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Research report

Robustness of gene expression profiling in glioma specimen samplings and derived cell lines

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Abstract

One of the most promising applications of microarrays is class distinction through gene expression profiling as a diagnostic tool. However, as there is apparent spatial heterogeneity in the morphology of cancer cells within a tumor, it is unclear if tumor sampling can be applied and yield consistent signals. In this report, we examined six brain tumors, four glioblastoma, and two oligodendroglioma biopsies. The six brain tumor tissues from two distinct different classes were dissected in four distinct areas and gene expression was profiled using microarrays. We used hierarchical clustering to compare the variability of gene expression profiles between spatially distinct biopsies of the same tumor as compared to the variability between tumors of the same histologic group. We conclude that, in general, repeat spatially distinct samples are not needed for microarray experiments and the gene expression signatures are robust across the tumor. Predominantly, variation was much greater between samples from different patients than from the multiple samplings of given tumor. Further, we compared biopsy expression profiles to the cell lines derived from those tissues. In general, the tumor cell lines vary greatly from the parental tissues and cluster more strongly with each other than the parental tissue. We select and examine the set of genes altered in expression to allow adaptation to cell culture.

Theme: Disorders of the nervous system
Topic: Neuro-oncology

Keywords: Glioblastoma; Oligodendrioglioma; Gene expression profiling; Microarray; Brain tumor classification; Cell culture

1. Introduction

Gliomas are the most common primary central nervous system tumor in humans. These tumors are commonly classified into four histologic grades [5]. Among them, glioblastoma multiforme (GBM) is the most aggressive (WHO grade IV). Each grade of tumor is predominantly classified as well by the following three morphologic descriptors: astrocytic, oligodendrocytic, and mixed. While the histology-based classification of gliomas is important to clinical management of patients, there is growing recognition of the importance of molecular markers including large-scale analyses like microarray gene expression analysis [2,3,8,11]. Gene expression profiling is a modern and rapidly evolving molecular diagnostic tool with many potential future applications such as proper patient management. It has enormous

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promise for prediction of cancer development, aggressiveness, and response to therapy after diagnosis [12]. In fact, microarray analysis studies have already revealed novel molecularly based subclasses of gliomas [13] and correlations with survival [10] as well as also other malignancies such as gastric cancer [15], breast carcinoma, colon carcinoma, lymphoma, leukemia, and melanoma [1]. Thus, precise gene expression fingerprinting is likely to allow for accurate diagnosis of tumors for proper patient management. Furthermore, these studies may reveal novel fundamental features of the disease for rational drug targeting.

However, it is unclear if spatial sampling of tumors confounds its utility as a diagnostic tool. That is, are gene expression profiles constant across gliomas and independent on sampling strategy? Previously, others have performed studies comparing different biopsy techniques for the accuracy of gene expression in breast carcinoma [14]. The study of Symmans et al. [14] supports the notion of similarity of gene expression independent of biopsy technique. The objectives of this study were: (1) to evaluate the similarity of gene expression fingerprint within tissue samples of gliomas, (2) to test the reproducibility of the gene expression based classification, and (3) to test the similarity of gene expression profiling between a tissue specimen and a cell culture line derived from it. For this purpose, four distinct areas of four different primary glioblastomas and two oligodendrogliomas were collected by micro-dissection. Gene expression profiles of each region and cell cultures derived from the same tissue were generated and compared using the human Affymetrix Hu6800 gene chip.

