β-1,4-Galactosyltransferase III Enhances Invasive Phenotypes Via β1-Integrin and Predicts Poor Prognosis in Neuroblastoma

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Abstract

Purpose: Neuroblastoma (NB) is a neural crest-derived tumor that commonly occurs in childhood. β-1,4-Galactosyltransferase III (B4GALT3) is highly expressed in human fetal brain and is responsible for the generation of poly-N-acetyllactosamine, which plays a critical role in tumor progression. We therefore investigated the expression and role of B4GALT3 in NB.

Experimental Design: We examined B4GALT3 expression in tumor specimens from 101 NB patients by immunohistochemistry and analyzed the correlation between B4GALT3 expression and clinicopathologic factors or survival. The functional role of B4GALT3 expression was investigated by overexpression or knockdown of B4GALT3 in NB cells for in vitro and in vivo studies.

Results: We found that B4GALT3 expression correlated with advanced clinical stages ($P = 0.040$), unfavorable Shimada histology ($P < 0.001$), and lower survival rate ($P < 0.001$). Multivariate analysis showed that B4GALT3 expression is an independent prognostic factor for poor survival of NB patients.

B4GALT3 overexpression increased migration, invasion, and tumor growth of NB cells, whereas B4GALT3 knockdown suppressed the malignant phenotypes of NB cells. Mechanistic investigation showed that B4GALT3-enhanced migration and invasion were significantly suppressed by β1-integrin blocking antibody. Furthermore, B4GALT3 overexpression increased lactosamine glycans on β1-integrin, increased expression of mature β1-integrin via delayed degradation, and enhanced phosphorylation of focal adhesion kinase. Conversely, these properties were decreased by knockdown of B4GALT3 in NB cells.

Conclusions: Our findings suggest that B4GALT3 predicts an unfavorable prognosis for NB and may regulate invasive phenotypes through modulating glycosylation, degradation, and signaling of β1-integrin in NB cells. Clin Cancer Res; 19(7): 1705–16. ©2013 AACR.

Introduction

Neuroblastoma (NB), which accounts for 10% of pediatric cancers, is a common solid malignant tumor derived from embryonic neural crest cells of sympathetic nervous system, and most frequently from adrenal medulla (1–3). The disease exhibits extreme heterogeneity, from spontaneous regression as in differentiated ganglioneuroma to malignant progression as in undifferentiated NB (UNB), in relation to the diverse biological features of the tumor (1, 4). Metastasis, an NB staging factor, is found in 50% to 60% of all NB cases; and advanced NBs typically metastasize to distant lymph nodes, bone marrow, bone, liver, or other organs. Although the overall prognosis of NB patients has remarkably improved with the advancement in recent therapies, the long-term survival of high-risk NB remains only 40% despite intensive multimodal therapy (1–3). Finding new prognostic factors is necessitated to further classify and tailor therapy for improving outcome of patients with unfavorable NB.
Changes in carbohydrates on integrins. Among which, 12 contain B4GALT3 tissues and the fetal brain expresses much higher levels of (ECM) proteins and consist of ever, roles of B4GALT3 in NB and its effects on NB tumor study showed that B4GALT3 prefers adding galactose to the (LacNAc; ref. 10). Ocosamine (GlcNAc)-terminated oligosaccharides on B4GALTs transfer galactose from UDP-Gal to acceptor preferences, and biological functions. The b the (7–9). play critical roles in modulating cancer metastasis, intra- and human natural killer-1 carbohydrate. These glycans mines are often further modified to present tumor-associ- ferentiation, migration, invasion, and immune responses (5). Aberrant expression of glycans is observed in most human cancers and is associated with malignant transform- and tumor progression (6). Poly-N-acetyllactosa- mines are often further modified to present tumor-associated antigens, such as sialyl Lewis X, Lewis X, polysialic acid, and human natural killer-1 carbohydrate. These glycans play critical roles in modulating cancer metastasis, intra- cellular protein trafficking, and neuronal development (7–9).

The b,1,4-galactosyltransferase III (B4GALT3) belongs to the b,1,4-galactosyltransferase (B4GALT) family, which consists of 7 members with distinct tissue distributions, acceptor preferences, and biological functions. The B4GALTs transfer galactose from UDP-Gal to N-acetylglu- cosamine (GlcNAc)-terminated oligosaccharides on N-glycan, O-glycan, or glycolipid to form N-acetyllactosamine (LaNAc; ref. 10). B4GALT3 is widely expressed in human tissues and the fetal brain expresses much higher levels of B4GALT3 than does adult brain (11, 12). Previous in vitro study showed that B4GALT3 prefers adding galactose to the beginning of a poly-N-acetyllactosamine chain (13). How- ever, roles of B4GALT3 in NB and its effects on NB tumor cells are still unclear.

