. Invasion involves modulation of adhesion, controlled degradation of matrix for generating space for cellular movement which utilizes matrix as the traction and generated ECM fragments as chemoattractants [3]. Cell surface molecules play a major role in negotiating most steps in invasion and metastasis. Tumor cells show several surface modifications associated with metastatic and invasive phenotype. One such consistently observed cell surface modification is the increased expression of β1,6 branched N-linked oligosaccharides. The formation of such oligosaccharides is catalysed by the Golgi resident enzyme N–acetylglucosaminyl transferase V (GnT-V) [4]. Expression of such oligosaccharides correlates positively with invasive phenotype. These are expressed not

only on invasive cancer cells [5-8] but also on the normal cells involved in invasive function [9, 10].

Previous work from our laboratory has investigated the role of these oligosaccharides in regulating processes critical for cancer cell invasion by comparing invasive variant B16BL6 with its parent cell line B16F10 which differ in the expression of these oligosaccharides and by using N-glycosylation inhibitor Swainsonine (SW) or by using antisense to the enzyme GnT-V. By all these approaches it was shown that expression of these oligosaccharides regulates adhesion positively [11]. This contradicted several other reports where their expression although correlated positively with invasiveness, showed negative correlation with cellular adhesion [12, 13]. The expression of these oligosaccharides had no effect on basal secretion of Matrix metalloproteinases (MMPs). Their expression appeared to positively regulate chemotaxis (motility in response to soluble chemoattractants like components of Extra Cellular Matrix [ECM] and Basement Membrane [BM]) however; it regulates haptotaxis (motility in response to substratum bound chemoattractants) in a complex manner. Expression of such oligosaccharides promotes haptotaxis on ECM component (fibronectin) whereas their expression inhibits haptotaxis on reconstituted BM (matrigel) [11].

Above studies raise several key questions,

- How do these β1,6 branched oligosaccharides regulate adhesion both positively and negatively? Is it because of differential substitutions of terminal sugars on the oligosaccharides?

- Degradation of matrix is very crucial for invasion. The expression of oligosaccharides has no effect on MMP secretion, then how do they regulate matrix degradation?
- How do these oligosaccharides differentially regulate motility on ECM and BM components?

Objectives:

- To investigate the role of terminal substitutions on β1,6 branched N-linked oligosaccharides in regulating cellular adhesion and thus invasion.
- To investigate the role of β1,6 branched N-linked oligosaccharides in regulating matrix degradation.
- To investigate the role of these oligosaccharides in regulating the motility of cells on ECM and basement membrane components.

Methodology:

Cell culture

Melanoma cells were routinely cultured in Minimum Essential Medium (MEM) containing 5% fetal bovine serum supplemented with vitamins, non-essential amino acids, sodium pyruvate, L-glutamine and antibiotics at 37°C in CO_2 incubator. Glycosylation inhibitor (SW) (2 µg/ml) was added for 48 h at 25% confluency for the inhibition of formation of β1,6 branched N-linked oligosaccharides.

Preparation of total cell lysate and Western blotting

Total cell lysates was prepared by lysing the melanoma cells in 20 mM Tris Chloride buffer containing 1% NP-40, 0.5% Sodium deoxycholate, 150 mM Sodium Chloride and 1mM each of Magnesium Chloride and Calcium Chloride and protease inhibitor cocktail followed by sonication and centrifugation. Proteins were resolved on 10 % SDS PAGE and blotted on PVDF membrane. Blots were probed either with biotinylated lectins (L-PHA for β1,6 branched N-oligosaccharides, SNA for α2,6 linked sialic acids, MAL-II for α2,3 linked sialic acids, LEA for polylacNAc and AAL for fucose) or antibodies against integrins (β1, α3, α5), tetraspanins (CD82, CD151), proteases or their receptor (MMP-9, MT1-MMP, uPAR), tagged proteins (GFP, FLAG) and for loading control (β-actin) were used.

Purification of β1,6 branched –N oligosaccharides using lectin L-PHA precipitation

B16F10 and B16BL6 cells were lysed in 10 mM Tris chloride buffer containing 30 mM N-octyl β-D-glucopyranoside, 3 mM protamine sulphate and protease inhibitor cocktail. Cell lysates were incubated with L-PHA agarose beads overnight at 4°C. Beads were washed and eluted with 1X non reducing sample buffer containing 4M urea.

Flow cytometric analysis

Paraformaldehyde fixed melanoma cells were incubated with biotinylated lectins (L-PHA, SNA, MAL-II, LEA and AAL) followed by Extravidin FITC. Surface expression of membrane proteins were studied by incubating melanoma cells with antibodies against hyaluronan receptor CD44, integrins (β1, α3, α5, α6), tetraspanins (CD82, CD151), proteases (MT1-MMP, uPAR) followed by their respective FITC tagged secondary antibodies. Cells treated with Extravidin FITC or FITC tagged secondary antibody alone

served as control. Fluorescent cells were acquired by BD FACSCalibur at 488 nm and analysed by cell quest software.

Gelatin zymography

B16BL6 and the same cells treated either with Swainsonine or transduced with non-targeting shRNA or shRNA to GnT-V were grown on uncoated (plastic), fibronectin and matrigel coated culture dishes under serum free conditions. The medium collected after 24 h (conditioned medium) were concentrated and it was loaded on 10% SDS-PAGE resolving gel containing 0.1% Gelatin under non-reducing conditions at 4°C. The gel was renatured by soaking the gel in 2.5% TritonX-100, washed and incubated for 36 h in Tris buffer (pH7.5) containing 50 mM $CaCl_2$ at 37°C. Gels were stained with 0.2% Coomassie brilliant blue and destained to visualize the zone of lysis.

Cell spreading assays

Melanoma cells were seeded on fibronectin and matrigel coated and BSA blocked coverslips, in serum free medium for 45 min at 37°C in a CO_2 incubator. Coverslips only blocked with BSA served as control. After incubation cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% TritonX-100. Phalloidin FITC or Phalloidin TRITC was used for F-actin staining and DAPI was used for nuclear staining. Coverslips were mounted on glass slides using vectashield and observed under confocal microscope. Cell spreading was quantitated by calculating cytoplasmic to nuclear area ratio.

Colocalization by indirect immunostaining

Melanoma cells were seeded on coverslip and grown overnight in complete medium up to 70-80% confluency. Cells were washed with PBS (pH7.5) and fixed with 2% paraformaldehyde. Cells blocked with 3% BSA in PBS and incubated with primary antibody for 1h in a humidified chamber followed by three washes with PBS to remove excess or non-specifically bound antibody. Cells were further incubated with fluorescent tagged secondary antibody for 1h followed by three washes. Cell incubated only with fluorescent tagged secondary antibody served as iso-control. Nuclei were stained with DAPI and coverslips were mounted on slides using vectashield. Images were acquired using a Carl Ziess Laser confocal microscope.

Adhesion Assays

Adhesion assays of tritium labeled melanoma cells were performed on 96 well plate coated overnight at 4°C with fibronectin (representative of ECM component) and matrigel (representative of BM components) at a concentration of 10 µg/ml. The number of bound B16BL6 or untreated cells on each substrate was taken as 100%.

Haptotaxis assays

Haptotaxis assays were performed using 24-well transwell units (Boyden chambers) with 8-µm pore size polycarbonate filter coated with either fibronectin or matrigel (each 10 µg/ml) on the lower surface, in triplicates. Briefly, 0.2 x 10^6 tritium labeled cells suspended in 300 µl MEM were added to the upper compartment of the Boyden chambers, and 600 µl of plain medium were added to the lower compartment. Cells were allowed to migrate for 6 h at 37°C in a humidified atmosphere containing 5% CO_2.

Invasion assays

Invasion assays were performed using Modified Boyden chambers with a layer of matrigel (coated using 30 µl of 1mg/ml matrigel/insert). Briefly, 0.2 x 10^6 tritium labeled cells suspended in 300 µl MEM were added to the upper compartment of the Boyden chamber, and 600 µl of conditioned medium were added to the lower compartment. Cells were allowed to invade for 36 h at 37°C in a humidified atmosphere containing 5% CO_2.

Wound healing assays

Melanoma cells were cultured on 6 well plates precoated with fibronectin and matrigel, blocked with BSA and grown for 24 h in MEM. Cells were washed and serum starved for 24 h. A uniform straight wound was made using a 2 µl tip. Cells were washed to remove unbound cells and maintained in serum free MEM and the wound closure was measured under time lapse inverted microscope for 24 h at 37°C and 5% CO_2.

Reverse transcription and semi-quantitative–PCR

Total RNA was isolated from melanoma cell lines using Trizol reagent. The first strand cDNA was synthesized by Protoscript First cDNA synthesis kit using oligo (dT) primers and M-MuLV reverse transcriptase. Transcript level for GnT-V, tetraspanins CD9, CD63, CD81, CD82 and CD151 were compared in melanoma invasive variants using their specific primers. Transcript levels of GAPDH served as loading control.

Cloning of GFP tagged and FLAG tagged CD151

Murine CD151 gene was PCR amplified from total cDNA and it was cloned into pJET1.2 cloning vector (Fermentas), from which it was sub cloned into pEGFP-N1 vector (Clontech) between HindIII and KpnI restriction sites. Sequence of pEGFP–CD151

construct was verified by sequencing. To clone CD151 in FLAG tagged vector, CD151 gene from pEGFP-CD151 clone was excised by HindIII and KpnI digestion and cloned into C-terminal p3X FLAG-CMV vector.

Transfection & selection of clones

Culture dishes (35 mm) were seeded with exponentially growing B16BL6 cells and transfected with CD151-GFP or CD151-FLAG construct using lipofectamine 2000. Cells were put under Neomycin (G418) selection at 1300 µg/ml 48 h post transfection. Medium was replaced with fresh medium every 3 days, till transfected cells formed isolated colonies. These colonies were picked up by trypsin digestion and cultured in 24 well plates and maintained as separate stocks. Later, these clones were checked for expression of transgene and maintained at a G418 concentration of 1000 µg/ml.

Designing and cloning of short hairpin RNA (shRNA) constructs for downregulating ST6Gal-I and GnT-V enzyme on melanoma cells

Downregulation of α2,6 linked sialic acids and β1,6 branched N-linked oligosaccharides in B16BL6 cells was carried out by shRNA against enzyme ST6Gal-1 and GnT-V, respectively. For the same, shRNA was designed against both the enzymes as per guidelines outlined by [14]. ShRNA cassettes were PCR amplified using specific primers. PCR products were digested with XhoI and EcoRI. Digested products were gel purified and ligated into XhoI and EcoRI linearized inducible lentiviral vector pTRIPz.

Preparation of lentiviral particle and Transduction

For the generation of lentivirus particle shRNA constructs in inducible vector pTRIPz along with helper constructs psPAX2 and pMD2.G were co-transfected into HEK293FT cells using Calcium Phosphate method of transfection. Viral supernatant were collected 24 h post changing of transfection medium and spun at 5000 rpm for 20 min at 4°C.

B16BL6 cells were transduced at 50% confluency in a 35 mm plate with 1ml of viral supernatants using polybrene 8 µg/ml. Transduced cells were then placed under puromycin selection at a concentration of 1 µg/ml. Stably transduced cell lines were maintained at puromycin concentration of 0.75 µg/ml. Expression of shRNA was induced by the addition of doxycycline at a concentration of 4 µg/ml for 96 h.

Purification of FLAG tagged CD151 using anti-FLAG M2 affinity gel

B16BL6 cells expressing FLAG tagged CD151 (CD151-FLAG) were treated with SW for 48 h. Swainsonine treated and untreated cells were homogenized in the lysis buffer containing 20 mM Tris HCl pH 7.4, 100 mM NaCl, 4 mM EDTA, 1% NP-40 detergent, and cocktail of protease inhibitors using Down's homogenizer. Cell lysates was incubated overnight with 100µl anti-FLAG M2 affinity gel (50% suspension) at 4°C. Bound proteins were eluted using 100 µg/ml of 3x FLAG peptides.

Results:

Objective I: To investigate the role of terminal substitutions on β1,6 branched N-linked oligosaccharides in regulating cellular adhesion and thus invasion.

The expression of β1,6 branched N-oligosaccharides was reconfirmed in the parent cell line B16F10 and its invasive variant B16BL6 cells. Increased expression of such

oligosaccharides positively correlates with their invasive phenotype as inhibition of their expression in B16BL6 cells by α-mannosidase II inhibitor, SW, inhibited the invasive ability of these cells. To investigate if the terminal substitutions on β1,6 branched N-oligosaccharides regulate adhesion both positively as well as negatively, they were analysed as follows.

Analysis of terminal substitutions associated with increased β1,6 branched N-linked oligosaccharides on melanoma invasive variants

Beta1,6 branched N-linked oligosaccharides have been shown to be the preferred site for addition of various terminal sugars. Substitution of probable terminal sugars on such oligosaccharides were compared between murine melanoma invasive variants by Western blotting and flow cytometry. Terminal sugars could be sialic acids (SA), poly-N-acetyllactosamine (polylacNAc), or fucose. Results showed that sialic acids in α2,6 linkage and polylacNAc were the major substitution that were associated with increased β1,6 branching and the invasive phenotype.

Confirmation that proteins carrying β1,6 branched N-linked oligosaccharides are substituted with α2,6 linked sialic acid and polylacNAc

Sialic acid and polylacNAc are present on both N-linked and O-linked oligosaccharides. Their increased substitution on β1,6 branched N-oligosaccharides was further confirmed by purifying the proteins carrying β1,6 branched oligosaccharides using the lectin L-PHA precipitation from both B16F10 and B16BL6 cells. Results showed that proteins carrying β1,6 branched N-oligosaccharides from B16BL6 cells indeed have increased substitution of α2,6 linked SA and polylacNAc as compared to B16F10 cells.

Removal of α2,6 linked SA by sialidase treatment or inhibition of enzyme ST6Gal-I by shRNA decreases adhesion

Desialylation of B16BL6 cells either by sialidases that specifically cleave either α2,3 or both α2,3 and α2,6 linked sialic acids, showed that removal of both α2,3 and α2,6 linked SA decreases adhesion to ECM and BM components. However, removal of only α2,3 linked SA does not have much effect on adhesion. This was further confirmed by assessing adhesion, after shRNA mediated down regulation of ST6Gal-I (enzyme that catalyzes addition of α2,6 linked sialic acids on N-linked oligosaccharides) in B16BL6 cells.

Down regulation of ST6Gal-I decreases the invasive ability of B16BL6 cells

Increased expression of α 2,6 linked SA has been observed to mediate adhesion of B16BL6 cells. The effect of increased adhesion mediated by α2,6 linked SA in regulating invasion was studied using ST6Gal-I down regulated B16BL6 cells. Down regulation of α2,6 linked SA decreases the ability of B16BL6 cells to invade through reconstituted BM (matrigel).

Objective II: To investigate the role of β1,6 branched N-linked oligosaccharides in regulating matrix degradation.

Matrix degradation is very crucial for invasion and Matrix metalloproteinase (MMPs) are the major contributors of matrix degradation. However, the expression of these oligosaccharides does not have any effect on basal secretion of MMPs. Does increased adhesion mediated by these oligosaccharides have any role in MMP secretion?

Increased adhesion mediated by β1,6 branched N-linked oligosaccharides induces MMP-9 secretion as seen by Gelatin zymography and MMP-9 blotting

Expression of β1,6 branched N-oligosaccharides have been shown to regulate adhesion to both ECM and BM components. Increased adhesion mediated by these oligosaccharides induces the secretion of MMP-9 by the B16BL6 cells when these cells are grown on fibronectin and matrigel coated culture dishes as compared to uncoated (plastic) dishes. However, inhibition of expression of these oligosaccharides either by SW or shRNA to enzyme GnT-V decreases the adhesion and thus inhibited the induction of MMP-9 secretion. In order to facilitate invasion, tumor cells also regulate matrix degradation by regulating the expression and localization of proteases with motility receptors.

Role of β1,6 branched N-oligosaccharides on motility receptors (β1 integrin and CD44) in regulating their association with urokinase Plasminogen Activation Receptor (uPAR) and membrane tethered MMPs (MT1-MMP) in melanoma invasive variants

Motility receptors β1 integrin and hyaluronan receptor CD44 were found to be carriers of β1,6 branched N-oligosaccharides and the role of these oligosaccharides in regulating the association of motility receptors (β1integrin and CD44) with MT1-MMP and uPAR was studied. MT1-MMP and uPAR are the major regulators of the proteolytic cascade and their expression has been reported to be altered as the cells become invasive and metastatic. Before investigating the role these oligosaccharides play in regulating association between them, expression of these motility receptors, MT1-MMP and uPAR were studied. Results showed that in B16 murine melanoma invasive variants, expression of motility receptors (β1integrin and CD44) and uPAR was found to be unchanged. However, the expression of MT1-MMP was significantly higher in the invasive B16BL6 cells. It was also found by confocal microscopy that glycosylation of β1 integrin regulates its association with MT1-

MMP. However, association of β1 integrin with uPAR and CD44 with either MT1-MMP or uPAR was unaffected by the presence of these oligosaccharides. However, these results would need to be confirmed by co-immunoprecipitation which was not possible because of non-availability of good quality antibodies.

Objective III: To investigate the role of these oligosaccharides in regulating the motility of cells on ECM and basement membrane components.

Beta 1,6 branched N-oligosaccharides on melanoma cells not only regulate motility but also their spreading, positively on ECM (fibronectin) but negatively on BM (matrigel) components

Cell spreading is the post adhesion event that dictates cell motility. We show that glycosylation also regulates spreading of B16 melanoma cells differentially on fibronectin and matrigel. The presence of these oligosaccharides enhanced spreading of B16BL6 cells on fibronectin, but attenuated it on matrigel as seen by F-actin staining using phalloidin TRITC and by comparing ratio of cytoplasmic/nuclear area. Differential spreading and motility of B16BL6 cells on fibronectin and matrigel could be due to differential expression of their respective receptors or presence of such oligosaccharides on them.

Both α and β subunits of fibronectin (α5β1) and laminin (α3β1) receptors carry β1,6 branched N-oligosaccharides, however their presence has no effect on their cell surface expression

Purification of proteins carrying β1,6 branched N-oligosaccharides on L-PHA agarose beads from total cell lysates of B16BL6 cells, showed presence of not only β1 integrin but

also the α subunits of both fibronectin (α5β1) and laminin (α3β1) receptors by Western blotting. Laminin is the major component of matrigel. Comparison of B16BL6 cells either with B16F10 cells or with the cells treated with N-glycosylation inhibitor SW showed that glycosylation status does not impact expression of integrin receptors (α3β1 and α5β1) on the cell surface. Other mechanism by which tumor cells regulate invasion and metastasis is by regulating the sequestration of these receptors on specific membrane microdomains formed by tetraspanin superfamily of proteins known as Tetraspanin Enriched membrane Micrdomains (TEMs). TEMs formed by CD82 regulate the fibronectin receptor (α5β1) whereas laminin receptors (α3β1, α6β1) are regulated by TEMs formed by CD151. Invasive tumors down regulate most tetraspanins except CD151 and CO-029. Do B16 melanoma invasive variants regulate motility differentially on ECM and BM components by regulating the expression of tetraspanins?

Expression of tetraspanins does not correlate with the invasiveness of B16 melanoma cells

Analysis of transcript levels of the tetraspanins CD151, CD82, CD81, CD63 and CD9 in B16F10 and B16BL6 cell lines by semi quantitative RT-PCR did not show any correlation between the invasive ability of the cell lines and transcript levels of any of these tetraspanins. The total levels and surface levels of CD82 and CD151 which regulate fibronectin and laminin receptors respectively were comparable in B16F10 and B16BL6 cells as seen by Western blotting and flow cytometry. Thus, suggesting that altered motility on ECM and BM component is not due to alteration in expression of tetraspanins. Other probable mechanism by which tumor cells regulate motility is by regulating the association of tetraspanins with motility receptor in glycosylation dependent manner.

Glycosylation regulates association of laminin receptor integrin α3β1 with tetraspanin CD151

The effect of glycosylation on the association of CD151 and α3β1 was evaluated by co-localization studies using CD151 cells tagged with GFP. Results showed that inhibition of glycosylation promotes the association of CD151 with β1 integrin. It was further confirmed by co-immunoprecipitation of FLAG tagged CD151, using anti-FLAG M2 affinity gel, from cell lysate of B16BL6 cells and its SW treated counterpart expressing FLAG tagged CD151.

CD151 modulates spreading, haptotactic cell migration and invasion in glycosylation dependent manner

Cells require an optimum level of cellular adhesion for migration. Likewise, cells also require an optimum spreading for motility. CD151 transfected B16BL6 cells showed marginal increase in cell spreading on matrigel as compared to vector control, however, after inhibition of glycosylation using SW, both the cell types showed significantly higher spreading as compared to untreated cells. This is also evident in the higher ratio of cytoplasmic to nuclear area. Overexpression of CD151 in B16BL6 melanoma cells had very marginal effect on their haptotactic motility on matrigel. However, after inhibition of glycosylation, motility of CD151 transfected cells on matrigel increased significantly as compared to the cells transfected with vector alone. Overexpression of CD151 further increased the invasive ability of B16BL6 cells as compared to those transfected with vector alone. However, upon SW treatment invasive ability of both the cell types decreased to almost same level which was significantly low for both.

Discussion:

Invasion is the key process involved in most steps of metastasis. For invasion, tumor cells need to modulate their adhesion to ECM and BM components, degrade them for creating space and need to be motile. Earlier work in the lab has shown the involvement of β1,6 branched N-linked oligosaccharides in regulating the steps critical for invasion. Increased expression of these oligosaccharides has been consistently shown to positively regulate invasion. However, expression of such oligosaccharides does not always correlates with adhesion. Their expression has been shown to modulate adhesion either positively or negatively [11, 12, 15]. Beta 1,6 branched N-linked oligosaccharides have been shown to be the preferred site for the addition of various terminal substitutions. Role of the terminal sugars in modulating adhesion was studied by comparing their levels between melanoma invasive variants. Sialic acids in α2,6 linkage was shown to be the major substitution associated with β1,6 branched N-oligosaccharides on B16BL6 cells. Effect of increased substitution of α2,6 SA on regulating adhesion was studied by enzymes that remove them. However, as there is no sialidase which specifically removes only α2,6 linked SA, we have used two sialidases, one which removes only α2,3 linked SA and another which removes SA in both α2,3 and α2,6 linkage. Our results showed that removal of α2,3 linked SA did not have much effect on adhesion, but removal of SA in both linkages significantly decreased the adhesion to ECM and BM component. Role of α2,6 linked SAs in positively modulating adhesion was further demonstrated by shRNA mediated specific inhibition of ST6Gal-1 (enzyme which adds α2,6 linked sialic acid). Specific inhibition of α2,6 linked SA decreases the adhesion of B16BL6 cells on ECM and BM component.

To be invasive, tumor cells require an optimum level of cellular adhesion. Neither the cells adhering tightly nor those adhering too loosely to the substratum are able to move. Tumor cells possibly achieve an optimum level of adhesion for invasion by regulating terminal substitutions on β1,6 branched N-oligosaccharides. For instance, in human bladder carcinoma cell line T24, increased substitution of α2,3 linked SA on these oligosaccharides decreases adhesion but increases invasion. Similarly, in breast cancer and human melanoma decreased adhesion associated with increased expression of β1,6 branched N–oligosaccharides could be because of the differences in the linkage of terminally substituted sialic acids [16]. Expression of α2,6 linked SA substituted β1,6 branched N-oligosaccharides was associated not only with increased adhesion but also enhanced invasive potential of B16 melanoma cells.

Matrix degradation is very crucial for invasion and it is a highly regulated process as the same matrix serves as substratum for movement. Tumor cells regulate indiscriminate degradation of matrix by secreting the MMPs in zymogenic form. Increased expression of MMPs has been shown to correlate with the invasive phenotype of the cancer cells. However, in murine melanoma invasive variants, the basal expression of MMPs does not correlate with invasion [11]. Secretion of MMPs has also been shown to be induced in response to extracellular cues like adhesion to different matrices [17]. Increased adhesion of melanoma cells as a result of expression of β1,6 branched N-linked oligosaccharides resulted in significant increase in MMP-9 secretion. Inhibition of glycosylation either by SW or shRNA to GnT-V decreases the adhesion and thus concomitant inhibition in MMP-9 secretion.

Another mode of regulating matrix degradation is by regulating activation of zymogenic MMPs in a space and time dependent manner. MT1-MMP and uPA-uPAR system are key players in such regulation. The expression of MT1-MMP and uPAR correlates with invasive properties of cancer cells. Our result showed that in melanoma invasive variants expression of MT1-MMP correlates positively with invasion. However, the expression of uPAR is unaltered. Motility receptors such as integrins, hyaluronan receptor CD44 have been shown to be involved in focalized localization of these receptors towards invasive front [18, 19]. Beta1 integrin and CD44 were found to be the carriers of such oligosaccharides and their role in regulating the association of these receptors with the MT1-MMP and uPAR was studied in B16 murine melanoma cells. By confocal microscopic studies we showed that the presence of such oligosaccharides regulates the association of β1 integrin with MT1-MMP. However, expression of these oligosaccharides does not influence the association of β1 integrin and CD44 with uPAR. These results need to be confirmed by immunoprecipitation studies and can be performed with good quality antibodies for immunoprecipitation which are at present not commercially available.

Cell migration is an indispensable step in cancer cell invasion and metastasis. Cell spreading is prerequisite for cell movement. Even spreading of the cells would differ depending on the substratum. Less spread, in contrast to well spread morphology appeared to be favored by cancer cells for breaching the BM [20]. Our results showed that the expression of these oligosaccharides has the same differential effect on spreading as that seen on motility. Inhibition of these oligosaccharides inhibited both spreading and motility of B16BL6 cells on fibronectin but increased it on matrigel. Invasive cancer cells need to optimize their interaction with the substratum. They need to be more motile when invading

the ECM but more stabilized on BM. The cells possibly optimize their adhesion and spreading once they reach the BM barrier. Highly motile cells may not be able to stabilize their interactions to induce secretion of matrix degrading enzymes required to breach the BM. Similar observations were reported earlier by Leppa et al. who showed that the steroid induced transformation of S115 mammary epithelial cells is associated with expression of complex N-glycans substituted with poly-N-acetyllactosamine on β1 integrin subunit of laminin receptors. Inhibition of these oligosaccharides increased the spreading of cells on laminin-1 but was unaffected on fibronectin [21].

The differential spreading and motility on fibronectin and matrigel thus could be due to differential glycosylation of respective receptors. However, both the subunits of the major receptors for fibronectin (α5β1) and laminin (α3β1), (laminin is the major component of matrigel >50%), were found to carry β1,6 branched N-oligosaccharides. The other cause of differential motility could be due to differences in surface localization of respective integrin receptors. Tumors cells do show altered surface expression of certain integrin receptors [22]. Even altered glycosylation appears to dictate the levels of integrins on cell surface [23]. However, in B16 melanoma cells the expression of α5β1 and α3β1 and even α6β1 remained almost identical on B16F10, B16BL6 and SW treated B16BL6 cells.

The other possibility is differential regulation of fibronectin and laminin receptors in the tetraspanin (TSP) enriched membrane micro domains (TEMs). TEMs appear to modulate invasion associated processes by regulating the availability of TEM associated molecules, particularly integrins. Expression of most TSPs except CD151 and CO-029 is down regulated by highly invasive and metastatic cells [24]. However, there was no significant

change in the transcript levels of major TSPs, which are important from the point of invasion, in the parent B16F10 and its invasive variant B16BL6 cells. The motility of cells on fibronectin is largely regulated by the association of fibronectin receptor (α5β1) with TSP CD82 while that on matrigel/laminin of laminin receptor (α3β1) by TSP CD151. The levels of both these TSPs also remained almost identical on melanoma invasive variants as assessed by flow cytometry and Western blotting.

Glycosylation of both the tetraspanin (CD82) and fibronectin receptor (α5β1) has been shown to regulate their association and thus motility [25]. It is possible that expression of β1,6 branched N-oligosaccharides on the laminin receptor α3β1 also regulates its association with CD151 to influence its motility for invasion of basement membrane. Both α3 and β1 subunits express these oligosaccharides on B16BL6 cells and treatment with SW resulted in significantly higher association of β1 integrin with CD151. Since no other α subunit has been reported to carry these oligosaccharides (except α3 shown by us), the effects of SW on the association of β1 integrin with CD151 and cellular properties (on matrigel) should be as a result of inhibition of glycosylated structures on α3β1integrin. In spite of enhanced spreading and motility on BM (matrigel), SW treated cells showed significant reduction in their invasion of matrigel. We thus demonstrate that the differential motility mediated by β1,6 branched N-oligosaccharides on ECM and BM component, indeed aids in melanoma cells invasive ability by regulating the association of integrin receptors with specific TEMs.

In conclusion, these investigations demonstrate the mechanism by which β1,6 branched N-oligosaccharides regulate invasion in melanoma cells. It showed that even the terminal

substitutions on these oligosaccharides may be critical for influencing adhesion to matrices. Increased adhesion appeared to induce the secretion of MMPs and thus invasion which was reversed on their inhibition of expression of these oligosaccharides. Their presence on integrins also influences the association of the receptors with membrane tethered forms of matrix degrading enzymes. This study also brings an insight into the complex mechanism by which these oligosaccharides regulate the spreading and movement of cells on ECM and BM components to achieve optimum invasion.

References:

1. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144 5): 646-74
2. Spano D, et al. (2012) Molecular networks that regulate cancer metastasis. Semin Cancer Biol 22 3): 234-49
3. Stetler-Stevenson WG, Aznavoorian S, Liotta LA (1993) Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 9: 541-73
4. Takahashi N, et al. (2009) High expression of N-acetylglucosaminyltransferase V in mucinous tumors of the ovary. Oncol Rep 22 5): 1027-32
5. Yagel S, et al. (1989) Evidence that beta 1-6 branched Asn-linked oligosaccharides on metastatic tumor cells facilitate invasion of basement membranes. International journal of cancer Journal international du cancer 44 4): 685-90
6. Fernandes B, et al. (1991) Beta 1-6 branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasia. Cancer Res 51 2): 718-23
7. Takano R, et al. (1990) Increase of beta 1-6-branched oligosaccharides in human esophageal carcinomas invasive against surrounding tissue in vivo and in vitro. The American journal of pathology 137 5): 1007-11
8. Niimi K, et al. (2012) High expression of N-acetylglucosaminyltransferase IVa promotes invasion of choriocarcinoma. Br J Cancer 107 12): 1969-77
9. Pili R, et al. (1995) The alpha-glucosidase I inhibitor castanospermine alters endothelial cell glycosylation, prevents angiogenesis, and inhibits tumor growth. Cancer Res 55 13): 2920-6
10. Tomiie M, et al. (2005) Elevated expression of N-acetylglucosaminyltransferase V in first trimester human placenta. Biochem Biophys Res Commun 330 3): 999-1004
11. Reddy BV, Kalraiya RD (2006) Sialilated beta1,6 branched N-oligosaccharides modulate adhesion, chemotaxis and motility of melanoma cells: Effect on invasion and spontaneous metastasis properties. Biochimica et biophysica acta 1760 9): 1393-402
12. Demetriou M, et al. (1995) Reduced contact-inhibition and substratum adhesion in epithelial cells expressing GlcNAc-transferase V. The Journal of cell biology 130 2): 383-92

13. Guo HB, et al. (2002) Aberrant N-glycosylation of beta1 integrin causes reduced alpha5beta1 integrin clustering and stimulates cell migration. Cancer Res 62 23): 6837-45
14. Dykxhoorn DM, Novina CD, Sharp PA (2003) Killing the messenger: short RNAs that silence gene expression. Nature reviews Molecular cell biology 4 6): 457-67
15. Yoshimura M, et al. (1995) Suppression of lung metastasis of B16 mouse melanoma by N-acetylglucosaminyltransferase III gene transfection. Proceedings of the National Academy of Sciences of the United States of America 92 19): 8754-8
16. Cui H, et al. (2011) Differential expression of the alpha2,3-sialic acid residues in breast cancer is associated with metastatic potential. Oncol Rep 25 5): 1365-71
17. Saito Y, et al. (2010) Potentiation of cell invasion and matrix metalloproteinase production by alpha3beta1 integrin-mediated adhesion of gastric carcinoma cells to laminin-5. Clinical & experimental metastasis 27 4): 197-205
18. Marrero-Diaz R, et al. (2009) Polarized MT1-MMP-CD44 interaction and CD44 cleavage during cell retraction reveal an essential role for MT1-MMP in CD44-mediated invasion. Cell motility and the cytoskeleton 66 1): 48-61
19. Ahmed N, et al. (2003) Downregulation of urokinase plasminogen activator receptor expression inhibits Erk signalling with concomitant suppression of invasiveness due to loss of uPAR-beta1 integrin complex in colon cancer cells. Br J Cancer 89 2): 374-84
20. Chu YW, et al. (1993) Expression of complete keratin filaments in mouse L cells augments cell migration and invasion. Proceedings of the National Academy of Sciences of the United States of America 90 9): 4261-5
21. Leppa S, Heino J, Jalkanen M (1995) Increased glycosylation of beta 1 integrins affects the interaction of transformed S115 mammary epithelial cells with laminin-1. Cell Growth Differ 6 7): 853-61
22. Mierke CT, et al. (2011) Integrin alpha5beta1 facilitates cancer cell invasion through enhanced contractile forces. Journal of cell science 124 Pt 3): 369-83
23. Kim LT, et al. (1992) Altered glycosylation and cell surface expression of beta 1 integrin receptors during keratinocyte activation. Journal of cell science 103 (Pt 3): 743-53
24. Richardson MM, Jennings LK, Zhang XA (2011) Tetraspanins and tumor progression. Clinical & experimental metastasis 28 3): 261-70
25. Ono M, et al. (2000) Glycosylation effect on membrane domain (GEM) involved in cell adhesion and motility: a preliminary note on functional alpha3, alpha5-CD82 glycosylation complex in ldlD 14 cells. Biochem Biophys Res Commun 279 3): 744-50

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ABBREVIATIONS

AAA : *Aluria aurantia* agglutinin

AVP : Avidin HRPO

BLAST : Basic Local Alignment Search Tool

BSA : Bovine Serum Albumin

DAPI : Diamidino-2-phenylindole dihydrochloride

DEPC : Diethyl Pyrocarbonate

ECL : Enhanced Chemiluminiscent

EDTA : Ethylene Diamine Tetra Acetate

ECM : Extracellular matrix

BM : Basement Membrane

FITC : Fluorescein isothiocynate

FACS : Fluorescent Activated Cell Sorter

FBS : Fetal Bovine Serum

FN : Fibronectin

GFP : Green Fluorescent Protein

GnT-V : N-acetylglucosaminyltransferase-V

HRPO : Horse Radish Peroxidase

kDa : Kilo Dalton

LAMP1 : Lysosomal Associated Membrane Protein-1

LB : Luria Bertani

LEA : *Lycopersicon esculentum* Agglutinin

L-PHA : Leucoagglutinin Phytohemagglutinin

MAA : *Maackia amurensis* Agglutinin

MAT : Matrigel

MMP : Matrix Metalloproteinase

NP-40 : Nonidet P-40

PBS : Phosphate Buffered Saline

POPOP : 1,4-bis(5-phenyloxazol-2-yl) benzene

PPO : 2, 5-Diphenyl-oxazole

PIPES : Piperazine-N,N′-bis(2-ethanesulfonic acid)

PMSF : Phenyl Methyl Sulfonyl Fluoride

PCR : Polymerase Chain Reaction

PVDF : Poly Vinylene DiFlouride

RT : Reverse Transcriptase

SA : Sialic Acids

SDS : Sodium Dodecyl Sulphate

ST6Gal-I : β-galactoside α2,6-sialyltransferase

SW : Swainsonine

SNA : *Sambucas nigra* Agglutinin

TRITC : Tetramethyl Rhodamine Isothiocynate

TIMP : Tissue Inhibitor of Matrix metalloproteinase

TEMED : N, N, N', N',-Tetramethylethylenediamine

TTBS : Tween- Tris Buffered Saline

UN : Uncoated

uPA : Urokinase Plasminogen Activator

uPAR : Urokinase plasminogen Activator Receptor

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CHAPTER 1

INTRODUCTION

1.1 Cancer

Despite recent advancement in diagnostic tools and medicines that are able to detect and cure diseases, **Cancer** continues to be second most leading cause of mortality, after heart disease. However recently, cancer has ousted the heart disease and has become the major cause of death in Hispanic Americans [1]. Cancer can be defined as uncontrolled division of cells due to defects in normal regulatory signalling pathways. The development of cancer involves complex, dynamic changes in the genome that initially lead to the generation of pre-neoplastic lesions and eventually gives rise to clonal variants that proliferate into tumors, usually over many decades. Completion of human genome project has further assisted in better understanding of genes that are involved in cancer initiation and progression [2]. There are three major classes of genes which are involved in maintenance of cellular physiology in the normal cells namely (1) **tumor suppressors** are the gene which are mainly involved in cell cycle check points but upon mutation these gene lose their ability to control cell cycle progression e.g. **R**etino**b**lastoma (RB) TP53, APC etc. Each cell in the body except for germ cells has two copies of a particular gene. The mutation in the tumor suppressor genes are recessive in nature i.e. mutation in one copy of the gene is not sufficient (haploinsufficiency) to overcome its ability to control its function; hence it requires mutation in both the copy of the gene. Mutation in both the copy of the tumor suppressor gene is analogues to a non-functional brake of an automobile. Driver of the vehicle with dysfunctional brake fails to stop it even after applying brake; similarly mutation in tumor suppressor gene cannot withhold the cells from continuous division. (2) **Proto-oncogenes** are the normal genes which are involved in cell cycle progression, inhibition of cell differentiation and apoptosis but upon mutation even in a single copy of

gene makes it constitutively active (dominant) as an oncogene e.g. **ra**t **s**arcoma (RAS), MYC, B-RAF etc. Mutation in proto-oncogene is analogous to automobile with stuck accelerator; even if the driver tries to de-accelerate he fails. Similarly, mutation in proto-oncogenes cannot prevent the cells from dividing. (3) **Stability or caretaker** genes are responsible for repairing the DNA damage which occurs during exposure to mutagens or while repairing the error during normal DNA replication. Thus stability genes control the rate of mutation. Mutation in these genes leads to the impairment in DNA repair and therefore, increases the frequency of mutation e.g. BRCA1/2, ATM, MLH1 etc. [3].

Any **qualitative** or **quantitative** alterations in these three classes of genes lead to breakdown of their normal functioning. These genetic alterations are known to occur by point mutation, deletion, gene amplification, chromosomal translocation or other mechanisms. Besides, qualitative or quantitative changes in the genes, structural alterations in gene can also occur by epigenetic mechanisms. Methylation of cytosine bases in DNA or modification in the expression of genes by changes in histone profile are the common epigenetic changes that also play vital role in tumor progression. For instance, there is change in global DNA methylation pattern during cancer progression leading to genomic instability [4]. Deregulated expression of microRNAs (miRNAs) is another emerging epigenetic mechanism involved in carcinogenesis. Several of miRNAs are found to be upregulated or downregulated in numerous cancers [5].

It is predicted that during the process of tumorigenesis, cells must acquire a minimum of six mutations to become abnormal or malignant [6]. These mutations are included in those genes that make the cells malignant. The Six basic properties required for malignant transformation are,

1. **Self-sufficiency in growth signals:** Normal cells require growth factors for their growth and proliferation. However, tumor cells are able to synthesize their own growth factors or they amplify the growth factor signalling by overexpressing growth factor receptors such as HER-2/neu in stomach and breast cancer; epidermal growth factor receptor, erbB in stomach, brain and breast tumors [7].

2. **Insensitive to growth-inhibitory signals**: In order to maintain the tissue homeostasis many anti proliferative or anti-growth signals operate within the normal tissue. However, tumor cells evade these anti proliferative signals by downregulating the receptors through which these signals transmit. For instance, TGFβ signalling is an important anti proliferative signal, to avoid such signalling tumor cells downregulate TGFβ receptors [8] or they expresses mutant or dysfunctional receptors [9].

3. **Extensive replication potential**: According to Hayflick, normal cells have definite replicative potential, after that cells show senescence which is due to loss of 50-100 bp telomeric DNA from ends of each chromosome after every cycle of cell division. Tumor cells overcome the problem of replicative senescence by overexpressing the enzyme telomerase which prevents the loss of telomeric DNA by maintaining the ends of chromosomes [10].

4. **Ability to avoid apoptosis or programmed cell death:** Expansion of tumor cells depend not only on their rate of proliferation but also on the rate of cell attrition. Apoptosis is leading cause of cell attrition and it is regulated by Bcl2 family of proteins which has both pro and anti-apoptotic functions. Tumor cells evade the apoptosis by down regulating the expression of death receptor proteins, for instance

reduced expression of CD95 has been reported in neuroblastoma and lymphoma, or by overexpression of inhibitor of apoptotic proteins, for example survivin, its overexpression has been reported in several cancers [11].

5. **Sustained angiogenesis:** In normal tissues the process of angiogenesis is regulated by the balance between angiogenesis inducer and their countervailing inhibitors. However, in tumor cells the balance is shifted towards angiogenic switches. At the primary site tumors do not grow beyond the size of 2 mm in diameter due to lack of nutrients and oxygen. Hypoxic condition within the tumor, induces the expression of hypoxia inducible factor (HIF) which in turn, initiates the process of angiogenesis by regulating the expression of molecules like Vascular Endothelial Growth Factor (VEGF) which is involved in endothelial cell proliferation [12].

6. **Competent to invade and produce distant tumors**: The tumors that remain confined to the boundaries of an organ are called benign tumors and can generally be taken care of by clinical intervention. However, tumors often attain the property to invade the surrounding normal tissue, breach the organ basement membrane and disseminate to distant organ sites via lymphatics or blood circulation and form tumors at these sites. This property of dissemination is referred to as metastasis. Tumors that attain this property of invasion and metastasis are called malignant and it is the major cause of mortality in cancer patients. More than 90% of the patients die because of the invasive and metastatic cancers rather than the primary tumors. Although important, this is the least understood aspect of tumor biology because of the complexity and multiple step nature of this process [13].

All these six critical features are hailed as the hallmarks of cancer development [6]. Over the years the ability of tumor cells to evade host immune defence mechanisms has also been recognised and researched [14]. **Evasion of immune response** is now also considered to be an additional hallmark of cancer. Cancer cells are highly proliferating cells and they have increase demand of energy for the generation of new cells. Normal cells depend on glycolysis, Krebs cycle and mitochondrial oxidative phosphorylation pathway for glucose metabolism. However, cancer cells can reprogram the glucose metabolism mainly to glycolysis even in presence of oxygen. Cancer cells have devised several strategies to fulfil the energy demand. For instance, they show increased expression of glucose transporter (Glut1 and Glut3) which facilitate the import of glucose into cytoplasm. The glycolytic pathway intermediates are utilized by the tumor cells for the generation of nucleosides and amino acids which in turn help in biosynthesis of macromolecules and organelles required for assembling new cancer cells [15]. **Reprogramming of energy metabolism is now considered to be an additional hallmark to the existing list** [16]. Besides these genetic alterations, various factors in the host microenvironment are also believed to influence malignant cell growth. Although, invasion and metastasis are the major cause of mortality in cancer patients, the molecular mechanisms underlying these processes are still poorly understood [17].

1.2 Metastasis

Metastasis is defined as the dissemination of tumor cells from the primary site of origin to non-contiguous organ sites and formation of secondary metastatic foci [18]. It is believed to be a complex multistep process. Clinical observations provided initial clues

about the possible mechanisms involved in metastasis. Pathological examinations showed that tumors that are contained within the boundaries of basement membrane (carcinoma in situ) have a better prognosis as compared to those that breach the basement membrane (invasive). The latter generally metastasize to lymph nodes and even distant organs. Similarly, the tumors that metastasize were found to be well vascularised. Clinical observations also showed that some tumors metastasize only regionally to lymph nodes or to organs in the anatomic vicinity. It also revealed that highly invasive and aggressive tumors are highly metastatic, although, some like gliomas aggressively invade the surrounding normal tissues but do not metastasize to other organ sites. These studies very clearly showed that invasion and metastasis are the most lethal aspects of malignancy and provided clues about the possible steps required for a tumor to be metastatic [19].

It was realized that for continuous growth the tumor cells require both oxygen and nutrients and thus they do not grow beyond a certain size unless well vascularised. For tumors to metastasize, the first requirement would be to detach from the primary and invade the surrounding normal tissue towards the source of oxygen and nutrients. The tumors thus need to be motile and be able to degrade surrounding matrix and basement membrane (BM). The newly formed blood vessels are poorly formed, the endothelial lining is sometimes discontinuous and they often lack BM. This offers an easy escape route for their entry into circulation, referred to as intravasation. Invasive tumor cells have the ability to actively intravasate by degrading the vascular basement membrane and displacing endothelium. Not all tumor cells are equipped to survive the high shear forces in circulation and the host immune defences. However, many tumor cells have evolved mechanisms to survive in circulation. The next step is the organ colonization. Several tumors metastasize

only to the organs in the anatomic vicinity like the regional lymph nodes or the organs receiving the afferent blood vessel from the primary. However, many tumors bypass several organs in the blood flow path and very specifically colonize distinct organ sites [20]. Although clinical studies provided insight about the different steps, it did not provide clues to the possible mechanism or the molecules that are involved at each step.

To overcome this, several in vivo and in vitro experimental models and cell lines with specific metastatic characteristic were developed for each step of metastasis and each step was studied in isolation [21, 22]. This not only helped in confirming the involvement of steps in metastasis but also resulted in identification of large number of host and tumor derived molecules that participate in these step of metastasis [23].

1.3 Metastatic cascade

The sequence of events which are involved in dissemination of tumor cells from primary site to a new distant organ site is often referred to as metastatic cascade.

1.3.1 Detachment of tumor cells from primary tumor

Normally cells are held in place by their interactions with the neighbouring cells and with the underlying matrix or basement membrane. Cell-cell interactions are mediated by tight junctions, adherens junctions and the desmosomes. Claudins, occludins and junctional adhesion molecules (JAMs) are the transmembrane proteins involved in the formation of **tight junctions** that are connected to the actin cytoskeleton via adaptor proteins like zona occludins (ZO-1, 2 or 3) proteins and several others. The classical and non-classical cadherins are involved in the formation of **adherens junctions** and

desmosomes, respectively. E-cadherin mediates cell-cell adhesion by mediating homophilic interactions between E-cadherins on neighbouring cells. The adhesive state is maintained by the interaction of cadherins to the actin based cytoskeleton via the adaptor proteins referred to as catenins. Defects in any one of them results in non-adhesive state. Tumor cells are known to often downregulate cadherins and modulate the functions of catenins like β-catenins by phosphorylating it. The non-classical cadherins like desmogleins and desmocollins form desmosomal complexes by homophilic interactions between them on the neighbouring cells. They are connected to intermediate filaments via adaptor proteins like plectins [24]. Alteration in any of these components impairs cell-cell adhesion.

Cell to matrix interactions are primarily via two types of structures, **focal adhesions and hemidesmosomes**. The dense BM that surrounds the organs and blood vessels prevents passive movement of cells across it and the extracellular matrix (ECM) has very similar composition. They consist of collagenous proteins of several different types like Collagen-I (ECM) and Collagen-IV (BM), non-collagenous glycoproteins like fibronectin (ECM) and laminin (BM), glycosaminoglycans (GAGs) and proteoglycans [25, 26]. The ECM and BM may differ in the types of these components and the proportions of each to provide its unique property and specificities. Integrins and cell surface proteoglycans are the major receptors that participate in cellular adhesion and movement.

Both **focal adhesion** and **hemidesmosomal junctions** are formed by integrin receptors. The laminin receptor, integrin α6β4 is the key component of hemidesmosomes. The long cytoplasmic tail of β4 integrin appears to aid the interaction of this integrin receptor with the underlying intermediate filaments formed by keratins via the adaptor proteins. These interactions with underlying BM provide stability to the epithelial cells.

Altered expression of these receptors, adaptor proteins, or changes in cytoskeletal intermediate filament proteins, results into changes in adhesive state. There are 18α and 8 β integrin subunits which combine variously to form 24 different heterodimeric integrin receptors that together are able to recognise all the major collagen and non-collagenous glycoprotein components of ECM and BM. Integrins are connected to actin based cytoskeleton (microfilaments) via adaptor proteins like talin, vinculin and so on. Tumor cells modulate the expression of these receptors, adaptor proteins or the organization of the cytoskeleton to achieve altered adhesive and motile state [27, 28].

Recent studies have highlighted that detachment of cells during metastasis is further augmented by a process similar to that seen in embryonic cells termed as Epithelial to Mesenchymal Transition (EMT). During EMT, cancer cells express various transcription factors such as snail, slug, twist, ZEB1 and ZEB2 which directly or indirectly regulate E-cadherin expression. E-cadherin is the major molecule of adherens junction, with the loss of E-cadherin there is concomitant loss of apical and basal polarity of cells. The adherent epithelial morphology of the cells changes to a more motile mesenchymal phenotype with the gain of mesenchymal marker like N-cadherin and vimentin [29]. The transition of epithelial cells into mesenchymal state is crucial initial step in the cascade of metastatic events.

1.3.2 Invasion into the surrounding normal tissue

The cells that have broken free form the primary tumor need to be motile and are able to create space for movement. The surrounding matrix is used as traction for the movement of cells. Integrin receptors together cover all the major collagen and non-

collagenous glycoproteins and thus serve as a major class of molecules involved in movement of cells. Modulation of the integrin receptor expression, post translational modifications (PTMs) on them or their association with membrane microdomains all regulate cellular movement. Tumor cells utilize all these mechanisms to achieve a motile state. The cell surface proteoglycans may also have an important role in mediating movement on GAGs and proteoglycan components of the matrix. CD44 the hyaluronate receptor is a good example of receptors that mediate interactions with the GAGs and play a key role in cancer metastasis. Multiple splice variants of CD44 exist, and some of these variants have been shown to be specific for the metastatic phenotype.

Tumor cells must create space for movement. Tumor cells secrete a whole range of matrix degrading enzymes; however, as the same matrix is used as traction for movement, the degradation is highly regulated. Most of the enzymes are secreted in zymogenic form and their activation occurs in a cascade in a space and time dependent manner. Urokinase plasminogen activator (uPA) that converts plasminogen into plasmin is one of the key enzymes that control such regulation. Plasmin converts most of the proenzymes like Pro-MMPs into active MMPs whose substrates include the protein core of proteoglycans, collagenous protein and non-collagenous glycoprotein components of ECM and BM. The enzymes uPA gets associated with the cell surface via its receptor uPAR which often associates with the receptors involved in motility. The uPA/uPAR system and the membrane tethered forms of MMPs (MT1-MMP) that get activated during transport to the cell surface and associated with motility receptors together with uPAR are the major regulators that couple matrix degradation and cellular movement. The other components that participate in tumor cell invasion include the lysosomal enzymes cathepsins, the

ADAM (A Disintegrin and Matrix metalloproteinase) family of proteins, glycosaminoglycanases like heparanases and several others [30]. Each of these molecules helps tumor cells in their invasion and dissemination.

1.3.3 Intravasation

The process of entry of tumor cells into the lumen of lymphatic or blood vessels is termed as intravasation. Intact vascular BM lining the blood vessels acts as obstacle for the entry of tumor cells in the circulation. For intravastion metastatic cells either produces degradative enzymes that disrupt the vascular BM or induces the process of angiogenesis. Tumors do not grow beyond a certain size unless well vascularized. Metastatic tumors are generally highly angiogenic. The newly formed blood vessels around tumors however, are poorly formed. They lack BM and even the endothelial lining is often discontinuous. This offers an easy escape route for tumor cells to get into circulation [31]. The process of intravasation can also be facilitated by the molecular alteration that supports the ability of the tumor cell to cross the endothelial barrier and pericyte. Recently, it has been shown that notch signalling can contribute to tumor cell intravastion. Tumor cells expressing notch receptors binds to its ligand jagged1 in the endothelial cells of tumor associated blood vessels and thus induces intravastion. The transcriptional regulator, amino terminal enhancer of split (Aes) has been shown to inhibit notch signalling in primary colon carcinoma and thus notch mediated tumor cell intravasation but in invasive colon carcinoma tumor cell downregulate Aes and thus promote intravasation [32]. Intravastion of breast carcinoma has been shown to be enhanced by the activation of the TGF-β receptors, probably by promoting the expression of Angiopoietin-like 4 (ANGPTL4). Its

expression in turn disrupts the vascular endothelial cell junction and thus increases the permeability of cancer cells to the micro vessels wall and thus augments local invasion [33]. Intravasation of the breast carcinoma cells has also shown to be enhanced by the perivascular tumor associated macrophages [34].

1.3.4 Survival in circulation

Once the tumor cells have successfully intravasated into the lumen of blood vessels, they can easily disseminate through venous and arterial circulation. During the circulation tumor cells must survive variety of stresses in order to reach distant sites. For e.g. anoikis, a form of apoptosis induced in absence of adhesion to substratum, they also seem to be deprived of integrin ECM interaction dependent survival signals, to overcome that tumor cells show increased expression of Tyrosine receptor Kinase (Trk receptor) expression that prevents anoikis and promotes their survival [35]. Tumor cells also must overcome the damage sustained by shear forces of blood flow, predation by natural killer cells, a component of innate immune system, and also the toxicity induced by high level of oxygen. In order to overcome these, tumor cells form large emboli by their interaction with platelets. Cell surface carbohydrates in the form of Lewis antigens and integrin receptors for fibrinogen on tumor cells and P-selectin and fibrinogen receptor integrin αIIβ3 on platelets have been implicated in tumor cell embolization. This not only aids in evading immune surveillance but also acts as shock absorber [36]. It helps tumor cells to lodge mechanically into fine vasculature of the secondary organ and platelet derived growth factors aid its sustained growth.

1.3.5 Organ colonization

Once in circulation tumor cells are able to reach almost all organ sites. However, some tumors metastasize only regionally either to the lymph nodes or to the organs in the anatomic vicinity, while several others metastasize to very specific distant organ sites. Most of the regional metastasis can be explained based on the lymphatic or blood flow patterns. Tumors often metastasize to the draining lymph nodes or to the organs receiving afferent blood vessels from the primary. Liver receives maximum number of colon cancer cells via portal vein from colon that drains into liver, and thus chances of colon cancers metastasizing to liver is very high. Similarly, prostate cancers are also believed to colonize vertebral bones through vertebral venous plexus of spine [37]. However this is not the exclusive mechanism of prostate cancer metastasis to bone. Tumors that are either released into circulation as multicellular emboli or are able to form hetrocellular emboli with platelets of leucocytes while in circulation also get trapped in the first vasculature that they encounter and thus metastasize in the regional vicinity. This is often referred to as **Anatomical or Mechanical mode of organ colonization** proposed by Dr. J. Ewing and is also referred to as Ewing's hypothesis [38].

