

Poly *N*-acetylactosamine substitutions on *N*- and not *O*-oligosaccharides or Thomsen–Friedenreich antigen facilitate lung specific metastasis of melanoma cells via galectin-3

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Abstract Galectin-3 on vascular endothelium has been shown to facilitate lung specific metastasis. Metastatic variants of B16 melanoma were chosen to identify specific ligands that mediate lung colonization via galectin-3. Flow cytometry showed that, galectin-3 binding to cells correlates with surface expression of poly *N*-acetylactosamine (polylacNAc) but not with other reported ligands, *e.g.* Thomsen-Friedenreich (T/Tn) antigen. Immobilized galectin-3 promoted adhesion of melanoma cells in a metastasis dependent manner. Moreover, adhesion and galectin-3 binding to cells were specifically inhibited with lactose. These properties together with lung metastasis were inhibited with *N*-glycosylation inhibitor Swainsonine (SW), whereas, *O*-glycosylation inhibitor Benzyl- α -*N*-acetylgalactosamine (BG) had no effect. BG treatment significantly increased expression of T/Tn antigen on low metastatic cells; however, had no effect on their metastatic potential. The studies very comprehensively demonstrate the importance of polylacNAc substitutions on *N*-oligosaccharides in galectin-3 mediated lung metastasis.

Keywords Galectin-3 · β 1,6 branched *N*-oligosaccharides · Poly *N*-acetyl lactosamine · Adhesion · Lung specific metastasis

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Introduction

Progression of tumors from benign to metastatic phenotype accounts for more than 90% of mortality associated with cancer patients. The complexities of the multi-step process coupled with numerous tumor-host interactions are major challenges in developing effective therapies for metastasis [1, 2]. The final outcome of metastasis depends on detachment of cells from the primary site, movement away from the primary, intravasation, survival in circulation, and finally organ homing [1]. Tumors often metastasize to the first organ encountered during circulation, which is facilitated by formation of multi-cellular emboli by many tumor cells. However, apart from this anatomical/mechanical phenomenon, many tumors metastasize to very specific organ sites, bypassing several organs in their blood flow path [2, 3]. Organ specific metastasis has intrigued scientists for over a century. Seed and Soil hypothesis proposed by Dr. Paget in 1889 based on autopsies of breast cancer patients, still appears to hold true [4]. Ability of cells to form adhesive interactions with the molecules on the target organ, and adapt to the new organ growth environment appear to be the key factors responsible for organ specific metastasis [2, 3]. However, over the years, chemokines and their receptors have also emerged as important determinants of organ specificity [5].

Molecules on the cell surface are expected to play a crucial role not just in mediating all the steps of organ specific colonization, but also in regulating the overall process of metastasis [6]. Tumors indeed show several cell surface modifications, each of which appears to aid them at a specific stage of metastasis, and alterations in their expression often impacts their metastatic properties [6, 7].

One such consistently observed modification on metastatic cells is increased expression of β 1-6 branched *N*-oligosaccharides on cell surface glycoproteins, which is catalysed by the golgi enzyme *N*-acetylglucosaminyl transferase-V (GnT-V) [8]. Expression of these oligosaccharides on several human tumors such as those of breast, colon, esophagus, endometrium and gliomas have been shown to be associated with the metastatic phenotype [9–11]. Their expression on several human and murine cancer cell lines has been shown to correlate with the metastatic potential [9]. Induction or inhibition of these oligosaccharides in such cell lines, using chemical or genetic methods has been shown to result in a corresponding acquisition or inhibition of the metastatic phenotype, respectively [12–15]. Their expression on highly invasive cancers [16, 17] and on normal cells involved in invasive functions [18] suggests their involvement in the process of invasion; however, the precise mechanism behind this remains unclear.

Another interesting fact is that a majority of cell lines expressing these oligosaccharides metastasize to either lungs or to the liver [12, 13, 19]. Adhesive interactions between the tumor cell surface molecules and organ vascular endothelium is emerging as one of the key determinants of organ specific metastasis [3, 20]. The role of β 1-6 branched *N*-linked oligosaccharides in organ specific metastasis is speculated for two main reasons. Firstly, larger substitutions on these oligosaccharides may alter structural and functional characteristics of proteins that carry them [21]. The small subset of proteins that carry these oligosaccharides include, integrins, cadherins, CD-44, EGF-R and Lysosome associated membrane proteins (LAMPs) [9, 21–24]. It is possible that some of these modifications aid organ specific metastasis. Secondly, β 1-6 branch serves as most preferred site for addition of several other terminal substitutions such as polyacNac, Lewis antigens and sialic acids [9, 25, 26]. These oligosaccharides may facilitate metastasis by providing novel ligands for many endogenous lectins like galectins, selectins and sialic acid binding lectins [9, 26]. It is thus possible that the terminal substitutions may ultimately dictate the choice of the target organ.

Low and high metastatic variants of B16 melanoma have been chosen as the model for these studies for three reasons. First is the availability of cell lines with vastly different metastatic potential. Second, they contain melanin, making it easier to quantitate the number of colonies. Third, the high metastatic (B16F10) cells, selected from low metastatic cells (B16F1) specifically colonize lungs irrespective of the route of administration (intravenous, where lungs would be the first site; or intra-aortic, where lungs would be the last organ encountered). They metastasize to the lungs even when implanted at an ectopic site such as the

subcutaneous, and in parabiotic pair of animals, both the mice showed lung metastasis, although only one of them received the injection [27, 28].

B16 melanoma showed metastasis dependent expression of β 1-6 branched *N*-oligosaccharides [29]. Translocation of the lysosomal protein LAMP1, carrying polyacNac substituted β 1-6 branched *N*-oligosaccharides to the surface, has been shown to correlate with the metastatic potential of these cell lines [29]. PolyacNac, the high affinity ligands for galectin-3 [30] are repeating units of the disaccharides *N*-acetylglucosamine and galactose [31]. Since each LAMP1 molecule carries about 17 to 19 *N*-glycan chains, it provides high density of high affinity ligands (polyacNac) for galectin-3 on the surface of B16F10 cells. We also showed that galectin-3 is expressed in high amounts in the mice lungs, and is expressed constitutively on the surface of lung vascular endothelium, facilitating lung homing [29]. Galectin-3 is a multi-functional beta-galactoside binding protein belonging to galectin family. Basically, a nucleo-cytoplasmic protein, but gets secreted out in a non-classical manner and becomes part of the cell surface and Extra Cellular Matrix (ECM) [32]. Galectin-3 may play a major role at different steps of metastasis. Although, polyacNac have the highest affinity for galectin-3, there are several reports that implicate T/Tn antigen in galectin-3 mediated organ colonization [33, 34]. The present paper attempts to characterize the precise ligands on melanoma cells that facilitate galectin-3 mediated processes promoting lung specific metastasis.

