Supporting Information

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SI Materials and Methods

Reagents. Anti-Lewis x (anti-Le^x), anti-sialyl Lewis x (anti-sLe^x), and anti-GD2 antibodies were purchased from BD Biosciences. Anti-GD1a, anti-GT1b, and Alexa Fluor 488 anti-A2B5 antibodies were purchased from Millipore. Antibodies against complex gangliosides GM1 and GM2 (anti-GM1 and anti-GM2, respectively) were purchased from Calbiochem. Anti-Lewis y (anti-Le^y) and anti-sialyl Tn (anti-sTn) antibodies were purchased from Abcam. Anti-Thomsen–Friedenreich (TF) antigen antibody was purchased from Thermo Scientific. Anti-Tn antibody was purchased from DakoCytomation. Fluorescence-labeled or purified MC813-70 (anti–SSEA-4 mAb) and MC631 were purchased from Biolegend. MC813-70 ascites was purchased from the Developmental Studies Hybridoma Bank at the University of Iowa. The use of these antibodies in individual experiments is described in the following paragraphs.

Cell Culture. U-251, U-138, LN-18, T98, LN-229, U87, U-373, Hs683, D54MG, GBM 8401, GBM 8901, G5T, G9T, SNB75, A172, and SF126 cells were routinely maintained in high-glucose DMEM (Life Technologies) supplemented with 10% (vol/vol) FBS (Biological Industries). DBTRG cells were maintained in RPMI 1640 (Life Technologies) with 10% (vol/vol) FBS.

Immunofluorescent Staining. Cells were plated on plastic tissue-culture chamber slides (Nunc) overnight to allow sufficient attachment, fixed with 4% (wt/vol) paraformaldehyde for 15 min at room temperature, washed three times with PBS, and then blocked with 3% (wt/vol) BSA in PBS. Cells then were incubated overnight with 10 μg/mL of mAb MC813-70 (Biolegend), washed three times with PBS, and incubated for 2 h at room temperature with 5 μg/mL FITC-conjugated anti-mouse IgG (eBioscience). Nuclei were counterstained with Hoechst 33342 (2 μg/mL) (Life Technologies). All images were acquired by an Olympus IX71 microscope.

Immunohistochemistry. For MC813-70 staining on normal brain and glioblastoma multiforme (GBM) specimens, three different tissue microarray slides (Biomax), comprising a total of 19 normal brain sections and 55 GBM sections, were tested. The slides were dried at 56 °C for 1 h, deparaffinized in xylene, and rehydrated in graded alcohols, followed by treatment with blocking buffer [2% (wt/vol) Blocking Reagent (Roche) in PBS with 0.1% Triton X-100] for 30 min at room temperature. The slides then were incubated overnight at 4 °C with mAb MC813-70 (10 μg/mL) in blocking buffer. After gentle washing with PBS and Tween-20 (PBST), the immunoreactivity on specimens was detected with the SuperSensitive Polymer-HRP IHC Detection System (BioGenex), and the slides were counterstained with hematoxylin and prepared for mounting.

Glycan Array Fabrication. Microarrays were printed (BioDot; Cartesian Technologies) by robotic pin (SMP3; TeleChem International Inc.) with the deposition of ∼0.6 nL per spot. Amine-containing glycans in printing buffer [300 mM sodium phosphate (pH 8.5), 0.01% Triton X-100] were spotted onto *N*-Hydroxysuccinimide (NHS)-activated glass slides. Each glycan was printed at 100 μM in a replicate of four or at 50 μM in a replicate of six for K_d determination. Printed slides were allowed to incubate in 80% humidity for 30 min, followed by desiccation overnight. Remaining NHS groups were blocked by immersing the slides for 1 h in SuperBlock (PBS) Blocking Buffer (Pierce).

Antibody-Binding Assay. mAb MC813-70 (Alexa Fluor 647; Biolegend) was prepared in 100 μL of PBS-BSA-Tween (pH 7.4, with 3% (wt/vol) BSA and 0.05% Tween-20) and applied to cover the grid. After incubation in a moist chamber for 30 min, the slides were rinsed with PBST and deionized water and were blow-dried. The slides were scanned at 635 nm in GenePix 4300A (Molecular Devices). Data were analyzed by GenePix Pro-6.0 (Molecular Devices).

Sialidase Treatment. Cells were washed and resuspended in PBS buffer at 1×10^7 cells/mL. Cells (10^6 cells/100 μL) were incubated with or without 500 mU α2,3 sialidase (New England BioLabs) for 1 h at 37 °C and were washed twice with FACS buffer followed by surface staining and flow cytometry. The efficiency of sialidase treatment was measured by biotinylated *Maackia amurensis* lectin II (MAL II; Vector Laboratories), which recognizes α2,3-linked sialic acids.

Extraction of Glycosphingolipids. Cells (4×10^7) were harvested, washed, with PBS, and homogenized in water. Methanol and chloroform were added to the homogenate at a ratio of 8:4:3 (vol/vol/vol), and the sample was incubated in a bath sonicator for 30 min. After centrifugation at 3,000 × *g* for 15 min, the pellet was repeatedly extracted with 4:8:3 (vol/vol/vol) chloroform/methanol/water, and the combined supernatant was dried under a stream of nitrogen. The total lipid extract then was dissolved in chloroform/methanol/water (30:60:8, vol/vol/vol), and gangliosides were purified by DEAE-Sephadex A-25 (GE Healthcare)-based anion-exchange chromatography. Unbound flow-through containing neutral glycolipids was collected and dried. After washing with chloroform/methanol/water (30:60:8, vol/vol/vol), gangliosides were eluted with chloroform/methanol/aqueous NaCl (0.02, 0.2, and 0.8 M stepwise) (30:60:8, vol/vol/vol), followed by desalting with Sep-Pak C18 Cartridges (Waters). The extracts were dried under nitrogen, and the ganglioside residues and neutral glycolipid residues were redissolved in 100 μL chloroform/methanol (2:1, vol/vol).