However, several tumors metastasize to very specific distant organ sites bypassing several organs during the course of their journey from the primary. Breast cancer cells metastasize only to lung, liver and bones, and some to the brain. Some of the melanoma cells metastasize to brain. Prostate cancer metastasizes mainly to bone and less frequently to other organs. Uveal melanoma metastasizes most frequently to liver [23]. The phenomenon of **Organ specific metastasis** has intrigued researchers for over a century. Dr. Paget, as early as 1889 proposed **Seed and Soil hypothesis,** based on autopsy study of almost 735 breast cancer patients. He compared cancer cells to the seeds and the target

organ as the soil, and proposed that like seeds which get dispersed in all directions when the tree comes to fruition but are able to grow only on the soil congenial for its growth [39]. Similarly, once the cells reach circulation they are able to reach almost all the organs but are able to grow and give rise to secondary metastasis only in the organs that support their growth. By late 1970's Prof. Fidler brought organ specific metastasis again into focus by developing several animal models and cell lines that metastasize in an organ specific manner. He showed that apart from **organ microenvironment, adhesive interactions** between molecules on tumor cells and those on the target organ are also important in **Organ Specific Metastasis** [40]. Over the years the **chemokines** expressed by the target organ and their **receptors** on tumor cells have also been shown to play a key role in organ specific metastasis of some cancers [41].

Irrespective of the mode of organ colonization, the first barrier for colonizing an organ is the organ endothelium. The arrested tumor cells would need to extravasate out of the circulation and adapt to the new growth environment in the target organ. For **extravasation,** the tumor cells need to interact with the endothelial cells to retract it. The next step is to degrade the exposed vascular basement membrane. Tumor cells utilize cell surface receptors to interact with basement membrane components and the matrix degrading enzymes to breach it to get into the organ parenchyma. The next major hurdle is the ability of the tumor cells to adapt to the organ environment.

However, very few tumor cells appear to be able to adopt the new organ microenvironment to form metastatic foci. As tumor cell population is highly heterogeneous; all the cells are not competent enough to form colonies and thus may remain dormant. For instance, breast carcinoma cells remain latent for several years to

decades, whereas lung adenocarcinomas cells remain dormant for months; however few cells known as tumor initiating cells are competent to grow. Initial growth of the tumor cells depends on both the autocrine and paracrine factors but latter on tumor cells become independent of any cytokine and growth factors [42]. For a cell to be metastatic it must be competent in all the above steps of metastasis. The cells fail to metastasize even if they are defective in any one of these steps of the metastatic cascade. This is termed as "**metastatic inefficiency**".

Invasion appears to be the major event in negotiating all the major steps of the metastatic cascade. Tumor cells defective in this property are unable to metastasize, and thus understanding the molecular mechanisms involved in the invasion process would yield information to tackle metastasis.

1.4 Tumor cell invasion

Tumor cell invasion is the hallmark of all malignant tumors. Invasion in itself is a complex and poorly understood process. The biochemical mechanism of tumor cell invasion appears to be identical, to the one used by the normal non-malignant cells, like endothelial cell during angiogenesis and trophoblast cells during implantation of embryo. A three step hypothesis has been proposed to illustrate the cascade of biochemical events during tumor cell invasion [43]. The initial step in cancer cell invasion is the modulation of tumor cell adhesion to extracellular matrix followed by proteolytic degradation of the ECM/BM components and migration of cells using the matrix components as traction for movement or their proteolytic degraded products as chemoattractants. However, all these steps are linked and interdependent.

A large number of both host and tumor derived molecules participate in this process. Previous studies have clearly demonstrated that invasive and metastatic tumor cells show distinct changes in the repertoire of cell surface molecules as compared to less invasive and non-metastatic cells. These changes are in the form of alteration in expression of, **i)** molecules involved in cellular adhesion like cadherins, integrins and their associated proteins, **ii)** molecules involved in proteolytic degradation like MMPs and other proteases, **iii)** molecules involved in motility like CD44, integrins and their associated proteins. However, all these processes are very finely regulated in spatio-temporal manner. The molecules involved in modulating cellular adhesion and motility are similar; therefore their roles in regulating adhesion and motility have been discussed together after matrix degradation.

1.4.1 Matrix degradation

Once the tumor cells get detached from the surrounding neighbouring cells, they need to gain access to blood circulation, for that tumor cells need to degrade the surrounding ECM and BM. Basement membrane forms the major barrier whose degradation would be a prerequisite for tumor cell dissemination. For degrading these matrices, tumor cells along with surrounding stromal cells produce several classes of proteases which aid in matrix degradation.

1.4.2 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases are one such class of proteases which are Ca^{+2} and Zn^{+2} ion dependent endopeptidase. These are involved in various physiological processes such as

tissue remodelling, in the inflammatory responses, organ development and in various diseases like cancer. There are about 23 known human MMPs [44]. Each MMP consists of mainly four domains: the signal peptide domain (pre-domain), pro-peptide domain, catalytic and c-terminal hemopexin domain (except for MMP-7, MMP-23 and MMP-26 which lack hemopexin domain). Besides, these domains few MMPs also have transmembrane (TM) and short cytoplasmic tail domain. Based on the presence or absence of TM domain MMPs are grouped into secreted and membrane anchored type proteases. Secreted MMPs are in catalytically inactive form (zymogen) due to interaction of the cysteine residue of the pro-domain with Zinc ion of catalytic site. Activation of MMP involves disruption of interaction between cysteine residue in the pro-domain and Zinc ion in the catalytic domain, a mechanism termed as '**Cysteine Switch**'.

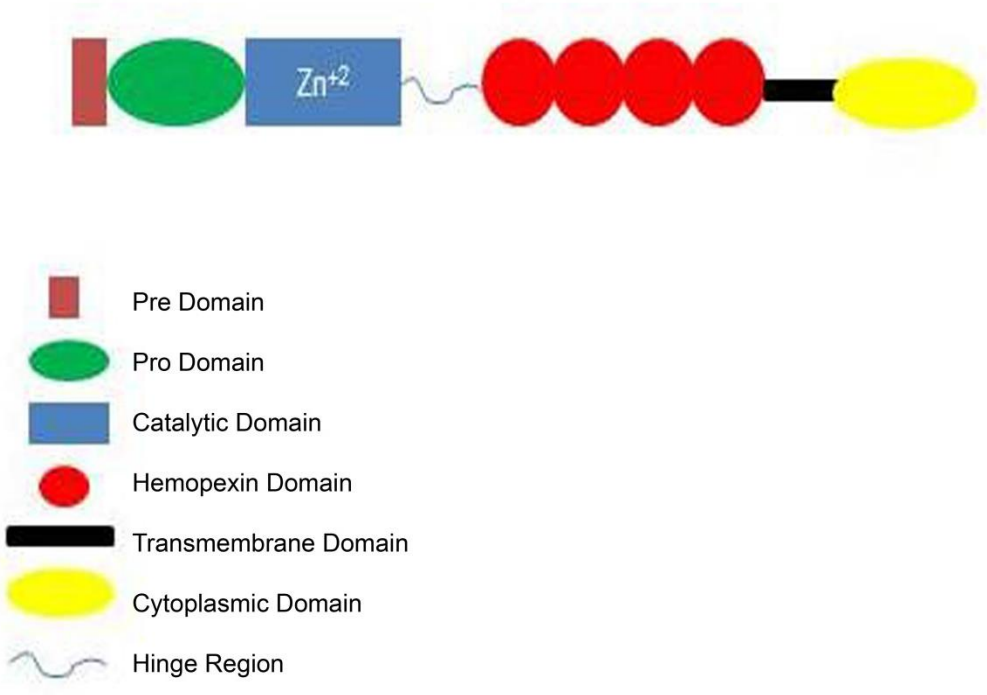


Illustration 1: Depicting the structural domains of matrix metalloproteinases (MMPs)

Depending on the substrate they cleave, secreted MMPs are further categorized into matrilysins, stromelysins, collagenase and gelatinases. MMP-2 and MMP-9 are major gelatinases, presence of collagen binding domain (CBD) within their catalytic domain, distinguish them from other MMPs. Gelatinases are overexpressed in several malignant tumors and their expression correlates with tumor aggressiveness and poor prognosis. Besides the secreted gelatinases recently, they are also shown to be associated with membrane proteins such as integrins, CD44 and Ku proteins via hemopexin or CBD domain. Their interaction with membrane proteins probably aids invasive cells in the focalized degradation of matrix [45-47]. The activity of the MMPs are regulated by their endogenous inhibitors knows as Tissue Inhibitor of Matrix Metalloproteinase (TIMPs) there are four TIMPs have been reported so far, TIMP1, 2, 3 and 4. TIMPs reversibly inhibit MMPs in1:1 stoichiometric ratio. Their overexpression or inhibition markedly inhibited or increased the metastatic and invasive potential of several cancer cell lines, respectively [48].

Membrane tethered MMPs are attached to the cell membrane either by glycosylphosphatidylinositol (GPI) anchored or transmembrane domain. MT1-MMP is the major protease which has been shown to be localized in the invadopodia. Like other MMPs MT1-MMP is also synthesised in zymogenic form and their activation involves removal of prodomain by the action of Golgi resident pro protein convertase furin, during its trafficking to the cell membrane. Activation of MT1-MMP is very essential because it is one of the key molecules involved in the cascade of MMP activation. Increased expression of MT1-MMP correlates with the invasive potential of several cancers [49]. Manipulation of their expression either by downregulation or overexpression concomitantly decreased or

increased the invasive potential of cell lines [50, 51]. As MT-MMPs exist in the already activated form on the cell surface, they promote invasion either by degrading the matrix directly or by activating other MMPs like MMP-2 in the vicinity of the cells, thus highlighting their importance in overall tumor cell invasion.

1.4.3 uPA/uPAR Proteolytic system

Urokinase-type plasminogen activator (uPA) is 45-55 kDa serine protease secreted in inactive form (pro-uPA). Activation of pro-uPA occurs by the binding to its receptor, urokinase Plasminogen Activator Receptor (uPAR). Upon activation uPA converts plasminogen into plasmin, a broad substrate specificity protease which can degrade several extracellular matrix proteins. Plasmin can also directly activate the zymogenic form of MMPs into active MMPs. uPAR is a glycosylphosphatidylinositol (GPI) anchored membrane protein, enhanced expression of uPAR towards invasive front has been reported in several cancers such as gastric carcinoma [52]. Increased expression of uPAR towards invasive front may facilitate invasion by the localized degradation of matrix through the activation of plasmin and MMPs. Moreover, uPAR in association with integrins also regulates tumor cell adhesion and motility. Recently, it has been shown that downregulation of uPAR decreases the tumor cell invasion by modulating their adhesion and migration. Not only the expression but the localization of uPAR in lipid rafts has also been shown to play an important role in regulating migration and invasion [53].

1.4.4 Cathepsins

Cathepsins are a group of lysosomal cysteine and aspartic proteinases namely cathepsin B, L and cathepsin D present in almost all mammalian cells. Cathepsins are

involved in the degradation of intracellular or endocytosed proteins. During the process of carcinogenesis cathepsins may be secreted outside by the tumor cells or translocated to the cell membrane for degrading the components of ECM and basement membrane. In order to prevent indiscriminate degradation of matrices, cathepsins may be secreted as procathepsins and can be converted into active form by other proteases. Activity of cathepsins can be regulated by their natural inhibitors known as cystatins, a, 10-13 kDa protein. Besides their direct role in matrix degradation they can directly activate MMPs and uPA, and thus facilitate matrix degradation [54]. Expressions of several cathepsins have been reported to be associated with malignant transformation. For instance, cathepsin D expression has been shown to be associated with the aggressiveness of breast and prostate cancer. Cathepsin B activity has been shown to be elevated in metastatic variant of murine melanoma cells. Inhibition of both intracellular and extracellular cathepsins has been shown to inhibit the invasive ability of cancer cells, indicating that both the secretion of enzyme and intracellular degradation pathways are important for invasion [55].

1.4.5 Proteoglycanase

Proteoglycans are the major constituent of the ECM and BM. In order to invade through these matrices tumor cell produces several enzymes that are capable of degrading them. Hyaluronidase, a hyaluronic acids degrading enzyme, whose expression has been shown to be enhanced in breast cancer [56]. Recently, overexpression of hyaluronidase expression in breast cancer cell line has shown to increase the invasive potential of these cell lines [57].

Heparanase is another proteoglycanase capable of cleaving the carbohydrate chain of heparan sulphate (HS) proteoglycan. As the secreted growth factors remain anchored to the proteoglycans of the ECM, heparanase mediated remodelling of the ECM by the cleavage of HS results in the release of GAG anchored growth factors that play an important role in tumor cell growth and angiogenesis. Increased expression of this enzyme has been reported in several cancers [58, 59] and has also shown to be localized towards the invasive front in esophageal carcinoma [60].

1.4.6 A Disintegrin And Metalloproteinases (ADAMs)

ADAMs are a family of proteins characterized by presence of a prodomain, metalloproteinase domain, a disintegrin domain having integrin receptor binding activities, cysteine rich domain, EGF like domain, transmembrane domain and cytoplasmic domain. They are involved in shedding of cell membrane proteins as well as degradation of extracellular matrices. Expression of ADAMs have shown to be involved in several steps of cancer cell progression and they also serve as a biomarker for several cancers [61, 62]. Increased expression of ADAM17 has been shown to be involved in hypoxia induced invasiveness of glioma cell lines. shRNA mediated inhibition of ADAM17 inhibited the ability of glioma cells to invade [63]. Closely related proteases to ADAMs are ADAMTS (A disintegrin and metalloproteinase with thrombospondin motifs) unlike ADAMs which are membrane bound (except for variant form of ADAM 12 and 28) ADAMTS is secreted. Overexpression of some ADAMTS likes ADAMTS-4 and 5 have been reported in glioblastoma, these ADAMTS may contribute to invasiveness of glioblastoma by the cleavage of brevican, a brain specific proteoglycan [64].

Degradation of ECM and BM components by the above protease paved the way for the migration of the tumor cells.

1.4.7 Modulation of cellular adhesion and motility

Cell motility is one of the crucial steps in cancer cell invasion and it requires dynamic interaction between tumor cell and the substratum on which it adheres and moves. Though, matrix degradation is an important event during tumor cell invasion, it is highly regulated and generally occurs towards the invading front of the cell. The degraded products of ECM generated as a result of matrix lysis serve as chemoattractant for directional movement of the tumor cells (Chemotaxis). The regulated degradation is important as the same matrix is used by the tumor cells as traction for forward motility (Haptotaxis) [65]. ECM is highly heterogeneous and consists of proteoglycans, collagens and non-collagenous glycoproteins. Integrins participate in motility by serving as receptors for all the major collagens and non-collagenous glycoproteins. The interactions of cell surface proteoglycans with GAGs and proteoglycans on the ECM/BM play a key role in the motility. Hyaluronate receptor CD44 is one of the examples of these interactions which play a key role in metastasis of certain cancers.

1.4.8 Hyaluronate receptor - CD44

CD44 is the major receptor for the hyaluronic acids (HA), one of the key constituent of ECM. CD44 has been shown to mediate both cell to cell as well as cell to matrix interaction. In order to be metastatic and invasive, tumor cells have been shown to modulate the expression of CD44. CD44 pre-mRNA consists of 20 exons, due to alternative splicing of introns it exist in several isoforms (CD44v6-v10, v = splice variants).

CD44s is the standard CD44 isoform formed by the splicing of introns between fifth and sixteenth exon. In the metastatic prostate cancer, tumor cells downregulate CD44s as compared to their benign counterpart or they overexpress other isoforms (CD44v7-10) [66]. Increased expression of CD44v6 isoform has been reported in several aggressive cancers such pancreatic adenocarcinoma, head and neck squamous cell carcinoma and breast cancer. Interaction of HA with CD44 has been shown to regulate the cancer cell motility in ovarian carcinoma cell lines, interaction of CD44 with HA promotes their motility. Inhibition HA binding site on CD44 by monoclonal antibody against CD44 prevents the HA mediated increased cell motility of human ovarian carcinoma cell lines [67].The extracellular domain of CD44 is also extensively modified by N-glycans, O-glycans which has been shown to regulate HA binding [68]. In addition to these CD44 is also modified by the addition of proteoglycans like chondrotin sulphate and heparin sulphate which adds in its interaction with growth factors [69]. Besides CD44, integrins are the major receptor of large components of ECM and BM and their interaction with these components have been shown to play an important role in regulating cancer cell adhesion and motility.

1.4.9 Integrins

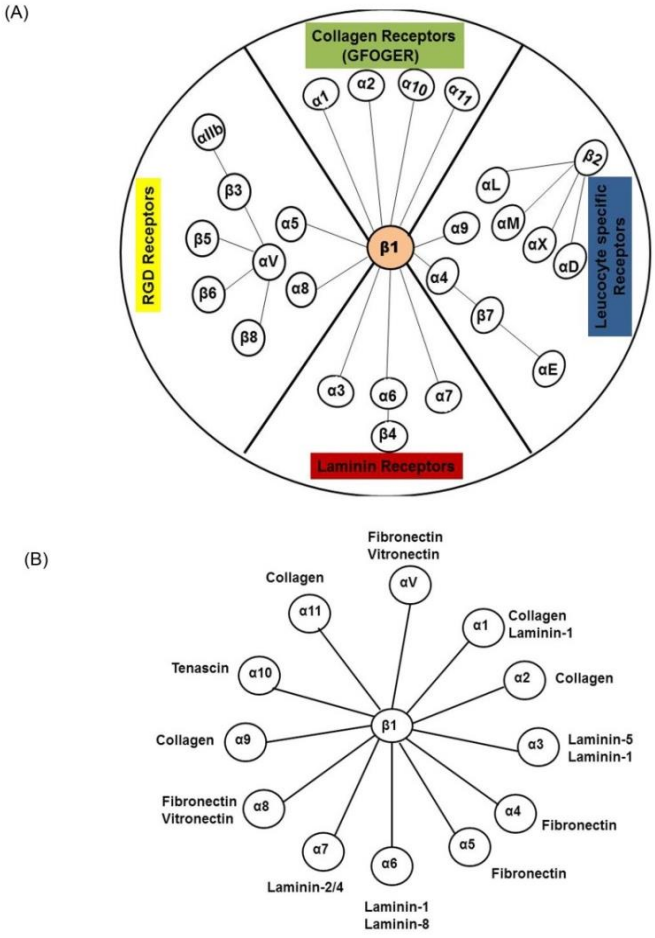
Integrins are obligate heterodimeric transmembrane glycoprotein receptors composed of non-covalently associated α and β subunits. There are about 18α and 8β subunits known so far which combine variously to form 24 different heterodimeric integrin receptors [70]. Integrins are the major receptors for both collagens and non-collagenous glycoproteins. Each integrin receptor has distinct substrate specificity but often they have overlapping specificity with varied affinity for ligands **(Illustration: 2)**. For instance, α5β1

is the major fibronectin receptor; besides this fibronectin also serve as ligand for several integrin heterodimers.

Each integrin subunit has one large N-terminal extracellular domain (approx. 800 amino acids), a transmembrane domain (approx. 20 amino acids) and except for β4 integrin which has large cytoplasmic tail (approx. 1000 amino acids) all others have a short cytoplasmic tail (approx. 13-17 amino acids). The cytoplasmic tail of integrin connects to actin cytoskeleton by several adaptor proteins, except for β4 integrin whose cytoplasmic tail is connected to intermediate filament proteins via adaptor proteins like plectins. The extracellular region of both α and β subunits contain distinct subdomains. The alpha subunit comprises of β propeller head domain, a thigh and two calf domains. Beta propeller domain of α subunits contains an additional inserted (I) domain of about 200 amino acids. The extracellular domain of β subunits also contains αI domain, a PSI (plexin/semaphorin/integrin) domain, a hybrid domain, four EGF repeats and membrane proximal β tail domain (βTD). I domain of integrin subunit is crucial for ligand binding [71].

Depending on the ligand binding, integrin exists in three different conformations such as bent, active and clustered. When integrins are not bound to the ligand they are called as inactive, or in rested/bent conformation. In the rested conformation, transmembrane domains of both the subunits are in close proximity to their cytoplasmic domain. In active conformation integrins are bound to ligand, which results in extension of extracellular domain and separation of cytoplasmic and transmembrane domain. Upon ligand binding when many activated integrins are clustered together at the plasma membrane they are said to be in clustered conformation [71]. Clustering results in the

formation of focal adhesion, which is essential for actin cytoskeleton assembly and activation of downstream signalling for several cellular functions. When integrins are bound to ligand they are capable of both inside out and outside in (bidirectional) signalling across the plasma membrane and thus regulate processes such as differentiation, survival, adhesion and motility [70].



Modified and adapted from [72]

Illustration 2: (A) Different types of integrin receptors. (B) Heterodimers formed by β1 integrin and their substrate specificity.

Integrins play an important role in regulating adhesion and protrusions in migrating normal as well as tumor cells. Cellular migration requires discrete steps: polarization i.e., formation of distinct front and rear end, cellular protrusion, transmembrane connection of cytoskeleton to ECM for the generation of traction for forward propulsion of the cells and retraction from the rear end [73]. Several signalling molecules are involved in these processes. Downstream Focal Adhesion Kinase (FAK) signalling is very crucial for integrin mediated directional cell movement. FAK serves as a scaffold to recruit src, a tyrosine-protein kinase, to focal adhesion, directing several pathways to promote cell migration. Continuous rearrangement of actin cytoskeleton regulates cell spreading which is a key requirement for cell movement. Actin cytoskeletal reorganization is mainly mediated by Rho GTPase family of proteins. Tumor cells can achieve optimum adhesion required for invasion and movement by either upregulating or downregulating certain integrin receptors. In malignant melanomas increased expression of αVβ3 integrin has been reported towards invasive front as compared to pre neoplastic tumors [74]. Besides, the alteration in expression of integrins during the process of invasion, metastatic tumor cells show increased level of activated β1integrin in contrary to primary tumor which show lowered level of activated βl integrin [75].

Integrins are highly glycosylated molecules; each subunit contains several N-glycosylation sites. Glycosylation on integrin has been shown to affect integrin structure, dimerization, affinity, clustering and stability of different integrin receptors [76-80]. Besides alteration in their expression, altered glycosylation of integrins also plays an important role in regulating its functions, probably by influencing its interaction with ECM

or other membrane proteins in its vicinity. The motility regulating functions of integrins may be modulated by another family of cell surface proteins known as tetraspanins.

1.4.10 Tetraspanins

Tetraspanins are the transmembrane proteins that transverse the membrane four times. Tetraspanins associate among themselves or with proteins from other families like integrins, growth factor receptors and proteases to form a membrane microdomain called **T**etraspanin **E**nriched **M**icrodomains (TEMs) [81]. TEMs are quite distinct from the rafts in terms of their disruption by Triton X-100 at 4°C, solubility because of palmitolylation in non-ionic detergent, insensitivity to cholesterol depletion [82]. Tetraspanins modulate the function of their associated proteins such as immunoglobulin superfamily proteins (IgSF), growth factor receptors and integrins [83]. Tetraspanins modulate the function of integrin receptors by regulating their compartmentalization or localization on the cell membrane, modulating their signalling and trafficking [84]. Tetraspanins are a family of 33 known human proteins of 25-50 kDa in size and made up of about 230 amino acids, it has certain characteristic residues such as CCG motif in outer large extracellular loop, disulphide bridge, and polar residues in transmembrane domains **(Illustration 3)**. Majority of animal cells contain several tetraspanins. While tetraspanin CD81 is widely expressed, few tetraspanins like CD151 have very limited distribution which is found mainly in epithelial, endothelial, neuronal and fibroblastic cells. However, some tetraspanins like RDS/peripherin and uroplakin are expressed only in outer segment of rod cells in retina and in urethra, respectively.

Most of the tetraspanins are posttranslationally modified by glycosylation or palmitolylation. Glycosylation is mainly present on the large extracellular domain of tetraspanins, except for CD9 which has glycosylation on small extracellular domain and CD81 which lacks glycosylation. Palmitolylation of tetraspanins on the other hand, occurs at the membrane proximal cysteine residues towards cytoplasmic leaflet. Tetraspanins are known to play an important role in wide variety of biological processes such as fertilization, viral and protozoan infection, cell proliferation, immune cell activation, tumorigenesis, cell motility and invasion [85].

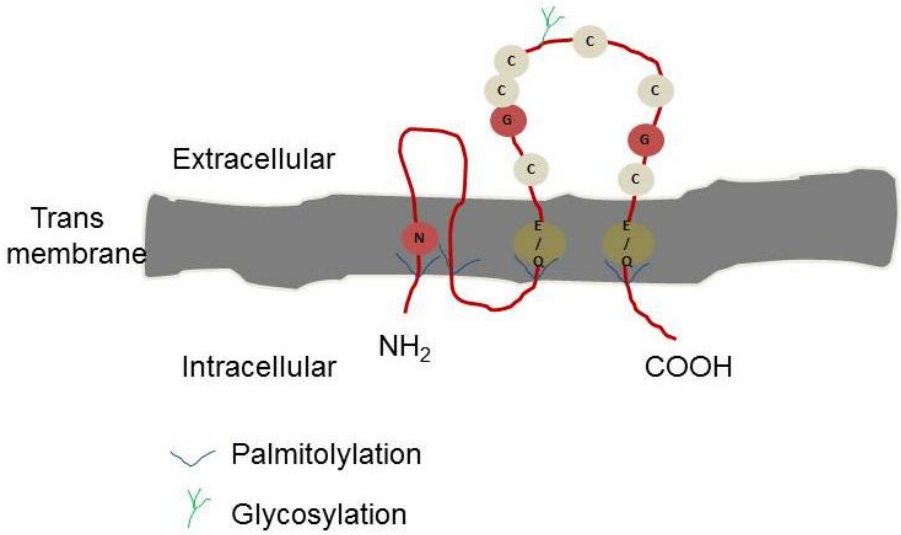


Illustration 3: Depicting the structural representation of tetraspanins

Invasive and metastatic tumors downregulate most tetraspanins except for CD151 and CO-029, in the process of tumorigenesis, tetraspanins such as CD82 and CD9 act as tumor suppressor proteins [86]. Over expression of these tetraspanins in invasive cell lines inhibits their invasion and metastasis by regulating adhesion, motility and matrix degradation. Expression of CD82 in metastatic prostate cancer cell lines inhibits cellular

protrusions and retraction crucial for cell movement by attenuating actin reorganization [87]. Tetraspanins such as CO-029 and CD151 promotes invasion and metastasis by regulating cell migration and matrix degradation. CD151 is the major laminin receptor associated tetraspanin. Knockdown of CD151 in highly invasive breast cancer cell line MDA MB231 inhibits cellular migration. CD151 has been shown to increase MMP-9 secretion by their homophilic interaction [88]. Thus tetraspanins can perform both pro as well as antimetastatic functions [89].

Microdomains formed by the tetraspanin CD151 and CD82 modulate the function of laminin and fibronectin receptors respectively. Glycosylation on both integrin and tetraspanin have been shown to regulate motility [90]. Recently, it has been found that tetraspanin can modulate the integrin function by regulating its glycosylation [91]. Besides, these alterations in expression of molecules involved in the processes of invasion, tumor cells also have been found to exhibit several membrane modifications in the form of altered glycosylation.

1.5 Altered cell surface glycosylation associated with invasion and metastasis

1.5.1 Glycosylation

Glycosylation is the most abundant posttranslational modification. More than 50% of all the known proteins and about 80% of secreted and membrane proteins are glycosylated. About 1-2% of the human genome encodes for the enzymes such as glycosyltransferases (enzymes which can add sugars) or glycosidases (enzymes which can remove sugars) [92]. Glycosylation involves covalent addition of oligosaccharides to the

lipids or proteins forming glycoconjugates which include **glycolipids, proteoglycans and glycoproteins.**

Glycolipids

Glycolipids are the molecules containing one or more monosaccharide units attached to ceramide by a glycosidic linkage. In glycosphingolipid, the monosaccharide unit is glucose which is attached to the terminal primary hydroxyl group of lipid moiety. Based on the oligosaccharides composition the glycolipids are divided into seven families these are lacto, neolacto, globo, isoglobo and ganglio series in vertebrates; mollu and artho series in invertebrates [93]. Altered expression of gangliosides has also been observed in several cancers for instance, in melanoma increased expression of gangliosides such as GD3, GM3 have been observed, while GD2 in neuroblastoma [94]. Recently increased expression of GD2 and GD3 has been reported in breast cancer stem cells as compared to non-cancer stem cells [95].

Glycolipids are an important constituent of membrane microdomains called Rafts. These form a platform for sorting and signalling involved in cancer cell adhesion and migration [96].

Proteoglycans

Proteoglycans comprise of a core protein and one or more covalently attached glycosaminoglycan (GAGs). GAGs are composed of repeating units of disaccharides where one sugar is usually uronic acid (D-glucuronic acid or L-Iduronic acid) and other sugar is N-acetyl glucosamine (GlcNAc) or N-acetyl galactosamine (GalNAc). Depending on the

type of GAGs proteoglycans are mainly of five types these are chondroitin sulphate, dermatan sulphate, keratan sulphate, heparan sulphate and hyaluronic acid. Proteoglycans are either secreted into the extracellular matrix to form hydrated gels, which support the tissues to withstand compressional forces or localize on the cell membrane. The cell membrane associated proteoglycan serve as co-receptor to help the cells in binding to the extracellular matrix or affect the growth factor signalling. Changes in the expression of heparan sulphate proteoglycans (HSPG) such as syndecans and glypicans have been shown to be altered in several cancers. For instance, syndecan-1, a membrane anchored HSPG has been shown to be elevated in stroma of breast cancer cells and its expression correlates with poor prognosis [97]. Similar, overexpression of glypican-1, a GPI anchored HSPG has been observed in gliomas and breast cancer and their expression has been shown to affect metastasis by modulating growth factor signalling [98, 99].

Glycoproteins

These are another type of glycoconjugates where oligosaccharides are covalently attached to the proteins. In glycoproteins glycosylation can be 'O' linked if sugars are attached to the hydroxyl group of serine or threonine or 'N' linked if sugars are attached to the amide group of asparagine residues having a consensus sequence (N-X-S/T) where X is any amino acid except for proline or aspartic acids.

O-Linked oligosaccharides or O-Glycans

Formation of O-linked glycans occurs by the covalent addition of the N-acetylgalactosamine (GalNAc) to the hydroxyl group of the serine and threonine residues. Biosynthesis of O-glycans occurs in the lumen of Golgi apparatus and it involves sequential

addition of monosaccharides to the growing polypeptide chain by the action of glycosyltransferase, the resulting product becomes the acceptor substrate for the subsequent glycosyltransferase. Besides, addition of GalNAc, less commonly other sugars (galactose, mannose and xylose) can also be added to OH-group of ser/thr residues of polypeptide chain forming different types of O-glycans. Mucins are the extensively O glycosylated proteins. Polypeptide sequence of mucins has variable number of tandem repeat (VNTR) region rich in serine, threonine and proline. The presence of proline residues within VNTR region facilitates formation of O-glycans. These are further elongated by the action of other glycosyltransferases resulting in the formation of elongated, branched structures known as core I, II, III and IV. Elongated mucin type O-glycans are found in normal epithelium of gastrointestinal, genitourinary and respiratory tract. Expression of altered mucin type of O-glycans has been reported in several cancers. For instance, in carcinoma of breast and colon mucin has truncated (incompletely glycosylated) O-glycan structure T/Tn antigen [100]. Recently, it has been shown that O-glycans have both metastatic and antimetastatic role [101]. O-glycans with core 2 structure have metastasis promoting function in several cancers, such as bladder cancer. Bladder cancer cells expressing enzyme which catalyses core 2 structures (C2GnT) forms more lung metastatic foci as compared to non-expressing cells. O-glycans with core2 structure in bladder cancer cells prevent NK cell mediated tumor killing. However, expression of core3 synthesizing enzyme (core3 synthase) in prostate cancer cell lines decreased their metastatic ability by regulating integrin expression and thus tumor cell invasion and motility [102].

N-Linked oligosaccharides or N-Glycans

The biosynthesis of N-linked oligosaccharides occurs in lumen of endoplasmic reticulum and continued in Golgi by the sequential action of several resident glycosyltransferases and glycosidases. The N-glycan biosynthesis involves several discrete steps, these are [103]-

1. **Formation of N-glycan precursor molecule and its addition to Dolichol pyrophosphate.**

Synthesis of N-glycan precursor begins in the cytosolic face of the ER by the transfer of GlcNAc-P from UDP-GlcNAc to the dolichol phosphate (DP) to form dolichol pyrophosphate N-acetylglucosamine (GlcNAc-DPP). Dolichol is a polyisoprenol lipid carrier consisting of five isoprene units attached linearly in head to tail manner. Sequential addition of another GlcNAc and five mannose residues from UDP-GlcNAc and GDP-Man, respectively, to the GlcNAc-DPP generates ($Man_5GlcNAc_2$ DPP). The $Man_5GlcNAc_2$-DPP residues translocate across the lipid bilayer from cytosolic side of ER by the action of flippase, so that glycan can be exposed to the luminal face of ER. The fourteen sugar precursor molecule ($Glc_3Man_9GlcNAc_2$-DPP) is formed by the further addition of four mannose and three glucose residues to the $Man_5GlcNAc_2$-DPP [103].

2. **Transfer of precursor molecule from dolichol pyrophosphate to growing polypeptide chain.**

The $Glc_3Man_9GlcNAc_2$ is transferred enbloc from DPP on to the Asn residue of Asn-X-Ser/Thr consensus sequence of the nascent growing polypeptide chain in the lumen of ER by the action of multisubunit protein complex, oligosaccharyl-transferase [103].

3. Processing of N-glycans

It involves sequential removal of glucose molecules by the action of glucosidase I and II. Further processing of N-glycans is mediated by ER resident α-mannosidase I which removes one mannose. Glycoproteins with this type of sugars ($Man_8GlcNAc_2$) are referred as **high mannose type.** Subsequent removal of three mannose residues from $Man_8GlcNAc_2$ in the Golgi by the action of Golgi resident α-mannosidase-IA, B, C results in the formation of $Man_5GlcNAc_2$. The $Man_5GlcNAc_2$ is the common molecule for the formation of hybrid and complex type of oligosaccharides. Addition of N-acetylglucosamine residue to the C-2 of the mannose α1,3 in the core of $Man_5GlcNAc_2$ by the action of N-acetyl glucosaminyl transferase (GnT)-I, form GlcNAc-$Man_5GlcNAc_2$. Glycoproteins with this type of sugars are referred to as **hybrid type**. Removal of two mannose form $GlcNAcMan_5GlcNAc_2$ by the action of Golgi resident α-mannosidase II results in the formation of $GlcNAcMan_3GlcNAc_2$. By the subsequent action of GnT-II on C2 of the mannose α1,6 in the core of $GlcNAcMan_3GlcNAc_2$, form the precursor molecule for biantennary complex type of sugars. Tri and tetra antennary structure are formed by the action of GnT-IV and GnT-V, respectively. Another Golgi resident enzyme GnT-III, adds N acetylglucosamine to the β mannose of the core resulting in the formation of bisected hybrid and complex type of sugars [103].

4. Maturation of N-glycan

It involves addition of sugars **a)** mainly fucose to the to the core (first GlcNAc attached to the asparagine residue **b)** elongation of the hybrid and complex N-glycan by the addition of β linked galactose to the terminal GlcNAc of each antennae resulting in the formation of N

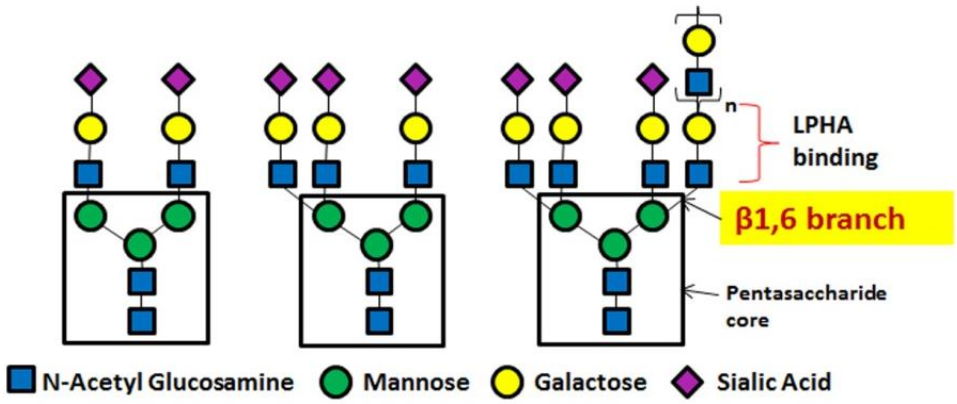
acetyllactosamine (LacNAc) structure **c)** Maturation of N-glycans by the addition of terminal sugars like sialic acids, fucose, galactose, N-acetyllactosamine and sulphate to antennary structures known as capping or decoration of sugars.

Thus, all N-Glycans have a common pentasaccharides core ($Man_3GlcNAc_2$). Depending on the action of specific enzymes, which act in proper order and time and their expression in respective Golgi compartment, N-linked glycoproteins formed are of three types, namely **high mannose**, **hybrid** and **complex type** [103].

Several observations and experimental evidences over the past few decades have generated the current opinion that N-glycans play very crucial role in progression and metastasis of cancer. By the comparative analysis of the surface glycoproteins from the virus transformed and non-transformed cells, Warren and Glick observed that glycans from the transformed cells were highly branched and sialylated [104, 105]. This phenomenon was established as "**Warren and Glick phenomena**". Later on it was found that the enzyme N-acetylglucosaminyl transferases-V (GnT-V) was responsible for the formation of highly branched N glycans on polyoma virus transformed baby hamster kidney (BHK) cells [106]. GnT-V is a Golgi resident enzyme, encoded by the Mgat5 gene, catalyses the formation of highly branched complex N-linked oligosaccharides (**Illustration 4**) and its increased expression has been observed in several cancers.

1.5.2 Beta1,6 branched N-linked oligosaccharides and cancer metastasis

Neoplastic transformations have been associated with several changes in glycan profile.



(Adapted and modified from essentials of Glycobiology [103])

Illustration 4: Typical structure of N-linked oligosaccharides (bi, tri and tetra antennary)

Significance of increased expression of the β1,6 branched N-oligosaccharides in metastatic progression has emerged from several clinical evidences observed in human cancers. Increased expression of β1,6 branched N-oligosaccharides has been reported in the atypical hyperplasia and breast carcinoma as compared to normal and benign tissues. Expression of these oligosaccharides in human colorectal carcinoma serves as an independent prognostic marker for tumor recurrence and patient survival [107]. Moreover, enhanced expression of these oligosaccharides has been also demonstrated in gliomas, melanomas, esophageal, mucinous tumor of ovary, gastric carcinomas and endometrial cancer [108-111].

Clinical observations showing the association between expression of these oligosaccharides and human cancers, was further supported by numerous experimental evidences from human and murine cancer cell lines. Transformation of Rat2 fibroblast cells

with oncogene like T24H-ras or v-fps have been shown to increase both, the GnT-V activity and metastatic potential of these cells [112]. Similarly, transformation of NIH3T3 cells by expression of her2/neu oncogene induces the expression and activity of GnT-V enzyme which in turn increased the expression of β1,6 branched N-oligosaccharides on specific sets of proteins [113]. Glycosylation mutant of highly metastatic MDAY-D2 cell line was associated with their loss in their metastatic potential due to loss in the activity of GnT-V [114]. Inhibition of the formation of these oligosaccharides in metastatic cell lines by N-glycosylation inhibitor Swainsonine, which results in the formation of hybrid instead of complex N-glycans showed significant reduction in metastatic potential [115]. By manipulating the expression of these oligosaccharides in the cell lines either by overexpression or downregulation of GnT-V showed concomitant increase and decrease in their metastatic potential. Expression of GnT-V in colon cancer cell lines increased their ability to metastasize to distant organ by promoting the ability of these cells to attach to vascular endothelium [116]. Expression of GnT-III, a competitive inhibitor of GnT-V in B16F10 cells inhibited the ability of these cells to form metastatic colonies in the lung [117].

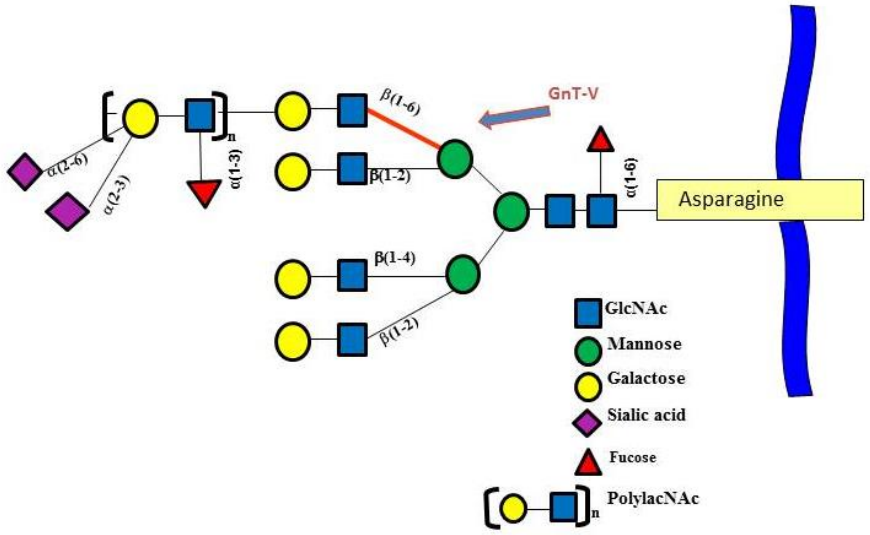
Besides, exemplifying the association of these oligosaccharides and metastasis in cell lines, their association with metastasis had been also demonstrated in mgat5 knockout mice. Polyoma middle T antigen induced experimental mammary cancer and lungs metastasis was significantly inhibited in mgat5 (-/-) mice as compared to mgat5 (+/+) counterparts [118].

Thus, evidences obtained from the increased association of β1,6 branched N-linked oligosaccharides in several human cancers, from their chemical inhibition or glycosylation

mutant and also from genetic manipulation of their expression conclusively established that β1,6 branched N-oligosaccharides play an important role in metastasis. Expression of these oligosaccharides was not only associated with progression of several cancers but also they have been shown to play a pivotal role in organ specific metastasis.

1.5.3 Beta1,6 branched N-linked oligosaccharides and organ specific metastasis

Organ specific metastasis involves specific interaction between the tumor cells and vascular endothelium of the target organ, extravasation and adaption of the tumor cells in new microenvironment. Beta1,6 branched N-oligosaccharides is the preferred site for the addition of various terminal sugars like sialic acids (SA), fucose or poly-N-acetyllactosamine (polylacNAc) (**Illustration 5)**. These substitutions serve as novel ligands for the several endogenous lectins expressed on organ vascular endothelium. For instance, sialyl Lewis X antigen (sLe^x) substituted β1,6 branched N-oligosaccharides promotes the metastasis of melanoma to the liver by facilitating their attachment to the liver vascular endothelium via E-selectins which serve as ligand for sLe^x antigen [116].



(Adapted and modified from essentials of Glycobiology [103])

Illustration 5: Depicting the β1,6 branched N-linked oligosaccharides and probable associated terminal sugars.

Recently, it has been shown that the presence of poly-N-acetyllactosamine substitutions on β1,6 branched N-oligosaccharides facilitate the lung colonization of B16 murine melanoma cells. Galectin-3, a beta galactoside binding lectin has been shown to be expressed in highest amounts on the lungs of mice. It was shown to be expressed on all the compartment of the lungs, including the surface of lung vascular endothelium. Expression on the vascular endothelium serves as a ligand for the polylacNAc substituted β1,6 branched N-oligosaccharides and which in turn helps in mediating lung specific metastasis [119, 120]. Besides, their role in mediating initial interaction these oligosaccharides also participate in all the steps of extravasation and possibly survival and proliferation of cancer cells in the secondary site. Several growth factor receptors have been shown to be modified

by the β1,6 branched N-linked oligosaccharides their contribution in the growth of the tumor cells as the secondary metastatic site could not be rule out. [121] The expression of these oligosaccharides appears to influence majority of the steps of metastasis and thus may also have a role in regulating tumor cell invasion.

1.5.4 Beta1,6 branched N-linked oligosaccharides and invasion

Expression of β1,6 branched N-linked oligosaccharides have been shown to be strongly associated with invasive normal as well as metastatic cancer cells. Endothelial cells express these oligosaccharides when they need to be invasive during angiogenesis [122]. Cells of the immune system like activated granulocytes, macrophages and lymphocytes express them to extravasate and reach the inflamed site [123]. Even trophoblast express these oligosaccharides during implantation of embryo into uterus [124]. Increased expression of these oligosaccharides is reported in several invasive cancers such as gliomas [125] and towards invading front in esophageal carcinoma [126]. Increased expression of β1,6 branched N-oligosaccharides correlates with invasive potential of metastatic sublines as compared to non-metastatic murine mammary carcinoma cell line SP1 [127]. Expression of the enzyme GnT-V in non-invasive human fibrosarcoma cells and murine fibroblast cells made them invasive [128]. Moreover, downregulation of the GnT-V or overexpression of its competitive inhibitor GnT-III in highly invasive cancer cell lines significantly decreased their ability to invade [129].

1.5.5 Possible mechanisms by which β1,6 branched N-oligosaccharides affect cancer cell invasion

Expression of these oligosaccharides possibly regulates processes critical for invasion like adhesion, matrix degradation and motility. Invasive tumor cells mediate all these process either by the modification of the proteins carrying them or the signalling mediated by them. Expression of these oligosaccharides on cell adhesion molecules like integrins, cadherins CD44 or ECM components like laminin regulates cell to cell or cell to ECM interaction. Expression of such oligosaccharides has been shown to regulate adhesion to both ECM and BM components. Expression of GnT-V in an immortalized lung epithelial cell line, Mv1Lu and HT1080 inhibits adhesion to Col-IV and FN possibly by altering the glycosylation of integrin receptors [79, 130]. E-cadherin is the one of the molecule that serves as substrate for both GnT-V and GnT-III. Addition of bisecting N-glycans on E-cadherin by GnT-III expression has been shown to stabilize adherens junction and thus cell to cell adhesion possibly by preventing its endocytosis. However, addition of β1,6 branched N-oligosaccharides on E-cadherin alters its localization and thus it promotes invasion by reducing cell to cell adhesion [131].

In addition to regulating adhesion, presence of these oligosaccharides on proteins involved in matrix degradation like matriptase has been shown to regulate the invasive ability of prostate cancer cell lines by regulating its activity [132], and stability of matriptase in human gastric cancer cell line MKN45 [133]. Inhibition of the expression of these oligosaccharides has been shown to inhibit matrix degradation by enhancing the expression of Tissue Inhibitor of Matrix metalloproteinase-1 (TIMP-1) [134]. MMP-2 and 9 are the major collagenases which are involved in the matrix degradation. Recently, it has been shown that presence of such oligosaccharides on TIMP-1 negatively modulates its ability to inhibit MMP-9 activity [135].

Besides regulating adhesion and matrix degradation, expression of these oligosaccharides also regulates cancer cell motility. Increased expression of these oligosaccharides has been shown to be involved in melanoma progression by up regulating cell motility [136]. Beta1,6 branched oligosaccharides served as high affinity ligand for galectin-3. The interaction between galectin-3 and such oligosaccharides on α5β1 integrin causes its activation and promote motility of mammary carcinoma cells on fibronectin [137]. Similarly, expression of GnT-III and GnT-V in MKN45 cell line concomitantly decreased and increased the motility of these cells on laminin-5 by modulating the glycosylation of the α3 integrin [138]. Recently, it has been shown that downregulation of GnT-V gene in gastric carcinoma cell line BGC823 inhibited invasion and metastasis by inhibiting EGFR signalling mediated EMT and MMP-9 secretion [139].

Thus, β1,6 branched N-oligosaccharides appears to influence metastasis by affecting the most crucial aspects in metastasis that is invasion. Despite considerable amount of research, the mechanism by which these oligosaccharides regulate invasion is not very clear and it requires further investigation.

1.6 RATIONALE OF THE STUDY

Metastasis is the major cause of cancer related mortality and invasion is involved in majority of the steps in metastasis cascade. Cancer cell invasion thus, is the hallmark of metastasis. Cell surface molecules play an important role in negotiating most steps in invasion and metastasis. Tumor cells show several surface modifications associated with metastasis and invasive phenotype. One such consistently observed cell surface modification is the expression of β1,6 branched N-linked oligosaccharides. Expression of

such oligosaccharides correlates positively with both invasive cancer cells as well as the normal cells involved in invasive functions.

B16 murine melanoma variant B16BL6, selected specifically for the invasive characteristics, and its parent cell line B16F10 have been used as a model system to investigate the mechanism by which these oligosaccharides regulate invasion **(illustration 6)**. Previous work in lab has explored their role in invasion by comparing invasive variants B16BL6 and B16F10 which differ in the expression of these oligosaccharides, by using N-glycosylation inhibitor Swainsonine (SW) and by using antisense to the enzyme GnT-V. It was shown that their expression **i)** correlates positively with adhesion to most ECM and BM components and regulates chemotaxis (motility in response to soluble chemoattractant) positively on both ECM (fibronectin as one representative) and BM (matrigel) components however, **ii)** it regulates haptotaxis (motility in response to substratum bound chemoattractant) in a complex manner, enhances it on fibronectin but attenuates it on matrigel **iii)** although, the expression of these oligosaccharides has no effect on the secretion of MMPs, it always correlated with invasiveness [140].

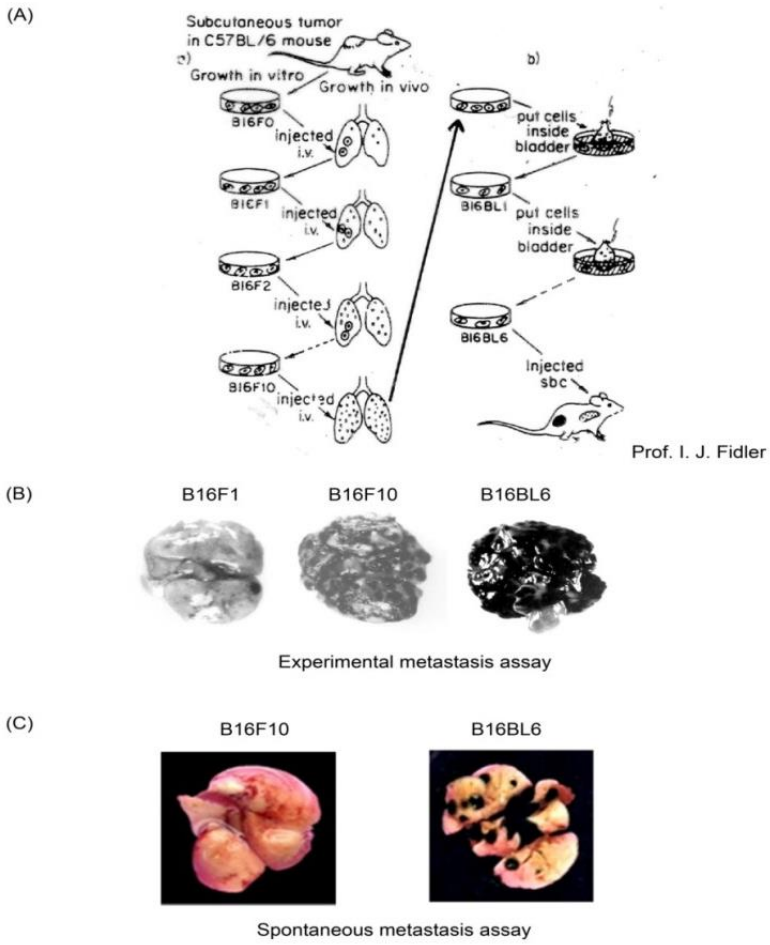


Illustration 6: The B16 murine melanoma model. (A) Generation of B16 murine melanoma cell lines. (B) Experimental metastasis assay. (C) Spontaneous metastasis assay

Above studies raise several key questions,

- Expression of β1,6 branched oligosaccharides are always associated with invasion and with increased adhesion on different matrix components, however, there are reports where their expression correlates negatively with adhesion. **How do these oligosaccharides regulate adhesion both positively and negatively? Is it because of differences in terminal substitutions on them?**

- Although, the expression of these oligosaccharides correlates with the invasiveness of melanoma cells, it does not correlate with the secretion of MMPs. Degradation of matrix is a key component of invasion process, it is important to investigate the mechanism by which these oligosaccharides regulate matrix degradation. **How do these oligosaccharides regulate matrix degradation?**

- The expression of these oligosaccharides regulates motility differentially on ECM and BM components. **What is the mechanism and its significance in terms of invasion?**

1.6.1 AIMS AND OBJECTIVE OF THE STUDY

- **To investigate the role of terminal substitutions on β1,6 branched N-linked oligosaccharides in regulating cellular adhesion and thus invasion.**

- **To investigate the role of β1,6 branched N-linked oligosaccharides in regulating matrix degradation.**

- **To investigate the role of these oligosaccharides in regulating the motility of cells on ECM and basement membrane components.**

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell lines and Reagents

Murine melanoma cell lines B16F10 and B16BL6 were a kind gift from Prof. I. J. Fidler, MD Anderson Cancer Center, Houstan, USA [21]. Minimal Essential Medium (MEM), Fetal Bovine Serum (FBS), L-glutamine, Antibiotic-Antimycotic solution, Non-essential amino acids, Vitamins, Sodium pyruvate, Lipofectamine™ 2000, TRIzol and Calcein AM was obtained from GIBCO, Invitrogen Corporation, USA. Aprotinin, Leupeptin, Pepstatin, PMSF, Trypsin, Tween-20, Bovine Serum Albumin (BSA), TEMED, β-mercaptoethanol, Glycine, Coomassie Brilliant Blue, Ponceau-S, N-hydroxysuccinimido biotin, Gluteraldehyde, Paraformaldehyde, Glass fibre filters, POPOP, PPO, anti-FLAG® M2 affinity gel, 3X FLAG® peptide, Lysolecithin, Phalloidin TRITC, Phalloidin FITC, DAPI, Trypan Blue, Gelatin, N-octyl β-D-glucopyranoside, Protamine sulphate, PIPES, Lysozyme, Cesium Chloride, RNase A, Ethidium Bromide, Diethyl Pyrocarbonate (DEPC), Primers for PCR, Polybrene, Puromycin and Doxycycline were obtained from Sigma Chemical Co, USA. Ampicillin, Kanamycin, Yeast extract, Tryptone and Agar powder were obtained from HIMEDIA, India. Tris, NP-40, Sodium Deoxycholate, Sodium Dodecyl Sulphate (SDS), Bisacrylamide, Triton X-100, Neomycin (G418) were obtained from USB, USA. Taq Polymerase, T4 Polynucleotide Kinase (PNK) buffer and kinase, ATP, dNTPs, T4 DNA ligase enzyme, restriction enzymes were purchased either from New England BioLabs *inc* (NEB), USA or Fermentas, USA. Protoscript first strand cDNA synthesis kit and sialidase were obtained from NEB, USA. Plasmid DNA extraction, DNA gel extraction kits were obtained from either Sigma or Qiagen, USA. Acrylamide, PVDF membrane, Enhanced Chemiluminiscent (ECL) plus Western blot detection reagent and

protein G sepharose beads were acquired from Amersham-Pharmacia Biotech Ltd., England. Fibronectin and matrigel were purchased from BD Pharmingen, USA. Biotinylated lectins were obtained from Vector Labs, USA were employed for all studies. Tissue culture plastic ware was obtained from BD Falcon or Nunc, USA. Radioactive tritiated thymidine for labelling cells was obtained from Board of Radiation and Isotope Technology (BRIT), India. All other fine chemicals were obtained locally and were of AR and GR grade. Water used to prepare all reagents was of Milli-Q grade.

