β-1,4-Galactosyltransferase III suppresses β1 integrin-mediated invasive phenotypes and negatively correlates with metastasis in colorectal cancer

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Metastasis often occurs in colorectal cancer (CRC) patients and is the main difficulty in cancer treatment. The upregulation of poly-N-acetyllactosamine-related glycosylation is found in CRC patients and is associated with progression and metastasis in cancer. β-1,4-Galactosyltransferase III (B4GALT3) is an enzyme responsible for poly-N-acetyllactosamine synthesis, and therefore, we investigated its expression in CRC patients. We found that B4GALT3 negatively correlated with poorly differentiated histology (P < 0.001), advanced stages (P = 0.0052), regional lymph node metastasis (P = 0.0018) and distant metastasis (P = 0.0463) in CRC patients. B4GALT3 overexpression in CRC cells suppressed cell migration, invasion and adhesion, whereas B4GALT3 knockdown enhanced malignant cell phenotypes. The β1 integrin-blocking antibody reversed the B4GALT3-mediated increase in cell invasion. B4GALT3 expression altered glycosylation on the N-glycan of β1 integrin probably through changes in poly-N-acetyllactosamine synthesis. Furthermore, overexpressed β1 integrin along with the activation of its downstream signaling transduction were found in B4GALT3 knockdown cells, whereas overexpression of B4GALT3 suppressed the expression of active β1 integrin and inhibited its downstream signaling. Our results suggest that B4GALT3 is negatively associated with CRC metastasis and suppresses cell invasiveness through inhibiting activation of β1 integrin.

Introduction

Colorectal cancer (CRC) is the fourth leading cause of death from cancer worldwide and is responsible for 8% of all cancer deaths (1). CRC patients without metastasis could be easily cured with surgery, but the possibility of cure through surgical resection is applicable to only a small portion of CRC patients once the tumors metastasize and spread to distant sites of the body (2). Metastasis is a common disease progression in CRC patients. Thirty-five percentage of CRC patients have metastatic tumors at the time of diagnosis and 33–50% of patients without metastases will further progress to stage IV during the course of their disease (1,3). Further understanding of tumor metastasis mechanisms would help to predict disease progression, develop new therapies and personalize systemic therapy.

Aberrant glycosylation often exists in human cancers and is associated with malignant transformation and tumor progression (4). Several carbohydrate-related structures in serum such as carcinoembryonic antigen and carbohydrate antigen 19-9 are used as tumor markers for cancer detection, for example, in the diagnosis of CRC (5). Poly-N-acetyllactosamines are normal glycan structures in organisms that are often modified to express tumor-associated antigens, such as sialyl Lewis x (6,7), which is highly expressed in CRC and is associated with cancer metastasis (8,9). Moreover, poly-N-acetyllactosamines can be recognized by galectins, like galectin-1 and galectin-3, which are upregulated in CRC and correlated with cancer progression (10). These poly-N-acetyllactosamine-associated glycan structures and lectins also play essential roles in intracellular protein trafficking, cell–cell and cell–matrix adhesion, immune cell homeostasis, inflammation and cancer metastasis (10,11).

β-1,4-Galactosyltransferases III (B4GALT3) belongs to the family of B4GALTs, which catalyze the biosynthesis of poly-N-acetyl-lactosamines. B4GALTs transfer galactose (Gal) from uridine diphosphate galactose to N-acetylgalactosamine (GlcNAc)-terminated oligosaccharides to form N-acetyllactosamine. Repeating units of N-acetyllactosamines then extend to form poly-N-acetyllactosamines on N-glycans, O-glycans, glycolipids or glycosaminoglycan chains (12). The B4GALT family consists of seven members with different tissue distributions, acceptor preferences and enzyme activities. Previous studies show that the extension of poly-N-acetyllactosamine on N-glycans and O-glycans is mainly achieved by B4GALT1 and B4GALT4 (13), whereas B4GALT5 with B4GALT6 and B4GALT7 act as enzymes for lactosylceramide and glycosaminoglycan chain biosynthesis, respectively (14–16). Although in vitro studies show that B4GALT3 has poor poly-N-acetyllactosamine extension ability, preferring to add the first Gal to the beginning of a poly-N-acetyllactosamine chain (13,17), the biological functions of B4GALT3 in CRC and its impacts on tumor cells are poorly understood.