High-Performance TLC. Glycosphingolipids (GSLs) were separated on high-performance TLC (HPTLC) plates precoated with glass-packed silica gel 60 (Merck). Gangliosides were chromatographed in chloroform/methanol/water (120:85:20, vol/vol/vol). and neutral GSLs were chromatographed in chloroform/methanol/water (120:70:17, vol/vol/vol), each supplemented with 2 mM $CaCl_2$. For analytic purposes, GSLs were stained with 0.3% orcinol in 3 M H_2SO_4 and then were transferred to a preheated heating plate (110 °C) until blue/purple spots appeared.

MALDI-MS Profiling and MS/MS Analysis. MALDI-MS analysis of permethylated glycans was conducted in an ABI 4700 Proteomics Analyzer (Applied Biosystems) using 2,5-dihydroxybenzoic acid (DHB) as the matrix (10 mg/mL). MALDI-MS/MS sequencing with low- and high-energy collision-induced dissociation was performed in a Q/TOF Ultima MALDI (Waters Micromass) and a 4700 Proteomics Analyzer using the DHB matrix as described above.

Complement-Dependent Cytotoxicity Assay. The complement-dependent cytotoxicity activity of anti–stage-specific embryonic antigen-4 (anti–SSEA-4) (MC813-70) mAb was measured by lactate dehydrogenase (LDH)-release assay using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega). Cells (1×10^4) were plated in each well of 96-well plates and were washed with PBS twice after overnight growth. The cells then

were incubated with 1 μg MC813-70 or mouse IgG3 isotype (control) in 50 μL phenol red-free DMEM or RPMI with rabbit complement (1:5 dilution) (Life Technologies). After incubation in a 5% CO_2 incubator at 37 °C for 1 h, the degree of cell lysis was determined by measuring the amount of LDH released into the culture supernatant. Maximum LDH release was determined by lysing the cells with the LYSIS Solution provided with the kit. Percentage of specific lysis was calculated according to the equation: % lysis = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