Materials and methods

Cell lines and reagents

B16F1 and B16F10 murine melanoma cell lines were obtained from National Centre for Cell Science, Pune, India. Cell culture reagents, TRIzol and Superscript TM amplification system for RT-PCR were obtained from Invitrogen, USA. Anti-mouse galectin-3 antibody raised in rat was purchased from R&D Biosystems, USA. Anti-Rat HRPO was obtained from Santa Cruz Biotechnology, USA. PVDF membrane and the ECL kit were purchased from GE Healthcare, Amersham, UK. Culture ware and Fibronectin were obtained from BD Falcon and BD Biosciences, USA, respectively. *E. coli* BL 21 with pET3C plasmid containing a full-length human galectin-3 was a kind gift from Dr. Hakon Leffler, Lund's University, Sweden. IPTG was obtained from USB Corporation, USA. Biotinylated lectins like Leuco-Phyto Haem Agglutinin (L-PHA), *Lycopersicon esculentum* lectin (LEA), and Jacalin, avidin–peroxidase, and streptavidin–FITC were either from Sigma Chemical Company, USA or Vector Labs, USA. Purified rh-galectin-

3 was biotinylated as described in [35]. Reagents for bacterial culture were purchased from Hi Media India, while all other chemicals were purchased locally and were of analytical grade.

Cell culture and experimental metastasis assay

Melanoma cells were routinely cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 0.03% glutamine, 10 units/ml of Penicillin G-sodium, 10 µg/ml of Streptomycin sulphate and 25 µg/ml of Amphotericin B and 10% fetal bovine serum (FBS). For treatment of cells with glycosylation inhibitors, melanoma cells were allowed to grow to 50% confluency before adding SW (2 µg/ml) and BG (2 mM). The cells were harvested after 24 h with 0.25% trypsin, washed once in DMEM containing 10% FBS, and twice in DMEM. For metastasis assays, it was ensured that the cells existed as single cell suspension and had greater than 95% viability. Cells (0.15×10^6 in number, contained in 100 µl) were injected intravenously (i.v.) in inbred strains of female C57BL6 mice via the lateral tail vein. The animals were sacrificed after 21 days and melanoma colonies on the surface of the lungs were counted using a dissecting microscope.

Purification of recombinant human galectin-3

Expression of recombinant human (rh) galectin-3 was induced in *E. coli* BL-21 containing pET3C plasmid using 50 mg/l of Isopropyl Thio D-Galactopyranoside (IPTG) as described in [36]. The protein was purified from the bacterial cell lysates using a Lactose-Sepharose affinity column and stored after vacuum drying.

Raising antibodies against L-PHA and rh galectin-3

L₄-PHA and rh galectin-3 were used as immunogens to immunize New Zealand rabbits (8–12 weeks old). ~50 µg of each immunogen in complete Freund's adjuvant was injected intra-dermally at multiple sites. Booster injections in incomplete Freund's adjuvant were given at subcutaneous sites, at 2 weeks interval. Immune serum was collected 8–10 days after the second booster, and IgG was purified by ammonium sulfate precipitation and DEAE ion exchange chromatography as described in [37].

Flow cytometric analysis

For flow cytometry, melanoma cells were first fixed by overnight incubation with 1.5% glutaraldehyde in PBS (pH 7.4) followed by lectin staining as described in [29]. Briefly, 0.5×10^6 melanoma cells were incubated with 20 µg of biotinylated (LEA) or 10 µg of biotinylated

Jacalin in 40 µl of FACS buffer (PBS pH 7.4, containing 1% FBS) followed by Extra Avidin FITC diluted 1:50 in FACS buffer. For determination of L-PHA and galectin-3 binding, melanoma cells were incubated with 20 µg each of L-PHA, or rh-galectin-3. This was followed by incubation with 20 µg Anti-LPHA antibody or 1 µg anti-rh galectin-3 antibody respectively. Finally, the cells were treated with 10 µg secondary Anti-Rabbit FITC in 40 µl FACS buffer. Cells treated with Extra-Avidin-FITC or anti-rabbit FITC alone served as control. Specificity of galectin-3 binding was confirmed by incubating the cells in presence or absence of specific competitive disaccharide lactose (50 mM). Fluorescent cells were acquired at 488 nm and analyzed on FACS Calibur using Cell Quest software.

Preparation of total cell lysates and Western blotting

Preparation of total cell lysates was done exactly as described in [29] using lysis buffer containing 10 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). Protein in the lysates was estimated as per [38] resolved on 10% SDS polyacrylamide gels according to [39], and electro-blotted on PVDF membrane as per [40]. The status of β1-6 branched *N*-oligosaccharides, polyacNAc and T-antigen on melanoma cells were checked by probing with biotinylated L-PHA, LEA and Jacalin, respectively followed by avidin peroxidase. The blots were developed using ECL kit.

Adhesion assays

Melanoma cells were radio-labeled with 30 µCi/ml of tritiated thymidine at 50% confluency and the cells allowed to grow overnight. Cells were harvested, washed, assessed for efficiency of labeling, and CPM/cell was calculated. Adhesion assays were performed in 96 well plates. Briefly, the wells were coated with galectin-3 (50 µg/ml) and Fibronectin (10 µg/ml) in 0.1 ml of serum free DMEM overnight at 4°C. Forty thousand tritium labeled cells, suspended in 100 µl of serum free DMEM were added to each coated well with or without lactose and sucrose (50 mM each) in triplicate and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 1 h. Wells were gently washed three times with 100 µl of PBS to remove the unbound cells. The bound cells were detached by 0.25% trypsin and harvested on glass fibre filters using Combi Cell Harvesting machine from Molecular Devices. Radioactive counts of the bound and initially seeded cells were taken using beta scintillation counting. The percentage adhesion was calculated as the percentage of bound cells

with respect to B16F10 cells bound to galectin-3 which was taken as 100%.

