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Ganglioside, Disialosyl Globopentaosylceramide (DSGb5), Enhances the Migration of Renal Cell Carcinoma Cells

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About one third of renal cell carcinoma (RCC) patients exhibit metastasis upon initial presentation. However, the molecular basis for RCC metastasis is not fully understood. A ganglioside, disialosyl globopentaosylceramide (DSGb5), was originally isolated from RCC tissue extracts, and its expression is correlated with RCC metastatic potential. DSGb5 is synthesized by GalNAc α2,6-sialyltransferase VI (ST6GalNAcVI) and is expressed on the surface of RCC cells. Importantly, DSGb5 binds to sialic acid-binding Ig-like lectin-7 (Siglec-7) expressed on natural killer (NK) cells, thereby inhibiting NK-cell cytotoxicity. However, the role of DSGb5 in RCC progression remains obscure. To address this issue, we used ACHN cells derived from malignant pleural effusion of a patient with metastatic RCC. Using the limiting dilution method, we isolated three independent clones with different DSGb5 expression levels. Comparison of these clones indicated that the cloned cells with high DSGb5 expression levels exhibited greater migration potential, compared to the clone with low DSGb5 expression levels. In contrast, DSGb5 expression levels exerted no significant effect on cell proliferation. We then established the ACHN-derived cell lines that stably expressed siRNA against ST6GalNAcVI mRNA or control siRNA. Importantly, the ST6GalNAcVI-knockdown cells expressed low levels of DSGb5. We thus demonstrated the significantly decreased migration potential of the ST6GalNAcVI-knockdown cells with low DSGb5 expression levels, compared to the control siRNA-transfected cells expressing high DSGb5 levels, but no significant difference in the cell proliferation. Thus, DSGb5 expression may ensure the migration of RCC cells. We propose that DSGb5 expressed on RCC cells may determine their metastatic capability.

Keywords: disialosyl globopentaosylceramide; ganglioside; metastasis; migration; renal cell carcinoma
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Introduction

About one third of renal cell carcinoma (RCC) patients exhibit metastasis upon initial presentation, with 40% of individuals undergoing radical nephrectomy developing post-surgery metastases (Bukowski 1997). Thus, RCC is characterized by its highly metastatic potential; however, the mechanism of metastasis is not fully understood.

A ganglioside, disialosyl globopentaosylceramide (DSGb5), was originally identified from RCC tissue extracts (Saito et al. 1991, 1994) and its expression in a primary RCC was correlated with the metastatic potential (Ito et al. 2001b, c). We previously reported that Siglec-7 is expressed as an inhibitory receptor on NK cells, and binds to internally branched α2,6-linked disialic gangliosides, such as DSGb5 (Ito et al. 2001a). Thus, DSGb5 inhibits NK cell cytotoxicity to RCC cells in a DSGb5-Siglec7-dependent manner (Kawasaki et al. 2010). Consequently, DSGb5 has been thought to produce a favorable environment for the survival and metastases of RCC cells (Ito et al. 2001a; Kawasaki et al. 2010). However, the role of DSGb5 in RCC metastasis has not been clarified. Therefore, to elucidate the functional roles of DSGb5 in RCC cells, we examined the ability of DSGb5 to affect cell proliferation and migration.

Materials and Methods

Cell lines

ACHN cells were originally derived from malignant pleural effusion of a patient with widely metastatic RCC and were purchased from Dainipponseiyaku Co. (Tokyo, Japan). The ACHN cells were maintained in DMEM containing glucose (4.5 g/L) (Gibco-Invitrogen, Rockville, MD, USA) supplemented with 10% heat-inactivated FBS (ICN Biomedicals, Aurora, OH, USA) in 5% CO_2 incubators at 37°C.

Monoclonal antibody (mAb)

Anti-DSGb5 mAb 5F3 was established using ACHN cells in

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our laboratory (Ito et al. 2001c). Normal mouse IgM was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Flow cytometric analysis

Cells were incubated with 20 μg/ml normal mouse IgM (Syntesome, Munich, Germany) or anti-DSGb5 mAb 5F3 for 1 h on ice. After washing with PBS containing 1% BSA, cells were incubated with fluorescein-labeled antibodies directed to mouse IgG and IgM (Jackson ImmunoResearch Laboratories) for 30 min on ice, washed, and then analyzed using a FACScan flowcytometer (BD Biosciences, San Jose, CA, USA) with CellQuest software (BD Biosciences).