Integrins are cell surface receptors of extracellular matrix (ECM) proteins and consist of a1- and b-subunits. Eighteen a-subunits and 8 b-subunits assemble into 24 different integrins. Among which, 12 contain b1-subunit (14). Changes in carbohydrates on b1-integrin have been reported to regulate integrin activity, resulting in altered cell adhesion and metastasis. Overexpression of N-acetylglucosaminyltransferase III (GnT-III) increases the synthesis of bisecting GlcNAc structures on N-glycans and inhibits a5b1-mediated cell spreading and migration (15). In contrast, increase in b1,6-GlcNAc branching on N-glycans by N-acetylgalactosaminyltransferase V (GnT-V) enhances cell migration toward fibronectin and cell invasion (16). B3GNT6 (core3 synthase) adds core 3 O-glycan to a2 and b1-integrin subunits to suppress tumor formation and metastasis in prostate cancer (17). However, effects of B4GALT3 on the glycosylation and function of b1-integrin have not been reported.

The fetal brain expresses much higher levels of B4GALT3 than does adult brain (11) and the public microarray datasets from Oncogenomics: Oberthuer Lab (http:// home.ccr.cancer.gov/oncology/oncogenomics/) reveals that, after P value minimization, only B4GALT3, but not other B4GALTs, predicted poor survival of NB patients. We therefore hypothesize that B4GALT3 could be a prognostic factor contributing to the pathogenesis of NB. In this study, we showed that B4GALT3 expression was upregulated in NB and its expression correlated with advanced stage, unfavorable histology, and predicted a poor prognosis in NB patients. In addition, B4GALT3 expression increased cell migration, invasion, and tumor growth of NB cells. Mechanistic investigation showed that B4GALT3-modified glycosylation of b1-integrin through increasing terminal galac- tose, and regulated the expression of mature form of b1- integrin and its signal transduction. These findings suggest that B4GALT3 enhances malignant properties of NB cells probably via modifying glycosylation and signaling of b1-integrin. This study is the first to report the pathologic and biologic roles of B4GALT3 in NB and suggests that B4GALT3 may be a potential therapeutic target for NB treatment.

Materials and Methods

Patients and tissue samples
Tissue samples were collected from 101 NB patients receiving treatment at the National Taiwan University Hospital between December 1, 1990 and December 31, 2009 with sufficient tumor tissues and complete follow-up were included in this study. Tumor tissues were obtained from resection or biopsy of the primary or metastatic tumors before any chemotherapy or radiotherapy. The use of human tissues for this study was approved by the National Taiwan University Hospital Ethics Committee and written consent was obtained from patients before collection of sample. Tissue specimens were fixed in 4% (w/v) parafor- maldehyde/PBS. The details of tumor staging, histological classification, and patient treatment have been described elsewhere (18). The demographics of this patient cohort can be found in Table 1.

Immunohistochemistry
Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in a series of graded alcohols. After incubation of 3% H2O2 in PBS with 0.1% Triton X-100 at...
Super Sensitive Link-Label immunohistochemistry Detection room temperature for 1 hour. After rinsing twice with PBS, BSA/PBS at 4°C for 10 minutes, the sections were blocked with 5% bovine serum albumin (BSA) in PBS for 30 minutes. Sections were incubated with a rabbit polyclonal antibody against B4GALT3 (Sigma-Aldrich) at 1:100 in 1% BSA/PBS at 4°C for 16 hours and followed by incubation at room temperature for 1 hour. After rinsing twice with PBS, Super Sensitive Link-Label immunohistochemistry Detection System (BioGenex) was applied to tissue sections. The specific immunostaining was then visualized with 3,3′-diaminobenzidine liquid substrate system (Sigma-Aldrich). All sections were counterstained with hematoxylin and mounted with UltraKitt (J.T. Baker). Negative controls were done by replacing primary antibodies with a control non-immune IgG at the same concentration. The immunoreactivity of B4GALT3 in NB tumors was categorized into 4 groups: "0" (no expression); "1+" (weak expression, expression in ~10–35% of neuroblastic cells); "2+" (moderate expression, expression in ~35–70% of neuroblastic cells); and "3+" (strong expression, expression in >70% of neuroblastic cells).

Table 1. B4GALT3 expression and clinicopathologic and biologic characteristics of neuroblastomas

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases</th>
<th>Positive B4GALT3 expression (%)</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤1.5 year</td>
<td>36</td>
<td>17 (47.2)</td>
<td>0.216</td>
</tr>
<tr>
<td>&gt;1.5 year</td>
<td>65</td>
<td>39 (60.0)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>58</td>
<td>29 (50.0)</td>
<td>0.201</td>
</tr>
<tr>
<td>Female</td>
<td>43</td>
<td>27 (62.8)</td>
<td></td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1, 2, 4S</td>
<td>34</td>
<td>14 (41.2)</td>
<td>0.040&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3, 4</td>
<td>67</td>
<td>42 (62.7)</td>
<td></td>
</tr>
<tr>
<td>Primary tumor site</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenal</td>
<td>60</td>
<td>37 (61.7)</td>
<td>0.128</td>
</tr>
<tr>
<td>Extra-adrenal</td>
<td>41</td>
<td>19 (46.3)</td>
<td></td>
</tr>
<tr>
<td>Shimada histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unfavorable</td>
<td>49</td>
<td>37 (75.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Favorable</td>
<td>52</td>
<td>19 (36.5)</td>
<td></td>
</tr>
<tr>
<td>MYCN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplified</td>
<td>25</td>
<td>18 (72.0)</td>
<td>0.055</td>
</tr>
<tr>
<td>Nonamplified</td>
<td>76</td>
<td>38 (50.0)</td>
<td></td>
</tr>
<tr>
<td>COG risk group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>33</td>
<td>13 (39.4)</td>
<td>0.012</td>
</tr>
<tr>
<td>Intermediate</td>
<td>9</td>
<td>3 (33.3)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>59</td>
<td>40 (67.8)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: COG, Children’s Oncology Group.  
<sup>a</sup>χ² test.  
<sup>b</sup>Stages 1, 2, and 4S versus stages 3 and 4.