2. Materials and methods

2.1. Tissue and RNA isolation

All patients participating in this study gave informed consent for gene profiling analyses prior to surgery. At the time of resection, the tumor was examined by a neuropathologist and dissected into two portions, one for tissue diagnosis by histopathology and the other for RNA extraction. This procedure was done within 15 min of surgical resection while the tissue was kept on ice. The specimen portion destined for RNA extraction was snap frozen in liquid nitrogen and stored at −80 °C. The tumor was positioned such that four spatially distinct samples were taken as far away from each other as permitted by tissue parameters. Most samples were about 3 cm in diameter. Total RNA was extracted as previously described [9]. Only samples with sufficient quality and quantity of RNA were further analyzed. 10 μg of total RNA was used to generate double stranded cDNA using Superscript choice for cDNA synthesis (Invitrogen, Carlsbad, California, USA). Biotin-labeled antisense cRNA was synthesized by in vitro transcription using the ENZO BioArray HighYield kit (Enzo Diagnostics, Farmingdale, New York, USA). 20 μg cRNA was chemically fragmented and was then hybridized to Affymetrix Hu6800 GeneChip arrays (Affymetrix, Santa Clara, California, USA), containing 7129 targets including more than 5000 well-characterized human genes. These arrays were washed, stained with streptavidin–phycoerythrin, and scanned to generate an image file. The quality, yield, and size distribution of total RNA, labeled transcripts, and fragmented cRNA were estimated by spectrophotometric analysis at 260 and 280 nm and electrophoresis on RNA 6000 Nano-LabChips (Agilent Technologies, Palo Alto, California, USA).

2.2. Cell culture

Primary human brain tumor cultures were established from three surgically resected tissue specimens as described previously (Table 1) [4]. Tumors were taken directly from the operating room at the time of surgery. Tissues were finely minced using sterile scissors, rinsed with PBS, and dispersed with trypsin-EDTA. Monolayer cells were plated in T75 flasks (Costar) and cultured in DMEM/Ham's F12 (Irvine Scientific Santa Ana, California, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, California, USA), L-glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were grown for about 10 passages in culture. RNA was extracted from cells grown to 80% confluence.

2.3. Data analysis

Image files from the arrays were visually inspected to assure accurate grid placement. Images were analyzed with Affymetrix MASv 5.0 software, using default settings, to generate cel files and determine absence/presence calls. Gene expression values were calculated in dCHIP (1.2) using model-based expression and the perfect match minus mismatch algorithm implemented in dCHIP. Each array was scaled to the median array.

Normalized gene expression data for 18 samples (cf. Fig. 1) were imported and visualized in dCHIP [7]. For these analyses, of the 7129 probesets, probesets showing little or no variation across samples were filtered out using a coefficient of variation (standard deviation/mean) of 0.5, leaving 931 genes that represent significant expression

Table 1
Characteristics of the tumor specimen and cell lines used in this study

Cell bank #	Tumor biopsy histology	Cell line immunocytochemistry
405	GBM, grade 4 astrocytoma	GFAP +, synaptophysin −
131	GBM, grade 4 astrocytoma	N/A
137	GBM, grade 4 astrocytoma	N/A
53	GBM, grade 4 astrocytoma	N/A
64	Grade 2 oligodendroglioma	GFAP −, synaptophysin +
52	Grade 2 oligodendroglioma	GFAP −, synaptophysin +