Integrins are cell surface receptors for extracellular matrix (ECM) molecules and consist of heterodimers of α- and β-subunits. Among all subunits, β1 integrin is known to interact with almost all common ECM components found in human tissues (18). Differential glycosylation of β1 integrin may result in distinct effects on integrin activation and modulates cell adhesion and cancer metastasis. O-glycans and N-glycans are both found on β1 integrin. The addition of core 3 O-glycan to α2 and β1 integrin subunits has been shown to suppress tumor formation and metastasis (19). Also, increased bisecting GlcNAc structures on N-glycans inhibit α5β1-mediated cell spreading and migration (20). In contrast, increased β1,6-GlcNAc branching on N-glycans enhances cell migration toward fibronectin and cell invasion through matrigel (21). N-glycans carry poly-N-acetyllactosamines preferentially on the Manα1–6 rather than the Manα1–3 arm of complex N-glycan, and extensive studies have focused on the β1,6-GlcNAc branching on the Manα1–6 arm (22,23).

To investigate the role of B4GALT3 in CRC tumor metastasis, we examined the expression level of B4GALT3 in CRC tumors and its correlation with clinicopathologic factors. B4GALT3 expression is negatively correlated with poorly differentiated histology, advanced stages and metastasis in CRC patients. B4GALT3 knockdown increased cell migration, invasion and the activation of β1 integrin and its downstream signaling. Furthermore, B4GALT3 modulated glycosylation changes on the N-glycan of β1 integrin. The results indicate that B4GALT3 expression suppresses invasive cell phenotypes by inhibiting β1 integrin activation through altering glycosylation on β1 integrin. Our findings suggest that B4GALT3 may function as a metastasis suppressor in CRC through modulating β1 integrin glycosylation and activation.

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Abbreviations: B3GNT, β3-N-acetylgalactosaminyltransferase; B4GALT3, β-1,4-galactosyltransferase III; BSA, bovine serum albumin; CRC, colorectal cancer; ECL, Erythrina cristagalli lectin; ECM, extracellular matrix; FAK, focal adhesion kinase; FBS, fetal bovine serum; Gal, galactose; GlcNAc, N-acetylgalactosamine; LEL, Lycopersicon esculentum lectin; mRNA, messenger RNA; MTI, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PBS, phosphate-buffered saline.
Materials and methods

**Immunohistochemistry**

Tissue array BC051110 and CO2161 (US Biomax, Rockville, MD) contained 110 and 208 non-overlapping CRC patient tissues, respectively. Tissue sections were deparaffinized in xylene and rehydrated in a series of graded alcohols. After incubation in 3% H₂O₂ for 10 min, tissues were incubated with 1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) to prevent antibodies from binding non-specifically. A 1:100-diluted rabbit polyclonal anti-B4GALT3 antibody (Sigma–Aldrich, St Louis, MO) was applied to sections at room temperature overnight, followed by Super Sensitive Link-Label immunohistochemistry Detection System (BioGenex, Fremont, CA). Specific immunostaining was visualized with a 3,3-diamobenzidine (DAB) substrate solution (Sigma–Aldrich). All slides were counterstained with hematoxylin (Sigma–Aldrich) and mounted with UltraKitt (J.T. Baker, Phillipsburg, NJ) mounting medium.

**Cell culture**

HCT116, SW480, SW620, colo205 and HT29 were obtained from the Biosource Collection and Research Center (BCRC, HsinChu, Taiwan). HCT15 and Caco2 were kindly provided by Prof. M.-S.Lee (National Taiwan University, Taipei, Taiwan). HCT116, SW480, SW620, colo205 and HT29 were maintained with Dulbecco’s modified Eagle’s medium (Gibco, Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Gibco, Life Technologies), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco, Life Technologies) in a humidified tissue culture incubator at 37°C and 5% CO₂ atmosphere. Caco2 was maintained in Dulbecco’s modified Eagle’s medium containing 20% FBS and HCT15 was cultured in RPMI1640 (Hyclone, Thermo Scientific, Logan, UT) with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco, Life Technologies).

**Stable expression of B4GALT3**

HCT116 and SW480 cells were transfected with B4GALT3/pDNA3.1A or pcDNA3.1A/myc-His (Invitrogen, Life Technologies, Camarillo, CA) using Lipofectamine 2000 (Invitrogen, Life Technologies) according to the manufacturer’s protocol. The transfected cells were selected in 600 μg/ml G418 for further studies. The overexpression of B4GALT3 was confirmed by western blotting.