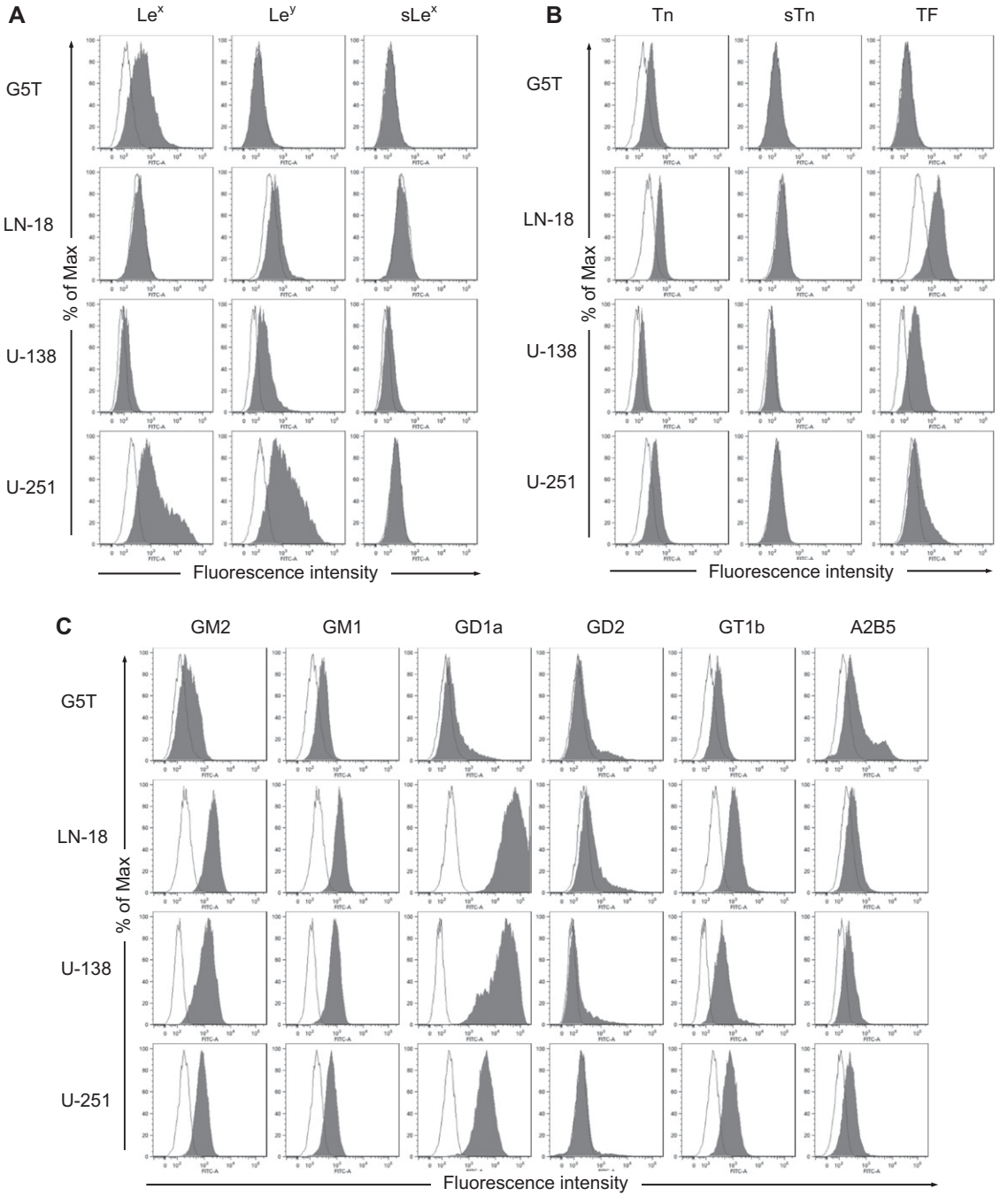


Fig. S1. (Continued)

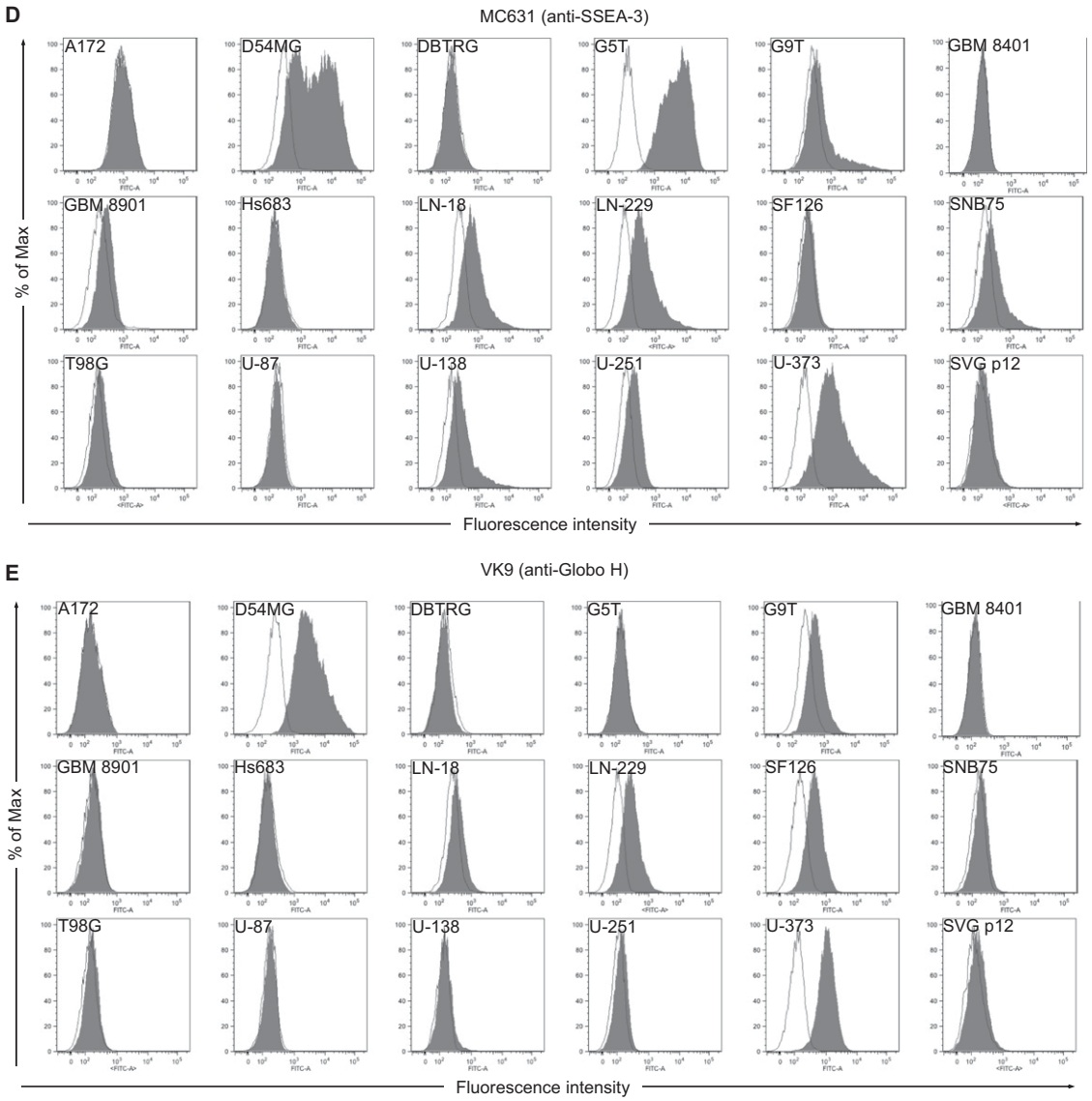


Fig. S1. Flow cytometric analyses of glycan-related molecules on GBM cell lines. Expression of Lewis antigens Le^x, Le^y, and sLe^x (*A*), O-linked glycans Tn, sTn, and TF (*B*), complex gangliosides GM2, GM1, GD1a, GD3, GD2, GT1b, and A2B5 (c-series gangliosides) (*C*), stage-specific embryo antigen 3 (SSEA-3) (*D*), and fucosyl Gb5Cer (Globo H) (*E*) were analyzed. Cells were incubated with optimal concentrations of the primary antibodies and fluorescent-labeled secondary antibodies or Alexa Fluor 488-conjugated MC631 or allophycocyanin-conjugated VK9 and were analyzed by a FACSCanto flow cytometer. All cells examined were GBM cell lines, except for SVG p12, which is a normal human fetal glial cell line transformed with SV40 large T antigen. The histograms of the cells stained with specific antibodies and isotype control are shown in gray and white, respectively.