Cell culture and transfection

NB cell lines SH-SY5Y and SK-N-DZ from American Type Culture Collection (ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Thermo scientific) containing 10% FBS (Invitrogen, Life Technologies Inc.), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Life Technologies Inc.) in a humidified tissue culture incubator at 37°C and 5% CO2 atmosphere. Genomic profiles of NB cell lines used in this study were listed in Supplementary Table S1. For stable transfection, SH-SY5Y was transfected with B4GALT3/pDNA3.1A (B4GALT3) or pcDNA3.1A/myc-His (Vector; Invitrogen, Life Technologies Inc.) using Lipofectamine 2000 (Invitrogen, Life Technologies Inc.) according to the manufacturer’s protocol. The transfected cells were selected with 400 µg/mL of G418 for 14 days and pooled for further studies.

Knockdown of B4GALT3 expression

Duplex siRNA against B4GALT3 (siB4GALT3) and non-targeting control siRNA (siCtrl) were purchased from Invitrogen. SK-N-DZ cells were transfected with siRNAs using Lipofectamine RNAiMAX (Invitrogen, Life Technologies Inc.) at a final concentration of 10 nmol/L siRNA for 48 hours before experiments. For stable knockdown of B4GALT3, SK-N-DZ cells were infected with shB4GALT3/pLKO.1-puro (shB4GALT3) or shCtrl/pLKO.1-puro (shCtrl; RNAi Core, Academia Sinica) using lentivirus-based infection system. After selected with 1 µg/mL

Table 2. Clinicopathologic and biologic factors affecting survival rate

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Age at diagnosis: &gt;1.5 y versus ≤1.5 year</td>
<td>3.091 (1.495–6.391)</td>
<td>0.002</td>
</tr>
<tr>
<td>Clinical stage: advanced (3, 4) versus early (1, 2, 4S)</td>
<td>21.126 (6.101–67.493)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MYCN: amplified versus nonamplified</td>
<td>4.224 (2.431–7.337)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B4GALT3 expression: positive versus negative</td>
<td>3.979 (1.969–8.039)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Shimada histology: unfavorable versus favorable</td>
<td>6.407 (3.259–12.593)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Primary tumor site: adrenal versus nonadrenal</td>
<td>1.296 (0.740–2.273)</td>
<td>0.365</td>
</tr>
</tbody>
</table>

Abbreviations: RR, risk ratio; 95% CI, 95% confidence interval.
puromycin (Millipore) for 1 week, cells were injected subcutaneously into mice for in vivo cell growth observation.

**Plasmid construction**

Human full-length B4GALT3 was cloned from SH-SY5Y cells. The sense primer was 5’-GGAAGCAGAAGATGTTGCCGAGGCTGCTGGA-3’ and antisense primer was 5’-TCTAAGATCTGGAGGGCGCGCT-3’. The sequence of RT-PCR products was confirmed by DNA sequencing and completely matched human B4GALT3 sequence (NM_003779.2). The DNA was cloned into pcDNA3.1A/myc-His (Invitrogen, Life Technologies Inc.) plasmid.

**MTT assay**

Cells were seeded in hexaplicate wells of 96-well plates, and each well contained 2 × 10³ cells in 100 µL complete DMEM. After incubation for indicated time, 10 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide solution (MTT; Sigma-Aldrich) was added to each well. Cells were incubated with MTT solution at 37°C for 4 hours. The MTT formazan crystals were dissolved with a solution containing 10% (w/v) SDS and 0.01N HCl and the colorimetric intensity was measured at the dual wavelengths of 570 and 630 nm using a spectrophotometer.

**Tumor growth in vivo**

For in vivo tumor growth analysis, 6-week-old female SCID mice and 7-week-old female nude mice (National Laboratory Animal Center, Taiwan) were injected subcutaneously with 2 × 10⁵ of SH-SY5Y transfectants (n = 7 mice for each group) and SK-N-DZ transfectants (n = 6 mice for each group). At day 61 for SH-SY5Y and day 31 for SK-N-DZ after injection, mice were sacrificed and tumors were excised for further analyses. Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University College of Medicine.

**Cell differentiation assay**

Cells were seeded on plates for 1 day and serum-starved on the following day. Cells were lysed and cell lysates were collected for analyses of βIII-tubulin expression by Western blotting.