**Knockdown of B4GALT3 expression**

Two B4GALT3 small interfering RNAs (HSS112777 and HSS189507) and control scramble small interfering RNA (siCtrl) (Invitrogen, Life Technologies) were used. HT29 and Caco2 cells were transfected with small interfering RNAs using Lipofectamine RNAiMAX (Invitrogen, Life Technologies) according to the manufacturer’s instruction to a final concentration of 20 nM for 2 days.

**Immunofluorescence**

Cells were seeded on cover slides coated with poly-L-lysine and allowed to grow for 2 days. Upon ~80% confluence, cells were fixed with 3% formaldehyde solution and incubated in 0.5% BSA/PBS containing 0.1% Triton X-100. Rabbit polyclonal anti-B4GALT3 and Alexa Fluor® 555 mouse anti-GM130 antibodies (BD Biosciences, Franklin Lakes, NJ) were added to cells followed by goat anti-rabbit IgG–fluorescein isothiocyanate antibodies. 4',6-Diamidino-2-phenylindole was used for cell nuclei staining.

**MTT assay**

Cells were seeded in 96-well plates and each well contained 2 × 10³ cells. A solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma–Aldrich) was added to each well. Cells were incubated at 37°C for 4 h to allow the reduction of MTT by mitochondrial dehydrogenases. MTT formazan crystals were dissolved in a solution containing 10% (wt/vol) sodium dodecyl sulfate and 0.01 N HCl. Colorimetric intensity was measured at the dual wavelengths of 570 and 630 nm with a spectrophotometer.

**Transwell migration assay**

Transwell migration assays were performed using cell culture inserts with 8 μm PET track-etched membranes (BD Biosciences). Cells were detached and resuspended in serum-free medium and seeded into inserts. Cells were allowed to migrate toward a medium containing 10% (vol/vol) FBS as a chemotactant. After 48 h, migrated cells were fixed and stained with 0.5% (wt/vol) crystal violet (Sigma–Aldrich). Cell numbers were counted from three random fields.

**Matrigel invasion assay**

Matrigel (BD Biosciences) was coated on the upper surfaces of insert membranes. The following experimental procedures were the same as for the transwell migration assay. To inhibit β1 integrin activation, detached cells were incubated with a β1 integrin-blocking antibody (clone P4C10; Millipore, Billerica, MA) for 10 min before seeding onto inserts. After incubation for 2 days, invaded cells were fixed and stained with crystal violet.

**Cell adhesion assay**

Cell adhesion assays were performed on 96-well culture dishes coated with 5 μg/ml BSA, fibronectin, collagen IV or laminin (Sigma–Aldrich), and blocked by 1% BSA. Cells were detached from culture plates using trypsin and ethylenediaminetetraacetic acid followed by resuspension in serum-free Dulbecco’s modified Eagle’s medium. Cells (10⁵ per well) were seeded into the coated 96-well plates in xeplicate and incubated at 37°C for 1 h (30 min of incubation for Caco2). Wells were washed with PBS to remove unattached cells. The adherent cells were then fixed with 100% methanol and stained with 0.5% (wt/vol) crystal violet. Excess stain was washed away by ddH₂O and the wells were allowed to dry completely. The staining of crystal violet was revealed by cells from release by addition of 2% sodium dodecyl sulfate and the optical density was measured at 550 nm. At least four independent experiments were performed on each cell line.

**Immunoblotting and lectin pull-down assay**

B4GALT3 proteins were detected with a rabbit anti-B4GALT3 polyclonal antibody (Sigma–Aldrich). Antibodies against total paxillin, p-actin (BD Biosciences), pTyr397-FAK, pTyr118-paxillin (Cell Signaling Technology, Danvers, MA) and total FAK (Santa Cruz Biotechnology, Dallas, TX) were used to analyze β1 integrin signaling. The detection of glycan structures on glycoproteins was achieved by lectin pull-down assays using biotinylated Ricinus communis agglutinin I (RCA I), Erithrina cristagalli lectin (ECL), Eucytopersicum esculentum (tomato) lectin (LEL) and Phaseolus vulgaris leucoagglutinin (PHA-L) (Vector Laboratories, Burlingame, CA). Total cell lysates were incubated with biotinylated lectins at 4°C for 16 h. Streptavidin-conjugated agarose beads (Vector Laboratories) were then added and lysates were further incubated for additional 6 h. The precipitated proteins were then subjected to western blotting. A β1 integrin antibody (clone 18/CD29; BD Biosciences) was used to detect β1 integrin protein expression in immunoblots.