Determination of GnT-V transcript using semi-quantitative RT-PCR

Total RNA was extracted from cells using TRIzol and 1st strand cDNA synthesis was initiated using Superscript TM amplification system using oligo (dT) primers and M-MuLV reverse transcriptase as per manufacturer's protocol. The following primers were used to check the transcript levels of GnT-V in melanoma variants in which GAPDH served as loading control-

GnT-V Forward primer-5'-TCCTCGAGGTTGAAAA TTGGTGTCC-3'

GnT-V Reverse primer-5'-ACCTCGAGCATGCACTG GTAATGAAC-3'

GAPDH forward primer-5'-TGAAGGTCGGTGTGAA CGGATTTG-3'

GAPDH reverse primer-5'-CATGTAGGCCATGAGG TCCACCAC-3'

The product size of GnT-V is 561 bps, while that of GAPDH is 983 bps. After completion of the RT-PCR, 10 μ l products were applied to 1.2% agarose gel containing ethidium bromide for electrophoresis.

Statistical analysis

All data are represented as mean \pm SE unless stated. When two groups were compared, the Student's *t* test was employed. Comparison within a group was done by performing One way ANOVA by Dunnett Postdoc test using SPSS software. (*p* value < 0.05 was considered significant).

Results

Binding of galectin-3 to melanoma cells is carbohydrate dependent, which in turn dictates their adhesion to immobilized galectin-3 and lung metastasis

Earlier studies have shown that Galectin-3 expressed on lung vascular endothelium facilitates lung specific metastasis of melanoma cells. Flow cytometry performed to assess binding of galectin-3 to the surface of melanoma cells showed that it is dependent on their metastatic potential (Fig. 1a), which was also confirmed by Western blotting (Fig. 1c). To check the specificity of binding, melanoma cells were incubated with galectin-3 in presence of the competitive disaccharide lactose. Flow cytometry showed that lactose reversed the binding of galectin-3 very significantly (Fig. 1b). To further confirm if the differential

binding of galectin-3 to melanoma cells also resulted in their differential adhesion to immobilized galectin-3, adhesion assays on galectin-3 coated dishes were performed. The results clearly demonstrated that melanoma cells show significantly increased adhesion to galectin-3 as compared to uncoated plastic. Furthermore, adhesion to galectin-3 correlated with their metastatic potential. Lactose at the 50 mM concentration significantly reversed adhesion, whereas the nonspecific disaccharide sucrose had no effect (Fig. 1d). The results suggest that binding of galectin-3 to the cells is mediated specifically by the carbohydrates on the cell surface.

Binding of galectin-3 to melanoma cells is dependent upon the expression of polyacNAc on β 1-6 branched *N*-oligosaccharides and not T/Tn-antigen

PolyacNAc on cell surface are the major high affinity ligands for galectin-3, although, T/Tn antigens have also been shown to participate in galectin-3 mediated metastasis. Therefore, it was important to investigate the contribution of each of these oligosaccharides in lung-specific metastasis. Flow cytometry showed that high metastatic cells displayed significantly increased expression of polyacNAc, which correlated with increased binding of galectin-3 to their cell surface (Figs. 1a, and 2a). This, in turn, correlated with β 1-6 branched *N*-oligosaccharides expressed on melanoma cells in a metastasis dependent manner (Fig. 2b). T/Tn antigen expression, however, did not show any correlation with galectin-3 binding or metastasis (Fig. 2c). The results thus suggest that increased polyacNAc substitutions on β 1-6 branched *N*-oligosaccharides and not T/Tn antigen, enhance binding of galectin-3 to high metastatic melanoma cells.

Inhibition of *N*- but not *O*-oligosaccharides significantly reduces, surface expression of polyacNAc, adhesion to galectin-3 and metastasis of melanoma cells to the lungs

Although, β 1-6 branch of *N*-oligosaccharides is one of the most preferred sites for polyacNAc substitutions, even *O*-oligosaccharides often carry them. Therefore, it was important to check the possible contribution of polyacNAc on *O*-linked oligosaccharides in galectin-3 mediated lung metastasis. The glycosylation inhibitors BG and SW, which inhibit biosynthesis of *O*-linked oligosaccharides and complex *N*-oligosaccharides, respectively, were employed. Flow cytometry showed that SW treatment leads to a significant loss in the expression of polyacNAc, whereas inhibition of *O*-oligosaccharides had only a marginal effect on the same (Fig. 3a). This was also confirmed by western blotting. Very similar LEA staining pattern was obtained for B16F10 and BG treated B16F10 cells; whereas SW treated