2.1.1 Antibodies

Rat anti-mouse β1integrin monoclonal antibody (clone 9EG7) and mouse anti-mouse α3 integrin monoclonal antibody (clone42/CD49c) was obtained from BD Pharmingen, USA. Rat anti-mouse β1 integrin monoclonal antibody (clone Mab1.2) and mouse anti-mouse MT1-MMP monoclonal antibody (clone 113-5B7) was obtained from Calbiochem, USA. Goat anti-mouse MMP-9, anti-β actin antibody (clone AC-74), anti GFP N-Terminal antibody, polyclonal anti-rabbit FLAG antibody, anti-rat HRPO, anti-mouse HRPO, anti-rabbit FITC conjugate, anti-mouse FITC, anti-rat TRITC conjugate, Extra-Avidin FITC conjugate, Avidin Peroxidase were obtained from Sigma Chemical Co, USA. Goat anti-mouse uPAR antibody was obtained from R and D systems, USA. Rat anti-mouse α5 integrin monoclonal antibody (clone MFR5/5H10), rabbit anti-human α5 integrin polyclonal antibody (clone H104), rabbit anti-human CD151 polyclonal antibody (clone H-80), anti-goat HRPO and anti-rabbit HRPO were purchased from Santacruz Biotechnology, Inc., USA. Rabbit polyclonal CD82 antibody obtained from IMGENEX biotech Pvt. Ltd.,

India. Rabbit alexa 568, mouse alexa 568 and goat alexa 568 were obtained from molecular probes, Life technologies, USA.

2.2 METHODS

2.2.1 Maintenance of cell lines in-vitro

B16 murine melanoma variants were routinely cultured in Minimal Essential Medium (MEM) supplemented with sodium pyruvate, non-essential amino acids (1X), vitamins (2X), 0.03% L-glutamine, antibiotic-antimycotic solution, (100 units/ml of Penicillin G-sodium, 100 µg/ml of Streptomycin sulphate and 250 µg/ml of Amphotericin B), 0.04 mg/ml gentamycin and 5% FBS (Complete medium). Cell suspension ($1x10^6$ cells/ml) in 8 ml of complete medium was cultured in a 90 cm^2 tissue culture discs and incubated in a humidified atmosphere containing 5% CO_2 at 37ºC to achieve 90% confluency.

2.2.2 Cell harvesting, subculturing and cryopreservation

Confluent monolayer cell culture was washed with Phosphate Buffered Saline (PBS – 10 mM Sodium Phosphate and 150 mM NaCl, pH 7.4), and cells were harvested with trypsin solution (0.25% trypsin, 0.02% EDTA and 0.05% glucose in PBS) for one min. Action of trypsin was inhibited by addition of complete medium and it was removed by washing once with complete medium. The cell number and viability was checked by dye exclusion with 0.04% Trypan blue in TBS. Cells with greater than 95% viability were used for all the assays or sub cultured for next passage. Cells of early passage were preserved in cryopreservent (FBS containing 10% DMSO) in liquid Nitrogen. Each frozen stock contained about 2-3 x 10^6 cells/ml.

2.2.3 Revival of cell lines from frozen stocks kept in liquid Nitrogen

A vial from the frozen stocks of cell lines stored in liquid nitrogen was quickly thawed in water bath at 37°C followed by two washes with complete medium by centrifuging the cells at 1500 g for 10 min at 37°C. After centrifugation cell pellet was resuspended into 5 ml of complete medium and seeded into 25 cm^2 cell culture flask and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Next day medium was changed to remove nonviable, non-adherent cells.

2.2.4 Preparation of total cell lysates

Melanoma cells were harvested, washed thrice with chilled PBS pH 7.4 and solubilized in chilled lysis buffer containing 10 mM Tris chloride, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM magnesium chloride and 1 mM calcium chloride and protease inhibitors (1µg/ml of each of pepstatin, leupeptin, aprotinin and 0.3 mM PMSF). About 15 x 10^6 cells were lysed in 1 ml of lysis buffer by six cycles of sonication each cycle is of 30 sec with 30 sec intervals at 50% output on ice. The clarified supernatant (lysate) obtained by centrifugation at 20,000 rpm for 1 h at 4°C was aliquoted and stored at -20°C.

2.2.5 Protein estimation

Protein was estimated according to Peterson's modification of Lowry method [141]. Samples (5µls) were taken in 1 ml of distilled water and mixed with 1 ml each of Copper-Tartarate-Carbonate (CTC) reagent [Mixture of equal volumes of solution A (10% Sodium Carbonate) and Solution B (0.1% Copper Sulphate containing 0.2% Sodium Potassium

Tartrate)], 10% Sodium Dodecyl Sulphate (SDS), 0.8 N NaOH, and distilled water and incubated for 10 min. 0.5 ml of six times diluted Folin and Ciocalteau's reagent was mixed and incubated for 30 min in dark. Absorbance of solution was measured at 750 nm using a UV Spectrophotometer. Bovine Serum Albumin was used as the standard.

2.2.6 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as per the method described by [142]. 30% Acrylamide (29.2% acrylamide and 0.8% N,N' methylene bisacrylamide) was mixed with 1M Tris base (pH8.8) and 20% SDS, in order to obtain the desired resolving gel concentration containing 0.37 M Tris-Base and 0.1% SDS. Fifty micro litres each of ammonium persulphate (20% w/v) and TEMED (Tetraethylmethyl ethylene diamine) 10% (v/v) were added and the gel was poured between sealed glass plates with spacers. A stacking gel (4.5% Acrylamide, 0.0625 M Tris pH 6.8, 0.2% SDS), was overlaid on the polymerized resolving gel. The protein samples were boiled for 5 min with or without β-mercaptoethanol in sample buffer (0.0625 M Tris pH-6.8, 2% SDS, 10% glycerol (w/v), 750 mM β-mercaptoethanol and 0.05% Bromo Phenol blue) and loaded into wells. Electrophoresis was done using electrode buffer (0.025 M Tris-chloride, 0.2% SDS (w/v) and 0.192 M glycine) at 100 V (constant voltage). Finally, protein bands on the gels were detected by staining with either 0.2% Coomassie brilliant blue (in 50% methanol, 10% acetic acid and 40% miliQ water), or by Silver staining or proteins was transferred to polyvinylidene diflouride (PVDF) membrane for blotting.

2.2.7 Western Blotting

The transfer of proteins from the gel to a polyvinylidene diflouride (PVDF) membrane was done as per [143]. The resolving gel was equilibrated in chilled transfer buffer (0.025 M Tris, 0.192 M Glycine, and 20% Methanol) for 15 min. The membrane was pre-treated, super-imposed on the gel and the transfer apparatus was set as per manufacturers' instructions. Transfer of proteins to the membrane was carried out with chilled transfer buffer using a constant voltage of 90 Volts, for 3 h. Amount of protein transferred to the membrane was checked by soaking the membrane in 0.2% (v/v) Ponceau–S stain in 3% trichloroacetic and 3% sulphosalicylic acid, for 2 min. The stain was later washed off with Tris Buffered Solution (TBS-20 mM Tris and 500 mM NaCl).

2.2.8 Probing of Western blots with lectins or specific antibodies

Cell surface oligosaccharides on glycoproteins were studied by using carbohydrate specific lectins. Biotinylated lectin such as L-PHA (Leukoagglutinin phytohemagglutinin) is used as a probe for β1,6 branched N-linked oligosaccharides, LEA *(Lycopersicon esculentum)* for Poly-N-acetyllactoasmine, MAA/MAL-II *(Maackia amurensis)* and SNA *(Sambucas nigra* agglutinin*)*, respectively for α2,3 and α2,6 linked sialic acids and AAL (*Aleuria aurantia* lectin) for fucose. Lectin blots were subsequently developed using avidin peroxidase (AVP). Concentration of lectins and AVP used for lectin blotting is listed in **Table 1**.

Table 1: Shows the biotinylated lectins used for the study, their concentration and incubation time.

Lectin	Blocking (1hour)	Lectin incubation (1hour)	Avidin peroxidase (AVP) incubation (1hour)
Biotinylated L-PHA	T-TBS	2µg/ml	1:30,000
Biotinylated LEA	T-TBS	.5µg/ml	1:30,000
Biotinylated SNA	T-TBS	.25µg/ml	1:30,000
Biotinylated MAL-II	T-TBS	2µg/ml	1:30,000
Biotinylated AAL	T-TBS	2µg/ml	1:30,000

T-TBS Tris Buffered saline containing 0.1% Tween 20, 20 mM Tris pH 7.4 and 500 mM NaCl

The PVDF membranes blotted with protein were blocked with either 3% BSA or 5% milk and probed with their specific primary antibodies and secondary HRPO conjugated antibody. Concentration of primary antibodies and their respective secondary HRPO conjugated antibody is listed in **Table 2**. The blots were developed using Enhanced Chemiluminiscent (ECL plus) reagent.

Table 2: Shows the primary antibodies and their respective secondary antibodies used for the study, their concentration and incubation conditions.

Antibody	Blocking	*Primary Antibody Incubation (1h at RT)	Secondary Antibody Incubation (1h) at RT
β1 Integrin	5% Skimmed milk	1:1000 in 1.6% Skimmed milk	1µg/ml Anti-Rat HRPO in1.6% Skimmed milk
α3 Integrin	5% Skimmed milk	1:250 in 1.6% Skimmed milk (O/N)	1µg/ml Anti-mouse HRPO in1.6% Skimmed milk
α5 Integrin	5%Skimmed milk	1:1000 in 1.6% Skimmed milk	1µg/ml Anti-Rat HRPO in1.6% Skimmed milk
CD44	3% BSA	1:1000 in 1% BSA	1µg/ml Anti-Rat HRPO in1%BSA
CD82	3% BSA	1:2500 in1% BSA	1µg/ml Anti- Rabbit HRPO in1%BSA
CD151	5% Skimmed milk	1:1000 in 1.6% Skimmed milk	1µg/ml Anti- Rabbit HRPO in1.6% Skimmed milk
GFP	5% Skimmed milk	.5µg/ml in 2.5% Skimmed milk	1µg/ml Anti- Rabbit HRPO in1.6% Skimmed milk
uPAR	5% Skimmed milk	1:1000 in 1.6% Skimmed milk	1µg/ml Anti- goat HRPO in1%BSA
MT1-MMP	3% BSA	1:1000 in 1% BSA	1µg/ml Anti-mouse HRPO in1%BSA
MMP-9	3% BSA	.3µg/ml in1%BSA (O/N)	2µg/ml Anti- goat HRPO in1%BSA
β-actin	3% BSA	.1µg/ml in1%BSA	.5µg/ml Anti-mouse HRPO in1%BSA
FLAG	3%BSA	.1µg/ml in1%BSA	.5µg/ml Anti-mouse HRPO in1%BSA

*Primary antibody incubation was for 1h unless mentioned overnight (O/N) at 4°C.

2.2.9 Purification of L-PHA reactive proteins

For purification of L-PHA reactive proteins, total cells suspended in buffer containing 10 mM Tris-HCl (pH 7.5), containing 150 mM NaCl and protease inhibitor (1μg/ml) each of pepstatin, leupeptin and aprotinin, and 0.3 mM PMSF) were sonicated five times for 30 sec each at (50% output control) at 4°C. The lysate was supplemented with N-octyl-β-D-glucopyranoside and protamine sulphate to attain a final concentration of 30 mM and 0.3%, respectively and further incubated for 1 h on ice. Cells supernatant was collected by centrifugation at 16,000 g for 1 h at 4°C as described in [144]. 2000 μg Lysate from B16BL6 cells were then incubated with 100 μl L-PHA agarose beads (Vector labs) overnight at 4°C. Unbound and non-specifically bound proteins were removed by giving five washes with wash buffer (10 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, 3 mM N-octyl-β-D-glucopyranoside). Bound proteins were then eluted using non-reduced Laemmli sample buffer, containing 4M urea.

2.2.10 Purification of FLAG tagged CD151 using anti-FLAG M2 affinity gel

B16BL6 cells expressing FLAG tagged CD151 (CD151-FLAG) were treated with SW for 48 h. Swainsonine treated and untreated cells were homogenized in the lysis buffer containing 20 mM Tris HCl pH 7.4, 100 mM NaCl, 4 mM EDTA, 1% NP-40 detergent, and cocktail of protease inhibitors (1μg/ml each of pepstatin, leupeptin and aprotinin, and 0.3 mM PMSF) using Down's homogenizer (20 strokes of tight fit homogenizer) and kept at 4°C for 5 h, followed by centrifugation at 16,000 g for 30 min. Two thousand microgram of cell lysates was incubated overnight with 100 μl anti-FLAG M2 affinity gel (50%

suspension) at 4°C on rocker shaker. The beads were washed four times with the lysis buffer. Bound proteins were eluted using 100 µg/ml of 3x FLAG peptides.

2.2.11 Flow cytometric analysis

For flow cytometry, 90% confluent melanoma cells were harvested as described above. Cells were washed thrice with PBS and then fixed with 1.5% glutaraldehyde or 1% paraformaldehyde in PBS (pH 7.4) by overnight incubation at 4°C. The cells were pelleted and washed 3 times with PBS to remove fixative followed by blocking with FACS buffer (1% FBS in PBS). Subsequently, surface expression of β1,6 branched N-oligosaccharides, poly-N-acetyllactosamine, sialic acids, fucose and tetraspanins CD82, CD151 and their associated integrin receptors (α3, α5 and β1), MT1-MMP and uPAR was analysed by incubating with either biotinylated lectins or specific primary antibodies, respectively for 1 h. This was followed by incubation with fluorophore tagged streptavidin or secondary antibody, respectively. Concentration of biotinylated lectins and antibodies used for flow cytometric analysis are listed in **Table 3** and **4**, respectively. Labeled cells were acquired using FACSCalibur (BD Biosciences) and data was analysed using CellQuest Pro software.

Table 3: Shows the concentration of biotinylated lectins and streptavidin FITC used for the flow cytometric analysis of cell surface oligosaccharides.

S. No.	Lectins	Amount/40µl	Streptavidin FITC
1	Biotinylated L-PHA	20µg	1:50
2	Biotinylated LEA	20µg	1:50
3	Biotinylated SNA	20µg	1:50
4	Biotinylated MAL-II	20µg	1:50
5	Biotinylated AAL	20µg	1:50

Table 4: Show the concentration of primary antibodies and secondary FITC conjugated antibodies used for the flow cytometric analysis of cell surface proteins.

S. No.	Antibodies	Dilution	Secondary FITC conjugated antibody	Dilution
1	β1 Integrin	1:50	Anti-Rat	1:100
2	α3 Integrin	1:25	Anti-mouse	1:50
3	α5 Integrin	1:50	Anti-Rat	1:50
4	CD44	1:50	Anti-Rat	1:50
5	CD82	1:50	Anti-Rabbit	1:50
6	CD151	1:50	Anti-Rabbit	1:50
8	uPAR	1:10	Anti-Goat	1:50
9	MT1-MMP	1:50	Anti-mouse	1:50

2.2.12 Colocalization by indirect immunostaining

Cell were seeded on coverslip and grown overnight in complete medium up to 70-80% confluency. Cells were washed thrice with PBS (pH 7.5) and fixed with 2% paraformaldehyde at RT for 5 min. Cells were again washed with PBS and blocked with 3% BSA in PBS for 1h at RT in humidified chamber. BSA blocked cells were incubated with primary antibody for 1h in humidified chamber followed by three washes with PBS to remove excess or non-specifically bound antibody. Cells were further incubated with fluorescent tagged secondary antibody for 1h followed by three washes. Cell incubated only with fluorescent tagged secondary antibody serve as iso-control. Nuclei were stained with DAPI and coverslips were mounted on slides using vectashield mounting medium. Images were acquired using a Carl Ziess Laser confocal microscope.

2.2.13 Cell spreading assays

Cell spreading assays were done as described by [137]. Briefly, melanoma cells were harvested, washed thrice with plain medium (MEM without FBS) to remove FBS and seeded at a cell density of 0.5 x 10^6 cells/ml in plain medium on the coverslips coated overnight with 10 μg/ml fibronectin and matrigel each in serum free MEM at 4°C. The coverslips were blocked with 2% BSA for 1h at 37°C. Coverslips coated with BSA only, served as control. Bound cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 15 min and stained with 2 μg/ml Phalloidin FITC or TRITC staining solution made in PBS (containing 1μg/ml of Lysolecithin, 10% Methanol, 0.5% BSA in PBS) for 15 min at 37°C. Nuclei were stained with 5 μg/ml of 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS for 1-2 min. The stained cells were mounted using vectashield (Vector lab) and images were acquired using a Carl Zeiss Laser confocal Microscope at 63x magnification.

2.2.14 Wound healing assays

Wound healing assays were performed in 6 well plates coated overnight with fibronectin and matrigel each at a conc. 10 μg/ml (as representatives of ECM and BM components, respectively) in plain medium at 4°C followed by blocking of non-specific sites with 2% BSA for 1 h at 37°C. Melanoma cells were harvested and seeded at a cell density of 0.5 x 10^6 cells and incubated at 37°C for 24 h in a CO_2 incubator. The cells were subsequently washed free of serum and serum starved for 24 h for cell synchronization. A straight, uniform wound (approx. 400 μm in width) was made using a 2.5 μl micropipette tip on the plate upon reaching 95% confluency. The dislodged cells were washed off and

the cells were maintained in MEM. Wound closure of cells in response to the above ECM and BM was measured for 24 h by time lapse video imaging of at least three different positions across the length of the wound using a Carl Ziess Inverted Microscope. Uncoated culture dishes, blocked only with BSA served as control. Mean values of triplicate wound area closure for each position was analysed using the ImageJ 1.43 (NIH) software.

2.2.15 Sialidase treatment

Briefly, 1 x 10^6 tritium (H^3) labelled cells were treated with 2,3 and 2,3/2,6 linkage specific sialidase 50 U/ml and 200 U/ml respectively, in plain medium pH 7.4 and incubated at 37°C for 1 h with intermittent tapping at interval of 15 min. Same number of cells incubated in plain medium at 37°C without sialidase for 1 h served as control. After incubation, cells were washed with plain medium thrice and one wash with PBS. These cells were further used for adhesion assay as described below.

2.2.16 Adhesion assays

Melanoma cells were either radiolabeled with tritiated thymidine or labelled with Calcein AM. For radiolabeling 10 μCi/ml of tritiated thymidine in complete MEM was added at 50% confluency and the cells allowed to grow overnight at 37°C in CO_2 incubator. Cells were harvested, washed with plain medium (MEM without serum) to remove serum, and radioactivity incorporation (CPM/cell) was assessed. For Calcein AM labelling 95% confluent cells were incubated with Calcein AM 3 μg/ml for 30 min at 37°C in humidified CO_2 incubator. Labelled cells were harvested and washed with plain medium to remove serum. Adhesion assays were performed in 96 well plates. Briefly, the wells were coated with fibronectin (representative of ECM component) and matrigel (representative of BM

component) overnight at 4°C, each at a concentration of 10 µg/ml in 0.1 ml plain MEM. Forty thousand tritium labeled cells, suspended in 100 µl of plain MEM were added to each well in triplicate and incubated at 37°C for 1 h in a CO_2 incubator. Wells were gently washed five times with 100 µl of PBS to remove the unbound cells. A separate plate (control plate) was also seeded in triplicate with 40,000 cells from each cell type/treatment group, which was not washed, served as a control for checking initial cell seeding. The bound cells were detached by 0.25% trypsin and harvested on glass fiber filters using Combi Cell Harvesting machine (Molecular Devices). Radioactive counts of the bound and initially seeded cells were taken using beta scintillation counting. In case of Calcein AM labeled cells, the fluorescence was captured by Mithras LB 940 multimode microplate reader (Berthold technologies). The percent adhesion was calculated as percentage of bound cells with respect to adhesion of B16BL6 or untreated cells which was taken as 100%.

2.2.17 Haptotaxis assays

Haptotaxis assays were performed using, polycarbonate filter with 8-µm pore size, the lower surface of the filter is coated with fibronectin and matrigel (each 10 µg/ml) and blocked with 0.1% BSA, in triplicates on 24-well transwell units. Briefly, 0.2 x 10^6 tritium labelled cells suspended in 300 µl MEM were added to the upper compartment of the Boyden chamber, and 600 µl of plain medium were added to the lower compartment. Cells were allowed to migrate for 6 h at 37°C in a humidified atmosphere containing 5% CO_2. After incubation, the cells from the upper side of inserts were removed by cleaning with

cotton swabs. The filters were cut from the inserts and radioactive counts were measured using β scintillation counter.

2.2.18 Invasion assays

Invasion assays were performed using matrigel coated inserts (30 μg of 1 mg/ml matrigel per insert) with 8 μm pore size polycarbonate filter on 24 well transwell units. Briefly, 0.2 x 10^6 tritium labelled cells suspended in 300 μl MEM were added to the upper compartment of the Boyden chamber, and 600 μl of spent medium (complete medium collected from 50 % confluent culture of B16BL6 cells grown in complete medium) were added to the lower compartment. The spent medium served as attractant for the cells. Cells were allowed to invade for 32 h at 37°C in a humidified atmosphere containing 5% CO_2. After incubation the cells from the upper side of inserts were removed by cleaning with cotton swabs. The filters were cut from the inserts and radioactivity was measured using β scintillation counter.

2.2.19 Gelatin zymography

Melanoma cells were seeded at a density of 1 x 10^6 cells in 3 ml of complete MEM, in a 60 mm culture dish overnight coated with fibronectin (10 μg/ml) and matrigel (50 μg/ml) in serum free MEM at 4°C. Cells seeded on uncoated culture dish served as control. The cells were incubated for 24 h at 37°C in a CO_2 incubator. The cells were washed free of serum and incubated in plain MEM for 24 h. The medium obtained after 24 h of culture (conditioned medium) was collected, centrifuged at 5000 rpm for 10 min to remove cellular

debris and further concentrated 10 times using 10 kDa cut off filter (Amicon, Millipore). Concentrated samples were dried by speed vac. and stored in -20°C till use.

To detect gelatinase activity, each dried conditioned medium was reconstituted in equal volume 1x Laemmli buffer was loaded on 10% SDS-PAGE containing 0.1% gelatin and run under non-reduced conditions at 4°C. After electrophoresis, the gel was rinsed in renaturing solution (2.5% Triton X-100) for 1 h under mild shaking conditions. The gel was washed thrice with 0.1 M Tris-HCl (pH 7.5) to remove Triton X-100 and gel was further incubated in a buffer containing 0.1 M Tris (pH 7.5) and 50 mM $CaCl_2$ for 36 h at 37°C in a water bath. The gel was stained in 0.2% Coomassie Blue for 1 h and destained with 50% destaining solution (50% methanol, 10% acetic acid and 40% miliQ water) to visualize the clear zone of lysis.

2.2.20 Reverse transcription and semiquantitative-PCR

Total RNA was isolated from melanoma cell lines using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was determined by measuring absorbance at 260 nm using Nano Drop 1000 spectrophotometer. The first strand cDNA was synthesized by Protoscript First strand cDNA synthesis kit (New England Biolabs, USA) using oligo (dT) primers and M-MuLV reverse transcriptase as per manufacturer's protocol. Primers sequences which were used to check the expression of tetraspanins, GAPDH and GnT-V by semiquantative PCR are mentioned in **Table 5**, GAPDH served as loading control. The primer sequence for the expression of MT1-MMP and uPAR is mentioned in **Table 6**.

Table 5: Shows the sequence of primers used for semiquantative RT- PCR for tetraspanins, GnT-V, GAPDH and CD151 amplification.

A	**Primer sequences used for expression of tetraspanins (5'-3')**
1	CD151 Forward: TAAAGTGGAGGTGGCTGTAT Reverse: GTAGTGGGGCTGGCACATAG
2	CD82 Forward: TTCCGTCCTACAAACCTCAT Reverse: TTCCGTCCTACAAACCTCAT
3	CD81 Forward: TCCATGAGACGCTCAACTGT Reverse: GCACCATGCTCAGAATCATC
4	CD63 Forward: CAAGGACAGAGTCCCGATT Reverse: TCCCAAGACCTCCACAAAAG
5	CD9 Forward: CATGCTGGGATTGTTCTTCG Reverse: GCTCCAAAGGACCAGCTATG
B	**GAPDH (House Keeping gene)** Forward: TGAAGGTCGGTGTGAACGGATTTG Reverse: CATGTAGGCCATGAGGTCCACCAC
C	**CD151 (Primer for gene amplification)** Forward: AAGCTTATGGGTGAATTCAATGAGAAGAAG Reverse: GGTACCCAGTAGTGTTCCAGCTTGAGGCTTC
D	**GnT-V** Forward: TCCTCGAGGTTGAAAATAGGTGTCC Reverse: ACCTCGAGCATGCACTGGTAATGAAC

Table 6: Shows list of sequences of primer used for studying the expression of protease and their receptor.

A	Primer for the expression of protease (5'-3')
1	MT1-MMP Forward: AGTAAAGCAGTCGCTTGGGT Reverse: TGGGTAGCGATGAAGTCTTC
2	uPAR Forward: CACAAACCTCTGCAACAGGC Reverse: GTAGCCACCAGGCACTGATT
B	**Real time primer sequence for MMP-9 (5'-3')** Forward: TCATTCGCGTGGATAAGGAG Reverse: AGGCTTTGTCTTGGTACTGG

Table 7: Shows list of primer sequences used for sequencing

A	**pJET1.2 (5'-3')** Forward: CGACTCACTATAGGGAGAGCGGC Reverse: AAGAACATCGATTTTCCATGGCAG
B	**CMV (5'-3')** Forward: TGACGTCAATGGGAGTTTGT
C	**pTRIPz (5'-3')** Forward: GGAAAGAATCAAGGAGG

2.2.21 Quantative RT–PCR

For detecting the transcript levels of GnT-V, primers specific for it were synthesized. Ribosomal protein L4 (RPL4) was used as housekeeping gene for relative quantification of transcript levels. The primers sequence for detecting GnT-V and RPL4

transcript levels are mentioned in **Table 8**. The real time PCR reaction was carried out in 7900 HT system (ABI Prism) and for detecting amplicons Power SYBR green (ABI) was used. The data represents mean of two different experiments carried out in duplicates with different batches of cDNA. The Ct values obtained were normalized to RPL4 values which served as housekeeping control. Analysis was performed using $2^{-\Delta\Delta Ct}$ method [145].

2.2.21 Cloning of GFP tagged and FLAG tagged CD151

Murine CD151 gene was PCR amplified from total cDNA using forward and reverse primers for CD151 using high fidelity polymerase Pfu. A forward and reverse primer sequence used for CD151 amplification is mentioned in **Table 5**. Forward and reverse primers incorporated HindIII and KpnI sites respectively, the PCR product was cloned into pJET1.2 cloning vector (Fermentas) (**Illustration 7**), from which it was subcloned into pEGFP-N1 vector (Clontech) (**Illustration 7**) between HindIII and KpnI restriction sites. PCR conditions for CD151amplification (50 µl reaction) is mentioned below.

Temp.	Time (Min)	Components	Volume (µl)
95(Initial denaturation)	5	Forward primer	2
95 (Denaturation)	0.30	Reverse primer	2
55 (Annealing)	0.30	10X buffer	5
72 (Extension)	0.30	dNTPs (10mM)	2
72 (Final extension)	10	Taq polymerase	0.5
No. of cycles: 30		MiliQ H_2O	38.5

Sequence of pEGFP–CD151 construct was verified by sequencing, using specific primers for vectors mentioned in **Table 7**. To clone CD151 in FLAG tagged vector, CD151 gene from pEGFP-CD151 clone was excised by HindIII and KpnI digestion and cloned into C-terminal p3X FLAG-CMV vector (Sigma) (**Illustration 7**).

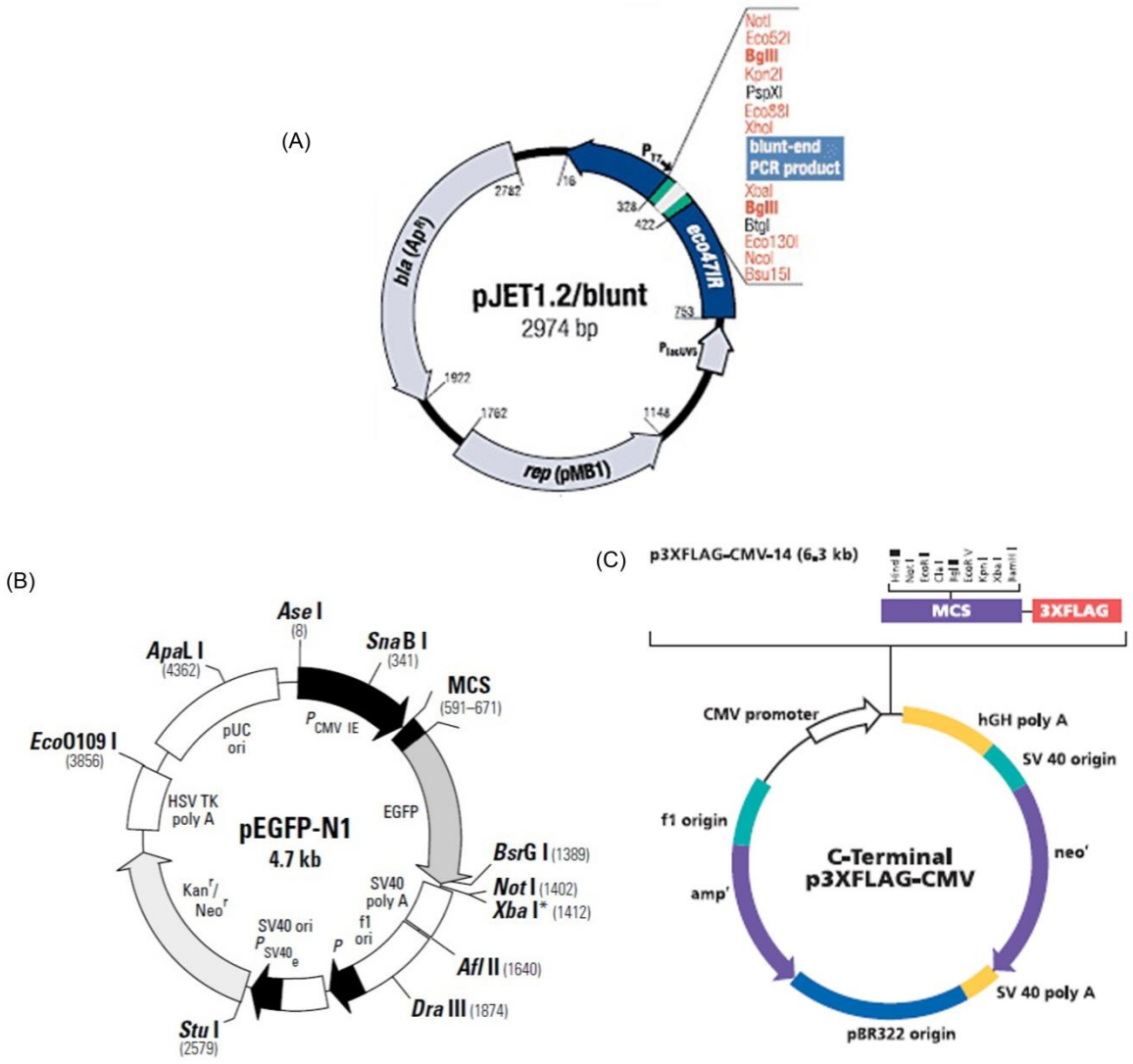


Illustration 7: Vector maps of the vectors used for CD151 cloning

2.2.22 Transfection & selection of clones

Exponentially growing culture of B16BL6 cells were harvested and seeded on 60 mm culture dishes and grown up to approximately 80% confluency. Typically 8 µg of plasmid DNA and 24 µl of lipofectamine™ 2000 solution, each was diluted in 0.5 ml plain MEM in sterile microcentrifuge tube. The DNA and lipofectamine solution was gently mixed with pipette, and added to the culture dishes drop wise, the plate was gently swirled and incubated overnight at 37°C. The medium was removed and culture was rinsed twice with PBS or plain MEM. The cells were then grown for 24 h in complete medium.

The cells were harvested and seeded into 100 mm petri dishes and grown in complete medium containing Neomycin (G418) at 1300 µg/ml for selecting the transfectants. Medium was replaced with fresh medium every 3 days, till transfected cells formed isolated colonies. These colonies were picked up by spot trypsinization and cultured in 24 well plates and maintained at a G418 concentration of 1000 µg/ml.

2.3 Strategy to downregulate ST6Gal-1 and GnT-V enzymes on melanoma cells and analysis of its effect on cellular and metastatic properties

2.3.1 Designing and cloning of shRNA constructs

Downregulation of α2,6 linked sialic acids and β1,6 branched N-linked oligosaccharides in B16BL6 cells were carried out by short hairpin RNA against enzymes ST6Gal-1 and GnT-V, respectively. The shRNAs were designed against both the enzymes as per guidelines outlined by [146]. A 22 nucleotide sequence from the open reading frame of murine ST6Gal-I and GnT-V were searched and their specificity was checked by

performing an online BLAST search of the mouse NCBI sequence database. ShRNA cassette was PCR amplified using forward primer having XhoI site followed by sense sequence and 15 nucleotide loop sequence. The reverse primer contained EcoRI site, sense sequence and 15 nucleotide loop sequence. 11 nucleotide loop sequences are complementary to each other (**Illustration 7**). Primer sequences used for shRNA amplification are mentioned in (**Table 8**).

PCR conditions for shRNA amplification (100 µl reaction)

Temp.	Time (Min)	Components	Volume (µl)
94(Initial denaturation	5	Forward primer	4
94 (Denaturation)	5	Reverse primer	4
50 (Annealing)	1.30	10X buffer	10
72 (Extension)	1	DMSO (10%)	10
72 (Final Extension)	20	dNTPs (10mM)	2
No. of cycles: 30		Taq polymerase	2
		Milliq H_2O	68

PCR products were run on agarose gel cut purified and digested with XhoI and EcoRI. Digested products were again run on agarose gel and DNA was purified from the cut bands using gel extraction kits. Inducible lentiviral vector pTRIPz (**Illustration 8**) was linearized by XhoI and EcoRI digestion. The linearized vector and XhoI and EcoRI digested PCR product was ligated in 1:9 ratios (vector: insert) using T4 DNA ligase Enzyme (NEB), overnight at 22°C.

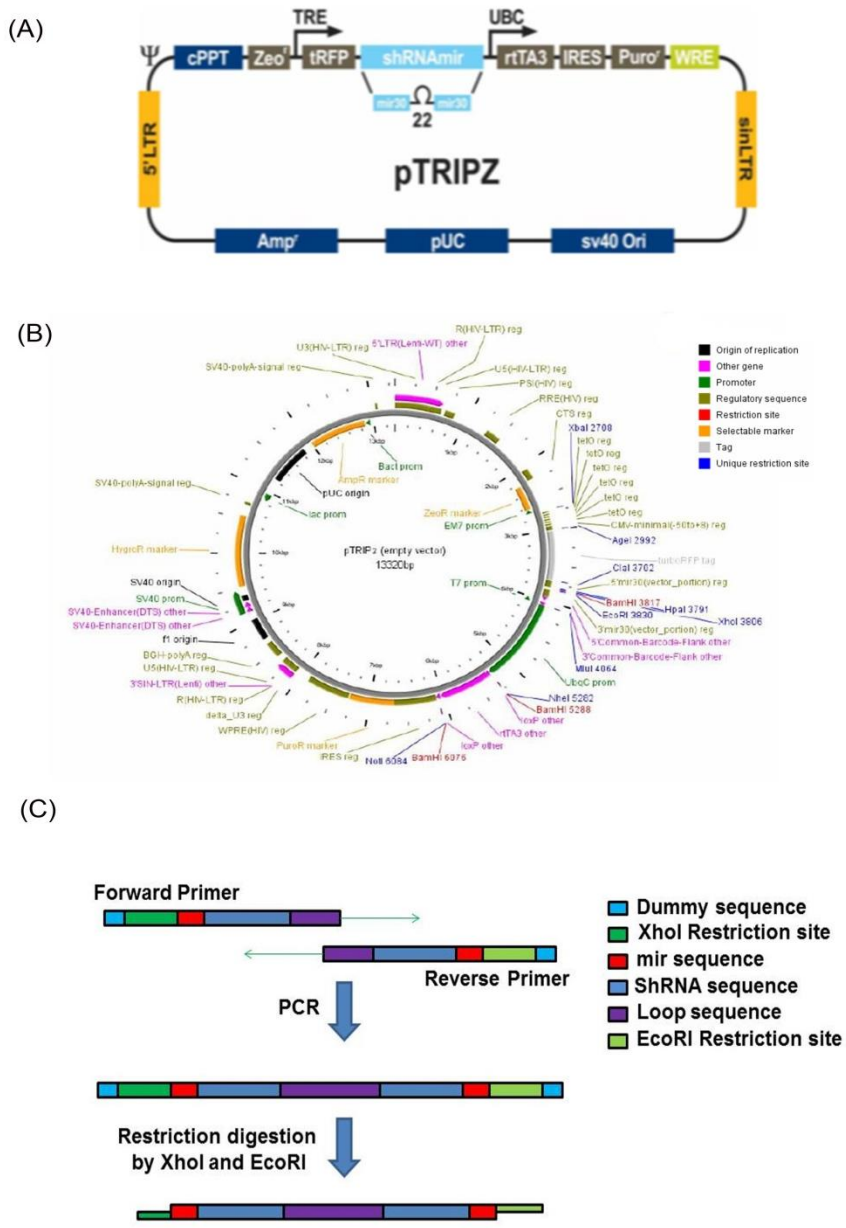


Illustration 8: (A) pTRIPz inducible lentiviral vector (B) pTRIPz inducible lentiviral vector map (C) PCR strategy.

Table 8: Show the sequence of primers used for shRNA amplification and primer used for their validation of knockdown at transcript level

A	**shRNA amplification primer (5'-3')**
1	ST6Gal-I shRNA Forward: GAACTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCGAGAGAT TGATAATCATGATTAGTGAAGCCACAGA Reverse: TTGAATTCCGAGGCAGTAGGCACGAGAGATTGATAATCATGATT ACATCTGTGGCTTC
2	GnT-V shRNA-I Forward: GAACTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCTGGAAGAATAA GAAAATCTATAGTGAAGCCACAGA Reverse: GTTGAATTCCGAGGCAGTAGGCACTGGAAGAATAAGAAAATCTAT ACATCTGTGGCTTC
3	GnT-V shRNA-II Forward: GAACTCGAGAAGGTATATTGCTGTTGACAGTGAGCGGCAGATATCAT TAATGGAGTTTAGTGAAGCCACAGA Reverse: GTTGAATTCCGAGGCAGTAGGCAGCAGATATCATTAATGGAGTTT ACATCTGTGGCTTC
B	**Real time Primers (5'-3')**
1	GnT-V Forward: CCTGGGGAAAGTGGAATCTG Reverse: GCTGTTGTCTGGGGTATGAG
2	RPL4 (Housekeeping gene for real time PCR) Forward: GACAGCCCTATGCCGTCAGTG Reverse: GCCACAGCTCTGCCAGTACC

The ligated plasmid was transformed into ultra-competent DH5α cells. Competent cells transformed with linearized vector alone, served as negative control. Briefly ultra-competent cells were thawed and ligation mixture was added to it and incubated on ice for 30 min. Competent cells mixed with plasmids were given heat shock at 42°C for 90 sec and the mixture were subjected to cold shock on ice for 5 min. The cells were then mixed with 1ml sterile LB broth and incubated with at 37°C for 1 h in orbital shaker incubator. Cells were spun at 5000 rpm for 1 min cell pellet was resuspended in 200 µl of LB broth and spread on low salt LB agar plate containing 100 µg/ml, ampicillin and incubated overnight at 30°C.

2.3.2 Screening of the recombinant colonies

About ten Individual colonies that were grown on LB agar plates after transformation were picked up and inoculated into 1ml LB broth and allowed to grow overnight at 37°C. Plasmids were extracted from them by alkaline lysis method and recombinant clone were screened by restriction digestion.

2.3.3 Isolation of plasmids by alkaline lysis method

Plasmid isolation from the overnight grown culture was carried by the alkaline lysis method [147]. The bacterial culture of 1.5 ml was distributed in sterile microcentrifuge tubes and centrifuged at 5000 rpm, at 4°C for 5 min the medium was removed and the pellets were dried as possible. To the dried bacterial pellets 100 µl of alkaline lysis solution I (GTE buffer: 50 mM Glucose, 25 mM Tris pH8.0 and 10 mM EDTA) was added and vortexed till the pellets were completely dissolved. Then 200 µl of alkaline lysis solution II (0.2 NaOH and 1%SDS) was added, mixed gently by inverting and kept for 2 min 150 µl of

ice-cold alkaline lysis solution III (3M potassium acetate pH 4.8 in glacial acetic acid) was then added and kept on ice for 10 min the tubes were then centrifuged at 13000 rpm, 4°C for 15 min and the supernatant containing the renatured plasmid was transferred to a fresh tube. Then equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added for removal of proteins and saccharides, mixed by vortexing and centrifuged as above for 5 min. The aqueous phase was transferred to a fresh microcentrifuge tube and double the volume of absolute alcohol was added for precipitation of plasmid DNA and mixed well. Tubes were kept on ice for 15 min and centrifuged at the above conditions for 20 min. The ethanol was removed and the pellets were washed with 70% ethanol (chilled) to remove salts, centrifuged as above for 5 min and all the traces of ethanol was removed. The pellets were completely dried at 37°C for 30 min. The dried pellets was reconstituted in 20 µl of autoclaved distilled water and 0.3 µl of RNase (1µg/ml) was added to each tube and incubated at 37°C for 45 min to degrade RNA molecules.

Positive colonies containing the shRNA were screened or checked by XhoI and EcoRI digestion, positive clones gave an insert release of 120 bp. Presence of positive clones was also confirmed by EcoRI and AgeI digestion positive clones gave an insert release of 958 bp whereas empty vector without shRNA gave an insert release of 838 bp.

2.3.4 Maxi prep of plasmid DNA

Maxi prep is different from miniprep as the starting *E.coli* culture volume is 100-200 ml LB broth. Maxi prep was performed using kit based method from Sigma Aldrich. 200 ml of overnight culture was pelleted by centrifugation at 5000 g for 10 min. The pellet was resuspended in 12 ml resuspension solution and vortexed until the pellets were

completely dissolved. Cells were lysed by adding 12 ml of lysis solution and mixed thoroughly by gently inverting the tubes for 6-8 times and the tubes are kept at RT for 3 min. 12 ml of chilled neutralization solution was added to neutralize the lysed cells resulting in the formation of a white aggregate (cell debris, proteins, lipids, SDS and chromosomal DNA). Then 9 ml of binding solution was added to the pellet and poured into the barrel of the filter syringe and were incubated for 5 min at RT. The white aggregate should float at the top. During incubation the binding column was prepared by adding 12 ml of column preparation solution and was centrifuged at 5000 g for 5min. By holding the filter syringe barrel over binding column the plunger was gently inserted to expel the clear lysate into the column. The binding column was then spun in a swinging bucket rotor at 3000 g for 2 min and the eluent were discarded. The column was then washed with wash solution 1 and 2 and centrifuged at 5000 g for 2 min and 5 min respectively. The column was then transferred to a fresh 50 ml collection tube and 3ml of elution solution was added and were kept at RT for 20 min and were then centrifuged at 5000 g for 5min for the elution of DNA.

2.3.5 Preparation of ultracompetent E.coli Dh5α

Reagents required

SOB (300 ml)

Tryptone -6 gm

Yeast extract-1.5 gm

NaCl- 0.15 gm

KCl- 5.6 gm

The pH of the SOB medium was adjusted to pH 7 with 1N NaOH, followed by the addition of 4 ml 1 M glucose and 1ml of 2 mM $MgCl_2$, just prior to inoculation of bacteria.

Transformation Buffer (200ml)

PIPES- 0.6 gm

$CaCl_2$ 0.4 gm

KCl- 3.7 gm

The pH was adjusted to pH 6.7 with 10N KOH and 2.18 gm $MnCl_2$ and sterilized by filtering it through 0.22µm filter.

Ultra competent *E. coli* Dh5α cells were prepared as described [147]. A single colony of Dh5α strain of *E. coli*, from an overnight grown LB agar plate was inoculated into 250 ml of SOB medium and incubated at 18°C with mild shaking till the optical density (OD) at 600 nm reaches between0.3 to 0.5. The culture was incubated on ice for 10 min followed by centrifugation at 2500 g for 10 min at 4°C to pellet the bacterial culture. The culture supernatant was discarded and the bacterial cells pellets were gently resuspended in 80 ml of ice cold transformation buffer. The cell suspension was incubated on ice for 10 min followed by centrifugation at 2500 g for 10 min at 4°C. The supernatant was discarded and the bacterial cell pellet was resuspended in 20 ml of ice cold transformation buffer containing 7% DMSO. This suspension of cells was incubated on ice for 10 min and subsequently aliquoted nearly 200 µl into 1.5 ml micro centrifuge tubes, snap freezed into liquid nitrogen and immediately stored at -80°C until further use.

2.3.6 Preparation of lentiviral particle and transduction

For the generation of lentivirus particle, empty vector or non-target shRNA or the cloned shRNA constructs in inducible vector pTRIPz along with helper constructs psPAX2 and pMD2.G which codes for viral capsid and envelop proteins, respectively were co-transfected into HEK293FT cells using Calcium Phosphate method of transfection (**Illustration 9**), viral supernatant were collected 24 h post changing of transfection medium, it was spun at 5000 rpm for 20 min at 4°C.

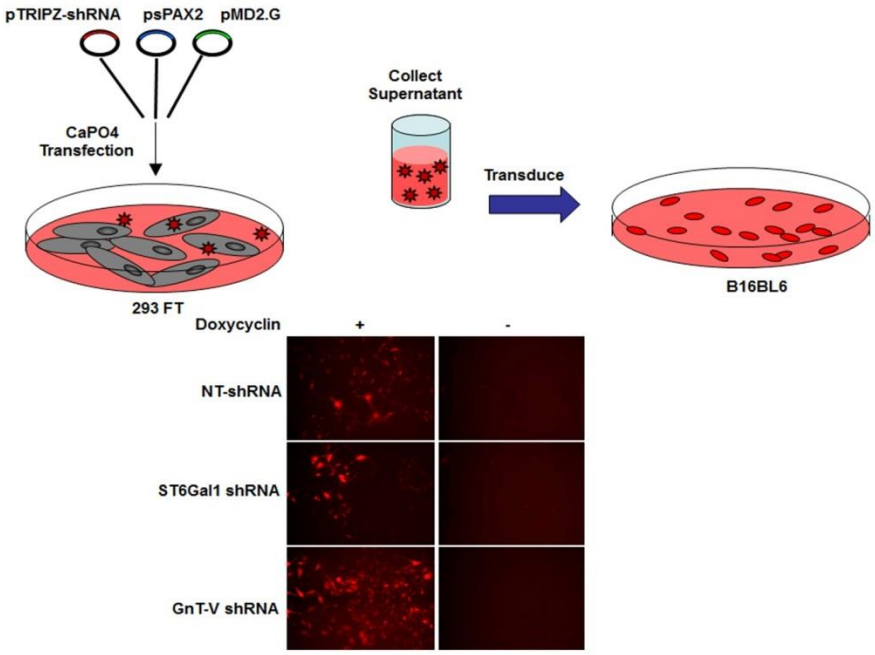


Illustration 9: Schematic representation of generation of lentiviral particle and transduction into B16BL6 cells

B16BL6 cells were transduced at 50% confluency with 1ml of viral supernatants using polybrene 8 µg/ml. Transduction medium was replaced by complete medium after giving three washes with plain medium. Transduced cells were then put under puromycin selection at a concentration of 1µg/ml. stably transduced cell lines were maintained at

puromycin concentration of 0.75 µg/ml. Expression of shRNAs were induced by the addition of doxycycline at a concentration of 4 µg/ml for 96 h.

2.4 Densitometric and statistical analysis

Densitometric quantitation of scanned images of Western blots and gelatin zymography gel images were done by ImageJ 1.43 software (NIH). Statistical analysis was performed using Graphpad Prism 5. The unpaired student's t-test was employed when two groups were compared. One way ANOVA was used to analyse more than two groups P value < 0.05 was considered as significant.

CHAPTER 3

RESULTS

3. RESULTS

Previous work in the lab has shown that the expression of β1,6 branched N-linked oligosaccharides correlates positively with their adhesive and invasive phenotype. This was established by using different approaches like, **i)** by comparing cell lines that differ in the expression of β1,6 branched N-linked oligosaccharides, **ii)** by inhibiting their formation using glycosylation inhibitor Swainsonine (SW) and **iii)** by inhibiting addition of these oligosaccharides by using antisense to enzyme GnT-V which catalyses addition of β1,6 branch [140].

The ability of B16 melanoma cells to adhere to most ECM/BM components also correlated with the expression of these oligosaccharides. However, in some other cell types its expression correlates negatively with adhesion. Their expression thus appears to regulate adhesion both positively and negatively. Depending on their basic adhesive ability, tumour cells possibly regulate adhesion to reach an optimum required for motility and invasion. Cells adhering too tightly would be rendered immobile and loosely adhered cells may not be able to generate sufficient traction for motility. Since the expression of these oligosaccharides almost always correlates with invasiveness, the cells possibly achieve optimum adhesion by altering the terminal substitutions on these oligosaccharides apart from altering the expression of the receptors themselves. This leads to the first objective of the thesis.

Objective I: To investigate the role of terminal substitutions on β1,6 branched N-linked oligosaccharides in regulating cellular adhesion and thus invasion

3.1 Characterization of the murine melanoma invasive variant B16BL6 and its parent cell line B16F10 for the expression of β1,6 branched N-linked oligosaccharides

Expression of β1,6 branched N-oligosaccharides as assessed by flow cytometry and Western blotting between parent cell line B16F10 and its invasive variant B16BL6 cells showed that it correlates with their invasiveness. The level of the transcript for the enzyme GnT-V, which catalyses the addition of β1,6 branched N-linked oligosaccharides also correlated with the invasiveness of the cell lines **(Figure 1A and B)**. Inhibition of their expression by α-mannosidase II inhibitor SW, significantly inhibited the expression of β1,6 branched N-oligosaccharides **(Figure 1C)** with the concomitant decrease in their ability to invade through reconstituted BM (matrigel) as compared to untreated B16BL6 cells **(Figure 1D)**.

3.2 Analysis of terminal substitutions associated with increased β1,6 branched N-linked oligosaccharides on melanoma invasive variants

Beta1,6 branched N-linked oligosaccharides have been shown to be the preferred site for the addition of various terminal sugars. Substitution of probable terminal sugars on such oligosaccharides were compared between murine melanoma invasive variants by Western blotting and flow cytometry **(Figure 2A and B)**.Terminal sugars could be sialic acids (SA), poly-N-acetyllactosamine (polylacNAc), or fucose. Sialic acids may be substituted in different linkages it may be either α2,3 or α2,6 linked. Sialic acids in these two linkages were studied by MAL-II and SNA blotting, respectively. Similarly, polylacNAc and fucose substitution were compared by probing with LEA and AAL, respectively. Result showed that SA in α2,6 linkage and polylacNAc were the major

substitution that were associated with increased β1,6 branched N-oligosaccharides and the more invasive, B16BL6 cells.