**Flow cytometry**

Cells were detached from culture dishes and resuspended in 2% BSA/PBS. Total β1 integrin antibody (clone TDM29; Chemicon, Temecula, CA) or active β1 integrin antibody (clone HUTS-21; BD Biosciences) was added to the cells. Cells were then rotated at 4°C for 30 min followed by incubation of fluorescein anti-mouse IgG antibodies at 4°C for 30 min. The fluorescence intensity was analyzed by flow cytometry (FACS Calibur; BD Biosciences). β1 integrin antibodies were replaced by mouse IgG in negative controls for each cell.

**Statistical analysis**

Statistical analyses were performed using SPSS 10.0 for Windows (SPSS, Chicago, IL). The Student’s t-test was used to compare the differences between two experimental groups. Pearson’s chi-squared test was used to assess the association between pairs of categorical variables. All statistical tests were two sided, and P < 0.05 was considered statistically significant.

**Results**

B4GALT3 expression correlates with clinicopathological factors in CRC patients

B4GALT3 expression in CRC tissues was determined by immunohistochemical staining. Tissues with incomplete clinical information or that were damaged during staining were excluded from further analysis. A total 281 patient tissues were analyzed in this study. Figure 1A shows a typical supranuclear Golgi staining of B4GALT3 in CRC cells. B4GALT3 expression was categorized into four groups (+0, +1, +2 and +3) according to an expression percentage and intensity in CRC tumors (Figure 1A). B4GALT3 expression was observed in most well and moderately differentiated tumors (Figure 1A: +2 and +3) and decreased in expression and intensity with undifferentiated histology (Figure 1A: +0 and +1). For clinicopathological analysis, CRC tumors were divided into low B4GALT3 expression (groups +0 and +1) and high B4GALT3 expression (groups +2 and +3) categories. A low expression of B4GALT3 is associated with poorly differentiated histology (P < 0.001), advanced tumor stages (P = 0.0052), regional lymph node metastasis (P = 0.0018) and distant metastasis (P = 0.0463) (Table I). We further analyzed the correlation between B4GALT3 expression intensity and regional lymph node metastasis. The expression of B4GALT3 is weaker in patients with regional lymph node metastasis than without lymph node metastasis.
metastasis (Figure 1B; \(P = 0.0086\)). Taken together, our data suggest that a decrease in B4GALT3 expression in CRC tumors predicted poorly differentiated histology, advanced tumor stages and cancer metastasis.

**Expression of B4GALT3 in CRC cells suppresses cell migration and invasion**

CRC cells showed variable levels of B4GALT3 protein expression (Figure 1C). To investigate B4GALT3 functions in CRC cells, we chose HCT116 and SW480 to overexpress B4GALT3 because the two cell lines expressed B4GALT3 relatively low. HT29 and Caco2 were used for B4GALT3 knockout because the B4GALT3 expression in these cells was relatively high among all cell lines. B4GALT3 expression in overexpressed or knockeddown cells was confirmed by western blotting (Figure 1D). We further verified messenger RNA (mRNA) expression of other enzymes responsible for poly-N-acetyllactosamine synthesis. The mRNA levels of B4GALTs and \(\beta_3\)-N-acetylglucosaminyltransferase 2 (B3GNT2) have no significant changes in B4GALT3-overexpressed or knockdown cells, except for in \#B4GALT3-2 knockout HT29 that showed decrease in B4GALT2-, -4 and -6 mRNA expressions (Supplementary Figure 1, available at Carcinogenesis Online). Immunofluorescence staining shows that the expression of B4GALT3 in CRC cells was colocalized with Golgi marker GM130 (Figure 1E).

No significant difference was found in B4GALT3-mediated cell viability except a decrease in \#B4GALT3-2-transfected Caco2 cells (Figure 2A and 2B) using an MTT assay. B4GALT3 overexpression in HCT116 and SW480 significantly suppressed cell migration and invasion using transwell migration and matrigel transwell invasion assays, respectively (Figure 2C; \(P < 0.05\)). B4GALT3 knockdown in HT29 and Caco2 enhanced cell migration and invasion (Figure 2D; \(P < 0.05\)). These results indicate that B4GALT3 significantly suppressed cell migration and invasion, whereas its effect on cell proliferation is relatively minor.

