Different enzyme activities of sialyltransferases in gynecological cancer cell lines

P. H. Wang[1], *M.D.*; W. L. Lo[2], *M.S.*; C. C. Hsu[2], *M.S.*; T. W Lin[2], *M.S.*; W. L. Lee[3], *M.D.*; C. Y. Wu[4], *Ph.D.*; C. C. Yuan[1], *M.D.*; Y. C. Tasi[2], *Ph.D.*

[1]*Departments of Obstetrics & Gynecology, Taipei Veterans General Hospital, Institute of Clinical Medicine, National Yang-Ming University;*
[2]*Institute and Department of Biochemistry, National Yang-Ming University;*
[3]*Department of Medicine, Cheng Hsin Rehabilitation Center-Taipei, Institute of Clinical Medicine, National Yang-Ming University;*
[4]*Institute of Biological Chemistry, Academia Sinica, Taipei (Taiwan)*

Summary

Purpose: Due to rarity of a relationship between sialyltransferase enzyme activities and gynecological cancers, we arranged the study to evaluate sialyltransferase enzyme activity in the various kinds of gynecological cancer cell lines.

Methods: Ten cell lines from various kinds of gynecological cancers and two cell lines from normal tissue were enrolled in this study. The activities of each subtype of sialyltransferases were detected using Galβ1,3GalNAc-acetyl-lactosamine)-Obzl (acceptor for ST2,3Gal I), Galβ1,3GlcNAcβ1,3Galβ1,4GlcNAc (acceptor for ST2,3Gal III), Galβ1,4GlcNAc (acceptor for ST2,3Gal IV), asialo-bovine submaxillary mucin (acceptor for ST2,6GalNAc I), asialo-fetuin (acceptor for STα2,6GalNAc II), and fetuin (acceptor for ST2,6GalNAc III), respectively. The amounts of sialic acids were measured using fluorescein-conjugated *Sambucus nigra agglutinin* (SNA) specific for α2,6-sialic acids and fluorescein-conjugated *Maackia Amurensis agglutinin* (MAA) specific for α2,3-sialic acids.

Results: The activities of two sialyltransferase subtypes, ST3Gal I & ST6GalNAc II, were significantly higher in nearly all cell lines. More specifically, cervical cancer cell line-ME180, ovarian cancer cell line-ES-2, and choriocarcinoma cell line-BeWo showed high levels of ST3Gal I enzyme activity; all gynecological cancer cell lines except endometrial cancer cell line-RL95-2 had high levels of ST6GalNAc II enzyme activity when compared with a normal control cell line - fibroblast cell line (CCD-966Sk). Cell lines tested in this study have diverse levels of surface α2,6-sialic acid sugar chains (enhanced SNA binding) when compared with α2,3-sialic acid sugar chains (enhanced MAA binding) but we found that some cell lines such as Ca Ski (cervical cancer cell line), CC7T (cervical cancer cell line), PA-1 (ovarian cancer cell line), and BeWo showed significantly altered cell surface α2,6-sialic acid sugar chains.

Conclusion: Increasing enzyme activity of ST3Gal I and ST6GalNAc II might be important in various kinds of gynecological cancers. More specifically, enhanced activity of sialyltransferases involving α2,6-sialic acid sugar chains might be more important in cancer development. Future studies will investigate whether the enzyme activity of these sialyltransferases can be helpful for clinical practice.

Key words: Enzyme activity; Gynecological cancer cell lines; Sialyltransferase.

Introduction

Sialic acids are widely distributed in nature as terminal sugars on oligosaccharides attached to protein or lipid moieties, including a number of derivatives of the nine-carbon acid amino sugar neuraminic acid [1-5]. They and their derivatives play a role in a variety of biological processes including cell-cell communication, cell-matrix interaction, adhesion, and protein targeting [2]. There is a large body of evidence suggesting that tumor cells have changed surface properties from their normal counterparts, and that these changes are partially due to altered sialo-glycoconjugates expressed on the plasma membrane [6]. Although much evidence shows the important role of altered sialylation in various kinds of cancers [7-13], the altered sialylation of gynecological cancers has been little evaluated, except for choriocarcinoma and cervical cancer [1, 14]. In this study, we detected enzyme activity of six different types of sialyltransferases (STs) in ten cell lines from various kinds of gynecological cancers and two cell lines from normal tissues to assess the extent of sialylation associated with the establishment of the carcinoma.