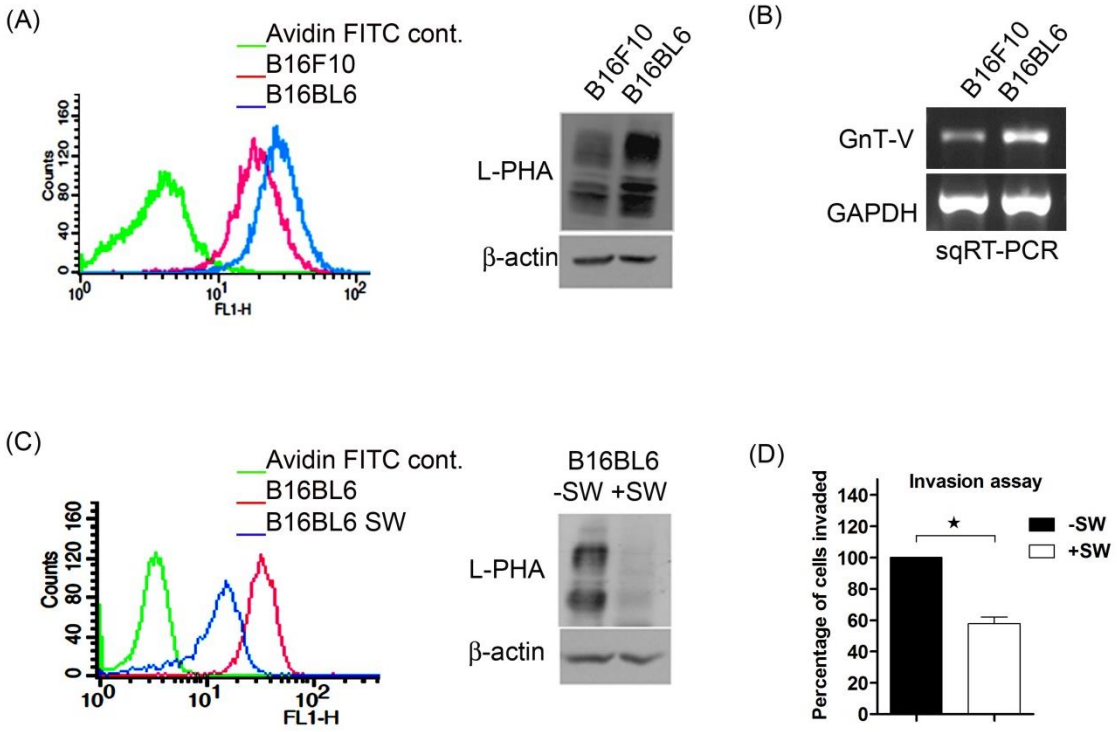


Figure 1: Expression of β1,6 branched N-linked oligosaccharides correlates with invasion. Expression of β1,6 branched N-linked oligosaccharides was compared between B16F10 and B16BL6 cells; and B16BL6 cells treated with Swainsonine (SW) (A and C) by flow cytometry and Western blotting using biotinylated lectin L-PHA as a probe. (B) Expression of enzyme GnT-V at transcript level by semiquantative RT-PCR (sqRT-PCR). B16BL6 cells treated with only avidin FITC served as control for flow cytometry. β-actin and GAPDH served as loading control for Western blotting and sqRT-PCR respectively. (D) Comparison of invasive ability of B16BL6 cells (-SW) and the same cells treated with Swainsonine (+SW). Number of invaded B16BL6 cells was taken as 100 percent. Values are mean ± SE of two independent experiments carried out in triplicate. Student's t test was performed to compare significance between two cell lines * $p<0.01$.

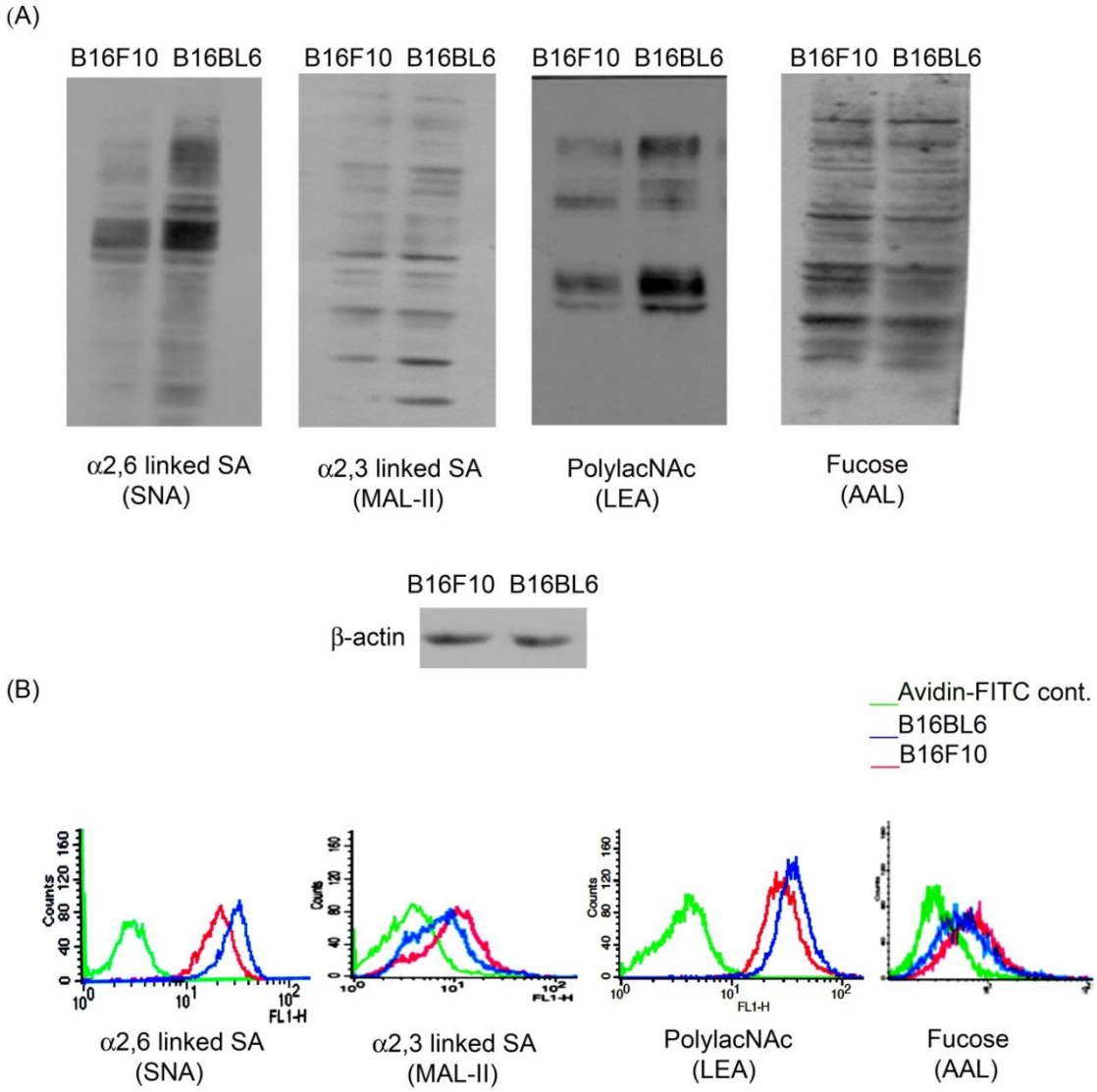


Figure 2: Comparison of terminal substitutions in melanoma invasive variants. Parent cell (B16F10) and its invasive variant (B16BL6) were compared for expression of terminal sugars. **(A)** By Western blotting and **(B)** by flow cytometry using biotinylated lectins as probe. β-actin served as loading control for Western blotting and cells treated only with avidin FITC served as control for flow cytometry.

3.3 Confirmation that proteins carrying β1,6 branched N-linked oligosaccharides are substituted with α2,6 linked sialic acid and polylacNAc

Sialic acid and polylacNAc are present on both N-linked and O-linked oligosaccharides. Their increased substitution on β1,6 branched N-oligosaccharides were further confirmed by purifying the proteins carrying β1,6 branched N-oligosaccharides using L-PHA–Agarose from cell lysates of both B16F10 and B16BL6 cells. L-PHA binding proteins from cell lysates of B16F10 and B16BL6 was normalized with respect to expression of β1,6 branch using lectin L-PHA as a probe for Western blots **(Figure 3 panel 1).** Comparison of proteins from both these cell lines carrying equal amounts of β1,6 branched N-oligosaccharides **(Figure 3 panels 2-5)** showed that oligosaccharides on B16BL6 cells carried significantly increased substitution of α2,6 linked SA **(Figure 3 panel 2**). These proteins also carried higher polylacNAc **(Figure 3 panel 4)**. If α2,6 linked SA on β1,6 branched N-oligosaccharides regulates adhesion their removal should have an effect on it.

3.4 Effect of enzymatic desialylation of α2,6 linked sialic acid on adhesion of B16BL6 cells to ECM and BM components

Sialic acids on glycoproteins are mainly present in either α2,3 or α2,6 linkages. Although, SA in α2,6 linkage are the major terminal substitution on β1,6 branched N-oligosaccharides on B16BL6 cells, there is no sialidase that specifically removes only α2,6 SA. There are sialidases that remove either α2,3 or both α2,3/6 linked SA. In order to study the effect of α2,6 linked SA on modulating adhesion, we have used these sialidases to remove either only α2,3 or both α2,3/6 linked SA to assess their effect on adhesion, assuming that this combination would help in deciphering the role of α2,6 SA in regulating

adhesion. Concentration of sialidase to be used for efficient desialylation without affecting the viability of cells are 50 U/ml and 200 U/ml for α2,3 and α2,3/α2,6 sialidase, respectively. Removal of sialic acids was checked by Western blotting (**Figure 4A and B**). Our result showed that removal of α2,3 linked sialic acids did not have much effect on adhesion whereas removal of sialic acids in both α2,3/6 linkage significantly decreased the adhesion of B16BL6 cells on fibronectin and matrigel (**Figure 4C**). This result indicates that sialic acids in α2,6 linkage have a positive role in promoting adhesion of B16BL6 cells. However, approaches that specifically target α2,6 SA on N-oligosaccharides would be required to confirm these results.

3.5 Effect of down regulation of α2,6 linked sialic acids on N-glycans on B16BL6 cells in regulating their adhesion and ability to invade through reconstituted BM (matrigel)

Induction of B16BL6 cells transduced with non-targeting (NT) shRNA with doxycycline did not have any effect on the expression of α2,6 linked SA as assessed by flow cytometry and Western blotting (**Figure 5A**), whereas induction in cells transduced with shRNA against ST6Gal-I, significantly inhibited the expression of α2,6 linked SA (**Figure 5B**). Decreased expression of α2,6 SA upon induction (ST6Gal-I shRNA +DOX) significantly decreased the adhesion of these cells on fibronectin and matrigel as compared to uninduced cells (ST6Gal-I shRNA -DOX). However, induction of non-targeting shRNA in B16BL6 cells (NT shRNA +DOX) did not alter the adhesion of cells to fibronectin and matrigel when compared with uninduced cells (NT shRNA -DOX cells) (**Figure 6A and B**).

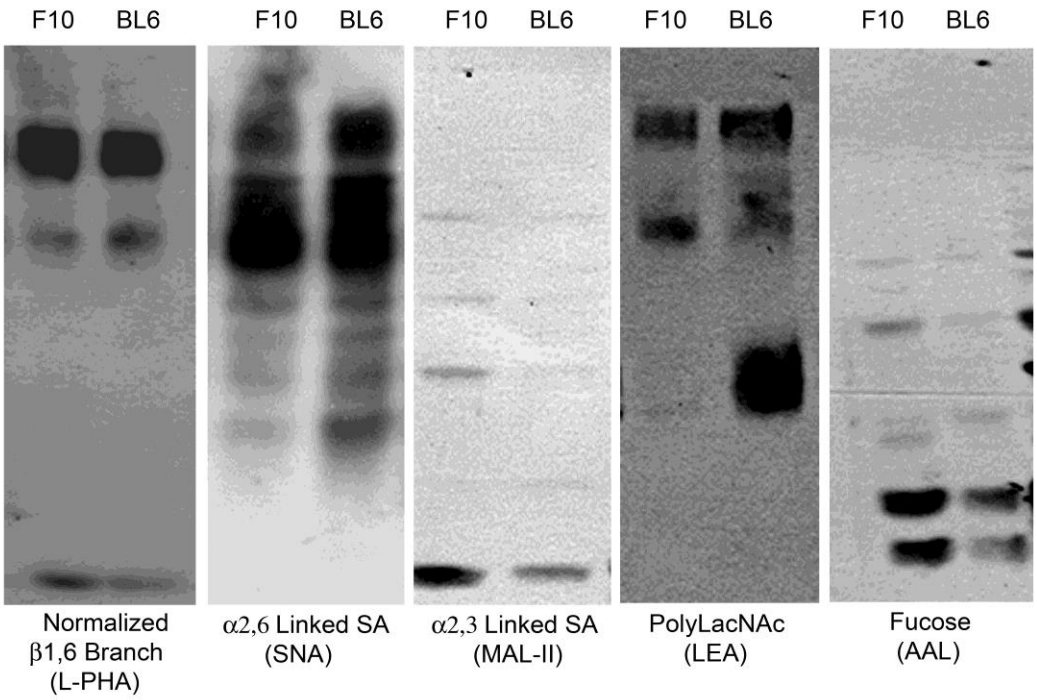


Figure 3: Increased expression of α2,6 linked SAs is associated with increased expression of β1,6 branched N-linked oligosaccharides. L-PHA binding proteins purified from whole cell lysates of B16F10 (F10) and B16BL6 (BL6) cells were normalized with respect to expression of β1,6 branched oligosaccharides by blotting and probing using biotinylated lectin L-PHA (Panel 1). Such normalized proteins from both the cell lines were blotted and probed for the terminal sugars with respective biotinylated lectins, SNA, MAL-II, LEA and AAL (panels 2-5).

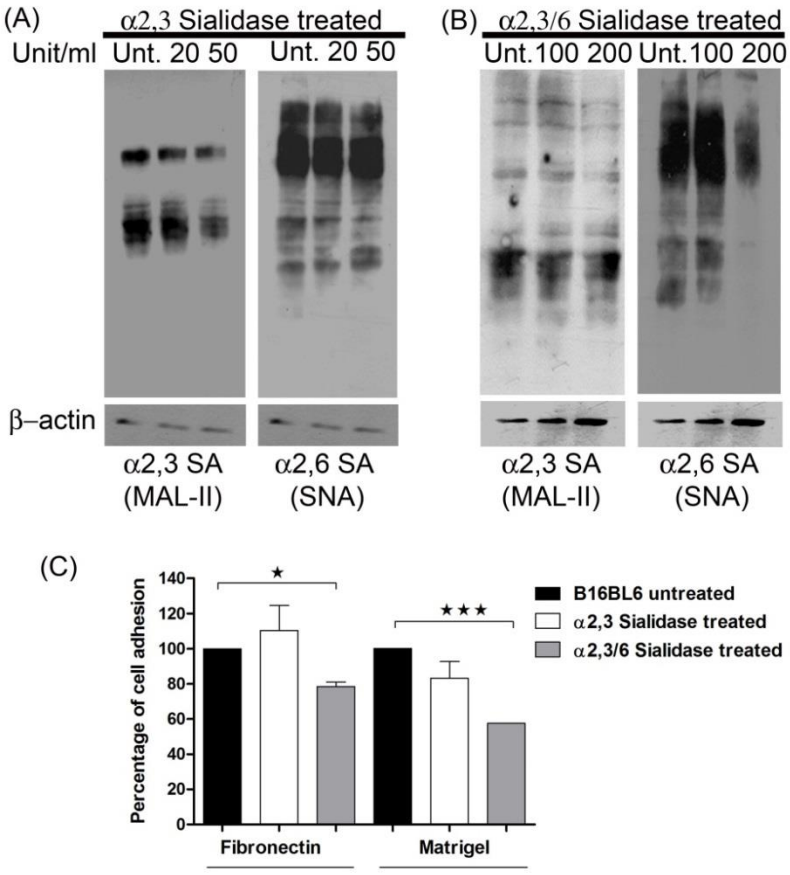


Figure 4: Enzymatic desialylation of α2,6 linked sialic acids decreases adhesion. Cell lysates from untreated (Unt) B16BL6 cells and those treated with **(A)** α2,3 linkage specific sialidase (20 and 50 unit/ml) and **(B)** α2,3/6 linkage specific sialidase (100 and 200 unit/ml) were Western blotted and probed with biotinylated lectin SNA and MAL-II. β-actin served as loading control for Western blotting. **(C)** Tritiated thymidine labeled B16BL6 cells were treated with 50 unit/ml and 200 unit/ml of α2,3 and α2,3/6 linkage specific sialidase respectively, and adhesion assay were performed as described in materials and methods. Untreated B16BL6 cells served as control and their adhesion on each substrate (fibronectin and matrigel) was taken as 100 percent. Values are mean ± SE of three independent experiments performed in triplicate. * and *** denotes $p<0.0133$ and $p<0.0001$, respectively.

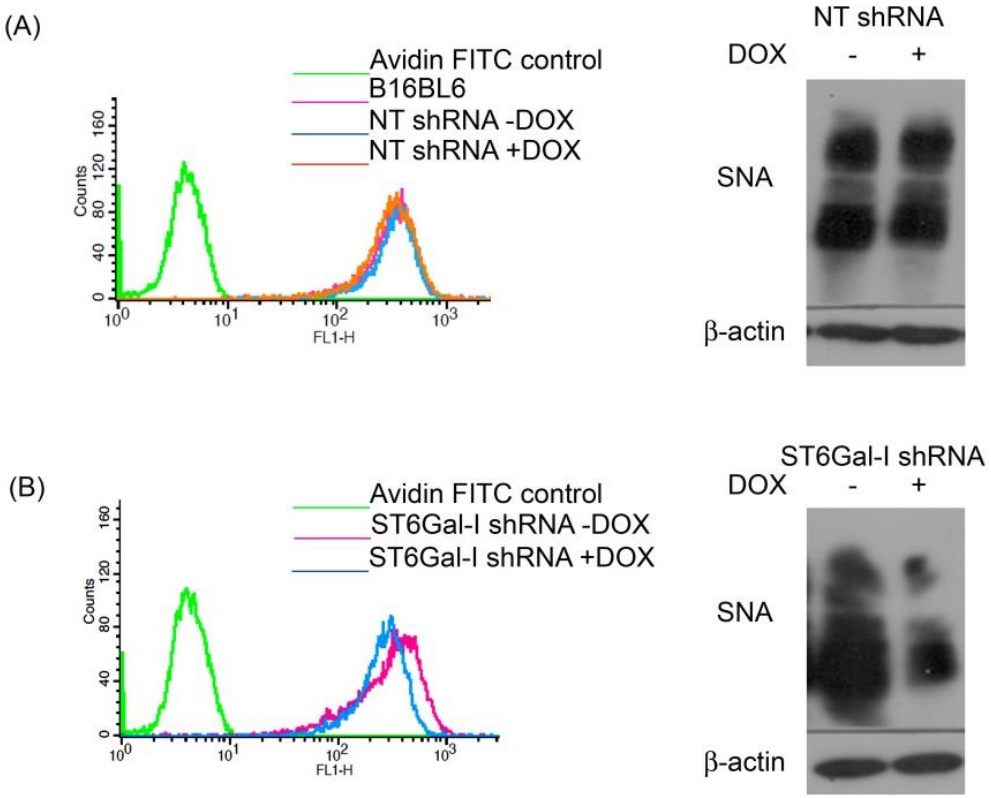


Figure 5: ShRNA mediated inhibition of ST6Gal-I decreased the expression of α2,6 linked SA. (A) Expression of α2,6 linked SA in B16BL6 cells and uninduced (NT shRNA –DOX) and doxycycline induced (NT shRNA +DOX) B16BL6 cells transduced with non-targeting shRNA by flow cytometry (*left panel*) and Western blotting (*right panel*). **(B)** Expression of α2,6 linked SA in ST6Gal-I shRNA transduced B16BL6 cells in uninduced (ST6Gal-I shRNA –DOX) and doxycycline induced (ST6Gal-I shRNA +DOX) cells, by flow cytometry (*left panel*) and Western blotting (*right panel*). For flow cytometric analysis cells were fixed with 1% paraformaldehyde and cells treated with only streptavidin FITC served as control. β-actin served as loading control for Western blotting.

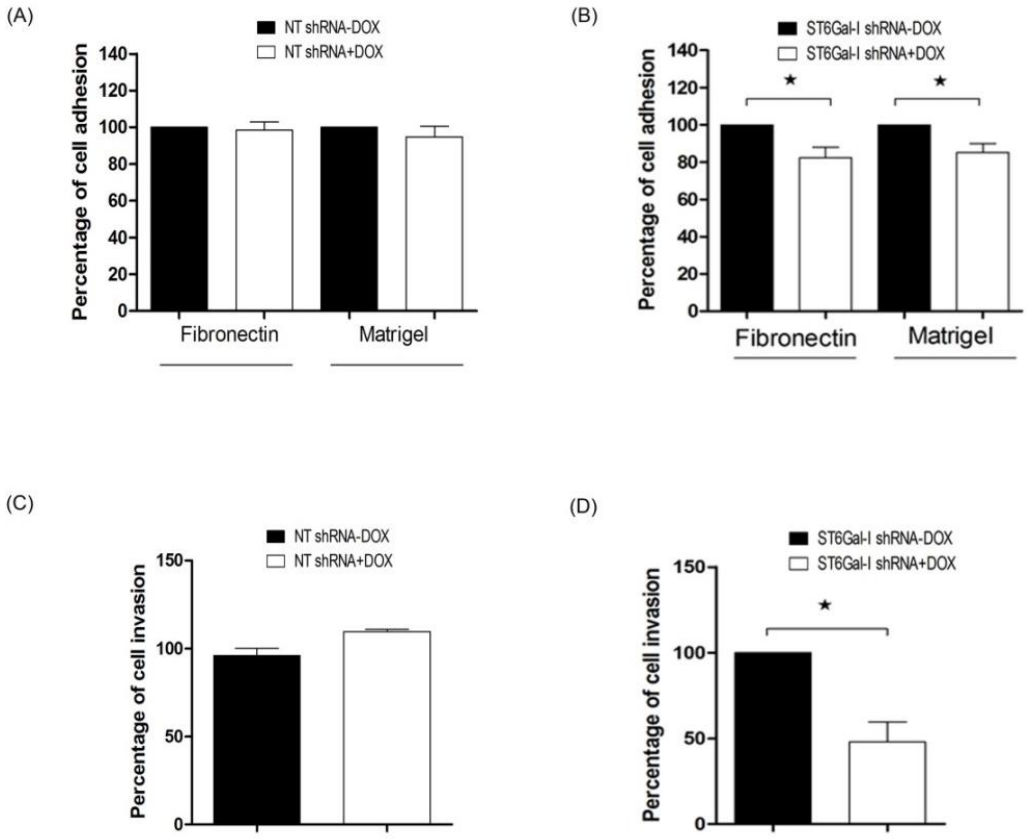


Figure 6: **Effect of downregulation of ST6Gal-I expression on adhesion and invasion of B16BL6 cells. (A)** Adhesion of induced (NT shRNA +DOX) and uninduced (NT shRNA –DOX) B16BL6 cells stably transduced with non-targeting shRNA or **(B)** ST6Gal-I shRNA, after induction (ST6Gal-I shRNA +DOX) or uninduced (ST6Gal-I shRNA -DOX), on fibronectin and matrigel. Values are mean ± SE of three independent experiments performed in triplicates.* denotes $p < 0.0369$. **(C)** Invasion assays of induced (NT shRNA +DOX) and uninduced (NT shRNA –DOX) non-targeting shRNA transduced B16BL6 cells. **(D)** Invasion assays of doxycycline induced (ST6Gal-I shRNA +DOX) and uninduced (ST6Gal-I shRNA –DOX) B16BL6 cells stably transduced with ST6Gal-I shRNA. Values are mean ± SE of three independent experiments done in triplicates. * denotes $p<0.0113$.

Inhibition of α2,6 SA also decreased the invasive potential of cells (ST6Gal-I shRNA +DOX) as compared to un-induced (ST6Gal-I shRNA –DOX) cells and induced/uninduced clones expressing NT-shRNA (NT shRNA +DOX)/(NT shRNA –DOX) (**Figure 6C and D**). These results clearly suggest that the increased substitution of α2,6 linked sialic acids regulates invasion by regulating adhesion.

Expression of β1,6 branched N-oligosaccharides shows a strong association with the invasive phenotype and matrix degradation is an important requirement for a cell to be invasive. To investigate if these oligosaccharides have any role in regulating matrix degradation is the second objective of this thesis.

Objective II: To investigate the role of β1,6 branched N-linked oligosaccharides in regulating matrix degradation

Earlier work done the in lab has also shown that the expression of these oligosaccharides did not correlate with the basal secretion of MMPs. However, matrix degradation is very crucial for invasion and is regulated in several ways and regulating the levels of matrix degrading enzymes is just one of them. Other modes include localization at the invasive front of the invading cells, activation of the pro-enzymes and induction of secretion of matrix degrading enzymes in response to extracellular cues. We need to investigate if these oligosaccharides regulate any of these processes, to understand their role in invasion.

3.6 Effect of increased adhesion to ECM/BM components promoted by β1,6 branched N-linked oligosaccharides on induction of MMP-9 secretion

B16BL6 cells show significantly higher adhesion to fibronectin or matrigel as compared to uncoated or BSA (to block non-specific adhesion) coated dishes **(Figure 7A)**. B16BL6 cells grown on fibronectin (10 µg/ml) and matrigel (50 µg/ml) coated plates also showed significant induction of secretion of MMP-9 as compared to cell grown on uncoated plates, as assessed by gelatin zymography and Western blotting **(Figure 7B, upper and lower panels, respectively)**. Induction of MMP-9 secretion was found to be glycosylation dependent. Treatment of cells with N-oligosaccharides inhibitor SW significantly decreased adhesion **(Figure 7A)** resulting in concomitant decrease in MMP-9 secretion **(Figure 7C)**. Glycosylation dependent MMP-9 induction was also observed at the transcript level as assessed by qRT-PCR **(Figure 7D)**.

This was also assessed after specific inhibition of expression of β1,6 branched N-oligosaccharides in B16BL6 cells by shRNA mediated downregulation of GnT-V. Real time PCR showed that doxycycline (DOX) induction efficiently knocked down the GnT-V transcript levels in GnT-V shRNA transduced clones as compared to B16BL6 cells transduced with non-targeting shRNA (**Figure 8A and B**). GnT-V downregulation resulted in significant reduction in the expression of β1,6 branched N-oligosaccharides upon induction in GnT-V shRNA transduced clones at the cell surface as assessed by flow cytometry, and on proteins by Western blotting using biotinylated lectin L-PHA as probe **(Figure 8C and D)**. Inhibition of β1,6 branched N-oligosaccharides by induction of ShRNA to GnT-V by doxycycline, inhibited adhesion as compared to uninduced cells **(Figure 9A)**. This in turn inhibited MMP-9 induction in GnT-V shRNA transduced clones induced with doxycycline as compared to B16BL6 cells or doxycycline induced B16BL6 cells transduced with NT shRNA **(Figure 9B and C)**.

Tumor cells may also regulate matrix degradation by regulating the expression and localization of proteases and their motility receptors on the cell surface.

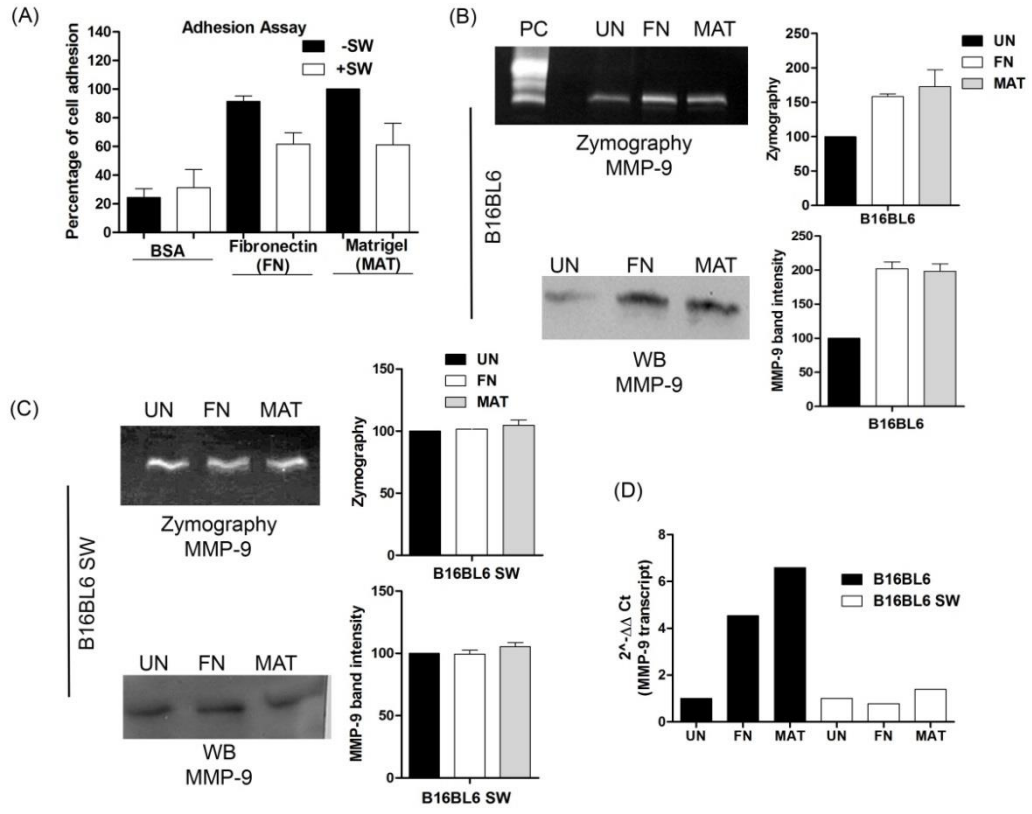


Figure 7: Expression of β1,6 branched N-oligosaccharides regulates adhesion dependent MMP-9 secretion. (**A**) Adhesion of B16BL6 cells before and after Swainsonine treatment was compared on BSA coated (BSA), fibronectin (FN) and matrigel (MAT) coated 96 well plates. Adhesion of B16BL6 cells on matrigel was taken as 100 percent. **(B)** Conditioned medium collected from B16BL6 and **(C)** Swainsonine treated B16BL6 cells (B16BL6 SW) grown on uncoated (UN), fibronectin (FN) and matrigel (MAT) coated culture dishes, were analysed by gelatin zymography (*upper panels*) and by Western blotting (*lower panel*). 1:20 diluted human blood served as positive control (PC). Graph represents densitometric quantitation of MMP-9 bands of two independent experiments. MMP-9 band intensity on uncoated dish (UN) was taken as 100 percent. (**D**) Analysis of MMP-9 transcript by real time PCR from untreated (B16BL6) and Swainsonine treated B16BL6 cells (B16BL6 SW).

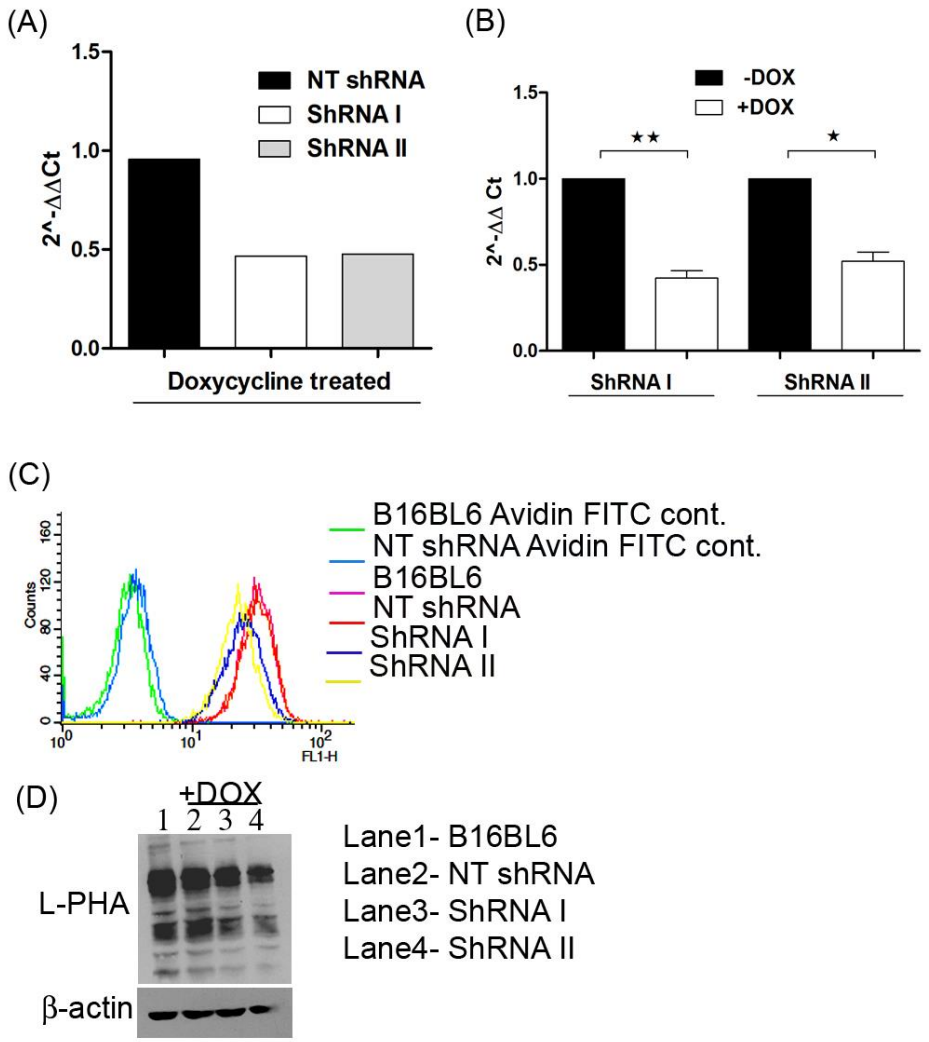


Figure 8: Characterization of B16BL6 cells stably expressing shRNAs to GnT-V in a doxycycline (DOX) inducible lentiviral vector pTRIPz. Comparison of the expression of GnT-V transcript by qRT-PCR **(A)** between B16BL6 cells transduced with, non-targeting (NT shRNA), GnT-V shRNA I and shRNA II upon doxycycline induction for 96 h, **(B)** between doxycycline treated (+DOX) and untreated (-DOX) GnT-V shRNA I and ShRNA II transduced B16BL6 cells. **(C)** Analysis of surface expression of β1,6 branched N-linked oligosaccharides in these clones by flow cytometry using lectin L-PHA staining, cells treated only with Avidin-FITC served as control. **(D)** Western blotting of cell lysates from these clones using lectin L-PHA. β-actin served as loading control.

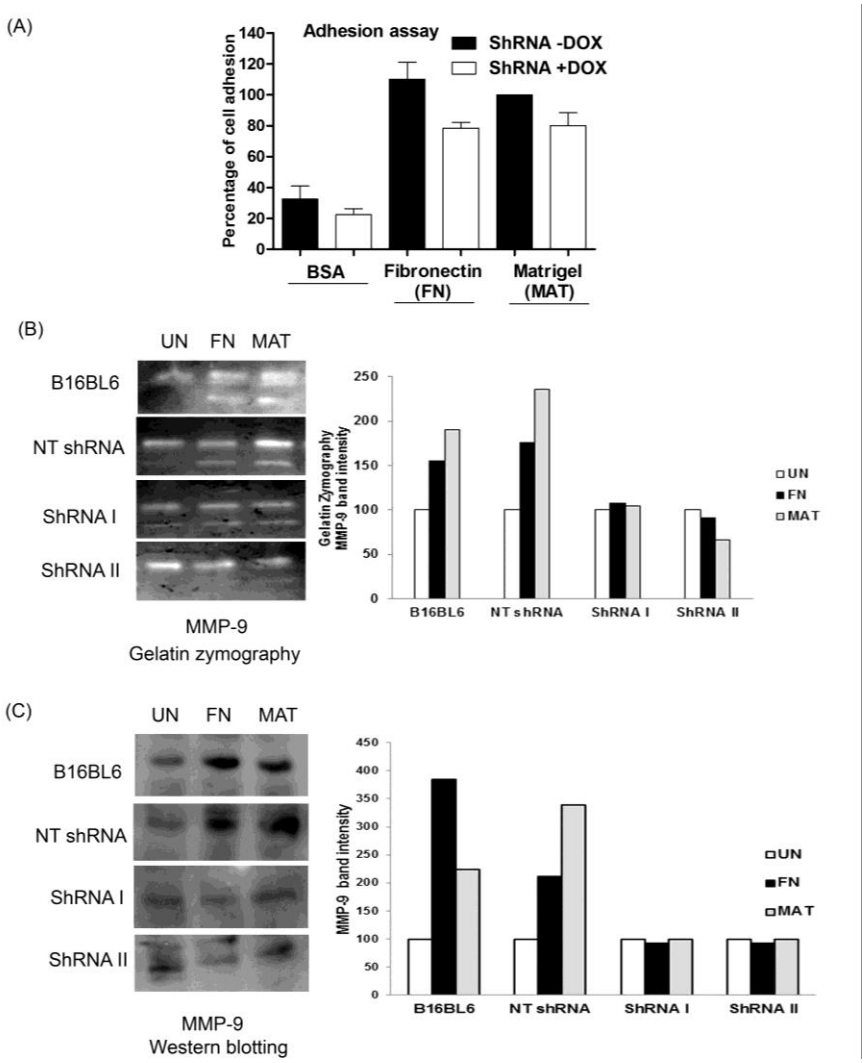


Figure 9: Inhibition of β1,6 branched N-oligosaccharides by shRNA to GnT-V inhibited MMP-9 induction. (**A**) Comparison of adhesion of B16BL6 cells transduced with shRNA to GnT-V with and without induction by doxycycline treatment on BSA coated (BSA), fibronectin (FN) and matrigel (MAT) coated 96 well plates. Adhesion of B16BL6 cells on matrigel was taken as 100 percent. Conditioned medium collected from B16BL6, NT shRNA and GnT-V shRNA transduced B16BL6 cells, after induction with doxycycline for 96 h were grown on uncoated (UN), fibronectin (FN) and matrigel (MAT) coated culture dishes, were analysed by **(B)** gelatin zymography and **(C)** by Western blotting. Graph represents densitometric quantitation of MMP-9 bands. MMP-9 band intensity on uncoated dish (UN) was taken as 100 percent.

3.7 Role of β1,6 branched N-oligosaccharides on motility receptors (β1 integrin and CD44) in regulating their association with urokinase Plasminogen Activator Receptor (uPAR) and membrane tethered MMPs (MT1-MMP) in B16 melanoma cells

Matrix degradation is a highly regulated process as the same matrix serves as substratum for movement. In order to avoid indiscriminate degradation of matrix, tumor cells co-localize the membrane tethered forms or the receptors of certain proteases with the receptors involved in motility of cells such that the matrix degradation is focalized at the site of movement. To investigate if β1,6 branched N-oligosaccharides on motility receptors have any role in this process, first the levels of motility receptors identified to carry them viz., β1 integrin and CD44 were compared in the invasive variants B16F10 and B16BL6 cells. The total levels as well as the levels of their expression on cell surface, as assessed by Western blotting and flow cytometry respectively, remained unaltered (**Figure 10A and B**). Precipitation experiments with the agarose bound lectin L-PHA confirmed that they indeed carried β1,6 branched N-oligosaccharides and the levels of glycosylated proteins carrying these oligosaccharides also correlated with invasive potential of the cells (**Figure 10C**). Glycosylation in B16BL6 cells was inhibited using SW to see if it regulates the surface expression of these receptors. Flow cytometry data showed that SW had no effect on their surface expression (**Figure 10D**).

MT1-MMP, the membrane tethered form of MMPs, and uPAR the receptor for uPA, are the major cell surface molecules that are involved in the activation of pro-MMPs in the vicinity of cancer cells. Results comparing expression on B16F10 and B16BL6 cells showed that the expression of MT1-MMP correlates positively with the invasive phenotype

as assessed by both Western blotting, flow cytometry and at the transcript level by semiquantative PCR (**Figure 11A-C)**. However, the expression of uPAR remained unaltered (**Figure 11A-C**). Both MT1-MMP and uPAR often associate with the motility receptors like integrins and CD44 such that the matrix degradation is restricted at the site of movement. Together they regulate invasion by coupling matrix degradation with cellular movement. Since β1 integrin and CD44 both carry β1,6 branched N-oligosaccharides, their role in regulating association with MT1-MMP and/or uPAR was analysed by laser confocal microscopy (LCM).

The results showed that both β1 integrin and CD44 associate with MT1-MMP and uPAR. The association of β1 integrin with MT1-MMP appeared to be regulated to some extent by the presence of these oligosaccharides as inhibition with SW affected this association (**Figure 12B**), however, SW had no effect on its association with uPAR (**Figure 12C)**. The presence or absence of β1,6 branched N-oligosaccharides on CD44 did not appear to affects its association with either MT1-MMP or uPAR (**Figure 13A and B**). However, these results would need to be confirmed by co-immunoprecipitation which was not possible because of non-availability of good quality antibodies for immunoprecipitation.

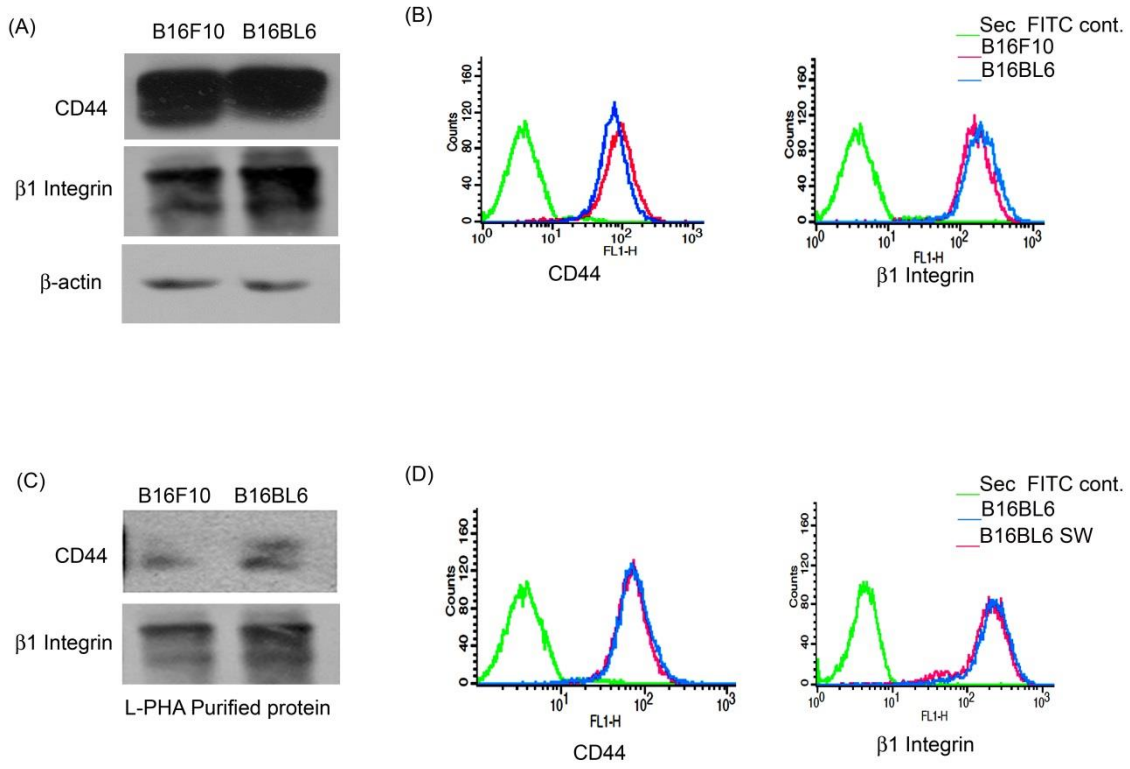


Figure 10: Comparison of B16 melanoma variants for the expression of motility receptors. (A) Cell lysate from B16F10 and B16BL6 cells were blotted and compared for the expression of β1 integrin, CD44, by Western blotting. **(B)** By flow cytometry using their specific antibodies. **(C)** Equal amount of proteins (2000 µg) from B16F10 and B16BL6 cell lysate were Lectin L-PHA precipitated and eluted bound proteins from these cells were blotted and probed with their specific antibodies. **(D)** Surface expression of CD44 and β1 integrin in B16BL6 and SW treated B16BL6 cells (B16BL6 SW). β-actin and cells treated with only secondary FITC served as control for Western blotting and flow cytometry, respectively.

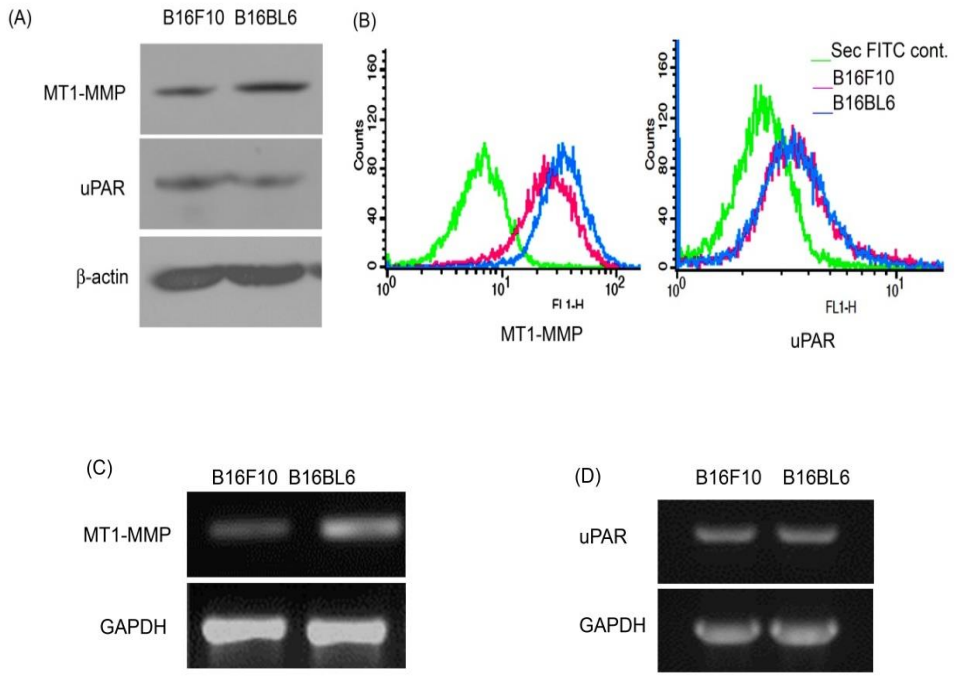


Figure 11: Expression of MT1-MMP and uPAR in B16 melanoma variants. (A) Cell lysates from B16F10 and B16BL6 cells were blotted and probed with MT1-MMP and uPAR specific antibody. **(B)** Flow cytometric analysis of the surface expression of MT1-MMP and uPAR. β-actin and cells treated with only secondary FITC served as loading control for Western blotting and flow cytometry, respectively. Analysis of the transcript levels of **(C)** MT1-MMP and **(D)** uPAR. GAPDH served as loading control.

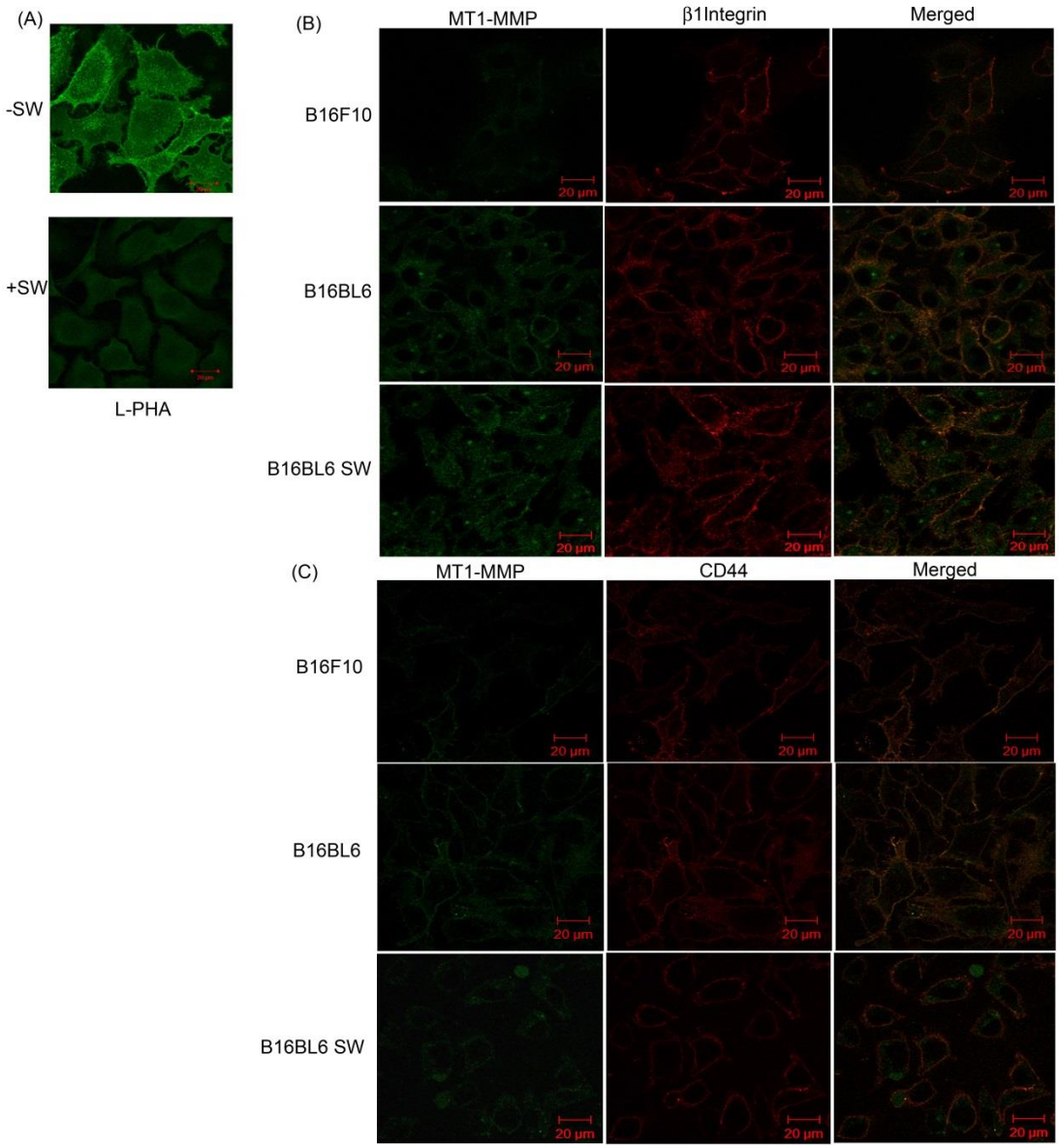


Figure 12: Effect of glycosylation in regulating association between motility receptors and MT1-MMP. (A) Lectin L-PHA staining of B16BL6 (-SW) and the same cells treated with Swainsonine (+SW). **(B)** Colocalization of MT1-MMP (green) with β1 integrin (red) in B16F10, B16BL6 and B16BL6 cells treated with SW (B16BL6 SW). **(C)** Colocalization of MT1-MMP (green) with CD44 (red) in B16F10, B16BL6 and B16BL6 SW cells. Colocalized or merged images were shown in yellow. Scale bar 20µm.

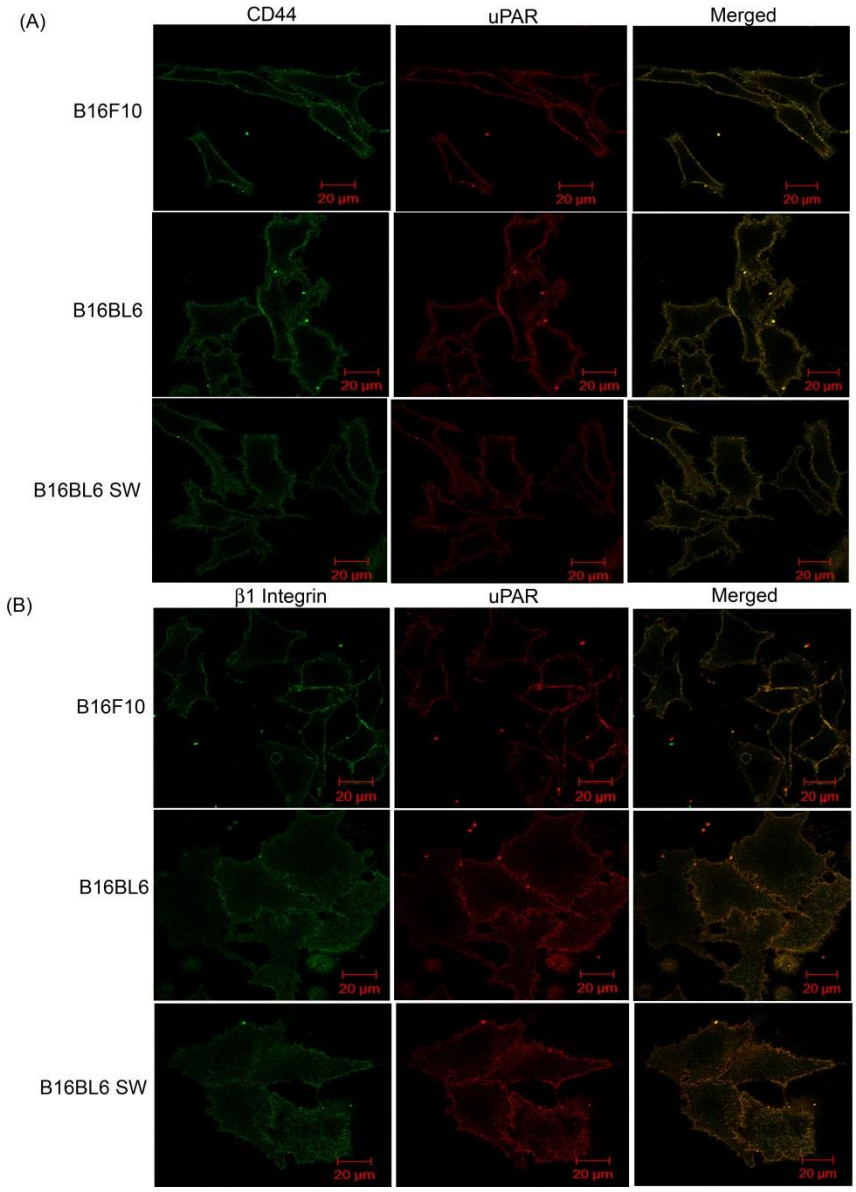


Figure 13: Effect of glycosylation in regulating association between motility receptors and uPAR. (A) Colocalization of CD44 (green) with uPAR (red) in B16F10, B16BL6 and B16BL6 SW cells. **(B)** Colocalization of β1 integrin (green) with uPAR (red) in B16F10, B16BL6 and B16BL6 SW cells. Colocalized or merged images were shown in yellow. Scale bar 20µm.

Another interesting aspect is that, the presence of these oligosaccharides on invasive cells appears to modulate their motility in a substrate specific manner. As demonstrated earlier their presence enhanced haptotactic motility on fibronectin a major component of ECM however, attenuated it on matrigel which is the reconstituted basement membrane (BM) [140]. The mechanism by which these oligosaccharides regulate motility differentially on these components forms the third objective of this thesis.

Objective III: To investigate the role of these oligosaccharides in regulating the motility of cells on ECM and basement membrane components

3.8 Effect of glycosylation in regulating cell spreading on ECM (fibronectin) and BM (matrigel) components

We confirmed our previous results and showed that SW treatment had a substrate dependent effect on the movement of cells, attenuated on fibronectin and enhanced on matrigel (**Figure 14A**). Motility of cells is dependent to a large extent on their spreading. Inhibition of expression by SW had similar effect on the spreading of the cells, attenuated on fibronectin, but enhanced on matrigel as seen by confocal images and by comparing ratio of cytoplasmic/nuclear area (**Figure 14B and C**). These results show that inhibition of glycosylation results in increased spreading of cells on matrigel (BM) and this morphology appears to promote haptotactic motility on this substrate, whereas, it inhibited cell spreading on fibronectin (ECM) which apparently affects their motility towards fibronectin. It would be important to investigate the mechanism of these differential substrate specific effects of glycosylation on the spreading and motility of cells, and to understand the implications of these observations in terms of tumor cell invasion.