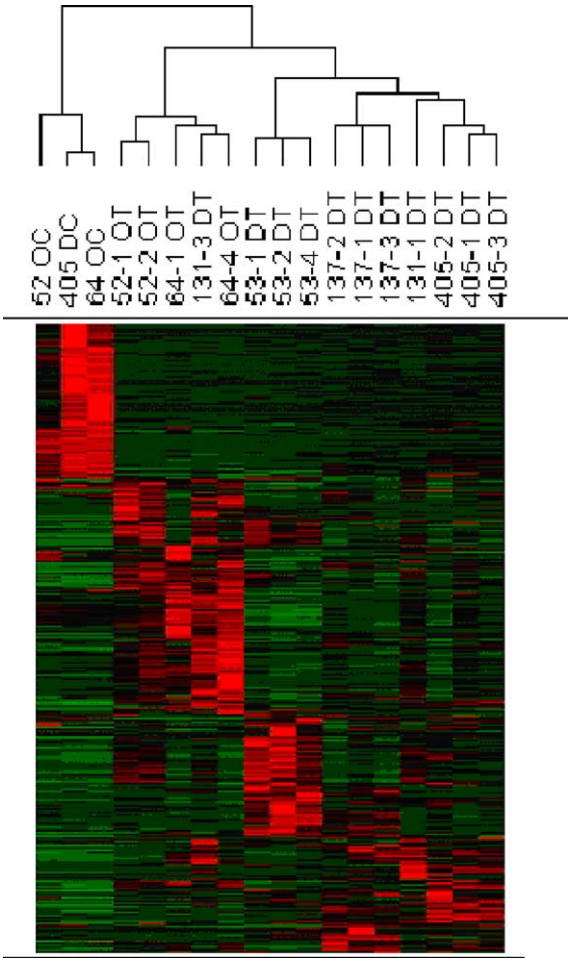

Fig. 1. Hierarchical clustering of the samples investigated in this report based on 931 differentially expressed genes with highest coefficient of variation across all the samples. C = cell culture, T = tissue, O = oligodendroglioma, D = de novo glioblastoma multiforme.

changes across samples. The samples were then clustered with the hierarchical clustering method implemented in the dCHIP software [7].

3. Results and discussion

3.1. Consistency of gene expression signatures in distinct spatial regions of gliomas

We first evaluated the similarity of gene expression among glioma specimen sections within tumors and across two different glioma histologies (Table 1). To this end, we analyzed in this study the expression profiling of four GBM tissue specimens (DT) (53,131, 137, and 405; Fig. 1), two oligodendroglioma tissue specimens (OT) (52 and 64; Fig. 1), and three cell lines derived from tissues CL-405, CL-52, and CL-64 (O/D C; Fig. 1). Initially, all tissue samples were dissected from four areas of the specimen but only a subset with sufficient quality and quantity of RNA was applied to microarray chips and analyzed for their gene expression; generally 2–3 areas were used (405-1,2,3; 137-1,2,3; 131-1,3; 53-1,2,4; 64-1,4; 52-1,2; cf. Fig. 1).

Our microarray analyses revealed that of the 7129 targets analyzed on the Affymetrix Hu6800 array, 931 exhibited significant changes in expression with a coefficient of variation across all the samples of greater than 0.5 (data not shown). Three genes were evaluated by quantitative RTPCR, specifically Angiotensinogen expression was dramatically reduced in cell lines, TAF 15 expression was reduced significantly in oligodendroglioma samples, and finally, Fibronectin 1 expression was more highly expressed in cell lines (data not shown). These data are consistent with our microarray findings. Clustering by similarity of expression profiles revealed that all samples of the same tumor with one exception cluster similarly, indicating that the expression profile within a tumor specimen is conserved across the specimen (Fig. 1). Furthermore, all the GBMs aggregated in a distinct cluster from the oligodendrogliomas. This suggests that the highest degree of gene expression variance occurs between different grades of tumor, than between samples of the same grade and the least variance occurs within any given sample. One GBM sample (131; Fig. 1) clustered with two different classes, once with the oligodendroglioma samples (131-3) and once with the rest of the GBMs (131-1). We histologically examined sections that were immediately adjacent to those used for the microarray analysis and found that the sample indeed included a mix of cells from both classes (glioblastoma and oligodendroglioma) (Fig. 2). Histologically, GBM tumors are characterized by high proliferation indices, dense cellularity, and focal necrosis. A small fraction of GBMs may contain areas with histological feature of oligodendroglial differentiation [6]. This finding (Fig. 2) suggests that, generally, the clustering is precise. However, there might be exceptions where mixtures of several lineages contribute to the tumor burden. This word of caution also has important implications for prognosis,