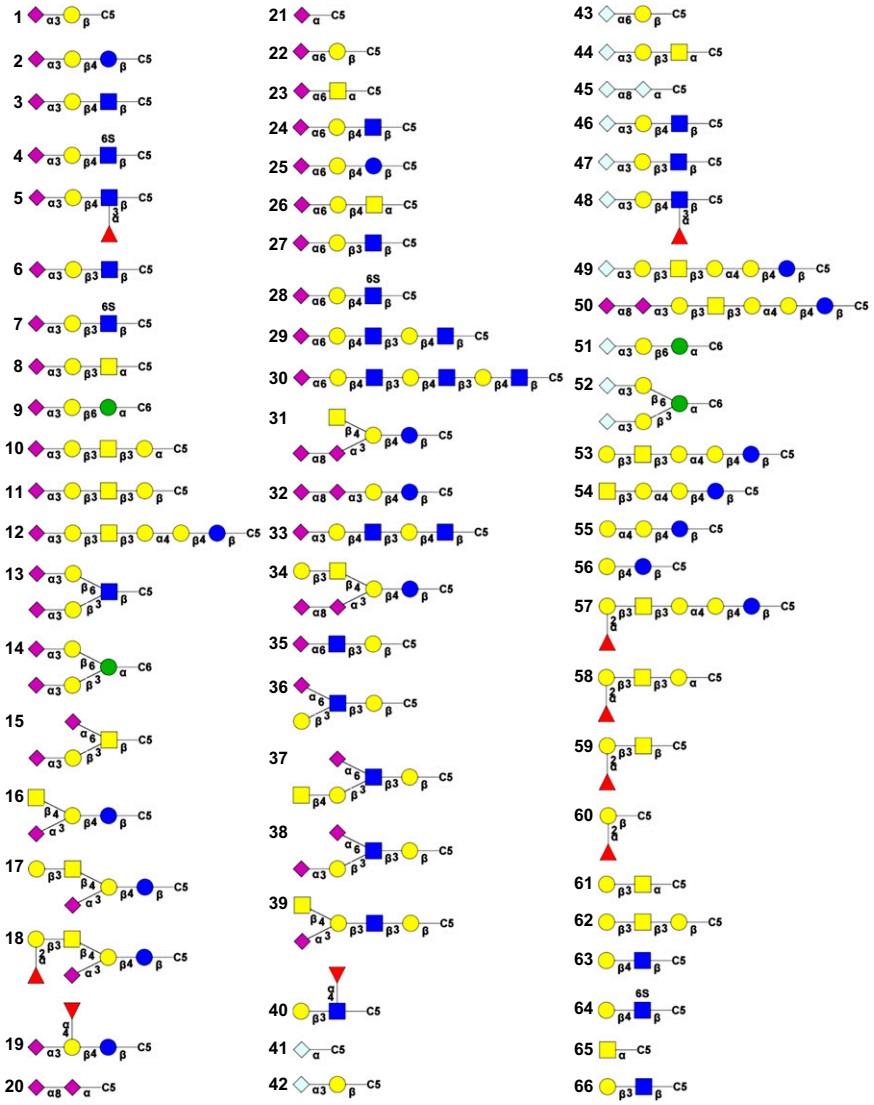
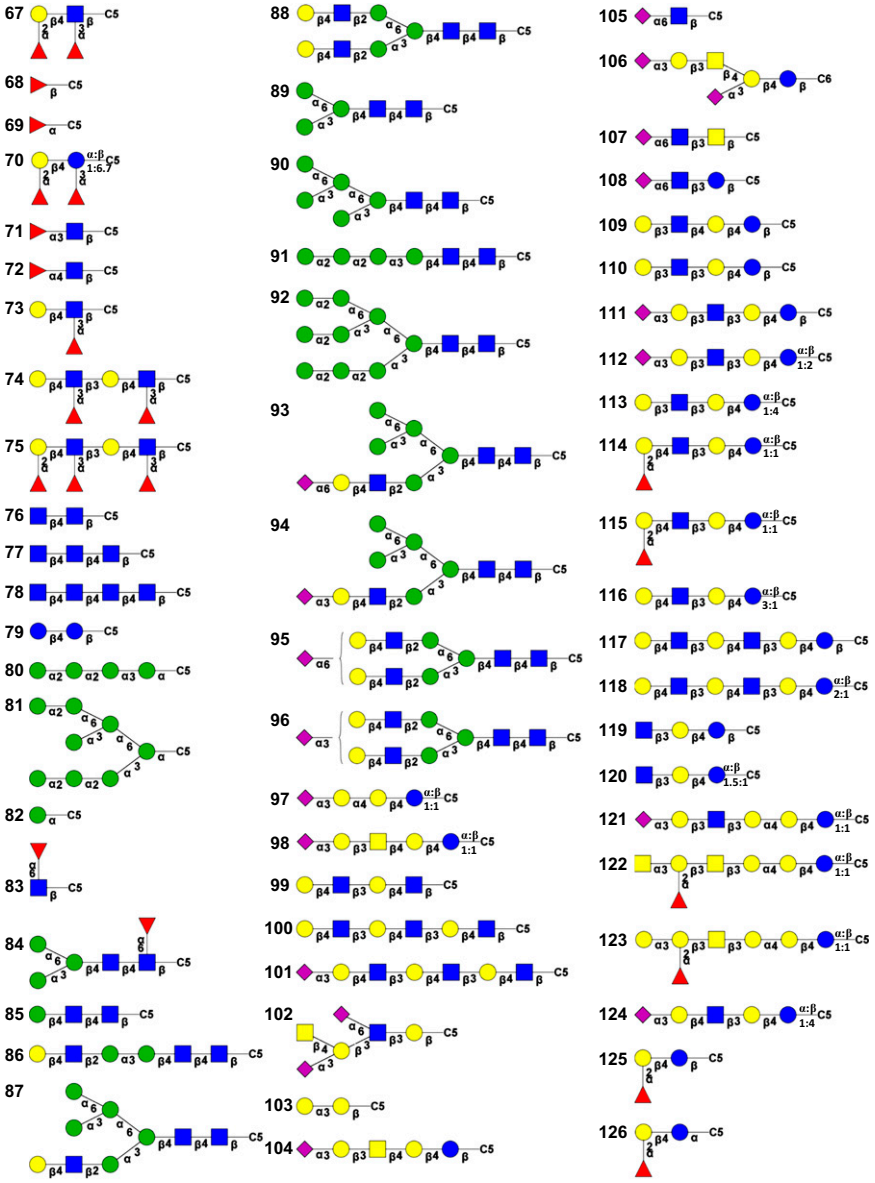