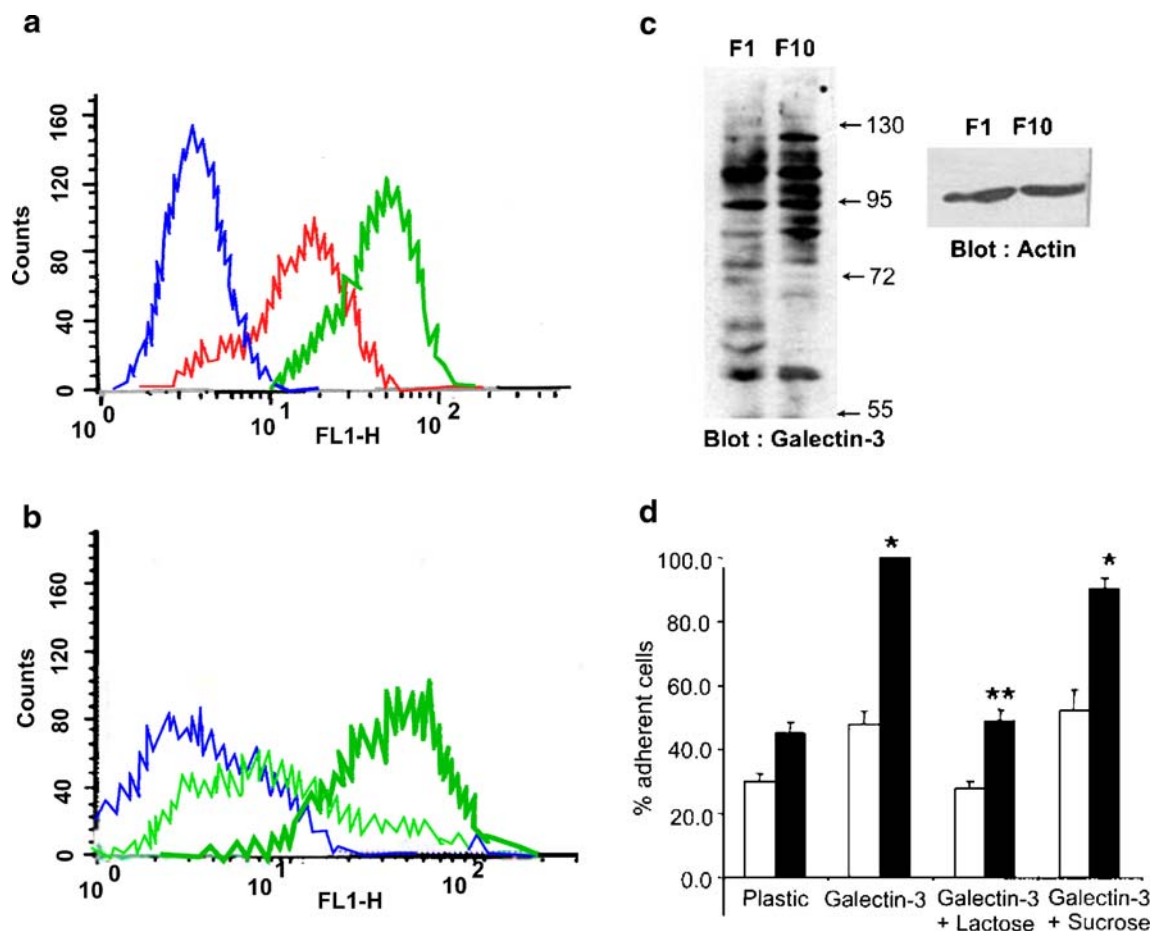


Fig. 1 Flow cytometric analysis of galectin-3 binding to **a** B16F1 (red line) and B16F10 cells (thick green line) and **b** to B16F10 cells in presence (thin green line) and in absence (thick green line) of 150 mM lactose. B16F10 cells treated only with anti rabbit FITC (blue line) served as control. **c** Western blotted lysates of melanoma variants probed either with biotinylated galectin-3 or anti-beta actin antibody. **d** Analysis of adhesion of B16F1 (empty bars) and B16F10 cells (filled bars) to galectin-3 coated wells in presence or absence of lactose and

sucrose. Percent adhesion for each cell type was calculated by comparing their adhesion with that of B16F10 cells, which was taken as 100%. Values are mean \pm SE of three independent experiments carried out in triplicate. Student's *t* test was performed to compare significance between two cell lines (denoted by *asterisk*, *p* value < 0.05), while one way ANOVA was used to compare within the same cell line (denoted by *double asterisks*, *p* value < 0.02)

cells showed a different set of proteins that carry poly-lacNAc (Fig. 3b). Although lysates from all the three cell types showed very similar overall intensity of staining with the lectin LEA, staining with β actin indicated much higher loading in the SW lane, suggesting lower LEA binding on SW treatment. The extent of reduction of LEA binding on SW treated cells appears much more by flow cytometry (Fig. 3a). Loss of poly-lacNAc on *N*-oligosaccharides possibly results in the shorter poly-lacNAc chains on *O*-oligosaccharides, which may be inaccessible for lectin binding on intact cells as compared to proteins on the blots.

Reduction in poly-lacNAc on SW treated cells appeared to reduce their adhesion to galectin-3, whereas, BG treatment had no effect. The effect of inhibitors on adhesion to galectin-3 was very similar to that seen on fibronectin

(Fig. 3c). Their effect was also reflected on the metastatic properties of the cells. Experimental metastasis assays with 0.3 million cells, almost completely filled the lungs (Fig. 3d) and the number of colonies were uncountable. Mice were injected with 0.15 million cells to get countable number of colonies (Fig. 3e). The results showed that SW treatment significantly reduced the metastatic potential of B16F10 cells, whereas, BG treatment appeared to have no effect on the number of colonies (Fig. 3d and e). However, the mice injected with BG treated B16F10 cells showed increase in the size of the lungs and the melanoma colonies were larger and fused (Fig. 3d and supplementary data). The data thus clearly indicates that poly-lacNAc substitutions only on *N*- and not *O*-oligosaccharides facilitate adhesion of melanoma cells to galectin-3 and their metastasis to the lungs.

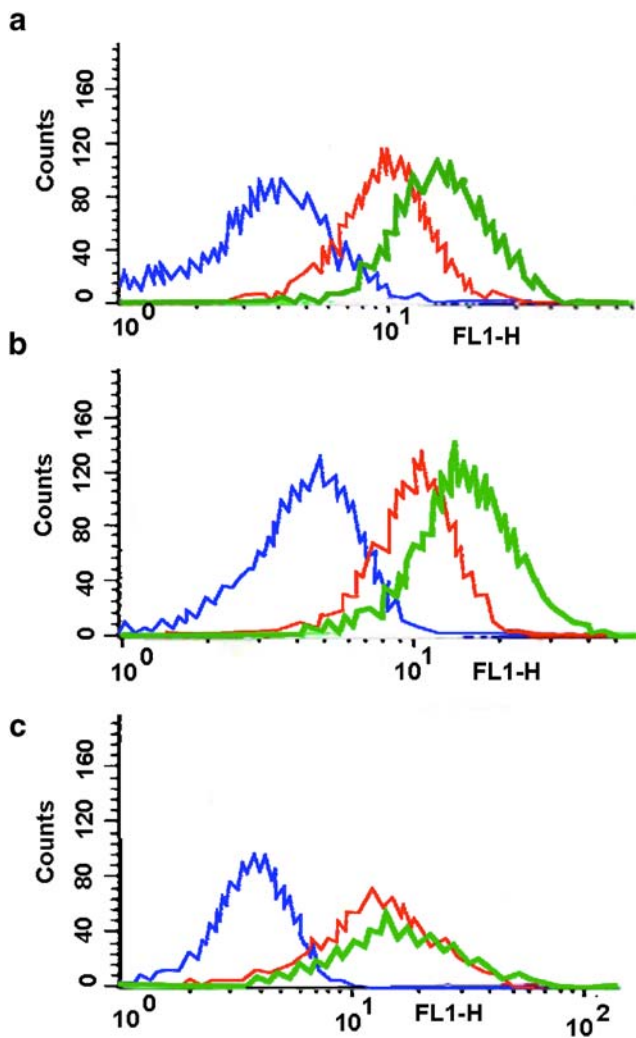


Fig. 2 Comparison of surface expression of **a** polyacNAc using biotinylated LEA, **b** β 1-6 branched *N*-oligosaccharides using L-PHA and anti-L-PHA and **c** T-antigen using biotinylated Jacalin, in B16F1 (red line) and B16F10 cells (green line) using flow cytometry. B16F10 cells treated only with either Avidin-FITC (**a** and **c**) or Anti-rabbit FITC **b** (blue line) served as control

