B4GALNT1 induces angiogenesis, anchorage independence growth and motility, and promotes tumorigenesis in melanoma by induction of ganglioside GM2/GD2

Hideki Yoshida1, Lisa Koodie1, Kari Jacobsen1, Ken Hanzawa2, Yasuhide Miyamoto2 & Masato Yamamoto1,3,4*

β-1,4-N-Acetyl-Galactosaminyltransferase 1 (B4GALNT1) encodes the key enzyme B4GALNT1 to generate gangliosides GM2/GD2. GM2/GD2 gangliosides are surface glycolipids mainly found on brain neurons as well as peripheral nerves and skin melanocytes and are reported to exacerbate the malignant potential of melanomas. In order to elucidate the mechanism, we performed functional analyses of B4GALNT1-overexpressing cells. We analyzed ganglioside pattern on four melanoma and two neuroblastoma cell lines by high performance liquid chromatography (HPLC). We overexpressed B4GALNT1 in GM2/GD2-negative human melanoma cell line (SH4) and confirmed production of GM2/GD2 by HPLC. They showed higher anchorage independence growth (AIG) in colony formation assay, and exhibited augmented motility. In vitro, cell proliferation was not affected by GM2/GD2 expression. In vivo, GM2/GD2-positive SH4 clones showed significantly higher tumorigenesis in NOD/Scid/IL2Rγ-null mice, and immunostaining of mouse CD31 revealed that GM2/GD2 induced remarkable angiogenesis. No differences were seen in melanoma stem cell and Epithelial-Mesenchymal Transition markers between GM2/GD2-positive and -negative SH4 cells. We therefore concluded that B4GALNT1, and consequently GM2/GD2, enhanced tumorigenesis via induction of angiogenesis, AIG, and cell motility. RNA-Seq suggested periostin as a potential key factor for angiogenesis and AIG. These findings may lead to development of novel therapy for refractory melanoma.

Malignant melanoma is the most common and lethal skin cancer1,2. It is a cancer with one of the highest rise in incidence3, and the overall 5-year survival rate is less than 10% for patients with stage IV disease4. There have been major advances in the treatment of advanced melanoma including Ipilimumab, an antibody to cytotoxic T-lymphocyte-associated-antigen-4 (CTLA-4), and BRAF inhibitor5–9. However, the anti-CTLA-4 antibody shows benefit in less than 50% of patients10. While BRAF inhibitors increased survival compared to other chemotherapies, its indication is limited to about half of patients with BRAF V600 mutations, and almost all patients develop resistance to these inhibitors11. While the combination of Nivolumab (monoclonal antibody against programmed death 1, PD-1) and Ipilumumab has demonstrated an impressive 2-year overall survival rate of 63.8% in stage III-IV patients12, further improvement of therapy is still needed for the treatment of advanced melanoma patients.

β-1,4-N-Acetyl-Galactosaminyltransferase 1 (B4GALNT1) encodes B4GALNT1 (GM2/GD2 synthase), and it works as the key enzyme which transfers a N-acetylgalactosamine (GalNAc) to GM3/GD3, yielding gangliosides GM2/GD2 as part of their stepwise synthesis (Fig. 1A). Gangliosides, including GM2 or GD2, belong to the family of glycosphingolipids ( GSL) and contain one or more sialic acids, N-acetyl derivatives of neuraminic acid, in their hydrophilic oligosaccharide chain.13 Gangliosides are sialic acid-containing glycosphingolipids that are most

1Department of Surgery, University of Minnesota, Minneapolis, Minnesota, USA. 2Department of Molecular Biology, Osaka International Cancer Institute, Osaka, Japan. 3Masonic Cancer Center, University of Minnesota, Minneapolis, Minnesota, USA. 4Stem Cell Institute, University of Minnesota, Minneapolis, Minnesota, USA. *email: yamam016@umn.edu
abundant in the nervous system, especially brain neurons. They also exist in peripheral nerves and skin melanocytes. These molecules are reported to have important biological functions, such as intercellular communication, cell cycling, cell growth, adhesion, differentiation, and cell motility. Gangliosides are not only detected at high levels in tumors of neuroectodermal cell origin but also related to the biological and clinical behavior of many kinds of tumors. Recently, some analysis revealed that patients with higher expression of B4GALNT1 and GM2/GD2 correlated with poorer prognosis in renal cell carcinoma (TCGA data set; Human Protein Atlas), neuroblastoma, and melanoma. Thus, B4GALNT1 gene is considered to be key tumor-associated antigens, indicating that their expression is a meaningful marker for metastatic condition and are potential therapeutic targets for melanoma.

