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Correlation between the sialylation of cell surface Thomsen-Friedenreich antigen and the metastatic potential of colon carcinoma cells in a mouse model

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The cell surface glycosylation profiles of a liver metastatic colon carcinoma variant cell line, SL4 cells previously selected from colon 38 cells *in vivo* for liver colonization were investigated. Flowcytometric analysis was performed with 7 plant lectins and 10 carbohydrate specific monoclonal antibodies. The results showed that peanut agglutinin (PNA), Sambucus nigra agglutinin, Ulex europeus agglutinin-I, anti-Le^x, anti-Le^y, and anti-Le^b antibodies bound to the parental colon 38 cells but not to SL4 cells. Another variant cell line was selected *in vitro* for the paucity of cell surface PNA-binding sites using a magnetic cell sorter and was designated as 38-N4 cells. The binding profiles of plant lectins and carbohydrate-specific antibodies to 38-N4 cells were very similar to those of SL4 cells. After intrasplenic injections, metastatic ability of 38-N4 cells was higher than that of colon 38 cells. PNA binding to SL4 cells and 38-N4 cells was detected after sialidase treatment of these cells, indicating increased sialylation of Thomsen-Friedenreich antigen in these cells. The mRNA levels of sialyltransferases, ST3Gal I, ST3Gal II, ST6GalNAc I, and ST6GalNAc II, were compared. The level of ST3Gal II mRNA was elevated in both SL4 cells and 38-N4 cells, whereas the level of ST6GalNAc II mRNA was elevated in 38-N4 cells compared with colon 38 cells. According to the expression array analysis, there are other glycosyltransferase genes differentially expressed between SL4 and colon 38 cells, yet their involvement in the altered glycosylation in these cells is unclear.

Keywords: **colon carcinoma, metastasis, mucin, Thomsen-Friedenreich antigen**

Abbreviations: **ABA, Agaricus bisporus agglutinin; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; mAb, monoclonal antibody; mMGL, mouse macrophage galactose and N-acetylgalactosamine-specific C-type lectin; PBS, phosphate-buffered saline; PHA-L_4, Phaseolus vulgaris agglutinin-leukoagglutinating subunit 4; PNA, Arachis hypogaea (Peanut) agglutinin; RT-PCR, reverse transcription-polymerase chain reaction; SNA, Sambucus nigra agglutinin; ST3Gal I, ST3Gal II, ST6GalNAc I, and ST6GalNAc II, sialyltransferases are designated according to the recommendation (Tsuji et al., 1996); T-antigen, Thomsen-Friedenreich antigen; UEA-I, Ulex europaeus agglutinin I; VVA-B_4, Vicia villosa agglutinin isolectin B_4; WGA, Triticum vulgaris (Wheat germ) agglutinin.**

Introduction

Varieties of cell surface glycans were reported to alter during tumor progression into metastatic stages [1–3]. Differential expressions of glycans between primary tumors and metastatic foci were reported based on clinical investigations in a variety of carcinomas [4]. The glycans may have functions in the regulation of tumor cell recognition and trafficking as seen with sialylated and fucosylated lactosamine repeats [5]. Another putative marker glycan for carcinoma progression is the Thomsen-Friedenreich antigen (T-antigen), which consists of the Galβ1-3GalNAc sequence on mucins [6,7]. Arachis hypogaea (Peanut) agglutinin (PNA) recognizes this structure [8,9] and has been used in immunohistochemical studies to elucidate the expression of T-antigen in normal, premalignant, and malignant human colonic tissues [10–13]. According to their reports, normal colonic mucosas do not express PNA-binding sites or PNA-binding sites were detected only in the supranuclear (Golgi) regions of epithelial cells. The majority of colon carcinoma tissues express PNA-binding sites. In a murine experimental system, effective immunotherapy of mammary adenocarcinoma

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was achieved using synthetic T-antigen [14]. There are conflicting reports with regard to differential expression of T-antigen between primary colorectal tumors and liver metastases. Cao and co-workers reported that liver metastases expressed higher levels of T-antigen epitopes than primary carcinomas [15]. Bresalier and co-workers reported that primary carcinomas expressed a greater quantity of T-antigen than metastases [16]. Despite these accumulating evidences, whether T-antigen plays any role in metastasis formation remains to be elucidated.

To study such a causal relationship, it is crucial to use syngeneic experimental systems because cell surface glycans often function through the immune system. We have selected highly metastatic colon carcinoma variant cells from the colon 38 cells of C57BL/6 mice. The metastatic variant cell line obtained by four cycles of *in vivo* selection for liver metastasis after intrasplenic injection was named SL4 cells (Morimoto-Tomita et al., to be published). In the present report, we investigated the cell surface expressions of carbohydrate chains by using these variant cells. The parental colon 38 cells were strongly stained with PNA as revealed by flowcytometric analysis, whereas SL4 cells were not. To assess whether cell surface PNA binding sites have a causal effect on liver metastasis, we established another cell line, 38-N4, from colon 38 parental cells by repeated selection of PNA-negative cells with a magnetic cell sorter. The 38-N4 cells showed high metastatic potential *in vivo* in syngeneic mice after intrasplenic injection but the degree was not as high as SL4 cells. Elevated levels of sialylation of T-antigen seemed to be the mechanism responsible for reducing PNA-binding to SL4 cells and 38-N4 cells.

Table 1. Lectins used in this study and their carbohydrate specificity

	Epitope	
Lectin	*Name*	*Carbohydrate structure*
PNA	T	Galβ1-3GalNAc-Ser/Thr
ABA	T + sialyl T	($\pm$Siaα2-3)Galβ1-3($\pm$Siaα2-6) GalNAc-Ser/Thr
VVA-B_4	Tn	GalNAc-Ser/Thr
SNA		Siaα2-6Gal/GalNAc-
UEA-I	H	Fucα1-2Galβ1-4GlcNAc-
WGA		(GlcNAc)2-5; Sia
PHA-L_4		*N*-linked tri/tetra-antennary sugars (GlcNAcβ1-6Man)

Table 2. Monoclonal antibodies used in this study and their carbohydrate specificity

Monoclonal antibody	*Epitope*	
	Name	*Carbohydrate structure*
73-30	Le^X	Fucα1-3(Galβ1-4)GlcNAc
H18A	Le^Y	Fucα1-3(Fucα1-2Galβ1-4)GlcNAc
7LE	Le^a	Galβ1-3(Fucα1-4)GlcNAc
2-25LE	Le^b	Fucα1-2Galβ1-3(Fucα1-4)GlcNAc
TKH2	sialy-Tn	Siaα2-6GalNAc-Ser/Thr
FH6, KM93, CSLEX-1	sialyl-Le^X	Fucα1-3(Siaα2-3Galβ1-4)GlcNAc
CA19-9	sialyl-Le^a	Siaα2-3Galβ1-3(Fucα1-4)GlcNAc
91.9H	sulfo-Le^a	HSO_3-3Galβ1-3(Fucα1-4)GlcNAc

Materials and methods

Lectins and monoclonal antibodies

Biotin-conjugated lectins and monoclonal antibodies used in the present study are listed in Tables 1 and 2. Biotin-PNA [8,9,17], biotin-ABA [18,19], biotin-UEA-I [20] and biotin-PHA-L_4 [21] were purchased from Seikagaku Corp. (Tokyo, Japan). Biotin-SNA [22] was purchased from E.Y. Labs, Inc. (San Mateo, CA). Biotin-VVA-B_4 [23,24] and biotin-WGA [25] were purchased from Vector (Burlingame, CA). mAb 73-30, mAb H18A, mAb 7LE, and mAb 2-25LE were purchased from Seikagaku Corp. mAb CSLEX-1 [26], mAb FH6 [27] and mAb CA19-9 [28] hybridoma cells were purchased from ATCC (Rockville, MD). mAb KM93 [29] was from Kyowa Hakko Kogyo (Tokyo, Japan). mAb TKH2 [30] was from Otsuka pharmaceutical Co., Ltd. (Tokushima, Japan). mAb 91.9H was prepared as previously described [31,32].