Fig. S2. (Continued)



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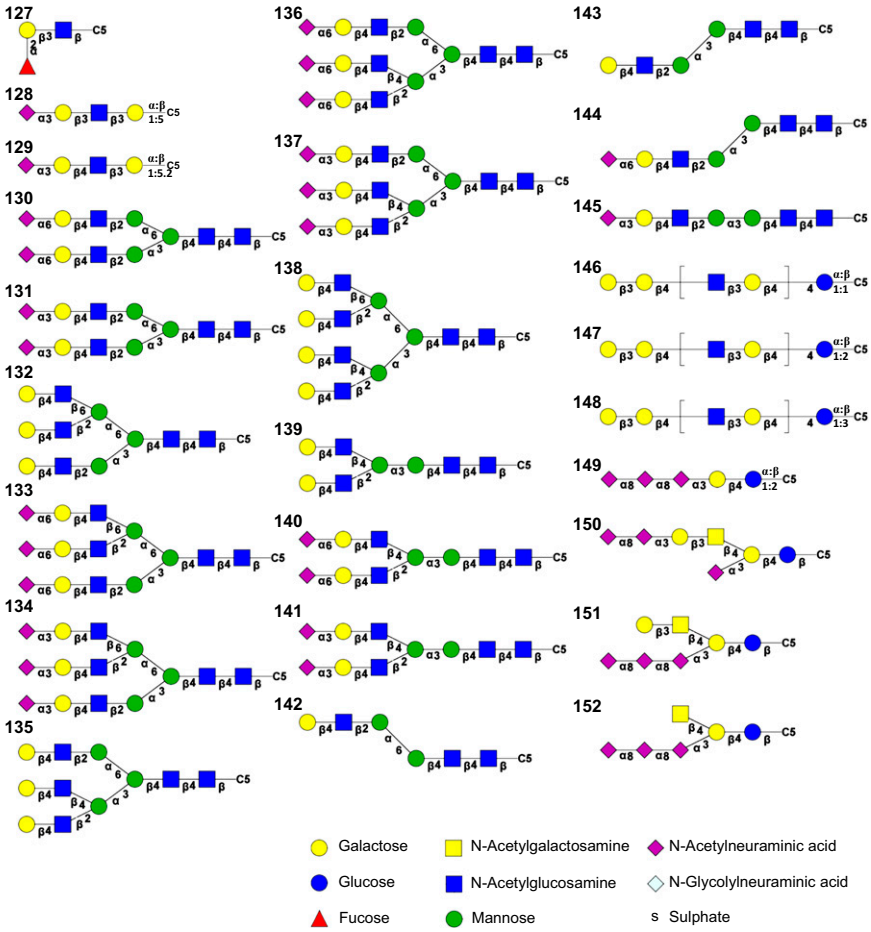

Fig. S2. Chemical structures of 152 oligosaccharides on glycan microarray glass slides. The graphical notation of glycan structures in this figure is based on the symbols proposed by the Consortium for Functional Glycomics. Enantiomeric ratios are indicated for the glycans containing enantiomers. C5, C5H10NH2; C6, C6H12NH2.