Our findings indicate the involvement of B4GALNT1 and GM2/GD2 in tumor establishment and progression as well as a potential direction of therapeutic approach via controlling B4GALNT1, and consequently GM2/GD2 expression in cancers such as melanoma.

Results

GM2/GD2 expression status in melanoma and neuroblastoma cell lines. To assess the GM2/GD2 expression level, four melanoma (A-375, RPMI-7951, WM115 and SH4) and two neuroblastoma cell lines (IMR32 and RTBM1) were measured by flow cytometry. One melanoma (WM115) and both of two neuroblastoma cell lines (IMR32 and RTBM1) expressed high level of GM2/GD2 (Fig. 1B).

Because gangliosides including GM2/GD2 require stepwise synthesis reactions (Fig. 1A), a model for induced expression of GM2/GD2 on cell surface via overexpression of B4GALNT1 needs the following conditions: 1) both GM3 and GD3 are positive, and 2) both GM2 and GD2 are negative. To evaluate these conditions accurately in the six cell lines, HPLC-based high-specificity analysis of gangliosides was performed (Fig. 1C). Being that SH4 melanoma cell line showed high expression of both GD3 and GM3 (black arrows) and no expression of GD2 and GM2 (white arrows), SH4 fulfilled the aforementioned conditions and was used in the following study. Other results of neuroblastoma cells were shown in Fig. S1.
Generation of GM2/GD2-positive SH4 melanoma clones. The SH4 cells were transfected with expression vectors with or without B4GALNT1 gene cassette, to establish GM2/GD2-positive and -negative SH4 clones. Two GM2/GD2-high clones were selected by single cell isolation (#4 and #5, Fig. S2A). These two clones showed significant expression of GD2, whereas Mock (pcDNA3.1(−) alone) and two clones showed no GD2 expression. The expressions of B4GALNT1 in mRNA level were in correspondence with those by flow cytometry (Fig. S2B). Additionally, HPLC revealed that the clones #4 and #5 expressed GM2/GD2 at high level (Fig. 1D). The reason that GD2 level in the transfected clones is very low compared to the GD3 level in the parental cells was interpreted that B4GALNT1 and ST8Sia1 competes GM3 as a substrate. It is known that GD2 is not synthesized from GM228.

Induction of morphological change, anchorage independence growth, and cell motility. The SH4 clones overexpressing GM2/GD2, #4 and #5, exhibited a distinct morphological appearance compared to SH4 Wild type (WT) or the mock transduced cells. The cells were round and formed aggregation. More than half of them were detached from the bottom of flask, but still capable of survival and proliferation after detachment (Fig. 2A). No significant difference was seen between the proliferation of GM2/GD2-positive SH4 clones and control (Fig. 2B). A soft agar colony formation assay demonstrated that GM2/GD2-positive SH4 clones formed larger and greater number of colonies than GM2/GD2-negative cells (#4; 86.6 ± 13.9, #5; 82.5 ± 6.5, Mock; 32.7 ± 6.6, #4 vs Mock; p < 0.0001, #5 vs Mock; p < 0.0001, Fig. 3A). There was no significant difference between the two GM2/GD2-positive SH4 clones (#4 vs #5; P = 0.15). In addition, faster wound closure was observed in the GM2/GD2-positive SH4 clones than the control cells (#4: 49.7 ± 16.4 vs #5: 56.5 ± 25.3 vs Mock: 85.9 ± 14.8, #4 vs Mock: p < 0.0001, #5 vs Mock: p < 0.0001, #4 vs #5: p = 0.98, Fig. 3B,C), indicating enhanced motility.

Enhancement of tumor incidence and growth speed in vivo. To assess the in vivo effect of GM2/GD2 induced by B4GALNT1 overexpression, the two GM2/GD2-positive SH4 clones and Mock were inoculated in NOD/Scid/IL2Rγ-null (NSG) mice to assess tumor initiation and growth. After transplanting 2 × 10⁶ GM2/GD2-positive and -negative SH4 cells, all mice receiving #4 and #5 cells developed tumors, whereas only three out of six mice injected with Mock cells developed tumors (P = 0.038, Fig. 4A, Table 1). In the NSG mice transplanted with lower number (2 × 10⁵) of GM2/GD2-positive or -negative SH4 cells, seven out of eight mice injected with #4 and #5 cells developed tumors, whereas two out of six mice injected with Mock cells developed tumors (P = 0.038, Fig. 4B, Table 1). Moreover, in the NSG mice transplanted with 2 × 10⁶ GM2/GD2-positive or -negative SH4 cells, tumors derived from GM2/GD2-positive cells grew to be approximately three times larger than the GM2/GD2-negative Mock at day 54 (9.4 ± 1.9 vs 3.2 ± 3.5 mm, P = 0.003, Fig. 4A). In the NSG mice transplanted with 2 × 10⁵ GM2/GD2-positive or -negative SH4 cells, tumors derived from GM2/GD2-positive cells grew to be over three times as large as those from GM2/GD2-negative Mock in 54 days (4.0 ± 1.8 vs 1.1 ± 1.8 mm, P = 0.026, Fig. 4B). Every tumor was solid, firm, and partially fibrotic.

