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Focused microarray analysis of glyco-gene expression in human glioblastomas

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Abstract

Altered glycosylation has been linked to cancer cell metastasis and invasivity. We have previously shown that expressing a specific sialyltransferase gene in gliomas inhibited tumor formation, *in vivo*. In order to identify other "glyco-gene" targets with therapeutic potential, focused 45-mer oligonucleotide microarrays were constructed containing all of the cloned human glyco-genes. Gene expression profiles of normal human brain and malignant gliomas were compared and microarray datasets analyzed using significance analysis of microarrays algorithms. There were 11 genes more highly expressed in gliomas compared to normal brain and 25 genes more highly expressed in normal brain compared to gliomas. Among the most noteworthy were high levels of MAN2A2 and ST6GalNAcV in normal brain tissue and high levels of POFUT1 and CHI3L1 in the gliomas, all changes corroborated by qRT-PCR. Historically, identification of changes in tumor-associated glycoconjugate expression was obtained by measuring individual enzyme activities or structural changes of specific molecules. With microarray technology, it is possible to measure all of genes associated with glycoconjugate biosynthesis and degradation simultaneously. Our data demonstrate that there are many significant and novel differences in glyco-gene expression that represent potential targets for the development of therapeutics for the treatment of brain tumors.

Keywords: gene expression, glioblastoma, glycosylation, glycosyltransferase, microarray.

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Virtually all tumor cells display aberrant cell-surface glycosylation patterns. These are most often caused by alterations in the biosynthesis and degradation of the oligosaccharide chains found in the glycoproteins, glycosphingolipids, proteoglycans, and glycosaminoglycans that comprise the cell-surface glycoconjugates associated with adhesion and migration in normal cellular function and metastasis and invasivity in tumor cells (Hakomori 2002).

There are many reports of aberrant cell-surface glycoconjugate expression in brain tumors. These include altered ganglioside expression (Traylor and Hogan 1980; Fredman *et al.* 1986; Jennemann *et al.* 1990, 1994; Wikstrand *et al.* 1991; Shinoura *et al.* 1992; Chang *et al.* 1997; Comas *et al.* 1999; Hamasaki *et al.* 1999; Markowska-Woyciechowska *et al.* 2000; Pan *et al.* 2000; Popko *et al.* 2002; Mennel and Lell 2005), tumor cell-associated ganglioside shedding (Ladisch *et al.* 1987, 1997; Nakamura *et al.* 1991; Li *et al.* 1996), β1-integrins, and N-CAM (Paulus *et al.* 1996; Gratsa *et al.* 1997). For recent reviews, see Fredman *et al.* (2003), Fish (1996), Pilkington (1992), Yates (1988) and Mikkelsen *et al.* (1998). The first report implicating aberrant glycosyltransferase gene expression in human brain tumors was a survey of α2,6ST mRNA expression in panel of astrocytic, meningeal, and metastatic primary human brain tumors by Kaneko *et al.* (1996). No α2,6ST mRNA expression was found in the astrocytic or metastatic tumors but robust expression in meningeal tumors was observed. This was followed by a report by Yamamoto *et al.* (1997b) showing that α2,3ST mRNA and α2,3-linked glycoprotein sialylation were markedly over-expressed in human glioma specimens, fetal astrocytes and a panel of human glioma cell lines. α2,6ST gene transfection studies, using the highly invasive human glioma cell line U373MG, resulted in marked inhibition of tumor cell invasivity in both an *in vitro*

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Abbreviations used: FDR, false discovery rate; FUT, fucosyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBMs, glioblastomas; SAM, significance analysis of microarrays.

matrigel-based invasion assay system and *in vivo* using a severe combined immunodeficient mouse model with direct intracranial injection of transfected tumor cells (Yamamoto *et al.* 1997a, 2001). The inhibition of invasivity was attributed to the generation of α,26-linked sialic acids on the glycoprotein N-linked oligosaccharides of the glioma-associated integrin, α3β1, leading to alteration in adhesivity and alterations in the signal transduction capability of the α3β1 integrin as shown by altered focal adhesion kinase activities in these glioma cells as well.

In a more recent report, Yamamoto *et al.* (2000) examined both GnT-III and GnT-V mRNA expression in gliomas and found that normal tissues expressed substantial GNTIII mRNA but negligible amounts of GnT-V mRNA. On the other hand, highly variable amounts of both GnT-III and GnT-V mRNA were found in the glioma specimens. Marked and consistent expression of GnT-III mRNA but variable expression of GnT-V mRNA in a panel of four human glioma cell lines and five human neuroblastoma cell lines was also observed. Stable GnT-V transfectants made with the human glioma cell line, SNB-19, showed marked changes in both cell morphology and focal adhesions along with a significant increase in invasivity *in vitro*. It was concluded that the marked expression, albeit quite variable, in the primary brain tumors and tumor cell lines taken as a whole suggested that β1,6-*N*-acetylglucosamine-bearing *N*-glycans play a role in glioma invasivity, consistent with earlier reports showing a strong correlation between metastatic potential and the expression of tri- and tetra-antennary β1,6NAG-bearing *N*-glycans (Dennis *et al.* 1987; Asada *et al.* 1997). And most recently, Dawson *et al.* (2004), examining the stable transfectants described above, found marked increases in apoptotic cell death as a result of the increased sensitivity to staurosporine, C2-ceramide or etoposide in each cell line.