Cell lines

The mouse colon carcinoma cell line, colon 38 [33], was kindly provided by Dr. Takashi Tsuruo (Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan). The preparation of the variant colon 38 cell lines, SL4, will be described elsewhere (Morimoto-Tomita et al., to be published). Briefly, tumor foci in the liver of mice injected into the spleen with 1.75×10^6 cells of colon 38 cells were harvested, established in culture, and designated as colon 38-SL1 cells. One million colon 38-SL1 cells were injected into the spleens of mice. Liver tumor lesions were harvested and established in culture. This procedure was repeated two more times, and the cell line obtained in this way was designated as colon 38-SL4 (SL4). The preparation of 38-N4 cells is described below. These cell lines were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing 10% fetal calf serum. For all experiments, except for RT-PCR analysis and microarray analysis, cells were harvested from subconfluent cultures by a 5-min treatment with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA).

Establishment of variant cell lines with low PNA binding to cell surface

Colon 38 cells were washed with MACS buffer (phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin

(BSA) and 2 mM EDTA). The cells were incubated at 8°C for 10 min in 12.5 μg/ml biotin-PNA diluted in PBS containing 2 mM EDTA at 1×10^7 cells/100 μl. The cells were washed with MACS buffer, and then incubated with Microbeads-Streptavidin (Miltenyi Biotec GmbH, Germany) at 8°C for 10 min. The suspension was washed with MACS buffer and the negative fraction was obtained using a BS column with a magnetic cell sorter I (Miltenyi Biotec GmbH). The obtained cell line was designated as 38-N1. The same procedure was repeated 3 more times to obtain 38-N4 cells.

Assay for *in vitro* growth

In vitro growth of colon 38, SL4, and 38-N4 cells was assayed using WST-1 reagent (Roche Diagnostics, Mannheim, Germany). Three thousand cells were seeded in triplicate in 96 well plate and incubated with culture media containing fetal calf serum descried above. After incubation time indicated, WST-1 reagent was added, incubated, and the absorbance was measured according to the manufacturer's protocol.

Sialidase treatment of cells

For flowcytometric analysis after sialidase treatment, cells were incubated with or without 0.1 U/ml sialidase from Clostridium perfringens [34,35] (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (HEPES buffered, pH 7.2) at 37°C for 1 hour.

Flowcytometric analysis

Cells were incubated with or without the biotin-conjugated lectins or the monoclonal antibodies listed in Tables 1 and 2 in fluorescence-activated cell sorter buffer (PBS containing 0.1% BSA and 0.1% sodium azide) for 30 min on ice. Cells were washed and incubated with fluorescein isothiocyanate-streptavidin (Zymed, South San Francisco, CA) for 15 min on ice or fluorescein-conjugated goat affinity-purified antibody to mouse immunoglobulins (Cappel, Inc., West Chester, PA) for 30 min on ice. Cells were washed and then treated with propidium iodide to stain dead cells. Data were analyzed on an EPICS® XL flowcytometer by collecting data only from propidium iodide-unstained cells using WinMDI software (Beckman Coulter, Fullerton, CA).

Assays for metastatic ability

Cells were washed in PBS twice and then resuspended in Hanks' balanced salt solution for injection. C57BL/6 mice were purchased from Charles River Japan Inc. (Yokohama, Japan). Mice (7 weeks old, female) were anesthetized with pentobarbital sodium (Nembutal, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). Single-cell suspensions (1.2×10^6 viable tumor cells in 50 μl Hanks' balanced salt solution) were injected into the spleens of mice. Twenty-four days after injection of the cells, mice were sacrificed, spleens and livers were removed and their weights were measured as indicators of tumor formation in spleens and hepatic metastases.

Statistical analysis

Data were analyzed by Student's *t* test for unpaired samples using StatView software (Abacus Concepts Inc., Berkeley, CA).

RT-PCR analysis of the expression of sialyltransferases

Total RNA was isolated with Ultraspec RNA isolation reagent (Biotecx Lab., Inc., Houston, TX). To avoid contamination with DNA, total RNA was treated with 100 U/ml of RNase-free DNase I (Boehringer Mannheim, Mannheim, Germany) for 1 hour at 37°C. Total RNA was reverse transcribed into cDNA using Super Script II RNase H^- reverse transcriptase (Gibco BRL, Gaithersburg, MD) and oligo(dT) primers (Pharmacia Biotech, Upsala, Sweden) and followed by treatment with RNase H (Toyobo Co., Ltd., Osaka, Japan). PCR amplification was carried out using 1.25 unit of AmpliTaq DNA polymerase (Perkin Elmer, Emeryville, CA) in a 50 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 200 μM dNTPs in the presence of specific primer pairs (500 nM each). Each cycle of PCR consisted of 30 sec of denaturation at 94°C, 30 sec of annealing at temperatures described below, and 1 min of extension at 72°C. Ten microliters of each reaction mixture was electrophoresed through 1% agarose gels containing ethidium bromide, and the signals were detected by using a Fluor-S MultiImager (Bio-Rad, Hercules, CA). The intensities were quantified with NIH Image software (Bio-Rad) and the results were shown in Figure 7. To ensure that the amount of the PCR products corresponded to the amount of template, PCR was conducted using templates at different dilutions. Control experiments were performed to determine the optimal PCR cycle number (Data not shown). The oligonucleotides used as primers, the annealing temperatures, and the PCR cycles were as follows.

Glyceraldehyde-3-phosphate dehydrogenase; 5′-CCT TCA TTG ACC TCA ACT AC-3′, 5′-AGT GAT GGC ATG GAC TGT GGT-3′, 59°C, 20 cycles, ST3Gal I [36,37]; 5′-AGC TGG GAG AGA ATG TCA AC-3′, 5′-GAG GTG TGA TGT GTT GTG TC-3′, 58°C, 25 cycles, ST3Gal II [38]; 5′-GAT GAA GTG CTC TCT TCG GG-3′, 5′-CAG GCA CGA TCT GGA ACA GT-3′, 55°C [39], 30 cycles, ST6GalNAc I [40]; 5′-CAT GAC GAG ATA TTG CAG AGG-3′, 5′-CTG CCT TGC TCT GAG GAT TC-3′, 55°C [39], 35 cycles, ST6GalNAc II [41]; 5′-TCC AGC ATC CGT GAC TAC CTG-3′, 5′-TTG TGC AGG TCT CGC CAT AGC-3′, 63°C, 30 cycles.

Analysis with DNA expression Genechip oligonucleotide microarrays

GeneChips were purchased from Affymetrix, Santa Clara, CA, USA. Target preparation and microarray processing was performed according to the manufacturer's recommendation. Total

RNA was prepared from the cells by using a Qiagen RNeasy kit. Nine micrograms of total RNA was used for the preparation of double-stranded cDNA with Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) and a T7-(dT)24 primer containing a T7 RNA polymerase promoter site (Amersham Biosciences). Biotinylated cRNA was synthesized from 5 μl of cleaned up double stranded cDNA with RNA transcript labeling kit (Enzo Diagnostics). A total of 20 μg of the cRNA product in a buffer solution of 40 mM Tris/acetate (pH 8.1)/100 mM potassium acetate/30 mM magnesium acetate was fragmented at 94°C for 35 min into approximately 35–200 nucleotides.

Targets for hybridization were prepared by combining 15 μg of fragmented cRNA with sonicated herring sperm DNA (0.1 mg/ml) plus four control bacterial and phage cRNA (1.5 pM BioB, 5 pM BioC, 25 pM BioD, and 100 pM Cre) samples, which served as internal controls for hybridization efficiency, in a buffer containing 100 mM MES, 1 M [Na+], 20 mM EDTA, and 0.01% Tween 20. The target was hybridized to an Affymetrix Mouse Genome U74Av2 chip containing probes for 12,000 murine genes for at 45°C 16 h with constant rotation at 60 rpm. The chips were washed at 25°C with 6X SSPE (0.9 M NaCl, 60 mM NaH_2PO_4, 6 mM EDTA), 0.01% Tween 20, then at 50°C with 100 mM MES, 1 M [Na^+], containing 0.01% Tween 20. The staining solution included streptavidin-phycoerythrin (10 μg/ml; Molecular Probes) and biotinylated goat anti-streptavidin (3 μg/ml; Vector Laboratories). Spots on the chips were washed and visualized by using the EukGE-WS2 protocol on an Affymetrix fluidics station. Fluorescence intensity was captured with an argon-ion laser GeneArray^{TM}Scanner (Agilent Technology, Palo Alto, CA, USA) with a 488-nm emission and detection at 570 nm.