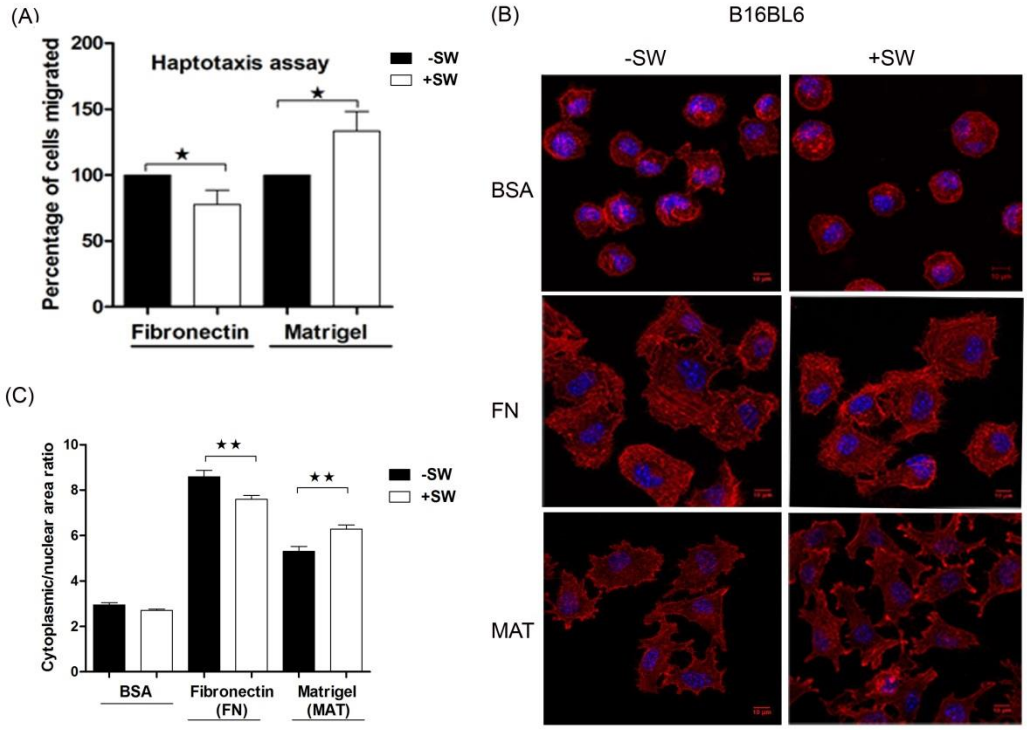


Figure 14: Expression of β1,6 branched N-oligosaccharides regulates spreading and motility positively on ECM (fibronectin) but negatively on BM (matrigel) components. **(A)** Haptotactic motility of untreated (-SW) and Swainsonine (+SW) treated B16BL6 cells was compared on 24 well trans-well units coated with fibronectin and matrigel. The number of B16BL6 cells migrated on each substrate was taken as 100 percent. Values are Mean ± SE of three independent experiments, * denotes $p<0.02$. **(B)** Comparison of cell spreading on BSA, fibronectin (FN) and matrigel (MAT) coated coverslips by F-actin staining (red) with Phalloidin-TRITC using laser confocal microscopy at 63x magnification between Swainsonine treated (+SW) and untreated (-SW) B16BL6 cells. Scale bar 10µm. **(C)** The graph represents quantitation of cell spreading by determining the mean of ratio of cytoplasmic to nuclear area of more than 100 cells, about 40 cells in each independent experiment done thrice. One way ANOVA was employed to check the statistical significance. Values represents mean ratio of all the cells ± SE ** denotes $p<.05$.

3.9 Effect of inhibition of β1,6 branched N-linked oligosaccharides specifically by shRNAs to GnT-V in regulating cell spreading on fibronectin and matrigel

Induction of non-targeting shRNA with doxycycline (NT shRNA +DOX) did not have any effect on cell spreading either on fibronectin or matrigel (**Figure 15A**). However, induction of shRNAs to GnT-V with doxycycline (ShRNA I +DOX and ShRNA II +DOX) in B16Bl6 cells decreased the spreading of cells on fibronectin as compared to uninduced cells (ShRNA I –DOX and ShRNA II –DOX) (**Figure 15B and C, upper panels**). In contrast, induction of shRNAs increased the spreading of B16BL6 cells on matrigel as compared to uninduced cells (**Figure 15B and C, lower panels**).The results thus confirm that the differential effect of SW on the spreading and motility are most likely due to loss of β1,6 branched N-oligosaccharides. Laminin is the major component of matrigel (>50%), it is thus likely that integrin receptors for laminin and fibronectin carry β1,6 branched N-oligosaccharides which apparently influence the cellular spreading and motility properties.

3.10 Analysis of the presence of β1,6 branched N-oligosaccharides on fibronectin (α5β1) and laminin (α3β1) receptors and its effect on their cell surface expression

Purification of proteins carrying β1,6 branched N-oligosaccharides on L-PHA agarose beads from total cell lysates of B16BL6 cells, showed that β1 integrin as well as the α subunits of both fibronectin (α5β1) and laminin (α3β1) receptors also carry such oligosaccharides as checked by Western blotting (**Figure 16A**).

Surface expression of these receptors on B16BL6 cells was compared with either B16F10 cells (which express lower levels of β1,6 branched N-oligosaccharides) or with SW treated B16BL6 cells (B16BL6 SW) or B16BL6 cells transduced with shRNA to GnT-V, with

(+DOX) and without (-DOX) induction with doxycycline, by flow cytometry. The results showed that glycosylation status did not impact surface expression of integrin receptors (α3β1 and α5β1) (**Figure 16B-D**). Other mechanism by which tumor cells regulate invasion is by manipulating the levels of tetraspanins or by modulating the association of integrin receptors with tetraspanins. Invasive tumors downregulate most tetraspanins except CD151 and CO-029 [86].

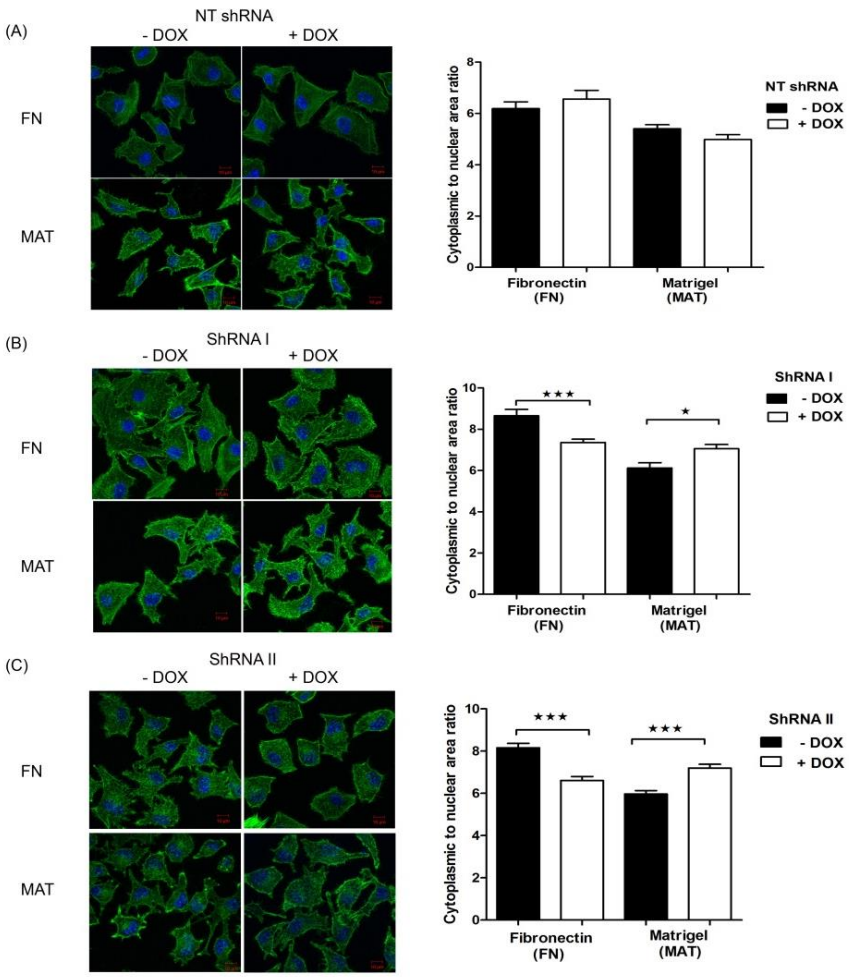


Figure 15: Specific inhibition of β1,6 branched N-linked oligosaccharides by shRNAs to GnT-V had similar effect on cell spreading. Cell spreading of doxycycline induced (+DOX) and uninduced (–DOX) **(A)** NT shRNA, **(B)** ShRNA I and **(C)** ShRNA II transduced B16BL6 cells on Fibronectin (FN) and Matrigel (MAT) coated coverslips was compared by F-actin staining (Green) with Phalloidin-FITC using laser confocal microscopy at 63 x magnification. Nucleus was shown in blue *(DAPI)* scale bar 10 µm. The Graph represents quantitation of cell spreading by calculating mean of the ratio of cytoplasmic to nuclear area of more than 75 cells, about 25 cells in each independent experiment done thrice. One way ANOVA was employed to check the statistical significance. Values represents mean ratio of all the cells ± SE * denotes $p<0.05$.

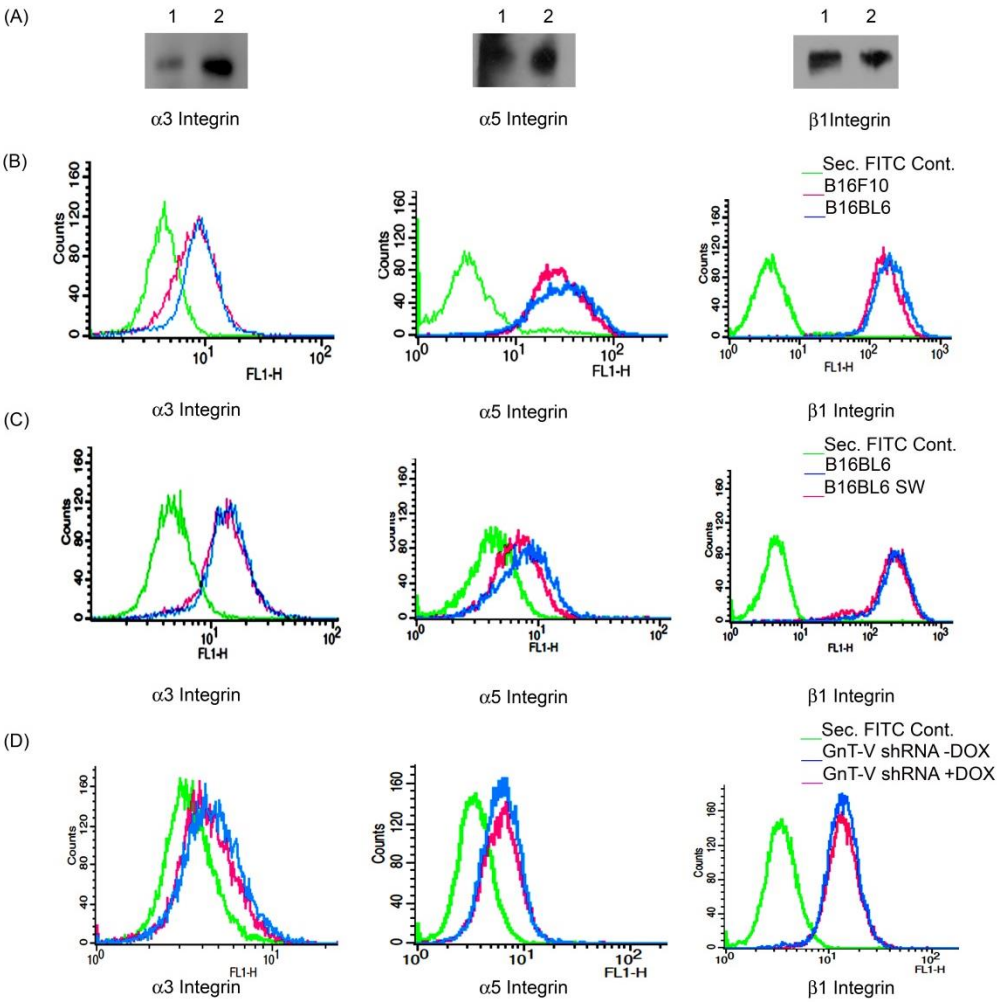


Figure 16: Presence of β1,6 branched N-oligosaccharides on both the subunits of fibronectin (α5β1) and laminin (α3β1) receptors has no effect on their cell surface expression. (A) Total cell lysate from B16BL6 cells (100µg) (lane1) and L-PHA binding proteins purified from (100µg cell lysate for β1 and 300µg for α3, α5 integrin (lane2) were blotted and probed with their specific antibodies. Comparison of surface expression of integrin α3, α5 and β1 integrin on **(B)** B16F10 and B16BL6 cells, **(C)** untreated (B16BL6) and Swainsonine treated (B16BL6 SW) cells, **(D)** ShRNA to GnT-V transduced B16BL6 cells with (ShRNA +DOX) and without induction with doxycycline (ShRNA –DOX) by flow cytometry using specific antibodies. Cells treated only with FITC labeled secondary antibody served as control.

3.11 Analysis of the expression of tetraspanins in B16 melanoma invasive variants

Transcript levels of the major tetraspanins CD151, CD82, CD81, CD63 and CD9 which are reported to be up/down regulated by the invasive and metastatic cancers were compared in B16F10 and B16BL6 cell lines by semi quantitative RT-PCR. The transcript levels of these tetraspanins were comparable in the parent and the invasive B16BL6 cells (**Figure 17A**). The total levels of CD82 and CD151 which regulate fibronectin and laminin receptors respectively remained unaltered in these (B16F10) and (B16BL6) cells, as seen by Western blotting (**Figure 17B**) and flow cytometry (**Figure 17C**). This suggests that the differential motility on fibronectin and matrigel (laminin) associated with invasive phenotype, is not due to the altered expression of tetraspanins.

Another mechanism by which cancer cells may regulate motility is by glycosylation. Glycosylation on the integrin receptor as well as tetraspanins has been reported to prevent the association of CD82 with α5β1 integrin and promote movement [90]. Thus enhanced motility on fibronectin could be due to the presence of β1,6 branched N-oligosaccharides on α5β1 integrin preventing their association. Does glycosylation of laminin receptor(s) regulate its association with CD151 and thus motility on matrigel?

3.12 Effect of Glycosylation in regulating association of laminin receptor integrin α3β1 with tetraspanin CD151

Although, both the subunits of laminin receptor α3β1 are carriers of β1,6 branched N-oligosaccharides, their role in regulating association with CD151 was evaluated. Due to the non-availability of antibody against murine CD151 for immunoprecipitation or confocal studies, B16BL6 cells were transfected with GFP-tagged CD151. In contrast to the diffused

fluorescence in the pEGFP-vector transfected cells, CD151-GFP transfected cells showed distinct membrane localization (**Figure 18A**).

The effect of glycosylation on the association of CD151 and α3β1 was evaluated in the clones of B16BL6 cells stably expressing GFP tagged CD151 grown on matrigel coated coverslips and assessed by confocal microscopy. The results showed that treatment of cells with SW results in significantly increased association of β1 integrin subunit (most likely α3β1) with CD151 (**Figure 19A and B**). However, co-immunoprecipitation with anti GFP antibody or β1integrin did not work as the immunoprecipitation always gave a band of IgG heavy chain that co-migrated with the tagged CD151. To overcome this problem, B16BL6 cells were transfected with FLAG tagged CD151 (CD151-FLAG). Lysates of cells expressing FLAG tagged CD151were used for immunoprecipitation employing FLAG M2 affinity gel, and the bound protein was eluted with 3x FLAG peptide. Immunoprecipitates from cell lysates of SW treated (+SW) and untreated cells (-SW) indeed showed that the association of CD151 with β1 and even α3 subunit of integrin receptor for laminin is dependent on their glycosylation status (**Figure 19C and D**).

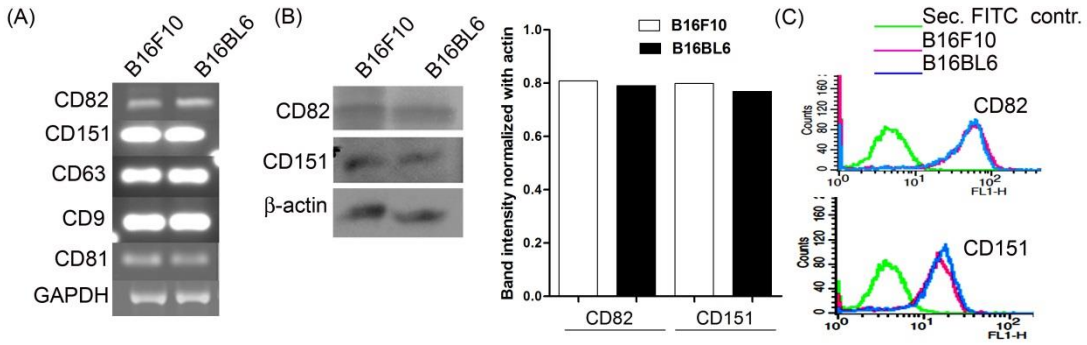


Figure 17: Expression of tetraspanins did not correlate with the invasiveness of B16 melanoma cells. Comparison of B16 melanoma invasive variants for the expression of **(A)** the transcript levels of major tetraspanins by semiquantitative RT-PCR, and the expression of CD82 and CD151 by **(B)** Western blotting using specific antibodies (left panel) and densitometric quantitation of band intensity normalized with β-actin (right panel), and on the cell surface by **(C)** flow cytometry using specific antibodies, where cells treated only with secondary anti rabbit FITC served as control. GAPDH and β-actin served as loading control for semiquantitative RT-PCR and Western blotting, respectively.

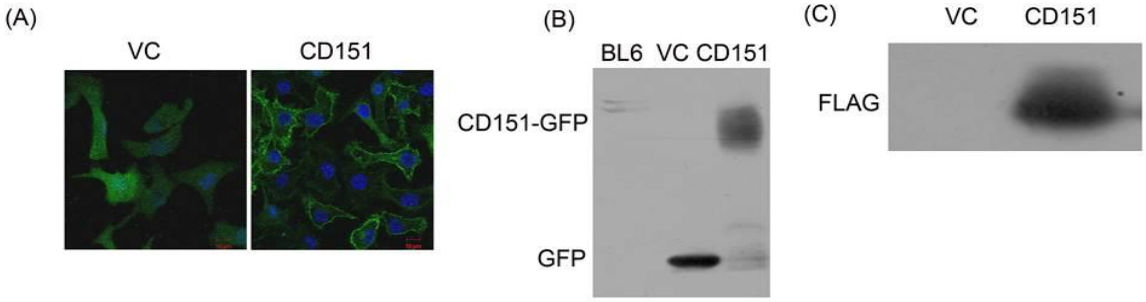


Figure 18: Characterization of B16BL6 cells transfected with Vector alone (pEGFP) and GFP/FLAG tagged CD151. (A) The expression of GFP (green) by laser confocal microscope, nucleus was shown in blue (DAPI) scale bar 10 µm. **(B)** Cell lysates from untransfected (B16BL6), vector alone (VC) and CD151 transfected B16BL6 (CD151) cells were blotted and probed with anti-GFP antibody. **(C)** Cell lysate from vector alone (pCMV 3x FLAG vector) (VC) and FLAG tagged CD151 transfected B16BL6 cells (CD151-FLAG) were blotted and probed using anti-FLAG antibody.

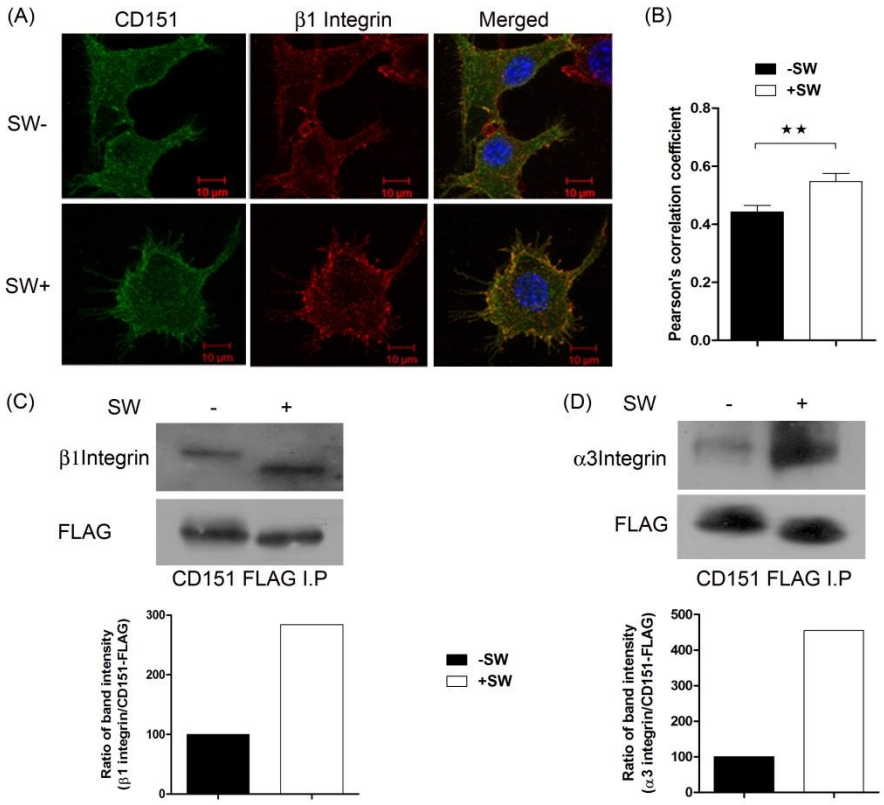


Figure 19: Glycosylation regulates association of CD151 and laminin receptor integrin. **(A)** Comparison of co-localization of GFP tagged CD151 (green) with β1 integrin (red) in Swainsonine treated (+SW) and untreated (-SW) (CD151-GFP transfected) B16BL6 cells. Merged image (yellow) depicts co-localization. Scale bar10 µm. **(B)** Graph represents the analysis of co-localized images by Pearson's correlation coefficient using Image J 1.43 software (NIH). Values are mean ± SE of three independent experiments (~10 different fields in each experiment). Coimmunoprecipitation of FLAG tagged CD151 from cell lysate of Swainsonine treated and untreated B16BL6 cells stably expressing FLAG tagged CD151 (CD151-FLAG) were blotted and probed with **(C)** β1 integrin and anti-FLAG antibody and **(D)** α3 integrin and anti-FLAG antibody. Graph represents quantitation of the ratio of band intensities of β1 integrin and CD151-FLAG (C, *lower panel*) or α3 integrin and CD151-FLAG (D, *lower panel*) in Swainsonine treated (+SW) and untreated (-SW) cells. Ratio of band intensity between β1 or α3 integrin and CD151-FLAG in untreated cells was taken as 100 percent.

3.13 Effect of glycosylation in regulating cell spreading, migration and invasion in CD151 overexpressing B16BL6 cells

Cells require an optimum level of cell adhesion for migration. Cells are unable to move when they adhere, either loosely or too tightly to the substratum. Similarly, cells require an optimum spreading for motility. As compared to vector control, CD151 transfected cells marginally increased the cell spreading on matrigel, however, after inhibition of glycosylation using SW, both the cell types showed significantly higher spreading as compared to untreated cells (**Figure 20A and B**). This is also evident in the higher ratio of cytoplasmic to nuclear area (**Figure 20C**).

Overexpression of CD151 in B16BL6 melanoma cells had marginal effect on their cell migration as seen by scratch wound assay and haptotactic motility towards matrigel. However, after inhibition of glycosylation, motility of CD151 transfected cells on matrigel increased significantly as compared to the cells transfected with vector alone as assessed by scratch wound assay (**Figure 21A and B**) and haptotactic motility assay (**Figure 21C**). Over expression of CD151 further increased the invasive ability of B16BL6 cells as compared to those transfected with vector alone. However, on SW treatment invasive ability of both the cell types decreased to almost same level which was significantly low for both vector alone and CD151 transfected B16BL6 cells treated with SW (**Figure 21D**).

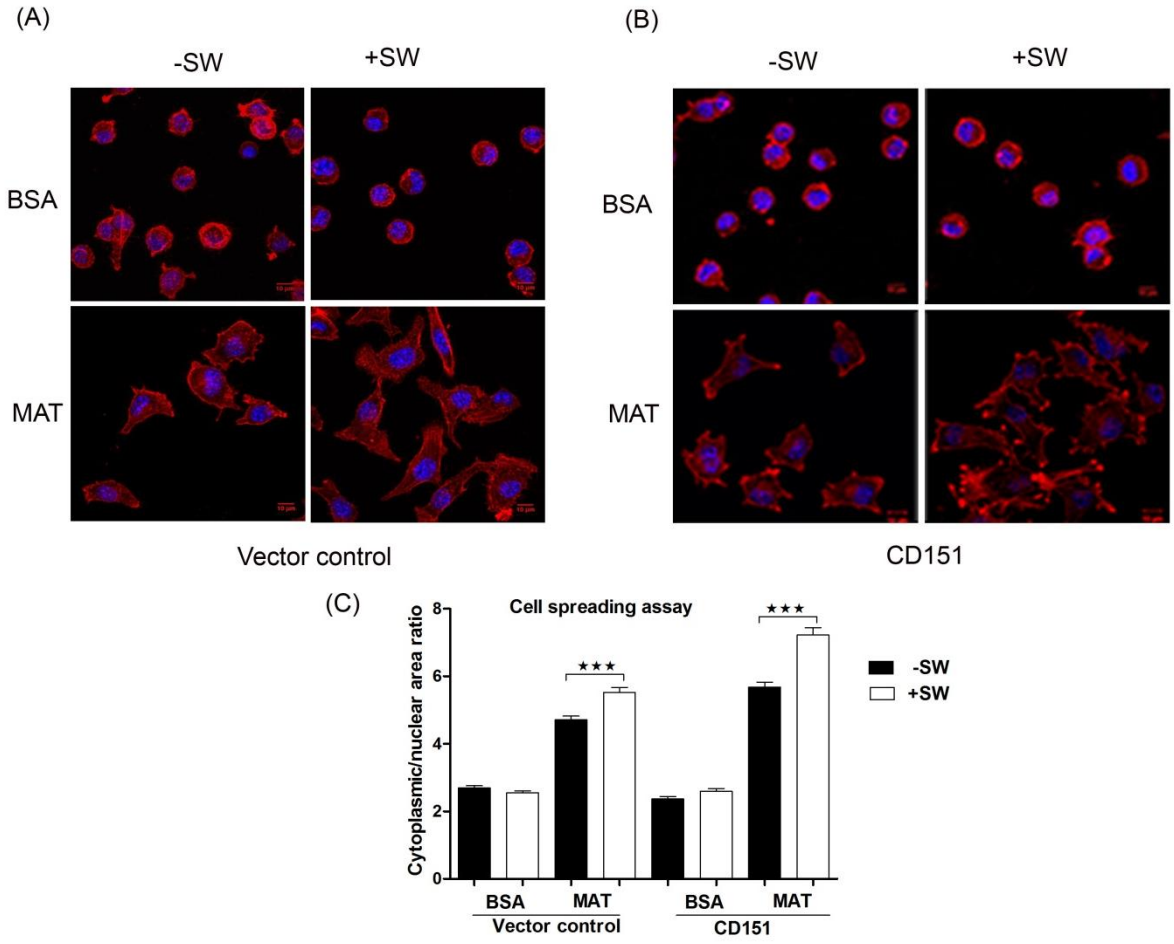


Figure 20: Glycosylation dependent association of integrin receptors with CD151 modulates cell spreading. Comparison of cell spreading of **(A)** untreated (-SW) and Swainsonine treated (+SW), vector transfected (Vector control) and **(B)** CD151 transfected B16BL6 cells (CD151) on BSA and matrigel (MAT) coated coverslips by F-actin staining (red) with Phalloidin-TRITC using laser confocal microscopy at 63x magnification. Nucleus was shown in blue (DAPI) scale bar 10 µm. **(C)** The Graph represents quantitation of cell spreading by calculating ratio of cytoplasmic to nuclear area of more than 100 cells, about 40 cells in each independent experiment done thrice. One way ANOVA was employed to check the statistical significance. Values represents mean ratio of all the cells ± SE *** denotes $p<0.05$.

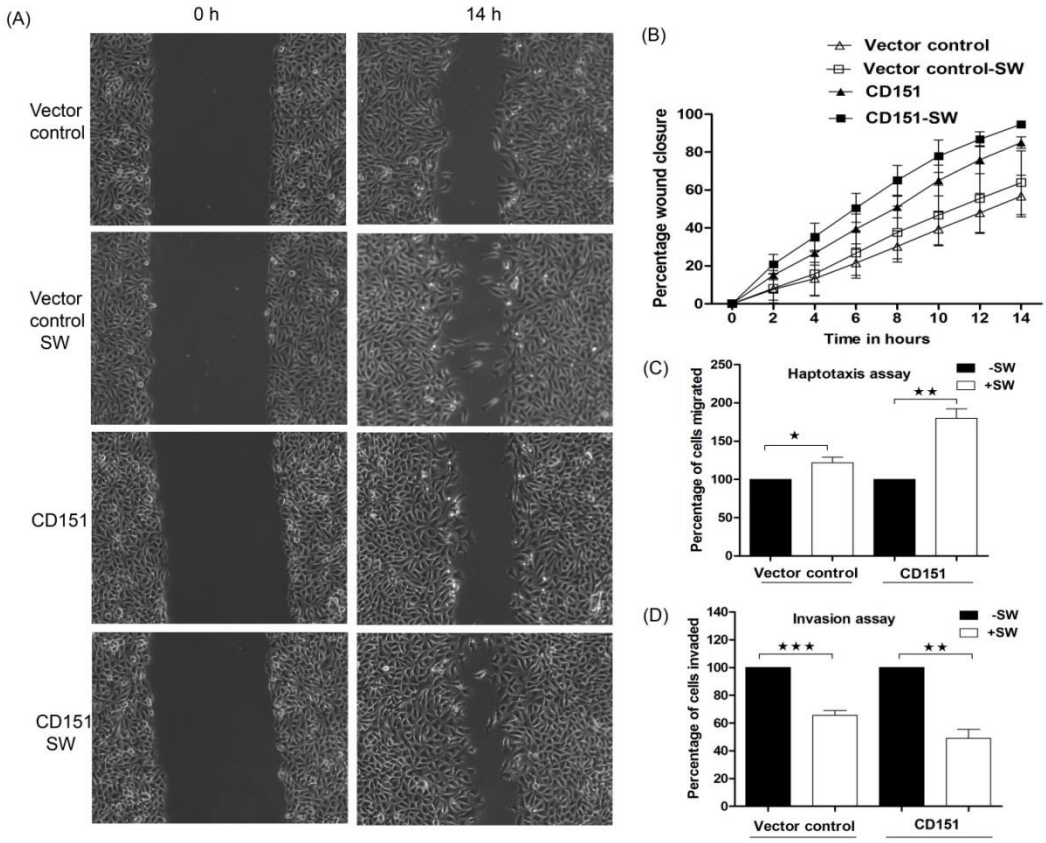


Figure 21: Glycosylation dependent association of integrin receptors with CD151 modulates cell motility and Invasion. (A) Comparison of cell motility between untreated and Swainsonine (SW) treated vector alone (Vector control) and GFP-tagged CD151 transfected B16BL6 cells (CD151) by scratch wound assay. **(B)** Graph represents the quantitation of the percentage wound closer of two independent experiments, each experiment at three different points. **(C)** Comparison of the haptotactic motility of untreated (-SW) and Swainsonine (+SW) treated vector transfected (Vector control) and GFP-tagged CD151 transfected B16BL6 cells (CD151) on 24 well transwell units coated with matrigel. The number of untreated cells that migrated in response to matrigel (Vector control or CD151) was taken as 100 percent. Values are mean ± SE of three independent experiments done in triplicate * and ** denote $p<0.039$ and $p<0.0028$ respectively. **(D)** Comparison of the invasive ability of untreated (-SW) and Swainsonine (+SW) treated B16BL6 cells transfected either with vector control or GFP tagged CD151. Invasion was compared by taking the number of untreated vector control (VC) or CD151transfected (CD151) B16BL6

that invaded the matrigel barrier was taken as 100%. Values are mean ± SE of three independent experiments carried out in triplicate. Student's t test was performed to compare significance between two cell lines. *** and ** denote $p<0.0005$ and $p<.0095$, respectively.

CHAPTER 4

DISCUSSION

4. Discussion

Cancer is one of the lethal diseases of the 21st century. Development of cancer involves complex dynamic changes in the genome. These changes in the genome can be established not only by the genetic mechanism but also by epigenetic mechanism. Normal cells remain confined within tissue boundary however, once the cancer cells become metastatic, they overcome tissue confinement and spread to other organ sites referred to as metastasis. Metastasis is the most fatal aspect of cancer and accounts for majority of the cancer related mortality. Despite metastasis being a clinically significant process, it is not clearly understood because of its complexity and being multistep in nature.

Invasion is involved in almost each step of metastasis i.e., while breaking free from the primary site during intravasation and extravasation and for getting established at the secondary target organ site. Thus, invasion is considered to be the key hallmark of metastasis. Invasion involves modulation of cellular adhesion to ECM/BM components, degradation of the ECM/BM and movement of the cells using matrix as traction or its degraded products as chemoattractants [148].

Cell surface molecules are the major participants in almost all the steps of invasion and metastasis and thus cancer cells show several surface modifications associated with it. Some of these modifications are **i)** alteration in the expression of molecules involved in cell to cell interaction like tight, adherens junctional and desmosomal proteins. **ii)** Molecules involved in cell to matrix interaction and motility like integrin superfamily proteins, proteoglycans and also **iii)** the alteration in the expression and localization of molecules involved in matrix degradation like several classes of proteases and their inhibitors.

Besides, these, invasive and metastatic tumor cells also show surface modification in the form of altered cell surface glycosylation.

Expression of β1,6 branched N-linked oligosaccharides is one such consistently observed cell surface modification associated with metastatic and invasive cancer cells. Increased expression of these oligosaccharides correlates with malignant potential of several human cancers such as breast, colon, melanoma, gliomas, esophageal, ovarian, endometrial [108-111]. Enhanced expression of such oligosaccharides also correlates with the metastatic potential of several human and murine cancer cell lines. Manipulation of their expression by using glycosylation mutants or chemical inhibition or by genetic manipulation resulted in loss/gain of metastatic phenotype [114-117]. Increased expression of these oligosaccharides is observed not only in invasive cancer cells but also in the normal cells involved in invasive functions like invading trophoblast cells during the implantation of embryo, endothelial cells during angiogenesis and even activated lymphocyte, granulocytes, macrophages during inflammation [122-124]. However, the precise mechanism by which these oligosaccharides regulate invasion is not very clear. Since, invasion involves modulation of adhesion, matrix degradation and motility of cancer cells; these oligosaccharides probably regulate the steps in invasion. Previous work from the lab using B16 murine melanoma invasive variants as model system has shown that, irrespective of the strategies used (by comparing invasive variants B16F10 and B16Bl6, or the cells in which glycosylation was inhibited with SW or antisense to GnT-V), expression of these oligosaccharides correlates with the invasive potential of Bl6 melanoma cell lines (**Figure 1**). The expression of β1,6 branched N-oligosaccharides in these B16 melanoma invasive variants always correlated positively with their adhesion on most ECM and BM

components and chemotactic motility towards representative members of ECM (Fibronectin) and BM (matrigel). Irrespective of the levels of expression of these oligosaccharides these cells showed no difference in the basal secretion of MMPs when grown on plastic. Their expression appeared to regulate haptotactic motility differentially on ECM (fibronectin) and BM (matrigel) components, enhanced on fibronectin but attenuated on matrigel [140]. However, the expression of β1,6 branched N-oligosaccharides always correlated with invasiveness.

Regulation of adhesion to matrix by β1,6 branched N-oligosaccharides:

These oligosaccharides appear to regulate adhesion to matrix components, both positively and negatively. In some cell lines like in B16 melanoma and in HT-29 they promote adhesion [117, 140, 149] while on many others their expression has an exactly opposite effect on adhesion [130, 150]. Adhesion is the key step in cancer cell invasion and tumor cells require an optimum level of cellular adhesion for movement and invasion. Cells that adhere too tightly are unable to move, and those adhering loosely are also immobile and thus non-invasive [151, 152]. Beta1,6 branched N-oligosaccharides possibly are able to regulate adhesion both positively and negatively by regulating the terminal substitutions associated with them as it is the preferred site for addition of various terminal sugars. Depending on their basal adhesive characteristics tumor cells possibly regulate adhesion by manipulating these terminal substitutions.

Among the various possible terminal substitutions, apart from polylacNAc, expression of α2,6 linked sialic acids was found to correlate with the expression of β1,6 branched N-oligosaccharides on the invasive variants of B16 melanoma cells (**Figure 2**).

Comparison of purified proteins normalised with respect to the expression of β1,6 branched N-oligosaccharides from B16F10 and B16BL6 cells confirmed that β1,6 branched N-oligosaccharides on invasive cells are indeed associated with α2,6 SA as the terminal substitution (**Figure 3**). Increased expression of α2,6 linked SA in general on mammary carcinoma cell line MDA-MB-435 has been shown to be associated with increased adhesion of cells to collagen-IV [153]. On the contrary, removal of α2,3 linked SA from these oligosaccharides on bladder carcinoma cell line T24 increased their adhesion to fibronectin and collagen-IV [154]. Similarly, in breast cancer and human melanoma cells negative correlation between expression of β1,6 branched N–oligosaccharides and adhesion appears to be due to substitution of SA in α2,3 linkage [150, 155]. It is thus possible that α2,6 SA substitutions on these oligosaccharides promotes adhesion and thus their removal should decrease adhesion.

Although, the enzyme that specifically removes α2,6 SA is not available, by using a combination of sialidases that remove only α2,3 or both α2,3 and α2,6 linked SA, β1,6 branched N-oligosaccharides have earlier been shown to be the preferentially substituted with α2,6 linked SA [156]. Using similar desialylation approach we showed that removal of α2,3/6 linked SA from B16BL6 cells significantly decreased their adhesion to fibronectin and matrigel (**Figure 4C**). However, removal of only α2,3 linked SA did not have much effect on adhesion. The role of terminal α2,6 linked SA in regulating adhesion of B16BL6 cells was further demonstrated by **s**pecific inhibition of α2,6 linked SA on proteins carrying N-linked oligosaccharides from B16BL6 cells, by shRNA against ST6Gal-I (**Figure 6B**). ST6Gal-1 is the only sialylaltransferases that adds SA in α2,6 linkage on N-glycans. Decreased adhesion to ECM component (fibronectin) and reconstituted BM (matrigel) also

resulted in significant reduction in the invasive ability of cells in which α2,6 SA was down regulated (**Figure 6D**).

Regulation of matrix degradation by β1,6 branched N-oligosaccharides:

Degradation of the matrix is a key feature of invasion process although; the whole process is highly regulated. The mechanisms to regulate matrix degradation involve, controlling secretion, activation and localization of matrix degrading enzymes. Matrix metalloproteinases are a class of enzymes that are capable of degrading the major components of ECM and BM viz., collagens, non-collagenous glycoproteins and the protein moiety of the proteoglycans. Previous work from the lab has shown that the expression of these oligosaccharides on B16 melanoma cells does not correlate with the secretion of MMPs when grown on uncoated or BSA coated culture dishes (basal secretion). However, since the expression of these oligosaccharides correlates with adhesion to ECM (FN) and BM (matrigel) [140], (**Figure 7A and 9A**), increased adhesion of these cells on FN and matrigel resulted in significant induction of MMP-9 secretion (**Figure 7B**). Inhibition of expression of these oligosaccharides either using glycosylation inhibitor SW or by transduction of shRNA to GnT-V (which catalyses the addition of β1,6 branch) inhibited adhesion **(Figure 7A and 9A)**. This was associated with significant reduction in MMP-9 secretion **(Figure 7B, 9B and C)**. Adhesion of Gastric carcinoma cells to laminin-5 have been shown to induce the secretion of MMP-9 [157]. Increased adhesion to matrices mediated by these oligosaccharides could be one of mechanism adopted by the tumor cells for inducing the secretion of MMP-9 and thus, facilitating the regulated degradation of matrices. Since α2,6 linked SA substitution on β1,6 branched N-

oligosaccharides regulates adhesion, its contribution in regulating induction of MMP-9 secretion cannot be ruled out.

In order to prevent the indiscriminate degradation of matrix, which is also used as traction for movement, tumor cells localize the degradation machinery with the motility receptors [158]. CD44 and β1 integrin are found to be the carrier of β1,6 branched N-oligosaccharides. The surface expression of motility receptors is often regulated by the extent of glycosylation [159]. However, comparison of invasive variants B16F10 and B16BL6 cells which differ in expression of such oligosaccharides or treatment of B16BL6 cells with SW did not affect their surface localization **(Figure 10)**. CD44 and β1 integrin have been shown to be associated with proteolytic machinery [160, 161]. To investigate whether presence of β1,6 branched N-linked oligosaccharides on motility receptors have any role in localizing receptors/membrane tethered forms of proteases with the receptors involved in motility. Membrane tethered form of proteases (MT1-MMP) or receptor for proteases (uPA) i.e. uPAR are the major regulators for conversion of pro-MMPs into active form. The expression of these proteins has often been shown to correlate with the invasive potential of cancer cells [162]. The expression of MT1-MMP on B16 melanoma invasive variants indeed correlated with the invasive potential **(Figure 11)**, however, expression of uPAR was unaltered in these cell lines **(Figure 11).**

Motility receptors like β1 integrin or CD44 were found to be associated with MT1-MMP and uPAR in B16 melanoma invasive variants **(Figure 12 and 13)**. Upon inhibition of glycosylation in B16Bl6 cells with SW, it only inhibited the association of β1 integrin with MT1-MMP **(Figure 12C)**. However, association of β1 integrin with uPAR and CD44 with MT1-MMP/uPAR was unaltered. These results need to be confirmed by

immunoprecipitation studies, but it was not possible due to non-availability of good commercial antibody for immunoprecipitation. Recently, it has been shown that glycosylation on αVβ3 and α3β1 integrin regulates the association of uPAR in human melanoma cell lines [163]. However, we did not observe glycosylation dependent association of β1 integrin with uPAR, this could be due to interaction of uPAR with α subunits.

Regulation of motility of cells on ECM/BM components by β1,6 branched N-oligosaccharides:

Besides, modulating the adhesion and matrix degradation, expression of these oligosaccharides appears to influence motility of cells in a substrate specific manner, enhanced on ECM (fibronectin) and attenuated on BM (laminin/matrigel) components [140]. Increased adhesion on ECM and BM components on one hand regulates matrix degradation and on the other hand possibly regulates movement [152]. To be invasive, tumor cells need to be more motile when surrounded by other cells and the ECM, however, once they reach the basement membrane they need to stabilize adhesion for sustained degradation followed by controlled motility. Even spreading of the cells would differ depending on the substratum and less spreaded morphology appears to play an important role in breaching the BM [164]. The expression of these oligosaccharides appeared to have the same differential effect on spreading as that seen on motility. Inhibition of these oligosaccharides inhibited spreading of B16BL6 cells on fibronectin but increased it on matrigel **(Figure 14A, B** and **Figure 15B, C**). Similar observations were reported earlier by Leppa et al. who showed that the steroid induced transformation of S115 mammary epithelial cells is associated with expression of complex N-glycans substituted with poly-N-

acetyllactosamine on β1 integrin laminin receptors. Inhibition of these oligosaccharides increased the spreading of cells on laminin-1 but was unaffected on fibronectin [165]. Integrin receptors α3, α5, αv, β1 and β3 have been shown to be the carriers of β1,6 branched N-oligosaccharides on different cell types [144]. These oligosaccharides have been shown to affect integrin structure, dimerization, affinity, clustering and stability of different integrin receptors [76-80].

Altered glycosylation of respective integrin receptors (for fibronectin and components of matrigel) could be responsible for the differential spreading and motility on these substrates. However, both the subunits of the major receptors for fibronectin (α5β1) and laminin (α3β1), (laminin is the major component of matrigel >50%), were found to carry β1,6 branched N-oligosaccharides (**Figure 16A**). Tumor cells do show altered surface expression of certain integrin receptors [166]. Even altered glycosylation appears to dictate the levels of integrins on cell surface [159]. The other cause of differential motility could thus be due to differences in surface localization of respective integrin receptors. However, the expression of α5β1 and α3β1 integrin (**Figure 16B, C and D**) remained almost identical on B16F10, B16BL6 and the cells in which glycosylation was inhibited.

The other possibility is differential regulation of fibronectin and laminin receptors in the tetraspanin (TSP) enriched membrane micro domains (TEMs). TEMs appear to modulate invasion associated processes by regulating the availability of TEM associated molecules, particularly integrins. Expression of most TSPs except CD151 and CO-029 is down regulated by highly invasive and metastatic cells [86]. However, there was no significant change in the transcript levels of major TSPs, which are important from the

invasion point of view, in the parent B16F10 and its invasive variant B16BL6 cells (**Figure 17A**). The motility of cells on fibronectin is largely regulated by the association of fibronectin receptor (α5β1) with TSP CD82 while that on matrigel/laminin by the association of laminin receptor (α3β1) with TSP CD151. The levels of both these TSPs, viz., CD82 and CD151, also remained almost identical in the melanoma invasive variants, as assessed by Western blotting and flow cytometry (**Figure 17B and C**). This data indicates that to be invasive it is not necessary to show alteration in expression of tetraspanin. There are a few instances where absence of correlation between the level of expression and invasiveness has been demonstrated. For example, in some of the invasive variants of human bladder carcinoma cell lines, there is no correlation between expression of CD82 and their invasive potential [167]. This shows that the cells possibly use an alternate mechanism to regulate integrin TSP interaction and thus invasion.

Glycosylation of both the tetraspanin (CD82) and fibronectin receptor (α5β1) has been shown to regulate their association and thus motility [90]. It is possible that expression of β1,6 branched N-oligosaccharides on the laminin receptor α3β1 also regulates its association with CD151 and influences its motility for invasion of basement membrane. The association of laminin receptors with tetraspanins is largely dictated by the interaction of α subunit with CD151 [168]. Among the laminin receptors α3β1 is the major carrier of β1,6 branched N-oligosaccharides which associates with CD151. Both, α3 and β1 integrin subunits express these oligosaccharides on B16BL6 cells and treatment with SW resulted in significantly higher association of β1 integrin with CD151 as demonstrated by laser confocal microscopy and co-immunoprecipitation studies **(Figure 19A, B and C)**. Integrin subunit β1 associates with several other α subunits, however, immunoprecipitation

experiments showed that SW treatment significantly promoted association of CD151 with not only β1 but also α3 subunit of integrin receptor **(Figure 19D)**. The enhanced spreading and motility on matrigel could thus be due to altered association of laminin receptor α3β1 with TSP CD151. Although, the use of CD151 transfected cells limited our options to use more specific methods of inhibition of β1,6 branched N-oligosaccharides (as multiple transfection and selection involves several passaging affects the metastatic potential of these cell lines), all our previous experiments and work have confirmed that SW is equally effective [140]. Therefore, we have used SW for the inhibition of glycosylation in CD151 transfected B16BL6 cells. A single N-glycosylation site on CD151 has been identified and SW treatment results in reduction in the size of FLAG tagged CD151 **(Fig. 19C and D)**, which may also have a role in regulating association of CD151 with integrins. However, immunoprecipitated CD151 showed no reactivity to either L- or E-PHA which recognise multiantennary/bisected complex N-glycans.

CD151 majorly contributes to the invasiveness of the cells, but its effect on motility is highly regulated, and glycosylation appears to play a key role in this regulation. Treatment of B16BL6 cells overexpressing CD151 with SW increased their motility significantly; however, it had an exactly opposite effect on their invasiveness. It suggests that cancer cells regulate their motility on BM to be invasive and glycosylation of receptors has a major role in dictating their association with tetraspanins and thus motility. In spite of enhanced spreading and motility on BM (matrigel) **(Figure 20 and 21)**, SW treated cells showed significant reduction in their invasion through matrigel **(Figure 21D)**. Spreading of the cells differs depending on the substratum and less spread morphology on BM appears to be an important determinant for breaching this barrier [164]. To be invasive, tumor cells

need to be more motile when surrounded by other cells and the ECM, however, once they reach the basement membrane they possibly need to stabilize adhesion for sustained degradation followed by controlled motility.

Expression of β1,6 branched N-oligosaccharides on human fibrosarcoma cells has been shown to result in increased movement on fibronectin and invasion of matrigel [79]. Increased expression β1,6 of branched N-oligosaccharides in human gastric cancer cells resulted in increased movement on laminin and could be reversed by overexpression of bisected N-glycans on laminin receptor α3β1 [138]. More recently, the association of the receptors with the tetraspanins and the membrane microdomains formed by them, TEMs, has also assumed importance, not only in regulating the receptor glycosylation, but also its function and cell motility. Downregulation of CD151 was shown to cause reduction of α1, 2 fucosylation and bisected N-glycans on α3β1 integrin. This resulted in reduced motility of cells on laminin [91]. These investigations bring an insight into the complex mechanism by which these oligosaccharides regulate spreading and movement of cells on ECM and BM components to achieve optimum invasion. Specifically, it highlights the importance of membrane micro-domains formed by tetraspanins in regulating their association with motility receptors like integrins and the role that β1,6 branched N-oligosaccharides play in modulating them and thus cancer cell movement and invasion.

In conclusion, these investigations highlight the complex mechanism of tumor cell invasion, a crucial requirement for their dissemination. It demonstrates the key role played by tumor cell surface modifications associated with invasion and metastasis in regulating different steps of the invasion process. Specifically, it delineates the mechanism by which

the invasion associated expression of β1,6 branched N-oligosaccharides regulate key events like adhesion, matrix degradation and migration. These studies showed that even the terminal substitutions like sialic acids associated with these oligosaccharides and the linkage in which they are attached may influence invasion by modulating adhesion to matrix components.

These studies have explored the significance of increased adhesion facilitated by β1,6 branched N-oligosaccharides in terms of invasion. It demonstrates that these oligosaccharides regulate adhesion which in turn regulates the secretion of MMPs. It also demonstrates the role that these oligosaccharides on motility receptors (like CD44 and β1 integrin) play in localization of receptor or membrane tethered forms of matrix degrading enzymes (uPAR and MT1-MMP) with them.

It brings an insight into the complex regulation of function of the receptors that are involved in regulating motility and possibly invasion. The level of receptor, the extent and type of glycosylation of the receptor, the levels and glycosylation of the tetraspanin(s) all appear to determine the extent of association of the receptor(s) with membrane microdomains - TEMs, and thus cell movement and invasion.

These studies demonstrate that invasion is indeed a very complex process where each step is regulated in multiple ways and altered cell surface glycosylation associated with the invasive phenotype plays an integral role in regulating all the processes of cellular invasion. Development of strategies to target such glycosylation would not only help in understanding but also tackling the disease better.

CHAPTER 5

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

- Expression of β1,6 branched N-linked oligosaccharides correlates with the invasive potential of B16 melanoma invasive variants.
- Sialic acids in α2,6 linkage and polylacNAc are the major terminal substitutions associated with these oligosaccharides.
- Increased substitution of α2,6 linked sialic acids promotes adhesion of cells to ECM and BM components.

  Increased substitution of α2,6 linked SA regulates adhesion positively to a level possibly optimum for its motility and invasion.
- Increased adhesion associated with increased expression of α2,6 linked SA substituted β1,6 branched N-linked oligosaccharides induces secretion of MMP-9.
- Expression of MT1-MMP correlates with invasive potential of B16 melanoma cell lines.
- MT1-MMP associates with β1 integrin and this association is regulated by the presence of β1,6 branched N-linked oligosaccharides on β1 integrin.

  Increased expression of β1,6 branched N-oligosaccharides and their associated terminal substitution like α2,6 linked SA on B16BL6 regulate adhesion induced secretion of MMP-9. Enhanced expression coupled with glycosylation dependent association of MT1-MMP with molecules like β1 integrin regulate matrix degradation and invasion.
- Expression of β1,6 branched N-oligosaccharides differentially regulates spreading and thus motility of B16BL6 cells, enhanced on fibronectin (ECM) and attenuated

on matrigel (BM). This is reversed on inhibition of expression of these oligosaccharides.

- Altered motility is neither due to alteration in expression of their respective integrin receptors nor the tetraspanins which are shown to modulate integrin functions.
- Both the subunits of fibronectin (α5β1) and laminin- major component of matrigel (α3β1) receptors carry β1,6 branched N-oligosaccharides.
- Glycosylation regulates the spreading/motility on matrigel (laminin) by modulating the association of tetraspanin CD151 with the integrin receptor for laminin α3β1 integrin, which impacts invasion of BM.

Cancer cells regulate invasion of basement membrane by modulating spreading and motility which in turn is regulated by the expression of β1,6 branched N-oligosaccharides. Presence of these oligosaccharides on α3β1 integrin promotes cellular morphology optimum for spreading and movement by regulating its association with tetraspanins (CD151), which impacts invasion.