From these studies and several other recent reports that suggested that glycosyltransferase mRNA levels can be used as diagnostic markers for detecting metastatic neuroblastoma cells (Hoon *et al.* 2001) in bone marrow and high grade glioblastomas (Oblinger *et al.* 2006), a more detailed analysis of glycosyltransferase and glycosyl hydrolase gene expression in human malignant gliomas was undertaken. A focused oligonucleotide microarray containing all of the cloned human "glyco-genes" was constructed and used to screen a panel of primary human gliomas, normal human brain and human glioma cell lines.

Materials and methods

Human tissue samples

For the microarray analysis, six surgical specimens and six normal human brain specimens were used. For the qRT-PCR analyses, four additional glioblastoma and four additional normal brain specimens were used.

Brain tumor tissue (GBM, WHO Grade IV) from patients was acquired from Field Neurosciences Institute, Saginaw, MI and from the tumor bank maintained by the Falk Center. None of the patients had been subjected to chemotherapy or radiotherapy prior to resection. Protocols for tissue accrual and use were approved by the appropriate Institutional Review Boards. Immediately upon resection, tissue was stored in RNA*later* RNA Stabilization Reagent (Qiagen, Valencia, CA, USA) and subsequently snap frozen and maintained in liquid nitrogen. All tissue samples used in these analyses were evaluated by a neuropathologist; all specimens selected were characterized by dense tumor cellularity and the presence of >90% tumor tissue. The average patient age at time of resection was 56.1 (±13.1) years. There were six males and four females represented, and the mean survival was 8.7 (±3.0) months from time of diagnosis.

Normal brain tissue was obtained from representative tissue punches of gray matter from 10 appropriately age-matched hemicoronal brain tissue sections obtained from autopsy specimens provided by the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, Maryland. All tissue sections were maintained in liquid nitrogen. The average age at time of death was 58.0 (±7.6) years. There were seven males and three females represented, and the mean post-mortem interval for sample preservation was 17.2 (±5.4) h.

Human glioma cell culture

The following cell lines were used for qRT-PCR analysis: human glioblastomas, SNB19 and D54MG (generously provided by Dr P. Kornblith, University of Pittsburgh, Pittsburgh, PA and Dr D. Bigner, Duke University, Durham, NC, USA respectively), U87MG, U373MG, U118MG, U251, and A172 (obtained from ATCC, Rockville, MD, USA). All established human brain tumor cell lines were maintained using Dulbecco's modified Eagle's medium (containing 4.5 g/L glucose) supplemented with 10% heat-inactivated fetal bovine serum (Whittaker BioProducts, Walkersville, MD, USA).

Microarray fabrication, validation, and quality control

The 359 genes comprising our focused Human Glycobiology microarray are compiled from currently available NCBI/EMBL/TIGR human sequence databases and the Consortium for Functional Glycomics-CAZy databases (available at http://www.cazy.org/CAZY/) and represented all of the cloned human glycosyltransferases, glycosylhydrolases, polysaccharide lyases, and carbohydrate esterases. Only those genes with fully curated Reference Sequence (RefSeq) ID's were used. Individual 45-mer oligonucleotides complementary to sequences within these human mRNAs were designed and prioritized using stringent selection criteria, including minimal secondary structure, minimal homology to other genes in the available human genomic databases, no low complexity or repeat regions, and all with a similar, yet well-defined T_m [ArrayDesigner v2.03, Premier Biosoft, Palo Alto, CA, USA] to provide optimal hybridization efficiency across all oligos on the array. Control oligonucleotides representing the most traditionally accepted and commonly utilized housekeeping genes (Lee *et al.* 2002) were similarly designed and prioritized.

These optimal oligonucleotides were individually synthesized with the addition of a 5′-amino linker (C6-TFA, Glen Research, Sterling, VA, USA) onto each oligonucleotide, as described (Kroes *et al.* 2006). The oligonucleotides were then robotically arrayed, covalently linked in quadruplicate to aldehyde-coated glass microscope slides, and quality controlled prior to use.

The dynamic range, discrimination power, accuracy, reproducibility, and specificity of the focused oligonucleotide microarrays used in these studies were evaluated by exogenous mRNA spiking experiments, exactly as detailed in our previous study (Kroes *et al.* 2006). The dynamic range, defined as the range of transcript abundance over which hybridization intensity was linearly correlated in six independent experiments and was found to be between two and three orders of magnitude. The data presented in this report fell within this dynamic range. We also used discrimination power, or the ability to discriminate authentic signal from background at the low end of the dynamic range, to set appropriate cutoffs prior to statistical analysis of the data. The reproducibility of both the raw, preprocessed data and appropriately normalized data was determined by comparison of the coefficients of variation across all levels of expression for each exogenous transcript, and was typically CV = 0.09. The accuracy of the microarray results was determined by direct comparison to individual mRNA abundance determined by qRT-PCR analysis of the spiked mRNA samples. Conformity between the two datasets (i.e., qRT-PCR and the spiked microarray samples) was measured, with a Pearson correlation coefficient of +0.96, which is in good agreement with reported values (Baum *et al.* 2003). Hybridization specificity was evaluated using a range of 1–6 sequence mismatches synthesized within the gene-specific 45-mer oligonucleotide immobilized on the array. The oligonucleotides used on the microarrays were gene specific since adverse effects on hybridization efficiency were not found with less than three mismatches.