Interpretation of Genechip data

The output files were visually inspected for hybridization artifacts and analyzed with Affymetrix® Microarray Suite ver.5 software (Affymetrix). Fluorescence intensity was measured for each probe. The detection score [R] was calculated for each probe pair and compared to the predefined threshold Tau (default value = 0.015). The one-sided Wilcoxon's signed-rank test was employed to determine the level of significance and each gene was assigned to be a present (P), marginal (M), or absent (A). The test was also used for the comparison analysis to determine whether the results were meaningful based on the intensity of each expression array. In the comparison analysis, each probe set on the array was compared to its counterpart on the baseline array, and a p-value was calculated to indicate an increase (i.e. high in SL4 cells), decrease (i.e. low in SL4 cells), or no change in the gene expression. Cutoff values (defaults g1 = 0.0025 and g2 = 0.003) were applied to generate change calls, i.e. different (D), no different (ND), or marginally low (ML), of low (L). The signal log ratio is supposed to represent the magnitude and direction of the difference of a transcript when two arrays were compared (experiment versus baselines). The value was calculated by a comparison of each probe pair on the experiment array and the probe pair on the baseline array. The difference is expressed as the log2 ratio.

Results

Cell surface carbohydrate expressions of colon 38 cells and SL4 cells

We previously established the highly metastatic variant cell line SL4 from a mouse colon carcinoma cell line, colon 38. SL4 cells apparently grew faster in spleens and livers after intrasplenic injection than parental colon 38 cells (Morimoto-Tomita et al., to be published). Colon 38 cells and SL4 cells were investigated for their cell surface carbohydrate chains by flowcytometric analysis using the lectins and monoclonal antibodies listed in Tables 1 and 2. As shown in Figures 1 and 2, PNA, Sambacus nigra agglutinin (SNA), Ulex europeus agglutinin-I (UEA-I) and anti-Le^X, Le^Y, Le^b antibodies bound to colon 38 cells but not to SL4 cells. Anti-Le^a, sialyl Tn, sialyl Le^X, sialyl Le^a, or sulfo Le^a antibodies did not bind to colon 38 or SL4 cells. Agaricus bisporus agglutinin (ABA), Vicia villosa agglutinin isolectin B_4 (VVA-B_4), Triticum vulgaris (Wheat germ) agglutinin (WGA),

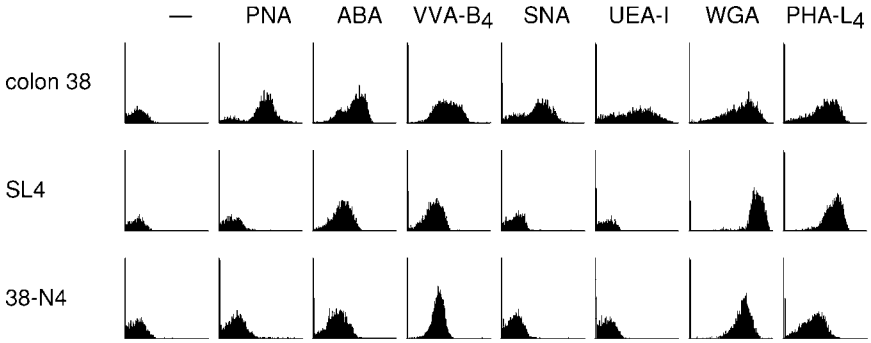


Figure 1. Flowcytometric analysis of colon 38 cells, SL4 cells, and 38-N4 cells with lectins listed in Table 1. Cells were incubated with or without biotin-conjugated lectins and then further incubated with fluorescein isothiocyanate-streptavidin. Abscissa and ordinate show relative fluorescence intensity and cell number, respectively.

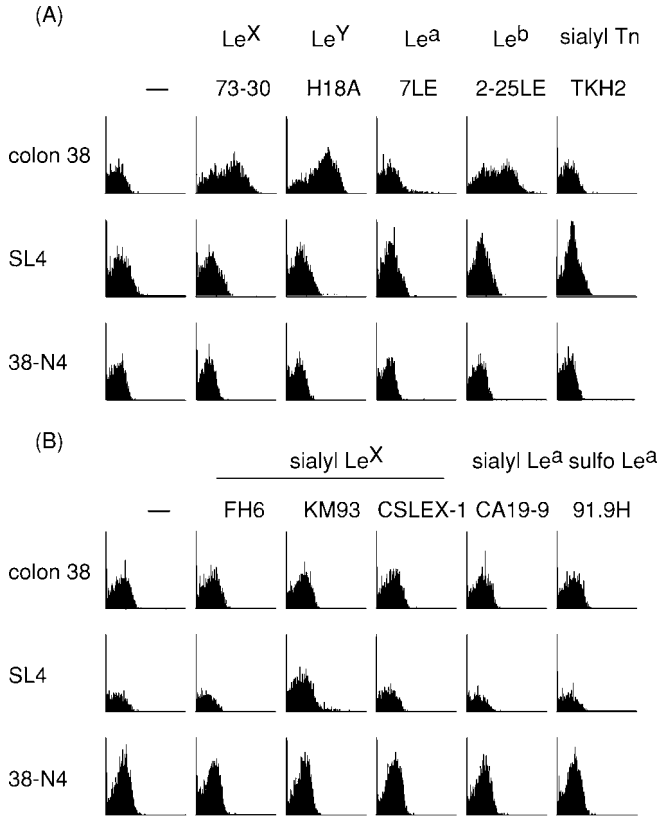


Figure 2. Flow cytometric analysis of colon 38 cells, SL4 cells, and 38-N4 cells with monoclonal antibodies listed in Table 2. Cells were incubated with or without mouse monoclonal antibodies and further incubated with fluorescein-conjugated antibody to mouse immunoglobulins. Abscissa and ordinate show the relative fluorescence intensity and cell number, respectively.

and Phaseolus vulgaris agglutinin-leucoagglutinating subunit 4 (PHA-L_4) bound to both colon 38 cells and SL4 cells to similar extents.

Selection and cell surface characterization of 38-N4 cells

To assess whether loss of cell surface PNA binding is causally related to the elevated metastatic potential, another cell line with reduced reactivity towards PNA was selected from the parental colon 38 cells. A magnetic cell sorter was used and four cycles of selections were applied (38-N4 cells). Flowcytometric analysis revealed that the profiles of lectin and antibody binding to 38-N4 cells were very similar to those of SL4 cells (Figures 1 and 2). These results suggested that biosynthetic pathways of *O*-glycans in SL4 cells and 38-N4 cells are similar. The anchorage-dependent growth rate of 38-N4 cells was slightly higher than colon 38 cells, but significantly slower than SL4 cells (Figure 3).

Metastatic ability of colon 38 cells and 38-N4 cells

To compare the metastatic ability of colon 38 cells with 38-N4 cells in a syngeneic mouse model, 1.2×10^6 cells were

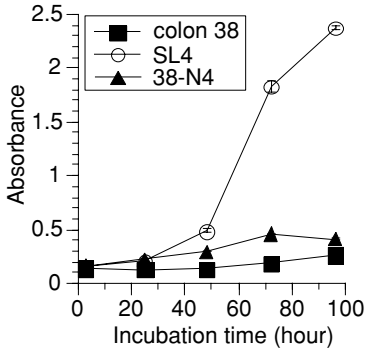


Figure 3. Anchorage-dependent growth of colon 38 cells, SL4 cells, and 38-N4 cells. Assays for *in vitro* growth were performed using WST-1 reagent as described in materials and methods. The mean absorbance measured after incubating with WST-1 reagent was shown. Bars indicate the standard deviation of triplicate analysis.

intrasplenically inoculated. Twenty-four days after inoculation, the incidence of macroscopic tumor formation in spleens was 8/8 and 0/10 for colon 38 cells and 38-N4 cells, respectively. The weights of spleen from mice inoculated with colon 38 cells were significantly greater ($p < 0.0001$) than those from mice inoculated with 38-N4 cells (Figures 4 and 5). The incidence of hepatic metastasis was 5/8 and 10/10 for colon 38 cells and 38-N4 cells, respectively. Liver weight was used as an indicator of the degree of metastatic growth of colon carcinoma cells. The mean liver weight of mice inoculated with 38-N4 cells was significantly greater ($p = 0.0009$) than that of mice inoculated with colon 38 cells. However, the biological properties *in vivo* of 38-N4 cells were different from those of SL4 cells because SL4 cells grew at the site of injection to form larger tumors than parental colon 38 cells (Morimoto-Tomita et al., to be published). Therefore, 38-N4 cells selected for decreased cell surface binding of PNA are similar to the metastatic variant, SL4, with regard to high metastatic capacity.