BG treatment results in increased avidity of galectin-3 binding, associated with increased expression of T/Tn antigen and β 1-6 branched *N*-oligosaccharides on B16F10 cells

Surface carbohydrates on melanoma cells were characterized to understand why BG—the inhibitor of polyacNAc synthesis on *O*-oligosaccharides, had no effect on galectin-3 mediated properties. Flow cytometry showed that BG treatment had no effect on the extent of galectin-3 binding to B16F10 cells (Fig. 4a and b). BG treated cells, however, bound galectin-3 with significantly higher avidity. While binding of galectin-3 to B16F10 cells could be substantially

reversed using 150 mM lactose (Fig. 4a), its binding to BG treated cells could not be reversed to the comparable levels even at 300 mM concentration (Fig. 4b). This increased avidity could be due to enhanced exposure of T/Tn antigen due to BG treatment. Alternatively, it could be due to increase in the number and repeating units of polyacNAc on β 1-6 branched *N*-oligosaccharides. Flow cytometric analysis and Western blotting using Jacalin confirmed that B16F10 cells showed significantly increased T/Tn antigen expression on BG treatment (Fig. 4c and e). Since, BG treatment did not reduce the expression of polyacNAc on melanoma cells (Fig. 3 a and b), flow cytometry and western blotting was performed to see if this is compensated by increase in β 1-6 branching on *N*-oligosaccharides. The results showed that BG treatment indeed leads to increased expression of these oligosaccharides (Fig. 4d and f). These observations suggest that increased avidity of galectin-3 binding could be due to both these types of oligosaccharides.

Increased expression of T/Tn antigen on BG treatment of B16F1 cells does not enhance their metastatic potential

While both polyacNAc and T/Tn antigens are potential ligands for galectin-3, T/Tn antigens have significantly lower affinity as compared to polyacNAc. BG treatment had no or slightly enhanced effect on the metastatic properties of B16F10 cells (Fig. 3d and e). Since, B16F10 cells are very highly metastatic, any enhancement in the expression of these carbohydrates may not be truly reflected in increased metastasis. Therefore, B16F1 cells, which have much lower basal metastatic potential were chosen to investigate the participation of T/Tn antigens in galectin-3 mediated properties. Although, BG treatment resulted in significantly enhanced expression of T/Tn antigen, as seen by Jacalin binding (Fig. 5a and c) both treated and untreated B16F1 cells showed comparable metastatic abilities by experimental metastasis assay (Fig. 5d). The expression of β 1-6 branched *N*-oligosaccharides was analyzed to explain the lack of any effect of BG on metastatic properties of B16F1 cells. Flow cytometry and western blotting using L-PHA confirmed that BG had no effect on the expression of these oligosaccharides in B16F1 cells (Fig. 5b and c). This is possibly because the enzyme GnT-V responsible for the addition of β 1-6 branch, is down regulated in these cells. This was clearly reflected in the significantly lower level of the transcript for GnT-V in B16F1 cells as compared to B16F10 cells obtained by RT-PCR analysis (Fig. 5e). These results further reiterate that over expression of polyacNAc as a result of increased β 1-6 branching on *N*-oligosaccharides, serve as the major ligands that facilitate galectin-3 mediated adhesion and metastasis.