Dilution cloning of ACHN cells according to DSGb5 expression levels

ACHN cells were detached, washed, and suspended to contain 50 cells in 10 ml of the culture medium. The cells were plated at 0.5 cells per well in a 96-well culture plate in a volume of 100 μL per well. The plates were maintained at 37°C and examined with an inverted phase contrast microscope. Observation of a single colony in a well was interpreted as the presence of monoclonal cells originating from a single cell. The monoclonal cells were isolated and analyzed by flowcytometry using mAb 5F3, with the clones subsequently segregated into two groups based on the level of DSGb5 expression (Grenman et al. 1989; Butterworth et al. 2011).

Establishment of ACHN-derived cells stably expressing siRNA for GalNAc α2,6-sialyltransferase VI

GalNAc α2,6-sialyltransferase VI (ST6GalNAcVI) is the sialyltransferase responsible for the synthesis of DSGb5 from monosialosyl globopentaosylceramide (MSGb5) (Fig. 1) (Senda et al. 2007). To obtain ACHN cell lines expressing low DSGb5 levels, cloned ACHN cells were transfected with a siRNA expression vector targeting ST6GalNAcVI mRNA and were selected as described previously (Kawasaki et al. 2010). Briefly, the siRNA expression plasmid vector was established using a protocol developed by iGENE (http://igene-therapeutics.co.jp/) (Sapporo, Japan) and was purchased from TAKARA Bio (Tokyo, Japan) along with a control plasmid vector. For plasmid DNA, pBAsi-hU6 Pur DNA was used for siRNA expression and the control siRNA vector. The medium was replaced with fresh medium 6 h after transfection and incubated with 5 ng/ml puromycin in DMEM+10% FBS to establish puromycin-resistant colonies. The siRNA sequence used for targeting ST6GalNAcVI was CCAAUGAGGUCUUCCAUUA. The sequence of the scrambled siRNA control was UAUCGUUAACUAGAUCCCG (Kawasaki et al. 2010).

RT-PCR analysis of ST6GalNAcVI expression levels

Total RNA was extracted using TRIzol reagent, according to the manufacturer's protocol (Life Technologies, USA). Concentrations and purities of total RNA were calculated by measuring absorbance at 260 nm with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA). ST6GalNAcVI mRNA expression was analyzed using a LightCycler ST300 (Roche, Mannheim, Germany) with LightCycler Software v3.5 (Roche). Primers used for ST6GalNAcVI amplification were 5′-TCAGCAGTGTTCGTGATCCT-3′ (forward) and 5′-GAAGTGGAGCATCACTGACG-3′ (reverse), as described previously (Senda et al. 2007).

Cell proliferation assay

Cells were seeded in 96-well plates (BD Biosciences, San Jose, CA, USA) at 2×10^3 cells per well in DMEM containing 10% FBS and cultured for various durations. The number of viable cells was measured daily.

Cell migration assay

Cell migration was determined using 24-well plates with Transwell 3422 membrane filters (Corning, NY, USA), which contain polycarbonate membranes with 8 μm pores. The cells were har-

