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

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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 by 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.

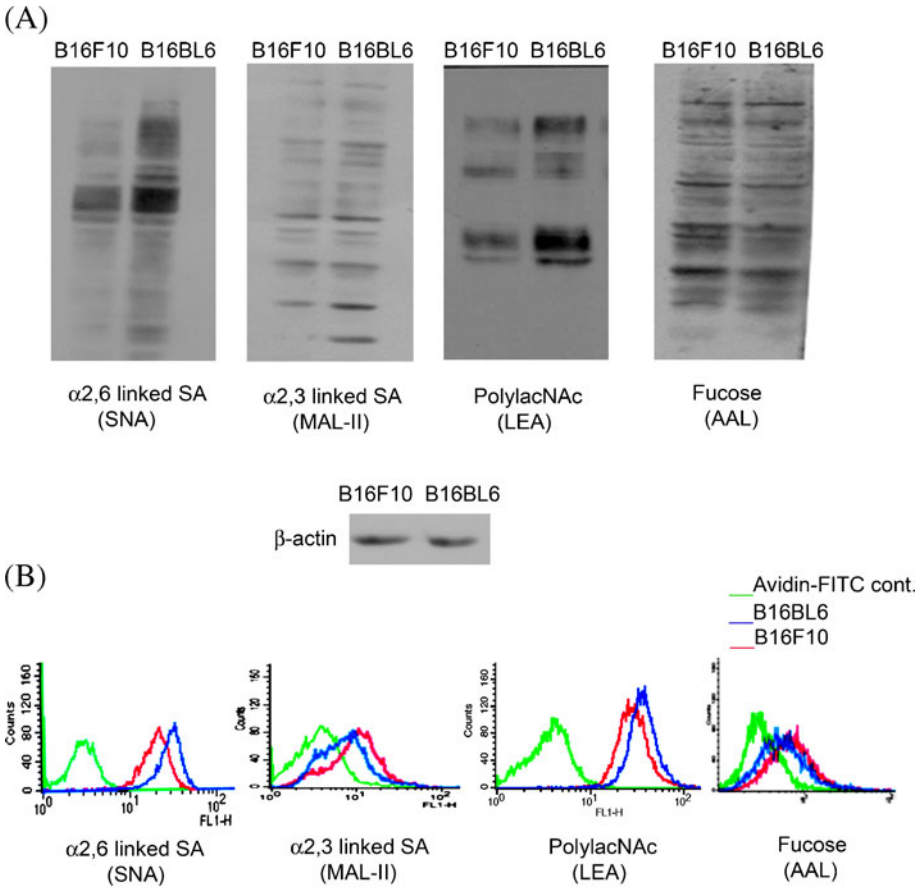
2.10 *Statistical analysis*

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).

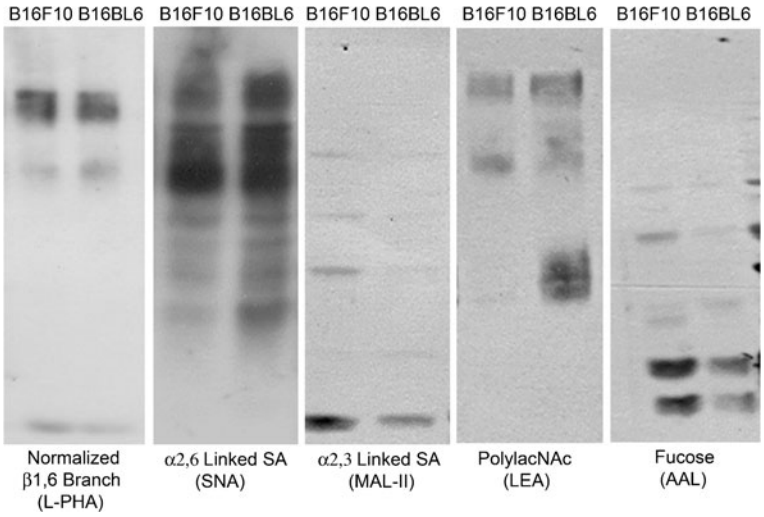


Figure 2. Increased expression of α2,6-linked SAs is associated with increased expression of β1,6-branched N-linked oligosaccharides. L-PHA binding proteins purified from whole cell lysates of B16F10 and B16BL6 cells were normalized with respect to expression of β1,6-branched *N*-oligosaccharides by 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