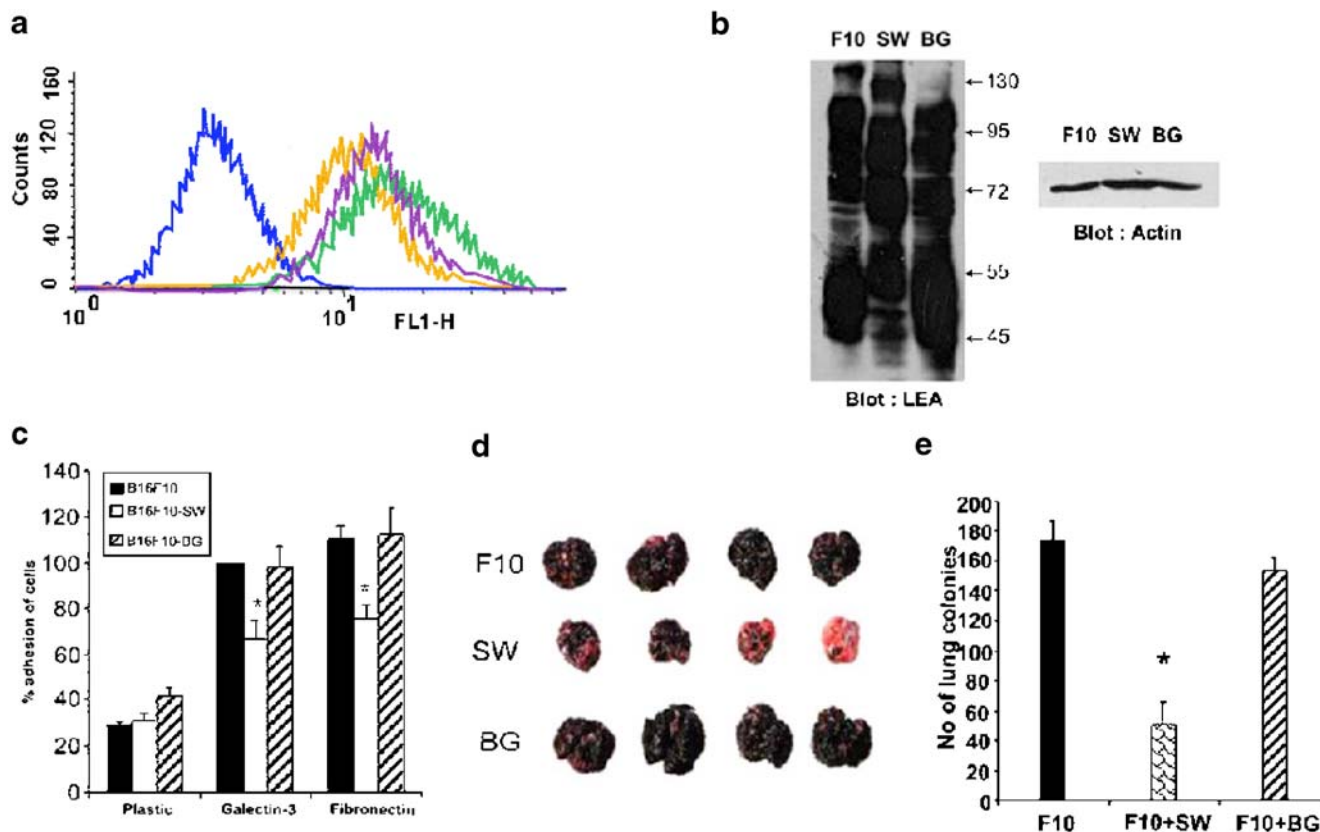


Fig. 3 **a** Comparison of expression of polyacNAc in untreated, SW and BG treated B16F10 cells by **a** flow cytometry, (*green line*) B16F10 cells, (*red line*) SW-, and (*purple line*) BG-treated cells. B16F10 cells treated only with Avidin-FITC served as control (*blue line*), and **b** Western blotting using biotinylated LEA as probe. **c** Comparison of adhesion of the same set of cells on galectin-3 and fibronectin coated wells. Percent adhesion for each cell type was calculated by comparing their adhesion with that of B16F10 cells, which was taken as 100%. Values are mean \pm SE of three independent

experiments carried out in triplicate. Student's *t* test was performed to compare significance between two cell lines (denoted by *asterisk*, *p* value < 0.05). **d** Comparison of melanoma colonies in lungs of mouse injected with (0.3 million) B16F10 cells and those treated with glycosylation inhibitors SW and BG under experimental metastasis assay conditions. **e** lung colonies of the same cells after injection of 0.15 million cells (to get distinct colonies) represented graphically. Significance obtained by performing one way ANOVA is denoted by *asterisk*, *p* value < 0.002

Discussion

Galectin-3 expressed on tumor cells, appears to promote malignant progression by facilitating homophilic cell-cell interactions [41]. However, overexpression of carbohydrate ligands on tumor cells also promotes metastasis by facilitating heterophilic interactions with host cells via galectin-3. This often results in adhesion of cancer cells to different cell and tissue spaces, for example, surface of the vascular endothelium expressing galectin-3 [29, 42]. Adhesion to such immobilized galectin-3 appears to be determined by the extent of its specific binding to carbohydrate ligands, present on cancer cell surface (Fig. 1).

Galectin-3 is member of beta-galactoside binding lectins, which exhibits differential binding to various galactoside derivatives [30]. PolyacNAc, both on *N*- and *O*-linked oligosaccharides, are the most preferred ligands for galectin-

3 [43]. Availability of metastatic variants of B16 melanoma and their comparison helps in identification of the potential ligands for galectin-3 on these cells. Increase in metastasis-dependent expression of polyacNAc, together with β 1-6 branched *N*-oligosaccharides (Fig. 2a and b), make polyacNAc on *N*-glycans, the most likely ligands for galectin-3. This is especially because, β 1,6 branch of *N*-oligosaccharides is one of the favored sites for polyacNAc substitutions [25].

Participation of polyacNAc on *N*-oligosaccharides in galectin-3 mediated effects is further corroborated by a significant loss in adhesion to galectin-3, metastasis to lungs and polyacNAc expression, on inhibition of *N*-oligosaccharides (Fig. 3). Although, the effect of inhibition on adhesion to galectin-3 was very similar to that seen on fibronectin, the mechanism of modulation is quite different. While adhesion to galectin-3 is dependent entirely on its