**Matrigel invasion assay**

Cell invasion assays were done in BioCoat Matrigel invasion chambers (BD Pharmingen) according to the manufacturer’s protocol. Briefly, SH-SY5Y (5 × 10⁵) or SK-N-DZ (2 × 10⁵) cells in 300 µL serum-free DMEM were seeded into upper part of the chamber, and 700 µL complete DMEM containing 10% (v/v) FBS as chemoattractant was loaded in lower part of the chamber. Cells were allowed to invade the matrigel for 48 hours. For β1-integrin functional blocking assay, cells were mixed with β1-integrin blocking antibodies (clone 4A10; Millipore) to a final concentration of 10 µg/mL and incubated at room temperature for 10 minutes before seeding into invasion chambers. Mouse IgG served as an isotype control. The invading cells were fixed with 100% methanol and stained with 0.5% (w/v) crystal violet (Sigma-Aldrich). The number of invaded cells per field was counted and presented as mean ± SD values from at least 3 independent experiments. The cell numbers were counted by 2 investigators.

**Transwell migration assay**

Transwell migration assays were done using 8-µm pore, 6.5-mm polycarbonate transwell filters (Corning Costar Corp.). SH-SY5Y (5 × 10⁵) or SK-N-DZ (2 × 10⁵) cells in 300 µL serum-free DMEM were seeded into upper surface of the filter and allowed to migrate toward complete DMEM. After migration for 48 hours, migrated cells at lower surface of the filter were fixed and stained with 0.5% (w/v) crystal violet (Sigma-Aldrich), and cell numbers were counted under a microscope at 3 random fields. For integrin-dependent cell migration, 5 µg/mL of laminin or fibronectin were coated on the upper surface of filters before seeding of cells. The mean ± SD values were calculated from the numbers of migrated cells/field from at least 3 independent experiments. The cell numbers were counted by 2 investigators.

**Flow cytometry**

SK-N-DZ cells were transfected with siCtrl or siB4GALT3 for 48 hours. Cells were detached using cold 2 mmol/L EDTA and resuspended in 500 µL PBS with 0.5% BSA. Cells (1 × 10⁷/100 µL) were incubated with anti-β1-integrin antibody (clone 18/CD29; BD Pharmingen) at 1:100 in 0.5% BSA/PBS on ice for 30 minutes. Cells were washed twice with ice cold 0.5% BSA/PBS and then incubated with a fluorescein anti-mouse IgG antibody (1:100 in 0.5% BSA/PBS) on ice for 30 minutes. The fluorescence intensity of 1 × 10⁴ cells for each sample was analyzed on a flow cytometer (FACS Calibur; BD Pharmingen). Samples without primary antibody served as negative controls for each cell.

**Western blot analysis and lectin pull-down assay**

B4GALT3 proteins were detected with a rabbit anti-B4GALT3 polyclonal antibody (Sigma-Aldrich). For detection of β1-integrin signaling, antibodies against total β1-integrin (BD Pharmingen), β-actin (Sigma-Aldrich), total-FAK (focal adhesion kinase; Santa Cruz Biotechnology, Inc.), and pY397-FAK (Cell Signaling Technology) were used. Detection of glycoproteins decorated with terminal-galactose or N-acetyllactosamine was achieved by lectin pull-down assays using biotinylated *Ricinus communis* agglutinin I (RCA I), *Datura Stramonium* Lectin (DSL), and *Lycopersicon Esculentum* (Tomato) Lectin (LEL; Vector Laboratories). Briefly, 500 µg of total cell lysates were incubated with biotinylated-RCA I, -DSL, or -LEL at 4°C for 16 hours, streptavidin agarose beads (Vector Laboratories) were then added and incubated for an additional 6 hours. The pulled down proteins were then subjected to Western blot analysis. Intensity of signals on Western blots was quantified by ImageJ 1.42q software (Wayne Rasband).
Integrin degradation assays
Cells were seeded on 12-well plates and adhered for 24 hours. Cycloheximide (10 μg/mL; Sigma-Aldrich) was added to the cells for the indicated time points. Cells were lysed in lysis buffer (Tris 20 mmol/L, pH 8.0, NaCl 137 mmol/L, 1% NP-40, 10% glycerol, Na3VO4 2 mmol/L, β-glycerophosphate 2 mmol/L, PMSF 2 mmol/L, and 1% protease inhibitor cocktail (Sigma-Aldrich) and β1-integrin protein levels were determined by Western blotting. β-Actin was used as an internal control. The 0 hour control was set to 100% and the detected level of β1-integrin was calculated as the percentage of 0 hour control for each time point.

Statistical analyses
Data are presented as mean ± SD. Statistical analyses are carried out using SPSS 10.0 for Windows software (SPSS Inc.). Student t-test was used to compare differences between 2 experimental groups. Pearson χ2 test was used to assess the association between pairs of categorical variables. Survival probabilities in various subgroups were estimated using Kaplan–Meier method, and the significant differences in each group were analyzed by log-rank tests. Each factor possibly affecting patient survival was further analyzed by univariate and multivariate Cox proportional hazard model. All statistical tests were 2-sided and P < 0.05 was considered statistically significant.