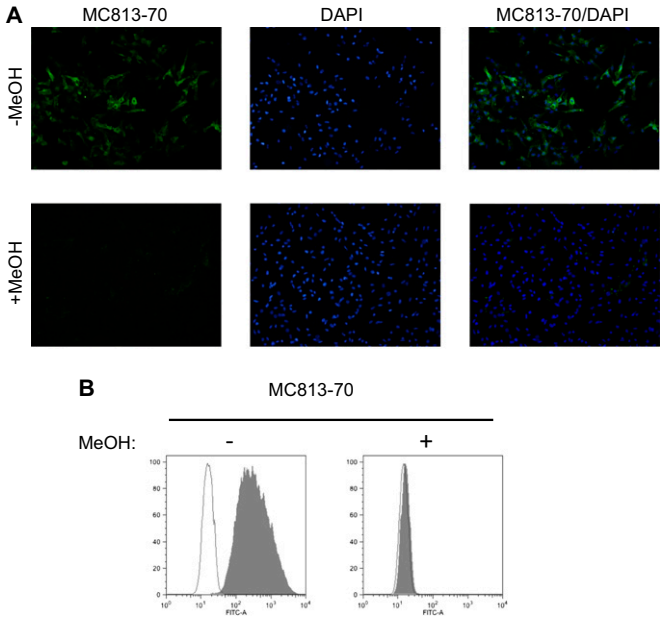

Fig. S3. Effect of methanol on MC813-70 immunoreactivity toward GBM cells. DBTRG cells with or without methanol (MeOH) treatment were stained with MC813-70 and subjected to immunofluorescent microscopy (*A*) and flow cytometry (*B*). DBTRG cells showed positive immunofluorescent staining (green in *A*, gray histogram in *B*), which disappeared after treatment with MeOH. For immunofluorescent microscopy, nuclei were stained with DAPI (blue); for flow cytometry, staining with isotype control is shown as a white histogram.

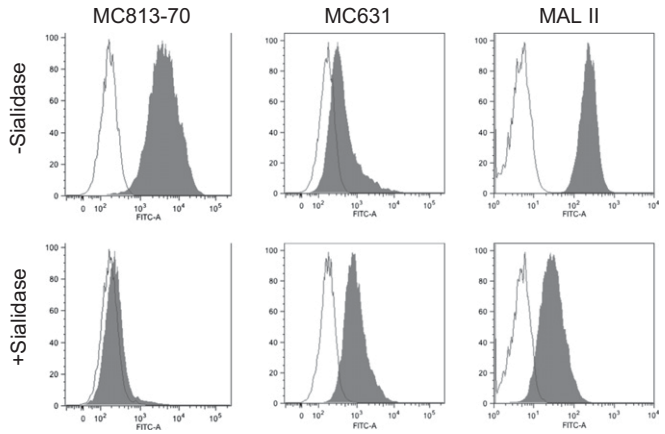

Fig. S4. Desialylation of GBM cells affected MC813-70 and MC631 staining. DBTRG cells were treated with α2,3-sialidase before staining with MC813-70 and MC631. Flow cytometric analysis showed that the intensity of MC631 staining increased and MC813-70 staining disappeared after sialidase treatment. The efficiency of sialidase treatment was monitored by staining with MAL II, which recognizes α2,3-linked sialic acids. The histograms of the cells stained with mAb and MAL II are shown in gray, and the histograms representing isotype control staining are shown in white.