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Materials and Methods

Cell Lines Culture

The five cervical carcinoma cell lines (SIHA, ME180, Ca Ski, and HeLa, and CC7T), two ovarian carcinoma cell lines (PA-1 and ES-2), one choriocarcinoma cell line (BeWo), uterine endometrial carcinoma cell line (RL95-2), breast cancer cell line (MCF7), fibroblast cell line (CCD-996Sk), and human umbilical endothelial cell line (HUV-ECC) were all except for one (CC7T) obtained from ATCC (American Tissue Culture Collection). The CC7T cell line was kindly provided from T. M. Chang of the Taipei Veterans General Hospital [15]. The cells were cultured in individual special media (GIBCO BRL) dependent on the user guide from ATCC. Briefly, the SIHA cells were cultured in minimal essential medium (MEM) with 10% fetal bovine serum (FBS); ME 180 cells were cultured in McCoy's 5A medium; CC7T cells and SIHA cells were cultu-

red in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS; Ca Ski cells were cultured in RPMI 1640 medium (2mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES and 1.0 mM sodium pyruvate supplemented with 10% FBS; HeLa cells were cultured in modified Eagle's medium (MEM) (2mM L-glutamine, Earle's BSS, 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate) supplemented with 10% FBS; PA-1 cells were cultured in MEM supplemented with 10% heat-inactivated FBS; ES-2 cells were cultured in McCoy's 5a medium supplemented with 10% FBS; MCF7 cells were cultured in MEM (2 mM L-glutamine, Earle's BSS, 1.5 g/l sodium carbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 0.01 mg/ml bovine insulin) supplemented with 10% FBS; BeWo cells were cultured in Han's F12K medium supplemented with 15% FBS; RL95-2 cells were cultured in 1:1 volume DMEM and Ham's F 12 medium (10 mM HEPEs, 0.005 mg/ml insulin, 2.0 g/l sodium bicarbonate) supplemented with 10% FBS; CCD-966 Sk cell were cultured in 1:1 volume Earles' BBS and 90% MEM (non-essential amino acids and 1 mM sodium pyruvate) supplemented with 10% FBS; HUV-EC-C cells were cultured in Ham's F12K medium (2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mg/ml heparin and 0.03 mg/ml endothelial cell growth supplement) supplemented with 10% FBS. All cell lines were cultured at 37°C in a 5% CO_2 atmosphere.

Substrate

Galβ1,3GalNAc-acetyl-lactosamine)-Obzl (acceptor for ST2,3Gal I), Galβ1,3GlcNAcβ1,3Galβ1,4GlcNAc (acceptor for ST2,3Gal III), Galβ1,4GlcNAc (acceptor for ST2,3Gal IV), asialo-bovine submaxillary mucin (acceptor for ST6GalNAc I), asialo-fetuin (acceptor for ST6GalNAc II), and fetuin (acceptor for ST6GalNAc III) were from Sigma (St. Louis, MO). CMP-[^{14}C]NeuAc (100 nCi/ul) was from Amersham (Amersham Pharmacia Biotech, U. K.). Fluorescein-conjugated *Maackia Amurensis leukoagglutinin* (MAA) [5, 16] and *Sambucuas nigra agglutinin* (SNA) [5, 17,18], which recognize the oligosaccharide species NeuAcα2-3Gal-R, and NeuAcα2,6Gal, respectively, and Con A which recognizes all non-specific linkages, were from Vector Labs Inc. (Burlingame, CA).