REFERENCES

REFERENCES

1. Siegel R, Naishadham D, Jemal A (2012) Cancer statistics for Hispanics/Latinos, 2012. CA: a cancer journal for clinicians 62 5): 283-98
2. Lander ES (2011) Initial impact of the sequencing of the human genome. Nature 470 7333): 187-97
3. Vogelstein B, Kinzler KW (2004) Cancer genes and the pathways they control. Nature medicine 10 8): 789-99
4. Sharma S, Kelly TK, Jones PA (2010) Epigenetics in cancer. Carcinogenesis 31 1): 27-36
5. Farazi TA, et al. (2011) miRNAs in human cancer. J Pathol 223 2): 102-15
6. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100 1): 57-70
7. Slamon DJ, et al. (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235 4785): 177-82
8. Marinari B, et al. (2008) The tumor suppressor activity of IKKalpha in stratified epithelia is exerted in part via the TGF-beta antiproliferative pathway. Proceedings of the National Academy of Sciences of the United States of America 105 44): 17091-6
9. Park I, et al. (2012) A novel gain-of-function mutation of TGF-beta receptor II promotes cancer progression via delayed receptor internalization in oral squamous cell carcinoma. Cancer letters 315 2): 161-9
10. Zhu CQ, et al. (2006) Amplification of telomerase (hTERT) gene is a poor prognostic marker in non-small-cell lung cancer. Br J Cancer 94 10): 1452-9
11. Kaufmann SH, Gores GJ (2000) Apoptosis in cancer: cause and cure. Bioessays 22 11): 1007-17
12. Folkman J (Year) Role of angiogenesis in tumor growth and metastasis. Seminars in oncology, 2002, vol 29. Elsevier, p. 15-8
13. Hart IR, Fidler IJ (1980) Cancer invasion and metastasis. Quarterly Review of Biology: 121-42
14. Rabinovich GA, Gabrilovich D, Sotomayor EM (2007) Immunosuppressive strategies that are mediated by tumor cells. Annual review of immunology 25: 267-96
15. Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324 5930): 1029-33
16. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144 5): 646-74
17. Spano D, et al. (2012) Molecular networks that regulate cancer metastasis. Semin Cancer Biol 22 3): 234-49
18. Cairns RA, Khokha R, Hill RP (2003) Molecular mechanisms of tumor invasion and metastasis: an integrated view. Curr Mol Med 3 7): 659-71
19. Nicolson GL (1988) Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. Cancer Metastasis Rev 7 2): 143-88
20. Brooks SA, et al. (2010) Molecular interactions in cancer cell metastasis. Acta Histochem 112 1): 3-25

21. Poste G, et al. (1980) In vitro selection of murine B16 melanoma variants with enhanced tissue-invasive properties. Cancer Res 40 5): 1636-44
22. Fidler IJ (1973) Selection of successive tumour lines for metastasis. Nature: New biology 242 118): 148-9
23. Nguyen DX, Bos PD, Massague J (2009) Metastasis: from dissemination to organ-specific colonization. Nature reviews Cancer 9 4): 274-84
24. Matter K, Balda MS (2003) Signalling to and from tight junctions. Nature Reviews Molecular Cell Biology 4 3): 225-37
25. Frantz C, Stewart KM, Weaver VM (2010) The extracellular matrix at a glance. Journal of cell science 123 Pt 24): 4195-200
26. Halfter W, et al. (2013) Protein composition and biomechanical properties of in vivo-derived basement membranes. Cell adhesion & migration 7 1): 64-71
27. Nagano M, et al. (2012) Turnover of focal adhesions and cancer cell migration. International journal of cell biology 2012: 310616
28. Bergstraesser LM, et al. (1995) Expression of hemidesmosomes and component proteins is lost by invasive breast cancer cells. The American journal of pathology 147 6): 1823-39
29. Gao D, et al. (2012) Microenvironmental regulation of epithelial-mesenchymal transitions in cancer. Cancer Res 72 19): 4883-9
30. Woodward JK, et al. (2007) The roles of proteolytic enzymes in the development of tumour-induced bone disease in breast and prostate cancer. Bone 41 6): 912-27
31. Dudley AC (2012) Tumor endothelial cells. Cold Spring Harbor perspectives in medicine 2 3): a006536
32. Sonoshita M, et al. (2011) Suppression of colon cancer metastasis by Aes through inhibition of Notch signaling. Cancer cell 19 1): 125-37
33. Padua D, et al. (2008) TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell 133 1): 66-77
34. Robinson BD, et al. (2009) Tumor microenvironment of metastasis in human breast carcinoma: a potential prognostic marker linked to hematogenous dissemination. Clinical cancer research : an official journal of the American Association for Cancer Research 15 7): 2433-41
35. Geiger TR, Peeper DS (2007) Critical role for TrkB kinase function in anoikis suppression, tumorigenesis, and metastasis. Cancer Res 67 13): 6221-9
36. Gay LJ, Felding-Habermann B (2011) Contribution of platelets to tumour metastasis. Nature reviews Cancer 11 2): 123-34
37. Geldof AA (1997) Models for cancer skeletal metastasis: a reappraisal of Batson's plexus. Anticancer Res 17 3A): 1535-9
38. Ewing J (1928) Neoplastic diseases: a treatise on tumors, vol. 721. WB Saunders Philadelphia,
39. Paget S (1989) The distribution of secondary growths in cancer of the breast. 1889. Cancer Metastasis Rev 8 2): 98-101
40. Langley RR, Fidler IJ (2007) Tumor cell-organ microenvironment interactions in the pathogenesis of cancer metastasis. Endocrine reviews 28 3): 297-321
41. Ben-Baruch A (2008) Organ selectivity in metastasis: regulation by chemokines and their receptors. Clinical & experimental metastasis 25 4): 345-56

42. Nicolson GL (1993) Paracrine and autocrine growth mechanisms in tumor metastasis to specific sites with particular emphasis on brain and lung metastasis. Cancer Metastasis Rev 12 3-4): 325-43
43. Stetler-Stevenson WG, Liotta LA, Kleiner DE, Jr. (1993) Extracellular matrix 6: role of matrix metalloproteinases in tumor invasion and metastasis. FASEB J 7 15): 1434-41
44. Kessenbrock K, Plaks V, Werb Z (2010) Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 141 1): 52-67
45. Yu Q, Stamenkovic I (1999) Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. Genes & development 13 1): 35-48
46. Monferran S, et al. (2004) The membrane form of the DNA repair protein Ku interacts at the cell surface with metalloproteinase 9. The EMBO journal 23 19): 3758-68
47. Brooks PC, et al. (1996) Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. Cell 85 5): 683-93
48. Jiang Y, Goldberg ID, Shi YE (2002) Complex roles of tissue inhibitors of metalloproteinases in cancer. Oncogene 21 14): 2245-52
49. Seiki M (2003) Membrane-type 1 matrix metalloproteinase: a key enzyme for tumor invasion. Cancer letters 194 1): 1-11
50. Ueda J, et al. (2003) Sequence-specific silencing of MT1-MMP expression suppresses tumor cell migration and invasion: importance of MT1-MMP as a therapeutic target for invasive tumors. Oncogene 22 54): 8716-22
51. Tsunezuka Y, et al. (1996) Expression of membrane-type matrix metalloproteinase 1 (MT1-MMP) in tumor cells enhances pulmonary metastasis in an experimental metastasis assay. Cancer research 56 24): 5678-83
52. Alpizar-Alpizar W, et al. (2012) Urokinase plasminogen activator receptor on invasive cancer cells: a prognostic factor in distal gastric adenocarcinoma. International journal of cancer Journal international du cancer 131 4): E329-36
53. Raghu H, et al. (2010) Localization of uPAR and MMP-9 in lipid rafts is critical for migration, invasion and angiogenesis in human breast cancer cells. BMC cancer 10: 647
54. Gocheva V, Joyce JA (2007) Cysteine cathepsins and the cutting edge of cancer invasion. Cell Cycle 6 1): 60-4
55. Mohamed MM, Sloane BF (2006) Cysteine cathepsins: multifunctional enzymes in cancer. Nature reviews Cancer 6 10): 764-75
56. Tan JX, et al. (2011) HYAL1 overexpression is correlated with the malignant behavior of human breast cancer. International journal of cancer Journal international du cancer 128 6): 1303-15
57. Tan JX, et al. (2011) Upregulation of HYAL1 expression in breast cancer promoted tumor cell proliferation, migration, invasion and angiogenesis. PloS one 6 7): e22836
58. Maxhimer JB, et al. (2002) Heparanase-1 expression is associated with the metastatic potential of breast cancer. Surgery 132 2): 326-33

59. Murry BP, et al. (2006) Heparanase mechanisms of melanoma metastasis to the brain: Development and use of a brain slice model. Journal of cellular biochemistry 97 2): 217-25
60. Ohkawa T, et al. (2004) Localization of heparanase in esophageal cancer cells: respective roles in prognosis and differentiation. Laboratory investigation; a journal of technical methods and pathology 84 10): 1289-304
61. Duffy MJ, et al. (2011) The ADAMs family of proteases: new biomarkers and therapeutic targets for cancer? Clinical proteomics 8 1): 9
62. Rocks N, et al. (2008) Emerging roles of ADAM and ADAMTS metalloproteinases in cancer. Biochimie 90 2): 369-79
63. Zheng X, et al. (2007) Inhibition of ADAM17 reduces hypoxia-induced brain tumor cell invasiveness. Cancer science 98 5): 674-84
64. Held-Feindt J, et al. (2006) Matrix-degrading proteases ADAMTS4 and ADAMTS5 (disintegrins and metalloproteinases with thrombospondin motifs 4 and 5) are expressed in human glioblastomas. International journal of cancer Journal international du cancer 118 1): 55-61
65. Aznavoorian S, et al. (1990) Signal transduction for chemotaxis and haptotaxis by matrix molecules in tumor cells. The Journal of cell biology 110 4): 1427-38
66. Yang K, et al. (2010) Stable alterations of CD44 isoform expression in prostate cancer cells decrease invasion and growth and alter ligand binding and chemosensitivity. BMC cancer 10: 16
67. Casey RC, Skubitz AP (2000) CD44 and beta1 integrins mediate ovarian carcinoma cell migration toward extracellular matrix proteins. Clinical & experimental metastasis 18 1): 67-75
68. Bartolazzi A, et al. (1996) Glycosylation of CD44 is implicated in CD44-mediated cell adhesion to hyaluronan. The Journal of cell biology 132 6): 1199-208
69. van der Voort R, et al. (1999) Heparan sulfate-modified CD44 promotes hepatocyte growth factor/scatter factor-induced signal transduction through the receptor tyrosine kinase c-Met. The Journal of biological chemistry 274 10): 6499-506
70. Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. Cell 110 6): 673-87
71. Srichai MB, Zent R (2010) Integrin structure and function. Cell-Extracellular Matrix Interactions in Cancer. Springer, pp 19-41
72. Barczyk M, Carracedo S, Gullberg D (2010) Integrins. Cell and tissue research 339 1): 269-80
73. Ridley AJ, et al. (2003) Cell migration: integrating signals from front to back. Science 302 5651): 1704-9
74. Felding-Habermann B, et al. (1992) Involvement of integrin alpha V gene expression in human melanoma tumorigenicity. The Journal of clinical investigation 89 6): 2018-22
75. Kato H, et al. (2012) The primacy of beta1 integrin activation in the metastatic cascade. PloS one 7 10): e46576
76. Zheng M, Fang H, Hakomori S (1994) Functional role of N-glycosylation in alpha 5 beta 1 integrin receptor. De-N-glycosylation induces dissociation or altered association of alpha 5 and beta 1 subunits and concomitant loss of fibronectin binding activity. The Journal of biological chemistry 269 16): 12325-31

77. Isaji T, et al. (2006) N-glycosylation of the beta-propeller domain of the integrin alpha5 subunit is essential for alpha5beta1 heterodimerization, expression on the cell surface, and its biological function. The Journal of biological chemistry 281 44): 33258-67
78. Isaji T, et al. (2004) Introduction of bisecting GlcNAc into integrin alpha5beta1 reduces ligand binding and down-regulates cell adhesion and cell migration. The Journal of biological chemistry 279 19): 19747-54
79. Guo HB, et al. (2002) Aberrant N-glycosylation of beta1 integrin causes reduced alpha5beta1 integrin clustering and stimulates cell migration. Cancer Res 62 23): 6837-45
80. Wang L, et al. (2007) Increase in beta1-6 GlcNAc branching caused by N-acetylglucosaminyltransferase V directs integrin beta1 stability in human hepatocellular carcinoma cell line SMMC-7721. Journal of cellular biochemistry 100 1): 230-41
81. Hemler ME (2005) Tetraspanin functions and associated microdomains. Nature reviews Molecular cell biology 6 10): 801-11
82. Le Naour F, et al. (2006) Membrane microdomains and proteomics: lessons from tetraspanin microdomains and comparison with lipid rafts. Proteomics 6 24): 6447-54
83. Romanska HM, Berditchevski F (2011) Tetraspanins in human epithelial malignancies. J Pathol 223 1): 4-14
84. Zoller M (2009) Tetraspanins: push and pull in suppressing and promoting metastasis. Nature reviews Cancer 9 1): 40-55
85. Hemler ME (2008) Targeting of tetraspanin proteins--potential benefits and strategies. Nat Rev Drug Discov 7 9): 747-58
86. Richardson MM, Jennings LK, Zhang XA (2011) Tetraspanins and tumor progression. Clinical & experimental metastasis 28 3): 261-70
87. Liu WM, et al. (2012) Tetraspanin CD82 inhibits protrusion and retraction in cell movement by attenuating the plasma membrane-dependent actin organization. PloS one 7 12): e51797
88. Hong IK, et al. (2006) Homophilic interactions of Tetraspanin CD151 up-regulate motility and matrix metalloproteinase-9 expression of human melanoma cells through adhesion-dependent c-Jun activation signaling pathways. The Journal of biological chemistry 281 34): 24279-92
89. Stipp CS (2010) Laminin-binding integrins and their tetraspanin partners as potential antimetastatic targets. Expert Rev Mol Med 12: e3
90. Ono M, et al. (2000) Glycosylation effect on membrane domain (GEM) involved in cell adhesion and motility: a preliminary note on functional alpha3, alpha5-CD82 glycosylation complex in ldlD 14 cells. Biochem Biophys Res Commun 279 3): 744-50
91. Baldwin G, et al. (2008) Tetraspanin CD151 regulates glycosylation of (alpha)3(beta)1 integrin. The Journal of biological chemistry 283 51): 35445-54
92. Preston RJ, et al. (2013) Elucidating the role of carbohydrate determinants in regulating hemostasis: insights and opportunities. Blood 121 19): 3801-10
93. Schnaar RL, Suzuki A, Stanley P (2009) Glycosphingolipids. In: Varki A, et al. (eds) Essentials of Glycobiology. 2nd edn, Cold Spring Harbor (NY)

94. Ruan S, Lloyd KO (1992) Glycosylation pathways in the biosynthesis of gangliosides in melanoma and neuroblastoma cells: relative glycosyltransferase levels determine ganglioside patterns. Cancer Res 52 20): 5725-31
95. Liang YJ, et al. (2013) Differential expression profiles of glycosphingolipids in human breast cancer stem cells vs. cancer non-stem cells. Proceedings of the National Academy of Sciences of the United States of America 110 13): 4968-73
96. Simons K, Toomre D (2000) Lipid rafts and signal transduction. Nature Reviews Molecular Cell Biology 1 1): 31-9
97. Barbareschi M, et al. (2003) High syndecan-1 expression in breast carcinoma is related to an aggressive phenotype and to poorer prognosis. Cancer 98 3): 474-83
98. Su G, et al. (2006) Glypican-1 is frequently overexpressed in human gliomas and enhances FGF-2 signaling in glioma cells. The American journal of pathology 168 6): 2014-26
99. Matsuda K, et al. (2001) Glypican-1 is overexpressed in human breast cancer and modulates the mitogenic effects of multiple heparin-binding growth factors in breast cancer cells. Cancer Res 61 14): 5562-9
100. Brockhausen I (2006) Mucin-type O-glycans in human colon and breast cancer: glycodynamics and functions. EMBO reports 7 6): 599-604
101. Tsuboi S, et al. (2012) Two opposing roles of O-glycans in tumor metastasis. Trends in molecular medicine 18 4): 224-32
102. Lee SH, et al. (2009) Core3 O-glycan synthase suppresses tumor formation and metastasis of prostate carcinoma PC3 and LNCaP cells through down-regulation of alpha2beta1 integrin complex. The Journal of biological chemistry 284 25): 17157-69
103. Stanley P, Schachter H, Taniguchi N (2009) N-Glycans. In: Varki A, et al. (eds) Essentials of Glycobiology. 2nd edn, Cold Spring Harbor (NY)
104. Buck CA, Glick MC, Warren L (1970) A comparative study of glycoproteins from the surface of control and Rous sarcoma virus transformed hamster cells. Biochemistry 9 23): 4567-76
105. Buck CA, Glick MC, Warren L (1971) Glycopeptides from the surface of control and virus-transformed cells. Science 172 3979): 169-71
106. Yamashita K, et al. (1985) Enzymatic basis for the structural changes of asparagine-linked sugar chains of membrane glycoproteins of baby hamster kidney cells induced by polyoma transformation. The Journal of biological chemistry 260 7): 3963-9
107. Seelentag WK, et al. (1998) Prognostic value of beta1,6-branched oligosaccharides in human colorectal carcinoma. Cancer Res 58 23): 5559-64
108. Dennis JW, Granovsky M, Warren CE (1999) Glycoprotein glycosylation and cancer progression. Biochimica et biophysica acta 1473 1): 21-34
109. Takahashi N, et al. (2009) High expression of N-acetylglucosaminyltransferase V in mucinous tumors of the ovary. Oncology reports 22 5): 1027-32
110. Ishibashi Y, et al. (2005) Expression of N-acetylglucosaminyltransferase V in the development of human esophageal cancers: immunohistochemical data from carcinomas and nearby noncancerous lesions. Oncology 69 4): 301-10
111. Yamamoto E, et al. (2007) Expression of N-acetylglucosaminyltransferase V in endometrial cancer correlates with poor prognosis. Br J Cancer 97 11): 1538-44

112. Dennis JW, et al. (1989) Oncogenes conferring metastatic potential induce increased branching of Asn-linked oligosaccharides in rat2 fibroblasts. Oncogene 4 7): 853-60
113. Chen L, et al. (1998) The her-2/neu oncogene stimulates the transcription of N-acetylglucosaminyltransferase V and expression of its cell surface oligosaccharide products. Oncogene 17 16): 2087-93
114. Cummings RD, Trowbridge IS, Kornfeld S (1982) A mouse lymphoma cell line resistant to the leukoagglutinating lectin from Phaseolus vulgaris is deficient in UDP-GlcNAc: alpha-D-mannoside beta 1,6 N-acetylglucosaminyltransferase. The Journal of biological chemistry 257 22): 13421-7
115. Humphries MJ, et al. (1986) Oligosaccharide modification by swainsonine treatment inhibits pulmonary colonization by B16-F10 murine melanoma cells. Proceedings of the National Academy of Sciences of the United States of America 83 6): 1752-6
116. Murata K, et al. (2004) Attachment of human colon cancer cells to vascular endothelium is enhanced by N-acetylglucosaminyltransferase V. Oncology 66 6): 492-501
117. Yoshimura M, et al. (1995) Suppression of lung metastasis of B16 mouse melanoma by N-acetylglucosaminyltransferase III gene transfection. Proceedings of the National Academy of Sciences of the United States of America 92 19): 8754-8
118. Granovsky M, et al. (2000) Suppression of tumor growth and metastasis in Mgat5-deficient mice. Nature medicine 6 3): 306-12
119. Krishnan V, et al. (2005) Altered melanoma cell surface glycosylation mediates organ specific adhesion and metastasis via lectin receptors on the lung vascular endothelium. Clinical & experimental metastasis 22 1): 11-24
120. Srinivasan N, et al. (2009) Poly N-acetyllactosamine substitutions on N- and not O-oligosaccharides or Thomsen-Friedenreich antigen facilitate lung specific metastasis of melanoma cells via galectin-3. Glycoconj J 26 4): 445-56
121. Guo HB, et al. (2010) Specific posttranslational modification regulates early events in mammary carcinoma formation. Proceedings of the National Academy of Sciences of the United States of America 107 49): 21116-21
122. Pili R, et al. (1995) The alpha-glucosidase I inhibitor castanospermine alters endothelial cell glycosylation, prevents angiogenesis, and inhibits tumor growth. Cancer Res 55 13): 2920-6
123. Lemaire S, et al. (1994) Expression of beta 1-6-branched N-linked oligosaccharides is associated with activation in human T4 and T8 cell populations. The Journal of biological chemistry 269 11): 8069-74
124. Tomiie M, et al. (2005) Elevated expression of N-acetylglucosaminyltransferase V in first trimester human placenta. Biochem Biophys Res Commun 330 3): 999-1004
125. Yamamoto H, et al. (2000) Beta1,6-N-acetylglucosamine-bearing N-glycans in human gliomas: implications for a role in regulating invasivity. Cancer Res 60 1): 134-42
126. Takano R, et al. (1990) Increase of beta 1-6-branched oligosaccharides in human esophageal carcinomas invasive against surrounding tissue in vivo and in vitro. The American journal of pathology 137 5): 1007-11

127. Yagel S, et al. (1989) Evidence that beta 1-6 branched Asn-linked oligosaccharides on metastatic tumor cells facilitate invasion of basement membranes. International journal of cancer Journal international du cancer 44 4): 685-90
128. Guo HB, et al. (2003) N-acetylglucosaminyltransferase V expression levels regulate cadherin-associated homotypic cell-cell adhesion and intracellular signaling pathways. J Biol Chem 278 52): 52412-24
129. Guo HB, Randolph M, Pierce M (2007) Inhibition of a specific N-glycosylation activity results in attenuation of breast carcinoma cell invasiveness-related phenotypes: inhibition of epidermal growth factor-induced dephosphorylation of focal adhesion kinase. The Journal of biological chemistry 282 30): 22150-62
130. Demetriou M, et al. (1995) Reduced contact-inhibition and substratum adhesion in epithelial cells expressing GlcNAc-transferase V. The Journal of cell biology 130 2): 383-92
131. Pinho SS, et al. (2013) E-cadherin and adherens-junctions stability in gastric carcinoma: functional implications of glycosyltransferases involving N-glycan branching biosynthesis, N-acetylglucosaminyltransferases III and V. Biochimica et biophysica acta 1830 3): 2690-700
132. Tsui KH, et al. (2008) Evaluating the function of matriptase and N-acetylglucosaminyltransferase V in prostate cancer metastasis. Anticancer Res 28 4A): 1993-9
133. Ihara S, et al. (2004) Addition of beta1-6 GlcNAc branching to the oligosaccharide attached to Asn 772 in the serine protease domain of matriptase plays a pivotal role in its stability and resistance against trypsin. Glycobiology 14 2): 139-46
134. Korczak B, Dennis JW (1993) Inhibition of N-linked oligosaccharide processing in tumor cells is associated with enhanced tissue inhibitor of metalloproteinases (TIMP) gene expression. International journal of cancer Journal international du cancer 53 4): 634-9
135. Kim YS, et al. (2008) Functional proteomics study reveals that N-Acetylglucosaminyltransferase V reinforces the invasive/metastatic potential of colon cancer through aberrant glycosylation on tissue inhibitor of metalloproteinase-1. Mol Cell Proteomics 7 1): 1-14
136. Przybylo M, et al. (2008) Beta1-6 branching of cell surface glycoproteins may contribute to uveal melanoma progression by up-regulating cell motility. Mol Vis 14: 625-36
137. Lagana A, et al. (2006) Galectin binding to Mgat5-modified N-glycans regulates fibronectin matrix remodeling in tumor cells. Mol Cell Biol 26 8): 3181-93
138. Zhao Y, et al. (2006) N-acetylglucosaminyltransferase III antagonizes the effect of N-acetylglucosaminyltransferase V on alpha3beta1 integrin-mediated cell migration. The Journal of biological chemistry 281 43): 32122-30
139. Huang B, et al. (2013) Downregulation of the GnT-V gene inhibits metastasis and invasion of BGC823 gastric cancer cells. Oncol Rep 29 6): 2392-400
140. Reddy BV, Kalraiya RD (2006) Sialilated beta1,6 branched N-oligosaccharides modulate adhesion, chemotaxis and motility of melanoma cells: Effect on invasion and spontaneous metastasis properties. Biochimica et biophysica acta 1760 9): 1393-402

141. Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83 2): 346-56
142. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 5259): 680-5
143. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences of the United States of America 76 9): 4350-4
144. Przybylo M, et al. (2007) Identification of proteins bearing beta1-6 branched N-glycans in human melanoma cell lines from different progression stages by tandem mass spectrometry analysis. Biochimica et biophysica acta 1770 9): 1427-35
145. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25 4): 402-8
146. Dykxhoorn DM, Novina CD, Sharp PA (2003) Killing the messenger: short RNAs that silence gene expression. Nature reviews Molecular cell biology 4 6): 457-67
147. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory press.
148. Stetler-Stevenson WG, Aznavoorian S, Liotta LA (1993) Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 9: 541-73
149. von Lampe B, Stallmach A, Riecken EO (1993) Altered glycosylation of integrin adhesion molecules in colorectal cancer cells and decreased adhesion to the extracellular matrix. Gut 34 6): 829-36
150. Litynska A, et al. (2006) Does glycosylation of melanoma cells influence their interactions with fibronectin? Biochimie 88 5): 527-34
151. DiMilla PA, et al. (1993) Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. The Journal of cell biology 122 3): 729-37
152. Palecek SP, et al. (1997) Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. Nature 385 6616): 537-40
153. Lin S, et al. (2002) Cell surface alpha 2,6 sialylation affects adhesion of breast carcinoma cells. Exp Cell Res 276 1): 101-10
154. Pochec E, et al. (2006) Characterization of the oligosaccharide component of alpha3beta1 integrin from human bladder carcinoma cell line T24 and its role in adhesion and migration. European journal of cell biology 85 1): 47-57
155. Cui H, et al. (2011) Differential expression of the alpha2,3-sialic acid residues in breast cancer is associated with metastatic potential. Oncol Rep 25 5): 1365-71
156. Suzuki O, et al. (2003) Alpha-2,6-sialylation of L-PHA reactive oligosaccharides and expression of N-acetylglucosaminyltransferase V in human diffuse large B cell lymphoma. Oncol Rep 10 6): 1759-64
157. Saito Y, et al. (2010) Potentiation of cell invasion and matrix metalloproteinase production by alpha3beta1 integrin-mediated adhesion of gastric carcinoma cells to laminin-5. Clinical & experimental metastasis 27 4): 197-205

158. Seftor RE, et al. (1991) Human melanoma cell invasion is inhibited in vitro by swainsonine and deoxymannojirimycin with a concomitant decrease in collagenase IV expression. Melanoma Res 1 1): 43-54
159. Kim LT, et al. (1992) Altered glycosylation and cell surface expression of beta 1 integrin receptors during keratinocyte activation. Journal of cell science 103 (Pt 3): 743-53
160. Cheng X, et al. (2009) ECRG2 regulates cell migration/invasion through urokinase-type plasmin activator receptor (uPAR)/beta1 integrin pathway. The Journal of biological chemistry 284 45): 30897-906
161. Mori H, et al. (2002) CD44 directs membrane-type 1 matrix metalloproteinase to lamellipodia by associating with its hemopexin-like domain. The EMBO journal 21 15): 3949-59
162. Wang X, et al. (2009) Increased aggressiveness of human prostate PC-3 tumor cells expressing cell surface localized membrane type-1 matrix metalloproteinase (MT1-MMP). Journal of andrology 30 3): 259-74
163. Janik ME, et al. Effect of alpha3beta1 and alphavbeta3 integrin glycosylation on interaction of melanoma cells with vitronectin. Acta Biochim Pol 57 1): 55-61
164. Chu YW, et al. (1993) Expression of complete keratin filaments in mouse L cells augments cell migration and invasion. Proceedings of the National Academy of Sciences of the United States of America 90 9): 4261-5
165. Leppa S, Heino J, Jalkanen M (1995) Increased glycosylation of beta 1 integrins affects the interaction of transformed S115 mammary epithelial cells with laminin-1. Cell Growth Differ 6 7): 853-61
166. Mierke CT, et al. (2011) Integrin alpha5beta1 facilitates cancer cell invasion through enhanced contractile forces. Journal of cell science 124 Pt 3): 369-83
167. Jackson P, Kingsley EA, Russell PJ (2000) Inverse correlation between KAI1 mRNA levels and invasive behaviour in bladder cancer cell lines. Cancer Lett 156 1): 9-17
168. Serru V, et al. (1999) Selective tetraspan-integrin complexes (CD81/alpha4beta1, CD151/alpha3beta1, CD151/alpha6beta1) under conditions disrupting tetraspan interactions. The Biochemical journal 340 (Pt 1): 103-11

PUBLICATIONS

α2,6 Sialylation associated with increased β1,6-branched *N*-oligosaccharides influences cellular adhesion and invasion

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Expression of β1,6-branched N-linked oligosaccharides have a definite association with invasion and metastasis of cancer cells. However, the mechanism by which these oligosaccharides regulate these processes is not well understood. Invasive variants of B16 murine melanoma, B16F10 (parent) and B16BL6 (highly invasive variant) cell lines have been used for these studies. We demonstrate that substitution of α2,6-linked sialic acids on multiantennary structures formed as a result of β1,6-branching modulate cellular adhesion on both extracellular matrix (ECM) and basement membrane (BM) components. Removal of α2,6 sialic acids either by enzymatic desialylation or by stably down-regulating the ST6Gal-I (enzyme that catalyses the addition of α2,6-linked sialic acids on N-linked oligosaccharides) by lentiviral driven shRNA decreased the adhesion on both ECM and BM components and invasion through reconstituted BM matrigel.

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1. Introduction

Metastasis is a multistep complex process responsible for >90% of cancer-related mortality (Spano *et al.* 2012). Invasion is a very crucial process for cancer cell metastasis and it involves modulation of cellular adhesion, degradation of matrix and motility (Aznavoorian *et al.* 1993). Cell surface molecules play a key role in mediating all these processes of invasion, and the post-translational modifications like aberrant glycosylation on these molecules regulate all these processes. Expression of β1,6-branched *N*-oligosaccharides is one such modification associated with metastatic and invasive phenotype of several cancer cells (Dennis *et al.* 1987; Takano *et al.* 1990; Fernandes *et al.* 1991; Yamamoto *et al.* 2000). Expression of these oligosaccharides always correlates positively with invasive ability of not only cancer cells (Yamamoto *et al.* 2000) but also normal cells involved in invasive functions for, e.g., the trophoblast cells during implantation of embryo into the uterus, granulocytes and macrophages during inflammation and endothelial cells during angiogenesis (Pili *et al.* 1995; Tomiie *et al.* 2005). Adhesion to matrix is a key step in invasion. Although the expression of these oligosaccharides is always associated with invasiveness, it appears to regulate adhesion both positively and negatively. The presence of these oligosaccharides on B16 murine melanoma cells appears to promote adhesion (Reddy and Kalraiya 2006; Yoshimura *et al.* 1995), whereas their expression on human melanoma and many other cancers of breast and bladder decreases adhesion to matrix components (Litynska *et al.* 2006; Pochec *et al.* 2006).

Adhesion is crucial for invasion and metastasis. Cells need to achieve an optimum level of adhesion to matrix for effective movement and invasion. Cells adhering either loosely or too tightly to substratum are neither able to move nor invade. Tumour cells possibly achieve this by altering the expression of adhesion receptors or glycosylation on these receptors. β1,6 Branching is the key step in the formation of highly branched multiantennary structures (Cummings *et al.* 1982). Each of these antennae can be substituted at the termini with different sugars like sialic acids (SA), repeating unit of *N*-acetyl glucosamine and galactose (polylacNAc) and fucose (Kawano *et al.* 1993, Pierce and Arango 1986). The sialic acids may be present in different linkages. It is possible that these terminal substitutions determine the final state of adhesion.

Keywords. Adhesion; β1,6-branched *N*-oligosaccharides; invasion; α2,6-linked sialic acid; poly-*N*-acetyllactosamine

Addition of sialic acids is catalysed by a family of 20 enzymes known as sialylaltransferases that catalyse the transfer of sialic acid from CMP sialic acid to glycoconjugates. About 15 of these sialylaltransferases have been cloned and characterized (Harduin-Lepers *et al.* 2001). The expression of sialylaltransferases has been shown to be altered in several cancers (Gretschel *et al.* 2003; Seales *et al.* 2005; Wang *et al.* 2005; Jun *et al.* 2012), for instance, increased expression of sialylaltransferases (ST6Gal-I and ST3Gal-III) has been reported in invasive cervical squamous cell carcinoma (Lopez-Morales *et al.* 2009). Like other glycosyltransferases they exhibit a notable specificity for both linkage and acceptor substrates. On N-linked glycoprotein, sialic acid is present either in α2,3 or α2,6 linkage. The addition of sialic acid in these linkages is mutually exclusive i.e. presence of sialic acid at one site in one specific linkage prevents addition of the other. Sialylation is known to alter the adhesive property of various cancer cells. Expression of β1,6-branched *N*-oligosaccharides always correlates with invasion but it modulates adhesion in a complex manner. This work investigated if the selective presence of different terminal sugars on β1,6-branched *N*-oligosaccharides have any role in differential regulation of adhesion.

2. Materials and methods

2.1 *Cell lines and reagents*

B16F10 and B16BL6 murine melanoma cell lines were obtained from Prof IJ Fidler, MD, Anderson Cancer Centre, Houston, USA. Cell culture reagents were from Invitrogen, USA. Culture ware and cell culture inserts was from BD Falcon, USA. Fibronectin and matrigel were purchased from BD Biosciences, USA. Restriction enzymes, T4 DNA ligase were from Fermentas International Inc., Canada. PVDF membrane and the ECL kit were purchased from GE Healthcare, Amersham, UK. Sialidases were obtained from New England Biolabs (NEB). Glutaraldehyde, Paraformaldehyde, Puromycin, Polybrene, *N*-octyl-β-D-glucopyranoside, Protamine sulphate, Bovine Serum Albumin (BSA), mouse monoclonal anti-β actin (AC-74), streptavidin-FITC, streptavidin-peroxidase conjugate were purchased from Sigma Chemical Co., USA. Biotinylated lectins Leuco-Phyto Haem Agglutinin (L-PHA) for β1,6-branched N-Linked oligosaccharides, *Lycopersicon esculentum* lectin (LEA) for poly-*N*-acetyllactosamine, *Sambucas nigra* agglutinin (SNA) for α2,6-linked sialic acid, *Maackia amurensis* agglutinin (MAL-II) for α2,3-linked sialic acids, *Aleuria aurantia* lectin (AAL) for fucose and L-PHA agarose beads was obtained from Vector labs, USA. Radioactive tritiated thymidine was obtained from Board of Radiation and Isotope Technology (BRIT), India. Reagents for bacterial culture were purchased from Hi Media, India, while all other chemicals were of analytical grade and purchased locally.

2.2 *Cell culture*

Melanoma cells were routinely cultured in minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, vitamins, L-glutamine and antibiotics (complete medium). The cells were grown in monolayer on plastic tissue culture flasks, incubated in humidified atmosphere of 5% CO_2 at 37°C. The cells were harvested with trypsin solution (0.25% trypsin, 0.02% EDTA and 0.05% glucose in PBS). Cells with greater than 95% viability were used for all the assays.

2.3 *Preparation of total cell lysates and Western blotting*

Total cell lysates were prepared exactly as described in (Krishnan *et al.* 2005) using lysis buffer containing 20 mM Tris chloride, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 1 mM each of Magnesium Chloride and Calcium Chloride and protease inhibitors (1 μg/mL each of pepstatin, leupeptin and aprotinin, and 0.3 mM PMSF). The protein content was estimated as per Peterson's modification of the Lowry's protocol, and were separated on SDS-PAGE according to Laemmli's method and transferred on to PVDF membrane as described in (Towbin *et al.* 1979). The expression of β1,6 N-linked oligosaccharides and probable terminal sugars on it like sialic acid (either in α2,3 or α2,6 linkage), polylacNAc and fucose on melanoma cells was checked by probing the Western blotted lysates with biotinylated L-PHA, MAL-II, SNA, LEA and AAL respectively followed by streptavidin peroxidase. The blots were developed using ECL kit.

2.4 *Flow cytometric analysis*

For flow cytometry, melanoma cells were first fixed by overnight incubation either with 1.5% gluteraldehyde or 1% paraformaldehyde in PBS (pH 7.4) at 4°C. Analyses of surface expression of β1,6-branched *N*-oligosaccharides and terminal substitution on melanoma invasive variants were performed using biotinylated L-PHA, SNA, MAL-II, LEA, and AAL respectively exactly as described in (Srinivasan *et al.* 2009).

2.5 *Purification of L-PHA reactive proteins*

For purification of L-PHA reactive proteins, total cell lysate from B16F10 and B16BL6 cells were prepared in buffer containing 10 mM Tris-HCl (pH7.5) containing 150 mM

NaCl and protease inhibitor (1 μg/mL each of pepstatin, leupeptin and aprotinin, and 0.3 mM PMSF) were sonicated five times for 30 s each at (50% output control) at 4°C. The lysate was supplemented with *N*-octyl-β-D-glucopyranoside and protamine sulphate to attain a final concentration of 30 mM and 0.3% respectively and further incubated for 1 h on ice. Cells supernatant was collected by centrifugation at 16,000*g* for 1 h at 4°C as described in (Przybylo *et al.* 2007). 2000 μg Lysate from B16BL6 cells were then incubated with 100 μL L-PHA agarose beads (Vector labs) overnight at 4°C. Unbound and nonspecifically bound proteins were removed by giving five washes with wash buffer (10 mM Tris-HCl pH 7.5), 500 mM NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, 3 mM *N*-octyl-β-D-glucopyranoside). Bound proteins were eluted using 1× non-reducing Laemmli buffer, containing 4 M urea.

2.6 *Sialidase treatment*

Briefly, 1×10^6 tritium (H^3) labeled cells were treated with either α2,3 or α2,3/6 linkage-specific sialidase 50 units/ml and 200 units/mL respectively in plain medium pH 7.4 and incubated at 37°C for 1 h with intermittent tapping at interval of 15 min. After incubation cells were washed with plain medium thrice and one wash with PBS. Sialidases treated cells were further used for adhesion assay.

2.7 *Adhesion assays*

For adhesion assays, melanoma cells were either labeled with tritiated thymidine or with a fluorescent dye Calcein AM (Invitrogen). Labeled cells (4×10^4 cells/well, in plain medium) were added to a 96 well plate coated overnight with fibronectin (representative of ECM component) and matrigel (reconstituted BM) at a concentration of 10 μg/mL exactly as described in (Reddy and Kalraiya 2006). The percentage adhesion was calculated as the percentage of bound cells with respect to B16BL6 cells or untreated cells, which was taken as 100%.

2.8 *Transduction and generation of stable cell lines*

Downregulation of α2,6-linked sialic acid in B16BL6 cells was performed by using short hairpin RNA (shRNA) against ST6Gal-I, enzyme which is involved in synthesis of α2,6-linked sialic acid. The shRNA was designed and cloned into pTRIPz vector (open biosystem). The shRNA containing plasmid together with helper plasmids p^{MD2G} and P^{PAX2} were co-transfected into 293 FT packaging cell line using $CaPO_4$ method of DNA transfection. Supernatant containing virus particles were collected at interval of 24 h and it was spun at 5000 rpm for 20 min at 4°C. Virus particles were then transduced into B16BL6 using 8 μg/mL polybrene (Sigma-Aldrich). Medium was changed 24 h post transduction and transduced cells were selected using puromycin (Sigma-Aldrich) at a concentration of 1 μg/mL and stable cell lines were maintained at a concentration of 0.5 μg/mL. Stably transduced cells were induced with doxycycline and these cells were further enriched using cell sorter, as these cells have Turbo RFP as reporter gene in them.

2.9 *Invasion assays*

Invasion assays were performed as described in (Reddy and Kalraiya 2006), using matrigel-coated (30 μg of 1 mg/mL matrigel per insert) 24-well transwell units with 8 μm pore size polycarbonate filter. Briefly, 0.2×10^6 tritium labelled cells suspended in 300 μL MEM were added to the upper compartment of the Boyden chamber, and 600 μL of conditioned medium (spent medium collected from 50% confluent B16BL6 cell culture) was added to the lower compartment which served as chemoattractant. Cells were allowed to invade for 36 h at 37°C in a humidified atmosphere containing 5% CO_2.

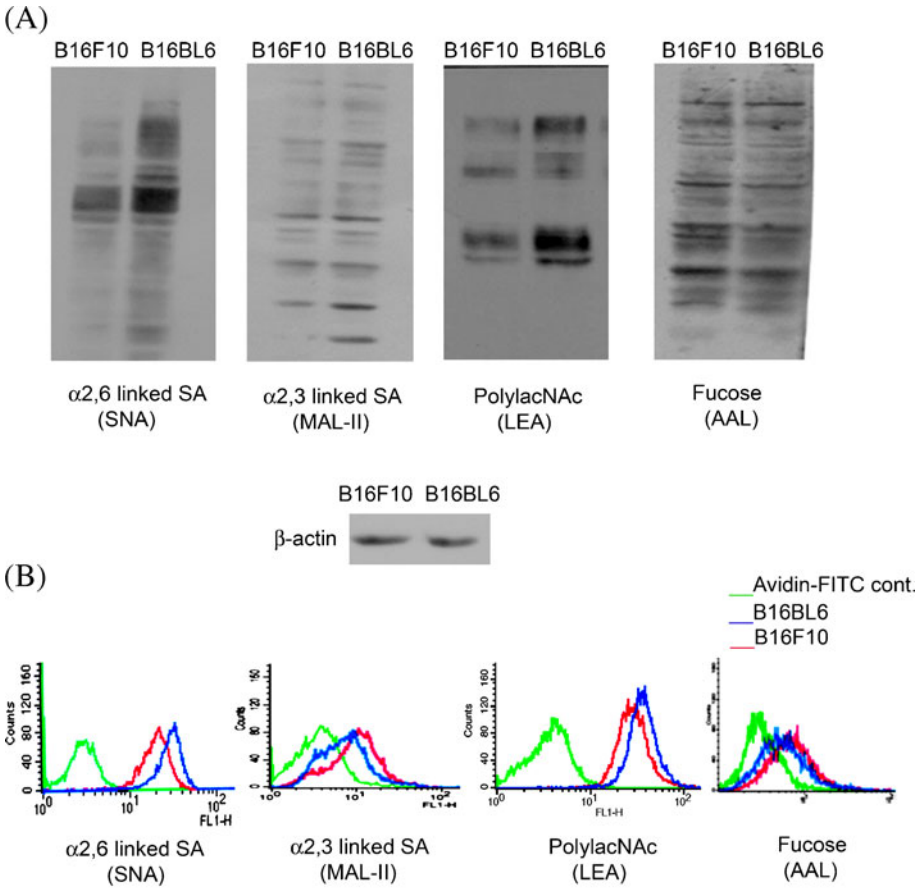
2.10 *Statistical analysis*

Statistical analysis was performed using Graphpad Prism 5. The unpaired Student's *t*-test was employed when two groups were compared. *P*-value<0.05 was considered as significant.

3. Results

3.1 *Analysis of terminal sugars on oligosaccharides of B16F10 and its highly invasive variant (B16BL6) cells*

Cell lysates were prepared from highly invasive B16BL6 and its parent cell line B16F10 as described by (Krishnan *et al.* 2005). Expression of β1,6-branched *N*-oligosaccharides have been shown to correlate with invasive potential of the B16 murine melanoma invasive variants (Reddy and Kalraiya 2006). The terminal substitutions on oligosaccharides such as sialic acids in either α2,3 or α2,6 linkage, polylacNAc and fucose were studied by Western blotting (figure 1A) and flow cytometry (figure 1B and C) using biotinylated lectins MAL-II, SNA, LEA and AAL respectively. Results showed that as compared to B16F10 cells, the increased expression of β1,6-branched *N*-oligosaccharides on B16BL6 cells was accompanied with significantly increased levels of α2,6-linked sialic acid and polylacNAc, as analysed in total cell lysates by Western blotting and on the cell surface by flow cytometry (figure 1A, B and C). Sialic acids are present on both O-linked and N-linked



(C)

Lectin	Mean fluorescence Intensity (MFI) in B16F10	Mean fluorescence Intensity (MFI) in B16BL6
SNA	20.48	32.64
MAL-II	17.14	17.64
LEA	29.55	36.44
AAL	20.92	16.31

Figure 1. Comparison of terminal substitutions on the oligosaccharides expressed on melanoma invasive variants. Parent cell line (B16F10) and its invasive variant (B16BL6) were compared for expression of the terminal sugars (**A**) on Western blotted cell lysates and (**B**) analysis of surface expression of terminal sugars on glutaraldehyde fixed cells by flow cytometry, using biotinylated lectins as probe. β-Actin served as loading control for Western blotting and cells treated only with FITC-labelled streptavidin served as control for flow cytometry and (**C**) table showing mean fluorescence intensity of data in (**B**).

oligosaccharides, it is thus important to confirm that the increase in α2,6-linked sialic acid is indeed due to increased expression of β1,6-branched *N*-oligosaccharides.

3.2 *Increased substitution of α 2,6 linked sialic acids on invasive cells is indeed on β1,6-branched N-oligosaccharides*

The multiantennary structures formed as a result of β1,6-branched N-linked oligosaccharides are the potential site(s) for the addition of various terminal sugars. Proteins carrying such oligosaccharides were purified from both B16F10 and B16BL6 cells using L-PHA agarose beads and bound proteins were eluted from beads by boiling them in 1× non-reducing sample buffer containing 4 M urea. Eluted proteins from B16F10 and B16BL6 were normalized with respect to β1,6-branched oligosaccharides and presence of terminal sugars on them were studied. Results showed that proteins carrying these oligosaccharides from B16BL6 were mainly substituted with α2,6-linked sialic acid (figure 2). Although, the overall levels of polylacNAc also increase, it was insignificant in the region of L-PHA reactive proteins (L-PHA specifically recognises β1,6-branched *N*-oligosaccharides).

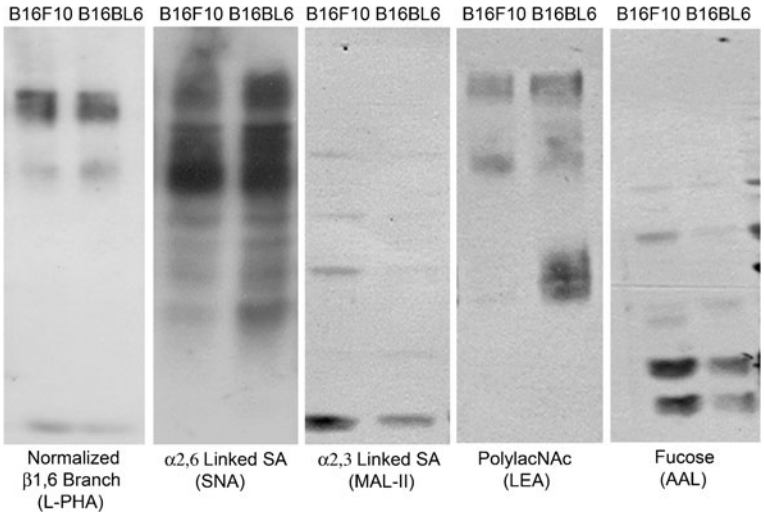


Figure 2. Increased expression of α2,6-linked SAs is associated with increased expression of β1,6-branched N-linked oligosaccharides. L-PHA binding proteins purified from whole cell lysates of B16F10 and B16BL6 cells were normalized with respect to expression of β1,6-branched *N*-oligosaccharides by blotting and probing using biotinylated lectin L-PHA (panel 1). Such normalized proteins from both the cell lines were blotted and probed for the terminal sugars with respective biotinylated lectins, SNA, MAL-II, LEA and AAL (panels 2–5).

3.3 *Effect of linkage specific sialic acids on adhesion of B16BL6 cells*

Sialic acids on glycoproteins are mainly present in either α2,3 or α2,6 linkage. Although, there is no sialidase that specifically removes only α2,6-linked sialic acid, there are sialidases that remove either α2,3- or both α2,3/6-linked SA. We have used enzymatic (sialidases) approach for desialylating either α2,3- or both α2,3/6-linked SA. Removal of sialic acids was checked by Western blotting (figure 3A and B).

Our result showed that removal of α2,3-linked sialic acids had very marginal effect on adhesion to fibronectin (ECM component) or the matrigel (BM). Interestingly it increased adhesion on fibronectin but decreased it on matrigel (figure 3C) and hyaluronic acid and collagen-IV (data not shown) but very marginally, whereas removal of sialic acids in both α2,3/6 linkage significantly decreased the adhesion of B16BL6 cells on fibronectin and matrigel (figure 3C) and even on hyaluronic acid and collagen-IV (data not shown), indicating that sialic acids in α2,6 linkage have a positive role in promoting adhesion of B16BL6 cells. However, approaches that specifically target α2,6 SA on *N*-oligosaccharides would confirm this.

3.4 *Down-regulation of α2,6-linked sialic acids on N-glycans on B16BL6 cells decreases their adhesion*

B16BL6 cells and the same cells transduced with non-targeting shRNA and shRNA against ST6Gal-I in an inducible lentiviral vector pTRIPz, were induced with doxycycline. Induction of non-targeting (NT) shRNA by doxycycline did not have any effect on expression of α2,6-linked sialic acid (figure 4A). Induction of ST6Gal-I transduced cells with doxycycline however, significantly inhibited the expression of α2,6-linked SA as assessed by flow cytometry and Western blotting (figure 4B).

B16BL6 cells transduced with NT shRNA, induced (NT shRNA +DOX) or un-induced (NT shRNA −DOX) with doxycycline, neither showed any changes in sialylation nor adhesion of cells to fibronectin or matrigel (figure 5A). However, decreased expression of α2,6 SA in ST6Gal-I transduced cells significantly decreased the adhesion of induced cells (ST6Gal-I shRNA +DOX) to fibronectin and matrigel as compared to un-induced cells (ST6Gal-I shRNA –DOX) (figure 5B).

3.5 *Down-regulation of α2,6-linked sialic acid decreased the invasive potential of B16BL6 cells*

To investigate if invasiveness of B16BL6 cells is indeed due to increased adhesion, the effect of down regulation of α2,6-linked SA on invasion was assessed. Induction of non-targeting (NT) shRNA (NT shRNA +DOX) did not have any significant effect on invasion (figure 5C). However, Inhibition of expression of α2,6-linked SA in ST6Gal-I shRNA transduced B16BL6 cells by inducing shRNA using doxycycline (ST6Gal-I shRNA +DOX), significantly decreased the ability of these cells to invade through reconstituted BM (matrigel), as compared to un-induced (ST6Gal-I shRNA −DOX) cells (figure 5D). These results

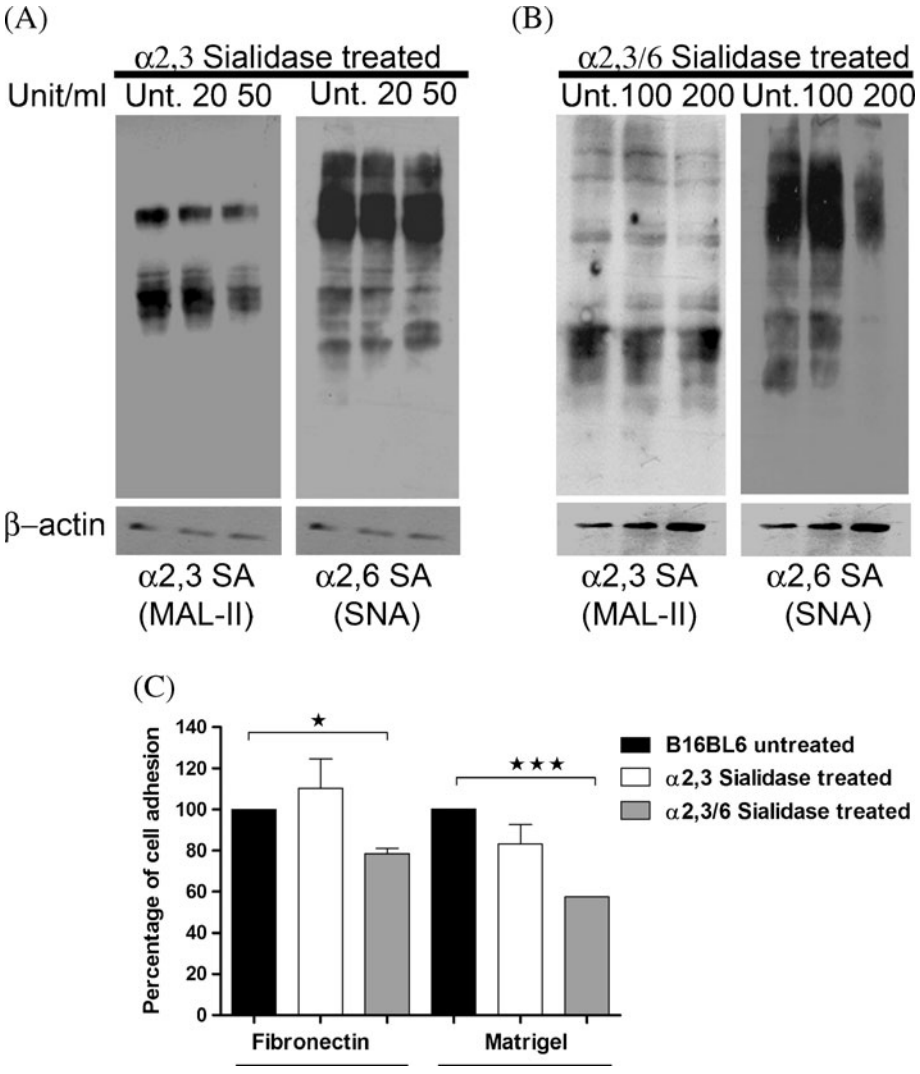


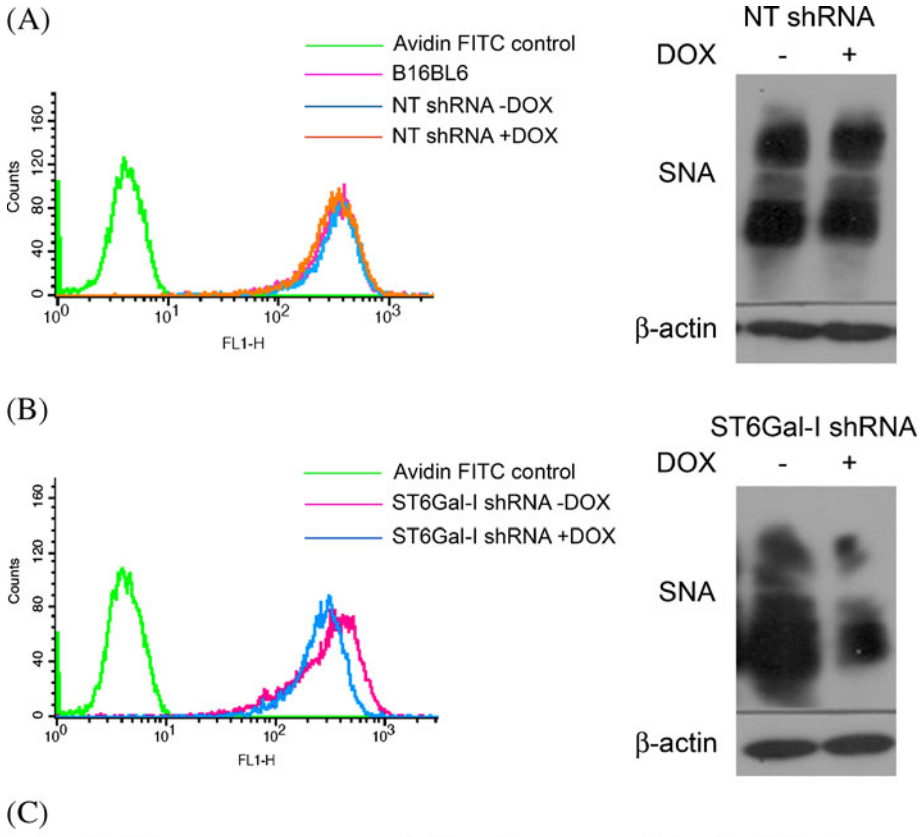
Figure 3. Enzymatic desialylation of α2,6-linked sialic acids decreases adhesion. Cell lysates from untreated (Unt) B16BL6 cells and those treated with (**A**) α2,3 linkage specific sialidase (20 and 50 unit/ml) and (**B**) α2,3/6 linkage specific sialidase (100 and 200 unit/ml) were Western blotted and probed with biotinylated lectin SNA and MAL-II. β-Actin served as loading control for Western blotting. (**C**) Tritiated thymidine labeled B16BL6 cells were treated with 50 unit/ml and 200 unit/ml of α2,3 and α2,3/6 linkage specific sialidase respectively, and adhesion assay were performed as described in materials and methods. Untreated B16BL6 cells served as control and their adhesion on each substrate (fibronectin and matrigel) was taken as 100%. Values are mean±SE of three independent experiments performed in triplicate. *p<0.0133 and ***p<0.0001.

clearly suggest that the increased substitution of α2,6-linked sialic acids regulates invasion by regulating the adhesion.