Results
B4GALT3 expression and clinicopathologic and biologic factors of NB tumors
We analyzed the public microarray datasets from Oncogenomics: Oberthuer Lab and found that, after P-value minimization, only B4GALT3, but not other B4GALTs, predicted poor survival of NB patients (Supplementary Fig. S1A). In addition, data from Oncogenomics: Seeger Lab also showed that B4GALT3 high expression predicted poor survival of NB patients (Supplementary Fig. S1B). To investigate the clinical importance of B4GALT3 and its correlation with clinicopathologic factors in NB, we examined the protein expression of B4GALT3 in NB tumors by immunohistochemical staining. B4GALT3 in NB cells showed typical staining of the Golgi apparatus (Fig. 1A), but not in Schwannian stromal cells. The immunoreactivity (from 0 to 3+) of B4GALT3 in NB tumors was shown in Fig. 1A. B4GALT3 expression (“1+-”, “2+-”, or “3+-”) was observed in most (71.7%) UNBs and was decreased in more differentiated tumors of differentiating NB (DNB, 45.2%) and ganglioneuroblastoma (GNB, 37.5%). Moreover, the intensity of B4GALT3 immunostaining was also decreased as the histology became differentiated, indicating that B4GALT3 expression inversely correlated with the histological grade of NB differentiation (Fig. 1B, P < 0.001, χ2-test). For further analysis, NB tumors were divided into 2 groups: negative (“0”, no expression of B4GALT3) and positive (“1+-”, “2+-”, “3+-”).
or "3−" expression of B4GALT3) B4GALT3 expression. The immunohistochemical staining revealed positive B4GALT3 expression in 55.5% (56/101) of NB tumors. In addition to histological grade of differentiation, positive B4GALT3 expression also significantly correlated with advanced clinical stages (stages 3 and 4; \( P = 0.04, \chi^2\)-test), and unfavorable Shimada histology (\( P < 0.001, \chi^2\)-test; Table 1). The percentage of positive B4GALT3 expression was also significantly higher in high-risk group patients than that in intermediate or low-risk group patients (\( P = 0.012, \chi^2\)-test; Table 1).

**B4GALT3 expression and patient survival**

Kaplan–Meier analysis showed that NB patients with positive B4GALT3 expression had a lower predictive 5-year survival rate compared to those with negative B4GALT3 expression (40.0% and 76.2% respectively; Fig 1C; \( P < 0.001, \log\)-rank test). Furthermore, univariate analysis showed that, in addition to B4GALT3 expression, patient age at diagnosis >1.5 years, advanced clinical stages (stages 3 and 4), MYCN amplification, and unfavorable Shimada histology strongly correlated with poor patient survival (Table 2). On multivariate analysis, only clinical stage, MYCN status, and B4GALT3 expression could predict patient survival independently (Table 2). To further evaluate the significance of B4GALT3 expression in prognostic discrimination, the impact of B4GALT3 expression on survival rate was analyzed against COG risk grouping. The result revealed that except in patients of low risk with a very good outcome, positive B4GALT3 staining in NB tumors constantly predicted poorer prognosis for patients with either intermediate risk (Fig. 1D; \( P = 0.045, \log\)-rank test) or high-risk disease (Fig. 1E; \( P = 0.015, \log\)-rank test). These results suggest that B4GALT3 expression is an independent prognostic factor of poor survival for NB patients and may provide complimentary prognostic information in addition to COG risk classification.

**Expression of B4GALT3 enhances malignant phenotypes of NB cells**

To elucidate effects of B4GALT3 on NB cell behaviors, we overexpressed B4GALT3 in SH-SY5Y and SK-N-AS cells (those do not express B4GALT3) or transiently knocked down B4GALT3 in SK-N-DZ and SK-N-BE cells (those express B4GALT3 endogenously). The overexpression or knockdown of B4GALT3 was confirmed by Western blotting and immunofluorescence staining (Fig. 2A; Supplementary Fig. S2A). Our results showed that B4GALT3 expression did not significantly affect cell growth by MTT assays observed for 4 days in all experimental cell lines (Fig. 2B; Supplementary Fig. S2B).

To examine the effect of B4GALT3 on tumor growth in vivo, mice were subcutaneously injected with vector- or B4GALT3-transfected SH-SY5Y cells as well as shCtrl- or shB4GALT3-transfected SK-N-DZ cells. We found that overexpression of B4GALT3 significantly increased tumor weights (Fig. 2C, top) and knockdown of B4GALT3 suppressed tumor growth in tumor sizes and weights (Fig. 2C, bottom). The results suggest that B4GALT3 could enhance NB tumor growth in vivo. Tumor xenografts were subjected to hematoxylin and eosin (H&E) staining and immunohistochemistry for erythrocytes, microvessel density, and cell proliferation observation. H&E staining showed less stromal tissues in SH-SY5Y/B4GALT3 tumors compared with control tumors (Supplementary Fig. S3A, top). In addition, B4GALT3 expression significantly increased microvessel density and percentage of Ki67-positive tumor cells (Supplementary Fig. S3). Conversely, SK-N-DZ/shB4GALT3 tumors showed that knockdown of B4GALT3 resulted in less blood perfusion, smaller diameter of blood vessels, and less Ki67-positive tumor cells compared with SK-N-DZ/shCtrl control tumors (Supplementary Fig. S4). These results suggest that B4GALT3 expression modulates tumor microenvironment in vivo.