No evident difference in major cancer stem cell markers. To evaluate the possibility that B4GALNT1 overexpression might enhance tumor incidence via induction of stemness, several melanoma stem cell markers were analyzed in GM2/GD2-positive and -negative cells by flow cytometry. Previous reports indicated that
Figure 3. Effect of B4GALNT1 overexpression to cell behavior. (A) Photographs of colonies of SH4 cells with and without B4GALNT1 overexpression 14 days later. Scale bar, 1,000 μm. Results represent the means ± s.d. of three independent experiments. (B) Light microscopic images of SH4 cells that were scratched and compared the wound width 6 and 24 h later. Scale bar, 1,000 μm. (C) Average wound widths, expressed as a percent of the original width, obtained from 30 measurements in each photo. Results represent the means ± s.d. of three independent experiments. *P < 0.01 compared with Mock. N.S.; Not Significant.

Figure 4. Effects of B4GALNT1 overexpressing on the proliferation of SH4 cells in xenograft models. (A) 2 × 10⁶, (B) 2 × 10⁵ SH4 cell were inoculated into lower flank of NSG mice subcutaneously.

| GM2/GD2 | cell | number | p-value |
|---------|------|--------|---------|
| positive | 2 × 10⁶ | 8/8 | 3/6 | P < 0.05 |
| negative | 2 × 10⁵ | 7/8 | 2/6 | P < 0.05 |

Table 1. Tumor incidence in vivo.
CD133, CD166, CD271, Nestin, and ABCB5 are potential melanoma stem cell markers. Aldehyde dehydrogenase (ALDH) activity has also been reported as a potential marker of melanoma stem cell. However, B4GALNT1 overexpression did not induce any meaningful change in any of these markers (Fig. S3A–F), there was no significant difference in CD133, CD166, and ABCB5 between GM2/GD2-positive and -negative cells. There was a small decrease in the expression of CD271 and Nestin in the #5 GM2/GD2-positive cell line, but this alone is unlikely to be the cause of enhanced tumor growth. That indicated that B4GALNT1 was unlikely to affect the stemness in the SH4 melanoma cell line.

Promotion of angiogenesis in vivo. We hypothesized that B4GALNT1 may enhance tumor induction and growth by increasing tumor vascularization. Tumors of similar size from each group were stained by hematoxylin and eosin (H&E) and immunofluorescence for murine-CD31. In H&E staining, the tumors induced by GM2/GD2-positive or -negative SH4 cells did not differ in any characteristics examined: cell shape, number of giant cells, nuclear-to-cytoplasm volume ratio reversal, and hyperchromatism (Fig. 5A). The major blood vessel in the GM2/GD2-positive tumors were more prominent than the one supplying the Mock tumor. Although some tumors showed evidence of local invasion, none of the tumor-bearing mice developed metastasis.

Immunofluorescence staining in tumors derived from GM2/GD2-positive clones by anti-mouse CD31 Ab exhibited many well-structured vessels, while GM2/GD2-negative Mock tumors had much less (Fig. 5B,C). Immunofluorescence performed using selective anti-human-CD31 antibody (non-reactive to mouse CD31) as a negative control did not show any staining of blood vessels (data not shown).

RNA-Seq revealed potential key molecule downstream of B4GALNT1. To identify the difference of the transcriptional profile between GM2/GD2-positive and -negative cells, we compared them by RNA-Seq and analyzed using edgeR. There were a total of 26,484 genes detected in the individual libraries (Table S1), and 472 genes showed over two-fold change between the two groups (Table S2). The heat map was shown in Fig. S4A. Among the 472 genes, 117 genes were up-regulated and 351 genes were down-regulated by B4GALNT1 overexpression. There was no significant difference between the two Mock clones (Fig. S4B), as well as between two GM2/GD2-positive samples (#4 and #5 clones; Fig. S4C). Genes exhibiting the Top 10 largest fold changes are listed in Table 2. The top up-regulated gene, PTPRD, is a member of the protein tyrosine phosphatase (PTP) family, and is known to be a signaling molecule that regulates a variety of cellular processes including cell growth, differentiation as a tumor suppressor, which was often down-regulated in a variety of tumors. The second highest was B4GALNT1, suggesting that our overexpression of the gene in SH4 had succeeded. The third highest, POSTN (peristin), functions as a ligand for alpha-V/beta-3 and alpha-V/beta-5 integrins to support adhesion and migration. In addition, it is known to increase angiogenesis. Furthermore, CPVL (Carboxypeptidase Vitellogenic Like), the top down-regulated gene was related to maturation of monocytes into macrophages. Carboxypeptidases are a large class of proteases that act to cleave a single amino acid from the carboxy termini.