**B4GALT3 regulates cell–ECM interaction and \(\beta_1\) integrin-mediated cell invasion**

Metastasis occurs when cancer cells acquire the ability to escape from original tumor sites. Cell–ECM interactions direct cell invasiveness and metastasis initiation (24). Therefore, we investigated the cell–ECM interaction by performing cell adhesion assays on fibronectin, laminin and collagen IV. B4GALT3 overexpression in HCT116 and SW480 decreased the cell attachment to ECM proteins, especially fibronectin and laminin (Figure 3A; \(P < 0.05\)), whereas B4GALT3 knockdown significantly enhanced cell adhesion to fibronectin and laminin in HT29 and all ECM in Caco2 (Figure 3B; \(P < 0.05\)).

Integrins are cell surface receptors that regulate cell invasion and adhesion to the ECM, and \(\beta_1\) integrin is the common receptor for collagen, laminin and fibronectin. A \(\beta_1\) integrin-blocking antibody, P4C10, significantly decreased cell invasion of mock-transfected cells but not B4GALT3-transfected cells (Figure 3C; \(P < 0.01\)). The increased invasion in B4GALT3 knockdown cells was reversed by P4C10 (Figure 3D; \(P < 0.05\)). Together, these results suggest that \(\beta_1\) integrin is involved in the B4GALT3-mediated decrease of cell–ECM interactions and the cell invasiveness of CRC cells.

**B4GALT3 modulates glycosylation of \(\beta_1\) integrin**

Since \(\beta_1\) integrin is an extensively glycosylated glycoprotein, we further examined whether B4GALT3 modifies glycan structures on \(\beta_1\)
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Fig. 2. The effect of B4GALT3 on malignant cell phenotypes in CRC cell lines. The effect of B4GALT3 overexpression (A) or knockdown (B) on cell growth was analyzed by MTT assay. Cells were cultured in growth medium and MTT reagents were applied to cells at indicated timepoints. The results are standardized to day 0 of each cell and presented as mean ± SD. **P < 0.01. (C and D) The significance of B4GALT3 on cell mobility and cell invasiveness was determined using transwell inserts. FBS (10%) served as a chemoattractant. Data are presented as mean ± SD from three independent experiments. *P < 0.05; **P < 0.01.

Table I. B4GALT3 expression and clinicopathologic characteristics of CRC

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases</th>
<th>B4GALT3 expression</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≦50 years</td>
<td>114</td>
<td>46 (40.4)</td>
<td>68 (59.6)</td>
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<tr>
<td>&gt;50 years</td>
<td>167</td>
<td>73 (43.7)</td>
<td>94 (56.3)</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>163</td>
<td>74 (45.4)</td>
<td>89 (54.6)</td>
</tr>
<tr>
<td>Female</td>
<td>118</td>
<td>45 (38.1)</td>
<td>73 (61.9)</td>
</tr>
<tr>
<td>Pathology</td>
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<td></td>
<td></td>
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<tr>
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<td>101 (40.9)</td>
<td>146 (59.1)</td>
</tr>
<tr>
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<td>16 (47.1)</td>
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<td>Grade</td>
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</tr>
<tr>
<td>Well differentiated</td>
<td>89</td>
<td>30 (33.7)</td>
<td>59 (66.3)</td>
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<tr>
<td>Moderately differentiated</td>
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<tr>
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<td>II</td>
<td>162</td>
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<td>III</td>
<td>78</td>
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<td>36 (46.2)</td>
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<tr>
<td>IV</td>
<td>18</td>
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<td>6 (33.3)</td>
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<tr>
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</tr>
<tr>
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<td>18</td>
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P value = chi-square test.
integrin. B4GALT3 overexpression in HCT116 and SW480 slightly decreased the molecular weight of β1 integrin on the western blot (Supplementary Figure 2A, available at Carcinogenesis Online), whereas knockdown of B4GALT3 in HT29 increased the molecular weight of β1 integrin (Supplementary Figure 2B, available at Carcinogenesis Online). Knockdown of B4GALT3 in Caco2 did not have obvious effect on β1 integrin molecular weight (Supplementary Figure 2B, available at Carcinogenesis Online). The data suggest that B4GALT3 expression may contribute to posttranslational modification on β1 integrin that resulted in a molecular weight shift of β1 integrin on western blots.