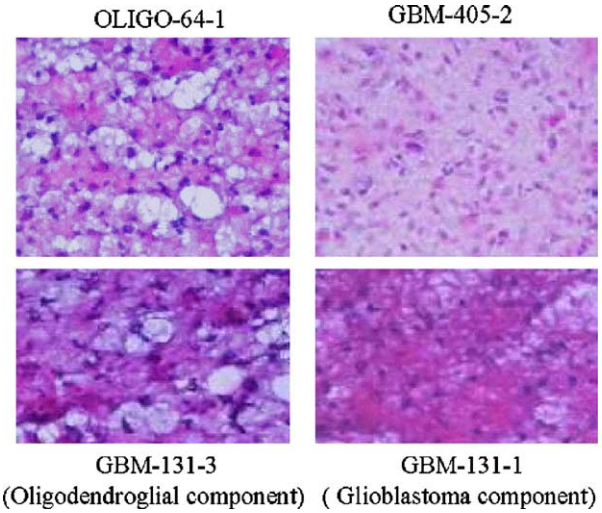

Fig. 2. Histopathologic staining of a representative oligodrendroglial ("oligo") and de novo glioblastoma ("GBM") tumors in the upper panel from this report and the two discordant samples (from oligo 131) in the lower panel.

since mixed oligodendrigliomas have a better outcome. Thus, gene expression profiles are robust to spatial sampling, although highly heterogenous tumors may be problematic. In other words, while tumors are heterogeneous and gene expression does vary over the landscape of the tumor, perhaps due to different admixtures of cells or differences in the malignant cells, the intratumor heterogeneity is sufficiently small that a robust and consistent gene expression image is represented within a single sample, and the differences between different patient biopsies remain apparent.

3.2. Strong alteration in gene expression in tumor cells adapted for growth in culture

Interestingly, all three cell lines derived from the tumors did not cluster with the parental tissues but rather clustered tightly together in a distinct "cell culture" group (Fig. 1). This effect suggests that cell cultures develop a discrete gene expression pattern that is largely shaped by their in vitro culture environment. We characterized our cell lines in tissue culture (Table 1). The immunocytochemistry showed that the cell cultures were indeed glioma cells and not stromal cells. They were negative for fibroblast markers. The GBM (#405) was positive for GFAP; and the grade 2 oligodendroglioma (#64) was negative for GFAP, but positive for synaptophysin. The growth characteristics are consistent with tumor cells; and the morphology resembled glioma, not fibroblasts. We have grouped other non-malignant cell lines and in general, they cluster with the malignant cell lines (data not shown). Thus, it is possible that there are such major gene expression responses to grow under the highly artificial environment of adherent cell

Table 2
List of the 40 most differentially expressed genes, with their fold change and functional group