Figure 5. Histological analyses of the tumors. (A) Images of tissue sections of SH4 (H&E). (B,C) Immunostaining with murine CD31. Scale bar, 1,000 μm (A; left), 200 μm (A; right), 400 μm (B), and 100 μm (C), respectively.
is associated with AIG by knocking out GM2/GD2 synthase42. AIG is often reported as a critical factor for tumori-

remarkable fold change in Akt/PKB pathway (Table S1).

detected in variety of solid tumors38,47. Kudo

invasion, and metastasis actively36–38. It is also known that high expression of periostin protein and/or mRNA is

in numerous biological processes, it sometimes contributes to tumorigenesis by promoting cancer cell survival,

Top 10 of the greatest genes expression changes in SH4 with vs without GM2/GD2.

Table 2. Top 10 of the greatest genes expression changes in SH4 with vs without GM2/GD2.

| Symbol | Gene name                  | Expr Fold change | p-value |
|--------|----------------------------|------------------|---------|
| PTPRD  | Protein tyrosine phosphatase receptor type D | 735.0563 | 0.005722 |
| B4GALNT1 | Beta-1,4-N-acetyl-galactosaminyltransferase 1 | 359.3801 | 1.4E-150 |
| POSTN  | Periostin                   | 286.3453 | 4.9E-26  |
| SERPINB2 | Serpin family B member 2   | 240.9988 | 7.94E-06 |
| HLA-DRB1 | human leukocyte antigen, class II, DR beta 1 | 228.4819 | 1.07E-05 |
| IL13RA2 | Interleukin 13 receptor alpha 2 | 191.2431 | 0.000616 |
| CSMD1  | CUB and sushi multiple domains 1 | 188.313  | 7.75E-05 |
| MYBPC1 | Myosin binding protein C type 1 | 182.0119 | 0.000805 |
| ADGRL2 | Adhesion G protein - coupled receptor L2 | 158.3556 | 4.73E-02 |
| TNFRSF14 | TNF receptor superfamily member 14 | 142.9792 | 0.011144 |

Down-regulation

| Symbol | Gene name                  | Expr Fold change | p-value |
|--------|----------------------------|------------------|---------|
| CPVL   | Carboxypeptidase vitellogenic like | −1370.3 | 3.27E-37 |
| MAGEA12 | Melanoma-associated antigen 12 | −1143.23 | 9.84E-16 |
| CSAG1  | Chondrosarcoma associated gene 1 | −676.619 | 8.41E-12 |
| GIPC3  | PDZ domain containing family Member 3 | −535.495 | 1.85E-20 |
| CPM    | Carboxypeptidase M           | −484.607 | 3.42E-15 |
| ZXD A  | Zinc finger, X-linked, duplicated A | −457.61 | 9.68E-09 |
| FAM83H | Family with sequence similarity 83 | −412.334 | 1.45E-08 |
| PRAME  | Preferentially expressed antigen in melanoma | −411.078 | 0.000851 |
| GABRQ  | Gamma-amino butyric acid type A receptor theta subunit | −402.747 | 1.57E-21 |
| LDB2   | LIM domain binding 2         | −371.463 | 0.000011 |

Discussion

In this study, we analyzed a variety of changes in the SH4 melanoma cell line upon overexpression of GM2/GD2 by

transfection of . One of remarkable findings

in vitro

is that GM2/GD2-positive SH4 cells showed

significant difference of AIG compared to Mock (Fig. 3A), interestingly, Mahata

et al. showed that periostin overexpression promoted invasion in head

and neck squamous cell carcinoma cells46 and to explore the genes that are coordinate-

ly expressed with periostin, they performed microarray analysis. Among the genes changed in their study,