To verify the glycosylation changes on β1 integrin, three lectins recognizing different Gal-related glycan structures were used in a lectin pull-down assay. RCA I binds preferentially to terminal Gal, ECL recognizes N-acetyllactosamine and LEL prefers poly-N-acetyllactosamines. B4GALT3 overexpression in SW480 decreased LEL binding to β1 integrin (Figure 4A; P < 0.05), whereas B4GALT3 knockdown in HT29 and Caco2 significantly increased LEL binding to β1 integrin (Figure 4B; P < 0.05). No significant differences were observed in binding of LEL to the β1 integrin in B4GALT3-overexpressed HCT116, but the binding of ECL to β1 integrin was decreased (Figure 4A; P < 0.05). Except HCT116, there were no significant differences in ECL and RCA I binding to β1 integrin (Figure 4A and 4B). To further confirm the effect of B4GALT3 on β1 integrin glycosylation, we overexpressed B4GALT3 in another CRC cell line, HCT15. The B4GALT3 expression level was examined and phenotypic changes were consistent with the findings for HCT116 and SW480 (Supplementary Figure 3, available at Carcinogenesis Online). In accordance with SW480, the overexpression of B4GALT3 in HCT15 decreased LEL binding to β1 integrin (Supplementary Figure 4, available at Carcinogenesis Online; P < 0.05). Together, our results suggest that B4GALT3 could modulate LEL-recognized carbohydrate structures on β1 integrin.

The LEL-recognized structures exist mainly on N-glycan of β1 integrin in CRC cells

To verify the existence of poly-N-acetyllactosamines on N-glycan of β1 integrin in CRC cells, PNGase F was used to remove N-glycan from glycoproteins. The binding of LEL to β1 integrin was almost completely eliminated by PNGase F treatment in all cells (Figure 4C and 4D). The effect of B4GALT3 on N-glycan structures was studied by PHA-L that binds to β1-1,6-GlcNAc branching of tri- and tetra-antennary oligosaccharides on complex-type N-glycans. No significant difference was found in the PHA-L pull-down assay (Supplementary Figure 5A and B, available at Carcinogenesis Online). The results reveal that the LEL-recognized structures mainly appear on the N-glycan of β1 integrin in CRC cells and that B4GALT3 expression has no significant effect on β-1,6-GlcNAc-branching N-glycans on β1 integrin.

B4GALT3 suppresses the activation of β1 integrin and its downstream signaling pathways

As β1 integrin is involved in B4GALT3-mediated cell invasion and B4GALT3 regulates the glycosylation of β1 integrin, we next
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examined the changes in mRNA and protein expression, active conformation and downstream signaling activation of β1 integrin. The mRNA expression of β1 integrin was measured by quantitative reverse transcription–polymerase chain reaction, and its protein level was analyzed by western blotting. Neither RNA levels (Supplementary Figure 6A and B, available at Carcinogenesis Online) nor protein expression of β1 integrin (Supplementary Figure 6C and D, available at Carcinogenesis Online) was changed by altering B4GALT3 expression. The surface expressions of total and active β1 integrin were analyzed by flow cytometry. B4GALT3 expression did not alter the surface expression of β1 integrin (Figure 5A and 5B, lower panels). However, the overexpression of B4GALT3 significantly decreased cell binding to HUTS-21, an antibody that specifically recognizes the active form of β1 integrin (Figure 5A and 5C, upper panels; P < 0.05). On other hand, B4GALT3 knockdown increased cell binding to HUTS-21 (Figure 5B and 5D, upper panels; P < 0.05). B4GALT3 overexpression inhibited the phosphorylation of focal adhesion kinase (FAK) and paxillin, β1 integrin downstream signaling molecules when cells adhered to fibronectin, laminin and collagen IV (Figure 5E). B4GALT3 knockdown increased FAK and paxillin phosphorylation in HT29 and Caco2 on fibronectin and laminin (Figure 5F). The results indicate that B4GALT3 expression decreases the active conformation of β1 integrin and therefore suppresses the activation of β1 integrin downstream signaling in CRC cells.

