Glycan profiling of gestational choriocarcinoma using a lectin microarray

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Abstract. Glycosylation is an important post-translational modification, in which attachment of glycans to proteins has effects on biological functions and carcinogenesis. Analysis of human chorionic gonadotropin, a glycoprotein hormone produced by placental trophoblasts and trophoblastic tumors, has contributed to the diagnosis and treatment of trophoblastic disease, resulting in reduced incidence and mortality. However, alterations of the glycan structure itself in choriocarcinoma have not been characterized. We established a new choriocarcinoma cell line, induced choriocarcinoma cell-1 (iC^3-1), which mimics the clinical pathohistology *in vivo*, to examine the tumorigenesis and pathogenesis of choriocarcinoma. In this study, the alterations of glycan structures in the development of choriocarcinoma were examined by performance of comprehensive glycan profiling in clinical samples and in iC^3-1 cells using a conventional microarray and the recently introduced lectin microarray. Microarray comparison showed significant upregulation of several characteristic glycogenes in the iC^3-1 cells as compared to the parental HTR8/SVneo cells. The lectin array showed increased α-2-6-sialic acid, Galβ1-4GlcNAc, GlcNAcβ1-3GalNAc, and decreased α-1-6 core fucose, high mannose, GalNacβ1-4Gal, GALNAc (Tn antigen) and Galβ1-3Gal in choriocarcinoma tissue compared to normal villi. This is the first report of a lectin array analysis in choriocarcinoma and provides useful information for understanding of the disease.

Introduction

Gestational choriocarcinoma, one type of gestational trophoblastic neoplasia, is a malignant epithelial trophoblastic tumor that can develop in the uterus after pregnancy (1). Half of the cases are preceded by a hydatidiform mole, while others occur after spontaneous abortion or normal term pregnancy (1). Treatment of a hydatidiform mole using a clinical marker, human chorionic gonadotropin (hCG), has significantly reduced the incidence of choriocarcinoma and improved survival (2). hCG is a glycoprotein that is produced by fused and differentiated placental syncytiotrophoblast cells and serves as a marker for placental function and assessment of treatment for malignant trophoblastic disease (3). The function and structure of hCG are well-established and hCG variants are known to be heavily glycosylated and sialylated glycoproteins (3).

Glycosylation is a common and versatile co- and post-translational modification that involves attachment of glycans to proteins, lipids or other organic molecules by glycosyltransferases encoded by glycogenes (4,5). Altered glycan structure by glycosylation is a common feature in cancer cells and promotes tumor cell invasion and metastasis. Certain glycans are markers for tumor progression (6,7), and thus it is important to analyze the structure and binding mechanisms of these structures in the context of cancer diagnosis and treatment. However, comprehensive glycan analysis has not been performed in choriocarcinoma although several molecular studies have revealed the expression of tumor-related proteins in choriocarcinoma (1). Indeed, conventional glycan profiling tools, such as capillary electrophoresis, liquid chromatography and mass spectrometry, may not be suitable for the initial detection of glycan alterations between samples because of the time-consuming, low-throughput and requirements for complex equipment and the generation of complex data (8,9). Conversely, lectin microarray has emerged in recent years and could be used for rapid and high-throughput analysis for protein glycosylation (8,9).

We previously established a new choriocarcinoma cell line, induced choriocarcinoma cell-1 (iC^3-1), that mimics the clinical pathohistology *in vivo*, and we used these cells to examine the tumorigenesis and pathogenesis of choriocarcinoma (10,11). The goal of the present study was to investigate alterations in glycan structure in the development of choriocarcinoma using comprehensive glycan profiling in clinical samples and in iC^3-1 cells using a conventional microarray and the recently

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developed lectin microarray. Herein, we report the initial data from a lectin microarray in choriocarcinoma tissue.

Materials and methods

Patients and samples. Formalin-fixed, paraffin-embedded tissues from 4 cases were used in the study. First trimester villi were obtained from induced-abortion cases (n=2) and choriocarcinoma samples were obtained from pregnancies that had gone to term and in which hysterectomy was performed piror to other treatment (n=2). The samples were obtained during surgery or delivery and were immediately fixed in formalin. All patients had been treated between 2008 and 2011 at Keio University Hospital. The samples were examined and diagnosed by two independent pathologists. This study was approved by the Institutional Review Board of Keio University School of Medicine (No. 20130115).