Sialyltransferase Assay

Cells were washed with ice cold phosphate buffered saline, pH 7.3, at 4°C. Then cells were pelleted and incubated on a rocking plate for 30 minutes at 4°C in 500 μl 0.25 M sodium cacodylate buffer, pH 6.5, containing 0.4% Triton X-100, and then sonicated with ten 1-s pulses on ice [19, 20]. Nuclei were removed by low speed centrifugation and supernatant was centrifuged at 30,000 x g at 4°C for 30 min. The pellet was suspended in ice-cold 100 mM sodium cacodylate buffer (pH 6.0) and the supernatant was concentrated 10-fold Amicon 30 filters and used as the enzyme source. Protein content in diluted supernatants was determined by the BIO-RAD protein Assay kit (Munich, Germany) using bovine serum albumin as a standard. The enzyme assay method was modified from Kurosawa's original design [20-22]. Briefly, the enzyme assays with glycoproteins, oligosaccharides, and glycolipids as acceptors were performed in the presence of 0.1 M sodium cacodylate buffer, pH 6.0, 10 mM $MgCl_2$, 0.5% Triton CF-54, 100 μM CMP-[^{14}C]NeuAc (100 nCi/μl), 2 μl of acceptor (oligosaccharide 1 mM, or glycoprotein 4 mg/ml), and 5 μl of individual cell line-cell lysate in a final volume of 10 μl with incubation at 37°C for one hour. At the end of the incubation period, the reaction mixtures were subjected to SDS-polyacryl-amide gel electrophoreses (SDS-PAGE) for glycoproteins as acceptors or were subjected to chromatography on high performance thin layer chromatography plates (Merck, Darmstadt, Germany) with a solvent system of ethanol/1-butonol/pyridine/acetic acid/water (100:10:10:2:30) for glycolipids and glycoprotein as acceptors and with a solvent system of ethanol/pyridine/n-butanol/water/acetic acid (100:10:10:30:5) for oligosaccharides as acceptors. The radioactivity of the sialylated acceptors was quantified with a BAS2000 radio image analyzer (Fuji Film, Japan). To get the quantitative data, experiments were re-examined under linear condition with time and enzyme concentrations (enzyme activity unit: nanomol sialic acid/mg protein/hr).

Lectin Blotting

Cells were homogenized by sonication in Tris/Mannitol buffer. Different amounts of homogenate used for the STs assay were applied to polyvinylidene difluoride (PVDF) membranes (ATTO Co., Ltd, Tokyo, Japan) in the Bio-Dot SF assembly (Bio-Rad, Munich, Germany) as recommended by the manufacturer. PVDF membranes were reported as stable against heating and various organic solvents in addition to blotting glycosphigolipids with high efficiency [23]. PVDF membranes were first impregnated by methanol, water and buffer (10 mM CAPS, 10% methanol, pH 11.0), and finally cleaned by 50% methanol for 1 sec [24]. The membrane was removed from the wetting solution, placed on Bio-Dot SF Apparatus (Bio-Rad, Munich, Germany), and then 100 μl PBS buffer was added to each well. The buffer was gently removed from the wells by a vacuum. As soon as the buffer solution drained from all the wells, the flow valve was adjusted so that the unit was exposed to atmospheric pressure and then the vacuum was disconnected. At that point, 200 μl homogenates were added to each well. Then the homogenates were removed from the wells by a vacuum and 200 μl PBS was added to each well again and the buffer gently removed. Membranes were incubated with 2% polyvinylpyrrolidone PBS buffer for 30 min at room temperature. Membranes were washed by water and followed by buffer A (50 mM Tris-HCl, ph 7.5, 0.15 M NaCl, 0.1% Tween 20) and dipped with buffer A for 5 min. Membranes were incubated with 20 μg/ml fluorescein isothiocyanate (FITC)-lectin (Vector Lab, Burlingame, CA) in buffer A for MAA, SNA and Con A for 60 min at room temperature. Then, the membranes were washed with buffer A twice. After washing, fluorescence labeled glycoproteins were analyzed by a BAS 1500 image analyzer (Fuji Film, Tokyo, Japan).

Results

The enzyme activity of STs in 12 human cancer cell lines was determined by sialyltransferase assay. As shown in Figure 1, all of the cell lines tested (Ca Ski, Me-180, HeLa, SiHa, Es-2, MCF-7, BeWo, RL95-2, CCD-966Sk, HUV-EC-C), except CC7T and PA-1, showed high enzyme activity of ST3Gal I. Interestingly, the enzyme activity of ST3Gal III and ST3Gal IV was low compared to that of ST3Gal I. By contrast, as shown in Figure 2, all of the cell lines except PA-1, and RL95-2, and CCD-966Sk showed high enzyme activity of ST6GalNAc II.