Discussion

In this study, we demonstrated that the expression of B4GALT3 in CRC patients is negatively correlated with poorly differentiated histology, advanced stages and metastasis. Knockdown of B4GALT3 in CRC cells enhanced cell migration, invasion and adhesion on ECM, especially on fibronectin and laminin, whereas overexpression of B4GALT3 suppressed these malignant cell phenotypes. Furthermore, knockdown of B4GALT3 in cells not only increased β1 integrin molecular weight on western blots and LEL binding to N-glycans of β1 integrin, but also increased the number of active β1 integrin on cell surfaces as well as its downstream signaling. Here, we report for the first time that B4GALT3 regulates CRC cell invasiveness through modification of the N-glycan structures on β1 integrin.

The N-glycosylation plays an important role in regulating β1 integrin activities. Aberrant expression of glycans on β1 integrin is frequently observed in various cancers and associated with metastasis (25,26). β1 integrin is extensively N-glycosylated as it possesses 12 potential N-glycosylation sites on its polypeptide backbone (27). We observed increased and decreased molecular weight of β1 integrin

Fig. 4. B4GALT3 modifies glycosylation of β1 integrin in CRC cell lines. (A and B) Lectins that recognize different Gal-related glycan structures were used in pull-down assays and an anti-β1 integrin antibody was used to determine the amount of lectin-binding β1 integrin. Cell lysates from mock and B4GALT3 transfectants (A) or siCtrl and siB4GALT3s transfectants (B) were pulled down with lectins and immunoblotted with an anti-β1 integrin antibody. The intensity of western blotting was quantified, normalized with total β1 integrin and presented as fold changes compared with mock or siCtrl transfectants in the lower panels. Error bar = standard deviation. * P < 0.05. (C and D) The existence of polylactosamine on N-glycans in CRC cell lines was analyzed by pull-down polylactosamine-carrying β1 integrin using LEL. N-glycans were removed from peptide chains by PNGase F. PF, PNGase F.
with B4GALT3 knockdown and overexpression, respectively. The molecular weight shift is due to different posttranslational modification, such as protein glycosylation. Since partial glycosylated precursor \( \beta_1 \) integrins form a stable pool in endoplasmic reticulum (23), the maturation of cell surface functional \( \beta_1 \) integrin is mainly dependent on the expression of glycosyltransferases when \( \beta_1 \) integrin leaves the Golgi apparatus. Our data revealed that the \( \beta_1 \) integrins pulled down by three different lectins were different in the molecular

**Fig. 5.** B4GALT3 inhibits the activation and downstream signaling of \( \beta_1 \) integrin. (A and B) The amount of cell surface-activated and total \( \beta_1 \) integrins were analyzed using flow cytometry. \( \beta_1 \) integrin antibodies were replaced by mouse IgG in negative controls. (A) The overexpression of B4GALT3 in HCT116 and SW480 decreased activated \( \beta_1 \) integrin levels, whereas surface expression of \( \beta_1 \) integrin remained unchanged. (B) The knockdown of B4GALT3 in HT29 and Caco2 increased activated \( \beta_1 \) integrin levels, whereas the surface expression of \( \beta_1 \) integrin was unchanged. (C and D) The fluorescence intensities of A and B are shown as mean ± SD from three independent experiments. *P < 0.05; **P < 0.01. (E and F) The activation of \( \beta_1 \) integrin downstream signaling pathways was analyzed using adhesion assays and western blotting. B4, B4GALT3; siB4-1, siB4GALT3-1; siB4-2, siB4GALT3-2.
weights, suggesting the presence of different β1 integrin glycoforms on cell surfaces. The LEL-recognized glycosylated β1 integrins had the highest molecular weight, and the results were coherent with the LEL-recognized carbohydrate structures, poly-N-acetyllactosaminose, which are relatively large structures on complex-type N-glycans.

The poly-N-acetyllactosaminose are mainly expressed on the β1,1,6-GlcNAc branch of N-glycans, whereas the β1,1,6-GlcNAc branches on β1 integrin modulate many cell behaviors, including cell migration and invasion. N-acetylgalactosaminyltransferase V catalyzes the synthesis of β1,1,6-GlcNAc branch on the Man1–6 arm of complex N-glycans (23). In previous studies, N-acetylgalactosaminyltransferase V overexpression in human fibrosarcoma HT1080 cells increased β1,1,6-GlcNAc branching of N-glycan on β1 integrin and enhanced cell migration and invasion (21). Because β1,1,6-GlcNAc branches could be further elongated to form poly-N-acetyllactosaminose, the effects of N-acetylgalactosaminyltransferase V may result from alteration of poly-N-acetyllactosaminose expression. In this study, we show that B4GALT3 knockdown may increase poly-N-acetyllactosaminose on β1 integrin N-glycans and enhance cell attachment to ECM, cell migration and invasion through matrigel. Our results further support that increasing poly-N-acetyllactosaminose expression on N-glycan β1 integrin may enhance cell invasiveness.