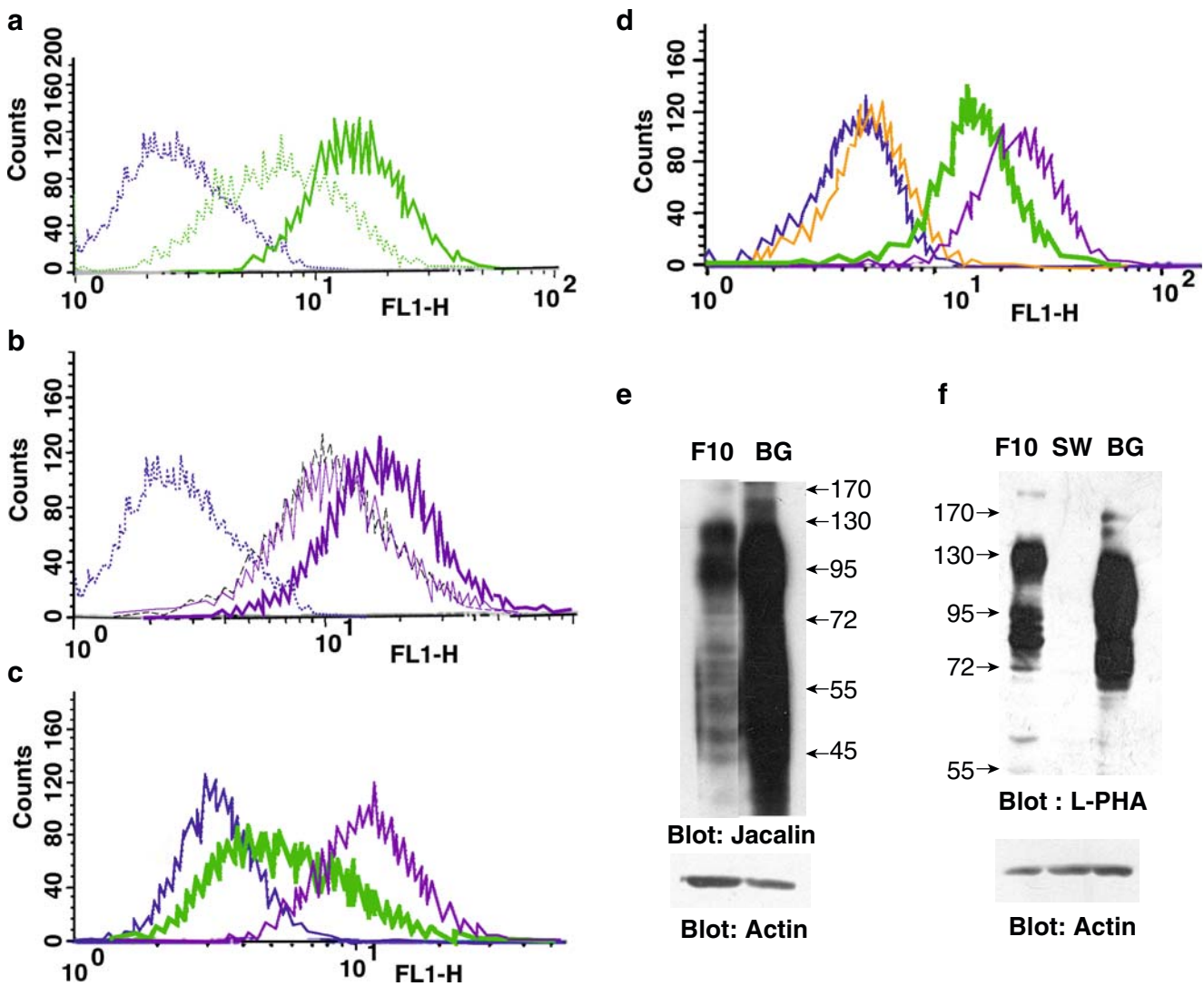


Fig. 4 Flow cytometric analysis of galectin-3 binding **a** to B16F10 cells in absence (*thick green line*) and presence (*thin green line*) of 150 mM lactose and **b** to BG-treated B16F10 cells in absence (*thick purple line*) and presence of 150 mM (*thin purple line*) and 300 mM lactose (*dotted line*). Fig. **a** and **b** represent histogram overlaps of the same experiment, split for clarity. Flow cytometric comparison of surface expression of (**c**) T-antigen on untreated (*thick green line*) and BG-treated B16F10 cells using (*thick purple line*) biotinylated jacalin, and **d** β 1-6 branched *N*-oligosaccharides on B16F10 cells (*thick green line*) and those treated

with SW (*red line*) and BG (*thick purple line*), using L-PHA and anti-LPHA antibody as probes. B16F10 cells treated with either anti-rabbit FITC (**a**, **b** and **d**), or Avidin-FITC only (**c**) (*blue line*) served as controls. Western blotting analysis of cell lysates of **e** untreated and BG treated B16F10 cells for expression of T-antigen using biotinylated Jacalin and **f** β 1,6 branched *N*-oligosaccharides on untreated, SW and BG treated B16F10 cells using biotinylated L-PHA as probe. Beta actin served as loading control

interaction with the carbohydrate ligands, adhesion to ECM components like fibronectin is modulated by the presence of carbohydrates on their receptors. Carbohydrates on such proteins has indeed been shown to alter not just adhesion, but several other cellular properties like movement and chemotaxis, critical for invasion and metastasis [17, 21]. Loss of only *N*-oligosaccharides appears to modulate both the types of adhesion (Fig. 3c).

Results with *O*-oligosaccharide inhibitor BG were quite intriguing. Although, treatment of B16F10 cells with BG resulted in a minor reduction in polyacNAc, it had no effect on their adhesion to galectin-3 or the number of metastatic colonies formed in the lungs (Fig. 3). However, the size of the lungs and the metastatic colonies were much bigger in case of BG treated cells as compared to untreated cells (Fig. 3d). Although, it ruled out the role of