Flowcytometric analysis after sialidase treatment

To determine the structural basis for the variable degree of PNA binding to these variant cells, PNA binding to these cells before and after sialidase treatment was compared by flowcytometric analysis. PNA strongly bound SL4 cells and 38-N4 cells treated with sialidase from Clostridium perfringens (Figure 6). Furthermore, ABA that bounds both T-antigen and sialylated T-antigen bounded untreated SL4 cells and 38-N4 cells as shown in Figure 1. Therefore, increased sialylation of Galβ1-3GalNAc is strongly suggested to be the mechanism responsible for the reduction of PNA-binding to SL4 cells and 38-N4 cells.

Sialyltransferase mRNA expression

Four of the mouse sialyltransferase [36], ST3Gal I [37], ST3Gal II [38], ST6GalNAc I [40], and ST6GalNAc II [41] were reported to transfer a sialic acid residue to Galβ1-3GalNAc,

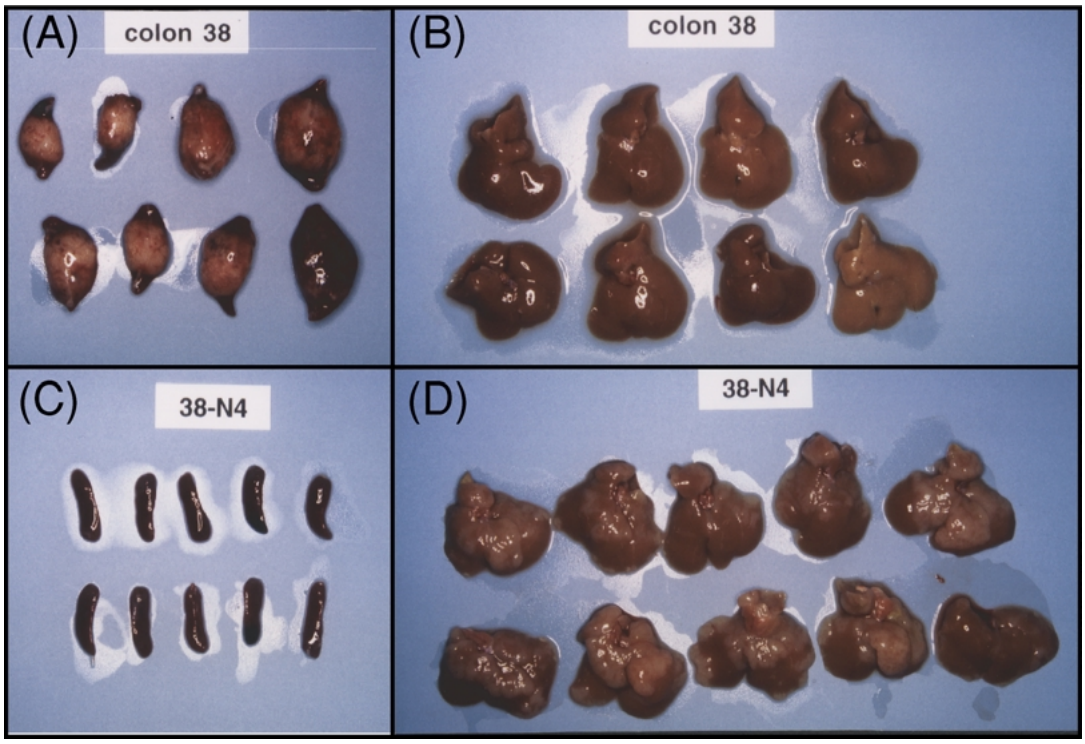
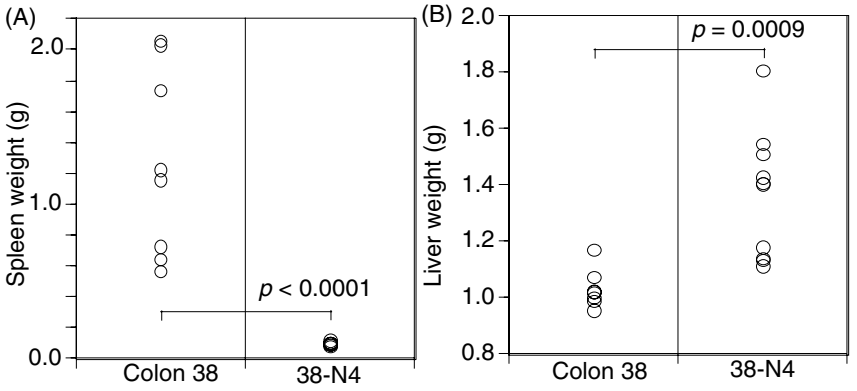


Figure 4. Tumors derived from colon 38 cells and 38-N4 cells at the site of inoculation and liver metastases. Viable tumor cells (1.2×10^6) were injected into the spleens of mice. Upper panels show spleens (A) or livers (B) of mice 24 days after intrasplenic injection of colon 38 cells. Lower panels show spleens (C) or livers (D) of mice 24 days after intrasplenic injection of 38-N4 cells.



(C)

Cells	Incidence of tumor growth		Weight (mean ± SD) g	
	Spleen	Liver	Spleen	Liver
Colon 38	8/8	5/8	1.27 ± 0.61	1.03 ± 0.07
38-N4	0/10	10/10	0.09 ± 0.01	1.37 ± 0.23

Figure 5. Summary of growth of tumors at the injection sites and liver metastases derived from colon 38 cells and 38-N4 cells. Viable tumor cells (1.2×10^6) were injected into the spleens of mice. Spleens and livers were removed at day 24 and their weights were measured as indicators of tumor formation in spleens and hepatic metastases. (A) Weights of spleens. (B) Weights of livers. (C) Incidence of tumor growth and mean weights of spleens and livers.

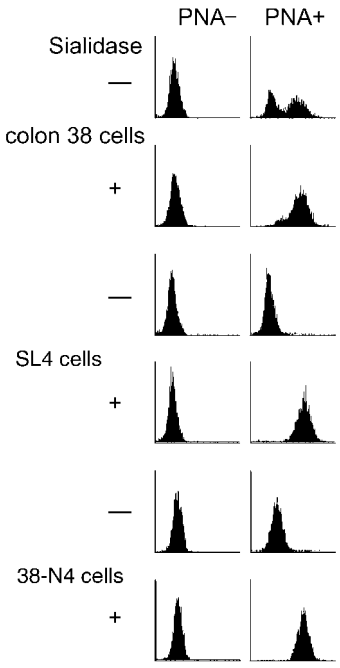


Figure 6. Flowcytometric analysis of colon 38 cells, SL4 cells, and 38-N4 cells untreated or treated with sialidase. Cells were incubated with or without 0.1 U/ml sialidase from Clostridium perfringens at 37°C for 1 hour and were analyzed by flowcytometry with biotin-conjugated PNA by the same method described in Figure 1. Abscissa and ordinate show the relative fluorescence intensity and cell number, respectively.

an acceptor substrate [42]. To assess whether increased sialyltransferase expression could explain the elevated level of T-antigen sialylation, we performed reverse transcription-polymerase chain reaction (RT-PCR) analysis of these sialyltransferases mRNA. One representative data was shown in Figure 7. The expression levels of ST3Gal II were shown to be higher in SL4 cells than in the parental cells. The expressions of both ST3Gal II and ST6GalNAc II were higher in 38-N4 cells than in the parental cells (Figure 7).

Elevated ST6GalNAc I expression in SL4 cells and 38-N4 cells was observed only when a large quantity of cDNA template was used.

Expression of genes involved in the biosynthesis of glycans

Mouse cDNA expression analysis was performed with DNA arrays by the use of Genechips. mRNA levels from colon 38 and SL4 cells were compared focusing on glycosyltransferase genes and other genes potentially involved in the modification of carbohydrate structures (Table 3). Among 37 glycosyltransferase genes tested, expressions of 3 genes, α2,3 SiaT (unpublished: gb D28942), β1,4-GalT (gb D37790), and α1,6-FucT (gb AB025198), were judged to be elevated in SL4 cells. ST3Gal II (gb X76989) was not judged as differentially expressed. Increase in the expressions of CMP-N-acetylneuraminic acid synthetase gene (gb AJ006215) and β-galactosidase gene (gb M57734) were indicated. These results strongly suggest that a wide range of glycosylation machinery is altered in SL4 cells as compared to colon 38 cells.