Besides of β1 integrin, many other adhesion-related receptors or receptor tyrosine kinases can modulate cell invasiveness. E-cadherin and integrin α-subunits are known to possess the β1,1,6-GlcNAc-branching structures, and changes of the complex-type N-glycan structures on these receptors could regulate malignant cell phenotypes (28–30). Moreover, increasing sialylation and fucosylation on epidermal growth factor receptor suppressed epidermal growth factor receptor-mediated invasion of lung cancer cells (31). Previously, we demonstrated that expression of B4GALT3 modified carbohydrate structures on both N-glycans and O-glycans of β1 integrin and regulated cell invasion in neuroblastoma cells (32). Therefore, it is still possible that B4GALT3 contributes to altering integrin signaling through modification of carbohydrate structures on other cell surface receptors, O-glycans or glycolipids.

Although extensive studies have focused on in vitro enzyme activity of B4GALT family, its in vivo function remains unclear. We found that suppressing B4GALT3 expression in CRC cells enhanced synthesis of LEL-recognized structures, probably poly-N-acetyllactosaminose, on β1 integrin. This finding is contradictory to the in vitro B4GALT3 enzyme activity that catalyzes poly-N-acetyllactosaminose synthesis on N-glycans, O-glycans and glycolipids (13,17). We showed that altering B4GALT3 expression neither had significant effect on the mRNA levels of poly-N-acetyllactosaminose synthesis-related genes nor changed the expression levels of terminal Gal, N-acetyllactosaminose and β1,1,6-GlcNAc-branching N-glycans on β1 integrins. However, the changes of LEL-recognized carbohydrate structures on β1 integrin suggest that the elongation of poly-N-acetyllactosaminose chains could be the main mechanism of B4GALT3 to regulate β1 integrin activation. The inconsistent results between in vitro and in vivo enzyme activity may have resulted from protein–protein interactions that were not revealed in in vitro enzyme activity assays. Given that B4GALT3 contains several consensus N-glycosylation sites (33) and glycosylation of glycosyltransferases is critical for proper enzyme activities and protein distributions (34), B4GALT3 may glycosylate itself or other glycosyltransferases, which may in turn cause enzyme activity changes or altered protein localization. In previous studies, B3GNT8 forms a protein complex with B3GNT2 and subsequently activates B3GNT2 for poly-N-acetyllactosaminose synthesis (35). B3GNT1 is physically associated with B4GALT1, and artificially relocating one of the enzymes will cause the relocation of the other (36). It is possible that B4GALT3 forms protein complexes with other glycosyltransferases or non-glycosyltransferase proteins, which in turn affects their enzyme activity or subcellular localization. Further investigation is required for fully understanding of the mechanisms by which B4GALT3 modulates poly-N-acetyllactosaminose synthesis and changes in β1 integrin glycosylation.

Aberrant expression of carbohydrate structures has been observed in many cancers and is associated with tumor progression and metastasis. Previous reports have shown that upregulation of several B4GALTs (37,38) as well as N-acetyllactosamine (38) and its derivatives (8,9) are associated with cancer progression, metastasis and poor survival in CRC patients (8,37–39). A recent liquid chromatography and mass spectrometry-based investigation showed differential expression of N-glycan modification, such as increased sialylated Lewis-type epitope expression, in CRC tumors compared with normal colon tissues (40). In this study, we are the first to report B4GALT3 expression is negatively correlated with tumor stages and metastasis of CRC patients. In accordance with the clinical analysis, knockdown of B4GALT3 expression in CRC cells enhanced cell migration and invasion abilities. Furthermore, suppressing B4GALT3 expression in CRC cells may promote poly-N-acetyllactosaminose synthesis on β1 integrins, which coincides with increased expression of N-acetyllactosaminose and its derivatives in metastatic CRC tissues.

Our findings open new insights into the regulation of cancer metastasis by aberrant expression of B4GALT3 through altering β1 integrin activation and glycan structures in CRC and provide a potentially new prognostic factor for prediction of metastatic CRC patients.

Supplementary material

Supplementary Figures 1–6 can be found at http://carcin.oxfordjournals.org/

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References