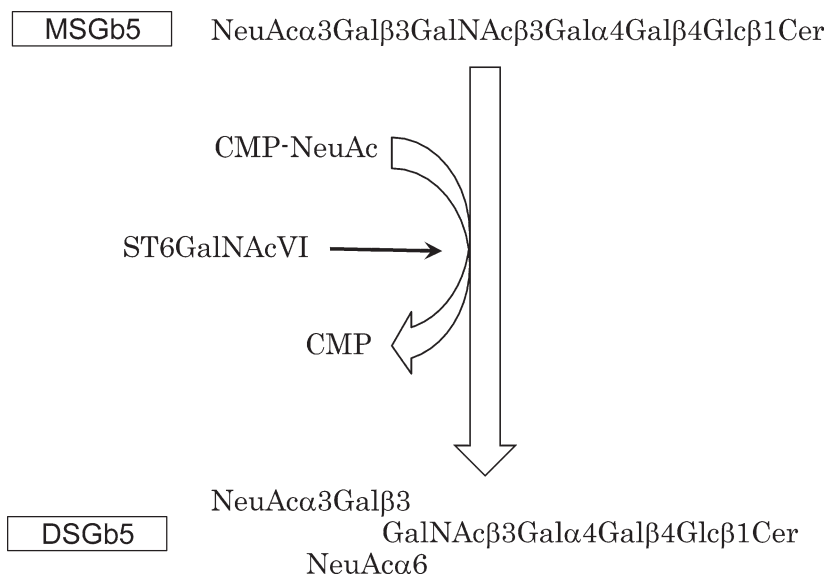

Fig. 1. The ganglioside structure used in the present study and the synthesis of DSGb5.
DSGb5 is synthesized from MSGb5 by the sialyltransferase "ST6GalNAc VI". The ganglioside structures used in the present study and the synthesis of DSGb5 from MSGb5 are summarized in this figure. ST6GalNAcVI belongs to a family of sialyltransferases that modify proteins and ceramides on the cell surface to alter cell-cell or cell-extracellular matrix interactions; the Gene Bank Accession No. of ST6GalNAcVI is M013443.3 (Gene ID 30815).
CMP-NeuAc, cytidine monophosphate-neuraminic acid.

vested, resuspended in DMEM containing 4.5 g/L of glucose with 0.1% FBS, and 100 μl of the cell suspension was seeded into the upper chamber of each filter at a density of 2×10^4 cells per well, with 600 μl of DMEM containing 50 μg/ml fibronectin (#354008, BD Biosciences) added to each lower well. The cells were then incubated at 37°C for 3 or 6 hours (Shang et al. 2012). When the assay was completed, the membrane was removed, fixed in 70% ethanol at 25°C for 30 min, and stained with hematoxylin. The upper side of the membrane was scraped with a cotton swab to remove cells that had attached but not migrated, and the membrane was then mounted onto a microscope slide. Cell migration was assessed in each well by counting the number of cells in 14 randomized fields at 200× magnification using a microscope. The experiments were performed at least 3 times.

Results

Dilution cloning of ACHN cells based on DSGb5 expression levels

Flow cytometric analysis of ACHN cells cultured in DMEM with a high glucose concentration (4.5 g/L), using anti-DSGb5 mAb 5F3, showed two DSGb5 expression peaks (Fig. 2A). Thus, the parent ACHN cell line contained a mixture of cell populations expressing high or low levels of DSGb5. To explore the function of DSGb5, we isolated ACHN cell clones expressing different levels of DSGb5 using the dilution cloning method. The two clones thus obtained, 1D1 and 1H4, expressed high levels of DSGb5, as determined by flow cytometric analysis (Fig. 2A), while the clone 1E2 expressed low levels of DSGb5. The 1E2 cell line was used to investigate the functional consequence of DSGb5 expression by comparing its features with the cell lines expressing high levels of DSGb5. RT-PCR analysis indicated that the expression levels of ST6GalNAcVI mRNA were similar between the parental ACHN cells and each of isolated ACHN clones (Fig. 2B). ST6GalNAcVI is the sialyltransferase responsible for DSGb5 synthesis. Moreover, all isolated clones expressed similar levels of ST6GalNAcVI mRNA, despite having different DSGb5 expression levels on their cell membranes.

Establishment of ACHN-derived cell lines with low DSGb5 expression levels

1D1 and 1H4 cells with high DSGb5 expression levels were used for this series of experiments. To explore the features attributed to differences in the DSGb5 expression