SULF1 was upregulated clearly in their result as well (9.30-fold; Table S2). On top of that, Kobuki et al. directly revealed that periostin increased cell proliferation and invasion in melanoma cell in vitro and in vivo using overexpression system48, and Fukuda et al. showed that periostin was a key factor in promoting melanoma cell metastasis using shRNA50. We therefore speculated that the findings support our conclusion that periostin and its downstream gene overexpression promoted migration induced by GM2/GD2. Furthermore, Bao et al. demonstrated that periostin activated the downstream Akt/PKB pathway via ctn33 integrin, by which they observed phosphorylation of Akt1/PKBα on Ser473 to promote cellular survival in colon cancer51. Their phosphorylation level, not the total amount, would therefore be contributing to the downstream effect of GM2/GD2. This may explain why our RNA-Seq result did not show a remarkable fold change in Akt/PKB pathway (Table S1).
In our observation, B4GALNT1 overexpression did not affect cell proliferation in vitro, while multiple genes related to tumor cell proliferation-promoting, such as POSTN, IL13RA2, NRP1 were up-regulated, and EMILIN2, which is a proliferation-suppressing gene, was down-regulated notably in mRNA level (Fig. 2B, Table 3). The relationship between ganglioside and cell proliferation is still controversial; some research suggest promotion of cell proliferation by gangliosides52–54, while others show inhibition55. These discrepancies may be explained by the difference in the ratio of GD2 + 3 vs GM2 + 3, as well as GD2 vs GD3, which may contribute to differences in cell proliferation. The report by Shibuya et al. which induced GM2/GD2 like ours showed enhanced cell migration55, which induced GM2/GD2 like ours showed enhanced cell migration55,while multiple genes related to tumor cell proliferation-promoting, such as POSTN, IL13RA2, NRP1 were up-regulated, and EMILIN2, which is a proliferation-suppressing gene, was down-regulated notably in mRNA level (Fig. 2B, Table 3).

Another finding was that GM2/GD2 strongly induced angiogenesis. The effect of B4GALNT1 for tumor incidence in vivo was assessed by injecting GM2/GD2-positive SH4 cells into NSG mice. As shown in Fig. 4A, B, the

Table 3. Gene expression characteristics. * means that the fold changes went the opposite direction compared with previous findings.

| Symbol | Expr Fold Change | findings | Symbol | Expr Fold Change | findings |
|--------|-----------------|----------|--------|-----------------|----------|
| **Invasion of tumor** | | | **Down-regulation** | | |
| POSTN | 286.345 | Increases | EDN3 | −248.929 | Decreases |
| NRP1 | 117.774 | Increases | VCAN* | −243.495 | Increases |
| GFRBP5* | 26.687 | Decreases | IL24 | −15.763 | Decreases |
| MMP1 | 20.329 | Increases | SERPINE1* | −9.371 | Increases |
| TGFBR2* | 6.627 | Decreases | SFRP1 | −6.729 | Decreases |
| ITGA1 | 4.868 | Increases | MMP2* | −6.046 | Increases |
| TXB2 | 2.561 | Increases | GP101* | −4.353 | Increases |
| HMGB3* | 2.021 | Decreases | CEACAM1* | −4.131 | Increases |
| SPHK1 | 2.012 | Increases | IGF* | −3.683 | Increases |
| CTSF* | −2.077 | Decreases | CSF1* | −2.879 | Increases |
| **Migration of melanoma cell** | | | **Cell proliferation of tumor cell lines** | | |
| NRP1* | 117.774 | Decreases | VCAN | −243.495 | Affects |
| SDC2 | 11.904 | Increases | LIGAM* | −20.041 | Increases |
| TNC | 2.217 | Increases | SERPINA5* | −10.969 | Increases |
| SPHK1 | 2.012 | Increases | SERPINE1* | −9.371 | Increases |
| | | | MMP2* | −6.046 | Increases |
| | | | IGF* | −3.683 | Increases |
| | | | FN1* | −2.15 | Increases |
| **Colony formation** | | | **Expr Fold Change** | **findings** | **Symbol** | **Expr Fold Change** | **findings** |
| POSTN | 286.345 | Affects | VCAN* | −243.495 | Increases |
| SERPINB2* | 240.999 | Decreases | VCAN* | −243.495 | Increases |
| IL13RA2 | 191.243 | Affects | EMILIN2 | −124.892 | Decreases |
| NRP1 | 117.774 | Affects | TRPM2* | −94.449 | Increases |
| HTN1 | 60.939 | Increases | SYNM* | −26.275 | Increases |
| MAF* | 50.99 | Decreases | PDGFB* | −22.149 | Increases |
| POU4F1 | 50.781 | Increases | LIGAM* | −20.041 | Increases |
| VIP | 39.551 | Increases | CARD10* | −16.191 | Increases |
| CDK14 | 30.195 | Increases | IL24 | −15.763 | Decreases |
| GFRBP5 | 26.687 | Increases | S100A4* | −15.312 | Increases |
| **Cell proliferation of tumor cell lines** | | | **Colony formation** | | |
| POSTN | 286.345 | Affects | VCAN* | −243.495 | Increases |
| HTN3* | 98.45 | Decreases | NUPR1 | −31.659 | Decreases |
| VIP | 39.551 | Increases | PDGFB* | −22.149 | Increases |
| TP63 | 8.144 | Increases | IL24 | −15.763 | Decreases |
| CDC47L | 4.871 | Increases | S100A4* | −15.312 | Decreases |
| ITGA1 | 4.868 | Increases | SERPINE1* | −9.371 | Decreases |
| ALDH1A1 | 4.81 | Affects | TRX1* | −7.463 | Increases |
| HAS2 | 4.155 | Increases | DUSP5* | −6.896 | Increases |
| TNC | 2.217 | Affects | SFRP1* | −6.729 | Increases |
| LIMA1* | 2.133 | Decreases | PTGES* | −6.519 | Increases |
tumors injected GM2/GD2-positive cells showed a higher tumor establishment rate. This result corresponds with the fact that B4GALNT1 is a clinical marker for advanced melanoma59. Liu Y et al. revealed that gangliosides accelerate tumor angiogenesis in murine cells and demonstrated that GM2/GD2-negative cells formed much smaller tumors, using GM3 synthase and GM2 synthase double knockout low ganglioside tumor model60. We assessed murine-CD31 expression in the tumors derived from GM2/GD2-positive cell by immunofluorescence staining and observed that B4GALNT1-overexpressing clones induced many CD31 positive endothelial cells and well-developed vessels. In addition, the surface of the GM2/GD2-positive tumors were better vascularized than that of Mock by observation of the recovered tumor with eyes. Tumor progression requires endothelial cells to be activated for the formation of a vascular system. Lang Z et al. found that the enrichment of human umbilical vein endothelial cell (HUVEC) membranes with gangliosides results in amplified VEGF-induced signaling that is important for angiogenesis, and concluded that gangliosides enhances VEGF-induced endothelial cell proliferation61. Liu Y et al. reported that reduction of gangliosides depleted vascularization, while addition of wild type gangliosides restored angiogenesis of ganglioside-poor tumor60. To clarify how gangliosides induce blood vessels, we assessed the relations of GM2/GD2 and VEGF. Some other reports indicate that gangliosides enhances VEGF and induces endothelial cell proliferation61,62. In addition, Liu Y et al. also reported that periostin induces angiogenesis via Erk/VEGF pathway61. However, in our result of RNA-Seq and real-time RT-PCR, the expression of VEGF did not show significant correlation with GM2/GD2 level (Table S1, Fig. S5). There is a possibility that interaction between periostin and integrins directly promoted angiogenesis49 or GM2/GD2 lowered the threshold for cytokine stimulation60,65.

