

Logical networks inferred from highly specific discovery of transcriptionally regulated genes predict protein states in cultured gliomas

Hassan M. Fathallah-Shaykh *

Department of Neurological Sciences, Section of Neuro-Oncology, Rush University Medical Center, 1725 West Harrison Street, Chicago, IL 60612, USA

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Abstract

Cultured glioma cells are motile and invasive. The phenotype of tumor cell motility is likely created by a complex system of molecular interactions because it requires the orchestration of molecular and physical events that modify the cytoskeleton, cell membrane, extracellular matrix, and signaling. Recent reports have described an algorithm for microarray data analysis that generates highly specific genome-scale discovery; these methods identify states of differential gene expression that are true to a high degree of certainty. Here, highly specific discovery of transcriptionally regulated genes combined with logical networks inferred from the functions of known genes predicts states of protein activation, which are validated in cultured glioma cells by independent laboratories. Highly specific discovery of transcriptionally regulated genes facilitates functional genomics of complex molecular systems.

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The phenotype of motility is not only critically relevant to the understanding and therapeutics of cancer but is also important in several pathological processes including vascular disease, osteoporosis, rheumatoid arthritis, and mental retardation. Tumor cell migration and invasion involves highly coordinated steps of dissociation of existing cellular adhesions, remodeling the actin cytoskeleton to project lamellipodium extensions, formation of new adhesions, and tail detachment along with proteolytic processing and secretion of extracellular matrix proteins along the trajectory [1]. Malignant gliomas are characterized by diffuse invasion of distant brain tissue; in addition, clinical and experimental data demonstrate that the phenotype of motility is generated by a complex combination of multiple molecular processes [2].

Cultured glioma cells retain the phenotype of invasiveness/motility [3–5]. Here, highly specific genome-scale dis-

covery is applied to analyze the expression datasets of 19,200 cDNAs in cultured glioma cells as compared to normal brain RNA, which appears to best represent genetic expression in normal adult glial cells. Embryonal human glial cultures are not readily available and genetic expression differs between embryonal and mature cells. Furthermore, the discovered genes are expected to be relevant to motility because unlike glioma cells, the various cell types of normal adult brain do not exhibit the motility phenotype.

The molecular mechanisms that create the phenotype of motility in cultured glioma cells may not be extrapolated to glioma cells *in vivo*; specifically, Camphausen et al. [6] study the expression profiles of 7680 human cDNA clones, gene expression profiles of human glioma cell lines. Using clustering algorithms, the authors find different molecular signatures in cells grown *in vitro* as compared to subcutaneous or as intracerebral xenografts. Similarly, Tatenhorst et al. [7] used oligonucleotide microarrays to study the expression patterns of 8832 genes in rat C6 glioblastoma

* Fax: +1 312 563 3562.

E-mail address: hfathall@rush.edu.

cultured in a monolayer as well as in brains of nude mice. Hoelzinger et al. [8] studied genetic expression patterns in glioblastoma cells laser captured from surgical samples.

Microarrays are very useful tools for studying and comparing gene expression; however, their data are very noisy and their molecular signatures may not be reproducible [9–11]. Recent reports describe a mathematical algorithm (MASH) that yields highly specific states of genetic expression from the direct genomic comparison of two samples by microarrays [12,13]. Because MASH significantly reduces false positive measurements, it discovers the transcriptionally regulated elements of molecular networks. Here, I study the idea that discovery of the transcriptionally regulated elements uncovers the molecular networks of biological systems and predicts protein states.

The novelty of this paper is in the use of genome-scale expression discovery (19,200 genes) and the new algorithm for microarray data analysis. Specifically, MASH models and filters technical noise inherent to each dataset [11,14]; its false discovery rate for the 19K microarray is only 1 per 192,000 measurements. The strategy of highly specific discovery differs from other methods for microarray data analysis in that: (1) it yields a significantly higher specificity and thus virtual certainty that the discovered genes are truly differentially expressed between samples, and (2) does not apply clustering methods to identify expression patterns or molecular signatures. Several groups have applied microarrays to discover patterns of genetic expression that correlate with clinical behavior of cancer [12,15–21]. Nevertheless, recent papers have reported poor reproducibility, variable prognostic performance, and bias in molecular signatures/patterns [22,10,23]. Here, the biological chemistry of the genes discovered by highly specific discovery uncovers several molecular pathways that have already been independently validated by other laboratories.

Materials and methods

Glioma cell lines. The experiment profile RNA isolated from six glioma cell lines as compared to normal brain RNA. Two glioma cell lines were purchased from ATCC (T98G and U373MG). The others were cultured from a glioblastoma, an oligodendroglioma, and two astrocytoma tumor samples (generous gift from Herbert Engelhard, University of Illinois, Chicago).

Microarrays. Normal brain RNA is obtained by pooling RNA from human occipital lobes harvested and pooled from four individuals with no known neurological disease whose brains are frozen less than 3 h post-mortem. Tumor RNA samples are extracted from six cultured glioma cell lines. The quality of RNA is assayed by gel electrophoresis, and only high quality RNA is processed. Tumor RNA is profiled as compared to aliquots from the same normal brain RNA. Microarray experiments use 19K microarrays (Ontario Cancer Institute, Ontario, Canada); the design includes probe switching (dye swapping) as described elsewhere [12,24]. Each 19K microarray contains 19,200 cDNAs spotted in duplicate. The experiments generate four replicate measurements per gene and tumor.

Data analysis. The algorithm for highly specific genome scale expression discovery (MASH) is applied to analyze the datasets [13]. The algorithm discovers states of genetic expression, up- or downregulation; its false discovery rate for the 19K microarrays in *same-to-same* comparisons or the probability that MASH discovers a false state of genetic expression

is only 1/192,000 measurements. The algorithm applies filters based on the slopes of the curve fits of the microarray data to a mathematical equation. The curve fits for the six cell lines are statistically significant ($R^2 > 0.99$). The following steps are followed in order [24]: (1) apply MASH to discover the genes differentially expressed in each cell line as compared to normal brain, (2) find the set of genes, *S*, that are discovered by the algorithm in at least one of the six-cell lines, and (3) identify the four 'raw' replicate ratios of each of the genes of *S* in each cell line. We then apply a filter consisting of the following fuzzy logic rules in sequence [12,24]: (1) all four replicate log₂ (ratios) of a gene in any cell line are of the same sign and different than 0 (all 4 show either up- or down-regulation) [12]. (2) The mean of the 4 replicate ratios is either > 1.5 or < 0.67 [12]. (3) If both rules 1 and 2 are true, compute the mean of the replicate log₂ expression values; otherwise, exclude the genes by transforming the log₂ expression to 0 [12]. (4) Exclude genes that are not resistant to both rules 1 and 2 in at least 5/6 cell lines [24]. And (5) exclude genes that are simultaneously upregulated in a cell line and downregulated in another [24]. The specificity and sensitivity of the filter that requires all four replicate measurements to be consistent in showing up- or down-regulation are discussed elsewhere [12].

Results

The experiments are designed to yield four replicate spots (genes) per sample with dye swapping. The four replicate measurements are imported into the software; MASH outputs a list of genes and their states of genetic expression within minutes. The results discover 268 genes consistently up- or down-regulated in at least 5/6 cultured glioma cell lines [13,25].

Fig. 1 and Table 1 (also Supplementary material) show the states of expression of the discovered genes, which predict a complex molecular system that controls: (1) actin polymerization, (2) membrane chemistry, (3) integrin-induced remodeling of the cytoskeleton, (4) actin assembly/branching, (5) signaling pathways, (6) contractility, (7) adherens junctions and cell adhesion, and (8) remodeling of the extracellular matrix. The states of genetic expression also predict activation of protein states, which have already been validated in cultured gliomas by independent laboratories. A complete bibliography is presented in Supplementary information.

Discovery of a molecular system for motility

Actin polymerization

Profilin, thymosin- β 4, ENAH, and MINK are upregulated in glioma cells as compared to normal brain cells. Profilin catalyzes the conversion of ADP-actin to ATP-actin. Thymosin- β 4 maintains a pool of sequestered ATP-actin that is ready to elongate any available actin filament barbed end. ENA/VASP binds to actin filaments in vitro and promotes actin nucleation and polymerization by recruiting profilin-actin to sites of cytoskeletal remodeling. MINK inactivates ADF/cofilin by phosphorylation at serine 3.