Because differentiation is an important figure of NB tissue grading, we examined the neuronal differentiation of SH-SY5Y and SK-N-DZ transfectants using βIII-tubulin as a neuronal differentiation marker and serum-starvation as a trigger for differentiation. Overexpression of B4GALT3 in SH-SY5Y suppressed serum starvation-triggered neuronal differentiation (Fig. 2D, left) and knockdown of B4GALT3 in SK-N-DZ directly increased neuronal differentiation without serum-starvation (Fig. 2D, right).

To examine effects of B4GALT3 on cell migration and invasion, transwell migration assay and matrigel invasion assay were done, respectively. Our results showed that B4GALT3 overexpression significantly enhanced migration and invasion in SH-SY5Y (Fig. 2E and F) and SK-N-AS cells (Supplementary Fig. S5A and S5B). Conversely, knockdown of B4GALT3 significantly suppressed migration and invasion in SK-N-DZ (Fig. 2D and E) and SK-N-BE cells (Supplementary Fig. S5A and S2B).

These results suggest that B4GALT3 expression enhances NB cell migration and invasion in vitro and contribute to tumor growth in vivo.

**β1-Integrin participates in B4GALT3-mediated cell migration and invasion**

β1-Integrins are crucial cell-ECM receptors that regulate numerous cell phenotypes including cell survival, migration, and invasion. In addition, glycosylation of β1-integrin has been found to modulate these biological functions (19). We therefore investigated whether β1-integrin plays a role in B4GALT3-enhanced cell migration and invasion. Our results showed that knockdown of B4GALT3 significantly suppressed cell migration toward β1-integrin ligands, fibronectin and laminin, in SK-N-DZ and SK-N-BE (Supplementary Fig. S6). Because SH-SY5Y and SK-N-AS cells could not migrate well toward ECMs under serum-free condition, 10% FBS was added to lower chamber as a chemoattractant. B4GALT3 overexpression significantly enhanced cell migration through fibronectin- or laminin-coated transwells in SH-SY5Y (Fig. 3A) and SK-N-AS cells (Supplementary Fig. S7A). In addition, β1-integrin blocking antibody, but not control IgG, inhibited migration of vector transfected cells and blocked the B4GALT3-enhanced cell migration toward...
ECMs. By contrast, knockdown of B4GALT3 inhibited cell migration toward fibronectin or laminin in SK-N-DZ (Fig. 3B) and SK-N-BE cells (Supplementary Fig. S7B). Furthermore, β1-integrin blocking antibody, but not control IgG, suppressed cell migration toward laminin in control siRNA knockdown SK-N-DZ (Fig. 3B) and SK-N-BE cells (Supplementary Fig. S7B). These results indicate that β1-integrin plays a role in regulating B4GALT3-enhanced migration toward ECM proteins. Moreover, we also observed that B4GALT3-increased invasion was significantly suppressed by β1-integrin blocking antibody in SH-SY5Y (Fig. 3C) and SK-N-AS cells (Supplementary Fig. S7C). In control siRNA-transfected SK-N-DZ (Fig. 3D) and SK-N-BE cells (Supplementary Fig. S7D), β1-integrin blocking antibody could reduce cell invasion through matrigel. Together, these data suggest that β1-integrin plays a role in NB cell migration and invasion and is involved in B4GALT3-enhanced migration and invasion in NB cells.

B4GALT3 modifies glycosylation and signaling of β1-integrin

Because β1-integrin is a crucial receptor for NB cell migration and invasion and B4GALT3-enhanced migration and invasion can be suppressed by β1-integrin blocking...
Effects of β1-integrin blocking antibody on B4GALT3-regulated migration and invasion. Cells were seeded onto upper surface of fibronectin-, laminin-, or matrigel-coated filters and 10% FBS was served as chemotactrant. β1-integrin blocking antibody (β1-integrin Ab, 10 μg/mL or isotype-matched control IgG (IgG) was applied to cells for observing the role of β1-integrin in cell migration and invasion. Cells were allowed to migrate or invade for 2 days. A, B4GALT3-enhanced migration on fibronectin or laminin was blocked by β1-integrin blocking antibody in SH-SY5Y cells. B, B4GALT3 knockdown suppressed cell migration on fibronectin or laminin in SK-N-DZ cells. C, effects of β1-integrin blocking antibody on B4GALT3-enhanced invasion in SK-N-SH SY5Y cells. D, effects of β1-integrin blocking antibody on invasion of SK-N-DZ cells transfected with siCtrl or siB4GALT3. All data are shown as mean ± SD from 3 independent experiments. *, P < 0.05; **, P < 0.01.