Using the lectins from SNA specific for α2,6-sialic acids and MAA specific for α2,3-sialic acids, it was shown that the cell lines tested have diverse levels of surface α2,6-sialic acid sugar chains (enhanced SNA binding) when compared α2,3-sialic acid sugar chains (enhanced MAA binding). The former showed that the

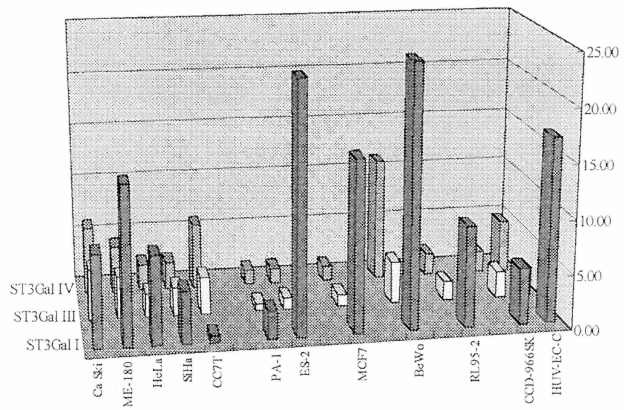

Figure 1. α2,3 sialyltransferases (ST2,3 Gal) I, III, and IV enzyme activities detected in the different cell lines (from the left to the right side: Ca Ski, ME180, HeLa, SiHa, CC7T, PA-1, ES-2, MCF7, BeWo, RL95-2, CCD-966Sk, and HUV-EC-C). All of the cell lines tested except CC7T and PA-1 showed high enzyme activity of ST3Gal I, especially ME-180, ES-2, MCF7, BeWo cell lines.

data of SNA/Con A ranged from 4.8% of SiHa cell lines to 72.4% of HUV-EC-C and the latter showed the data of MAA/Con A ranged from 1.3% of RL95-2 to 5.6% of PA-1 (Figures 3 and 4). However, generally the value of MAA/Con A from various kinds of gynecologic cancer cell lines was twice that of HUV-EC-C but was similar to that of fibroblast cell line - CCD-966Sk. Although the value of SNA/Con A from all cancer cell lines tested showed low levels of surface α2,6-sialic acid sugar chains compared to that of HUV-EC-C, we found that some gynecological cancer cell lines such as cervical cancer cell lines (Ca Ski, CC7T), ovarian cancer cell line (PA-1) and choriocarcinoma cell line (BeWo) had increased levels of surface α2,6-sialic acid sugar chains when compared with normal fibroblast cell lines (CCD-966Sk). Tables 1 and 2 show all the data in this study.

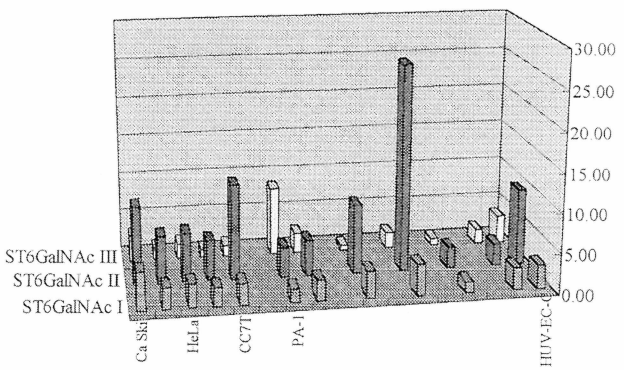

Figure 2. α2,6 sialyltransferases (ST2,6 GalNAc) I, II, and III enzyme activities detected in the different cell lines (from the left to the right side: Ca Ski, ME180, HeLa, SiHa, CC7T, PA-1, ES-2, MCF7, BeWo, RL95-2, CCD-966Sk, and HUV-EC-C). All of the cell lines tested except PA-1, and RL95-2, and CCD-966Sk showed high enzyme activity of ST2,3GalNAc II, especially CC7T and BeWo cell lines.

