1. **Name of the Student**: Amit Ranjan

2. **Name of the Constituent Institution**: Tata Memorial Centre

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4. **Title of the Thesis**: Role of beta1,6 branched N-linked oligosaccharides in regulating key cellular processes involved in cancer cell invasion

5. **Board of Studies**: Life science
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–acetylg glucosaminyl 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 CO₂ 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 CaCl₂ 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 CO₂ 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 (pH 7.5) 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% CO₂.

**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% CO₂.

**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% CO₂.

**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 α2,6 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 (β1 integrin 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 (β1 integrin 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 correlate 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 α3β1 integrin. 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:
Publications:

a. Accepted:

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   1. Ranjan A., Kalraiya R. D. “Glycosylation of the laminin receptor (α3β1) regulates its association with tetraspanin CD151: impact on adhesion, spreading, motility, degradation and invasion of basement membrane by tumor cells” has been communicated to the journal, Experimental Cell Research, and is under review.

Signature of Student: Amrit Ranjan
Date: 10/7/13
# Doctoral Committee:

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<td>Dr. Anita Borges</td>
<td>Member</td>
<td></td>
<td>6.7.13</td>
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**Forwarded through:**

Dr. S.V. Chiplunkar  
Chairperson  
Academics and Training  
Programme  
ACTREC

Dr. S.V. Chiplunkar  
Dy. Director  
ACTREC

Dr. R. Sarin  
Director  
ACTREC

Dr. K. Sharma  
Director Academics  
TMC

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**Advanced Centre for Treatment, Research and Education in Cancer (ACTREC)**  
Bhawan, Parel, Mumbai - 400 124, India