Cell culture. HTR8/SVneo, a human extravillous trophoblast cell line immortalized using SV40 T antigen (SV40Tag), was kindly provided by Dr C.H. Graham (Queen's University, Kingston, ON, Canada) (12). HTR8/SVneo/EGFP and iC^3-1 cells were obtained as previously described (10). Before culture, cell sorting was performed with a FACSVantage SE (BD Biosciences, Franklin Lakes, NJ, USA) (10) after staining with propidium iodide (P4170; Sigma-Aldrich, St. Louis, MO, USA). Sorting based on GFP and propidium iodide fluorescence with gating on forward and side scatter was used to obtain transfected cells and exclude non-viable cells. Sorted HTR8/SVneo/EGFP and iC^3-1 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Burlington, ON, Canada) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37˚C in a 5% CO_2 atmosphere (10).

Microarray analysis. Microarray analysis comparing iC^3-1 cells and parental HTR8/SVneo/EGFP cells were performed as previously reported (10). Briefly, total RNA from those cells was extracted using the Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. After being amplified, labeled and hybridized by the Human Whole Genome Oligo Microarray kit (G4112F; Agilent, Santa Clara, CA, USA) following the Agilent user's manual protocol, the arrays were scanned using the Agilent Dual-Laser DNA Microarray Scanner (G2565A; Agilent).

Lectin microarray analysis. Lectin microarray analysis was performed as previously described (13). Briefly, the membrane fractions of HTR8/SVneo and iC^3-1 cells were obtained using the ProteoExtract Subcellular Proteome Extraction kit (539790; Merck). Glycoproteins from formalin-fixed paraffin-embedded tissue of normal villi and choriocarcinoma were obtained as previously reported (14). The total protein content of each sample was determined using a Micro BCA Protein Assay kit (23235; Thermo Scientific, Hemel Hempstead, UK) and adjusted to 50 μg/ml with phosphate-buffered saline. A portion of each sample (1 μg) was added to 100 μg of Cy3 monoreactive dye pack (PA23001; GE Healthcare) and incubated for 1 h at room temperature in the dark. Cy3-labeled proteins were desalted using a Zeba Spin Desalting Column (89882; Thermo Scientific) and diluted to 2 μg/ml with probing solution (TBS containing 1% Triton X-100, 500 mM glycine).

A sample of 100 μl was added to each well on a lectin microarray glass slide (GP Biosciences, Yokohama, Japan) and incubated in a chamber (>80% humidity) for 150 min at room temperature in the dark until the binding reached equilibrium. Fluorescent images of the lectin microarrays were acquired using an evanescent-field fluorescence scanner (GlycoStation™ Reader 1200; GP Biosciences). The net intensity for each spot was calculated by subtracting the background value from the raw signal intensity, with averaging of the results from three spots. Acquired data were normalized by making the total fluorescence in each well (i.e. for 45 lectins) equivalent. All data were analyzed with Array-Pro Analyzer v. 4.5 (Media Cybernetics, Bethesda, MD, USA).

Results

Differences in glycogene expression in the iC^3-1 and HTR8/SVneo cells. Gene expression profiles of iC^3-1 cells and a parental trophoblast cell line, HTR8/SVneo, derived from first trimester placental tissue, were determined by microarray analysis to examine alterations in glycogenes in choriocarcinoma tumorigenesis. As previously reported, in iC^3-1 cells compared to HTR8/SVneo/EGFP cells, 3,094 genes were upregulated and 3,126 genes were downregulated among the 44,000 genes examined (10). To search the differences in glycol-gene expression, we referred to the GlycoGene DataBase (http://riodb.ibase.aist.go.jp/rcmg/ggdb/) for the upregulated or downregulated genes. This analysis showed that 20 glycogenes were significantly upregulated and 15 were significantly downregulated in iC^3-1 cells as compared to the HTR8/SVneo/EGFP cells (Table I). The upregulated genes included hyaluronan synthase (HAS2), which we previously found to show immunoreactivity in choriocarcinoma samples, but not in normal first trimester villi and term placenta (11).