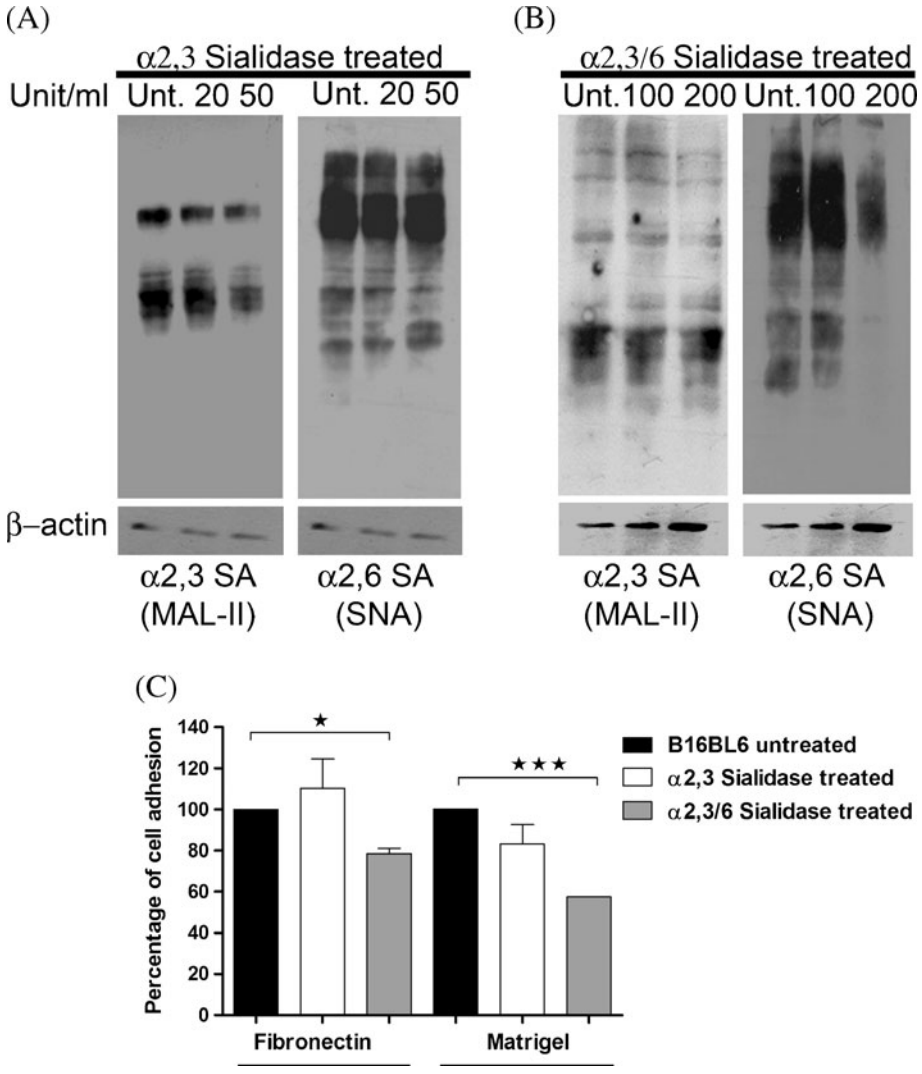


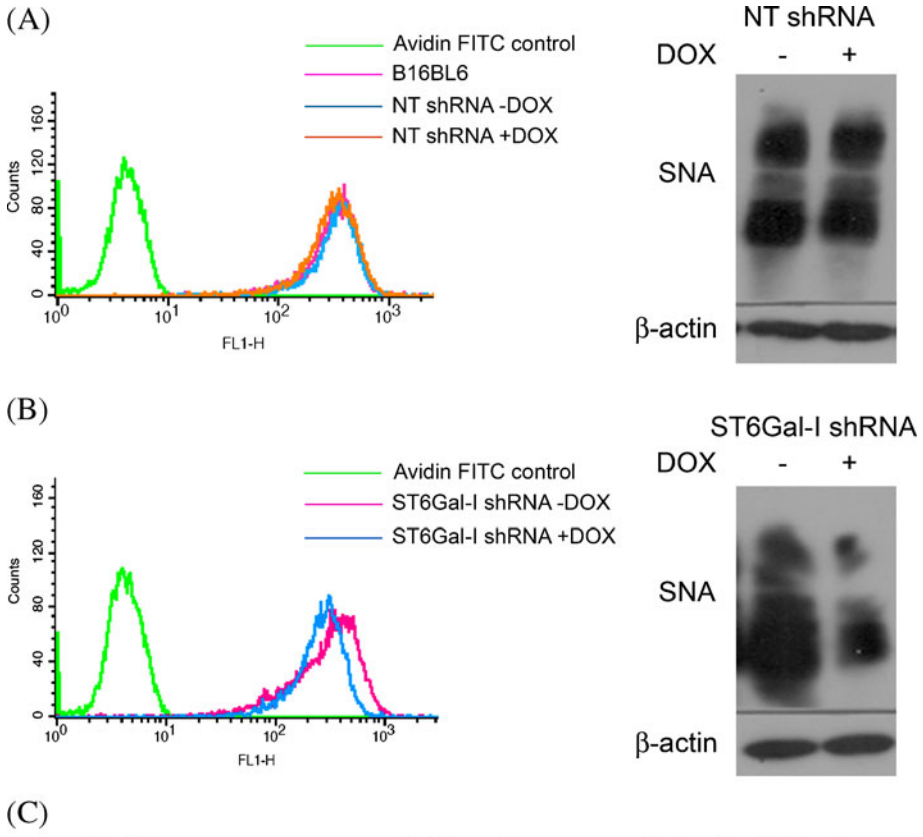
Figure 3. Enzymatic desialylation of α2,6-linked sialic acids decreases adhesion. Cell lysates from untreated (Unt) B16BL6 cells and those treated with (**A**) α2,3-linkage-specific sialidase (20 and 50 unit/ml) and (**B**) α2,3/6-linkage-specific sialidase (100 and 200 unit/ml) were Western blotted 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 the section on materials and methods. (**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-