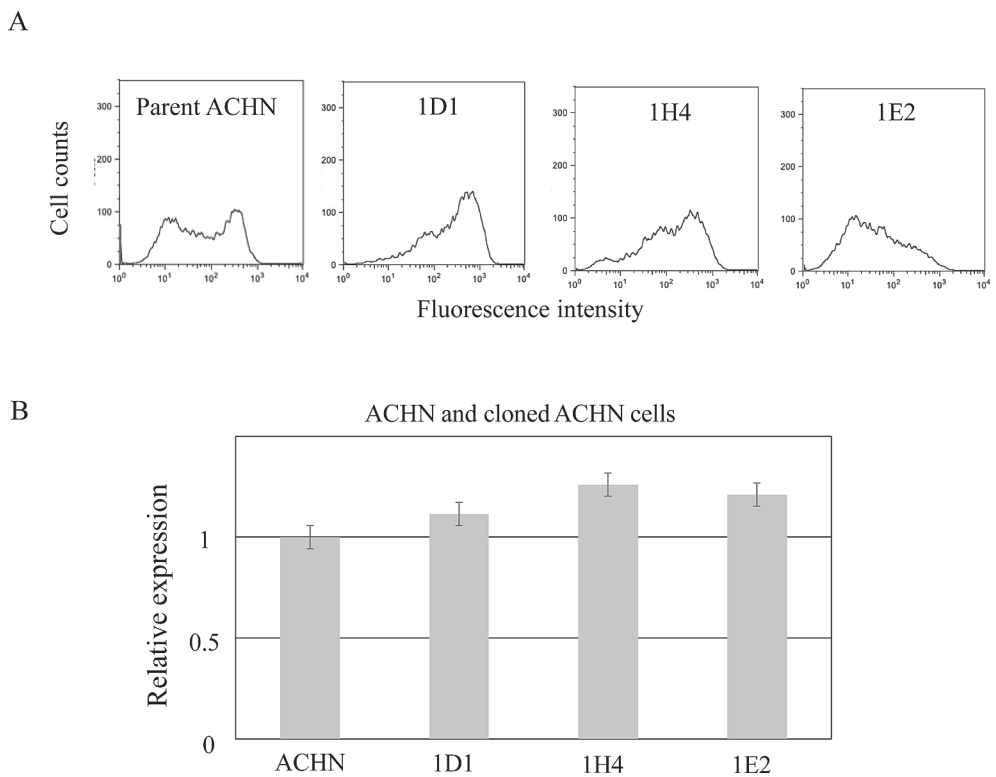

Fig. 2. Isolation of ACHN-derived cell clones with different expression levels of DSGb5.
Flow cytometric analysis was performed using mAb 5F3. The ordinate and abscissa show cell counts and fluorescence intensity, respectively. The cell surface expression of DSGb5 is proportional to fluorescence intensity; namely, these data show differences in the expression levels of DSGb5 among isolated cell lines. (A) Expression of DSGb5 in parental ACHN cells and individual ACHN clones (1D1, 1H4, and 1E2) determined with mAb 5F3. Clones were obtained from parental ACHN cells using the limiting dilution cloning method based on DSGb5 expression levels. (B) RT-PCR analysis of ST6GalNAcVI mRNA expression levels in isolated ACHN clones (1D1, 1H4, and 1E2). Note that there are no significant differences among parental ACHN cells and their isolated clones in relative expression levels of ST6GalNAcVI mRNA.