Target preparation; RNA extraction and labeling, and microarray hybridization

Total RNA was extracted from tissues with guanidine isothiocyanate and CsCl-ultracentrifugation, purified (Qiagen) and used as the substrate for RNA amplification and labeling, exactly as described (Kroes *et al.* 2006). We utilized universal human reference RNA (Stratagene, La Jolla, CA, USA) in our analyses and treated identical aliquots concurrently with the tissue samples. Equivalent amounts of Cy5-labeled (experimental) and purified Cy3-labeled (reference) amplified RNA (aRNA) targets (each labeled to 15–18% incorporation) were combined, denatured and hybridized at 46°C for 16 h. Following sequential high-stringency washes, individual Cy3 and Cy5 fluorescence hybridization to each spot on the microarray was quantitated by a high resolution confocal laser scanner.

Data acquisition and statistical analysis

Arrays were scanned (at 633 and 543 nm) at 5 μm resolution on the ScanArray 4000XL (Packard Biochip Technologies, Billerica, MA, USA) utilizing QuantArray software [v3.0] at the maximal laser power that produced no saturated spots. The adaptive threshold method was used to differentiate the spot from the background and spot intensity determined using median pixel intensity. Prior to normalization, quality confidence measurements (spot diameter, spot area, array footprint, spot circularity, signal: noise ratio, spot uniformity, background uniformity, and replicate uniformity) were calculated for each scanned array and spots were flagged that did not pass stringent selection criteria. The data from each channel were normalized using the LOWESS curve-fitting equation on a print-tip specific basis (GeneTraffic v2.8, Iobion Informatics, La Jolla, CA, USA). Statistical analyses were performed using the permutation-based SAM (significance analysis of microarrays) algorithm (v2.20, Stanford University, see Tusher *et al.* 2001), that reports the median false discovery rate (FDR) as the percentage of genes in the identified gene list (rather than in the entire cohort of genes present on the microarray) that are falsely reported as showing statistically significant differential expression. In our analyses, appropriately normalized data were analyzed utilizing the two class, unpaired analysis utilizing a minimum of 1000 permutations and was performed comparing expression data derived from glioblastomas (GBMs) vs. normal brain. The cutoff for significance in these experiments was set at a 0% FDR at a specified 1.5-fold change.

Quantitative real-time PCR analysis

The expression levels of selected genes were analyzed by real-time PCR using Brilliant SYBR Green qRT-PCR Master Mix (Stratagene) on an Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA). Reverse transcription of 1 μg of DNAsed, total RNA was primed with oligo(dT) and random hexamers and was performed exactly as described (Kroes *et al.* 2006). All primer sets were designed across intron:exon boundaries to derive ~100 bp amplicons (Table 1), with individual primer concentrations and final amplification conditions optimized for each gene. Dissociation curves were performed on all reactions to assure product purity. Original input RNA amounts were calculated by comparison to standard curves using purified PCR product as a template for the mRNAs of interest and were normalized to amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Experiments were performed in triplicate for each data point.

Table 1 qRT-PCR primers used in the study

Gene ID	Accession#	Primers (forward, reverse)	Amplicon size (bp)
MAN2A2	NM_006122	5′-TCAAGGACAACAAGAGAACC-3′, 5′-GATGGGTAGCTGGTAGAGTG-3′	100
POFUT1	NM_015352	5′-AACAGCTCTTCAAAGGGAAG-3′, 5′-ACAGTTGCCAATAAAGTGGT-3′	104
ST6GalNAcV	NM_030965	5′-TGGACGGATACCTCGGAGTG-3′, 5′-TCTGTCTGGTCAATCTGGGAG-3′	123
CHI3L1	NM_001276	5′-TGATGTGACGCTCTACGGC-3′, 5′-AATGGCGGTACTGACTTGATG-3′	161
GAPDH	NM_002046	5′-ATGGGGAAGGTGAAGGTCG-3′, 5′-GGGGTCATTGATGGCAACAATA-3′	108

Results

Focused microarray analysis identified aberrant glyco-gene expression in GBMs

We analyzed the expression of 359 glyco-related genes using our in-house fabricated focused oligonucleotide microarray platform in newly diagnosed GBMs and in normal tissue samples obtained from cortical sections from autopsy specimens. GBM (n = 6) and control (n = 6) RNA samples were studied in triplicate with three microarray slides for each sample. As each oligonucleotide is spotted in quadruplicate on the array, there are a total of 72 expression measurements for each gene in each group. To circumvent the inherent biological heterogeneity of clinical GBM specimens (Bigner *et al.* 1998), we employ a universal reference design (Churchill 2002) and comprehensive statistical analysis platforms to facilitate acquisition of expression profiles from a necessarily large number of biological and technical replicates. The tumor samples expressed between 77.3% and 86.7% of the genes present on the microarray. We used SAM analysis to determine statistically significant differences in the measured expression levels for each gene on the array and demonstrated that a number of genes can be identified with high confidence as differentially expressed between the two cell types (Fig. 1).