Discussion

A variant cell line, SL4, which is highly metastatic in livers, was recently selected *in vivo* from the mouse colon carcinoma colon 38 cells (Morimoto-Tomita et al., to be published). In the present study, we demonstrated that carbohydrate antigens such as T-antigen (PNA binding site), SNA binding site, Le^X Le^Y, Le^b, and H antigen were present on the surfaces of parental colon 38 cells but not on SL4 cells or 38-N4 cells that were selected *in vitro* for their deficiency in PNA binding sites. The T-antigen is a mucin-associated carbohydrate epitope that is considered to be a carcinoma-associated antigen. Although the majority of human colon carcinoma tissues are known to express T-antigen [10–12], conflicting observations about the correlation between T-antigen expression on primary colorectal tumors and liver metastases in humans were previously reported [15,16]. Our observation that metastatic variant cells showed decreased levels of T-antigen expression in mice is consistent with the results of Bresalier and co-workers with human materials [16]. With regard to studies on mice, the expression of sialic acid-containing proteins was shown to be higher in metastatic variant cell lines 51B LiM 5 and 51B LiM 6 than in the parental 51B cells [43]. MCA-38 LD cells, which are high liver-colonizing variant cells established from parental MCA-38 cells, expressed higher levels of β-galactoside α2,6-sialyltransferase mRNA and activity than non-colonizing MCA-38 CD cells [44]. However, the present study is the first to our knowledge to show that T-antigen is differentially expressed on the surfaces of metastatic carcinoma cells in a syngeneic experimental system. The 38-N4 cells selected for their deficiency in PNA-binding sites were metastatic in the same experimental system as that of SL4 cells. Thus, loss of cell surface T-antigen should at least in part causally related to increased potential of liver metastasis. We did not compare directly SL4 cells and 38-N4 cells in terms of metastatic ability *in vivo*. However, from the results shown in Figures 4 and 5, and the results of the experiment to compare metastatic ability of colon 38 cells and SL4 cells, it was clear that SL4 cells grew more rapidly in spleens and livers after intrasplenic injection into mice compared with 38-N4 cells. The reason for the decrease in tumorigenic capacity of 38-N4 cells at the site of inoculation (spleen) is not known.

Elevated sialylation of T antigen, but not decreased galactosylation or increased sialylation of GalNAc-Thr/Ser, can be attributed to the loss of PNA-binding sites on SL4 cells and 38-N4 cells because SL4 cells and 38-N4 cells became PNA-positive after sialidase treatment. Sialylation of T-antigen, i.e. the core 1 structure of *O*-glycan might prevent the extension of these carbohydrates. To study the mechanism of elevation of T-antigen sialylation, we analyzed the mRNA expressions of 4 sialyltransferases, ST3Gal I, ST3Gal II, ST6GalNAc I, and ST6GalNAc II,

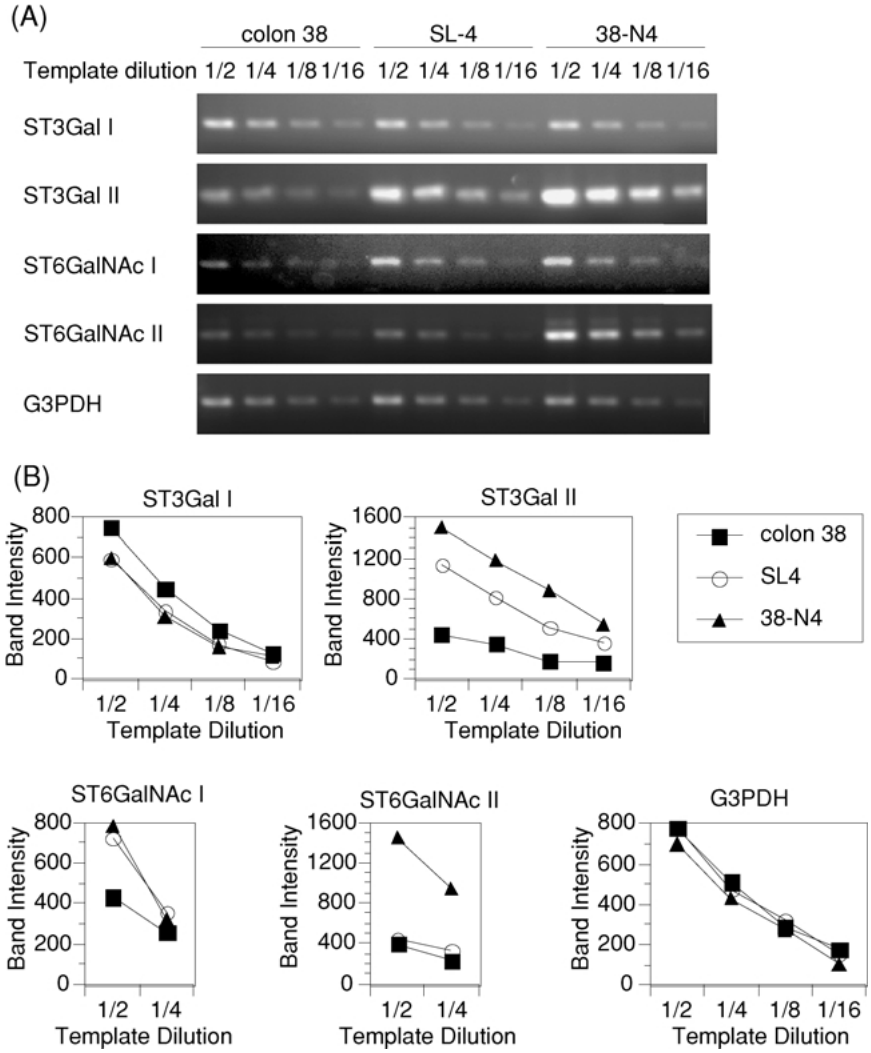


Figure 7. Semiquantitative RT-PCR analysis of sialyltransferase mRNA expression of colon 38 cells, SL4 cells, and 38-N4 cells. RT-PCR was conducted as described in materials and methods. The intensity of the band visualized after electrophoretically separating PCR products through 1% agarose gels containing ethidium bromide (A) was analyzed by Fluor-S MultiImager. The relative intensities of the band were shown in (B).

designated according to [36], which were reported to sialylate T-antigen [42]. Increases in the levels of ST3Gal II mRNA in SL4 cells, and ST3Gal II and ST6GalNAc II mRNA in 38-N4 cells were observed. There are three sialylated structures of T-antigen, Siaα2-3Galβ1-3GalNAc, Galβ1-3(Siaα2-6)GalNAc, and Siaα2-3Galβ1-3(Siaα2-6)GalNAc. ST3Gal II and ST6GalNAc II are the enzymes involved in the synthesis of Siaα2-3Galβ1-3GalNAc and Galβ1-3(Siaα2-6)GalNAc, respectively. According to our results, ST3Gal II seems to play more important role in hepatic metastasis in these mouse colon carcinoma systems because ST3Gal II mRNA was elevated in both SL4 cells and 38-N4 cells. Biochemical determinations of sialyltransferases expressed in these cells will be necessary to confirm these findings. It was also shown that SL4 cells and 38-N4 cells lost the expression of Siaα2-6Gal/GalNAc, Le^X, Le^Y, Le^b and H type antigen together with T-antigen. It is interesting to note that the cells selected for loss of reactivity with PNA also did not express fucosylated epitopes indicating that a common mechanism might be involved in the expression of these two types of cell surface antigens.