While GM2 and GD2 were reported to be increased greatly in cancer stem cells in breast cancer66,67, our data ruled out the possibility that GM2/GD2 enhanced tumor incidence via induction of cell stemness. We assessed some melanoma stem cell markers, such as CD133, CD166, CD271, Nestin, ABCB5, and ALDH activity by flow cytometry, and there was no evidence indicative of GM2/GD2 involvement in stemness (Fig. S3A–F).

In summary, our findings demonstrated that in the SH4 melanoma cell line, overexpression of B4GALNT1 as well as its main products GM2/GD2 promotes AIG and cell migration in vitro and enhances tumor incidence by inducing angiogenesis in vivo. To our best knowledge, this is the first time that RNA-Seq was performed to elucidate the influence of B4GALNT1. This result indicates that GM2/GD2 or B4GALNT1 is upstream regulator of periostin, and it might cause some change of characters related to tumorigenesis mentioned above in melanoma cell line. In this study, we have not only shown how GM2/GD2 exacerbates tumors’ malignant characters by using B4GALNT1 artificial expression system, but also reconfirmed RNA-Seq is useful tool to find novel potential target in cancer.

Materials and Methods
All experiments were performed in accordance with relevant guidelines and regulations.

Cell lines. Human melanoma cell lines (A375, RPMI-7951, SH4 and WM115) were purchased from American Type Culture Collection (Manassas, USA). Human neuroblastoma cell lines (IMR32 and RTBM1) were provided by Dr. Hajime Hosoi (Kyoto Prefectural University of Medicine, Japan). The melanoma cell lines were maintained in Dulbecco’s modified Eagle’s high-glucose medium (DMEM, Corning, USA) and neuroblastoma cell lines were maintained in Eagle’s minimal essential medium (EMEM, Corning) supplemented with 10% FBS, 100 U/ml penicillin and 10 mg/ml streptomycin (Corning). HUVEC was maintained in Endothelial Cell Growth Media (Sigma-Aldrich, USA). All cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂.