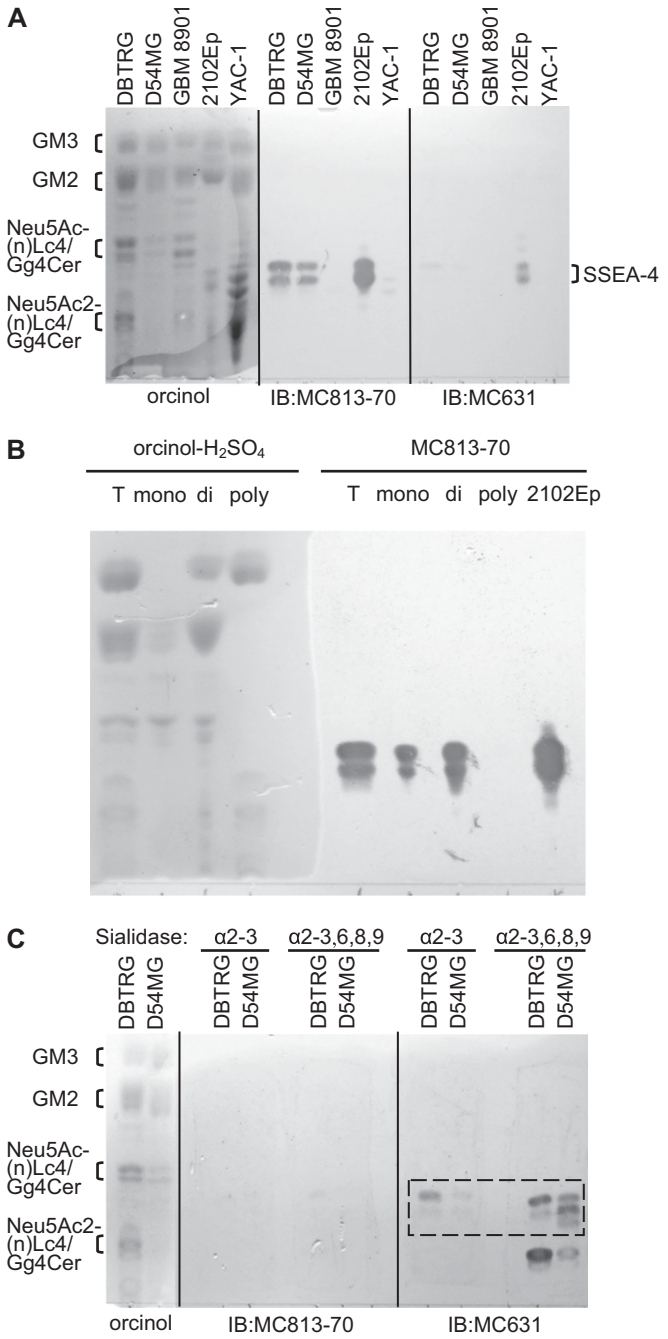

Fig. S5. HPTLC profiles and immunostaining of gangliosides from GBM cell lines. (*A*) Gangliosides were separated on an HPTLC plate and detected with orcinol (*Left*), MC813-70 mAb (*Center*), or MC631 mAb (*Right*). Gangliosides from 2012Ep (a human embryonal carcinoma cell line) and YAC-1 (a mouse lymphoma cell line) were applied to serve as the positive controls for SSEA-4 and GM1b, respectively. (*B*) Gangliosides from DBTRG cells were eluted by 0.8 M NaCl at once (T) or were fractionated into mono-, di-, and polysialylated gangliosides by stepwise elution with 0.02 M, 0.2 M, and 0.8 M NaCl. Gangliosides were separated on an HPTLC plate and detected by orcinol staining (*Left*) or MC813-70 immunostaining (*Right*). MC813-70^{+} signals were detected in the monosialylated ganglioside fraction. The gangliosides extracted from 2012Ep cells, which are known to express SSEA-4, were used as a positive control. (*C*) Detection of desialylated gangliosides on an HPTLC plate with orcinol (*Left*), MC813-70 mAb (*Center*), or MC631 mAb (*Right*). The GBM-associated ganglioside originally recognized by MC813-70 showed MC813-70 (–) and MC631 (+) after sialidase treatment, as marked in the dashed rectangle.

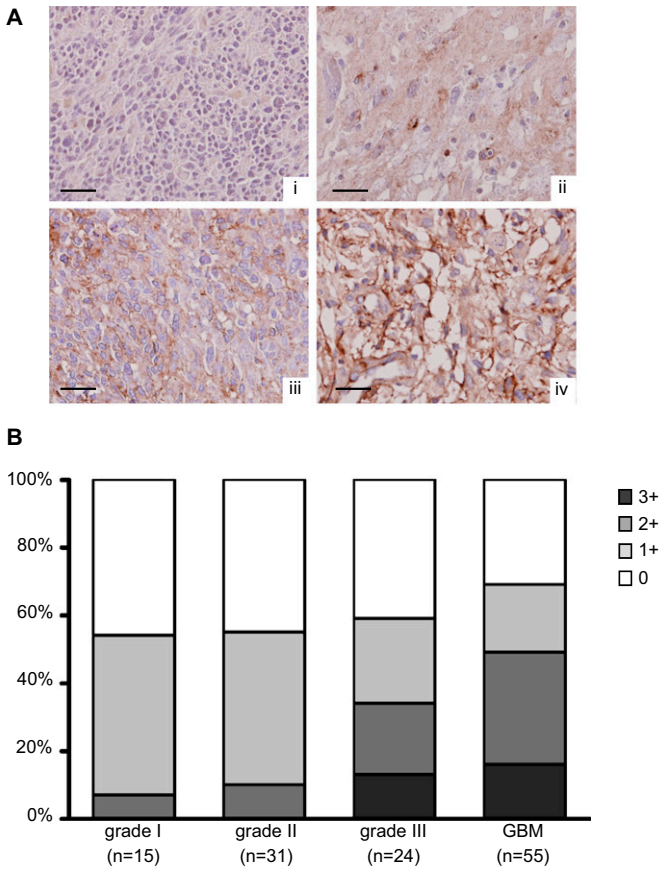

Fig. S6. Expression of SSEA-4 in grade I–IV astrocytoma. (*A*) GBM tissues were immunohistochemically stained with MC813-70. The staining intensity of GBM specimens was graded as 0 (negative, i), 1+ (weak, ii), 2+ (moderate, iii), and 3+ (strong, iv). (Scale bars, 100 μm.) (*B*) Statistical results of SSEA-4 immunohistochemistry (IHC). Grade I ($n = 15$), grade II ($n = 31$), grade III ($n = 24$), and grade IV (GBM, $n = 55$) specimens were counterstained with hematoxylin after IHC. The staining intensity of the tissues was graded as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong).

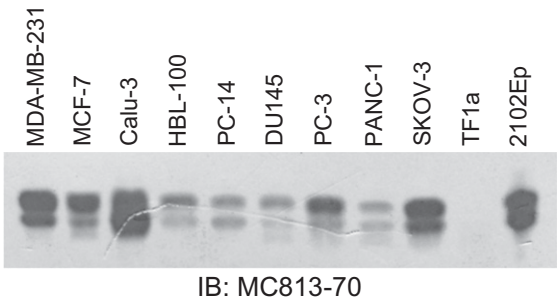

Fig. S7. Immunostaining of SSEA-4 on HPTLC-separated gangliosides from cancer cells. Representative breast (MDA-MB-231, MCF-7, and HBL-100), lung (Calu-3 and PC-14), prostate (DU145 and PC-3), pancreatic (PANC-1), ovarian (SKOV-3), erythroleukemia (TF1a), and embryonal carcinoma (2012Ep) cell lines are shown.

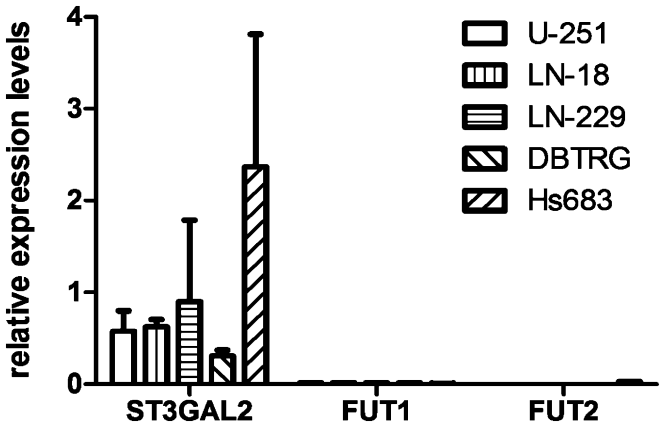

Fig. S8. Human GBM cell lines express a higher level of *ST3GAL2* than *FUT1* and *FUT2*. Total RNA was extracted from GBM cell lines and reverse-transcribed to cDNA. The expression levels of *ST3GAL2*, *FUT1*, and *FUT2* were determined by real-time PCR and normalized against the expression level of *GAPDH*.