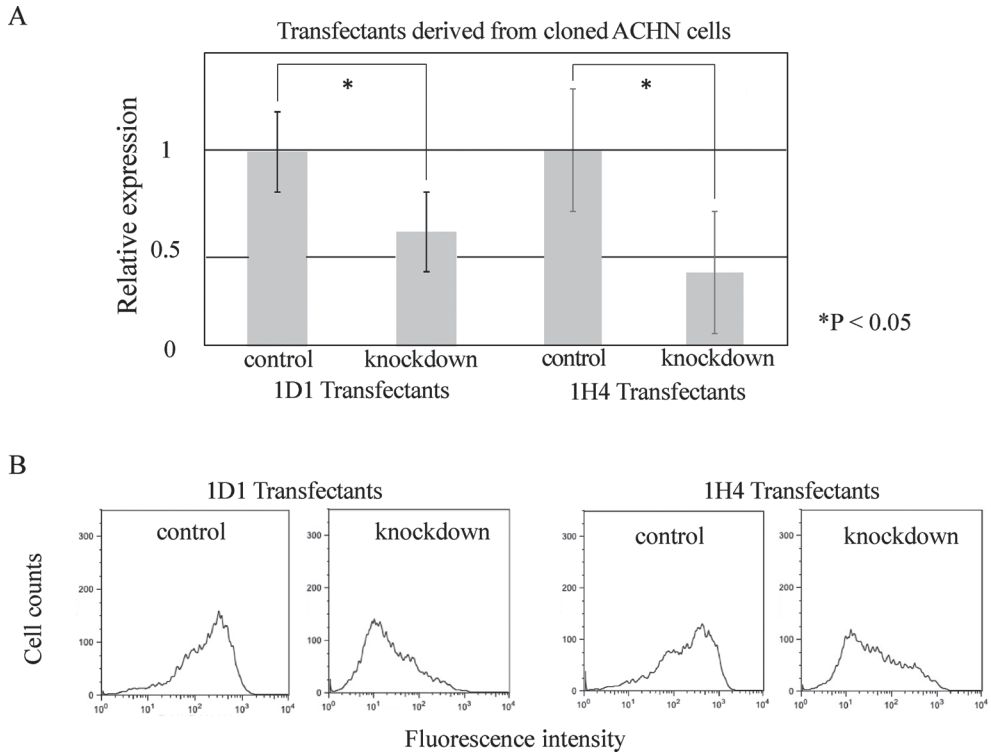

Fig. 3. Establishment of transfectants expressing different levels of DSGb5 from ACHN derived cell lines. (A) Knockdown of ST6GalNAcVI mRNA expression in transfectants expressing ST6GalNAcVI siRNA or control siRNA. 1D1 and 1H4 cells expressing high DSGb5 levels were stably transfected with ST6GalNAcVI siRNA or control siRNA. The transfectants were analyzed for ST6GalNAcVI mRNA expression by RT-PCR analysis. (B) Expression profiles of DSGb5 in transfectants expressing ST6GalNAcVI siRNA or control siRNA. Note that DSGb5 expression was down-regulated by knockdown of ST6GalNAcVI mRNA expression ($P < 0.05$).

levels, we established the cell lines expressing low levels of DSGb5 by ST6GalNAcVI mRNA knockdown. 1D1 and 1H4 cells were stably transfected with siRNA targeting ST6GalNAcVI mRNA or control siRNA using puromycin selection (Kawasaki et al. 2010). As expected, the expression level of ST6GalNAcVI mRNA was significantly decreased in 1D1 and 1H4 cells transfected with ST6GalNAcVI siRNA, compared to transfectants expressing control siRNA (Fig. 3A). Importantly, DSGb5 expression levels in 1D1 and 1H4 cells transfected with ST6GalNAcVI siRNA were greatly reduced, compared to control transfectants (Fig. 3B). We also confirmed that DSGb5 expression levels did not fluctuate over several passages (data not shown).

No noticeable effect of DSGb5 on cell proliferation

We next explored the difference in cell proliferation between ACHN-derived clones with high DSGb5 expression levels (1D1 and 1H4) and the clone with low DSGb5 expression levels (1E2). The cell proliferation of these three clones was not significantly different (Fig. 4A, left), suggesting that the DSGb5 expression level may be not a key determinant of cell proliferation. To confirm this notion, we also used the ST6GalNAcVI-knockdown cells derived from 1D1 and 1H4 cells, which expressed low levels of DSGb5 (see Fig. 3). Decreased expression levels of ST6GalNAcVI mRNA and DSGb5 did not significantly alter the cell proliferation (Fig. 4A, middle and right).

DSGb5 enhances cell migration

Using the same sets of the established clones, we next analyzed the role of DSGb5 in the cell migration (Fig. 4B). The transwell assay showed the significantly higher migration activities of the 1D1 and 1H4 cell lines with high DSGb5 expression levels, compared to the 1E2 cells with low DSGb5 expression levels (Fig. 4B, left). In addition, the 1D1 and 1H4 transfectants expressing control siRNA showed higher migration activities than those of the ST6GalNAcVI-knockdown transfectants with low DSGb5 expression levels (Fig. 4B, middle and right). Thus, the low expression levels of DSGb5 significantly decreased the degree of migration. These results suggest that cells with high DSGb5 expression had greater migration ability, compared to cells with low DSGb5 expression. Therefore, DSGb5 expression may be correlated with enhanced RCC cell migration.