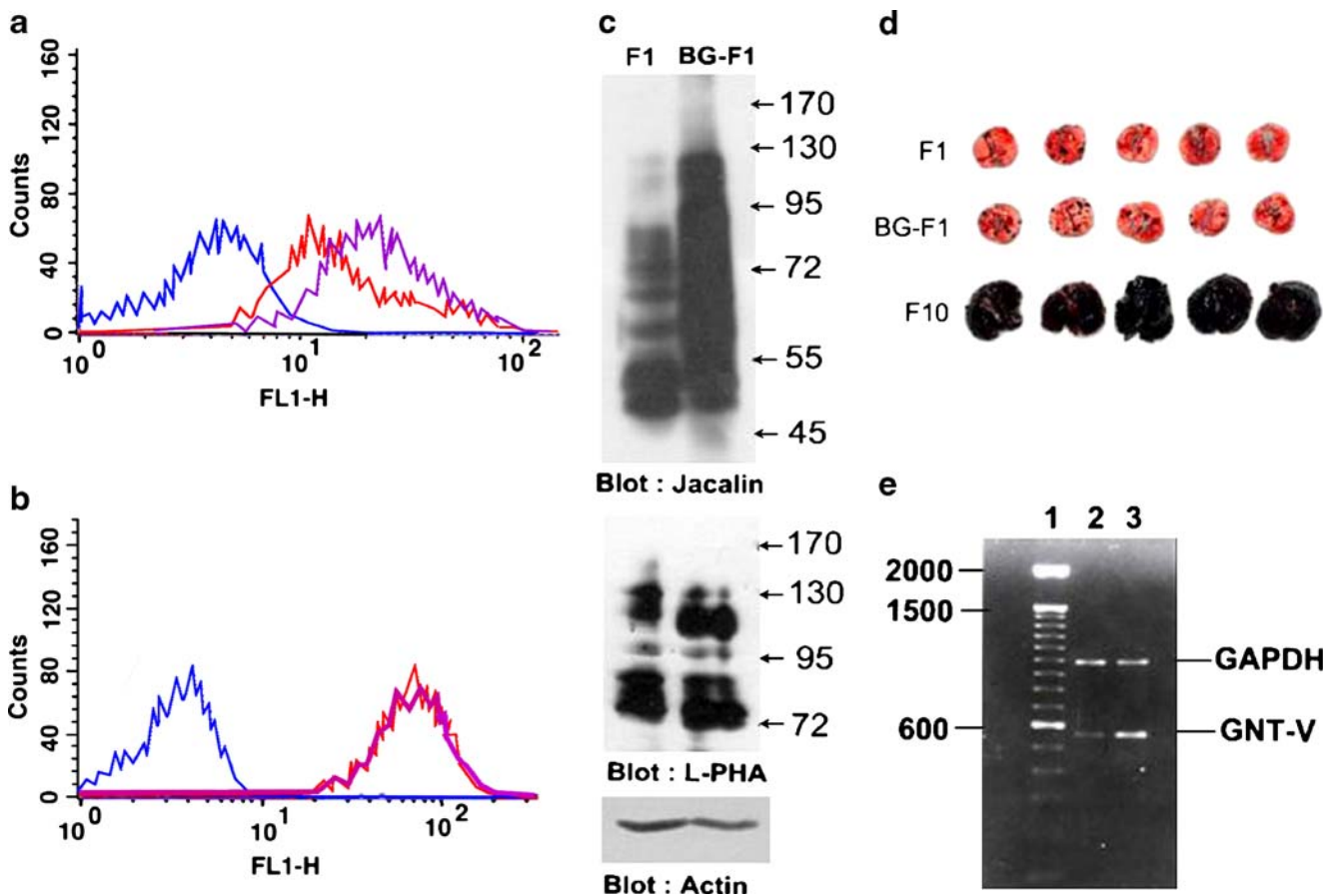


Fig. 5 Comparison of surface expression of **a** T-antigen on B16F1 (red line) and BG-treated-B16F1 cells (purple line) using biotinylated Jacalin as a probe, and **b** β 1-6 branched *N*-oligosaccharides on B16F1 cells (brown line) and BG-treated-B16F1 cells (purple line) using L-PHA and Anti-L-PHA as probes, by flow cytometry. B16F1 cells treated only with either **(a)** Avidin-FITC, or **(b)** Anti-rabbit FITC (blue line), served as controls. **c** Western blot analysis of cell lysates of

untreated and BG treated B16F1 cells for expression of T-antigen and β 1-6 branched *N*-oligosaccharides using biotinylated Jacalin and L-PHA respectively. Beta actin served as loading control. **d** Lungs of the mice injected with B16F1, BG-treated B16F1 and B16F10 cells, under experimental metastasis assay conditions. **e** Comparison of transcript levels of GnT-V enzyme in B16F1 (lane 2) and B16F10 cells (lane 3) using semi-quantitative RT-PCR

polylacNAc on *O*-oligosaccharides in galectin-3 mediated effects, it raised questions on the effect of BG on metastatic properties. Contrasting reports exist on the effect of BG in metastatic progression. While BG treatment inhibited metastasis in animal models of human colon cancer [44], it has been shown to augment metastasis of the highly invasive B16BL6 cells [45].

Surprisingly, BG treated B16F10 cells showed a significantly enhanced expression of β 1-6 branched *N*-oligosaccharides (Fig. 4d and f), which were possibly substituted with polylacNAc. The large sized metastatic colonies and almost identical adhesion of BG treated and untreated cells to galectin-3 (Fig. 3c and d), could thus be attributed to these oligosaccharides. Insignificant reduction in polylacNAc expression on BG treatment also points towards the same (Fig. 3a and b). Probably, loss of polylacNAc on *O*-oligosaccharides is compensated by these substitutions on additional β 1-6 branches seen on BG

treated cells. It is also possible that these branches carry polylacNAc with larger number of subunits. This would explain the increased avidity of galectin-3 binding to BG treated cells (Fig. 4a and b), and enhanced metastasis (Fig. 3d). Interaction of galectin-3 lattice with polylacNAc on proteins like EGF-R has been shown to result in their longer retention on the cell surface leading to sustained signaling [22]. This should explain the larger sized colonies seen with BG treated cells.

As expected, BG treatment also resulted in truncation of *O*-oligosaccharides, leading to overexpression or exposure of T/Tn antigen on B16F10 cells (Fig. 4c and e). There are several reports that implicate T/Tn antigens on tumor cells in mediating both homophilic, as well as heterophilic interactions with endothelial cells via galectin-3 [34, 42, 46]. Association of T/Tn antigens with malignant progression in gastric, colorectal and breast carcinomas has been observed, wherein it apparently participates in many of the

galectin-3 mediated processes [34]. Although, galectin-3 shows >200 fold higher affinity towards polyacNAc as compared to T/Tn antigens [30], when present in great numbers, the latter possibly provide stability to these interactions. However, the role of these antigens in metastasis has been ruled out for two reasons-i. the low and high metastatic variants do not differ in the expression of T/Tn antigens (Fig. 2c) and, ii. BG treatment although increased their expression on low metastatic cells (Fig. 5a and c), it did not enhance their metastatic potential (Fig. 5d). Significantly reduced levels of the transcript of GnT-V that catalyzes addition of β 16 branch in B16F1 cells (Fig. 5e) explains the absence of any effect of BG treatment on their metastasis. Although, these cells showed over expression of T/Tn antigen (Fig. 5a and c), the levels of β 1,6 branched *N*-oligosaccharides were comparable (Fig. 5b and c).

Available evidence for T/Tn antigens [34, 42], together with our observations on melanoma cells suggest that the level of expression, their accessibility and affinity towards galectin-3, possibly determine the effective ligand for galectin-3, on a cell. Presence of high affinity ligand-polyacNAc on carrier proteins like LAMP1, with a large extracellular domain and several repeating lacNAc units, make them easily accessible and, thus, the most likely galectin-3 ligand on melanoma cells. These results clearly rule out the participation of T/Tn antigens or polyacNAc on *O*-oligosaccharides in galectin-3 mediated lung metastasis of melanoma cells.

Galectin-3 is emerging as one of the key molecules, which regulate various cellular processes like adhesion, growth factor signaling and motility [22, 32]. It has been shown to be expressed in the rat, mice and human lungs [47, 48]. It not only regulates different steps in metastasis, but also impacts various other processes, like, recruitment and activation of neutrophils during pneumococcal infection [49], promotes pulmonary fibrosis [50], airway inflammation and bronchial hyper-responsiveness, in asthma [51]. Galectin-3 has been reported to be expressed on the vascular endothelium of the lungs from rat and mice [48], and could be a major player in lung specific metastasis [29]. Immunohistochemistry data showed that galectin-3 is also localized in all the major tissue compartments including epithelia of bronchioles, alveoli and vascular basement membrane (data not shown). Presence of galectin-3 beyond the barriers of vascular endothelium may be instrumental in mediating other critical events for successful organ colonization, and is being investigated.

Although, galectin-3 binds to several galactoside containing ligands, the level of expression, their accessibility and affinity for galectin-3 possibly ultimately determines its impact on metastatic properties. Identification of the potential ligand(s) on a cell type would not only aid in

predicting the disease progression, but also in designing specific strategies to target them for preventing metastasis. To our knowledge, this is the first comprehensive study, deciphering the precise ligands on tumor cells which mediate organ specific metastasis to lungs via galectin-3. It would, however, be important to confirm that the same mechanism operates in lung metastasis of human tumors with altered cell surface glycosylation.

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