Construction of a cDNA expression vector, gene transfection and selection. Human B4GALNT1 cDNA was cloned from IMR32 with the primers listed in Table S3. The fragment was first inserted into Topo vector using Zero Blunt TOPO PCR Cloning Kit (Invitrogen, USA). After confirmation of sequence, the cDNA cut out by BamHI and NotI was inserted into the cDNA3.1(+) expression vector (Invitrogen). SH4 cells were plated in a 60-mm plastic plate (Corning) and then transfected with the plasmids by using Superfect (Qiagen, Germany). Stable transfectants were isolated in the presence of 600 μg/ml G418 (Roche, Germany).

Flow cytometric analysis. Cells were trypsinized and washed twice with flow cytometry buffer (FCB, PBS supplemented with 1% FBS and 0.02% sodium azide (Sigma-Aldrich)). Cells were incubated with the anti-hGD2 mAbs (MAB2052, Millipore, USA) and anti-hABCBS mAbs (MA5-17026, Thermo Fischer Scientific, USA (1:100, 100 μl/10⁶ cells) for 1 h and then washed in FCB. The cells were subsequently incubated with FITC-labeled anti-mouse goat IgG (sc-2010: 1:1,000, 1 ml/10⁶ cells, Santa Cruz Biotechnology, USA) for 40 min, and washed twice with FCB. Cells were incubated in PE-conjugated anti-hCD133/2 mAbs (#130-090-853, Miltenyi Biotec, Germany), PE-conjugated anti-hALCAM/CD166 mAbs (#105902, R&D systems, USA) and FITC-conjugated anti-hCD2/1/NGFR antibody (#345103, BioLegend, USA) (1:200, 1 ml/10⁶ cells) for 15 min and then washed with FCB. With PE-conjugated anti-hNestin mAbs (#196908, R&D systems), cells (1 × 10⁶ cells/200 μl) were fixed with 200 μl of cold 4% paraformaldehyde (Sigma-Aldrich) at room temperature for 30 min, and then washed in FCB twice. After incubation with 500 μl 0.1% Triton X-100 (Sigma-Aldrich) for 10 min at room temperature, cells were washed with FCB twice with the antibody (1:200, 1 ml/10⁶ cells) for 30 min in the dark, and subsequently washed with FCB. ALDH activity was determined by ALDEFLUOR Kit (Stemcell Technologies, Canada) according to the manufacturer’s instructions. All procedures were performed at 4 °C. The samples were immediately analyzed by FACS Canto II flow cytometer (BD Biosciences, USA). In each sample at least 1 × 10⁴ events were collected. The data was analyzed with FlowJo software (FlowJo, LLC, USA).

High-performance liquid chromatography (HPLC). HPLC was carried out as described previously68-70. Briefly, the acidic glycosphingolipids were extracted from each melanoma and neuroblastoma cell
line (1 × 10⁶ cells) and digested with recombinant endoglucoceramidase II from Rhodococcus sp. (Takara Bio, Japan). The released oligosaccharides were labeled with 2-aminoypyridine and separated using a HPLC system equipped with a fluorescence detector. Normal-phase HPLC was performed on a TSK gel Amide-80 column ( Tosoh, Japan). The molecular size of each PA-oligosaccharide is given in glucose units (Gu) based on the elution times of PA-isomaltooligosaccharides. Reversed-phase HPLC was performed on a TSK gel ODS-80Ts column (Tosoh). The retention time of each PA-oligosaccharide is given in glucose units based on the elution times of PA-isomaltooligosaccharides. Thus, a given compound on these two columns provides a unique set of Gu (amide) and Gu (ODS) values, which correspond to coordinates of the 2-D map. PA-oligosaccharides were analyzed using LC/ESI MS/MS. Standard PA-oligosaccharides, PA-GM1 and PA-GD1a, were purchased from Takara Bio and PA-LST-a and PA-SPG were obtained from our previous study.

**Real time RT-PCR.** Total RNA was extracted from a tumor specimen with RNeasy mini kit (Qiagen) and complementary DNA (cDNA) was synthesized by the use of the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions, respectively. The primers used in this experiment are listed in Table S3. Real-time RT-PCR was carried out using LightCycler 480 System (Roche) with SYBR Green (Applied Biosystems, CA) as previously described. Thermal cycling conditions were: initial denaturation for 10 min at 95 °C, and 40 cycles of 15 sec at 95 °C, and 1 min at 60 °C. Data were analyzed with the Light Cycler software.

**Assay for cell proliferation.** Cells were seeded at 2 × 10⁴ cells/well in 12-well plate. Every 48 h, cells were dissociated by 0.25% trypsin (Corning) and neutralized by the same volume of 10% DMEM. After that 100 μl 0.5% trypsin blue (Sigma-Aldrich) and counted by Cellometer Auto T4 (Nexsolom Bioscience, USA) until days 6.