Table 1. — *Summary of surface α2,3-sialic acid sugar chains and enzyme activity of α2,3 sialyltransferase (ST2,3Gal I, III and IV) in the cell lines tested.*

Cell line	Con A	MAA	MAA/ Con A	ST3Gal I	ST3Gal III	ST3Gal IV
Ca Ski	1359	47	3.5%	8.44 (0.52)	4.62 (1.60)	6.22 (1.73)
ME-180	2027	58	2.9%	14.41 (0.68)	3.82 (0.78)	4.32 (0.59)
HeLa	2680	99	3.7%	8.13 (1.69)	2.12 (0.35)	2.45 (0.36)
SiHa	2707	151	5.6%	4.80 (0.14)	2.59 (0.26)	2.55 (0.27)
CC7T	4659	169	3.6%	0.60 (0.15)	3.42 (1.64)	6.08 (3.04)
PA-1	2520	141	5.6%	2.55 (0.53)	0.66 (0.35)	1.39 (0.38)
ES-2	2446	97	4.0%	23.05 (2.41)	1.07 (0.19)	1.46 (0.51)
MCF7	1542	51	3.3%	15.82 (1.30)	1.07 (0.40)	1.37 (0.25)
BeWo	3377	89	2.6%	24.18 (3.27)	3.88 (0.83)	11.46 (2.39)
RL95-2	2325	32	1.4%	9.23 (1.45)	1.70 (0.13)	2.02 (0.33)
CCD-966Sk	1326	35	2.6%	5.15 (0.47)	2.40 (0.11)	1.98 (0.15)
HUV-EC-C	2103	41	1.9%	17.06 (2.90)	2.68 (0.04)	4.48 (0.04)

Table 2. — *Summary of surface α2,6-sialic acid sugar chains and enzyme activity of α2,6 sialyltransferase (ST6GalNAc I, II and III) in the cell lines tested.*

Cell line	Con A	SNA	SNA/ Con A	ST6GalNAc I	ST6GalNAc II	ST6GalNAc III
Ca Ski	1359	243	17.9%	4.70 (1.01)	9.75 (1.60)	3.26 (0.87)
ME-180	2027	109	5.4%	2.62 (1.69)	5.97 (1.05)	1.48 (0.31)
HeLa	2680	151	5.6%	2.92 (1.02)	6.18 (1.93)	1.86 (0.53)
SiHa	2707	130	4.8%	2.32 (0.72)	5.08 (1.01)	0.93 (0.04)
CC7T	4659	1981	42.5%	2.68 (1.70)	11.90 (6.75)	1.32 (0.08)
PA-1	2520	945	37.5%	1.64 (0.64)	3.75 (0.47)	8.64 (1.23)
ES-2	2446	207	8.5%	2.62 (0.94)	4.52 (0.12)	2.48 (0.61)
MCF7	1542	104	6.7%	3.36 (1.37)	8.73 (0.46)	0.59 (0.12)
BeWo	3377	2290	67.8%	3.83 (1.59)	26.34 (0.20)	1.94 (0.17)
RL95-2	2325	150	6.5%	1.32 (0.27)	2.65 (0.22)	0.91 (0.07)
CCD-966Sk	1326	70	5.3%	2.79 (0.11)	2.82 (0.43)	1.77 (0.24)
HUV-EC-C	2103	1518	72.2%	2.97 (0.73)	9.69 (0.99)	3.53 (0.69)

Discussion

Sialyltransferases (STs) are a family of glycosyltransferase which catalyze the transfer of sialic acid from cystidine monophosphate N-acetylneuraminic acid (CMP-Neu5Ac) to non-reducing terminal positions on sugar chains of glycoconjugates (glycoproteins and glycolipids) [25,26]. A growing number of reports demonstrate that hypersialylation, which is observed in certain pathological processes such as oncogenic transformation, tumor metastases, and invasion, is associated with enhanced sialyltransferase (ST) activity [1-14]. More specifically, just like various kinds of cancers that demonstrate carbohydrate changes in breast cancer [8, 9, 27-29], colorectal cancer [10, 16, 30-35], lung cancer [36, 37], hepatic carcinoma [38, 39], gastric carcinoma [13], head and neck squamous cell carcinoma [12], brain tumor [11, 40], choriocarcinoma [14], squamous cell carcinoma of the cervix [1], and prostate cancer [41].