4. Discussion

Invasion is the key event in cancer cell metastasis. Molecules on the cell surface are the major participants in almost all the steps of invasion and metastasis. Tumour cells show several metastasis associated cell surface modifications. Some of these are in the form of post translational modifications of cell surface proteins. Many of these now serve as cancer biomarkers (Adamczyk *et al.* 2012). Expression of β1,6-branched N-linked oligosaccharides is one such modification associated with metastatic and invasive cancer cells. These oligosaccharides regulate invasion by modulating adhesion, matrix degradation and motility of cancer cells. However, the role of these oligosaccharides in modulation of adhesion is controversial.

Although, the expression of β1,6-branched *N*-oligosaccharides has always been shown to correlate with invasiveness, these



(C)

Cell Type	Mean fluorescence Intensity (MFI)
B16BL6	325
NT shRNA -DOX	337
NT shRNA +DOX	326
ST6Gal-I shRNA -DOX	335
ST6Gal-I shRNA +DOX	276

Figure 4. ShRNA mediated inhibition of ST6Gal-I decreased the expression of α2,6-linked SA. B16BL6 cells stably transduced with doxycycline (DOX) inducible shRNA against ST6Gal-I and non-targeting shRNA were treated with doxycycline for 96 h for shRNA expression and were fixed with 1% paraformaldehyde for flow cytometric analysis or lysed as described in material and method. (**A**) Expression of α2,6-linked SA in untransduced (B16BL6), non-targeting shRNA transduced B16BL6 cells in un-induced (NT shRNA −DOX) and doxycycline induced (NT shRNA +DOX) cells by flow cytometry (left panel) and Western blotting (right panel). (**B**) Expression of α2,6-linked SA in ST6Gal-I shRNA transduced B16BL6 cells in un-induced (ST6Gal-I shRNA −DOX) and doxycycline induced (ST6Gal-I shRNA +DOX) cells, by flow cytometry (left panel) and Western blotting (right panel). Cells treated with only streptavidin FITC served as control for flow cytometry. β-actin served as loading control for Western blotting. (**C**) Table showing mean fluorescence intensity of data in (**A**) and (**B**).

oligosaccharides appear to regulate adhesion to matrix components, both positively (Yoshimura *et al.* 1995; Reddy and Kalraiya 2006) as well as negatively (Demetriou *et al.* 1995; Litynska *et al.* 2006). Adhesion is the key step in cancer cell invasion. Tumour cells require an optimum level of cellular adhesion. Cells adhering either loosely or too tightly to the substratum are unable to move and invade. Expression of β1,6-branched *N*-oligosaccharides has been shown to result in the formation of complex multiantennary structures which can be substituted with different terminal sugars (Pierce and Arango 1986; Kawano *et al.* 1993). Depending on their basal adhesive characteristics, tumour cells possibly regulate adhesion by manipulating these terminal substitutions.

Among the various possible terminal substitutions, α2,6-linked sialic acids was found to be most predominantly associated with β1,6-branched N-oligosaccharides apart from polylacNAc substitutions on invasive B16BL6 cells (figure 1). Ongoing work in the lab has shown that polylacNAc on melanoma cells participate in lung specific metastasis via galectin-3 expressed on the lungs (Krishnan *et al.* 2005; Srinivasan *et al.* 2009). Comparison of terminal substitutions on purified glycoproteins carrying β1,6-

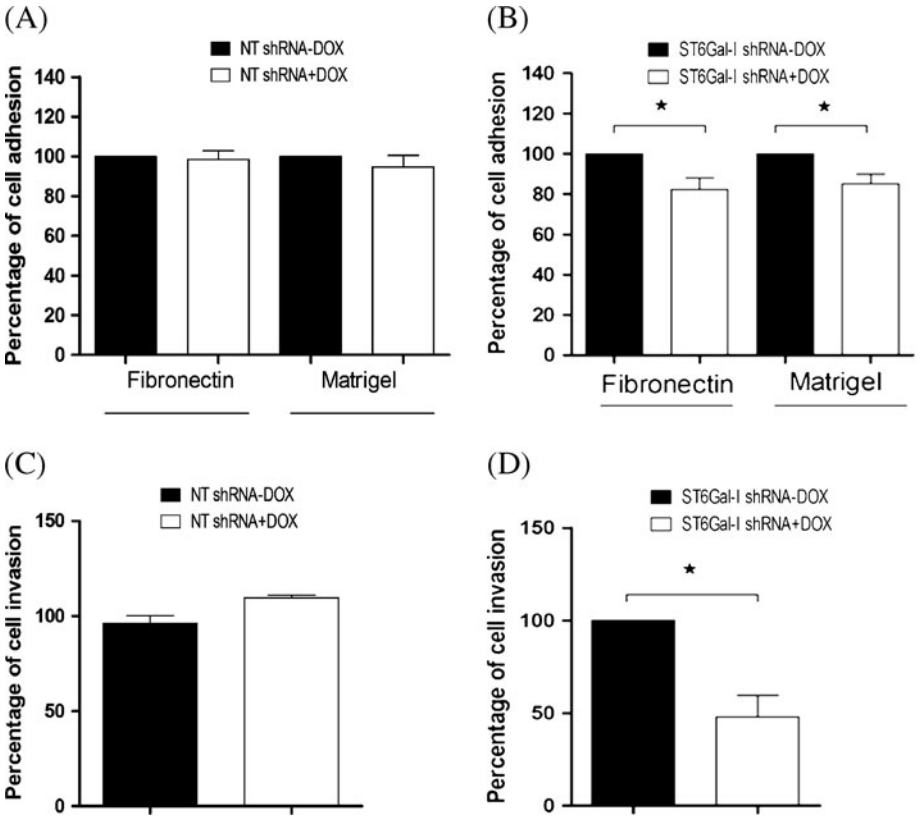


Figure 5. Effect of inhibition of ST6Gal-I expression on adhesion and invasion of B16BL6 cells. (**A**) Adhesion of induced (NT shRNA +DOX) and un-induced (NT shRNA −DOX) B16BL6 cells stably transduced with non-targeting shRNA or (**B**) ST6Gal-I shRNA, after induction (ST6Gal-I shRNA +DOX) or un-induced (ST6Gal-I shRNA −DOX), on fibronectin and matrigel. Values are mean±SE of three independent experiments performed in triplicates.*$p< 0.0369$. (**C**) Invasion assays of induced (NT shRNA +DOX) and un-induced (NT shRNA −DOX) non-targeting shRNA transduced B16BL6 cells. (**D**) Invasion assays of doxycycline induced (ST6Gal-I shRNA +DOX) and un-induced (ST6Gal-I shRNA −DOX) B16BL6 cells stably transduced with ST6Gal-I shRNA. Values are mean±SE of three independent experiments done in triplicates. *$p<0.0113$.

branched *N*-oligosaccharides from B16F10 and B16BL6 cells further substantiated the association of α2,6 SA substitution with invasive phenotype (figure 2).

Sialic acids indeed appear to regulate cellular adhesion. Increased overall expression of α2,6-linked sialic acids on mammary carcinoma cell line MDA-MB-435 has been shown to be associated with increased adhesion of cells to collagen-IV (Lin *et al.* 2002). On the contrary, removal of α2,3-linked SA from these oligosaccharides on bladder carcinoma cell line T24, or inhibition of ST3Gal-I (enzyme that adds α2,3 sialic acid using soyasaponin-1 in B16F10 cells, increased their adhesion to fibronectin and/or collagen-IV (Chang *et al.* 2006; Pochec *et al.* 2006). Similarly, in breast cancer and human melanoma cells, negative correlation between expression of β1,6-branched N-oligosaccharides and adhesion appears to be due to substitution of sialic acids in α2,3 linkage (Litynska *et al.* 2006; Cui *et al.* 2011).

The enzyme that specifically removes α2,6 SA is not available. Using a combination of sialidases that remove either only α2,3 or both α2,3 and α2,6-linked SA, β1,6-branched N-oligosaccharides were proposed to be substituted predominantly with α2,6-linked sialic acids, on human diffuse large B cell lymphoma (Suzuki *et al.* 2003). Using similar desialylation approach we showed, that removal of α2,3/6 linked SA from B16BL6 cells significantly decreased their adhesion to not only fibronectin and matrigel (figure 3C) but also hyaluronic acid and collagen-IV (data not shown). However, removal of only α2,3 linked SA had insignificant effect on adhesion (figure 3C). Its removal marginally decreased adhesion on hyaluronic acid, collagen-IV (data not shown) and matrigel, and marginal increase on fibronectin (figure 3C). Although, increased α2,6 sialylation on invasive cells correlated with the expression of β1,6-branched *N*-oligosaccharides, this approach does not rule out the contribution of sialylation on other oligosaccharides.

ST6Gal-I is a golgi enzyme that predominantly adds α2,6-linked SA on the termini of the antennary structures (Galβ1,4

GlcNAc) on N-linked oligosaccharides (Harduin-Lepers *et al*. 2001; Zhuo and Bellis 2011). The increased α2,6 sialylation associated with increased β1,6-branched N-oligosaccharides could be due to its substitution on multiple antennae formed as a result of β1,6 branching. Inhibition of expression of ST6Gal-I should inhibit expression of α2,6 SA and impact cellular adhesion. The cells transduced with a specific shRNA against ST6Gal-I not only inhibited expression of α2,6 SA but also adhesion to fibronectin and matrigel (figure 5B) accompanied with significant loss in their invasive ability (figure 5D). The cells transduced with non-targeting shRNA did not have any effect on adhesion or invasion of the transduced cells (figure 5A and C).

These results clearly highlight the importance of not only β1,6-branched *N*-oligosaccharides but also the associated terminal substitutions (especially sialylation) on them in the processes involved in invasion. The study demonstrates the importance of adhesion of cells to the substratum and the mechanisms that tumour cells adopt to achieve optimum adhesion to be invasive.

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References

Adamczyk B, Tharmalingam T and Rudd PM 2012 Glycans as cancer biomarkers. *Biochim. Biophys. Acta* **1820** 1347–1353

Aznavoorian S, Murphy AN, Stetler-Stevenson WG and Liotta LA 1993 Molecular aspects of tumor cell invasion and metastasis. *Cancer* **71** 1368–1383

Chang WW, Yu CY, Lin TW, Wang PH and Tsai YC 2006 Soyasaponin I decreases the expression of alpha2,3-linked sialic acid on the cell surface and suppresses the metastatic potential of B16F10 melanoma cells. *Biochem. Biophys. Res. Commun.* **341** 614–619

Cui H, Lin Y, Yue L, Zhao X and Liu J 2011 Differential expression of the alpha2,3-sialic acid residues in breast cancer is associated with metastatic potential. *Oncol. Rep.* **25** 1365–1371

Cummings RD, Trowbridge IS and Kornfeld S 1982 A mouse lymphoma cell line resistant to the leukoagglutinating lectin from Phaseolus vulgaris is deficient in UDP-GlcNAc: alpha-D-mannoside beta 1,6 N-acetylglucosaminyltransferase. *J. Biol. Chem.* **257** 13421–13427

Demetriou M, Nabi IR, Coppolino M, Dedhar S and Dennis JW 1995 Reduced contact-inhibition and substratum adhesion in epithelial cells expressing GlcNAc-transferase V. *J. Cell Biol.* **130** 383–392

Dennis JW, Laferte S, Waghorne C, Breitman ML and Kerbel RS 1987 Beta 1-6 branching of Asn-linked oligosaccharides is directly associated with metastasis. *Science* **236** 582–585

Fernandes B, Sagman U, Auger M, Demetrio M and Dennis JW 1991 Beta 1-6 branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasia. *Cancer Res.* **51** 718–723

Gretschel S, Haensch W, Schlag PM and Kemmner W 2003 Clinical relevance of sialyltransferases ST6GAL-I and ST3GAL-III in gastric cancer. *Oncology* **65** 139–145

Harduin-Lepers A, Vallejo-Ruiz V, Krzewinski-Recchi MA, Samyn-Petit B, Julien S and Delannoy P 2001 The human sialyltransferase family. *Biochimie* **83** 727–737

Jun L, *et al* 2012 Altered mRNA expressions of sialyltransferases in human gastric cancer tissues. *Med. Oncol.* **29** 84–90

Kawano T, Takasaki S, Tao TW and Kobata A 1993 Altered glycosylation of beta 1 integrins associated with reduced adhesiveness to fibronectin and laminin. *Int. J. Cancer* **53** 91–96

Krishnan V, Bane SM, Kawle PD, Naresh KN and Kalraiya RD 2005 Altered melanoma cell surface glycosylation mediates organ specific adhesion and metastasis via lectin receptors on the lung vascular endothelium. *Clin. Exp. Metastasis* **22** 11–24

Lin S, Kemmner W, Grigull S and Schlag PM 2002 Cell surface alpha 2,6 sialylation affects adhesion of breast carcinoma cells. *Exp. Cell Res.* **276** 101–110

Litynska A, Przybylo M, Pochec E, Kremser E, Hoja-Lukowicz D and Sulowska U 2006 Does glycosylation of melanoma cells influence their interactions with fibronectin? *Biochimie* **88** 527–534

Lopez-Morales D, Velazquez-Marquez N, Valenzuela O, Santos-Lopez G, Reyes-Leyva J and Vallejo-Ruiz V 2009 Enhanced sialyltransferases transcription in cervical intraepithelial neoplasia. *Invest. Clin.* **50** 45–53

Pierce M and Arango J 1986 Rous sarcoma virus-transformed baby hamster kidney cells express higher levels of asparagine-linked tri- and tetraantennary glycopeptides containing [GlcNAc-beta (1,6)Man-alpha (1,6)Man] and poly-N-acetyllactosamine sequences than baby hamster kidney cells. *J. Biol. Chem.* **261** 10772–10777

Pili R, Chang J, Partis RA, Mueller RA, Chrest FJ and Passaniti A 1995 The alpha-glucosidase I inhibitor castanospermine alters endothelial cell glycosylation, prevents angiogenesis, and inhibits tumor growth. *Cancer Res.* **55** 2920–2926

Pochec E, Litynska A, Bubka M, Amoresano A and Casbarra A 2006 Characterization of the oligosaccharide component of alpha3beta1 integrin from human bladder carcinoma cell line T24 and its role in adhesion and migration. *Eur. J. Cell Biol.* **85** 47–57

Przybylo M, Martuszewska D, Pochec E, Hoja-Lukowicz D and Litynska A 2007 Identification of proteins bearing beta1–6 branched N-glycans in human melanoma cell lines from different progression stages by tandem mass spectrometry analysis. *Biochim. Biophys. Acta* **1770** 1427–1435

Reddy BV and Kalraiya RD 2006 Sialilated beta1,6 branched N-oligosaccharides modulate adhesion, chemotaxis and motility of melanoma cells: Effect on invasion and spontaneous metastasis properties. *Biochim. Biophys. Acta* **1760** 1393–1402

Seales EC, Jurado GA, Brunson BA, Wakefield JK, Frost AR and Bellis SL 2005 Hypersialylation of beta1 integrins, observed in colon adenocarcinoma, may contribute to cancer progression by up-regulating cell motility. *Cancer Res*. **65** 4645–4652

Spano D, Heck C, De Antonellis P, Christofori G and Zollo M 2012 Molecular networks that regulate cancer metastasis. *Semin. Cancer Biol.* **22** 234–249

Srinivasan N, Bane SM, Ahire SD, Ingle AD and Kalraiya RD 2009 Poly N-acetyllactosamine substitutions on N- and not O-oligosaccharides or Thomsen-Friedenreich antigen facilitate lung specific metastasis of melanoma cells via galectin-3. *Glycoconj. J.* **26** 445–456

Suzuki O, Nozawa Y, Kawaguchi T and Abe M 2003 Alpha-2,6-sialylation of L-PHA reactive oligosaccharides and expression of N-acetylglucosaminyltransferase V in human diffuse large B cell lymphoma. *Oncol. Rep.* **10** 1759–1764

Takano R, Nose M, Nishihira T and Kyogoku M 1990 Increase of beta 1–6-branched oligosaccharides in human esophageal carcinomas invasive against surrounding tissue in vivo and in vitro. *Am. J. Pathol.* **137** 1007–1011

Tomiie M, Isaka S, Miyoshi E, Taniguchi N, Kimura T, Ogita K, Tsutsui T and Shimoya K 2005 Elevated expression of N-acetylglucosaminyltransferase V in first trimester human placenta. *Biochem. Biophys. Res. Commun.* **330** 999–1004

Towbin H, Staehelin T and Gordon J 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76** 4350–4354

Wang PH, Lee WL, Juang CM, Yang YH, Lo WH, Lai CR, Hsieh SL and Yuan CC 2005 Altered mRNA expressions of sialyltransferases in ovarian cancers. *Gynecol. Oncol.* **99** 631–639

Yamamoto H, Hurh J, Sweeley C, Leestma J, Mkrdichian E, Cerullo L, Nishikawa A and Ihara Y 2000 Beta1,6-N-acetylglucosamine-bearing N-glycans in human gliomas: implications for a role in regulating invasivity. *Cancer Res.* **60** 134–142

Yoshimura M, Nishikawa A, Ihara Y, Taniguchi S and Taniguchi N 1995 Suppression of lung metastasis of B16 mouse melanoma by N-acetylglucosaminyltransferase III gene transfection. *Proc. Natl. Acad. Sci. USA* **92** 8754–8758

Zhuo Y and Bellis SL 2011 Emerging role of alpha2,6-sialic acid as a negative regulator of galectin binding and function. *J. Biol. Chem.* **286** 5935–5941

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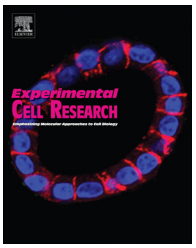

Research Article

Glycosylation of the laminin receptor (α3β1) regulates its association with tetraspanin CD151: Impact on cell spreading, motility, degradation and invasion of basement membrane by tumor cells

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ABSTRACT

Invasion is the key requirement for cancer metastasis. Expression of β1,6 branched N-oligosaccharides associated with invasiveness, has been shown to promote adhesion to most Extra Cellular Matrix (ECM) and basement membrane (BM) components and haptotactic motility on ECM (fibronectin) but attenuate it on BM (laminin/matrigel) components. To explore the mechanism and to evaluate the significance of these observations in terms of invasion, highly invasive B16BL6 cells were compared with the parent (B16F10) cells or B16BL6 cells in which glycosylation was inhibited. We demonstrate that increased adhesion to matrix components induced secretion of MMP-9, important for invasion. Further, both the subunits of integrin receptors for fibronectin (α5β1) and laminin (α3β1) on B16BL6 cells were shown to carry these oligosaccharides. Although, glycosylation of receptors had no effect on their surface expression, it had same differential effect on cell spreading as haptotactic motility. Absence of correlation between invasiveness and expression of most tetraspanins (major regulators of integrin function) hints at an alternate mechanism. Here we show that glycosylation on α3β1 impedes its association with CD151 and modulates spreading and motility of cells apparently to reach an optimum required for invasion of BM. These studies demonstrate the complex mechanisms used by cancer cells to be invasive.

Introduction

Metastasis is a complex multistep process responsible for majority of the cancer related deaths. The underlying molecular mechanisms, however, are still poorly understood [1–3]. Animal models have provided very useful clues about the different steps and the possible molecules involved at each step [4]. To metastasize, tumor cells induce angiogenesis, they must break free from the primary, invade the surrounding normal tissues and degrade organ/vascular basement membrane (BM) to disseminate via

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circulation, this is followed by survival in circulation, arrest in target organ vasculature, extravasation and acclimatization in the new organ environment [5]. Metastasis is believed to be very inefficient process and only cells proficient in all these properties are able to metastasize [6,7].

Invasiveness of cells is one of the key requirements for them to be metastatic. Invasion itself is a complex process involving multiple interactions between the host derived and tumor cell surface molecules. For a cell to be invasive it must, a) modulate its adhesion to the underlying matrix to a level optimum for movement, b) create space by degrading the matrix, however, in a highly regulated manner as the same matrix is used as the traction for forward motility and c) respond to the fragments of matrix components as chemoattractants for directional movement [8].

Tumor cell surface molecules play an important role in all these processes. Expression of β1,6 branched N-oligosaccharides has unequivocally been shown to be associated with the metastatic and invasive phenotype [9–13]. Highly invasive tumors like the gliomas express them in high amounts [14]. Even the normal cells involved in invasive functions express them for e.g. the trophoblast cells during implantation of embryo into the uterus, granulocytes and macrophages during inflammation and endothelial cells during angiogenesis [15,16]. Their expression on several cancer cells correlates with the disease progression [17]. Highly metastatic cancer cells express them on the invasive front [18]. However, how these oligosaccharides influence invasion is still poorly understood. Some of the proteins reported to carry these oligosaccharides include cell adhesion molecules like cadherins, some of the subunits of integrin receptor, CD44, growth factor receptor like Epidermal Growth Factor Receptor (EGFR), matriptase and other proteins like Lysosomal Associated Membrane Proteins (LAMPs) [13,19].

B16BL6 melanoma cells selected specifically for their invasive characteristics from highly metastatic B16F10 cells gain the ability to metastasize even from a subcutaneous site, as opposed to the parent cells that metastasize only when injected directly into circulation [20]. Using these as the model, and by employing different strategies to inhibit the expression of β1,6 branched N-oligosaccharides we previously showed that their expression on B16 melanoma variants correlates with their invasiveness. This in turn correlated with the ability of cells to adhere to most ECM and BM components [21]. However, in several other cell lines although, their expression almost always correlated with invasiveness, it resulted in decreased adhesion on ECM/BM components [22,23]. Expression of these oligosaccharides thus appears to modulate adhesion both positively [21,24] and negatively [22,23] and our unpublished results show that the terminal substitutions on them may play a key role in this regulation. The expression of β1,6 branched N-oligosaccharides appeared to positively regulate chemotaxis (motility in response to soluble chemoattractants like components of ECM and BM) however, it regulates haptotaxis (motility in response to immobilized matrix components) in a complex manner. It promoted haptotaxis towards ECM component (fibronectin) whereas it inhibited haptotaxis towards reconstituted BM (laminin/matrigel). Similarly, though their expression correlated very strongly with their invasiveness, it did not affect the basal secretion of MMPs [21]. Irrespective of the method of modulation, decreased expression of β1,6 branch N-oligosaccharides had same effect on cellular adhesion, chemotaxis, haptotactic motility and MMP secretion and thus invasion [21].

Integrins, the receptors for all the major collagenous proteins and non-collagenous glycoprotein components of ECM and BM play a major role in maintaining normal tissue homeostasis by stabilizing cellular interaction with the surrounding matrix. They are also involved in processes critical for invasion like adhesion, spreading and movement [25–27]. Tumor cells often regulate the surface expression of some of these integrin receptors to modulate their invasion associated properties. Normally, the integrin function is regulated by their lateral association with another family of cell surface proteins known as tetraspanins (TSPs) [28].

Tetraspanins apart from forming stable interactions with the integrins, often associate with themselves and with other members of tetraspanin family, growth factor receptors, immunoglobulin family of cell adhesion molecules, certain proteases, G-protein coupled receptors and its associated proteins, cholesterol and gangliosides. Together, they form a membrane microdomain termed Tetraspanin Enriched Microdomain (TEM) and provide a platform for regulating cellular events. TEMs appear to modulate invasion associated processes by regulating the availability of these molecules particularly integrins [29–31]. They also show specificity in terms of their association with particular integrins, like CD151 with laminin binding integrins [32] and CD82 with fibronectin receptors [33]. Tumor cells regulate cell motility and invasion either by modulating the expression of tetraspanins [34] or by regulating their association with integrins. Glycosylation has been shown to regulate their association, for instance, glycosylation of both CD82 and α5β1 has been shown to prevent their association and motility [35].

The present manuscript investigates the significance of enhanced adhesion in inducing secretion of MMP-9 and reduced spreading and motility on matrigel, associated with highly invasive cells expressing β1,6 branched N-oligosaccharides. Further, it identifies the integrin receptors that carry these oligosaccharides and attempt to explore the mechanism by which these oligosaccharides regulate α3β1 association with CD151 to reach an optimum level of adhesion, degradation, spreading and movement required to breach the basement membrane.

Materials and methods

Cell lines and reagents

B16F10 and B16BL6 murine melanoma cell lines were a kind gift from Prof. I.J. Fidler, MD Anderson Cancer Centre, Houston, USA which were generated in his laboratory [20]. Cell culture reagents, TRIzol® and Lipofectamine™ 2000 were from Invitrogen, USA. Protoscript® M-MuLV first Strand cDNA synthesis kit was from New England Biolabs (NEB), USA. Restriction enzymes, T4 DNA ligase, Pfu Polymerase and CloneJET™ PCR cloning Kit were from Fermentas International Inc., Canada. PVDF membrane and the ECL kit were purchased from GE Healthcare, Amersham, UK. Culture ware and cell culture inserts were from BD Falcon, USA. Fibronectin, matrigel, mouse anti-mouse α3 integrin (clone 42/CD49c), rat anti-mouse α6 integrin antibody (GoH3 for immunoprecipitation and function blocking experiments) and rat anti-mouse β1 integrin monoclonal antibody (9EG7 for flow cytometry and function blocking experiments) were purchased from BD

Biosciences, USA. Rat anti-mouse β1 integrin monoclonal antibody (clone MB1.2 for Western blotting and immunofluorescence) was purchased from Chemicon. Paraformaldehyde, Polybrene, Puromycine, Swainsonine, Phalloidin TRITC/FITC, 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI), N-octyl-β-D-glucopyranoside, Protamine sulfate, Biotinamido caproic acid 3 sulfo N hydroxy succinimide ester, Bovine Serum Albumin (BSA), Gelatin, Anti-FLAG® M2 Affinity gel, 3 × FLAG® peptide, rabbit polyclonal anti-FLAG, mouse monoclonal anti-β actin (AC-74), rabbit anti GFP N-terminal, goat anti-mouse MMP-9 antibodies, anti-rat HRPO, anti-rabbit FITC and anti-rat TRITC were purchased from Sigma Chemical Co., USA. Rabbit anti-human polyclonal CD151 antibody (H-80), rabbit anti-human polyclonal α5 integrin antibody (H-104) for Western blotting and rat anti-mouse monoclonal α5 integrin antibody (MFR5/5H10) for flow cytometry, anti-goat HRPO and anti-rabbit HRPO were obtained from Santacruz, Biotechnology, Inc., California, USA. Rabbit anti CD82 antibody and rabbit anti α6 integrin (for Western blotting) antibodies were developed by Imgenex Biotech Pvt. Ltd., India. Rat IgG used as control in function blocking assays was purified from normal rat serum using the same procedure as described in [36]. Vectashield mounting medium was obtained from Vector labs, USA. Radioactive tritiated thymidine was obtained from Board of Radiation and Isotope Technology (BRIT), India. Reagents for bacterial culture were purchased from Hi Media, India, while all other chemicals were of analytical grade and purchased locally.

Cell culture

Melanoma cells were routinely cultured in Minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, vitamins (MEM vitamin solution from GIBCO), L-glutamine, and antibiotics (Penicillin, streptomycin and amphotericin B) along with gentamycin (complete medium). The cells were grown in monolayer on plastic tissue culture flasks incubated in humidified atmosphere of 5% CO_2 at 37 °C. The cells were harvested and passaged for in vitro experiment by incubating monolayer with trypsin solution (0.25% trypsin, 0.02% EDTA and 0.05% glucose in PBS) for 1 min. Cells with greater than 95% viability were used for all the assays. For inhibition of glycosylation, cells were grown in presence of N-glycosylation inhibitor Swainsonine (SW) (2 μg/ml) for 48 h and harvested for in-vitro experiments on reaching 90% confluency.

Preparation of total cell lysates and Western blotting

Total cell lysates were prepared exactly as described in [37] using lysis buffer containing 20 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 1 mM each of $MgCl_2$ and $CaCl_2$ and protease inhibitors (1 μg/ml each of pepstatin, leupeptin and aprotinin, and 0.3 mM PMSF). The protein content was estimated as per [38], and was separated on SDS-PAGE according to [39] and transferred on to PVDF membrane as described in [40].

Purification of L-PHA reactive proteins

For purification of L-PHA reactive proteins, total cells suspended in buffer containing 10 mM Tris–HCl (pH 7.5), containing 150 mM NaCl and protease inhibitor (1 μg/ml each of pepstatin, leupeptin and aprotinin, and 0.3 mM PMSF) were sonicated five times for 30 s each at (50% output control) at 4 °C. The lysate was supplemented with N-octyl-β-D-glucopyranoside and protamine sulfate to attain a final concentration of 30 mM and 0.3%, respectively, and further incubated for 1 h on ice. Cells supernatant was collected by centrifugation at 16,000*g* for 1 h at 4 °C as described in [19]. 2000 μg Lysate from B16BL6 cells were then incubated with 100 μl L-PHA agarose beads overnight at 4 °C. Unbound and nonspecifically bound proteins were removed by giving five washes with wash buffer (10 mM Tris–HCl (pH 7.5), 500 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 3 mM N-octyl-β-D-glucopyranoside). Bound proteins were then eluted using non-reduced Laemmli sample buffer, containing 4 M urea.

Gelatin zymography

Tissue culture dishes (60 mm) were coated with 3 ml each of fibronectin (10 μg/ml) and matrigel (50 μg/ml) in plain MEM (MEM without FBS) by incubating overnight at 4 °C. One million melanoma cells in 3 ml of complete media were seeded in the uncoated and coated 60 mm dishes and incubated for 24 h at 37 °C in a CO_2 incubator. The cells were washed with serum free medium and incubated further in the same medium for 24 h, which (conditioned medium) was collected from each dish. This was centrifuged at 1500 rpm for 10 min to remove cellular debris and concentrated 10 times using 10 kDa cutoff filters (Amicon, Millipore). Concentrated samples were dried by speedvac and stored at −20 °C till use.

The dried concentrated conditioned medium was reconstituted in the original volume of non-reduced sample buffer and equal volumes of all samples were loaded on 10% SDS-PAGE gel copolymerized with 0.1% gelatin and run at 4 °C, for comparison. After electrophoresis, the gel was rinsed in renaturing buffer containing 2.5% Triton X-100 for 1 h under mild shaking conditions. The gel was freed of Triton X-100 by giving three wash with 0.1 M Tris–HCl (pH 7.4) and incubated in a buffer containing 0.1 M Tris–HCl (pH 7.4) and 50 mM $CaCl_2$ for 36 h at 37 °C in a water bath. The gel was stained in 0.2% Coomassie brilliant blue R-250 (Sigma) for 1 h, and destained with 50% destaining solution (50% methanol, 40% water and 10% acetic acid) to visualize the cleared zone of lysis. After collection of condition medium the cells were lysed in equal volume of 1 × Laemmli buffer and equal volume of cell lysates from UN, FN and MAT coated dishes was loaded on SDS PAGE and blotted. Blot was stained with Coomassie brilliant blue to check the similar number of cells.

Flow cytometric analysis

For flow cytometry, 90% confluent melanoma cells were harvested described as above. Cells were washed thrice with PBS and then fixed with 1% paraformaldehyde in PBS (pH 7.4) by overnight incubation at 4 °C. The cells were pelleted and washed three times with PBS to remove fixative followed by blocking with FACS buffer (1% FBS in PBS). Subsequently, surface expression of tetraspanin CD82, CD151 and their associated integrin receptors (α3, α5 and β1) was analyzed by incubating with specific primary antibodies for 1 h. This was followed by incubation with fluorophore tagged secondary antibody. Labeled cells were analyzed using FACSCalibur (BD Biosciences) and data was analyzed using CellQuest Pro software.

Colocalization using laser confocal microscopy

B16BL6 cells transfected with GFP tagged CD151 were seeded on matrigel (10 μg/ml) coated coverslips and grown overnight in complete medium up to 70–80% confluence. Cells were washed thrice with PBS (pH 7.4) and fixed with 2% paraformaldehyde at RT for 5 min. Cells were washed again with PBS and blocked with 3% BSA in PBS for 1 h at RT in humidified chamber. Cells were incubated with primary antibody (β1integrin) for 1 h in humidified chamber, followed by three washes with PBS to remove excess or non-specifically bound antibody. Cells were further incubated with fluorescent tagged secondary antibody (anti-rat TRITC) for 1 h followed by three washes with PBS. Cells incubated only with fluorescent tagged secondary antibody serve as isotype control. Nuclei were stained with 5 μg/ml of DAPI in PBS for 1–2 min and coverslips were mounted on slides using vectashield mounting medium. Images were acquired using a Carl Ziess Laser confocal microscope at 63× magnification. Images were analyzed using ImageJ 1.43 software (NIH).

Cell spreading assays

Cell spreading assays were done as per described in [41]. Briefly, coverslips were coated with 10 μg/ml fibronectin or matrigel in plain MEM overnight at 4 °C and blocked with 2% BSA for 1 h at 37 °C. The melanoma cells were harvested, washed thrice with plain medium to get rid of all the serum proteins and suspended at a cell density of 0.5×10^6 cells per ml in plain MEM. The cells were seeded on coated and BSA blocked coverslips and incubated for 45 min in a CO_2 incubator. Coverslips coated with BSA only, served as control. Bound cells were fixed in 4% paraformaldehyde, and permeabilized with 0.5% Triton X-100 for 15 min and stained with 2 μg/ml Phalloidin TRITC/FITC staining solution made in PBS (containing 1 μg/ml of lysolecithin, 10% methanol, and 0.5% BSA in PBS) for 15 min at 37 °C. Nuclei were stained with DAPI. The stained cells were mounted by vectashield and images were acquired using a Zeiss LSM510 META laser confocal microscope (Carl Ziess Microimaging, Inc., Thornwood, NY, USA) at 63× magnification. Spreading of cells was analyzed by calculating cytoplasmic to nuclear area ratio using ImageJ 1.43 software (NIH).

Invasion, motility and adhesion assays

All these assays were performed as described in [21]. Briefly, cells were labeled with 8 μCi/ml tritiated (H^3) thymidine. H^3 thymidine was added to cells when they at about 50% confluent and were allowed to grow overnight. Cells were then harvested and washed thrice with plain MEM. Invasion assays were performed using 24 well transwell units with 8 μm pore size polycarbonate filter (Modified Boyden chambers) coated with 1 mg/ml matrigel (30 μg/insert). Briefly, 0.2×10^6 tritium labeled cells suspended in 300 μl MEM were added to the upper compartment of the Boyden chamber, and 600 μl of spent medium (obtained by growing 50–60% confluent cultures of B16BL6 cells in complete medium for 24 h) added to the lower compartment served as the chemoattractant. Cells were allowed to invade for 32 h at 37 °C in a humidified atmosphere containing 5% CO_2. For invasion assays which involved blocking antibodies, 1×10^6 tritium labeled cells were incubated at RT for 1 h with intermittent tapping, with either control rat IgG, anti β1integrin (9EG7) or anti α6 integrin (GoH3) each at concentration of 30 μg/ml in 100 μl of plain MEM. After incubation 0.2×10^6 cells were seeded per insert for invasion as described above.

For haptotaxis assays, lower surface of Boyden chambers was coated with fibronectin and matrigel (each 10 μg/ml) in triplicates at 4 °C and blocked with 2% BSA for 1 h. Briefly, 0.2×10^6 cells suspended in 300 μl plain MEM were added into upper compartment, lower compartment in this case contains only plain MEM. Cells were allowed to migrate for 6 h at 37 °C in a humidified atmosphere in a CO_2 incubator at 5% CO_2. After performing these assays cells on the upper side of the membrane were removed by scrubbing thrice with cotton swabs. Filters were removed from the inserts and radioactivity was measured using a beta scintillation counter. The number of cells invaded and migrated was calculated based on the specific activity (counts per minute (CPM) per cell) of the cells.

Adhesion assays were performed in 96 well plates coated with 0.1 ml of fibronectin and matrigel each at a concentration of 10 μg per ml, overnight at 4 °C. Coated wells were washed and blocked with 2% BSA at 37 °C for 1 h. As described earlier, 50,000 tritiated H^3 labeled cells suspended in100 μl plain MEM were seeded into each well and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 1 h. Wells were washed four times with 100 μl PBS to remove unbound cells. Cells were harvested on glass fiber filters as described in [36]. Radioactive counts of the cells remaining bound to the substrates were taken using the beta scintillation counter. Identical number of cells seeded in a separate plate which was not washed served as the seeding control to calculate percent adhesion on each substrate.

Transduction and generation of stable cell lines

Downregulation of β1,6 branched N-linked oligosaccharides in B16BL6 cells was performed by using short hairpin RNA (shRNA) against enzyme GnT-V. The shRNAs were designed and cloned into pTRIPz vector (open biosystem) between XhoI and EcoRI site. The shRNA containing plasmid together with helper plasmids $p^{MD2.G}$ and ps^{PAX2} were co-transfected into 293 FT packaging cell line using $CaPO_4$ method of DNA transfection. Supernatant containing virus particles was collected at interval of 24 h and it was spun at 5000 rpm for 20 min at 4 °C. Virus particles was then transduced into B16BL6 using 8 μg/ml polybrene. Medium was changed 24 h post transduction and transduced cells were selected using puromycin at a concentration of 1 μg/ml and stable cell lines were maintained at a concentration of 0.5 μg/ml. Stably transduced cells were induced with 4 μg/ml doxycycline (Sigma) and these cells were further enriched using cell sorter, as these cells have Turbo RFP as reporter gene in them.

Reverse transcription, semiquantitative-PCR and quantitative real time PCR

Total RNA was isolated from melanoma cell lines using Trizol reagent according to manufacturer's instructions. RNA concentration was determined by measuring absorbance at 260 nm using a Nano Drop 1000 spectrophotometer. The first strand cDNA was synthesized by Protoscript First cDNA synthesis kit using oligo (dT) primers and M-MuLV reverse transcriptase as per manufacturer's protocol. Following primers were used to check the expression of tetraspanins

and GAPDH. GAPDH served as loading control. CD151 forward and reverse primer sequences were 5'-TAAAGTGGAGGTGGCTGTAT-3' and 5'-GTAGTGGGGCTGGCACATAG-3' respectively. CD82 forward and reverse primer sequence were 5'-TTCCGTCCTACAAACCTCAT-3' and 5'-TGTGTTCCCCATCTCCTTCT-3' respectively. CD63 forward and reverse primer sequence were 5'-CAAGGACAGAGTCCCGATT-3' and 5'-TCCCAAGACCTCCACAAAAG-3' respectively. CD9 forward and reverse primer sequence were 5'-CATGCTGGGATTGTTCTT-3' and 5'-GCTCCAAAGGACCAGCTATG-3' respectively. CD81 forward and reverse primer sequence were 5'-TCCATGAGACGCTCAACTGT-3' and 5'-GCAC-CATGCTCAGAATCATC-3' respectively. GAPDH forward and reverse primers were 5'-TGAAGGTCGGTGTGAACGGATTTG-3' and 5'-CATG-TAGGCCATGAGGTCCACCAC-3' respectively. For the analysis of MMP-9 transcript levels, quantitative real time PCR was performed. The real time PCR reaction was carried out in 7900 HT system (ABI Prism) and for detecting amplicons Power SYBR green (ABI) was used. The Ct values obtained were normalized to Ribosomal protein L4 (RPL4) values which served as housekeeping control. Analysis was performed using the $2^{-\Delta\Delta Ct}$ method. MMP-9 forward and reverse primers sequence were 5'-TCATTCGCGTGGATAAGGAG-3' and 5'-AGGCTTTG-TCTTGGTACTGG-3' respectively. RPL4 forward and reverse primer sequence were 5'-GACAGCCCTATGCCGTCAGTG-3' and 5'-GCCA-CAGCTCTGCCAGTACC-3' respectively.

Expression of GFP and FLAG tagged CD151

Murine CD151 gene was PCR amplified from total cDNA using forward and reverse primers for CD151 using high fidelity polymerase Pfu. Forward and reverse primers incorporated HindIII and KpnI sites respectively. CD151 forward and reverse sequences used were as follows. Forward primer sequence – 5'-AAGCTTATGGGTGAATTCAATGAGAAGAAG-3' and CD151 reverse primer sequence – 5'-GGTACCCAGTAGTGTTCCAGCTTGAGGCTTC-3'. The PCR product was cloned into pJET1.2 cloning vector (Fermentas), from which it was sub-cloned into pEGFP-N1 vector (Clontech) between HindIII and KpnI restriction sites. Sequence of pEGFP-CD151 construct was verified by sequencing. To clone CD151 in FLAG tagged vector, CD151 gene from pEGFP-CD151 clone was excised by HindIII and KpnI digestion and cloned into C-terminal p3× FLAG-CMV vector (Sigma Chemical Co.). B16BL6 cells were transfected either with, vectors alone (pEGFP and p3× FLAG-CMV) or with CD151 constructs (CD151-GFP and CD151-FLAG) using lipofectamine™ 2000, and stable transfected cell lines were selected by growing them in neomycin (1000 μg/ml) containing medium.

Purification of FLAG tagged CD151 using anti-FLAG M2 affinity gel

B16BL6 cells expressing FLAG tagged CD151 (CD151-FLAG) were treated with Swainsonine for 48 h. Swainsonine treated and untreated cells were homogenized in the lysis buffer containing 20 mM Tris–HCl pH 7.4, 100 mM NaCl, 4 mM EDTA, 1% NP-40 detergent, and cocktail of protease inhibitors (1 μg/ml each of pepstatin, leupeptin and aprotinin, and 0.3 mM PMSF) using Down's homogenizer (20 strokes of tight fit homogenizer) and kept at 4 °C for 5 h, followed by centrifugation at 16,000g for 30 min. Two thousand microgram of cell lysates were incubated overnight with 100 μl anti-FLAG M2 affinity gel (50% suspension) at 4 °C on a rocker shaker. The beads were washed four times with the lysis buffer. Bound proteins were eluted using 100 μg/ml of 3× FLAG peptides.

Densitometric and statistical analysis

Densitometric quantitation of scanned images of Western blots and zymography gel images was done by ImageJ 1.43 software (NIH). Statistical analysis was performed using Graphpad Prism 5. The unpaired student's *t*-test was employed when two groups were compared. One way ANOVA was used to analyze more than two groups, P value <0.05 was considered as significant.

Results

Increased expression of β1,6 branched N-oligosaccharides induces secretion of MMP-9 in melanoma cells grown on ECM and BM components

Expression of β1,6 branched N-oligosaccharides correlates with their ability to adhere to the substratum [21] (Supplementary Fig. 1A). Increased adhesion appears to induce secretion of MMP-9. Cells grown on fibronectin and matrigel coated culture dishes show significantly higher secretion of MMP-9 as compared to those grown on control uncoated culture dishes (Fig. 1A left panel and Fig. 1C). Inhibition of expression of β1,6 branched N-oligosaccharides either using SW or by doxycycline induction in clones expressing shRNAs to GnT-V (shRNA I and II) (Supplementary Fig. 2) inhibited such induction of MMP-9 secretion (Fig. 1A, right panel and Fig. 1D, middle and right panel). Whereas, uninduced clones expressing shRNAs to GnT-V or induced and uninduced clones expressing non-targeting shRNA (NT shRNA) showed similar induction of MMP-9 secretion as B16BL6 cells (Fig. 1C and D, left panel). Increased secretion of MMP-9 due to increased expression of the MMP-9 transcript was demonstrated in cell grown on fibronectin and matrigel, which was inhibited on inhibition of expression of β1,6 branched N-oligosaccharides (Fig. 1B and E). Coomassie brilliant blue staining of the equal volume of lysate prepared from cells remaining after collecting the conditioned media confirmed that cells seeded on each substrate were equal (Supplementary Fig. 1C). Together these results demonstrate that these oligosaccharides regulate invasion by modulating adhesion on different substrates, which apparently influences signals that induce secretion of MMP-9.

Expression of β1,6 branched N-oligosaccharides impacts cellular spreading in the same way as haptotactic motility on different substrates

Our earlier results showed that presence of these oligosaccharides regulates haptotactic motility differentially on fibronectin and on matrigel. Spreading of cells dictates their motility properties on different substrates. The effect of inhibition of these oligosaccharides on spreading was assessed by comparing B16BL6 with SW treated B16BL6 cells or among the doxycycline induced and uninduced B16BL6 cells transduced with either non-targeting shRNA or shRNAs to GnT-V. The results showed that like motility [21], the presence of these oligosaccharides enhanced spreading of cells on fibronectin, but attenuated it on matrigel as seen by confocal images and by comparing ratio of cytoplasmic/nuclear area (Figs. 2A and B and 3A–C).

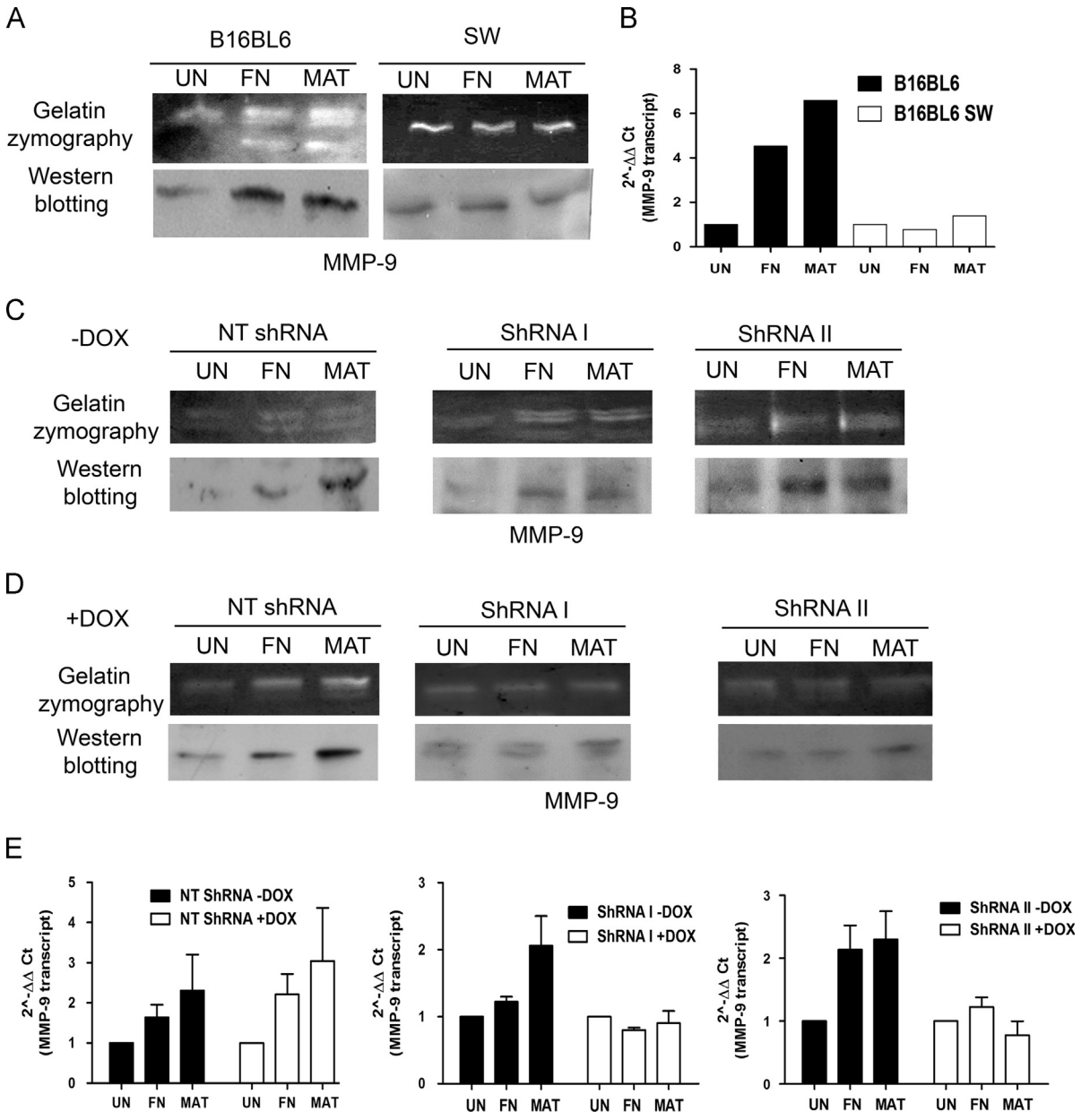

Fig. 1 – Expression of β1,6 branched N-oligosaccharides correlates with adhesion dependent MMP-9 secretion. (A) Levels of MMP-9 secretion and (B) transcripts were analyzed by collecting conditioned medium (CM) and by isolating RNA respectively, from untreated and SW treated cells grown on uncoated (UN), fibronectin (FN) and matrigel (MAT) coated culture dishes. MMP-9 secretion was analyzed by (A) gelatin zymography and Western blotting. (B) MMP-9 transcript levels were analyzed by quantitative PCR. Analysis of CM collected from cells transduced with shRNAs to GnT-V (shRNA I and ShRNA II) or non-targeting (NT shRNA) and grown on different substrates (C) without induction (−DOX) and (D) after induction with doxycycline (+DOX), by gelatin zymography (upper panels) and Western blotting (lower panels). (E) MMP-9 transcript levels were analyzed by quantitative PCR from these cells when grown on uncoated culture dishes (UN), fibronectin (FN) and matrigel (MAT).

Both the subunits of integrin receptors for fibronectin (α5β1) and laminin (α3β1) carry β1,6 branched N-oligosaccharides, however, their presence or absence has no effect on their cell surface expression

The differences in motility on fibronectin and matrigel could be due to differences in surface expression of the respective receptors, receptor glycosylation or inability of the receptors to translocate to the cell surface after inhibition of glycosylation. Almost complete binding of both the α and β subunits of the fibronectin (α5β1) and laminin receptor (α3β1) to L-PHA agarose beads from the lysates of B16BL6 cells showed that they carry β1,6 branched N-oligosaccharides (Fig. 4A). Unbound proteins showed no reactivity to these integrin subunits (data not shown). However, comparison of B16F10 and B16BL6 cells which differs in glycosylation or of B16BL6 cells with those in which glycosylation has been inhibited either using SW or with shRNA to GnT-V, had no effect on the surface expression of these receptors (Fig. 4B–D).

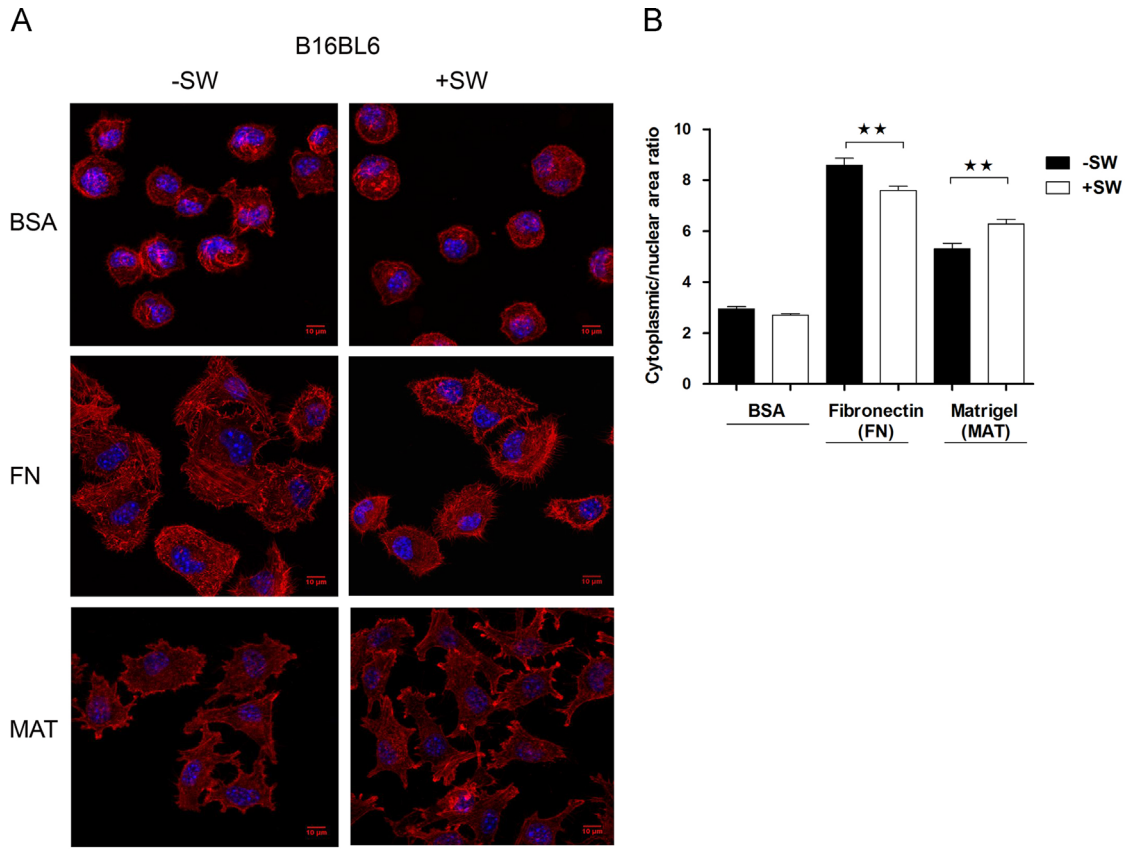

Fig. 2 – Expression of β1,6 branched N-oligosaccharides regulates spreading positively on ECM (fibronectin) but negatively on BM (matrigel) components. (A) Comparison of cell spreading on BSA, fibronectin (FN) and matrigel (MAT) coated coverslips by F-actin staining (red) with Phalloidin-TRITC using laser confocal microscopy at 63× magnification between Swainsonine treated (+SW) and untreated (−SW) B16BL6 cells. Scale bar 10 μm. (B) The graph represents quantitation of cell spreading by calculating ratio of cytoplasmic to nuclear area of more than 100 cells, about 40 cells in each independent experiment done thrice. One way ANOVA was employed to check the statistical significance. Values represent mean ratio of all the cells±SE and **denotes $p<0.05$.

