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

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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.

Keywords: Glioma; Microarrays; Expression discovery; Motility; Molecular systems; Systems biology

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 discovery 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

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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 postmortem. 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-β4, 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 four 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