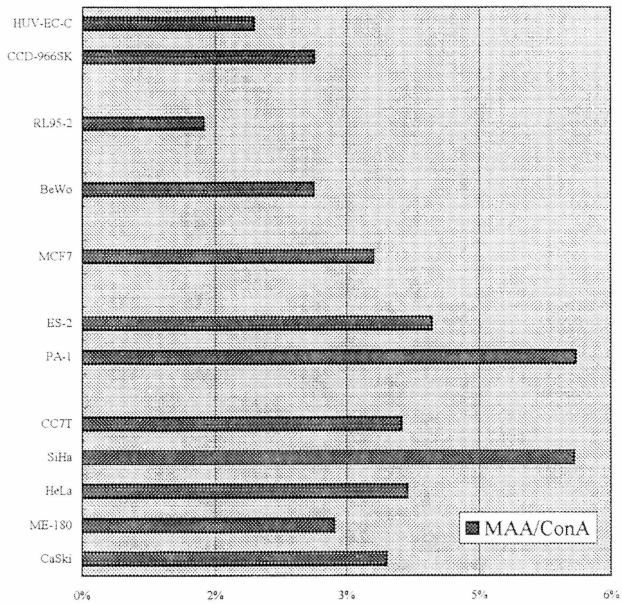

Figure 3. Levels of surface α2,3-sialic acid sugar chains (enhanced MAA binding) detected in the different cell lines (from the upper to the lower side: HUV-EC-C, CCD-966Sk, RL95-2, BeWo, MCF7, ES-2, PA-1, CC7T, SiHa, HeLa, ME-180, Ca Ski). The data of MAA/Con A ranged from 1.3% of RL95-2 to 5.6% of PA-1.

Although at least 18 distinct sialyltransferase genes exist, all involve tumor-associated changes in the expression of cell-surface sialoglycoconjugates [1]. For example, six human α2,3-sialyltransferase genes (hST3Gal I-VI) have so far been cloned [41]. In addition, the final sialyl-glycan structure is determined by a concerted action of all expressed sialyltransferases, just like ST3Gal II [17, 42, 43], which shares almost identical specificity with ST3Gal I; moreover, ST3Gal VI shares acceptor specificity with ST3Gal III and IV and competes with ST6Gal I. However, we did not study the other ten sialyltransferases. In contrast, in this report, we studied enzyme activity of six different types of sialyltransferases- ST2,3Gal I, ST2,3Gal II, ST2,3Gal IV, ST2,6GalNAc I, ST2,6GalNAc II, and ST2,6GalNac III in ten gynecological cancer cell lines to assess the candidate sialyltransferase to help in clinical practice.

ST3Gal I, being responsible for the α2,3-sialylation of Galβ1,3GalNAc on O-linked chains of glycoproteins and glycolipids, was proposed to have a decisive role in Sialyl-Le^x/Le^a biosynthesis [44] and sialylation of the Thomsen-Friedenreich antigen [45]. Enhanced ST3Gal I expression was found in lung cancers [36, 37], in breast cancer [27], and colon cancer [10, 30]. ST3Gal I mRNA expression was significantly increased in cases showing invasion of lymph vessels [10]. Transfer of sialic acid in the α2,3-linkage to Galβ1,(3)4GlcNAc on N-linked chains of glycoproteins is carried out by α2,3-sialyltransferase ST3Gal III or ST3Gal IV. The latter is also capable of adding sialic acid to Galβ1,3GalNAc found on O-linked chains of glycoproteins. The role of ST3Gal III or ST3Gal IV has also been discussed previously [36, 46]. Cancer cells have to adhere to vascular endothelial cells and then extravasate from the blood stream into the surrounding tissues in order to metastasize hematogeneously [36]. Altered glycosylation of malignant cell-surface lipids and proteins plays an important role in tumor cell adherence to endothelial cells by interaction mediated by E-selectin [36, 47, 48]. ST3Gal III is involved in the biosynthesis of sLe$^{(x)}$ and sLe$^{(a)}$ which are known as selectin ligands and tumor-associated carbohydrate structures [46]. Increased expression of ST3Gal III or ST3Gal IV may play a role in glial tumorigenesis [11]. Expression of sialyltransferase ST3Gal IV was significantly enhanced in gastric carcinoma tissue compared to normal tissue [13]. ST6Gal NAC I and ST6GalNac II were cloned as sialyltransferases that mainly utilize o-glycans as an acceptor. ST6GalNAc I exhibits the broadest substrate specificity for the following structure: GalNAC-O-Ser/Thr, Gal beta1,3GalNAc-O-Ser/Thr, and NeuAcalpha2,3Galbeta1,3GalNAc-O-Ser/Thr [18]. On the other hand, ST6GalNAc II exhibits a narrower substrate specificity requiring beta-galactosides linked to GalNAc residues, whereas sialic acid residues linked to galactose residues are not essential for its activity; i.e. this enzyme exhibits activity toward Galbeta1,2GalNac-O-Ser/Thr and NeuAcalpha2,3Galbeta1,3GalNac-o-ser/Thr [11]. Both genes are expressed in secretory organs such as the sub maxillary and mammary glands, thus these enzymes are considered to be involved in the