These results rule out the role of altered receptor glycosylation in modulating the surface localization of receptors and point towards an alternate mechanism by which glycosylation regulates motility of cells.

Level of expression of tetraspanins does not correlate with the invasiveness of B16 melanoma cell lines

Tetraspanins are the key regulators of integrin receptor localization and functions. Most tetraspanins except CD151 and CO-029 are downregulated in invasive cells. Transcript levels of the tetraspanins CD151, CD82, CD81, CD63 and CD9 were compared in B16F10 and B16BL6 cell lines by semiquantitative RT-PCR (Fig. 5A). Results did not show any appreciable changes in the transcript levels of most tetraspanins in the two melanoma invasive variants. The total levels of tetraspanin CD82 and CD151 which regulate fibronectin and laminin receptors respectively were compared in B16F10 and B16BL6 cells by Western blotting (Fig. 5B and C) and flow cytometry (Fig. 5D). The results showed that the total levels of these proteins and their surface expression remain unaltered in these variants.

Glycosylation regulates association of laminin receptor integrin α3β1 with tetraspanin CD151

Since both the subunits of laminin receptor α3β1 are carriers of β1,6 branched N-oligosaccharides, inhibition of expression of these oligosaccharides may alter the association of integrin receptor with CD151. Their role in regulating association of α3β1 with CD151 was evaluated. Due to the non-availability of good antibody against murine CD151 for immunoprecipitation or confocal studies, B16BL6 cells transfected with either GFP-tagged CD151 (for confocal studies) or FLAG tagged CD151 (for immunoprecipitation) had to be used. However, experiments with these cells limited the possibility of using shRNA to inhibit β1,6 branched N-oligosaccharides, as multiple transfections and selection of clones affected the basic invasive and metastatic characteristic of the cells. Thus for all the later experiments with these CD151 expressing clones, SW was used to inhibit N-glycosylation. Our previous studies have also shown that the effect of SW on motile characteristics of the cells is comparable to that seen with antisense to GnT-V [21].

B16BL6 cells transfected with pEGFP-vector alone showed diffused fluorescence, in contrast, the cells transfected with CD151-GFP showed distinct membrane localization (Supplementary Fig. 3A).

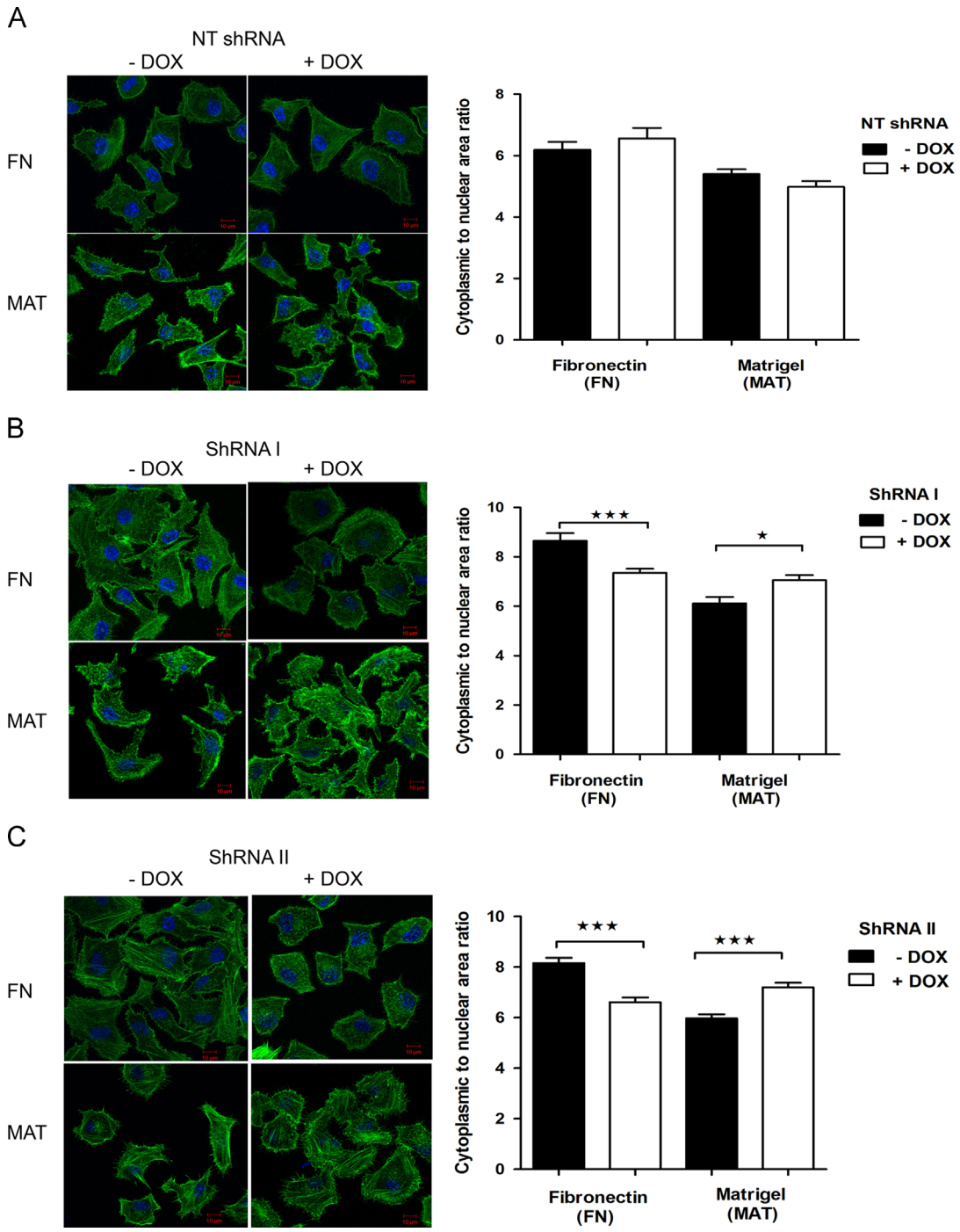

Fig. 3 – Specific inhibition of β1,6 branched N-linked oligosaccharides by shRNAs to GnT-V had similar effect on cell spreading. Cell spreading of doxycycline induced (+DOX) and uninduced (−DOX) (A) NT shRNA, (B) ShRNA I and (C) ShRNA II transduced B16BL6 cells on fibronectin (FN) and matrigel (MAT) coated coverslips was compared by F-actin staining (green) with Phalloidin-FITC using laser confocal microscopy at 63× magnification. Nucleus was shown in blue (*DAPI*). Scale bar 10 μm. The graph represents quantitation of cell spreading by calculating ratio of cytoplasmic to nuclear area of more than 75 cells, about 25 cells in each independent experiment done thrice. One way ANOVA was employed to check the statistical significance. Values represent mean ratio of all the cells ± SE and *denotes $p < 0.05$.

To explore if glycosylation on integrin receptors regulates their association with CD151 and thus motility on matrigel, clones of B16BL6 cells stably expressing GFP tagged CD151 were grown on matrigel coated coverslips and the association was evaluated by confocal microscopy. The results showed that treatment of cells with SW results in significantly increased association of β1 integrin with CD151 (Fig. 6A and B). However, the same could not be assessed for α3 integrin because of non-availability of good antibody against murine α3 integrin suitable for immunofluorescence. To overcome this immunoprecipitation experiments were performed.

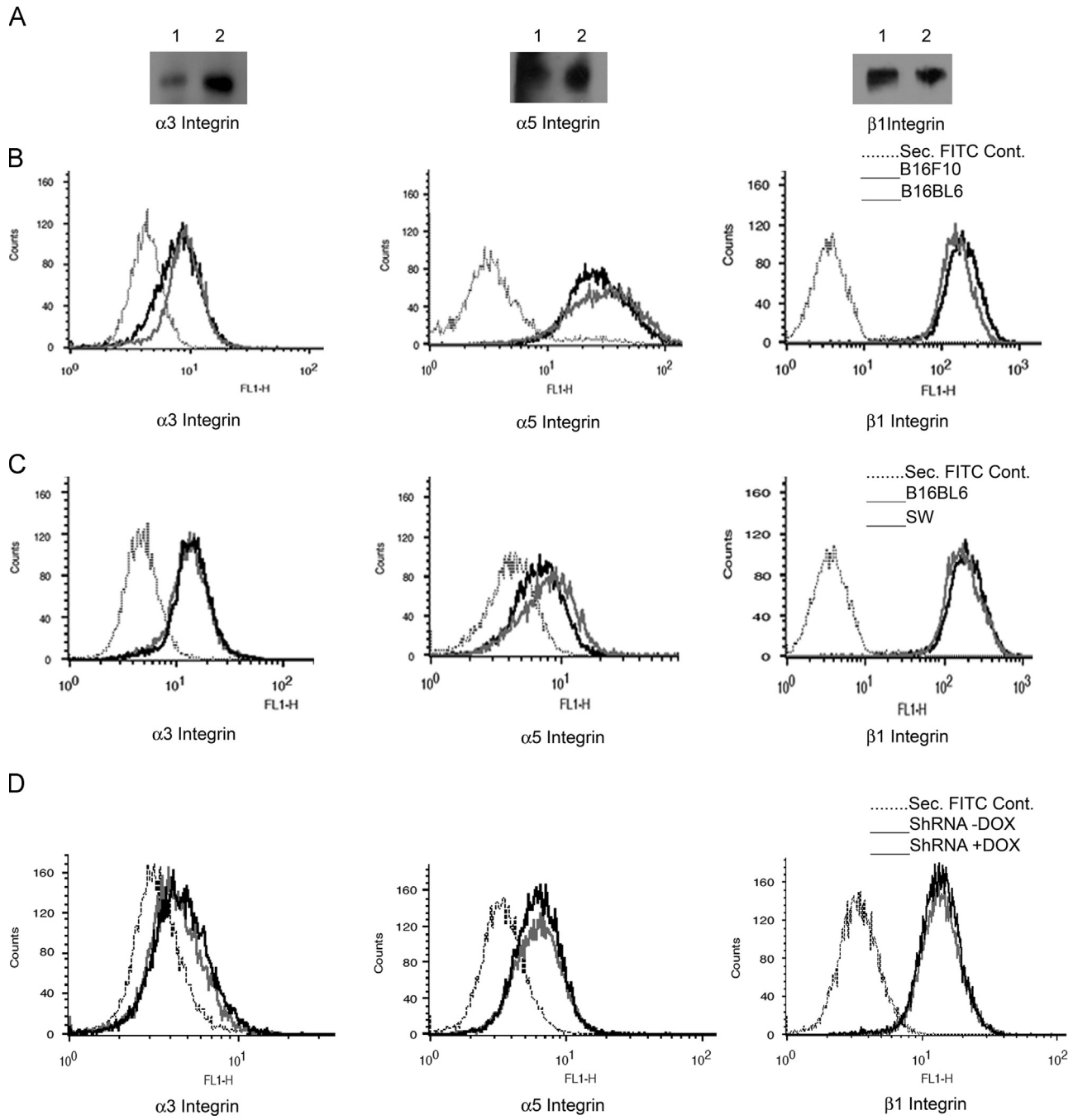

Fig. 4 – Presence of β1,6 branched N-oligosaccharides on both the subunits of fibronectin (α5β1) and laminin (α3β1) receptors has no effect on their cell surface expression. (A) Total cell lysate from B16BL6 cells (100 μg) (*lane1*) and L-PHA binding proteins purified from 100 μg cell lysate for β1 and 300 μg for α3, α5 integrin (*lane2*) were blotted and probed with their specific antibodies. Comparison of surface expression of integrin α3, α5 and β1 integrin on (B) B16F10 and B16BL6 cells and (C) B16BL6 cells and same cells treated with Swainsonine (SW) or (D) GnT-V shRNA transduced B16BL6 cells with and without induction of shRNA by doxycycline (ShRNA+DOX) and (ShRNA − DOX), respectively by flow cytometry using specific antibodies. Cells treated only with FITC labeled secondary antibody served as control.

Coimmunoprecipitation with anti GFP antibody or β1integrin did not work as the immunoprecipitates always gave a band of IgG heavy chain that co-migrated with the tagged CD151. To resolve this problem, lysates of cells expressing FLAG tagged CD151 were used for immunoprecipitation employing FLAG M2 affinity gel, and the bound protein was eluted with 3× FLAG peptide. Immunoprecipitates from cell lysates of SW treated and untreated cells indeed showed that SW treatment significantly augments the association of CD151 with both α3 and β1 integrin subunits of the major laminin receptor (Fig. 6C and D). Further, to show that the co-localization is indeed on the cell surface, SW treated and untreated B16BL6 cells were labeled with membrane

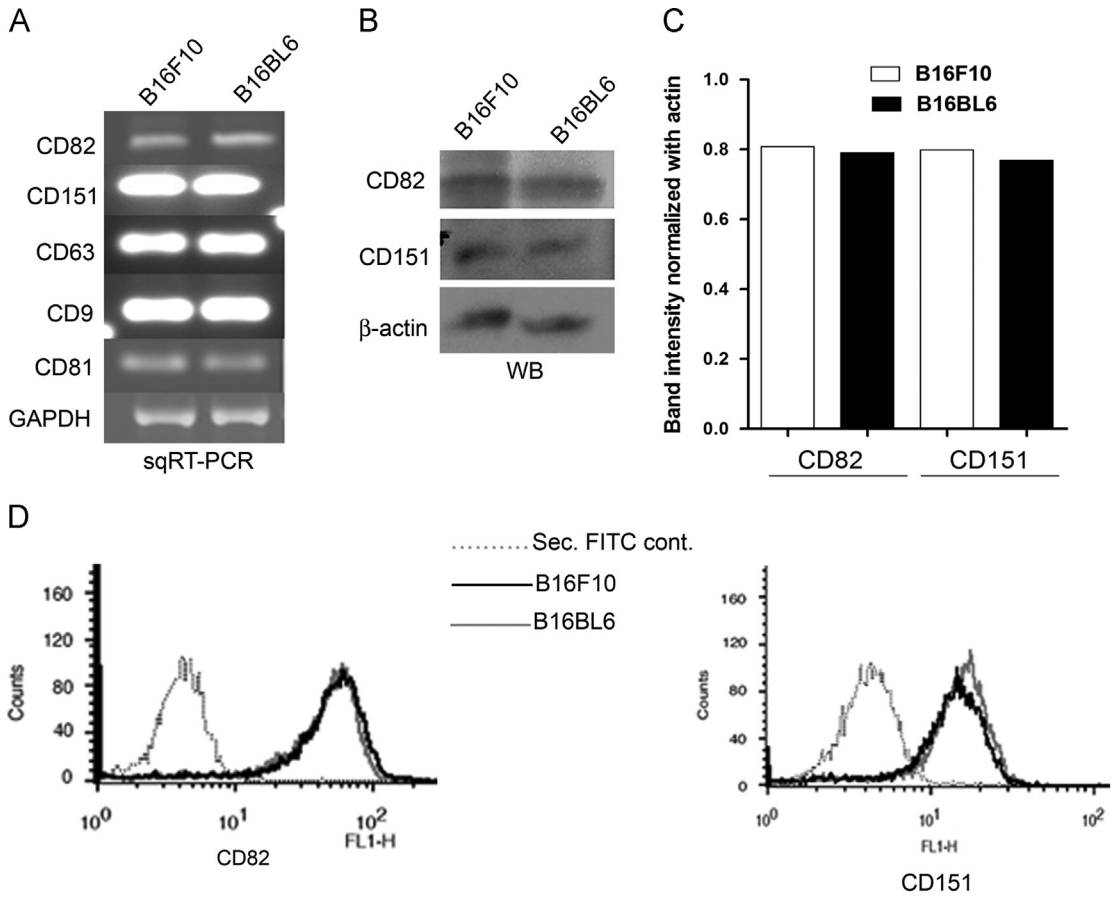

Fig. 5 – Expression of tetraspanins does not correlate with the invasiveness of B16 melanoma cells. (A) Comparison of B16 melanoma invasive variants for the expression of the transcript levels of major tetraspanins by semiquantitative RT-PCR. (B) The expression of CD82 and CD151 by Western blotting (WB) using specific antibodies, (C) densitometric quantitation of band intensity normalized with β-actin and (D) on the cell surface by flow cytometry using specific antibodies. Cells treated only with secondary anti-rabbit FITC served as control. GAPDH and β-actin served as loading control for semiquantitative RT-PCR (sqRT-PCR) and Western blotting respectively.

impermeable biotin and the lysates were immunoprecipitated with FLAG tagged CD151, Western blotted and probed with avidin peroxidase. The results confirmed that glycosylation significantly regulates association of CD151 with β1 and α3 integrin (Supplementary Fig. 4A). Immunoprecipitated α6 integrin from these lysates showed that it possibly exists as α6p form (70 kDa protein) which was found to be associated mainly with β1 integrin but not with β4 integrin as immunoprecipitates did not show β4 integrin even on longer exposure (Supplementary Fig. 4B). Although α6p showed a marginal reduction in size in SW treated cells, showed no reactivity to L-PHA in the immunoprecipitates of the lysates from untreated cells (Supplementary Fig. 4C). The association of α6 integrin (α6p form) with CD151 thus appears to be largely independent of their glycosylation status (Supplementary Fig. 4A).

Altered association of laminin receptor α3β1 with CD151 modulates spreading, haptotactic cell migration and invasion

Transfection of CD151 resulted in marginal increase in the cell spreading on matrigel as compared to cells transfected with vector control. However, inhibition of glycosylation using SW in both the cell types showed significantly higher spreading as compared to untreated cells (Fig. 7A) which is substantiated by the higher ratio of cytoplasmic to nuclear area in these cells (Fig. 7B). The higher spreading also appeared to augment their haptotactic motility properties. Overexpression of CD151 marginally increased the haptotactic motility of B16BL6 cells towards matrigel as compared to the cells transfected with vector alone (Supplementary Fig. 3D), however, after inhibition of glycosylation haptotactic motility increased significantly in both the vector and CD151 transfected B16BL6 cells (Fig. 7C). Interestingly, although, CD151 transfection increased motility towards matrigel only marginally, as expected, it significantly increased the invasive ability of the cells (Supplementary Fig. 3D). Further, the motility towards matrigel did not correlate with the invasiveness. SW treatment significantly increased the motility of cells towards matrigel; however, it had exactly opposite effect on their invasive ability (Fig. 7D). As β1 integrin associates with both α3 and α6 integrin subunits their contribution in regulating invasion was studied by blocking antibodies. Treatment of B16BL6 cells with β1 integrin blocking antibody (9EG7) showed that it inhibited invasion significantly as compared to α6 integrin blocking

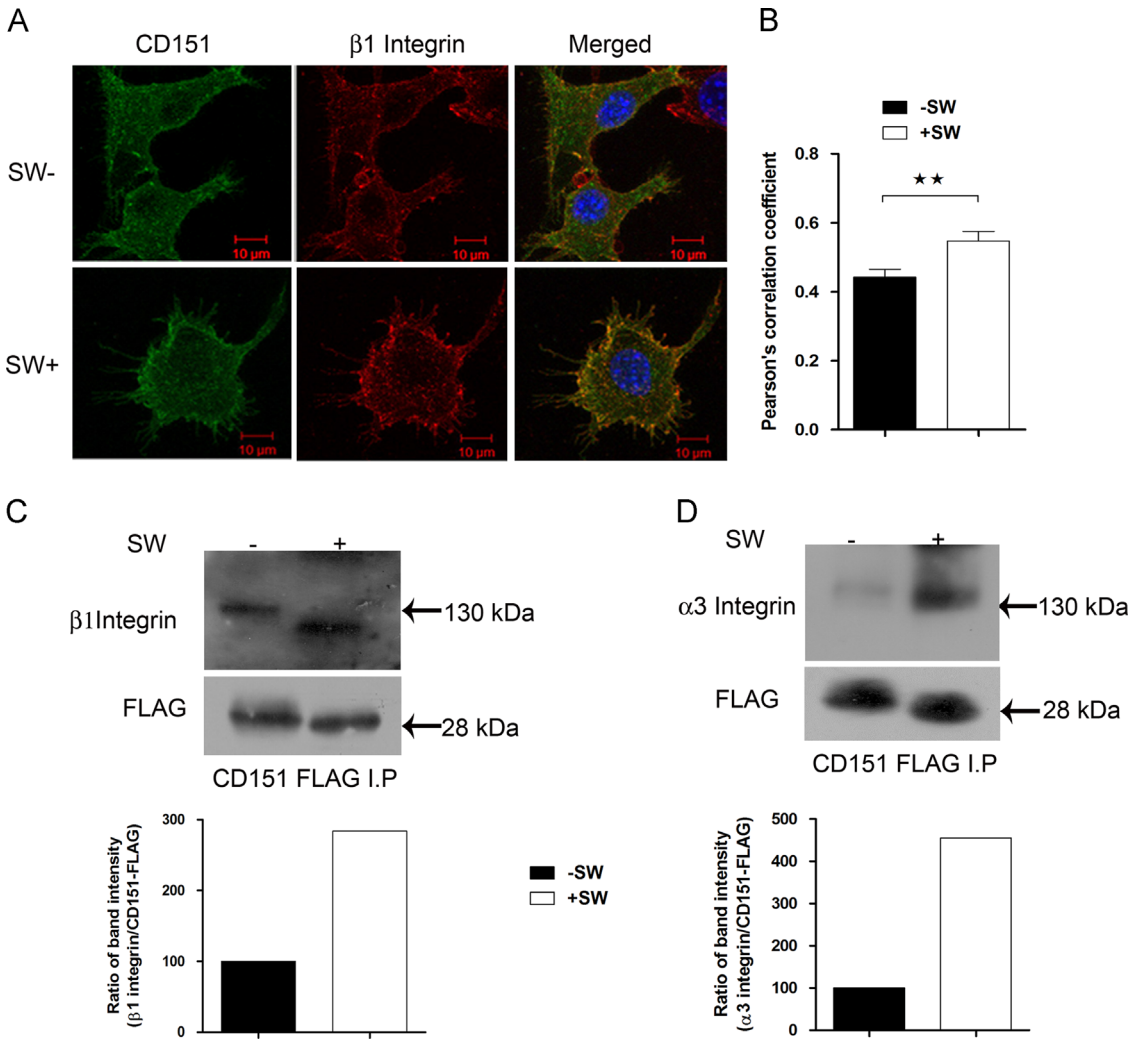

Fig. 6 – Glycosylation regulates association of laminin receptor integrin α3β1 with tetraspanin CD151. (A) Comparison of colocalization of GFP tagged CD151 (*green*) with β1 integrin *(red)* in Swainsonine treated (+SW) and untreated (−SW) CD151-GFP transfected B16BL6 cells. Merged image (*yellow*) depicts colocalization. Scale bar10 μm. (B) Graph represents the analysis of co-localized images by Pearson's correlation coefficient using ImageJ 1.43 software (NIH). Values are mean±SE of three independent experiments (~10 different fields in each experiment). Coimmunoprecipitation of FLAG tagged CD151 from cell lysate of Swainsonine treated and untreated B16BL6 cells stably expressing FLAG tagged CD151 (CD151-FLAG) were blotted and probed with (C) β1 integrin and anti-FLAG antibody, and (D) α3 integrin and anti-FLAG antibody. Graph represents quantitation of the ratio of intensity of band between β1 integrin and CD151-FLAG (C, *lower panel*) or α3 integrin and CD151-FLAG (D, *lower panel*) in Swainsonine treated (+SW) and untreated cells (−SW). Ratio of band intensity between β1 or α3 integrin and CD151-FLAG in untreated cells was taken as 100%.

antibody (GoH3) and control rat IgG treated cells (Fig. 7E). Although, the α3 integrin function blocking antibodies are not available, these results point towards its key role in invasion process. The cells treated with control IgG showed almost same level of invasion as untreated B16BL6 cells (Supplementary Fig. 4D).

Discussion

Invasion is the key requirement for malignant progression and β1,6 branched N-oligosaccharides on surface glycoproteins play a major role in regulating invasion associated processes. Degradation of matrix is a key feature of invasion although the whole process is highly regulated. One of the mechanisms to regulate matrix degradation is by controlling the secretion of matrix degrading enzymes. We have earlier shown that the expression of these oligosaccharides on B16 melanoma invasive variants, when grown in culture dishes has no effect on the secretion of MMPs. However, their expression correlates with adhesion of cells to most of the ECM and BM components. Increased adhesion of these cells was also associated with significant induction of MMP-9 secretion, when grown on these substrates (Fig. 1A). Inhibition of expression of these oligosaccharides has been shown to inhibit adhesion [21] (Supplementary Fig. 1A and B). The experiments using SW and the inducible clones expressing shRNA to GnT-V confirmed that the

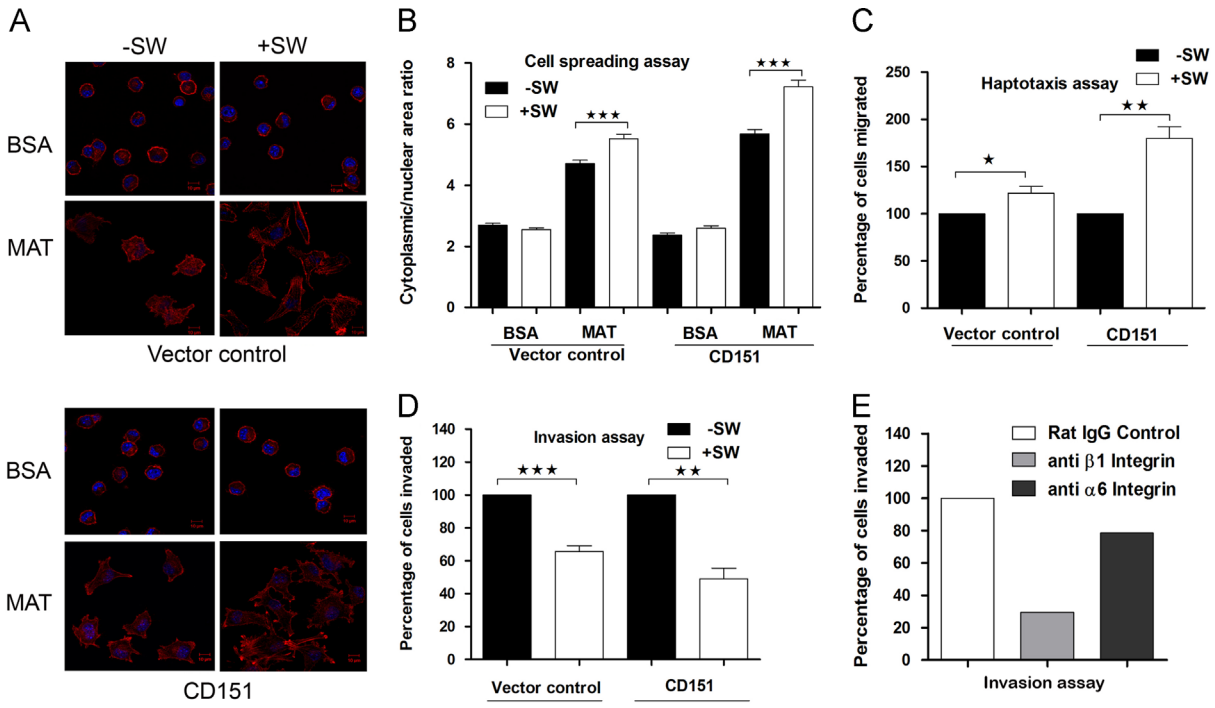

Fig. 7 – Glycosylation dependent association of integrin receptors with CD151 modulates spreading, haptotactic motility and thus invasion of B16 melanoma cells. (A) Comparison of cell spreading of untreated (−SW) and Swainsonine treated (+SW), vector transfected (Vector control) (*upper panels*) and CD151 transfected (CD151) B16BL6 cells (*lower panels*) on BSA and matrigel (MAT) coated coverslips by F-actin staining (red) with Phalloidin-TRITC using laser confocal microscopy at 63× magnification. Nucleus was shown in blue (*DAPI*). Scale bar 10 μm. (B) The graph represents quantitation of cell spreading by calculating ratio of cytoplasmic to nuclear area of more than 100 cells, about 40 cells in each independent experiment done thrice. One way ANOVA was employed to check the statistical significance. Values represent mean ratio of all the cells ± SE and ***denotes $p<0.05$. (C) Comparison of the haptotactic motility of untreated (−SW) and SW treated (+SW) vector control and GFP-tagged CD151 transfected B16BL6 cells (CD151) on 24 well transwell units coated with matrigel. The number of untreated cells that migrated in response to matrigel (Vector control or CD151) was taken as 100%. Values are mean ± SE of three independent experiments done in triplicate and *and **denote $p<0.039$ and $p<0.0028$ respectively. (D) Comparison of the invasive ability of untreated (−SW) and SW treated (+SW) B16BL6 cells transfected either with vector alone or GFP tagged CD151. Invasion was compared by taking the number of untreated; vector control and CD151 transfected B16BL6 that invaded the matrigel barrier were taken as 100%. Values are mean ± SE of three independent experiments carried out in triplicate. Student's *t* test was performed to compare significance between two cell lines. ***and **denote $p<0.0005$ and $p<0.0095$ respectively. (E) B16BL6 cells were treated with equivalent concentration (30 μg/ml) of control Rat IgG, anti β1 integrin (9EG7), or anti α6 integrin (GoH3) function blocking monoclonal antibody and invasion assay was performed. Number of cells treated with control IgG invaded through matrigel was taken as 100% which was comparable to untreated B16BL6 cells (Supplementary Fig. 4D).

expression of these oligosaccharides correlates with the induction of MMP-9 on fibronectin (ECM) and matrigel (BM) (Fig. 1A, C and D). The decreased levels of MMP-9 transcript on SW treatment or inhibition of GnT-V by shRNA induction (Fig. 1B and E) points towards the possible role of adhesion induced signaling in the induction of MMP-9 secretion, which is glycosylation dependent. Increased adhesion on ECM and BM components on one hand regulates matrix degradation and on the other hand possibly regulates movement [42]. It would be interesting to investigate if these oligosaccharides have any role in activating the pro-enzymes or their localization on the cell membrane in order to prevent indiscriminate degradation, as the same matrix components are also required for the movement of cells.

These oligosaccharides appear to influence motility of cells in a substrate specific manner, enhanced towards ECM (fibronectin) and attenuated towards BM (laminin/matrigel) components [21]. Laminin is the major component of matrigel and contributes to >50% of its weight. The attenuated motility on matrigel could thus be due to altered expression or modification of laminin receptors. The expression of these oligosaccharides appeared to have the same differential effect on spreading as that seen on motility. Inhibition of these oligosaccharides inhibited spreading of B16BL6 cells on fibronectin but increased it on matrigel (Fig. 2A and B). Similar observations were reported earlier by Leppa et al. [43] who showed transformation associated expression of complex N-glycans on β1 integrin, a subunit common to both laminin and fibronectin receptor(s). However, inhibition of these oligosaccharides increased spreading of cells on laminin-1 but was unaffected on fibronectin.

Integrin subunits α3, α5, αv, β1 and β3 have been shown to be the carriers of β1,6 branched N-oligosaccharides on different cell types [19]. These oligosaccharides have been shown to affect

integrin structure, dimerization, affinity, clustering and stability of different integrin receptors [23,44–47]. The differential spreading and motility on fibronectin and matrigel thus could be due to differential glycosylation of respective receptors. We find that β1 integrin is one of the major carriers of β1,6 branched N-oligosaccharides on melanoma cells, which partners with both fibronectin and laminin receptors. Among the laminin receptors β1 integrin forms dimers with α3, α6 and α7 subunits; however, α7 integrin has a relatively restricted distribution expressed largely on striated and cardiac muscle and certain endothelial and neuronal cell types [48]. Although it is also expressed on melanoma cells, its expression correlates inversely with the metastatic potential [49]. Lectin-precipitation experiments using immobilized L-PHA confirmed that both the subunits of the laminin receptor integrin α3β1 apart from fibronectin receptor α5β1 carry these oligosaccharides (Fig. 4A). Although, α6 integrin (α6p form) showed marginal reduction in its size on SW treatment (Supplementary Fig. 4A and B) it showed no reactivity towards L-PHA (Supplementary Fig. 4C). Previously, studies from other labs on B16F10 cells have also ruled out α6 integrin to be the carrier of β1,6 branched N-oligosaccharides [50].

The differential motility could also be due to differences in surface localization of respective integrin receptors. Tumor cells do show altered surface expression of certain integrin receptors [51]. Even altered glycosylation appears to dictate the levels of integrin receptors on cell surface [52]. However, the expression of α5β1 and α3β1 (Fig. 4B–D) and even α6β1 (data not shown) remained almost identical on B16F10, B16BL6 and on the cells in which glycosylation was inhibited. The other possible mechanism by which integrin function and thus motility could be regulated is by differential regulation of fibronectin and laminin receptors in the tetraspanin (TSP) enriched membrane micro-domains (TEMs). TEMs appear to modulate invasion associated processes by regulating the availability of TEM associated molecules, particularly integrins. Expression of most TSPs except CD151 and CO-029 is downregulated by highly invasive and metastatic cells [34]. However, there was no significant change in the transcript levels of major TSPs, which is important from the invasion point of view, in the parent B16F10 and its invasive variant B16BL6 cells (Fig. 5A). Motility of cells on fibronectin is largely regulated by the association of fibronectin receptor (α5β1) with TSP CD82 while that on matrigel/laminin by the association of laminin receptors with TSP CD151. The levels of both these TSPs also remained almost identical as assessed by Western blotting and flow cytometry (Fig. 5B–D). There are a few instances where absence of correlation between the level of expression and invasiveness has been demonstrated. For example, in some of the invasive variants of human bladder carcinoma cell lines, there is no correlation between expression of CD82 and their invasive potential [53]. The cells possibly use an alternate mechanism to regulate integrin TSP interaction and thus invasion.

Glycosylation of both tetraspanin (CD82) and fibronectin receptor (α5β1) has been shown to regulate their association and thus cell motility [35]. It is possible that altered glycosylation on laminin receptors and CD151 also modulates their association, to influence motility of cells and thus invasion of basement membrane. The association of laminin receptors with tetraspanins is largely dictated by the interaction of α subunit with CD151 [54]. Both α3 and β1 subunits of the laminin receptor express these oligosaccharides on B16BL6 cells (Fig. 4A). Treatment of cells with SW resulted in significant increase in the association of β1 integrin with CD151 as demonstrated by laser confocal microscopy (LCM) (Fig. 6A and B) and coimmunoprecipitation experiments (Fig. 6C and Supplementary Fig. 4A). Integrin subunit β1 associates with several other α subunits, however, immunoprecipitation experiments using total cell lysates of both unlabeled and those labeled with membrane impermeable biotin conclusively showed that SW treatment significantly promoted association of even α3 subunit of integrin receptor with CD151 (Fig. 6D and Supplementary Fig. 4A). However, glycosylation did not have significant effect in regulating association of CD151 with α6 integrin subunit (Supplementary Fig. 4A) which has been shown to exist as a 70 kDa protein referred to as α6p form, in many invasive cancer cells [55,56]. Although, the α6p form also associates with β1 (and not with β4) integrin subunit, the association appeared to be independent of their glycosylation status (Supplementary Fig. 4B).

The enhanced spreading and motility on matrigel could thus be due to altered association of laminin receptor α3β1 with TSP CD151. Although, the use of CD151 transfected cells limited our options to use more specific methods of inhibition of β1,6 branched N-oligosaccharides, all our previous experiments (Figs. 1, 3 and 4) and work [21] have confirmed that SW is as effective. A single N-glycosylation site on CD151 has been identified and SW treatment results in reduction in the size of FLAG tagged CD151 (Fig. 6C and D), which may also have a role in regulating association of CD151 with integrins, however, immunoprecipitated CD151 showed no reactivity to either L- or E-PHA (data not shown).

CD151 majorly contributes to the invasiveness of the cells, but its effect on motility is highly regulated, and glycosylation appears to play a key role in this regulation. Treatment of B16BL6 cells overexpressing CD151 with SW increased their motility significantly; however, it had an exactly opposite effect on their invasiveness. It suggests that cancer cells regulate their motility on BM to be invasive and glycosylation of receptors has a major role in dictating their association with tetraspanins and thus motility. In spite of enhanced spreading and motility on BM (matrigel) (Fig. 7A–C), SW treated cells showed significant reduction in their invasion through matrigel (Fig. 7D). In spite of non-availability of good function blocking antibodies to murine α3 integrin, using those against β1 and α6, these studies confirmed that the invasion of matrigel is predominantly mediated via α3β1 integrin as inhibition with anti α6 integrin function blocking antibody was only marginal (Fig. 7E). Spreading of the cells differs depending on the substratum and less spread morphology on BM appears to be an important determinant for breaching this barrier [57]. To be invasive, tumor cells need to be more motile when surrounded by other cells and the ECM, however, once they reach the basement membrane they possibly need to stabilize adhesion for sustained degradation followed by controlled motility.

Expression of β1,6 branched N-oligosaccharides on human fibrosarcoma cells has been shown to result in enhanced movement on fibronectin and invasion of matrigel [23]. Expression of β1,6 branched N-oligosaccharides in human gastric cancer cells resulted in increased movement on laminin and could be reversed by overexpression of bisected N-glycans on laminin receptor α3β1 [58]. More recently, the expression of CD151 was shown to regulate glycosylation of α3 integrin. Down regulation of CD151

resulted in reduced expression of bisected N-glycans on α3 integrin and decreased motility on laminin [59]. The above studies demonstrate a complex regulation of function of the receptors that are involved in regulating motility and possibly invasion. The level of receptor, the extent and type of glycosylation of the receptor appear to determine the extent of association of the receptor(s) with membrane microdomains – TEMs, and thus cell movement and invasion. Although, in these studies CD151 does not appear to carry complex multi-antennary type of N-oligosaccharides, their contribution in motility and invasion process would need to be investigated.

These investigations thus establish that the expression of β1,6 branched N-oligosaccharides on laminin receptor α3β1 is the key determinant of the association of the receptor with the tetraspanin CD151 and thus motility on BM. We also reveal that the motility of the cells on a particular substrate may not always be an indicator of its invasive ability. Our results clearly demonstrate that to be invasive cancer cells need to optimize their interaction with the substratum. They need to be more motile when invading the ECM but more stabilized on BM. The cells possibly optimize their adhesion and spreading once they reach the BM barrier. Highly motile cells may not be able to stabilize their interactions to induce secretion of matrix degrading enzymes required to breach the BM. These investigations provide insight into the complex regulation of the processes involved in invasion, and elucidate the role of receptor glycosylation in modulating their association with the components of the membrane microdomains to achieve an invasive phenotype.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2014.02.004.

REFERENCES

[1] D. Spano, C. Heck, P. De Antonellis, G. Christofori, M. Zollo, Molecular networks that regulate cancer metastasis, Semin. Cancer Biol. 22 (2012) 234–249.

[2] P.S. Steeg, Invasion and metastasis, Curr. Opin. Oncol. 4 (1992) 134–141.

[3] R.A. Cairns, R. Khokha, R.P. Hill, Molecular mechanisms of tumor invasion and metastasis: an integrated view, Curr. Mol. Med. 3 (2003) 659–671.

[4] I.J. Fidler, The organ microenvironment and cancer metastasis, Differentiation 70 (2002) 498–505.

[5] S.A. Brooks, H.J. Lomax-Browne, T.M. Carter, C.E. Kinch, D.M. Hall, Molecular interactions in cancer cell metastasis, Acta Histochem. 112 (2010) 3–25.

[6] I.J. Fidler, S.J. Kim, R.R. Langley, The role of the organ microenvironment in the biology and therapy of cancer metastasis, J. Cell. Biochem. 101 (2007) 927–936.

[7] K.J. Luzzi, I.C. MacDonald, E.E. Schmidt, N. Kerkvliet, V.L. Morris, A.F. Chambers, A.C. Groom, Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases, Am. J. Pathol. 153 (1998) 865–873.

[8] W.G. Stetler-Stevenson, L.A. Liotta, D.E. Kleiner Jr., Extracellular matrix 6: role of matrix metalloproteinases in tumor invasion and metastasis, FASEB J. 7 (1993) 1434–1441.

[9] J.W. Dennis, S. Laferte, C. Waghorne, M.L. Breitman, R.S. Kerbel, Beta 1-6 branching of Asn-linked oligosaccharides is directly associated with metastasis, Science 236 (1987) 582–585.

[10] S. Yagel, R. Feinmesser, C. Waghorne, P.K. Lala, M.L. Breitman, J.W. Dennis, Evidence that beta 1-6 branched Asn-linked oligosaccharides on metastatic tumor cells facilitate invasion of basement membranes, Int. J. Cancer 44 (1989) 685–690.

[11] M. Yanagi, Y. Aoyagi, T. Suda, Y. Mita, H. Asakura, N-Acetylglucosaminyltransferase V as a possible aid for the evaluation of tumor invasiveness in patients with hepatocellular carcinoma, J. Gastroenterol. Hepatol. 16 (2001) 1282–1289.

[12] Y.Y. Zhao, M. Takahashi, J.G. Gu, E. Miyoshi, A. Matsumoto, S. Kitazume, N. Taniguchi, Functional roles of N-glycans in cell signaling and cell adhesion in cancer, Cancer Sci. 99 (2008) 1304–1310.

[13] J.W. Dennis, M. Granovsky, C.E. Warren, Glycoprotein glycosylation and cancer progression, Biochim. Biophys. Acta 1473 (1999) 21–34.

[14] H. Yamamoto, J. Swoger, S. Greene, T. Saito, J. Hurh, C. Sweeley, J. Leestma, E. Mkrdichian, L. Cerullo, A. Nishikawa, Y. Ihara, N. Taniguchi, J.R. Moskal, Beta1,6-N-acetylglucosamine-bearing N-glycans in human gliomas: implications for a role in regulating invasivity, Cancer Res. 60 (2000) 134–142.

[15] M. Tomiie, S. Isaka, E. Miyoshi, N. Taniguchi, T. Kimura, K. Ogita, T. Tsutsui, K. Shimoya, T. Nakagawa, A. Kondo, M. Koyama, Y. Murata, Elevated expression of N-acetylglucosaminyltransferase V in first trimester human placenta, Biochem. Biophys. Res. Commun. 330 (2005) 999–1004.

[16] R. Pili, J. Chang, R.A. Partis, R.A. Mueller, F.J. Chrest, A. Passaniti, The alpha-glucosidase I inhibitor castanospermine alters endothelial cell glycosylation, prevents angiogenesis, and inhibits tumor growth, Cancer Res. 55 (1995) 2920–2926.

[17] B. Fernandes, U. Sagman, M. Auger, M. Demetrio, J.W. Dennis, Beta 1-6 branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasia, Cancer Res. 51 (1991) 718–723.

[18] R. Takano, M. Nose, T. Nishihira, M. Kyogoku, Increase of beta 1-6-branched oligosaccharides in human esophageal carcinomas invasive against surrounding tissue in vivo and in vitro, Am. J. Pathol. 137 (1990) 1007–1011.

[19] M. Przybylo, D. Martuszewska, E. Pochec, D. Hoja-Lukowicz, A. Litynska, Identification of proteins bearing beta1-6 branched N-glycans in human melanoma cell lines from different progression stages by tandem mass spectrometry analysis, Biochim. Biophys. Acta 1770 (2007) 1427–1435.

[20] G. Poste, J. Doll, I.R. Hart, I.J. Fidler, In vitro selection of murine B16 melanoma variants with enhanced tissue-invasive properties, Cancer Res. 40 (1980) 1636–1644.

[21] B.V. Reddy, R.D. Kalraiya, Sialilated beta1,6 branched N-oligosaccharides modulate adhesion, chemotaxis and motility of melanoma cells: effect on invasion and spontaneous metastasis properties, Biochim. Biophys. Acta 1760 (2006) 1393–1402.
[22] M. Demetriou, I.R. Nabi, M. Coppolino, S. Dedhar, J.W. Dennis, Reduced contact-inhibition and substratum adhesion in epithelial cells expressing GlcNAc-transferase V, J. Cell Biol. 130 (1995) 383–392.
[23] H.B. Guo, I. Lee, M. Kamar, S.K. Akiyama, M. Pierce, Aberrant N-glycosylation of beta1 integrin causes reduced alpha5beta1 integrin clustering and stimulates cell migration, Cancer Res. 62 (2002) 6837–6845.
[24] M. Yoshimura, A. Nishikawa, Y. Ihara, S. Taniguchi, N. Taniguchi, Suppression of lung metastasis of B16 mouse melanoma by N-acetylglucosaminyltransferase III gene transfection, Proc. Natl. Acad. Sci. USA 92 (1995) 8754–8758.
[25] C. Margadant, H.N. Monsuur, J.C. Norman, A. Sonnenberg, Mechanisms of integrin activation and trafficking, Curr. Opin. Cell Biol. 23 (2011) 607–614.
[26] R.O. Hynes, Integrins: bidirectional, allosteric signaling machines, Cell 110 (2002) 673–687.
[27] B.H. Luo, T.A. Springer, Integrin structures and conformational signaling, Curr. Opin. Cell Biol. 18 (2006) 579–586.
[28] F. Berditchevski, Complexes of tetraspanins with integrins: more than meets the eye, J. Cell Sci. 114 (2001) 4143–4151.
[29] M. Zoller, Tetraspanins: push and pull in suppressing and promoting metastasis, Nat. Rev. Cancer 9 (2009) 40–55.
[30] M.E. Hemler, Targeting of tetraspanin proteins—potential benefits and strategies, Nat. Rev. Drug Discov. 7 (2008) 747–758.
[31] R.P. Devbhandari, G.M. Shi, A.W. Ke, F.Z. Wu, X.Y. Huang, X.Y. Wang, Y.H. Shi, Z.B. Ding, Y. Xu, Z. Dai, J. Fan, J. Zhou, Profiling of the tetraspanin CD151 web and conspiracy of CD151/integrin beta1 complex in the progression of hepatocellular carcinoma, PLoS One 6 (2011) e24901.
[32] C.S. Stipp, Laminin-binding integrins and their tetraspanin partners as potential antimetastatic targets, Expert Rev. Mol. Med. 12 (2010) e3.
[33] R. Bass, F. Werner, E. Odintsova, T. Sugiura, F. Berditchevski, V. Ellis, Regulation of urokinase receptor proteolytic function by the tetraspanin CD82, J. Biol. Chem. 280 (2005) 14811–14818.
[34] M.M. Richardson, L.K. Jennings, X.A. Zhang, Tetraspanins and tumor progression, Clin. Exp. Metastasis 28 (2011) 261–270.
[35] M. Ono, K. Handa, D.A. Withers, S. Hakomori, Glycosylation effect on membrane domain (GEM) involved in cell adhesion and motility: a preliminary note on functional alpha3, alpha5–CD82 glycosylation complex in ldlD 14 cells, Biochem. Biophys. Res. Commun. 279 (2000) 744–750.
[36] N. Srinivasan, S.M. Bane, S.D. Ahire, A.D. Ingle, R.D. Kalraiya, Poly N-acetyllactosamine substitutions on N- and not O-oligosaccharides or Thomsen–Friedenreich antigen facilitate lung specific metastasis of melanoma cells via galectin-3, Glycoconj. J. 26 (2009) 445–456.
[37] V. Krishnan, S.M. Bane, P.D. Kawle, K.N. Naresh, R.D. Kalraiya, Altered melanoma cell surface glycosylation mediates organ specific adhesion and metastasis via lectin receptors on the lung vascular endothelium, Clin. Exp. Metastasis 22 (2005) 11–24.
[38] G.L. Peterson, A simplification of the protein assay method of Lowry et al. which is more generally applicable, Anal. Biochem. 83 (1977) 346–356.
[39] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[40] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.
[41] A. Lagana, J.G. Goetz, P. Cheung, A. Raz, J.W. Dennis, I.R. Nabi, Galectin binding to Mgat5-modified N-glycans regulates fibronectin matrix remodeling in tumor cells, Mol. Cell. Biol. 26 (2006) 3181–3193.
[42] S.P. Palecek, J.C. Loftus, M.H. Ginsberg, D.A. Lauffenburger, A.F. Horwitz, Integrin–ligand binding properties govern cell migration speed through cell–substratum adhesiveness, Nature 385 (1997) 537–540.
[43] S. Leppa, J. Heino, M. Jalkanen, Increased glycosylation of beta 1 integrins affects the interaction of transformed S115 mammary epithelial cells with laminin-1, Cell Growth Differ. 6 (1995) 853–861.
[44] M. Zheng, H. Fang, S. Hakomori, Functional role of N-glycosylation in alpha 5 beta 1 integrin receptor. De-N-glycosylation induces dissociation or altered association of alpha 5 and beta 1 subunits and concomitant loss of fibronectin binding activity, J. Biol. Chem. 269 (1994) 12325–12331.
[45] T. Isaji, Y. Sato, Y. Zhao, E. Miyoshi, Y. Wada, N. Taniguchi, J. Gu, N-glycosylation of the beta-propeller domain of the integrin alpha5 subunit is essential for alpha5beta1 heterodimerization, expression on the cell surface, and its biological function, J. Biol. Chem. 281 (2006) 33258–33267.
[46] T. Isaji, J. Gu, R. Nishiuchi, Y. Zhao, M. Takahashi, E. Miyoshi, K. Honke, K. Sekiguchi, N. Taniguchi, Introduction of bisecting GlcNAc into integrin alpha5beta1 reduces ligand binding and down-regulates cell adhesion and cell migration, J. Biol. Chem. 279 (2004) 19747–19754.
[47] L. Wang, Y. Liang, Z. Li, X. Cai, W. Zhang, G. Wu, J. Jin, Z. Fang, Y. Yang, X. Zha, Increase in beta1-6 GlcNAc branching caused by N-acetylglucosaminyltransferase V directs integrin beta1 stability in human hepatocellular carcinoma cell line SMMC-7721, J. Cell. Biochem. 100 (2007) 230–241.
[48] T. Velling, G. Collo, L. Sorokin, M. Durbeej, H. Zhang, D. Gullberg, Distinct alpha 7A beta 1 and alpha 7B beta 1 integrin expression patterns during mouse development: alpha 7A is restricted to skeletal muscle but alpha 7B is expressed in striated muscle, vasculature, and nervous system, Dev. Dyn. 207 (1996) 355–371.
[49] B.L. Ziober, Y.Q. Chen, D.M. Ramos, N. Waleh, R.H. Kramer, Expression of the alpha7beta1 laminin receptor suppresses melanoma growth and metastatic potential, Cell Growth Differ. 10 (1999) 479–490.
[50] R. Chammas, S.S. Veiga, L.R. Travassos, R.R. Brentani, Functionally distinct roles for glycosylation of alpha and beta integrin chains in cell–matrix interactions, Proc. Natl. Acad. Sci. USA 90 (1993) 1795–1799.
[51] C.T. Mierke, B. Frey, M. Fellner, M. Herrmann, B. Fabry, Integrin alpha5beta1 facilitates cancer cell invasion through enhanced contractile forces, J. Cell Sci. 124 (2011) 369–383.
[52] L.T. Kim, S. Ishihara, C.C. Lee, S.K. Akiyama, K.M. Yamada, F. Grinnell, Altered glycosylation and cell surface expression of beta 1 integrin receptors during keratinocyte activation, J. Cell Sci. 103 (Pt. 3) (1992) 743–753.
[53] P. Jackson, E.A. Kingsley, P.J. Russell, Inverse correlation between KAI1 mRNA levels and invasive behaviour in bladder cancer cell lines, Cancer Lett. 156 (2000) 9–17.
[54] V. Serru, F. Le Naour, M. Billard, D.O. Azorsa, F. Lanza, C. Boucheix, E. Rubinstein, Selective tetraspan–integrin complexes (CD81/alpha4beta1, CD151/alpha3beta1, CD151/alpha6beta1) under conditions disrupting tetraspan interactions, Biochem. J. 340 (Pt. 1) (1999) 103–111.
[55] T.L. Davis, I. Rabinovitz, B.W. Futscher, M. Schnolzer, F. Burger, Y. Liu, M. Kulesz-Martin, A.E. Cress, Identification of a novel structural variant of the alpha 6 integrin, J. Biol. Chem. 276 (2001) 26099–26106.
[56] M.C. Demetriou, M.E. Pennington, R.B. Nagle, A.E. Cress, Extracellular alpha 6 integrin cleavage by urokinase-type plasminogen activator in human prostate cancer, Exp. Cell Res. 294 (2004) 550–558.

[57] Y.W. Chu, R.B. Runyan, R.G. Oshima, M.J. Hendrix, Expression of complete keratin filaments in mouse L cells augments cell migration and invasion, Proc. Natl. Acad. Sci. USA 90 (1993) 4261–4265.
[58] Y. Zhao, T. Nakagawa, S. Itoh, K. Inamori, T. Isaji, Y. Kariya, A. Kondo, E. Miyoshi, K. Miyazaki, N. Kawasaki, N. Taniguchi, J. Gu, N-acetylglucosaminyltransferase III antagonizes the effect of N-acetylglucosaminyltransferase V on alpha3beta1 integrin-mediated cell migration, J. Biol. Chem. 281 (2006) 32122–32130.
[59] G. Baldwin, V. Novitskaya, R. Sadej, E. Pochec, A. Litynska, C. Hartmann, J. Williams, L. Ashman, J.A. Eble, F. Berditchevski, Tetraspanin CD151 regulates glycosylation of (alpha)3(beta)1 integrin, J. Biol. Chem. 283 (2008) 35445–35454.