Supplementary Materials & Methods

Patients and treatment

The patient cohort in this study was somewhat different from that of our previous study (1). Two patients without sufficient tissues for study were excluded, while 16 new patients treated during 2007 and 2009 were added, which made the total patient number into 101 in the current study. Male/Female distribution of NB patients in this study was 58:43, and a median age at diagnosis was 2.5 years (range, 0-13.5 years). Most tumors originated primarily from adrenal gland (52 cases), with others from retroperitoneum (19 cases), mediastinum (7 cases), neck (5 cases), and pelvis (4 cases). The tumor histology was categorized into 4 types according to the criteria of the International NB Pathology Classification (ref): i) NB (including undifferentiated, poorly differentiated, and differentiating subtypes); ii) ganglioneuroblastoma (GNB), intermix; iii) GNB, nodular; iv) GNB, maturing subtype. GNB, maturing subtype is a benign lesion and is not included in this study. For simplifying the description of correlation between B4GALT3 expression and histologic feature of NB tissues, we defined undifferentiated NB (UNB), poorly differentiated and differentiating NB (DNB), and GNB, intermixed for tumor histologic statement in this study. For classification of GNB, nodular subtype, the tumor behavior of this subtype depends mainly on the NB nodules, so nodular subtype was classified into either UNB or DNB.
according to the morphologic features of nodules. For prognostic analysis, tumors were classified into favorable or unfavorable histologic types. GNB, intermixed was classified as a favorable histologic type, and UNB and DNB were classified as either favorable or unfavorable according to the mitosis-karyorrhexis index and patient age at diagnosis based on the criteria of the Shimada’s classification system (2). The distinction of tumor staging was based on the International NB Staging System. MYCN status of the tumor tissue was evaluated by fluorescence in situ hybridization analysis of formalin-fixed paraffin-embedded tissues or fresh tumor single cells. Patients were treated with surgery only or a combination of multiple modalities including chemotherapy, radiotherapy, and/or autologous bone marrow transplantation according to the patient’s risk grouping based on the risk classification of the Children’s Cancer Group. The median follow-up after diagnosis was 70.6 months with a range of 1 to 204 months, and the overall predictive 5-year survival rate for this cohort was 48.7%.

**Immunohistochemistry**

Immunohistochemistry was performed as described previously in this article. Rabbit polyclonal anti-CD31 antibodies (LifeSpan Biosciences, Inc.) and anti-Ki67 antibodies (Vector Laboratories) were used for blood vessel and proliferating cell
staining.

cDNA synthesis and quantitative PCR

Total RNA from cultured cells was collected using TRIzol reagent (Invitrogen, Life Technologies Inc.) according to manufacturer’s protocol. Quantitative PCR System Mx3000P (Stratagene) was used for quantification of RNA levels. Primers for ITGB1 (β1 integrin) (sense: 5’-CATCTGCGAGTGTGGTGTCT-3’; antisense: 5’-GGGGTAATTTGTCCCGACTT-3’), and GAPDH (sense: 5’-ACAGTCAGCCGCATCTTCTT-3’; antisense: 5’-GACAAGCTTCCGGCTTCAG-3’) were designed by Primer 3 (v.0.4.0). Gene expression levels were normalized to GAPDH using MxPro Software (Stratagene).

Cell culture and transfection

NB cell lines SK-N-AS and SK-N-BE 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. For transient transfection, SK-N-AS was transfected with B4GALT3/pcDNA3.1A
(B4GALT3) or pcDNA3.1A/myc-His (vector) (Invitrogen, Life Technologies Inc.) using Lipofectamine 2000 (Invitrogen, Life Technologies Inc.) according to the manufacturer’s protocol. SK-N-BE cells were transfected with siRNAs using Lipofectamine RNAiMAX (Invitrogen, Life Technologies Inc.) at a final concentration of 20 nM siRNA for 48 h according to the manufacturer’s instruction.

**Matrigel invasion assay**

Cell invasion assays and β1 integrin functional blocking assays were performed as described previously in this article. SK-N-AS (5 × 10⁴ cells/chamber) or SK-N-BE (1 × 10⁵ cells/chamber) cells in serum-free DMEM were seeded into upper part of the chamber and 10% (v/v) FBS as chemoattractant was loaded in lower part of the chamber. After invasion for one day, cells were fixed and stained, and the number of invaded cells was counted.

**Transwell migration assay**

Transwell migration assays were performed as described previously in this article. SK-N-AS (5 × 10⁴ cells/chamber) or SK-N-BE (1 × 10⁵ cells/chamber) cells in serum-free DMEM were seeded into upper part of the chamber and 10% (v/v) FBS as chemoattractant was loaded in lower part of the chamber. Cells were fixed and stained.
after invasion for one day. For ECM-triggered cell migration, 1 \( \mu \)g/mL of laminin or fibronectin were coated on upper part of the filter for cell attachment and 5 \( \mu \)g/mL of laminin or fibronectin in serum-free DMEM were loaded to lower part of chamber as chemoattractants. Integrin-dependent cell migration was performed as described previously.

**Figure legends of supplementary figures**

**Figure S1.** Kaplan-Meier survival analysis from Oncogenomics database according to the expression of B4GALT3 in NB patients. A, data from Oberthuer Lab. The neuroblastoma prognosis database – Oberthuer lab (3) in Oncogenomics (http://pob.abcc.ncifcrf.gov/cgi-bin/JK) was analyzed. B, data from Seeger Lab. The data were retrieved from the neuroblastoma prognosis database – Seeger lab (4). \( P \) value was calculated using log-rank test.