B4GALT3 increases mature β1-integrin through delayed protein degradation

To investigate the effect of B4GALT3-altered glycosylation on β1-integrin, we examined the expression of β1-integrin in NB cells. Two different glycoforms of β1-integrin have been reported (20). The mature form of fully glycosylated β1-integrin exhibits a molecular weight of about 130 kDa, whereas the immature form with partial glycosylation is around 115 kDa. Our data showed that overexpression or knockdown of B4GALT3 in NB cell lines had no significant influences on mRNA levels of β1-integrin (Supplementary Fig. S9). Interestingly, we found that overexpression of B4GALT3 increased mature form, but not immature form, of β1-integrin in SH-SY5Y (Fig. 5A) and SK-N-AS (Supplementary Fig. S10A) cells. By contrast, knockdown of
B4GALT3 decreased protein expression of β1-integrin mature form in SK-N-DZ (Fig. 5A) and SK-N-BE cells (Supplementary Fig. S10B). To further verify effects of B4GALT3 on the expression level of mature form of β1-integrin, flow cytometry was done. Consistently, our data showed that the surface level of β1-integrin was increased in B4GALT3-transfected SH-SY5Y cells compared with vector-transfected cells, but was decreased in SK-N-DZ cells knocked down with B4GALT3 siRNA compared with control siRNA (Fig. 5B).

To determine whether changes in the level of mature form of β1-integrin was because of altered protein stability, we performed protein degradation assays in cells treated with cycloheximide which blocks protein synthesis. After 9 h treatment of cycloheximide, mature form of β1-integrin decreased to 53 ± 8% and 84 ± 10% in vector- and B4GALT3-transfected SH-SY5Y cells, respectively (Fig. 5C), suggesting that B4GALT3 overexpression delayed the degradation of mature form of β1-integrin. Moreover, knockdown of B4GALT3 in SK-N-DZ cells decreased the mature form of β1-integrin to 75 ± 8% compared with 106 ± 7% for control siRNA knockdown (Fig. 5C). Collectively, these results suggest that B4GALT3 stabilizes the mature form of β1-integrin to increase its surface expression on NB cells.

Discussion

GnT-V catalyzes the synthesis of β1,6GlcNAc branches on N-glycans that are elongated with poly-N-acetyllactosamine and is able to enhance tumor growth and metastasis (21). Lack of GnT-V activity in mice results in delayed tumor growth and is able to enhance tumor growth and metastasis (21). Therefore, it is suggested that GnT-V-modified poly-N-acetyllactosamine on N-glycans increases cancer malignancy. However, poly-N-acetyllactosamine levels on N-glycans, O-glycans, and glycolipids are dependent on the activity of both B4GALT and β-1,3-N-acetylgalcosaminyltransferase (B3GNT) family.
enzymes. The individual roles of these enzymes in the formation of poly-N-acetyllactosamine structures in vivo and in modulating cancer cell behaviors still remain unclear. Here we show that B4GALT3 is an unfavorable prognostic factor for NB and B4GALT3 expression can increase poly-N-acetyllactosamine levels on β1-integrin to enhance NB cell migration and invasion. Poly-N-acetyllactosamine structures have been found on β1 subunit of α3β1-integrin (22, 23), a receptor for laminin, fibronectin, and collagen. In vitro enzyme activity assays indicate that B4GALT3 catalyzes the formation of poly-N-acetyllactosamine (13). Consistent with these results, we found that B4GALT3 changes the amount of poly-N-acetyllactosamine on β1-integrin as revealed by altered RCA1 and LEL binding. We also observed that the B4GALT3-modified poly-N-acetyllactosamines are present on both N-glycans and O-glycans of β1-integrin. Our findings therefore suggest that B4GALT3 is one of the determinants that modulate the formation of poly-N-acetyllactosamine on β1-integrin.

It has been reported that protein levels of β1-integrin are decreased in more invasive NB cells (24). Reduced β1-integrin is suggested to release cells from ECM attachment and increase cell migration and invasion. However, we found that in NB cells B4GALT3 overexpression elevates expression of the mature form of β1-integrin and enhances migration and invasion. Conversely, B4GALT3 knockdown in NB cells decreases mature β1-integrin levels and suppresses these phenotypes. Furthermore, β1-integrin blocking antibody significantly suppresses NB cell migration and invasion, which is consistent with our previous study indicating that β1-integrin blocking antibody suppresses SK-N-SH cell migration and invasion (18). These results suggest that although low levels of β1-integrin are expressed on surfaces of invasive NB cells, activation of β1-integrin and enhancement of its downstream signaling are required for migration toward ECM and invasion through matrigel. Interestingly, several lines of evidence suggest that cell movement rates are maximal only at intermediate levels of...
adhesion (25). Strong adhesion to the ECM blocks migration, whereas at low adhesion the traction force generated is not enough to increase movement (25–27). Our results showed that B4GALT3-enhanced NB cell migration and invasion could be inhibited by β1-integrin blocking antibody. These findings suggest that the enhancement of β1-integrin activity to an optimal level is involved in the B4GALT3-facilitated migration and invasion of NB cells.