Sialyltransferases have been implicated with hepatic metastasis of colon carcinomas and lymphomas in a number of previous reports. For example, elevated ST6Gal I expression was observed with a poorly liver metastatic variant cell line of MDAY lymphoblastoid cell line [45]. ST6Gal I was, however, reported to be responsible for reduced homotypic aggregation and metastatic dissemination of colon carcinoma cells [46]. ST3Gal III and ST3Gal IV were shown to be responsible for the formation of epitopes involved in human colon carcinoma cell adhesion to endothelial cells and the authors claim that the mechanism play an important role in angiogenesis and metastasis [47]. Gessner and co-workers studied 2,3 and

Table 3. Expression of genes involved in the biosynthesis of glycans in colon 38 and SL4 cells

Name	*GenBank*	*Exp. 1*	*Fold*	*Exp. 2*	*Fold*	*RT-PCR*
ST3Gal I	X73523	**L** (A/P)		ND (P/P)	−0.3	ND
ST3Gal II	X76989	ND (A/A)		ND (A/A)		**H**
ST3Gal III	X84234	ND (A/A)		ND (P/A)		
ST3Gal V: GM3 synthase	Y15003	**L** (A/M)		ND (P/P)	−0.3	
alpha 2,3ST (unpublished)	D28941	**H** (P/M)	+0.4	**H** (P/A)		
ST6GalNAc I	Y11274	ND (A/A)		ND (A/A)		ND
ST6GalNAc II	X94000	ND (A/P)		**L** (P/P)	−0.4	ND
ST6GalNAc III	Y11342	ND (A/A)		ND (A/A)		
ST6GalNAc IV	AJ007310	ND (A/A)		ND (P/M)	+0.5	
ST6GalNAc IV	Y15780	ND (A/M)		ND (P/P)	+0.7	
ST6GalNAc V	AB030836	ND (A/A)		ND (A/M)		
ST6GalNAc VI	AB035174	ND (A/A)		ND (A/A)		
ST6Gal I	D16106	ND (A/A)		ND (A/A)		
ST8Sia I	X84235	ND (A/M)		ND (A/A)		
ST8Sia III	X80502	ND (A/M)		ND (A/A)		
ST8Sia IV	X86000	ND (A/A)		**H** (P/A)		
ST8Sia V	X98014	ND (A/A)		ND (A/A)		
Core 2 GlcNAc T	U19265	ND (A/A)		ND (A/A)		ND
I GlcNAc T	U68182	ND (A/A)		ND (A/A)		
N-acetylglucosaminyl transferase component Gpi1 (GPI1)	AF030178	ND (A/A)		ND (A/A)		
beta-1,3-N-acetylglucosaminyltransferase	AF092050	ND (P/P)	+0.2	ND (P/P)	−0.2	
Mannoside acetyl glucosaminyl transferase 1	L07037	ND (A/A)		ND (P/P)	−0.5	
Mannoside acetyl glucosaminyl transferase 3	L39373	ND (A/A)		**L** (A/A)		
acetylglucosaminyltransferase-like protein “Large”	AJ006278	ND (A/M)		**L** (P/P)	−0.9	
polypeptide GalNAc transferase-T1 (ppGaNTase-T1)	U73820	**H** (P/P)	+0.7	ND (P/P)	−0.3	
polypeptide GalNAc transferase-T3 (ppGaNTase-T3)	U70538	ND (A/A)		**ML** (A/P)		
polypeptide GalNAc transferase-T4 (ppGaNTase-T4)	U73819	ND (A/A)		ND (P/P)	−0.2	
alpha1,3 GalT	M85153	ND (M/A)		ND (P/P)	−0.3	
beta1,4 GalT	D37790	**H** (P/P)	+0.2	**H** (P/P)	+0.4	
beta1,4 GalT	M27923	ND (P/P)	+0.1	ND (P/P)	0	
beta1,4 GalT II	AB019541	ND (P/A)		**H** (P/P)	+0.2	
beta 1,3-galactosyltranferase-I (b3GT1)	AF029790	ND (A/A)		ND (A/A)		
beta 1,3-galactosyltranferase-II (b3GT2)	AF029791	ND (A/A)		ND (A/A)		
beta 1,3-galactosyltranferase-III (b3GT3)	AF029792	ND (A/A)		ND (A/A)		
CMP-N-acetylneuraminic acid synthetase	AJ006215	**H** (P/P)	+1.2	**H** (P/P)	+1.4	
CMP-N-acetylneuraminic acid hydroxylase	D21826	ND (A/A)		ND (A/A)		
CMP-sialic acid transporter	Z71268	ND (A/A)		**H** (P/P)	+0.9	
UDP-galactose transporter 1	AB027147	ND (A/A)		ND (P/P)	−0.6	
UDP-galactose transporter related isozyme 1	D87990	ND (P/P)	−0.1	**H** (P/P)	−0.7	
UDP-N-acetylglucosamine 2-epimerase/N-acylmannosamine kinase	AJ132236	ND (P/P)	+0.1	**H** (P/P)	−0.8	
N-Acetylglucosamine kinase	AJ242909	ND (A/A)		ND (P/P)	0	
Dolichyl-phosphate alpha-N-acetylglucosaminephosphotransferase 2	X65603	ND (P/P)	+0.1	**L** (P/P)	−0.8	
N-acetylglucosamine-6-O-sulfotransferase	AB01145	**L** (A/P)		**H** (P/P)	+0.6	
Heparan sulfate glucosaminyl 3-O-sulfotransferase	AF019385	ND (A/A)		**L** (A/A)		
glucosaminyl N-deacetylase/N-sulfotransferase	X75885	ND (A/A)		ND (M/A)		
Fuc-TIX	AB015426	ND (A/A)		ND (A/A)		
alpha-1,6-fucosyltransferase	AB025198	**H** (P/P)	+1.1	**H** (P/P)	+1	
GDP-L-fucose-beta-D-galactoside 2-alpha-1-fucosyltransferase	AF064792	ND (A/A)		ND (A/A)		
Fucosyltransferase 4	U33457	ND (A/A)		ND (A/A)		
Fuc-TVII	U45980	ND (A/A)		ND (A/A)		
Beta-galactosidase complex	M57734	**H** (P/A)		**H** (P/P)	+0.5	

(*Continued on next page.*)

Table 3. (*Continued.*)

Name	*GenBank*	*Exp. 1*	*Fold*	*Exp. 2*	*Fold*	*RT-PCR*
beta-D-galactosidase fusion protein	M60510	ND (A/A)		ND (A/A)		
alpha-D-galactosidase (Ags)	L46651	ND (A/A)		ND (A/A)		
alpha-D-galactosidase A (Ags)	U58105	ND (A/A)		**H** (P/P)	+0.5	
Protective protein for beta-galactosidase	J05261	**L** (P/P)	−0.7	ND (P/P)	0	

In this table, results of the one-sided Wilcoxon's signed-rank test was employed to determine the level of significance of cDNA hybridization in the microarray analyses and each gene was assigned as present (P), marginal (M), or absent (A). Subsequently, the expression levels of the gene in SL4 cells were compared to those in colon 38 cells and the results were designated as high in SL4 (H), no difference (ND), marginally low (ML) in SL4 or low in SL4 (L). The signal log ratio (number) represents the magnitude and direction of differences in the value for a transcript after two arrays were compared. Thus the numbers are the log2 ratio. Results of RT-PCR analysis (Figure 7) are also indicated in this table.

2,6 sialyltransferases of colon carcinoma tissues using Galβ1-4GlcNAc as an acceptor. They found that the 2,3-specific sialyltransferase was equally high in tumor and mucosa samples, but 2,6-specific enzyme was higher in tumor tissues than mucosas [48]. Harvey and co-workers compared poorly differentiated cell lines and well differentiated cell lines for their sialyltransferase activity to conclude the enzyme level was high in well differentiated cells [49]. These studies did not focus on the sialylation of T-antigen. Recently, Schneider and co-workers reported that overexpression of ST6GalNAc II was related to poor patient survival in human colorectal carcinomas [50]. This report and our results suggest that 38-N4 cells are good model to study the mechanism of malignant behavior of colon carcinoma cells overexpressing ST6GalNAc II. Although it has been known that normal colonic mucosa contains *O*-glycans rich with the core 3 structure [51] and it was lost during colon carcinogenesis, it was not known whether a similar alteration occurs in mice. Furthermore, the *O*-glycan changes we observed are those occurring during malignant progression but not carcinogenesis.

There has been no clear demonstration of the mechanism of aggressive behavior of tumor cells with decreased T-antigen and elevated sialylated T-antigen. One possible mechanism is an escape from the recognition by cells in the immune systems such as macrophages through the carbohydrate recognition. We previously reported that a lectin expressed on immature macrophages, MGLs in mice, bind to highly branched galactose-terminated N-linked sugar chains, as well as to glycopeptides containing clustered *O*-linked chains with terminal GalNAc-Ser/Thr or terminal Galβ1-3GalNAc residues [52,53] and involved in the recognition of tumor cells expressing truncated *O*-glycans [54]. In our preliminary study, colon 38 cells were more strongly stained with recombinant mMGL1 than SL4 cells or 38-N4 cells by flowcytometry. These results suggest that mMGL-positive macrophages are involved in the suppression of the metastatic growth of colon 38 cells through the recognition of T-antigen.