Glycan profiling analysis in choriocarcinoma. Glycan profiling analysis of normal villi, choriocarcinoma, HTR8/SVneo and iC^3-1 cells was used to investigate changes in glycan structure in the development of choriocarcinoma, using a lectin microarray. Signals for Cy3-labeled glycoproteins on the LecChip, which includes 45 different lectins, were scanned using GlycoStation Reader 1200 independently four times (Fig. 1A). The net intensity of each lectin signal was quantified using Glycostation Tools Pro and Array-Pro Analyzer (Fig. 1B). Lectins with increased levels in choriocarcinoma samples (>20% increase in signal intensity compared to villi) were *Sambucus nigra* agglutinin (SNA), *Sambucus sieboldiana* agglutinin (SSA), *Trichosanthes japonica* agglutinin I (TJA-I), *Ricinus communis* agglutinin I (RCA120), erythroagglutinating isolectin of phytohemagglutinin (PHAE), *Datura stramonium* agglutinin (DSA), *Agrocybe cylindracea* galectin (ACG), TxLC-I, *Urtica dioica* agglutinin (UDA), Jacalin, and wheat germ agglutinin (WGA) (Fig. 1B and Table II). In contrast, those with decreased levels in choriocarcinoma samples (>20% decrease in signal intensity compared to villi) were *Pisum sativum* agglutinin (PSA), *Lens culinaris* agglutinin (LCA), *Aspergillus oryzae* lectin (AOL), *Narcissus pseudonarcissus* agglutinin (NPA), *Galanthus nivalis* agglutinin (GNA), *Bauhinia purpurea* lectin (BPL),

Table I. Glycogenes with altered expression in iC^3-1 cells as compared to HTR8/SVneo/EGFP cells.

Gene symbol	Family	Fold-change	P-value
Upregulated			
HAS2	Glucuronyltransferases N-acetylglucosaminyltransferases	664.90	1.22.E-04
HS6ST2	Sulfotransferases	14.56	4.94.E-03
MGAT4A	N-acetylglucosaminyltransferases	9.18	1.01.E-03
XYLT1	Xylosyltransferases	9.18	1.05.E-03
GALNT14	UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase	8.15	1.13.E-03
POFUT1	Fucosyltransferases	6.62	3.61.E-03
ST6GALNAC3	Sialyltransferases	5.92	1.58.E-03
UST	Sulfotransferases	4.27	2.38.E-03
GALNT3	UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase	3.87	2.89.E-03
ST3GAL4	Sialyltransferases	3.40	2.64.E-03
GCNT2	N-acetylglucosaminyltransferases	3.33	2.42.E-03
ST8SIA5	Sialyltransferases	3.11	3.04.E-03
CHST2	Sulfotransferases	2.70	5.01.E-03
GALNT6	UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase	2.65	5.22.E-03
SLC35D1	Nucleotide sugar transporters	2.48	6.01.E-03
HS3ST3A1	Sulfotransferases	2.47	6.02.E-03
MGAT5B	N-acetylglucosaminyltransferases	2.26	1.20.E-02
GALNT10	UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase	2.20	7.98.E-03
FUT8	Fucosyltransferases	2.12	8.67.E-03
HAS3	Glucuronyltransferases N-acetylglucosaminyltransferases	2.03	5.27.E-03
Downregulated			
B4GALNT3	N-acetylgalactosaminyltransferases	0.18	2.32.E-03
ST6GALNAC2	Sialyltransferases	0.19	6.96.E-03
B3GALT5	Galactosyltransferases	0.24	7.09.E-03
GAL3ST1	Sulfotransferases	0.27	4.03.E-03
GALNT9	UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase	0.27	1.06.E-02
B3GAT1	Glucuronyltransferases	0.28	3.79.E-02
LARGE	Glycosyltransferase-like	0.30	9.68.E-03
MFNG	N-acetylglucosaminyltransferases	0.31	3.78.E-03
CHST1	Sulfotransferases	0.36	1.58.E-03
ST3GAL5	Sialyltransferases	0.36	1.69.E-03
NDST1	Sulfotransferases	0.38	5.41.E-03
POMT2	Mannosyltransferases	0.43	6.87.E-03
CHST3	Sulfotransferases	0.44	7.25.E-03
B3GALT2	Galactosyltransferases	0.49	2.45.E-03
GALNT2	UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase	0.50	1.00.E-02

Wisteria floribunda agglutinin (WFA), *Amaranthus caudatus* agglutinin (ACA), *Helix pomatia* agglutinin (HPA) and *Maackia amurensis* hemagglutinin (MAH) (Fig. 1B and Table II). A comparison of iC^3-1 and parental HTR8/SVneo cells showed quite similar glycan profiles, suggesting that activation of the MAPK/PI3K pathway does not contribute to the alteration of glycan structures (Fig. 1C).