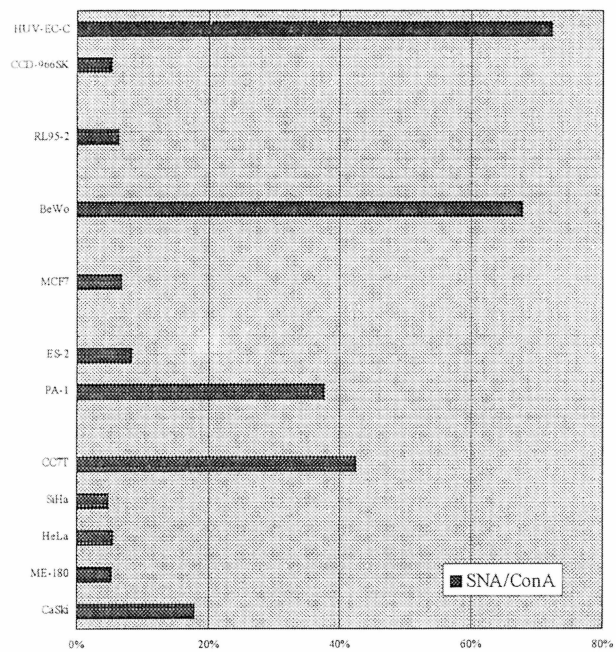

Figure 4. Levels of surface α2,6-sialic acid sugar chains (enhanced SNA binding) detected in the different cell lines (from the upper to the lower side: HUV-EC-C, CCD-966Sk, RL95-2, BeWo, MCF7, ES-2, PA-1, CC7T, SiHa, HeLa, ME-180, Ca Ski). The data of SNA/Con A ranged from 4.8% of SiHa cell lines to 72.4% of HUV-EC-C.

biosynthesis of o-glycans of mucin [11-12, 49]. ST6GalNAc I prefers GalNAc-Ser/Thr, and ST6GalNAc II acts on Galβ1,3GalNac-Ser/Thr. Expression of ST6GalNAc II was significantly increased in cases with metastases to lymph nodes along the vascular truck in colon cancer and provides a prognostic factor for patient survival [10]. ST6GalNAc III (which shows high homology in their primary structure with ST6GalNAc IV, and ST6GalNAc V) acts as a terminal sialic acid with an α2,3 linkage on galactose [49, 50].

In this study, we found that the cell lines from various kinds of gynecological cancers show abnormally elevated enzyme activity of ST3Gal I and ST6GalNAc, although the other four types of STs also showed a lesser degree of elevation. In addition, enhanced activity of sialyltransferases involving α2,6-sialic acid sugar chains might be more important in cancer development. In fact, in our previous work, we found that increased expression of β -galactoside α2,6-sialyltransferase (ST6Gal I) mRNA was found in squamous cell carcinoma of the cervix and high expression of ST6Gal I was associated with poor histological factors, such as deep stromal invasion, lymph-vascular space involvement, and poor differentiation [1]. Future studies will investigate whether the enzyme activity of these sialyltransferases can be helpful for clinical practice.

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Address reprint requests to:
Y.C. TSAI, M.D.
Institute of Biochemistry,
National Yang-Ming University,
155, Section 2, Li-Non Street,
Taipei 112 (Taiwan)