There were differences in the carbohydrate profiles other than T-antigen between colon 38 cells and SL4 cells (Figures 1 and 2) and genes potentially involved in the carbohydrate synthesis were found by the gene expression cDNA microarray analyses (Table 3). The results regarding the sialyltransferase genes did not appear to be consistent to the RT-PCR analysis. For example, the expression levels of ST3Gal II (gb X76989) were shown to be higher in SL4 cells than in the parental cells (Figure 7) by RT-PCR analysis whereas DNA microarray analysis did not indicate that the difference in the expression of this gene was significant. This is apparently due to the cutoff levels set by the analytical software, which ignores differences when the expression levels are low. Other results of DNA microarray analysis were also difficult to interpret. Increased expressions of β1,4 galactosyltransferase (gb D37790) [55] and α2,3 sialyltransferase (unpublished) (gb D28941) possibly enhance the expression of sialyl Le^X antigen. However, sialyl Le^X was expressed by neither colon 38 cells nor SL4 cells. Increased β-galactosidase (gb M57734) [56] expression in SL4 cells may be one mechanism of no sialyl Le^X expression and decreased Le^X expression. Increased β-galactosidase expression possibly causes the decrease of T-antigen. If it is the case, Tn antigen should be increased in SL4 cells. However, this was not observed i.e. VVA-B_4 binding was slightly lower in SL4 cells than colon 38 cells. We found the expression of CMP-Neu5Ac synthetase (gb AJ006215) [57] was higher in SL4 cells than colon 38 cells. However, not all of the sialic acid containing carbohydrates expressed higher in SL4 cells than colon 38 cells because SNA binding was lower in SL4 cells (Figure 1). In the case with sialyl T-antigen, CMP-Neu5Ac synthetase and ST3Gal II may cooperate to increase sialylation of T-antigen in SL4 cells. Increased expression of α1,6 fucosyltransferase (gb AB025198) [58] in human hepatoma tissue is well known [59]. However, in colonic tissue and colon cancer cells, their expression is poorly studied. In the present study, α1,6 fucosyltransferase expression was higher in SL4 cells than colon 38 cells. Further study is required whether α1,6 fucosyltransferase is involved in colon cancer progression and metastasis. We judge that the results of microarray analyses are reliable because differentially expressed mRNAs revealed by other techniques such as differential display PCR and subtractive hybridization were also shown by this technique (data not shown).

In the present study, we focused on the difference of T-antigen expression between colon 38 cells and SL4 cells. We also prepared 38-N4 cells, which were selected *in vitro* for their

deficiency of T-antigen. Using these cell lines we demonstrated that elevated sialylation of T-antigen is involved in the liver metastasis formation in the syngeneic mouse model. The mechanism of metastasis of tumor cells with increased sialylated T-antigen must be elucidated in the future study.

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References

1 Dennis JW, Laferte S, Tumor cell surface carbohydrate and the metastatic phenotype, *Cancer Metastasis Rev* **5**, 185–204 (1987).
2 Hakomori S, Aberrant glycosylation in tumors and tumor-associated carbohydrate antigens, *Adv Cancer Res* **52**, 257–331 (1989).
3 Dabelsteen E, Cell surface carbohydrates as prognostic markers in human carcinomas, *J Pathol* **179**, 358–69 (1996).
4 Irimura T, Nakamori S, Matsushita Y, Taniuchi Y, Todoroki N, Tsuji T, Izumi Y, Kawamura Y, Hoff SD, Cleary KR, Ota DM, Colorectal cancer metastasis determined by carbohydrate-mediated cell adhesion: Role of sialyl-Le^x antigens, *Sem Cancer Biol* **4**, 319–24 (1993).
5 Ota M, Takamura N, Irimura T, Involvement of cell surface glycans in adhesion of human colon carcinoma cells to liver tissue in a frozen section assay: Role of endo-β-galactosidase-sensitive structures, *Cancer Res* **60**, 5261–8 (2000).
6 Springer GF, T and Tn, general carcinoma autoantigen, *Science* **224**, 1198–206 (1984).
7 Springer GF, Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy, *J Mol Med* **75**, 594–602 (1997).
8 Irimura T, Kawaguchi T, Terao T, Osawa T, Carbohydrate binding specificity of the so-called galactose-specific phytohemagglutinin, *Carbohydr Res* **39**, 317–27 (1975).
9 Pereira ME, Kabat EA, Lotan R, Sharon N, Immunochemical studies on the specificity of the peanut (Arachis hypogaea) agglutinin, *Carbohydr Res* **51**, 107–18 (1976).
10 Ørntoft TF, Mors NP, Eriksen G, Jacobsen NO, Poulsen HS, Comparative immunoperoxidase demonstration of T-antigens in human colorectal carcinomas and morphologically abnormal mucosa, *Cancer Res* **45**, 447–52 (1985).
11 Yuan M, Itzkowitz SH, Boland CR, Kim YD, Tomita JT, Palekar A, Bennington JL, Trump BF, Kim YS, Comparison of T-antigen expression in normal, premalignant, and malignant human colonic tissue using lectin and antibody immunohistochemistry, *Cancer Res* **46**, 4841–7 (1986).
12 Boland CR, Lectin histochemistry in colorectal polyps, *Prog Clin Biol Res* **279**, 277–87 (1988).
13 Itzkowitz SH, Yuan M, Montgomery CK, Kjeldsen T, Takahashi HK, Bigbee WL, Kim YS, Expression of Tn, sialosyl-Tn, and T antigens in human colon cancer, *Cancer Res* **49**, 197–204 (1989).
14 Fung PYS, Madej M, Koganty RR, Longenecker BM, Active specific immunotherapy of a murine mammary adenocarcinoma using a synthetic tumor-associated glycoconjugate, *Cancer Res* **50**, 4308–14 (1990).
15 Cao Y, Karsten UR, Liebrich W, Haensch W, Springer GF, Schlag PM, Expression of Thomsen-Friedenreich-related antigens in primary and metastatic colorectal carcinomas. A reevaluation, *Cancer* **76**, 1700–8 (1995).
16 Bresalier RS, Ho SB, Schoeppner HL, Kim YS, Sleisenger MH, Brodt P, Byrd JC, Enhanced sialylation of mucin-associated carbohydrate structures in human colon cancer metastasis, *Gastroenterology* **110**, 1354–67 (1996).
17 Swamy MJ, Gupta D, Mahanta SK, Surolia A, Further characterization of the saccharide specificity of peanut (Arachis hypogaea) agglutinin, *Carbohydr Res* **213**, 59–67 (1991).
18 Presant CA, Kornfeld S, Characterization of the cell surface receptor for the Agaricus bisporus hemagglutinin, *J Biol Chem* **247**, 6937–45 (1972).
19 Sueyoshi S, Tsuji T, Osawa T, Purification and characterization of four isolectins of mushroom (Agaricus bisporus), *Biol Chem Hoppe Seyler* **366**, 213–21 (1985).
20 Sughii S, Kabat EA, Baer HH, Further immunochemical studies on the combining sites of Lotus tetragonolobus and Ulex europaeus I and II lectins, *Carbohydr Res* **99**, 99–101 (1982).
21 Cummings RD, Kornfeld S, Characterization of the structural determinants required for the high affinity interaction of asparagine-linked oligosaccharides with immobilized Phaseolus vulgaris leukoagglutinating and erythroagglutinating lectins, *J Biol Chem* **257**, 11230–4 (1982).
22 Shibuya N, Goldstein IJ, Broekaert WF, Nsimba-Lubaki M, Peeters B, Peumans WJ, The elderberry (Sambucus nigra L.) bark lectin recognizes the Neu5Ac(α2-6)Gal/GalNAc sequence, *J Biol Chem* **262**, 1596–601 (1987). sp.
23 Tollefsen SE, Kornfeld R, Isolation and characterization of lectins from Vicia villosa. Two distinct carbohydrate binding activities are present in seed extracts, *J Biol Chem* **258**, 5165–71 (1983).
24 Tollefsen SE, Kornfeld R, The B_4 lectin from Vicia villosa seeds interacts with N-acetylgalactosamine residues α-linked to serine or threonine residues in cell surface glycoproteins, *J Biol Chem* **258**, 5172–6 (1983).
25 Allen AK, Neuberger A, Sharon N, The purification, composition and specificity of wheat-germ agglutinin, *Biochem J* **131**, 155–62 (1973).
26 Fukushima K, Hirota M, Terasaki PI, Wakisaka A, Togashi H, Chia D, Suyama N, Fukushi Y, Nudelman E, Hakomori S, Characterization of sialosylated Lewis X as a new tumor-associated antigen, *Cancer Res* **44**, 5279–85 (1984).
27 Fukushi Y, Nudelman E, Levery SB, Hakomori S, Rauvala H, Novel fucolipids accumulating in human adenocarcinoma. III. A hybridoma antibody (FH6) defining a human cancer-associated difucoganglioside (VI3NeuAcV3III3Fuc2nLc6), *J Biol Chem* **259**, 10511–7 (1984).
28 Koprowski H, Steplewski Z, Mitchell K, Herlyn M, Herlyn D, Fuhrer P, Colorectal carcinoma antigens detected by hybridoma antibodies, *Somatic Cell Genet* **5**, 957–71 (1979).
29 Hanai N, Shitara K, Yoshida H, Generation of monoclonal antibodies against human lung squamous cell carcinoma and adenocarcinoma using mice rendered tolerant to normal human lung, *Cancer Res* **46**, 4438–43 (1986).