Table 2 shows the identities, functional annotations, and relative expression ratios of these differentially expressed genes. Of the 359 genes, 34 glyco-genes genes differed in their expression between GBM and normal brain by at least 1.5-fold (at 0% FDR). Importantly, at this stringent FDR, none of these changes was expected to be a false-positive. Of the 34 genes, 10 had increased and 24 had decreased measured expression levels in the grade IV tumors relative to normal brain. These differentially expressed transcripts fell into well-defined pathways for glycan biosynthesis and metabolism (Kanehisa *et al.* 2004; Table 2).

The expression pattern of four genes identified by stringent SAM analysis of the microarray data were further studied in a larger panel of tumors by quantitative real-time RT-PCR; α-mannosidase 2A2 (MAN2A2), protein *O*-fucosyltransferase 1 (POFUT1), ST6 (α-*N*-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-*N*-acetylgalactosaminide α2,6-sialyltransferase 5 (ST6GalNAcV), and chitinase 3-like 1 (CHI3L1). These genes represent key genes in *N*-glycan biosynthesis, *O*-glycan biosynthesis, ganglioside biosynthesis, and another glyco-related gene recently identified in high grade glioma.

qRT-PCR analysis corroborated the microarray results

We chose three genes that have not been previously associated with GBM, as well as one gene very recently described as up-regulated in gliomas and measured mRNA levels by real-time quantitative RT-PCR. Our core approach to transcriptome profiling has been to use the focused microarrays as a screening tool to identify statistically significantly differentially expressed genes followed by corroboration of a subset of these in larger sample panels using higher throughput methodology, including quantitative qRT-PCR. As such, we expanded the number of samples used for the qRT-PCR corroboration to analyze total RNA from 10 GBM samples, 10 age-matched normal brain controls, and seven glioma cell lines and analyzed the expression of MAN2A2, ST6GalNAcV, POFUT1, and CHI3 L1 relative to the level of GAPDH mRNA in each sample. GAPDH was chosen for a reference because its mRNA levels, measured by microarray analysis, were comparable between GBM and normal brain. Consistent with the microarray data, we measured significant down-regulation of MAN2A2 and ST6GalNAcV mRNAs and significant up-regulation of POFUT1 and CHI3L1 in the

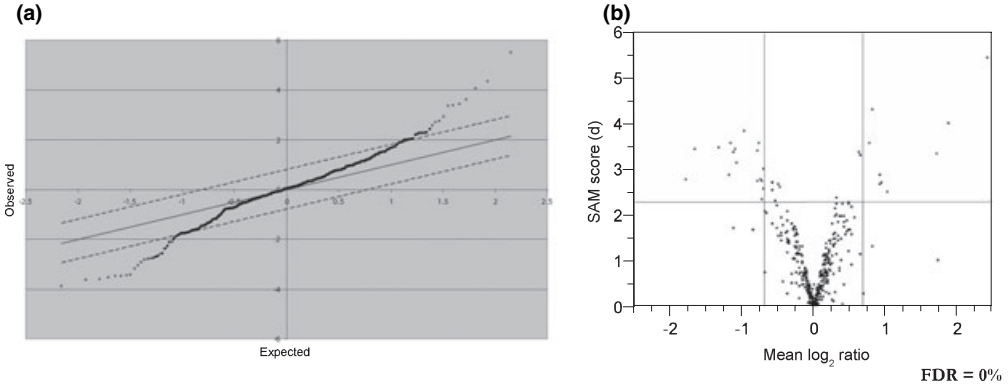

Fig. 1 Statistical Analysis of the Microarray Data. (a) SAM analysis of normalized expression data from six selected GBMs (red) *versus* six age-matched normal brain specimens (green). (b) Transformed data derived from SAM analysis at a false discovery rate (FDR) = 0%. The fold-change represented as a $\log_2$ ratio is plotted against the SAM (d) score, indicative of the significance of change. Thus, the largest, most significant changes lie in the upper left- and upper right-most quadrants of the plot.

Table 2 GBM-associated transcripts identified by microarray analysis (@ 0.0% FDR)