Membrane chemistry

MARCKS, HuR, and Net-5 are upregulated in glioma cells. Overexpression of HuD causes a striking stabilization of the MARCKS mRNA. MARCKS may regulate actin

Table 1

The genes discovered to be upregulated in cultured glioma cells as compared to normal brain that are relevant to the phenotype of motility/invasiveness

Image ID	Short name	Name	
504350	PFN1	Profilin 1	U
273224	TMSB4X	Thymosin, β 4, X chromosome	U
162646	ENAH	Enabled homolog ws	U
470942	MINK	Missshapen/NIK-related kinase	U
491272	SDC2	Syndecan 2	U
471209	CD2BP2	CD2 antigen (cytoplasmic tail)-binding protein 2	U
502184	Nexilin	Likely ortholog of rat F-actin binding protein nexilin	U
182358	ITGA3	Integrin, α 3	U
251172	ITGB5	Integrin, β 5	U
487442	NET-5	Tetraspan transmembrane four super family	U
291409	MARCKS	Myristoylated alanine-rich protein kinase C substrate	U
284461	HuR	ELAV (embryonic lethal, abnormal vision, and <i>Drosophila</i>)-like 1	U
343295	Galectin-1	Lectin, galactoside-binding, soluble, 1	U
274389	IGFBP7	Insulin-like growth factor-binding protein 7	U
266838	IGFBP3	Insulin-like growth factor-binding protein 3	U
258674	IGFBP4	Insulin-like growth factor-binding protein 4	U
491711	CTGF	Connective tissue growth factor	U
262752	CD44	CD44 antigen	U
263768	PTPN2	Protein tyrosine phosphatase, non-receptor type 2	U
282440	ARF4	ADP-ribosylation factor-4	U
161359	CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	U
133070	CALM1	<i>Homo sapiens</i> calmodulin-I (CALM1) mRNA,	D
252238	RhoC	Ras homolog gene family, member C	U
272210	RhoE	Ras homolog gene family, member E	U
301788	MYLC2A	Myosin, light polypeptide 7, regulatory	U
161468	ACTA2	Actin, α 2, smooth muscle, aorta	U
358484	ACTC	Actin, α , cardiac muscle	U
266074	CALD1	Caldesmon 1	U
130269	PAI1	Plasminogen activator inhibitor type 1	U
259127	MMP2	Matrix metalloproteinase 2	U
151144	FN1	Fibronectin 1	U
342926	LAMA5	Laminin, α 5	U
253460	LAMB1	Laminin, β 1	U
264960	COL1A2	Collagen, type I, α 2	U
418262	COL3A1	Collagen, type III, α 1	U
246281	COL4A2	Collagen, type IV, α 2	U
491106	COL5A2	Collagen, type V, α 2	U
110331	COL6A1	Collagen, type VI, α 1	U
136032	COL6A3	Collagen, type VI, α 3	U
41676	COL11A1	Collagen, type XI, α 1	U
255836	COL16A1	Collagen, type XVI, α 1	U
489524	COL18A1	Collagen, type XVIII, α 1	U

U and D imply up and downregulation in glioma cells as compared to normal brain, respectively.

of calmodulin combined with upregulation of caldesmon promotes the dissolution of stress fibers.

Adherens junctions and cell adhesion

ARF4 and CDH11 are upregulated in glioma cells and calmodulin is downregulated. ARF6 and ARF4 are members of the ADP-ribosylation factor (ARF) family of Ras-related GTPases, which cycle between active GTP-bound and inactive GDP-bound states. Activated ARF6 dissolves focal adhesions by inducing the redistribution of E-cadherin away from the cell surface into transferrin receptor containing endosomes.

Coexpression of wild-type CDH11 and the splice variant promotes a dramatic increase in the invasiveness of breast cancer cells as assayed by in vitro assays. IQGAP1 may either promote or inhibit motility. In the

presence calmodulin and Cdc42-GDP, IQGAP1 inhibits the binding of actin to the cadherin, β -catenin, α -catenin, and vinculin complex. However, when Cdc42 is activated and in the absence of calmodulin, IQGAP1 promotes motility by stabilizing the adhesive complex.