Based on the glycan-binding specificity of these lectins, among the N-type glycans, choriocarcinoma had higher levels of α-2-6 sialic acids (SNA, SSA, TJA-I and WGA), reduced levels of α-1-6 core fucoses (PSA, LCA and AOL), increases in Galβ1-4GlcNAc structures (RCA120, PHAE and DSA), and decreases in high mannose (NPA and GNA), compared to normal villi (Fig. 2 and Table II). In regards to O-type glycans, choriocarcinoma had increased GlcNAcβ1-3GalNAc structures (Jacalin) and decreases in GalNacβ1-4Gal (WFA), GALNAc (Tn antigen) (HPA) and Galβ1-3Gal (ACA), when compared to normal villi (Fig. 2 and Table II).

Discussion

The function of most proteins depends on post-translational modifications such as glycosylation, phosphorylation, methylation and sulfation (15). In glycosylation, glycan attachment modifies the function of proteins in areas such as cell recognition, cell-cell interactions and immune response, and is also

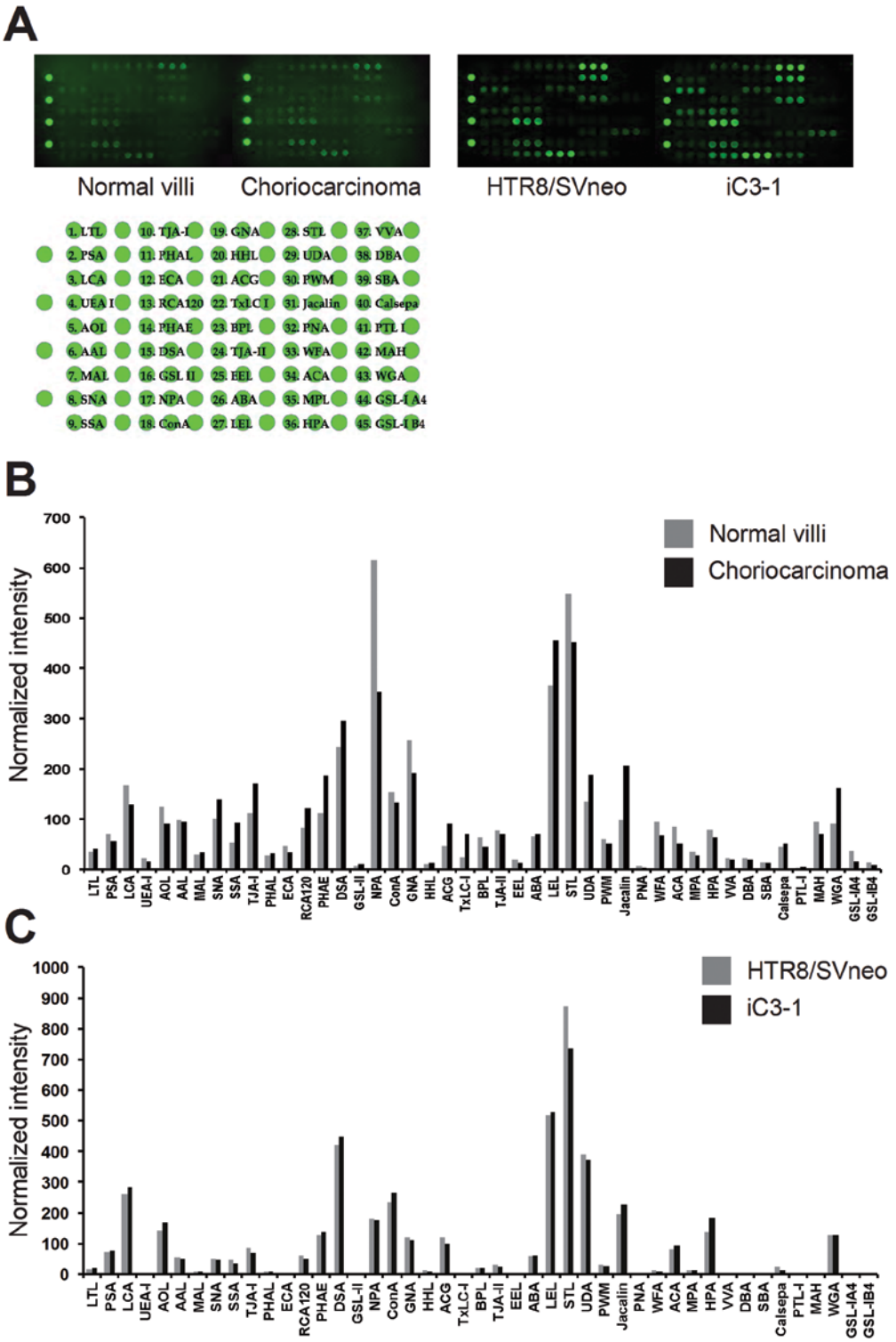

Figure 1. Comparison of glycan profiles by lectin microarray analysis of choriocarcinoma and villi samples. (A) Scans of the samples for all 45 lectins. Lectin spotting patterns are shown in the lower parts of the images. (B) Normalized glycan intensity in the samples of normal villi and choriocarcinoma. Signals were normalized using the mean of all lectins on the array. (C) Normalized glycan intensity in the samples of HTR8/SVneo and choriocarcinoma cell-1 (iC^3-1). Signals were normalized using the mean of all lectins on the array.

important in carcinogenesis, including proliferation, invasion, metastasis and tumor progression (16,17). Therefore, glycan analysis is a key to the understanding of physiological function and pathogenesis. However, progress in glycobiology has been slow due to the complexity of glycans and a lack of systematic methods with which to analyze their structures. This difficulty has been addressed by the introduction of technology for rapid, simple and high-throughput evaluation (18). In particular, the