**Figure S2.** Transient overexpression of B4GALT3 in SK-N-AS cells and knockdown of B4GALT3 in SK-N-BE cells. SK-N-AS cells were transiently transfected with pcDNA3.1 control plasmid (vector) or B4GALT3/pcDNA3.1 plasmid (B4GALT3). SK-N-BE cells were transiently transfected with non-targeting control siRNA (siCtrl)
or B4GALT3 siRNA (siB4GALT3). A, Temp overexpression or knockdown of B4GALT3 was confirmed by immunoblotting (IB) with anti-B4GALT3 antibody. Actin is an internal control. B, effects of B4GALT3 on cell growth by MTT assay. The results were standardized by day 0 of each cell to 1.0. Error bar = SD.

**Figure S3.** Xenografts from SCID mice subcutaneously injected with SH-SY5Y/vector or SH-SY5Y/B4GALT3 cells. A, tissues from tumor xenografts were subjected to H&E (upper panel) and immunohistochemical staining using anti-CD31 (middle panel) and anti-Ki67 (lower panel) antibodies. Images of H&E and CD31 staining were taken at 200X magnification and images of Ki67 staining were taken at 400X magnification. B, percentage of areas covered by blood vessels. C, average diameter of blood vessels. Blood vessel diameter was measured and calculated from all blood vessels in each image. D, microvessel density. Number of blood vessels (CD31-positive staining) was counted in each field. E, percentage of Ki67-positive cells. For statistical analyses, images of three random fields were taken from each mouse, and five mice were analyzed for each group. Data are represented as mean ± SD. *, *P < 0.05. Scale bar = 50 μm in H&E and CD31 staining. Scale bar = 10 μm in Ki67 staining.
**Figure S4.** Xenografts from nude mice subcutaneously injected SK-N-DZ/shCtrl and SK-N-DZ/shB4GALT3 cells. Tissues from tumor xenografts were subjected to H&E (upper panel) and immunohistochemical staining using anti-CD31 (middle panel) and anti-Ki67 (lower panel) antibodies. B, percentage of areas covered by blood vessels. C, average diameter of blood vessels. D, microvessel density. E, percentage of Ki67-positive cells. For statistical analyses, images of three random fields were taken from each mouse, and five mice were analyzed for each group. Data are represented as mean ± SD. *, \( P < 0.05; **, P < 0.01. \) Scale bar = 50 \( \mu m \) in images of H&E and CD31 staining. Scale bar = 10 \( \mu m \) in Ki67 staining.

**Figure S5.** Effects of B4GALT3 on NB cell migration and invasion. A, transwell migration assays were used for evaluating the effect of B4GALT3 on cell migration ability. Cells were seeded in serum-free DMEM and 10% FBS was used as chemoattractants. B, effects of B4GALT3 on NB cell invasion using matrigel invasion assays. Chemoattractant in the lower chamber was 10% FBS. Data are shown as mean ± SD from at least 3 independent experiments. *, \( P < 0.05; **, P < 0.01. \)

**Figure S6.** Effects of B4GALT3 on cell migration toward \( \beta1 \)-integrin substrates, fibronectin and laminin. Before cells were seeded in transwell chambers, one \( \mu g/mL \)
of fibronectin or laminin was coated on upper surface of chambers. Control siRNA and B4GALT3 siRNA transfected SK-N-DZ (A) and SK-N-BE (B) were seeded onto upper parts of chambers in serum-free DMEM. Five μg/mL of fibronectin or laminin were added to lower part of chambers which generate an ECM-gradient that triggered cell migration. Cells were allowed to migrate for one day. Data are shown as mean ± SD from at least 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$.

**Figure S7.** 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 chemoattractant. 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 one day. A, B4GALT3-enhanced migration on fibronectin or laminin was blocked by β1 integrin blocking antibody in SK-N-AS cells. B, B4GALT3 knockdown suppressed cell migration on laminin in SK-N-BE cells. C, effects of β1 integrin blocking antibody on B4GALT3-enhanced invasion in SK-N-AS cells. D, effects of β1 integrin blocking antibody on invasion of SK-N-BE cells transfected with Control or B4GALT3 siRNA. Data are shown as means ± SD. *, $P < 0.05$; **, $P < 0.01$. 
Figure S8. Poly-N-acetyllactosamines on both N-glycans and O-glycans of β1-integrin are modified by B4GALT3. SH-SY5Y cells transfected with mock (-) or B4GALT3 (+) were treated with 2 μM benzyl-α-GalNAc, a core 2 O-glycan synthesis inhibitor, or 25 μM swansonine, an N-glycan processing inhibitor for 3 days. β1-integrin decorated with poly-N-acetyllactosamines was pulled down by LEL lectin and detected by Western blotting with anti-β1-integrin antibody.

Figure S9. Quantitative RT-PCR analysis of mRNA levels of β1-integrin. The expression levels were normalized with the expression of GAPDH. Data are presented as means ± SD from at least 3 independent experiments.

Figure S10. B4GALT3 modulates protein levels of mature β1 integrin in SK-N-AS and SK-N-BE cells. Expression levels of β1 integrin were analyzed by Western blotting as shown in the upper panel. Thick and thin arrows indicate 125-kDa mature and 105-kDa immature forms of β1 integrin, respectively. Relative band intensities of total, mature form, and immature form of β1 integrin in SK-N-AS (A) and SK-N-BE (B) cells were analyzed by ImageJ software. Actin is an internal control. Effects of B4GALT3 on the level of total, mature form, and immature form of β1 integrin were
quantified and shown in the lower panels. All results are presented as means ± SD from 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$. 
Supplementary References:


