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Enhanced expression of the β4-N-acetylgalactosaminyltransferase 4 gene impairs tumor growth of human breast cancer cells

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ABSTRACT

Two β4-N-acetylgalactosaminyltransferases (β4GalNAcTs), β4GalNAcT3 and β4GalNAcT4, have been shown to be involved in the synthesis of the GalNAcβ1 → 4GlcNAc (LacdiNAc) group expressed on the outer branches of N- and/or O-glycans, and only β4GalNAcT4 is expressed in human mammary gland. We found that the expression level of the LacdiNAc group decreases as human breast cancers progress. To investigate biological significances of this disaccharide in human breast cancers, we transfected the FLAG-tagged β4GalNAcT4 cDNA into MDA-MB-231 cells, and obtained several clones showing enhanced expression of the gene. Clones 1 and 2 showed 15 and 9 times more transcript than mock-transfected cells. The FLAG-β4GalNAcT4 protein and its product, the LacdiNAc group, were detected in clone 1 and 2 cells. No change was observed in their growth rates while significant decreases in colony forming and invasive abilities were observed for clone 1 and 2 cells. When clone 1 cells were transplanted subcutaneously into nude mice, no tumors were formed while tumors were formed with mock-transfected cells. These results indicate that the expression of the LacdiNAc group is quite important for the suppression of malignancies of the MDA-MB-231 cells.

1. Introduction

N- and O-glycans play important roles in many biological systems including embryonic development and disease progression [1]. The GalNAcβ1 → 4GlcNAc (LacdiNAc) group is found in N- and O-glycans of both vertebrates and invertebrates, but its distribution appears to be limited in mammals [2,3]. However, recent progress in analytical methods for glycan structures has shown that the disaccharide group is distributed to a variety of mammalian tissues and cells. Regarding to the biosynthesis of the LacdiNAc group, two human β4-N-acetylgalactosaminyltransferases (β4GalNAcTs), β4GalNAcT3 (GeneBank™ accession number AB089940) and β4GalNAcT4 (GeneBank™ accession number AB089939) have been identified [4,5]. Both β4GalNAcTs can transfer GalNAc from UDP-GalNAc to the non-reducing terminal GlcNAc residues of N- and O-glycans in a β-1,4-linkage [6,7]. Although these enzymes exhibit high sequence homology and similar substrate specificities *in vitro*, they show different tissue distribution. Accordingly, the β4GalNAcT3 gene is expressed in human stomach, colon and testis, while the β4GalNAcT4 gene is expressed in human brain and ovary [5]. The LacdiNAc group is often modified further by sulfation [8], sialylation [9] and/or fucosylation [10], and these modified disaccharides play important roles in certain biological systems. For instance, the sulfated form expressed on a pituitary glycohormone, lutropin, is involved in the clearance by recognition with the mannose/GalNAc-4-SO_4 receptor on hepatic endothelial cells, and then, removed from the circulation [11].

Enhanced expression of the LacdiNAc group as well as β4GalNAcT gene has been reported to be associated with the progression of human prostate, ovary, liver and colon cancers [12–16]. Overexpression of the β4GalNAcT3 gene in HCT116 human colon cancer cells significantly increases malignant potentials such as colony formation in agarose gel and invasion [16]. In contrast, the increased expression of the β4GalNAcT3 gene correlates with favorable prognosis of human neuroblastomas [17]. Accordingly, over-expression of the β4GalNAcT3 gene in human neuroblastic cell lines, SK-N-SH and SK-SY5Y cells, results in decreases in potentials

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of cell migration, cell proliferation, colony formation, and invasion as compared to those of the control cells [17]. Thus, the expression of the LacdiNAc group appears to modulate malignant phenotypes of individual tumors in a tissue-dependent manner.

We previously reported the decreased expression of the LacdiNAc group on N-glycans in human primary breast carcinomas correlates with advanced clinical stages and prognostic statuses [18]. This suggests that the expression of the LacdiNAc group on N-glycans is important for keeping mammary epithelial cells in a normal condition. However, biological roles of the LacdiNAc group in human breast cancers remain to be elucidated. Therefore, in the present study, we cloned MDA-MB-231 human breast cancer cells showing the enhanced expression of the β4GalNAcT4 gene, and examined their potentials of cell proliferation, colony formation, tumor formation and invasion.

2. Materials and methods

2.1. Antibodies and lectins

The following antibodies and lectins were obtained; monoclonal anti-FLAG M2 antibody were from SIGMA-Aldrich (St. Louis, MO), anti-TGN38 antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Alexa Fluor dyes-conjugated mouse and rabbit IgGs from Life technologies (Waltham, MA), biotin-labeled *Wisteria floribunda* agglutinin (WFA) from Vector Laboratories (Burlingame, CA), FITC-conjugated WFA from EY Laboratories Inc. (San Metro, CA), HRP-conjugated *Ricinus communis* agglutinin 120 (RCA-120) from J-OIL MILLS, Inc. (Tokyo), respectively.

2.2. Cell culture and transfection of plasmid

MDA-MB-231 human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA), and cultured in DMEM containing 10% fetal calf serum at 37 °C.

A human full-length β4GalNAcT4 cDNA was isolated using the Marathon-Ready cDNAs from human brain as a template (Clontech Laboratories Inc., Mountain View, CA), and cloned into a p3 X FLAG-CMV-10 vector (SIGMA-Aldrich). The plasmid was transfected into MDA-MB-231 cells using a TransIT®-BrCa reagent according to the manufacturer's protocol (TAKARA BIO INC., Shiga, Japan). Single cell clones were obtained by the treatment with geneticin (250 μg/ml).

2.3. Quantitative real-time PCR analyses

Total RNA preparations were obtained by using an RNAiso Plus reagent (TAKARA BIO INC.). Then, cDNA was synthesized using an iScript Reverse Transcription Supermix reagent (Bio-Rad Laboratories Inc., Hercules, CA). Indicated amounts of a Kapa SYBR FAST reagent (KAPA Biosystems, Woburn, MA), sets of primers described below, and the obtained cDNA were mixed, and real-time PCR was carried out. PCR conditions were as follows; 95 °C for 10 s, followed by 35 cycles of 95 °C for 5 s, and 60 °C for 20 s. Primers used for real-time PCR were as follows; GAPDH (forward 5′-CTGACTTCAACAGCGACACC-3′; reverse 5′-CTGTAGCCAAATTCGTTGTCAT-3′), β4GalNAcT4 (forward 5′-GCCAGGACGTGATGGTTCACT-3′: reverse 5′-CATGTCCGCCAGGAACTGT-3′).

2.4. Biochemical and immunocytochemical analyses

Western blot analysis was performed as described previously [19]. Briefly, extracted proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (8%), and transferred to PVDF membrane. The blot was incubated with an anti-FLAG antibody, and after washing, incubated with an HRP-conjugated anti-mouse IgG antibody. For lectin blot analysis, the blot was incubated with biotin-labeled WFA, and then with an HRP-labeled streptavidin. In some cases, membrane proteins were treated with N-glycosidase F (Roche Applied Science, Penzberg, Germany) and neuraminidase from *Arthrobacter ureafaciensas* (Nacalai tesque, INC, Kyoto, Japan) prior to SDA-PAGE. Proteins were visualized with a chemiluminescent reagent. Immunofluorescent analysis was carried out as described previously [19]. For flow-cytometric analysis, trypsin-treated cells were washed with PBS containing 5 mM EDTA and 5 mg/ml of BSA, and then incubated with FITC-conjugated WFA. Likely, cells were incubated with biotin-conjugated RCA-120, and then treated with avidin-conjugated Alexa 488. Cells were analyzed by Cell Lab Quanta SC system (Beckman Courter Inc., Brea, CA).

2.5. Cell proliferation, invasion, and colony formation assays

Cell proliferation was assayed by using a MTT cell proliferation and cytotoxicity assay kit (BOSTER BIOLOGICAL TECHNOLOGY., LTD, Fremont, CA) according to manufacture's manual. For invasion assay, BD BioCoat Matrigel Invasion Chambers (Corning International, Corning, NY) were used. Each 25,000 cells were placed in the upper chambers with serum-free media and the lower chamber was filled with the media containing 10% FCS. Then, cells were allowed to invade the Matrigel membrane for 16 h and the invading cells were fixed in 70% ethanol and stained with a Giemsa solution. The number of the invading cells was counted, and ratios of cells invading Matrigel insert membrane and control insert membrane were calculated. In the case of colony formation assay, 0.5% of soft agar in DMEM containing 10% FCS was placed in 6-well plates as a bottom layer. Cells (5000 cells/well) in DMEM were mixed with a final concentration of 0.35% soft agar solution, and placed in the wells as an upper layer. Cells were cultured for 4 weeks until colonies were formed, and then colonies were stained with crystal violet.

2.6. Analysis of tumor growth activity of clone 1 cells

Each 2×10^6 cells were transplanted subcutaneously into both flanks of male Balb/c nu/nu nude mice (Japan SLC Inc., Hamamatsu, Japan). Animals were maintained under a specific pathogen-free condition, and sizes of tumors formed were measured periodically. When tumors reached to 5–8 mm long, animals were sacrificed. This study was performed in accordance with the recommendation of the Guidelines for the Care and Use of Laboratory Animals of the Japanese Society for Biochemistry, and the experimental procedures were approved by the Committees on the Ethics of Animal Experiments of Nagaoka University of Technology.

3. Results

3.1. Cloning of MDA-MB-231 cells showing higher expression of the β4GalNAcT4 transcript

The LacdiNAc group is expressed on N-glycans of human breast tissue, and its expression level decreases in the breast cancers [18]. The β4GalNAcT4 gene but not the β4GalNAcT3 gene is expressed in human breast tissue [5]. In order to enhance β4GalNAcT activity, human β4GalNAcT4 cDNA cloned into a 3xFLAG-CMV-10 vector was transfected into MDA-MB-231 cells. After selection with geneticin, several clones were obtained, and their mRNA levels were determined by quantitative real-time PCR analysis. The results showed that clone 1 and clone 2 cells express higher levels of the β4GalNAcT4 transcript than other clones, which are about 15 and 9 times higher than that of the mock-transfected cells,

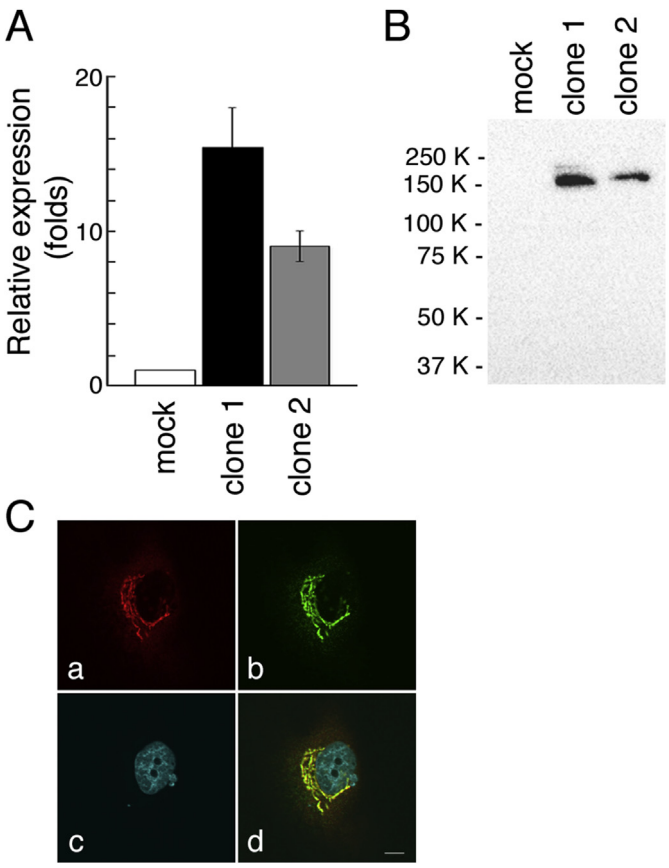

Fig. 1. Expression of β4GalNAcT4 protein in the cells. (A) Expression of the β4GalNAcT4 transcript in mock-transfected cells and cloned cells was quantified by real-time PCR as described in the text. The transcript levels were normalized against that of GAPDH. Results were obtained from three independent experiments, and are indicated as the mean ± S.E. (B) The expression of β4GalNAcT4 protein was determined by Western blot analysis using anti-FLAG antibody. (C) The distribution of β4GalNAcT4 protein in the cells is shown by double immunofluorescence using anti-FLAG antibody (a), anti-TGN38 antibody (b), DAPI (c), and they were overlaid (d). Scale bar, 10 μm.

respectively (Fig. 1A). Under the condition, there was no change in the expression level of the β4-galactosyltransferase 1 gene in the clones (unpublished data).

The expression of FLAG-β4GalNAcT4 protein was determined by Western blot analysis. When the blot was incubated with anti-FLAG antibody, an 150 K protein, which is one with an expected molecular weight based on the size of the cDNA transfected, was mainly detected in clone 1 and 2 cells (Fig. 1B). Clone 1 cells produced a 150 K protein much more than clone 2 cells (Fig. 1B), which is consistent to the transcript levels shown in Fig. 1A. Cytochemical analysis of clone 1 cells showed that FLAG-β4GalNAcT4 (Fig. 1C-a) co-localizes with TGN38 (Fig. 1C-b), which is a marker of the trans Golgi network (TGN), (Fig. 1C-d), indication that β4GalNAcT4 resides in the TGN.

To examine whether FLAG-β4GalNAcT4 can produce the LacdiNAc group in cell surface glycans of clone 1 and 2 cells, flow cytometric analysis using FITC-labeled-WFA was performed. The expression level of the LacdiNAc group at the cell surface increased in clones 1 and 2 cells (indicated in red and green, respectively) as compared with the mock-transfected cells (in blue) (Fig. 2A-a). Although the expression level of the β4GalNAcT4 transcript was higher in clone 1 cells than in clone 2 cells (Fig. 1A), there was no significant difference in the expression level of the LacdiNAc group at the cell surface between these two clones (Fig. 2A-a). These results indicate that clone 1 cells cannot produce much more the LacdiNAc group than clone 2 cells although the β4GalNAcT4 transcript and its protein levels in clone 1 cells are higher than clone 2 cells. When incubated with Alexa 488-labeled RCA-120, which interacts with the Galβ1 → 4GlcNAc/Glc group, no significant difference was detected between mock-transfected cells and clone 1 cells (Fig. 2A-b).

Membrane proteins were subjected to lectin blot analysis to detect individual glycoproteins carrying the LacdiNAc groups using WFA lectin. Proteins with molecular sizes of 150 K and 200 K, which are indicated with arrowheads, reacted with WFA in clone 1 and clone 2 cells but slightly in mock-transfected cells (Fig. 2B-d). Lectin binding was nullified when the blot was incubated in the presence of 100 mM GalNAc but not GlcNAc (data not shown), indicating that both proteins possess glycans with the LacdiNAc groups. When the blot was treated with PNGase F followed by incubation with WFA, the lectin-positive protein bands disappeared (Fig. 2B-d). Taken together, the LacdiNAc groups are shown to be expressed on N-glycans. After treatment with neuraminidase from *A. ureafaciensas*, WFA-positive 150 K and 200 K bands, which are indicated with arrows, were detected in both mock-transfected cells and two cloned cells, indicating that a part of the LacdiNAc groups produced in MDA-MB-231 cells are sialylated (Fig. 2B-e). The 75 K and 140 K bands detected in all samples reacted with HRP-streptavidin (data not shown), indicating that they are not binding to WFA lectin.

3.2. Proliferation rates and colony forming abilities of clone 1 and 2 cells

When proliferation rates of mock-transfected cells and clone 1 and 2 cells were determined by MTT assay, they showed similar proliferation rates (Fig. 3A). In order to examine whether the increased expression of the LacdiNAc group at the cell surface affects malignancies of clone 1 and 2 cells, anchorage-independent cell growth was examined by soft agar colony formation assay. Several large colonies were formed by mock-transfected cells, and the number of colonies with diameters more than 200 μm was 13.5 ± 2.0/well together with many other colonies with smaller sizes (Fig. 3B and C). In contrast, zero and a few numbers of colonies with diameters more than 200 μm were detected in clone 1 and 2 cells, respectively (Fig. 3B and C). Furthermore, the number of colonies with diameters less than 200 μm also decreased markedly in clone 1 and 2 cells (Fig. 3B). These results indicate that the expression of the LacdiNAc group in the cell surface glycoproteins attenuates the colony forming abilities of clone 1 and 2 cells.

3.3. Tumor growth activity of clone 1 cells

Mock-transfected cells and clone 1 cells were transplanted subcutaneously into nude mice. Two weeks later, tumors were formed in nude mice transplanted with mock-transfected cells but not clone 1 cells. Forty-five days after transplantation, tumors formed only with mock-transfected cells were excised, and their volumes were determined. The results showed that the average volume of tumors formed with mock-transfected cells is 107.1 ± 20.7 mm^3, while no tumors were formed with clone 1 cells (Fig. 4A). Likewise, clone 2 cells were transplanted into nude mice, and no tumors were formed (data not shown).

Since MDA-MB-231 cells possess a high invasive activity [20], effect of the expression of the LacdiNAc group at the cell surface on invasive activity of the cells was examined by Matrigel invasion assay. The results showed that clone 1 and 2 cells exhibit decreased invasive activities, which is about 60% that of mock-transfected cells (Fig. 4B). Thus, the expression of the LacdiNAc group at the cell surface also reduces an invasive activity of the cells.

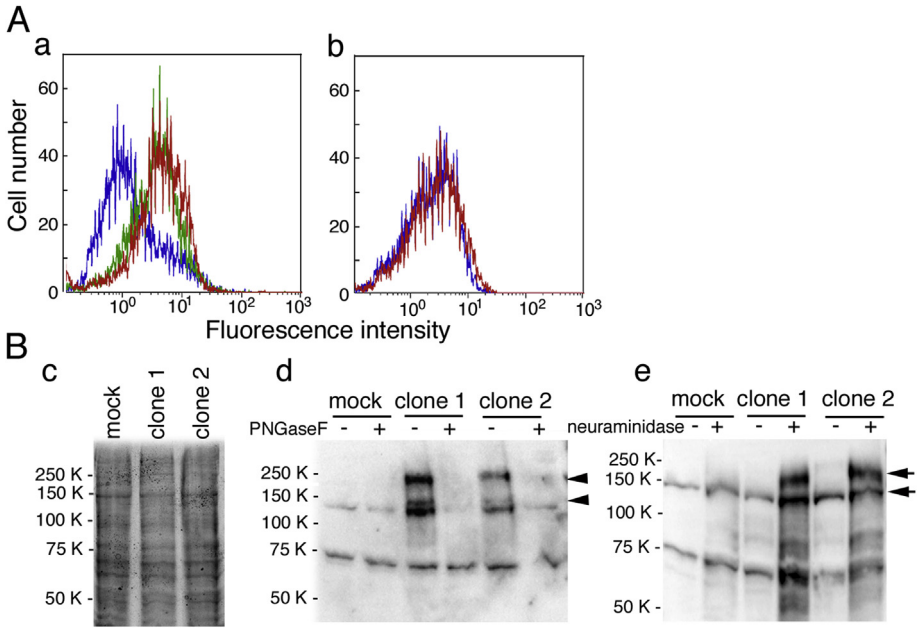

Fig. 2. Expression of the LacdiNAc group at the cell surface of the cells. (A) Expression of the LacdiNAc group at the cell surface of mock-transfected, clone 1 and 2 cells were analyzed by flow cytometry using fluorescence-labeled WFA lectin (a) and -RCA 120 lectin (b) as described in the text. Blue, red and green indicate mock-transfected, clone 1 and clone 2 cells. (B) Membrane glycoproteins prepared from mock-transfected, clone 1 and clone 2 cells were separated in SDS-PAGE and stained with CBB (c). The blots were treated with PNGase F (d) or neuraminidase (e) prior to incubation with WFA lectin as described in the text.

4. Discussion

Now, there are number of reports showing the occurrence of the LacdiNAc group in glycoproteins of mammalian tissues and cells, and the involvement of this disaccharide group in the progression of certain human tumors [12–18]. Interestingly, effect of the LacdiNAc group on tumor growth appears different from tissue to tissue [21]. We previously demonstrated that decreased expression of the LacdiNAc group attached to N-glycans correlates with advanced clinical stages of human breast cancers and with poor prognosis of the patients [18]. However, little is known about biological roles of the LacdiNAc group in tumor growth of human breast cancers. Our present study clearly demonstrated that the enhanced expression of the LacdiNAc group in MDA-MB-231 human breast cancer cells causes suppression of malignant

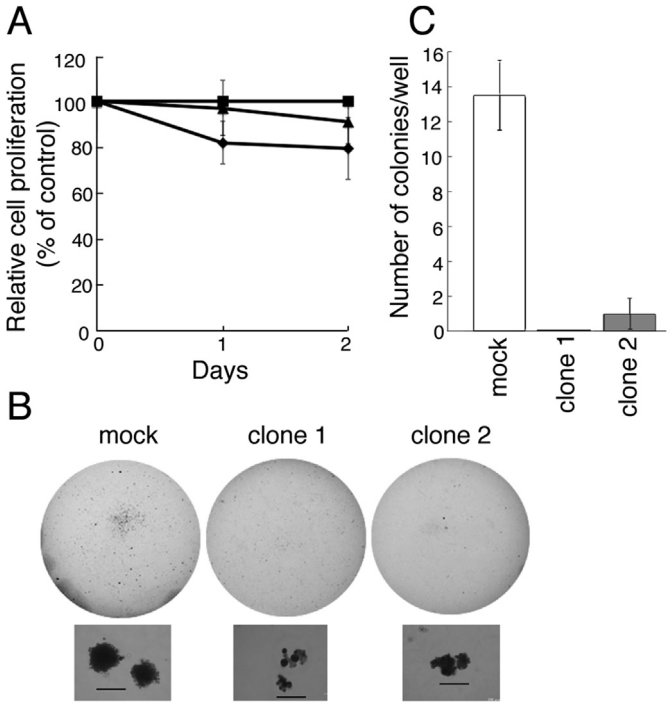

Fig. 3. Effects of increased expression of the LacdiNAc group on malignant phenotypes of the cells. (A) Cell proliferation was determined by MTT assay. Square, diamond and triangle indicate mock-transfected, clone 1 and clone 2 cells, respectively. (B) Typical colonies formed with mock-transfected, clone 1 and clone 2 cells are shown. Scale bars indicate 200 μm. (C) Numbers of colonies with diameters larger than 200 μm are shown.

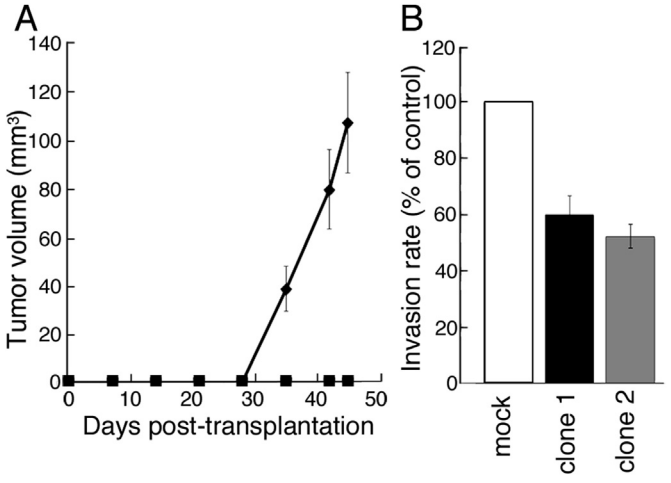

Fig. 4. Effects of increased expression of the LacdiNAc group on tumor growth and invasive activities of the cells. (A) Mock-transfected and clone 1 cells were transplanted into nude mice. Average volumes of tumors (calculated by $1/2 \times \text{length} \times \text{width}^2$) are shown. Diamond and square indicate mock-transfected and clone 1 cells. (B) Invasive activities of mock-transfected, clone 1 and clone 2 cells were determined using matrigel invasion chambers as described in the text.

phenotypes such as colony formation, cell invasion, and tumor formation in nude mice.