lectin microarray, based on an evanescent-field fluorescence detection principle, permits sensitive and quantitative real-time observation of multiple lectin-carbohydrate interactions unlike other conventional methods, e.g., mass spectrometry and chromatography (19). Even though a lectin microarray has emerged only in recent years, it has already been applied for

Table II. Lectins and their glycan-binding specificity.

Lectin	Signal ratio	Specificity
TxLC-I	3.010	Manα1-3(Manα1-6)Man, bi- and tri-antennary complex-type N-glycan, GalNAc
Jacalin	2.116	Galβ1-3GalNAc, GalNAc
ACG	1.973	Siaα2-3Galβ1-4GlcNAc
WGA	1.806	Chitin oligomers, Sia
SSA	1.729	Siaα2-6Gal/GalNAc
PHAE	1.666	Bi-antennary complex-type N-glycan with outer Gal and bisecting GlcNAc
TJA-I	1.533	Siaα2-6Gal/GalNAc
RCA120	1.455	Galβ1-4GlcNAc
UDA	1.408	GlcNAcβ1-4GlcNAc, mixture of Man5 to Man9
SNA	1.379	Siaα2-6Gal/GalNAc
DSA	1.206	(GlcNAcβ1-4)n, Galβ1-4GlcNAc
NPA	0.572	High-Mannose, Manα1-6Man
ACA	0.608	Galβ1-3GalNAc
BPL	0.717	Galβ1-3GalNAc, GalNAc
AOL	0.724	Fucα1-6GlcNAc (core fucose)
MAH	0.731	Siaα2-3Galβ1-3(Siaα2-6)GalNAc
GNA	0.744	High-Mannose, Manα1-3Man
LCA	0.774	Fucα1-6GlcNAc, α-D-Glc, α-D-Man
PSA	0.783	Fucα1-6GlcNAc, α-D-Glc, α-D-Man
HPA	0.793	α-linked terminal GalNAc

ACG, *Agrocybe cylindracea* galectin; WGA, wheat germ agglutinin; SSA, *Sambucus sieboldiana* agglutinin; PHAE, erythroagglutinating isolectin of phytohemagglutinin; TJA-I, *Trichosanthes japonica* agglutinin I; RCA120, *Ricinus communis* agglutinin I; UDA, *Urtica dioica* agglutinin; SNA, *Sambucus nigra* agglutinin; DSA, *Datura stramonium* agglutinin; NPA, *Narcissus pseudonarcissus* agglutinin; ACA, *Amaranthus caudatus* agglutinin; BPL, *Bauhinia purpurea* lectin; AOL, *Aspergillus oryzae* lectin; MAH, *Maackia amurensis* hemagglutinin; GNA, *Galanthus nivalis* agglutinin; LCA, *Lens culinaris* agglutinin; PSA, *Pisum sativum* agglutinin; HPA, *Helix pomatia* agglutinin. Signal ratio, the average of net intensity in choriocarcinoma samples/that in Villi samples.