Genbank accession	Fold change[a]	Gene ID
N-glycan biosynthesis		
NM_003032	1.91	Sialyltransferase 1 (β-galactoside α2,6-sialyltransferase) (SIAT1), transcript variant 2
NM_006122	−2.95	α-Mannosidase, class 2A, member 2 (MAN2A2)
NM_019109	−1.74	β-1,4 Mannosyltransferase (HMT-1)
NM_002372	−1.67	α-Mannosidase, class 2A, member 1 (MAN2A1)
O-glycan biosynthesis		
NM_015352	1.69	*O*-Fucosyltransferase 1 (POFUT1), transcript variant 1
NM_003605	−2.63	O-linked *N*-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-*N*-acetylglucosaminyltransferase)
(OGT), transcript variant 3		
AB032956	−1.65	UDP-*N*-acetyl-α-d-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase-like 1 (KIAA1130 protein)
Chondroitin/heparan sulfate biosynthesis		
NM_018644	−3.41	β1,3-Glucuronyltransferase 1 (glucuronosyltransferase P) (B3GAT1), transcript variant 1
NM_054025	−2.43	β1,3-Glucuronyltransferase 1 (glucuronosyltransferase P) (B3GAT1), transcript variant 2
NM_004455	−1.59	Exostoses (multiple)-like 1 (EXTL1)
Blood group glycolipid biosynthesis-neolactoseries		
NM_006876	−2.87	UDP-GlcNAc:β-Gal β1,3-*N*-acetylglucosaminyltransferase 6 (B3GNT6)
Blood group glycolipid biosynthesis-lactoseries		
NM_000149	1.51	Fucosyltransferase 3 (galactoside 3(4)-L-fucosyltransferase, Lewis blood group included) (FUT3)
Ganglioside biosynthesis		
NM_032528	1.85	β-galactoside a2,6-sialyltransferase II (ST6GalII)
NM_013443	−1.79	ST6 (α-*N*-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-*N*-acetylgalactosaminide α2,6-sialyltransferase 6 (ST6GALNAC6)
NM_030965	−1.62	ST6 (α-*N*-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-*N*-acetylgalactosaminide α2,6-sialyltransferase 5 (ST6GALNAC5)
Glycosphingolipid metabolism		
NM_003360	−1.65	UDP glycosyltransferase 8 (UDP-galactose ceramide galactosyltransferase) (UGT8)
NM_000153	−1.58	Galactosylceramidase (Krabbe disease) (GALC)
Globoside metabolism		
NM_033167	1.51	UDP-Gal:β-GlcNAc β1,3-galactosyltransferase, Polypeptide 3 (B3GALT3), transcript variant 2
Glycosaminoglycan degradation		
NM_153286	1.67	Hyaluronoglucosaminidase 1 (HYAL1), transcript variant 6
NM_012215	−1.89	Meningioma expressed antigen 5 (hyaluronidase) (MGEA5)
NM_033158	−1.60	Hyaluronoglucosaminidase 2 (HYAL2), transcript variant 2
Lipopolysaccharide biosynthesis		
NM_152312	−4.27	Glycosyltransferase-like 1B, LARGE2 (GYLTL1B)
NM_004737	−1.50	Glycosyltransferase-like, LARGE (MDC1D), transcript variant 1
Other		
Glyco-related		
NM_005228	2.02	Epidermal growth factor receptor (EGFR)
NM_002103	1.71	Glycogen synthase 1 (GYS1)
NM_001276	1.66	Chitinase 3-like 1 (cartilage glycoprotein-39, YKL-40) (CHI3 L1)
NM_031302	1.56	Glycosyltransferase 8 domain containing 2 (GLT8D2)
NM_173089	−1.85	Calpain 3, (p94) (CAPN3), transcript variant 5
NM_024344	−1.83	Calpain 3, (p94) (CAPN3), transcript variant 2
NM_173090	−1.81	Calpain 3, (p94) (CAPN3), transcript variant 6
NM_033309	−1.72	β1,3-galactosyltransferase-related protein
NM_173088	−1.69	Calpain 3, (p94) (CAPN3), transcript variant 4
NM_000188	−1.56	Hexokinase 1 (HK1), transcript variant 1

Table 2 Continue

Genbank accession	Fold change[a]	Gene ID
NM_020742	−1.55	Neuroligin 4 (NLGN4), transcript variant 1
Control/housekeeping		
NM_003380	4.11	Vimentin (VIM)
NM_005252	3.22	v-fos (FOS)
NM_000239	2.21	Lysozyme (renal amyloidosis) (LYZ)
NM_005594	2.07	Nascent-polypeptide-associated complex, α-polypeptide (NACA)
NM_000996	1.92	Ribosomal protein L35a
NM_001101	1.70	β-Actin (ACTB)
NM_002425	1.53	Matrix metalloproteinase 10 (stromelysin 2) (MMP10)
NM_005345	−2.69	Heat shock 70 kDa protein 1A (HSPA1A)
NM_001069	−2.43	Tubulin, β2A (TUBB2A)
NM_005438	−2.18	FOS-like antigen 1 (FOSL1)
NM_021130	−1.95	Peptidylprolyl isomerase A (cyclophilin A) (PPIA)

[a]The fold change was calculated between mean values of GBM (n = 6) and age-matched normal brain (n = 6) tissue. Positive values are indicative of an increase, and a negative a decrease, in gene expression in GBM relative to normal brain.