Extracellular matrix

Glioma cells produce nine chains of collagen, fibronectin, laminin, MMP2, and PAI1. High expression levels of PAI-1 in human brain and breast tumors are associated with malignant progression, a short recurrence-free survival and overall survival, and enhanced motility. MMP2 encodes an enzyme that degrades type IV collagen and cleaves laminin 5 to promote tumor cell migration.

Validation of molecular pathways/protein states in gliomas by independent laboratories

Membrane chemistry

Recent reports find upregulation and basal phosphorylation of MARCKS in cultured glioma cells [26,27]. In addition, Glanville et al. [28,29] find 10–15-fold greater PIP2 phosphodiesterase activity in glioma cells as compared to neuronal cells and Watt et al., find that PIP2 is concentrated over the lamellipodia of cultured astrocytoma cells. Several laboratories find baseline activation of PKC activity in glioma cells and Cho et al. [3,30–32] report that antisense PKC alpha inhibits glioma cell motility in vitro.

Actin assembly/branching

Rutka et al., and Jones et al. [4,33] find that FAK phosphorylation mediates motility in cultured glioma.

Signaling pathways

Murai et al. [34] show that engagement of CD44 promotes Rac activation and migration of the U251MG human malignant astrocytoma cell line. Camby et al. [35] find that adding galectin-1 to the culture media of cultured U87 and U373 astrocytoma cell lines leads to marked increase in motility. Ras activation plays a key role both in glioma formation and in mediating its motility [36–38]. Ding et al. [38] report baseline src-kinase activity and FAK phosphorylation in cultured glioma. In addition, Ichigotani et al. [39] find baseline activation of cdc42, Rac, and Ras in U87MG astrocytoma cells and baseline phosphorylation of PAK2 in U87MG astrocytoma cells.

Extracellular matrix

Salonen et al. [40] find that human glioma cell line U-251 contains PAI-1 in a rapidly releasable form, and Arai et al. [41,42] find high expression of PAI-1 in human gliomas in vivo. Cordes et al., Le et al., and Lu et al. [43–45] report elevated MMP2 expression and activity in cultured glioma cells.

Discussion

Gene array studies are increasingly being used to explore biological causes and effects, and even to diagnose diseases. However, patterns of genetic expression and molecular signatures may not be reproducible [22,46]. Michiels et al. [22] reanalyzed data from the seven largest published studies that have attempted to predict prognosis of cancer patients on the basis of DNA microarray analysis. The results reveal that the list of genes identified as predictors of prognosis was highly unstable. Ntzani and Ioannidis [10] examined 84 large-scale microarray expression datasets that address major clinical outcomes including death, metastasis, recurrence, and response to therapy. They find that these studies show variable prognostic performance.

Furthermore, Tan et al. [11] find little overlap and poor reproducibility between the states of genetic expression dis-

covered by three different microarray platforms. Kothapalli et al. [9] examined microarray data from two different systems; they report inconsistencies in variability of differential expression and discrepancy in fold-change calculations.

MASH significantly reduces false positive ratios (noise) and discovers the small number of genes whose states of differential gene expression are true to a high degree of certainty. The results of the present study support the idea that highly specific discovery of transcriptionally regulated genes combined with logical networks inferred from the functions of known genes predict the states of protein activation of molecular systems.

The results detailed in this paper uncover several interconnected molecular pathways with feedback loops. In addition, the molecular pathways related to membrane chemistry, actin assembly/branching, signaling pathways, and the extracellular matrix have been validated by independent laboratories (see figure and results). Nonetheless, the relevance of these models, based on glioma cells cultured in vitro, to human in vivo tumors is not known.

The findings raise the question whether glioma cells acquire this complex phenotype as a whole or piece-wise. The multiple hit theory of cancer stipulates that normal cells dedifferentiate because of the cumulative effects of several independent and random molecular events [47]. However, the complexity and apparent timely coordination of this system argue against a random piece-wise transformation from the normal phenotype. More likely than not, glioma cells acquire the phenotype of motility by turning on this molecular system as a unit and not piece-wise. This idea is supported by recent findings that glioma cells resemble neural stem cells in terms of phenotype, signaling, and behavior in vivo [48]. The system could have been active when motility was needed but is turned off in mature glial cells.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2005.08.254](https://doi.org/10.1016/j.bbrc.2005.08.254).

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