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

Chia-Hua Chen1, Shui-Hua Wang2, Chiung-Hui Liu1, Yi-Ling Wu1, Wei-Jen Wang1, Min-Chuan Huang2,3, Yi-Ling Wu1, Ji-Shiang Hung3,4, I-Rue Lai1,3, Jin-Tung Liang3 and Min-Chuan Huang5,6

1Graduate Institute of Anatomy and Cell Biology, National Taiwan University College of Medicine, Taipei 10051, Taiwan, 2Institute of Biological Chemistry, Academia Sinica, Taipei 11529, Taiwan, 3Department of Surgery and 4Department of Medical Research, National Taiwan University Hospital, Taipei 10048, Taiwan and 5Research Center for Developmental Biology and Regenerative Medicine, National Taiwan University, Taipei 10041, Taiwan

Abstract

Metastasis occurs often in colorectal cancer (CRC) patients and is the major 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 biosynthesis. Furthermore, more activated 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 percent 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.

Abbreviations: B3GNT, β3-N-acetylgalacosaminyltransferase; 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; MT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PBS, phosphate-buffered saline.

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-acetyllactosamines. 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 B4GALT3 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 Manβ1–6 rather than the Manβ1–3 arm of complex N-glycan, and extensive studies have focused on the β1,6-GlcNAc branching on the Manβ1–6 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.
The roles of B4GALT3 in colorectal cancer

Materials and methods

Immunohistochemistry

Tissue array BC051110 and CO2161 (US Biomax, Rockville, MD) contain 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-diaminobenzidine (DAB) solution in 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 removed from cells 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, f-actin (BD Biosciences), pSer397–paxillin, pSer118–paxillin (Cell Signaling Technology, Danvers, MA) and total FAK (Santa Cruz Biotechnology, Dallas, TX) were used to analyze f1 integrin signaling. The detection of glycan structures on glycoproteins was achieved by lectin pull-down assays using binautomated Ricinus communis agglutinin I (RCA I), Erithrina cristagalli lectin (ECL), Lupinus albus lectin (LAL) and Phaseolus vulgaris leucohagglutinin (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 f1 integrin antibody (clone 18/CD29; BD Biosciences) was used to detect f1 integrin protein expression in immunoblots.

Flow cytometry

Cells were detached from culture dishes and resuspended in 2% BSA/PBS. Total f1 integrin antibody (clone TDM29; Chemicon, Temecula, CA) or active f1 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). f1 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 = 0.005), regional lymph node metastasis (P = 0.0018) and distant metastasis (P = 0.0463) (Table 1). 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 knockdown because the B4GALT3 expression in these cells was relatively high among all cell lines. B4GALT3 expression in overexpressed or knockdown 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 β3-N-acetylglucosaminyltransferase 2 (B3GNT2) have no significant changes in B4GALT3-overexpressed or knockdown cells, except for in siB4GALT3-2 knockdown 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 siB4GALT3-2-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 β1 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 β1 integrin is the common receptor for collagen, laminin and fibronectin. A β1 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 β1 integrin is involved in the B4GALT3-mediated decrease of cell–ECM interactions and the cell invasiveness of CRC cells.

**B4GALT3 modulates glycosylation of β1 integrin**

Since β1 integrin is an extensively glycosylated glycoprotein, we further examined whether B4GALT3 modifies glycan structures on β1
The roles of B4GALT3 in colorectal cancer