Table S1. Expression of globo-series GSLs on various cancer cell lines

Tumor origin	Cell line	Antigen
Brain	A172	
Brain	D54MG	3, 4, H
Brain	DBTRG	4
Brain	G5T	3, 4
Brain	G9T	3, 4, H
Brain	GBM 8401	
Brain	GBM 8901	
Brain	Hs683	4
Brain	LN-18	3, 4, H
Brain	LN-229	3, 4, H
Brain	SF126	4, H
Brain	SNB75	3, 4
Brain	T95G	
Brain	U-138 MG	3, 4
Brain	U-251 MG	3, 4
Brain	U-373 MG	3, 4, H
Brain	U-87 MG	
Lung	A549	4, H
Lung	Calu-3	4
Lung	CL1	4
Lung	CL1-0	4, H
Lung	CL1-5	4, H
Lung	CL2	H
Lung	CL3	
Lung	H1299	
Lung	H1355	4, H
Lung	H157	3, 4, H
Lung	H441	4, H
Lung	H460	
Lung	H480	3, 4, H
Lung	H520	H
Lung	H661	3, 4, H
Lung	H928	3, 4, H
Lung	NuLi-1	3, 4, H
Lung	PC-13	H
Lung	PC-14	
Lung	PC-9	4
Breast	Au565	
Breast	BT-20	
Breast	BT-474	
Breast	BT-483	
Breast	BT-549	4
Breast	DU4475	4, H
Breast	HBL-100	4, H
Breast	HBL-435	
Breast	HCC1395	3, 4, H
Breast	HCC1599	4, H
Breast	HCC1806	3, 4, H
Breast	HCC1937	4
Breast	HCC38	4, H
Breast	Hs578T	3, 4, H
Breast	MCF-7	3, 4, H
Breast	MDA-MB-157	3, 4, H
Breast	MDA-MB-231	3, 4, H
Breast	MDA-MB-361	4, H
Breast	MDA-MB-453	4, H
Breast	MDA-MB-468	4
Breast	SK-BR-3	H
Breast	T47D	3, 4, H
Breast	ZR75	4, H
Colon	CX-1	4, H
Colon	DLD-1	H

Table S1. Cont.

Tumor origin	Cell line	Antigen
Colon	H3347	4, H
Colon	HCT1116	4
Colon	HT-29	H
Colon	SW480	4, H
Colon	SW620	4, H
Mouth	Ca922	4, H
Mouth	Cal27	4, H
Mouth	HSC3	4, H
Mouth	OC3	H
Mouth	OECM1	H
Mouth	SAS	H
Mouth	SCC25	4
Mouth	SCC4	3, 4, H
Mouth	Tu-183	H
Mouth	Tw1.5	4, H
Mouth	Tw2.6	4, H
Mouth	UMSCC-1	3, 4, H
Mouth	YD-15	3, 4, H
Esophagus	CE81T	H
Esophagus	KYSE70	4, H
Stomach	AGS	H
Stomach	AZ521	3, 4, H
Stomach	KATO III	3, 4, H
Stomach	NCI-N87	H
Stomach	SCM-1	3, 4, H
Stomach	SNU-1	4, H
Liver	59T	3, 4, H
Liver	Changliver	H
Liver	HA22T	H
Liver	Hep3b	3, 4, H
Liver	HepG2	4, H
Liver	Huh-7	4, H
Liver	J5	H
Liver	Mahlavu	
Liver	NTU-BL	3, 4, H
Liver	SK-HEP-1	3, 4, H
Bile duct	HuccT1	3, 4, H
Bile duct	SNU-1079	
Bile duct	SNU-1196	H
Bile duct	SNU-245	4, H
Bile duct	SNU-308	
Pancreas	AsPC1	4
Pancreas	BxPC3	4, H
Pancreas	HPAC	4, H
Pancreas	KP-4	3, 4, H
Pancreas	MIA PaCa-2	3, 4, H
Pancreas	Panc0203	4, H
Pancreas	PANC1	4
Pancreas	PL45	3, 4, H
Kidney	769-P	3, 4, H
Kidney	A498	4
Kidney	A704	H
Kidney	ACHN	3, 4, H
Kidney	Caki-1	3, 4, H
Kidney	Caki-2	3, 4, H
Cervix	HeLa	3, 4
Cervix	HeLa 229	3, 4
Cervix	HeLa S3	
Cervix	ME-180	4, H
Ovary	C33A	4
Ovary	CAOV3	4
Ovary	ES-2	4, H
Ovary	NUGCC	3, 4, H

Table S1. Cont.

Tumor origin	Cell line	Antigen
Ovary	OVCAR-3	4, H
Ovary	SiHa	
Ovary	SKOV3	4
Ovary	TOV-112D	4, H
Ovary	TOV-21G	3, 4, H
Prostate	22Rr1	4, H
Prostate	Du145	4
Prostate	hTERT-HPNE	3, 4
Prostate	PC-3	4

Antigen: 3, SSEA-3; 4, SSEA-4; H, Globo H.