Genbank	Gene name	C/T fold change	Gene symbol	Biological process
D00654	Actin, gamma 2, smooth muscle, enteric	−26.3	ACTG2	Cell motility and cytoskeleton maintenance
AF001294	Tumor suppressing subtransferable candidate 3	−10.1	PHLDA2	Apoptosis
D00017	Annexin A2	−9.6	ANXA2	Skeletal development
D17408	Calponin 1, basic, smooth muscle	−9.4	CNN1	Smooth muscle contraction
D83735	Calponin 2	−9.2	CNN2	Smooth muscle contraction
AF001548	Myosin, heavy polypeptide 11, smooth muscle	−8.4	MYH11	Contractile protein
D83174	Serine (or cysteine) protease inhibitor, member 2	−8.1	SERPINH1	Heat shock response
D31762	TRAM-like protein	−7.2	TRAM2	Protein targeting
D28124	Neuroblastoma, suppression of tumorigenicity 1	−7.2	NBL1	Negative regulation of cell cycle
D38583	S100 calcium binding protein A11 (calgizzarin)	−7.1	S100A11	Negative regul. of cell proliferation
D87116	Mitogen-activated protein kinase kinase 3	−4.5	MAP2K3	Signal transduction
D21261	Transgelin 2	−4	TAGLN2	Muscle development
M97935	Signal transducer and activator of transcription 1	−3.7	STAT1	Transcription activators
D86983	Melanoma associated gene	−3.7	D2S448	Response to oxidative stress
D25328	Phosphofructokinase, platelet	−3.2	PFKP	Glycolysis
D29963	CD151 antigen	−3.1	CD151	Cell adhesion
D25248	Actin filament associated protein	−3	AFAP	Modulator of actin filament
D14874	Adrenomedullin	−3	ADM	Signal transduction
D86425	Nidogen 2	−2.8	NID2	–
D87078	Pumilio (*Drosophila*) homolog 2	3	PUM2	–
D13146	2′,3′-Cyclic nucleotide 3′ phosphodiesterase	3.2	CNP	Synaptic transmission
D87463	Phytanoyl-CoA hydroxylase interacting protein	3.2	PHYHIP	–
D25217	KIAA0027 protein	3.4	MLC1	–
D31885	ADP-ribosylation factor-like 6 interacting protein	3.6	ARL6IP	–
D84361	Neuronal Shc	3.6	SHC3	Intracellular signaling cascade
D78012	Collapsin response mediator protein 1	3.8	CRMP1	Nucleotide metabolism
D26129	Ribonuclease, RNase A family, 1 (pancreatic)	4.1	RNASE1	–
D28114	Myelin-associated oligodendrocyte basic protein	4.2	MOBP	–
D38522	KIAA0080 protein	4.2	SYT11	Transport
X00351	Actin, beta	4.3	ACTB	Cell motility
D82346	Potassium voltage-gated channel, member 2	4.6	KCNQ2	Neurogenesis
D49958	Glycoprotein M6A	4.6	GPM6A	–
D87465	KIAA0275 gene product	5.1	SPOCK2	Regulation of cell differentiation
D82343	Olfactomedin 1	5.4	OLFM1	Neurogenesis
AF000959	Claudin 5	6	CLDN5	Tight junction strands
D13639	Cyclin D2	8.7	CCND2	Regulation of cell cycle
D21267	Synaptosomal-associated protein, 25kD	8.7	SNAP25	Regulation of synapse
D79990	Ras association (RalGDS/AF-6) domain family 2	9.2	RASSF2	Neuropeptide signaling pathway
D26443	Solute carrier family 1, member 3	9.6	SLC1A3	Synaptic transmission
D16181	Peripheral myelin protein 2	21.5	PMP2	–

C = cell line mean, T = tissue mean. Ratios with negative numbers denote genes with reduced expression in cell lines while positive numbers describe genes with increased expression.

culture, that this greatly obscures the expression pattern that is cancer specific. We have explored this issue using the gene expression data in more detail. We determined all of the genes that are differentially expressed between the tumor cell culture group and the tumor group to determine which components of the cancer cells were being most perturbed in tissue culture growth and selection. The most strongly differentially expressed genes are indicated in Table 2. EASE classification of all the up/down-regulated genes with more than a 2-fold change genes indicate a striking enrichment of genes involved in nucleotide and nucleic acid metabolism, cell proliferation, and signal transduction. We also observed loss of gene expression involved in development, signal transduction, and transporter activity including brain specific genes such as: synaptic transmission, neurotransmitter uptake, and neuropeptide signaling. Thus, there is a global reprogramming of the cancer cells or strong selection of the cancer cells that can survive and divide sufficiently rapidly in culture that may be grossly perturbing their utility as in vitro models of cancer. In cell culture, we have likely selected for cells with modifications that allow more rapid proliferation and a more undifferentiated phenotype relative to the primary tumor cell.

References

[1] A.A. Alizadeh, D.T. Ross, C.M. Perou, M. van de Rijn, Towards a novel classification of human malignancies based on gene expression patterns, J. Pathol. 195 (1) (2001 Sep.) 41–52.

[2] G. Bacciocchi, N. Gibelli, C. Zibera, et al., Establishment and characterization of two cell lines derived from human glioblastoma multiforme, Anticancer Res. 12 (1992) 853–861.

[3] C. Chauzy, B. Delpech, A. Olivier, et al., Establishment and characterisation of a human glioma cell line, Eur. J. Cancer 28 (1992) 1129–1134.