Discussion

In the present study, we isolated ACHN clones with high or low expression levels of DSGb5 and showed that clones expressing high levels of DSGb5 exhibited higher migration potential than the clone expressing low levels of

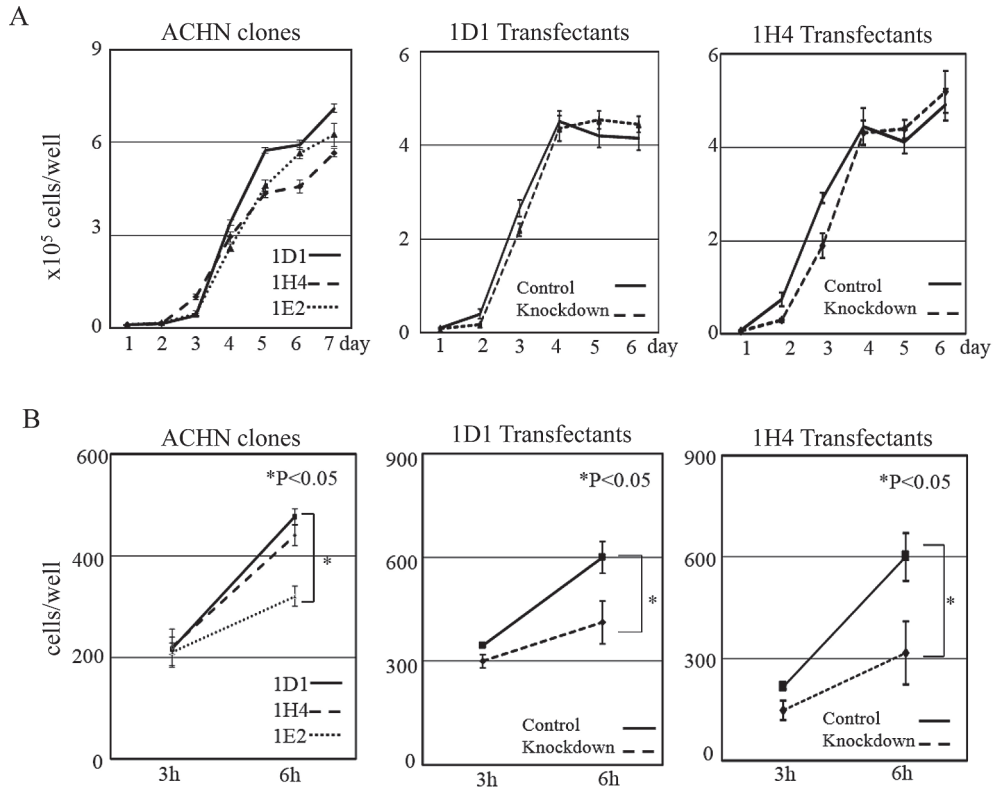

Fig. 4. Distinct role of DSGb5 in cell proliferation and migration.
(A) Role of DSGb5 in proliferation of three ACHN clones and the transfectants of 1D1 and 1H4 cells. Cells were seeded in 96-well plates at 2×10^3 cells per well and cultured, and the number of cells was measured daily. There were no significant differences among individual ACHN clones (left) and between the transfectants of 1D1 and 1H4 cells (middle and right). (B) Role of DSGb5 in cell migration. Cell migration was determined using 24-well plates with transwell membrane filters. Left panel shows the migration activity of 1D1 and 1H4 cells with high DSGb5 expression levels and 1E2 cells with low DSGb5 expression levels. Middle and right; control transfectants and ST6GalNAcVI-knockdown transfectants for 1D1 (middle) and 1H4 cells (right). The isolated clones with high DSGb5 expression levels (1D1 and 1H4) and the transfectants expressing control siRNA showed higher migration activities compared to the 1E2 cells with low DSGb5 expression levels and the ST6GalNAcVI-knockdown transfectants with low DSGb5 expression levels ($P < 0.05$), respectively.

DSGb5. We then established the stable transfectants with low expression levels of DSGb5 using siRNA for ST6GalNAcVI. In our experiments, we used stable transfectants expressing siRNA, instead of cells transiently expressing siRNA, because the knockdown effect seen in transient transfectants continued for only 74 hours, which was insufficient to examine proliferation.