**Anchorage-independent soft agar colony formation assay.** Cells were cultured in a two-layer soft agar system. It consisted of a 1% agarose (RPI, USA) underlayer and a 0.7% agarose overlay containing 1 × 10⁴ cells in 60-mm dishes (Corning). Colonies were allowed to form for 2 weeks with fresh media added every 3 days. Plates were stained with crystal violet and colonies more than 0.1 mm in diameter were counted.

**Wound-healing assay.** Wound-healing assays were carried out as described previously. Immediately after scratching (0 h), the plates were photographed and the distance between the edges of the wound area was measured. At 6 h and 24 h after scratching, the plates were photographed and the distance between the edges of the wound region was again measured.

**In vivo tumorigenesis.** Tumors were induced in 5–6 week old female and male NSG mice (Jackson Laboratory, USA). Each mouse was injected subcutaneously with SH4 cell lines transformed with pcdNA3.1(+) empty vector or the one expressing B4GALNT1 suspended in 0.1 ml of PBS at a single site (2 × 10⁶ cell; left, 2 × 10⁵ cell; right) to the lower flank. Tumor diameter was monitored every 2–3 days on onset of tumor formation. Mice were sacrificed when the largest tumor size reached 16 mm in diameter along with IACUC approved protocol. At the end point of the experiments, tumors were extracted. At least 4 mice were used in each group (Mock, #4 and #5). The animal experiments were performed in accordance with the institutionally approved animal experimental protocol.

**Histopathology and immunohistochemistry.** Histological specimens were fixed in 10% formalin and routinely processed for embedding in paraffin. The sections were stained with H&E. Some portions of tumors were embedded in OCT and frozen at −20 °C for immunofluorescence analyses. The tumors were sectioned 30-μm-thick by CM 1800 Cryostat (Leica Biosystems, Germany). The sections were fixed by 50 μl of ice-cold acetone for 5 min. After washing the slides in 1 x PBS, they were incubated in blocking buffer (2% bovine serum albumin (BSA) serum in PBS) for 1 h. The sections were incubated with PE-conjugated anti-mouse CD31 antibody (#102507, BioLegend, 1:500 at 4 °C overnight). After washing the slides in 1 x PBS three times, the slides were incubated by 20 μl VECTASHIELD Antifade Mounting Medium with DAPI (H-1200, Vector Laboratories, USA) and coverslipped. The tissues were observed with automated upright microscope System DM5500 B (Leica Biosystems).

**RNA sequencing (whole transcriptome shotgun sequencing, WTSS) and analysis.** RNAs derived from SH4 with (#4 and #5) or without (Mock; two samples) B4GALNT1 over-expression were analyzed by RNA sequencing as described in ref. 74–76. Quality tested with Bioanalyzer 2100 (Agilent Technologies, USA). Sequencing was accomplished on the MiSeq 500 (Illumina, USA). 50 bp FastQ paired-end reads (n = 23.6 Million per sample) were trimmed using Trimmomatic (v 0.33). Quality control checks on raw sequence data were performed with FastQC. Read mapping was performed via Hisat2 (2.1.0) using the Human UCSC genome (hg38) as reference. Differentially expressed genes were identified using the edgeR (Bioconductor, www.bioconductor.org) feature in CLCGWB (Qiagen) using raw read counts. The generated list was filtered based on a minimum 2 x absolute fold change and false discovery rate (FDR) corrected p < 0.05. Pathway analysis was performed in IPA (Qiagen) using fold change and FDR corrected values.

**Statistical analysis.** Statistical analysis was performed using the unpaired Student’s t-test. A P-value of less than 0.05 was considered statistically significant.

Received: 19 September 2019; Accepted: 22 November 2019; Published: 27 January 2020
Author contributions
Conception and design: H. Yoshida and M. Yamamoto. Development of methodology: H. Yoshida, L. Koodie, Y. Miyamoto, and M. Yamamoto. Acquisition of data (provide): H. Yoshida (All), L. Koodie (Immunostaining), K. Jacobsen (Animal experiments), and K. Hanzawa (Glycobiology). Analysis and interpretation of data: H. Yoshida and M. Yamamoto. Writing review, and/or revision of the manuscript: H. Yoshida and M. Yamamoto. Administrative, technical, or material support: L. Koodie and K. Jacobsen. Study supervision: M. Yamamoto.

Acknowledgements
This research was partly supported by grants from NIH/NCI (R01CA168448 and R01CA196215 to MY), and the Research Support from Department of Surgery, University of Minnesota.

www.nature.com/scientificreports/
Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-019-57130-2.
Correspondence and requests for materials should be addressed to M.Y.
Reprints and permissions information is available at www.nature.com/reprints.
Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020