30 Kjeldsen T, Clausen H, Hirohashi S, Ogawa T, Iijima H, Hakomori S, Preparation and characterization of monoclonal antibodies directed to the tumor-associated *O*-linked sialosyl-2,6 α-N-acetylgalactosaminyl (sialosyl-Tn) epitope, *Cancer Res* **48**, 2214–20 (1988).
31 Loveless RW, Yuen CT, Tsuiji H, Irimura T, Feizi T, Monoclonal antibody 91.9H raised against sulfated mucins is specific for the 3′-sulfated Lewis a tetrasaccharide sequence, *Glycobiology* **8**, 1237–42 (1998).
32 Tsuiji H, Hong JC, Kim YS, Ikehara Y, Narimatsu H, Irimura T, Novel carbohydrate specificity of a monoclonal antibody 91.9H prepared against human colonic sulfomucin: Recognition of sulfo-Lewis a structure, *Biochem Biophys Res Commun* **253**, 374–81 (1998).
33 Corbett TH, Griswold DP Jr, Roberts BJ, Peckham JC, Schabel FM Jr, Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure, *Cancer Res* **35**, 2434–9 (1975).
34 Corfield AP, Higa H, Paulson JC, Schauer R, The specificity of viral and bacterial sialidases for α2-3- and α2-6-linked sialic acids in glycoproteins, *Biochim Biophys Acta* **744**, 121–6 (1983).
35 Chien CH, Chang SC, Wei YH, Purification and characterization of neuraminidase from Clostridium perfringens, *Proc Natl Sci Counc Repub China B* **13**, 201–9 (1989).
36 Tsuji S, Datta AK, Paulson JC, Systematic nomenclature for sialyltransferases, *Glycobiology* **6**, v–vii (1996).
37 Lee YC, Kurosawa N, Hamamoto T, Nakaoka T, Tsuji S, Molecular cloning and expression of Gal β1,3GalNAc α2,3-sialyltransferase from mouse brain, *Eur J Biochem* **216**, 377–85 (1993).
38 Lee YC, Kojima N, Wada E, Kurosawa N, Nakaoka T, Hamamoto T, Tsuji S, Cloning and expression of cDNA for a new type of Gal β1,3GalNAc α2,3-sialyltransferase, *J Biol Chem* **269**, 10028–33 (1994).
39 Takashima S, Tachida Y, Nakagawa T, Hamamoto T, Tsuji S, Quantitative analysis of expression of mouse sialyltransferase genes by competitive PCR, *Biochem Biophys Res Commun* **260**, 23–7 (1999).
40 Kurosawa N, Takashima S, Kono M, Ikehara Y, Inoue M, Tachida Y, Narimatsu H, Tsuji S, Molecular cloning and genomic analysis of mouse GalNAc α2,6-sialyltransferase (ST6GalNAc I), *J Biochem* (Tokyo) **127**, 845–54 (2000).
41 Kurosawa N, Inoue M, Yoshida Y, Tsuji S, Molecular cloning and genomic analysis of mouse Galβ1, 3GalNAc-specific GalNAc α2,6-sialyltransferase, *J Biol Chem* **271**, 15109–16 (1996).
42 Tsuji S, Molecular cloning and functional analysis of sialyltransferases, *J Biochem* **120**, 1–13 (1996).
43 Bresalier RS, Rockwell RW, Dahiya R, Duh QY, Kim YS, Cell surface sialoprotein alterations in metastatic murine colon cancer cell lines selected in an animal model for colon cancer metastasis, *Cancer Res* **50**, 1299–307 (1990).
44 Piscatelli JJ, Cohen SA, Berenson CS, Lance P, Determinants of differential liver-colonizing potential of variants of the MCA-38 murine colon cancer cell line, *Clin Exp Metastasis* **13**, 141–50 (1995).
45 Lo NW, Dennis JW, Lau JT, Overexpression of the α2,6-sialyltransferase, ST6Gal I, in a low metastatic variant of a murine lymphoblastoid cell line is associated with appearance of a unique ST6Gal I mRNA, *Biochem Biophys Res Commun* **264**, 619–21 (1999).
46 Bosch J, Brossmer R, Kemmner W, Schlag P, Preparation and characterization of differently aggregated colorectal carcinoma cell subpopulations from surgical specimens, *Cancer Detect Prev* **22**, 319–29 (1998).
47 Dimitroff CJ, Pera P, Dall'Olio F, Matta KL, Chandrasekaran EV, Lau JT, Bernacki RJ, Cell surface N-acetylneuraminic acid α2,3-galactoside-dependent intercellular adhesion of human colon cancer cells, *Biochem Biophys Res Commun* **256**, 631–6 (1999).
48 Gessner P, Riedl S, Quentmaier A, Kemmner W, Enhanced activity of CMP-NeuAc:Gal β 1-4GlcNAc:α 2,6-sialyltransferase in metastasizing human colorectal tumor tissue and serum of tumor patients, *Cancer Lett* **75**, 143–9 (1993).
49 Harvey BE, Toth CA, Wagner HE, Steele GD Jr, Thomas P, Sialyltransferase activity and hepatic tumor growth in a nude mouse model of colorectal cancer metastases, *Cancer Res* **52**, 1775–9 (1992).
50 Schneider F, Kemmner W, Haensch W, Franke G, Gretschel S, Karsten U, Schlag PM, Overexpression of sialyltransferase CMP-sialic acid: Galβ1,3GalNAc-R α6-sialyltransferase is related to poor patient survival in human colorectal carcinomas, *Cancer Res* **61**, 4605–11 (2001).
51 Podolsky DK, Oligosaccharide structures of human colonic mucin, *J Biol Chem* **260,** 8262–71 (1985).
52 Yamamoto K, Ishida C, Shinohara Y, Hasegawa Y, Konami Y, Osawa T, Irimura T, Interaction of immobilized recombinant mouse C-type macrophage lectin with glycopeptides and oligosaccharides, *Biochemistry* **33**, 8159–66 (1994).
53 Tsuiji M, Fujimori M, Ohashi Y, Higashi N, Onami TM, Hedrick SM, Irimura T, Molecular cloning and characterization of a novel mouse macrophage C-type lectin, mMGL2, which has a distinct carbohydrate specificity from mMGL1, *J Biol Chem* **277**, 28892–901 (2002).
54 Sakamaki T, Imai Y, Irimura T, Enhancement in accessibility to macrophages by modification of mucin-type carbohydrate chains on a tumor cell line: Role of a C-type lectin of macrophages, *J Leukoc Biol* **57**, 407–14 (1995).
55 Uehara K, Muramatsu T, Molecular cloning and characterization of β-1,4-galactosyltransferase expressed in mouse testis, *Eur J Biochem* **244**, 706–12 (1997).
56 Nanba E, Suzuki K, Molecular cloning of mouse acid β-galactosidase cDNA: Sequence, expression of catalytic activity and comparison with the human enzyme, *Biochem Biophys Res Commun* **173**, 141–8 (1990).
57 Munster AK, Eckhardt M, Potvin B, Muhlenhoff M, Stanley P, Gerardy-Schahn R, Mammalian cytidine 5′-monophosphate N-acetylneuraminic acid synthetase: A nuclear protein with evolutionarily conserved structural motifs, *Proc Natl Acad Sci USA* **95**, 9140–5 (1998).
58 Hayashi H, Yoneda A, Asada M, Ikekita M, Imamura T, Molecular cloning of mouse α-1,6-fucosyltransferase and expression of its mRNA in the developing cerebrum, *DNA Seq* **11**, 91–6 (2000).
59 Miyoshi E, Noda K, Yamaguchi Y, Inoue S, Ikeda Y, Wang W, Ko JH, Uozumi N, Li W, Taniguchi N,The α1-6-fucosyltransferase gene and its biological significance, *Biochim Biophys Acta* **1473**, 9–20 (1999).

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