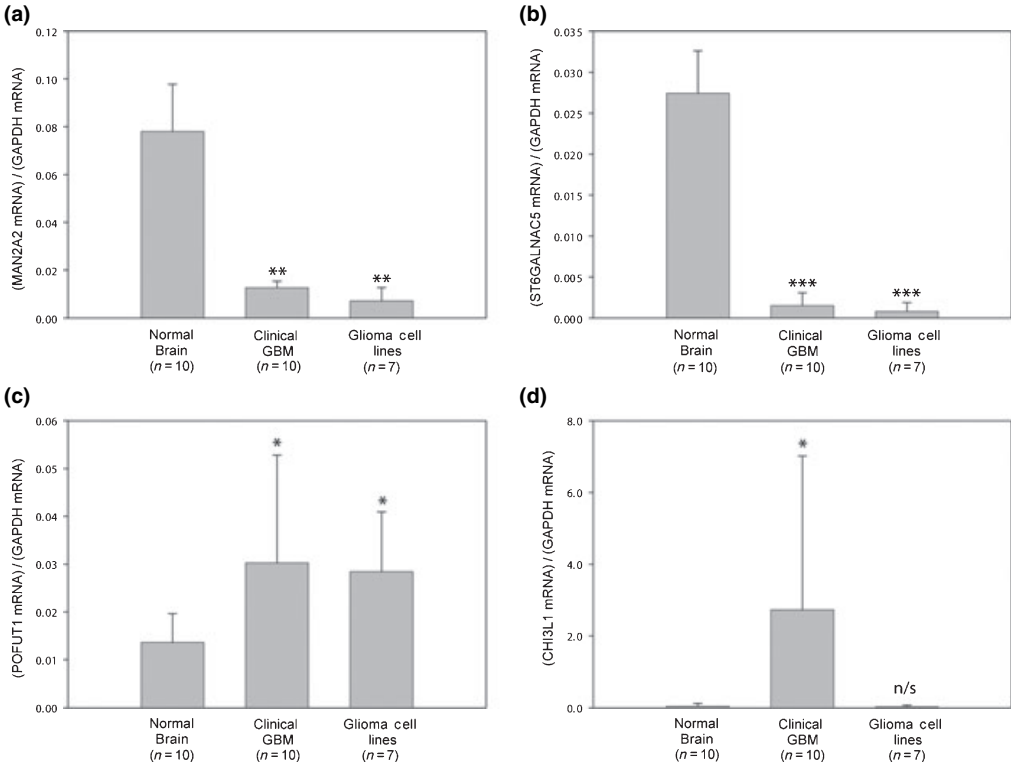

Fig. 2 qRT-PCR corroboration of selected glyco-targets identified by microarray analysis. For each mRNA, transcript abundance, normalized to GAPDH, was calculated by qRT-PCR, as described in Materials and methods. Data are presented for MAN2A2 (panel a), ST6GalNAcV (panel b), POFUT1 (panel c), and CHI3L1 (panel d) and represent mean (±SD). Significant differences between GBM and normal brain were observed for all genes (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed, unpaired student's t-test). n/s, not significant ($p > 0.05$).

GBMs relative to normal brain using qRT-PCR (Fig. 2). The glioma cell lines demonstrated comparable levels of MAN2A2, ST6GalNAcV, and POFUT1 to those observed in the primary tumor specimens.

Discussion

Glioblastomas (GBMs) are among the most devastating of all primary brain tumors. It is nearly uniformly fatal, with

median survival between 9 and 12 months from initial diagnosis (CBTRUS 2002-2003). Their highly invasive phenotype makes complete surgical resection virtually unachievable. The cornerstone of treatment for GBMs still includes surgery, radiation therapy (RT), and chemotherapy, but these interventions are still largely ineffective, as the majority of patients experience progression or recurrence (ABTA, American Brain Tumor Association 2004).

Cell-surface glycoconjugates play an important role in cellular differentiation and tumorigenesis: in particular, altered integrin glycosylation and the expression of specific glycosphingolipids have been shown in malignant brain tumors both *in vitro* and *in vivo*. Steady-state expression of the oligosaccharides associated with specific glioma glycoconjugates results from the orchestrated expression of all of the glycosyltransferases involved in its biosynthesis and the glycosidases involved in its degradation (reviewed in Varki 1999). Thus, in the context of processes as complex as gliomagenesis and invasivity, determining the global pattern of these changes seems as important as determining the individual, aberrantly expressed glyco-related gene changes. The precise biosynthetic and metabolic pathways of the oligosaccharides that decorate glycoproteins, glycosaminoglycans and glycosphingolipids have been characterized-essentially all of the enzymes involved in these processes have been cloned and sequenced.

We utilized custom-fabricated, focused oligonucleotide microarrays representing all of the known human glycosyltransferase and glyosylhydrolase genes. This provides a comprehensive platform of glyco-associated oligonucleotides, the flexibility to add new sequences to the arrays as they become available, and assures the most up-to-date coverage of these gene families. At the same time, we were developing our oligo-based microarray platform, the Consortium for Functional Glycomics also developed microarray-based resources for the analysis of glycoconjugate expression. It is an Affymetrix-based system available for use by investigators within the Consortium, and has recently begun to address some fundamental biological questions (Comelli *et al.* 2006). In order to begin to grasp the intrinsically complex regulation of genes responsible for the synthesis and degradation of specific carbohydrate structures essential for mediating glioma invasivity, we used our focused arrays to screen a panel of primary human brain tumors compared to normal human brain. To our knowledge, this technology has not yet been systematically applied to the global analysis of glyco-related gene expression in brain tumors, although small pilot studies have been recently reported in colon (Kemmner *et al.* 2003) and pancreatic tumors (Ide *et al.* 2006).