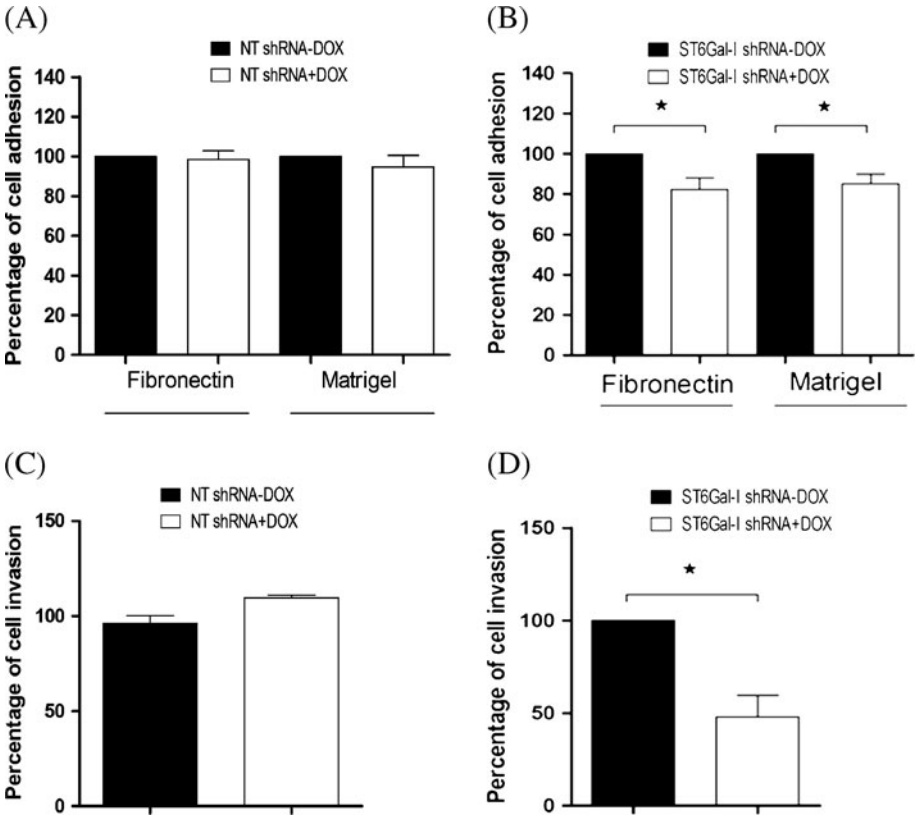


Figure 5. Effect of inhibition of ST6Gal-I expression on adhesion and invasion of B16BL6 cells. (**A**) Adhesion of induced (NT shRNA +DOX) and un-induced (NT shRNA −DOX) B16BL6 cells stably transduced with non-targeting shRNA or (**B**) ST6Gal-I shRNA, after induction (ST6Gal-I shRNA +DOX) or un-induced (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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