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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low (40.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>High (59.6)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>Low (43.7)</td>
<td>0.6236</td>
</tr>
<tr>
<td>≦50 years</td>
<td>114</td>
<td>68 (59.6)</td>
<td></td>
</tr>
<tr>
<td>&gt;50 years</td>
<td>167</td>
<td>94 (56.3)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>Low (54.6)</td>
<td>0.271</td>
</tr>
<tr>
<td>Male</td>
<td>163</td>
<td>89 (54.6)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>118</td>
<td>73 (61.9)</td>
<td></td>
</tr>
<tr>
<td>Pathology</td>
<td></td>
<td>Low (41.9)</td>
<td>0.1987</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>247</td>
<td>146 (59.1)</td>
<td></td>
</tr>
<tr>
<td>Mucinous adenocarcinoma</td>
<td>34</td>
<td>16 (47.1)</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td>Low (66.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Well differentiated</td>
<td>89</td>
<td>59 (66.3)</td>
<td></td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>134</td>
<td>82 (61.2)</td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>58</td>
<td>21 (36.2)</td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td>Low (69.6)</td>
<td>0.0052</td>
</tr>
<tr>
<td>I</td>
<td>23</td>
<td>16 (69.6)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>162</td>
<td>104 (64.2)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>78</td>
<td>36 (46.2)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>18</td>
<td>6 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Tumor invasion depth</td>
<td></td>
<td>Low (44.0)</td>
<td>0.5285</td>
</tr>
<tr>
<td>Submucosa</td>
<td>1</td>
<td>1 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Muscularis propria</td>
<td>25</td>
<td>14 (56.0)</td>
<td></td>
</tr>
<tr>
<td>Subserosa</td>
<td>134</td>
<td>82 (61.2)</td>
<td></td>
</tr>
<tr>
<td>Other organs or structures</td>
<td>121</td>
<td>65 (53.7)</td>
<td></td>
</tr>
<tr>
<td>Regional lymph node metastasis</td>
<td></td>
<td>Low (64.1)</td>
<td>0.0018</td>
</tr>
<tr>
<td>No</td>
<td>192</td>
<td>123 (64.1)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>89</td>
<td>39 (43.8)</td>
<td></td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
<td>Low (59.3)</td>
<td>0.0463</td>
</tr>
<tr>
<td>No</td>
<td>263</td>
<td>156 (59.3)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>18</td>
<td>6 (33.3)</td>
<td></td>
</tr>
</tbody>
</table>

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 β-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...
The roles of B4GALT3 in colorectal cancer

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
with B4GALT3 knockdown and overexpression, respectively. The molecular weight shift is due to different posttranslational modification, such as protein glycosylation. Since partial glycosylated precursor β1 integrins form a stable pool in endoplasmic reticulum (23), the maturation of cell surface functional β1 integrin is mainly dependent on the expression of glycosyltransferases when β1 integrin leaves the Golgi apparatus. Our data revealed that the β1 integrins pulled down by three different lectins were different in the molecular weight shift, which is consistent with the observation that the expression of glycosyltransferases is upregulated in cancer cells. The maturation of cell surface β1 integrin is largely dependent on the expression of glycosyltransferases when β1 integrin leaves the Golgi apparatus. Our data revealed that the β1 integrins pulled down by three different lectins were different in the molecular weight shift, which is consistent with the observation that the expression of glycosyltransferases is upregulated in cancer cells.

Fig. 5. B4GALT3 inhibits the activation and downstream signaling of β1 integrin. (A and B) The amount of cell surface-activated and total β1 integrins were analyzed using flow cytometry. β1 integrin antibodies were replaced by mouse IgG in negative controls. (A) The overexpression of B4GALT3 in HCT116 and SW480 decreased activated β1 integrin levels, whereas surface expression of β1 integrin remained unchanged. (B) The knockdown of B4GALT3 in HT29 and Caco2 increased activated β1 integrin levels, whereas the surface expression of β1 integrin remained 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 β1 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-acetyllactosamines, which are relatively large structures on complex-type N-glycans.

The poly-N-acetyllactosamines 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 Man9–6 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-acetyllactosamines, the effects of N-acetylgalactosaminyltransferase V may result from alteration of poly-N-acetyllactosamine expression. In this study, we showed that B4GALT3 knockdown may increase poly-N-acetyllactosamines 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-acetyllactosamine expression on N-glycan β1 integrin may enhance cell invasiveness.

Beside 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-acetyllactosamines, on β1 integrin. This finding is contradictory to the in vitro B4GALT3 enzyme activity that catalyzes poly-N-acetyllactosamine 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 terminal Gal, N-acetyllactosamine 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-acetyllactosamines 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 B4GALTs contain 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-acetyllactosamine synthesis (35). B3GNT1 is physically associated with B4GALT1, and artificially relocating one of the enzymes may cause the relocalization 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-acetyllactosamine 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-acetyllactosamine synthesis on β1 integrins, which coincides with increased expression of N-acetyllactosamine 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/

Funding

Acknowledgements
We thank Dr. M.-S. Lee (National Taiwan University, Taiwan) for kindly providing HCT15 and Cos2 cells and Dr. K.-H. Kho (Academia Sinica, Taiwan) for mass spectrometric analysis of carbohydrate structures.

Conflict of Interest Statement: None declared.

References
C.-H. Chen et al.

i-extension enzyme and different members of the beta 1,4-galactosyltransferase gene family. J. Biol. Chem., 275, 15868–15875.


Received August 30, 2013; revised November 25, 2013; accepted December 20, 2013