Microarray-based transcriptome profiling approaches *have* been very useful in elucidating many of the molecular underpinnings of glioma biology; from the identification of glioma-specific gene expression differences (Nutt *et al.* 2003; Tso *et al.* 2006), differences related to the invasive phenotype (Hoelzinger *et al.* 2005), to molecular classification of distinct GBM subtypes (Liang *et al.* 2005), identifying regiospecific expression differences within heterogeneous GBM tissue (Van Meter *et al.* 2006), to defining expression signatures predictive of patient survival (Rich *et al.* 2005). However, there is virtually no data regarding differential expression of genes involved in glycoconjugate biosynthesis and degradation generated in these studies. Until very recently (2006–2007, see http://www.affymetrix.com/products/arrays/specific/hgu133plus.affx), glyco-genes have been drastically under-represented on commercial arrays, including those used in the studies referenced above. Thus, despite the volume of data generated in these published reports, the fact that no glyco-genes have been identified in these analyses is not unexpected.

The differentially expressed glyco-genes identified in this study were distributed among several glycan biosynthetic and metabolic pathways (Table 1). Although the relationship to glioma-specific glycan structures may be inferred from these data, the identification of individual genes does provide clear molecular targets for the development of therapeutic strategies based on the modulation of their expression. For example, alpha mannosidase, class 2A, member 2 (MAN2A2) catalyzes the first committed step in the biosynthesis of complex *N*-glycans. The expression of complex *N*-glycan structures has been shown to be required for the expression of the metastatic phenotype (Dennis and Laferte 1987). Additionally, inhibition of the formation of complex *N*-glycan structures by swainsonine (a specific MAN2 inhibitor) reduces metastases. Swainsonine has gone to clinical trials in patients with advanced malignancies (Goss *et al.* 1997). However, based on our data demonstrating robust down-regulation of both α-mannosidase-2A2 and -2A1, the inhibition of mannosidase(s) by swainsonine would not appear to be an effective therapeutic strategy for the treatment of glioblastomas. In actuality, it would be molecular strategies targeting the over-expression of these enzymes that would be of clear advantage.

Of additional interest were the comparatively low levels of ganglioside-selective α2,6-sialyltransferase gene, ST6GalNAcV (GD1α synthetase), in all glioma and glioma cell lines relative to normal brain. This result extends our earlier studies showing the effective absence of the *glycoprotein-specific*, N-linked, α2,6-sialytransferase (α2,6ST) in all malignant brain tumors and metastases to the brain, direct modulation of its expression, and inhibition of tumor growth and invasivity in both *in vitro* and *in vivo* models. Thus, ST6GalNAcV may also have therapeutic potential for the treatment of malignant brain tumors. Interestingly, our microarray studies demonstrated that both ST6GalNAcV *and* ST6GalNAcV1 expression was significantly reduced in all of the human glioblastoma samples studied. Previous

studies have shown both to be involved primarily in the synthesis of GD1a-α series gangliosides using sialylLc4 as substrate (Okajima *et al.* 1999; Tsuchida *et al.* 2003) as well as GM1b, GD1a, and GT1b. In addition, our observed increased in fucosyltransferase 3 (FUT3) expression in gliomas, also found to be elevated in colon cancers (Tsuchida *et al.* 2003), may also interact with ST6GalNAcV and V1. FUT3 uses the same sialylLc4 substrate to generate disialyl-Lewis-a, a tumor-associated antigen (Miyazaki *et al.* 2004). In these studies, the authors demonstrate that ST6GalNAcV and V1 actually compete with FUT3 and thus our findings could be explained as increased disialyl-Lewis as being involved in the invasive phenotype in gliomas. Similarly, based on our findings, over-expression of GalNAcV (or VI) in gliomas may also be an efficacious therapeutic strategy in GBMs.

We also observed significant up-regulation of protein O-fucosyltransferase-1 (POFUT1), an enzyme that produces an *O*-fucose modification on epidermal growth factor-like repeats of a number of cell surface and secreted proteins. *O*-fucose glycans play important roles in ligand-induced receptor signaling. Work from several laboratories has established that POFUT1 is essential for Notch function (see Shi and Stanley 2003). The Notch family of receptors control cell fate during development and function as tumor suppressors in epithelial tumors (Nicolas *et al.* 2003). Several studies have demonstrated that lack of *O*-fucose alters the interaction of Notch with its ligands (Okajima *et al.* 2003; Sasamura *et al.* 2003). Notch-1 is present, albeit in small quantities, in both glioma cell lines and primary human gliomas (Purow *et al.* 2005); with markedly lower expression in the higher grade gliomas. The reason for lower expression of Notch-1 in most glioblastomas relative to other primary human gliomas is unclear. However, it has recently been shown that invasive cervical carcinomas also down-regulate Notch-1 expression relative to lower-grade, less invasive cervical tumors (Talora *et al.* 2002). Interestingly, *O*-fucosylation sites are also found on metalloproteases (uPA, tPA) which can also play key roles in brain tumor invasion and metastases (Harris *et al.* 1991; Rabbani *et al.*, 1992). Taken together, these observations raise the possibility that down-regulation of Notch-1 by altered fucosylation is important for progression to the more aggressive, highly invasive phenotype in glioblastomas.