Although glycosylation requires glycosyltransferases, the expression of glycolipids is not always correlated with glycosyltransferase RNA quantity. Glycosylation also depends on the amount of substrate as well as glycosyltransferase activity. In this regard, differences in the DSGb5 expression are thought to be influenced by glycosyltransferase activity as well as substrate quantity. However, in our experiments comparing ST6GalNAcVI-knockdown and control transfectants, DSGb5 levels were directly dependent on ST6GalNAcVI mRNA levels, because their cellular characteristics were equivalent, including substrate quantity.

Comparison of siRNA transfectants and control transfectants indicated that the functional role of DSGb5 was to enhance the cell migration. Several gangliosides have been reported to enhance cellular migration by associating with certain molecules. For instance, GD3 promotes cell growth and invasion of melanoma cells through the p130Cas and paxillin signaling pathway (Hamamura et al. 2005). GM3 promotes HGF-stimulated migration of murine hepatoma cells through enhanced phosphorylation of cMet at specific tyrosine sites, as well as PI3K/Akt-mediated migration signaling (Li et al. 2013). Additionally, GM1 association with homodimeric galectin-1 provides the molecular basis for growth regulation of human neuroblastoma cells (Kopitz et al. 2012). On the other hand, certain gangliosides have been reported to suppress cell migration in other cancers. GD3-synthase over expression inhibits survival and angiogenesis of pancreatic cancer cells through cell cycle arrest (Sarkar et al. 2014). The influence of DSGb5 on RCC cells via an intracellular pathway has been not elucidated. Therefore, we are now engaged in the analysis of signaling pathways related to RCC cell migration.

Gangliosides are known to exist in lipid rafts on cell membranes. Lipid rafts are cholesterol and sphingolipid-

enriched membrane microdomains that form compartmental platforms for cellular signaling (Zhou and Kramer 2005). Some molecules co-localizing with gangliosides on lipid rafts, such as integrins, vinculin, and paxillin, have been reported to induce cell migration and adhesion by regulating actin cytoskeleton dynamics (Hakomori 2008; Mitsuzuka et al. 2005; Wang et al. 2013). Protein kinases, such as the Src family, are present in lipid rafts and are important for the function of RhoA, which modulates actin cytoskeleton dynamics and cell morphology during cellular motility (Zhou and Kramer 2005). Further characterization of lipid raft constituents is essential to elucidate the regulatory mechanisms of DSGb5 on cellular migration in RCC.

In our recent report, immunohistochemical analysis of 130 radical prostatectomy specimens revealed that DSGb5 expression was significantly correlated with lymphovascular invasion of prostate cancer cells (Shimada et al. 2014). In the process of cancer metastasis, lymphovascular invasion is the critical step occurring subsequent to cellular migration. It is suggested that cancer cells with enhanced migration due to elevated DSGb5 expression could easily invade the lymphovascular system. The effect of DSGb5 in cancer metastases was observed in RCC cells, as well as clinicopathological findings in prostate cancer tissues.

It is noteworthy that knockout of ganglioside synthase, which affects lipid raft composition, reduced migration of normal human fibroblasts (Liu et al. 2010), and immunotherapy with anti-GD2 mAbs results in long term survival of patients with neuroblastoma (Ahmed and Cheung 2014). In the present study, we found that knocking down ST6GalNAcVI mRNA expression reduced the effect of DSGb5 on RCC cells; therefore, DSGb5 represents a potential anti-tumor treatment target for RCC patients. In fact, GD3 synthase knockdown completely abrogated *in vivo* tumor formation of cancer stem cells in breast cancer (Battula et al. 2012).

In conclusion, we have provided evidence that DSGb5 expressed on RCC cells may confer the metastatic capability, thereby enhancing cell migration. Down-regulation of the expression of DSGb5 or inhibition of the effect of DSGb5 represents a possible therapeutic avenue for RCC. However, further investigation of DSGb5 and related molecules is required to elucidate the regulatory mechanisms for cellular migration in RCC and to forward the development of DSGb5-based therapeutics.

Acknowledgments

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Conflict of Interest

The authors have no conflict of interest.

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