Glycosylation has been reported to regulate several properties of integrins, including protein conformation, receptor clustering, heterodimer formation, endocytosis, degradation rate, and recycling, as well as interactions between integrins and extracellular proteins (25). All of which are able to control integrin functions and cellular properties. We found that B4GALT3 increases expression of lactosamine sugars on the mature form of β1-integrin, which delays β1-integrin degradation and thereby enhances its downstream signaling. Therefore, we suggest that B4GALT3 modulates β1-integrin functions at least, in part, via regulating protein turnover of mature β1-integrin on cell surfaces. In contrast, in human fibrosarcoma HT1080 cells, GnT-V enhances migration by modulating integrin clustering and subsequent signal transduction pathways without changes in surface levels of α5- or β1-integrin (16). These findings suggest that the effects of B4GALT3 on β1-integrin are different from those generated by GnT-V, although both enzymes are able to increase poly-N-acetyllactosamine on β1-integrin. Because we found that B4GALT3 can generate poly-N-acetyllactosamine on both N- and O-glycans on β1-integrin, it is therefore reasonable to speculate that the differential effects mediated by B4GALT3 may result from the B4GALT3-modified poly-N-acetyllactosamine on O-glycans of β1-integrin. In addition to protein degradation, it remains possible that altered glycosylation of β1-integrin mediated by B4GALT3 can activate β1-integrin directly via conformational changes, which lead to increased cell migration and invasion. Because β1-integrin is not the only acceptor substrate for B4GALT3, it will be very interesting to identify other acceptor substrates for a complete understanding of the mechanisms by which B4GALT3 regulates NB cell behaviors.

β1-Integrins partner with α subunits to form 12 potential receptors, which bind to a wide range of RGD-containing ECM proteins, such as collagens, laminin, and fibronectin (28). Bidirectional signals of β1-integrin play a crucial role in apoptosis, differentiation, survival, migration, invasion, tumor progression, and metastasis (28). Interestingly, inhibition of β1-integrin or its downstream signaling partners has been shown to inhibit metastasis and to maintain tumor cells in a dormant state (28). Therefore, β1-integrin has been proposed to be a potential therapeutic target against cancer recurrence (28). This study showed that B4GALT3 expression predicts unfavorable outcomes of NB patients. In addition, knockdown of B4GALT3 suppresses integrin signaling and cell migration and invasion. These findings suggest that B4GALT3 may be a therapeutic target for NB treatment. To evaluate this possibility, we are currently synthesizing B4GALT3 inhibitors for treating mice xenografted with human NB tumor cells.

Glycosylation of surface and secreted molecules, such as ECM proteins, has been showed to modulate tumor progression via alterations in tumor microenvironment (29). Our results showed that B4GALT3 expression modulates tumor growth in an in vivo mouse model, but not in an in vitro cell growth system. We found that B4GALT3 over-expression results in less stromal tissues and higher blood vessel density in SH-SY5Y/B4GALT3 xenografts. In addition, B4GALT3 knockdown tumors have less blood perfusion and smaller blood vessels. These results therefore suggest that B4GALT3 expression increases tumor formation in vivo mainly via regulation of tumor microenvironment. Furthermore, B4GALT3 expression modulates neuronal differentiation of NB cells in vitro. It is therefore likely that more differentiated NB cells tend to undergo apoptosis early after tumor cell injection into mice. Interestingly, it has been suggested that inappropriate stromal tissues in tumor microenvironment can trigger apoptosis by unligated integrins via a process known as integrin-mediated death (IMD; ref. 30). Caspase-8 together with integrins regulates the IMD to modulate survival and invasiveness of NB cells (31). Because B4GALT3 can modulate protein levels and activity of β1-integrin as well as stromal tissues in tumor xenografts, further studies are required to establish the roles of B4GALT3 in alterations of tumor microenvironment.

Our results establish that B4GALT3 expression is a novel independent unfavorable prognostic factor for NB patients. The COG risk grouping is now widely accepted for prognostic discrimination and treatment allocation of NB patients (4). NB patients in the low-risk group have excellent outcomes, whereas NB patients in either the intermediate or high-risk group are 2 large groups showing prognostic heterogeneity. Additional prognostic factors may be required to further discriminate these 2 groups of patients. Here, we show that B4GALT3 expression predicts an unfavorable prognosis for NB patients in either intermediate or high-risk group. Therefore, assessing B4GALT3 protein expression in NB tumors could provide complementary prognostic information in addition to COG risk grouping, allowing clinicians to determine the most appropriate therapy or to develop personalized treatment for the NB patients.

In summary, this study shows that B4GALT3 protein expression is an independent prognostic factor and predicts poor outcomes in NB patients, complementary to clinical stage and MYCN status. Our results also indicate that B4GALT3 expression increases malignant phenotypes of NB cells in vivo and tumor growth in vivo, whereas knockdown of B4GALT3 suppresses NB cell invasive properties. Mechanistic investigation reveals that B4GALT3 expression modifies lactosamine structures on β1-integrin, delays protein degradation of β1-integrin, and enhances its downstream signaling. Our findings open novel insights into the role of lactosamine structures in NB development and suggest that B4GALT3 may be a potential target for NB treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
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