CHI3L1 (YKL-40), a secreted glycoprotein with a high degree of homology with glycosylhydrolases, was also over-expressed in the clinical gliomas. Its function is still poorly understood. It is over-expressed and implicated as a serum marker in aggressive breast (Johansen *et al.* 2003; Jensen *et al.* 2003), ovarian (Hogdall *et al.* 2003; Dehn *et al.* 2003), colorectal (Cintin *et al.* 1999), small cell lung, and melanoma. Markedly elevated levels of CHI3L1 were associated with worse prognosis in all of these studies (Johansen *et al.* 1995; Cintin *et al.* 1999; Dehn *et al.* 2003). It appears to be involved in tumor cell proliferation, survival, and invasiveness via remodeling of extracellular matrix. Furthermore, there is also evidence that CHI3L1 levels correlate with grade and potentially tumor burden in gliomas (Tanwar *et al.* 2002). Expression of the CHI3L1 protein has also been associated with a survival advantage of GBM cells in response to hypoxia, ionizing radiation, and p53 inhibition (Junker *et al.* 2005; Pelloski *et al.* 2005). Glioblastomas were found to have 3- to 62-fold elevation of CHI3L1 levels over normal brain (Tanwar *et al.* 2002), consistent with the highly variable over-expression that we observed in the clinical tumors used in this study. One recent study hypothesized that elevated levels of CHI3L1 in glioblastomas might be because of the presence of increased numbers of macrophages (Shostak *et al.* 2003), as the expression of CHI3L1 in macrophages has been well documented (reviewed in Rehli *et al.* 1997 and Renkema *et al.* 1998). Our observation that none of the glioma cell lines express CHI3L1 is consistent with these observations. Alternatively, the lack of CHI3L1 expression *in vitro* may simply be the result of the absence of extracellular matrix-associated signaling. Thus, it will be of great interest as to whether these cell lines grown as intracranial xenografts will differentially activate CHI3L1 expression.

The 10 primary tumors used in this study were representative of an exceptionally well-defined clinical cohort. We only selected tumors containing >90% (typically >95% by histopathological analysis) tumor with minimal to no necrotic or normal tissue contamination. Tumor tissue was frozen immediately upon resection, and normal tissue was collected with a minimal post-mortem interval to optimize RNA integrity. The intertumoral variability of the individual gene expression levels we experimentally quantitated in the corroborative qRT-PCR dataset reinforced our use of these 10 well-defined tumors to prioritize those most useful as potential clinical markers or therapeutic targets. While there was minimal intertumoral variability in the expression of ST6GalNAcV or MAN2A2, significant overlap in POFUT1 and CHI3L1 expression in individual tumors was clearly observed. Nevertheless, similar levels of heterogeneity in the expression of other well-characterized tumor markers have been described. Most germane are several recent studies of CHI3L1 expression in which marked individual heterogeneity exists in significantly larger clinical datasets, and differential CHI3L1 expression has still been demonstrated to be a useful marker of tumor grade, response to therapy, and overall survival in patients with high grade gliomas (Nutt *et al.* 2005; Pelloski *et al.* 2005; Hormigo *et al.* 2006).

The limitations of comparison of primary glioblastoma specimens to normal brain tissue in our analyses deserve elaboration. The diversity of cell types comprising normal brain, despite our exclusive use of gray matter, may not be totally representative of the cells from which gliomas are derived. Although there is no ''perfect'' control, normal brain

tissue consists of >90% glia (Terry *et al.* 1987) with normal cell-surface glycoconjugate profiles. The use of alternative controls, such as cultured normal human astrocytes or glioma stem cells suffer from significant limitations. Specifically (i) comparison of clinically resected tumor tissue to cultured, adherent astrocytes raises obvious concerns as to culture- or adhesion-related artifacts in cell-surface glycoconjugates, and (ii) whether the currently identified glioma stem cells are the actual cell-of-origin of glioblastomas is still an open question (Fan *et al.* 2007). In addition, differences in the relative amounts of actively dividing cells may potentially skew the gene expression profiles towards regulators of proliferation. However, the identification of several genes previously implicated in gliomagenesis (e.g., CHI3L1, EGFR, RPL35a, MMP-10) does provide a level of reliability to this approach. The significance of the individual genes identified here to gliomagenesis and invasivity will require detailed functional evaluation in gene targeting paradigms including RNA interference or gene delivery by gene transfection. The cell lines evaluated in the studies described in this report will clearly be useful in this functional evaluation.

In sum, focused microarray analyses comparing six Grade IV gliomas with six aged-matched normal brain specimens yielded 10 glyco-genes more highly expressed in gliomas compared to normal brain and 24 glyco-genes more highly expressed in normal brain compared to gliomas. Of these, four were strategically chosen and evaluated by qRT-PCR in an expanded panel of tissues and cell lines: each was found to corroborate the microarray data. These results demonstrate that our high quality, application-specific, low density microarray platform provides an efficient strategy that can pinpoint gene expression changes and provide a robust database for pursuing the creation of novel therapeutics. Moreover, this approach underscores the large qualitative and quantitative differences in the expression of glycoconjugate metabolic machinery in gliomas compared to normal brain.

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