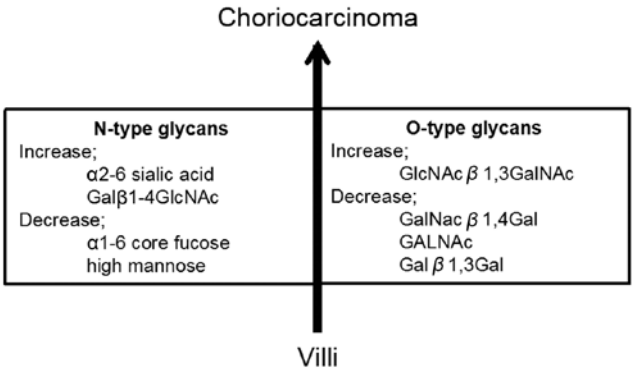

Figure 2. Alterations of glycan structure in the development of choriocarcinoma.

the development of disease-related glycoprotein markers, the evaluation of stem cells and the investigation of pluripotent cell markers (19).

Glycan analysis in choriocarcinoma has not previously been performed, in contrast to other cancers. Therefore, in the present study, we used microarrays for glycogenes and lectins in our established choriocarcinoma cell line and clinical samples to investigate glycan alterations in choriocarcinoma tumorigenesis. Conventional microarray comparison of iC^3-1 and parental HTR8/SVneo cells indicated changes in several significant glycogenes. These include: HAS2, which we have shown to be significantly overexpressed in choriocarcinoma samples (10); HAS3, which is correlated with tumor growth in colon cancer and esophageal squamous cell carcinoma (20,21); HS6ST2, which plays an important role in cell growth, invasion, migration and tumorigenicity in several types of cancers (22-24); FUT8, the expression of which is upregulated in lung, liver, ovarian, thyroid and colorectal cancer and correlates with tumor metastasis, disease recurrence and poor survival (25); POFUT1, which has higher expression in colon cancer and glioblastoma compared to normal tissue (26,27); GalNT14, a biomarker that is predictive of the response to PARA/extrinsic pathway-targeted therapy in non-small cell lung cancer, since high levels of GalNT14 mRNA and protein in tumor cell lines are associated with Apo2L/TRAIL sensitivity (28); MGAT5B, which is upregulated in prostate cancer cells and is a marker of tumor progression (29); CHST2, a clinical molecular marker for the outcome of osteosarcoma (30); and HS3ST3A1, expression of which is higher in cancer tissue compared to normal lung tissue (31).

The lectin microarray also produced novel findings for glycans in choriocarcinoma. Among the lectins with increased or decreased levels in choriocarcinoma as compared to normal villi, SSA, TJA-I, RCA120, SNA, ACA, BPL and HPA were found to exhibit the same binding pattern in colon cancer when compared to normal tissue (14). Thus, these lectins may

be useful as diagnostic and therapeutic markers in choriocarcinoma. In particular, the increased levels of SNA, SSA and TJA-I indicate the presence of a higher level of α-2-6 sialic acid, and also indicate the aggressive behavior of choriocarcinoma as these lectins were found to be highly expressed in poorly differentiated endometrial cancer cells compared to well-differentiated cells (32). In addition, SSA is a potential marker for isolation of cancer stem cells (CSCs) (33). iC^3-1 cells may contain components of choriocarcinoma CSCs and CD44 may be a marker for these CSCs (10). Thus, it will be of interest to attempt to isolate choriocarcinoma CSCs using CD44 and SSA.

The emerging technology of the lectin microarray is likely to permit elucidation of the precise glycan characteristics of proteins of importance in choriocarcinoma. Our data revealed that there are differences in glycogenes and lectin expression that represent potential therapeutic targets for choriocarcinoma.

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