It has been well known that alteration of glycan structures of the cell surface receptor glycoproteins such as β1-integrin is associated with the progression of human tumors and modulates cell-to-cell interaction, migration and invasion of tumor cells. In fact, enhanced expression of the β1,6-branched N-glycans in α5β1-Integrin by the transfection of the N-acetylglucosaminyltransferase V cDNA promotes the potential of migration and invasion of the human fibrosarcoma cells, and eventually attenuates E-cadherin-mediated cell-to-cell adhesion [22]. Furthermore, the sialylation and fucosylation of N-glycans of epidermal growth factor receptor (EGFR) inhibit its dimerization and the following its phosphorylation, which leads to the suppression of invasive potentials of human lung cancer cells [23]. When the β4GalNAcT4 gene is expressed in MDA-MB-231 cells, a significant increase of the LacdiNAc group in N-glycans of two membrane glycoproteins, whose molecular weights are 150 K and 200 K, is detected. Although we failed to identify these proteins, they might be cell surface receptors and involved in a signal transduction cascade associated with tumorigenesis of the breast cancer cells. Because previous studies demonstrated that overexpression of the β4GalNAcT gene results in the increases of the LacdiNAc group in N-glycans of glycoproteins such as β1-integrin [17], EGFR [24], leukemia inhibitory factor receptor (LIFR) and gp130 [25], and the LacdiNAc group in these proteins affects their underlying signal transduction pathways. In the case of SK-N-SH and SK-SY5Y neuroblastic cells with the higher expression level of β4GalNAcT3, the increased expression of the LacdiNAc group on N-glycans of β1-integrin causes a decrease in the phosphorylation level of tyrosine residues of FAK, Akt and ERK signaling molecules, and suppresses the migration, proliferation, colony formation and invasion of the cells [17]. The present study also showed that MDA-MB-231 cells with the higher expression of the β4GalNAcT4 gene exhibit the reduced colony forming and invasive activities like the β4GalNAcT3-transfected neuroblastic cells described above. Therefore, it is considered that growth and metastatic activities of human breast cancer cells are also regulated by the signaling molecules described above. To assess this, further studies are required.

We found that a part of this disaccharide produced in MDA-MB-231 cells is sialylated (Fig. 2). Recently, the sialylated and/or fucosylated forms of the LacdiNAc group are detected not only in the primary human mammary epithelia cells but also in MCF-7 and MDA157 human breast cancer cells [26]. Modifications of the LacdiNAc group have been well documented [8–10], and these modifications are considered to be important for the recognition by particular cell surface receptors. For examples, the sulfated form and sialylated form of the LacdiNAc group are recognized by the mannose receptor [11] and by the asialoglycoprotein receptor [27], respectively. On the other hand, the LacdiNAc group shows a specific interaction with galectin-3, which is a mammalian β-galactoside-binding lectin, and plays crucial roles in macrophage-mediated immune response [28]. Although it is not clear whether the modifications of the LacdiNAc group enhance malignancies of breast cancer cells, it is important to elucidate the physiological roles of these modifications.

The β4GalNAcT4 gene but not β4GalNAcT3 gene is expressed in human mammary gland and involved in the formation of the LacdiNAc group [5]. However, it is not clear whether that the expression level of the β4GalNAcT gene is altered in the progression of human breast cancers. Since our previous study showed that the expression level of the LacdiNAc group decreases in human breast cancers as revealed by histochemical analysis [18], it is assumed that the expression of the β4GalNAcT gene is down-regulated in the progression of human breast cancers. In the case of human neuroblastomas, the expression of β4GalNAcT3 correlated with a favorable tumor histologic profile and better patient-prognosis of this cancer [17]. To answer these questions, DNA microarray analysis using patients' specimens is further required.

Breast cancer is a common female cancer, and number of the patients increases worldwide. Elucidation of the molecular mechanisms underlying tumorigenesis and metastasis of this cancer makes it possible to provide suitable therapeutic methods to each patient in a particular subtype of this disease. However, there is no effective tool for patients with triple-negative breast cancer lacking hormone receptors and an epidermal growth factor receptor-2 [29]. Since the present study clearly demonstrated that the expression of the β4GalNAcT gene suppresses several malignant phenotypes of MDA-MB-231 cells, which is a triple-negative type breast cancer cell line, gene therapy using the β4GalNAcT4 cDNA might be effective to these patients.

Conflict of interest

The authors declare no conflict of interest.

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