[4] M.L. Estes, R.M. Ransohoff, J.T. McMahon, B.S. Jacobs, B.P. Barna, Characterization of adult human astrocytes derived from explant culture, J. Neurosci. Res. 27 (4) (1990 Dec.) 697–705.

[5] P. Kleihues, D.N. Louis, B.W. Scheithauer, L.B. Rorke, G. Reifenberger, P.C. Burger, W.K. Cavenee, The WHO classification of tumors of the nervous system, J. Neuropathol. Exp. Neurol. 61 (3) (2002 Mar.) 215–225 (discussion 226–9).

[6] J.A. Kraus, K. Lamszus, N. Glesmann, M. Beck, M. Wolter, M. Sabel, D. Krex, T. Klockgether, G. Reifenberger, U. Schlegel, Molecular genetic alterations in glioblastomas with oligodendroglial component, Acta Neuropathol. (Berl.) 101 (4) (2001 Apr.) 311–320.

[7] L. Cheng, W. Hung Wong, Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection, Proc. Natl. Acad. Sci. 98 (2001) 31–36.

[8] R. Maunoury, Establishment and characterization of 5 human cell lines derived from a series of 50 primary intracranial tumors, Acta Neuropathol. 39 (1977) 33–41.

[9] P.S. Mischel, R. Shai, T. Shi, S. Horvath, K.V. Lu, G. Choe, D. Seligson, T.J. Kremen, A. Palotie, L.M. Liau, T.F. Cloughesy, S.F. Nelson, Identification of molecular subtypes of glioblastoma by gene expression profiling, Oncogene 22 (15) (2003 Apr. 17) 2361–2373.

[10] C.L. Nutt, D.R. Mani, R.A. Betensky, P. Tamayo, J.G. Cairncross, C. Ladd, U. Pohl, C. Hartmann, M.E. McLaughlin, T.T. Batchelor, P.M. Black, A. von Deimling, S.L. Pomeroy, T.R. Golub, D.N. Louis, Gene expression-based classification of malignant gliomas correlates better with survival than histological classification, Cancer Res. 63 (7) (2003 Apr. 1) 1602–1607.

[11] S. Ramaswamy, P. Tamayo, R. Rifkin, S. Mukherjee, C.H. Yeang, M. Angelo, C. Ladd, M. Reich, E. Latulippe, J.P. Mesirov, T. Poggio, W. Gerald, M. Loda, E.S. Lander, T.R. Golub, Multiclass cancer diagnosis using tumor gene expression signatures, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 15149–15154.

[12] G. Russo, C. Zegar, A. Giordano, Advantages and limitations of microarray technology in human cancer, Oncogene 22 (42) (2003 Sep. 29) 6497–6507.

[13] R. Shai, T. Shi, T.J. Kremen, S. Horvath, L.M. Liau, T.F. Cloughesy, P.S. Mischel, S.F. Nelson, Gene expression profiling identifies molecular subtypes of gliomas, Oncogene 22 (31) (2003 Jul. 31) 4918–4923.

[14] W.F. Symmans, M. Ayers, E.A. Clark, J. Stec, K.R. Hess, N. Sneige, T.A. Buchholz, S. Krishnamurthy, N.K. Ibrahim, A.U. Buzdar, R.L. Theriault, M.F. Rosales, E.S. Thomas, K.M. Gwyn, M.C. Green, A.R. Syed, G.N. Hortobagyi, L. Pusztai, Total RNA yield and microarray gene expression profiles from fine-needle aspiration biopsy and core-needle biopsy samples of breast carcinoma, Cancer 97 (12) (2003 Jun. 15) 2960–2971.

[15] S.T. Tay, S.H. Leong, K. Yu, A. Aggarwal, S.Y. Tan, C.H. Lee, K. Wong, J. Visvanathan, D. Lim, W.K. Wong, K.C. Soo, O.L. Kon, P. Tan, A combined comparative genomic hybridization and expression microarray analysis of gastric cancer reveals novel molecular subtypes, Cancer Res. 63 (12) (2003 Jun. 15) 3309–3316.