Differential gene expression profiling in human brain tumors

JAMES M. MARKERT1, CATHERINE M. FULLER2, G. YANCEY GILLESPIE1, JAMES K. BUBIEN2, LEE ANNE MCLEAN2, ROBERT L. HONG3, KAILIN LEE3, STEVEN R. GULLANS3, TIMOTHY B. MAPSTONE4, AND DALE J. BENOS2

1Department of Surgery, 2Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Alabama 35294-0005; 3Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115; and 4Department of Neurosurgery, Emory University, Atlanta, Georgia 30322

Received 1 August 2000; accepted in final form 14 December 2000

Markert, James M., Catherine M. Fuller, G. Yancey Gillespie, James K. Bubien, Lee Anne McLean, Robert L. Hong, Kailin Lee, Steven R. Gullans, Timothy B. Mapstone, and Dale J. Benos. Differential gene expression profiling in human brain tumors. Physiol Genomics 5: 21–33, 2001.—Gene expression profiling of three human temporal lobe brain tissue samples (normal) and four primary glioblastoma multiforme (GBM) tumors using oligonucleotide microarrays was done. Moreover, confirmation of altered expression was performed by whole cell patch clamp, immunohistochemical staining, and RT-PCR. Our results identified several ion and solute transport-related genes, such as N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)-2 receptors, GABA receptor subunits α3, β1, β2, and β3, the glutamate transporter, the glutamate/aspartate transporter II, the potassium channel Kv2.1, hKvβ3, and the sodium/proton exchanger 1 (NHE-1), that are all downregulated in the tumors compared with the normal tissues. In contrast, aquaporin-1, possibly aquaporins-3 and -5, and GLUT-3 message appeared upregulated in the tumors. Our results also confirmed previous work showing that osteopontin, nicotinamide N-methyltransferase, murine double minute 2 (MDM2), and epithelin (granulin) are upregulated in GBMs. We also demonstrate for the first time that the cytokine and p53 binding protein, macrophage migration inhibitory factor (MIF), appears upregulated in GBMs. These results indicate that the modulation of ion and solute transport genes and heretofore unsuspected cytokines (i.e., MIF) may have profound implications for brain tumor cell biology and thus may identify potential useful therapeutic targets in GBMs.

Glioblastoma; glutamate transporters; amiloride; NMDA receptors; BNaC; potassium channels; gene microarrays; patch clamp; reverse transcription-polymerase chain reaction; immunohistochemistry

of primary brain tumors in adults and are divided by grade into two major categories: anaplastic astrocytoma and glioblastoma multiforme (GBM) (50). GBMs, the most malignant astrocytomas, are thought to arise either as a result of malignant transformation of lower grade (grade II) or anaplastic astrocytomas (secondary GBMs) or de novo (primary GBMs). Primary GBM refers to lack of an identifiable precursor tumor and is usually associated with a short clinical history and histologic features of GBM at first biopsy, whereas secondary GBM requires imaging or histologic evidence of evolution from a lesser grade glioma such as a grade II or grade III astrocytoma (anaplastic) (28). Despite aggressive surgical therapy, radiotherapy, and chemotherapy, malignant gliomas are almost always fatal; the overall 5-yr survival rate for patients with GBMs is less than 5.5%, and the median survival is ~52 wk from diagnosis (31).

Genetic alterations associated with GBM formation are somewhat different for primary and secondary GBMs (28). Typically, primary GBMs overexpress epidermal growth factor receptor (EGFR) and murine double minute 2 (MDM2) oncogene, occasionally with amplification (6, 22, 58). The tumor suppressor gene PTEN (MMAC1; i.e., “phosphatase and tensin homolog-mutated in multiple advanced cancers”) mutations are present in 30%, and p16 tumor suppressor gene deletions are found in another one-third of these primary GBM tumors (7, 15). Secondary GBMs are more likely to exhibit p53 mutations and platelet-derived growth factor-A/platelet-derived growth factor receptor-α (PDGF-A/PDGF-α) overexpression, which can also be demonstrated in low-grade tumor specimens (22, 58). Subsequently, these tumors accumulate loss of heterozygosity (LOH) for a segment of chromosomes 17p and 19q and alterations in the retinoblastoma gene, which are associated with progression to anaplastic astrocytoma. Further LOH of chromosome 10, PDGFR-α amplification, and DCC (i.e., “deleted in colon cancer”) gene mutations are seen in progression to GBM (28). Although some crossover occurs between primary and secondary GBMs that blur the distinction,

GLIOMAS ARE THE MOST COMMON primary tumor arising in the human brain. Malignant gliomas account for 30%
by and large the division by genetic aberrations appears significant.

With the advent of cDNA/oligonucleotide microarray analysis, a tool has become available for identifying clusters of genes whose expression may be altered in diseased states (20, 34, 47, 57). In this study, we used oligonucleotide microarray technology (Affymetrix) to define differences in gene expression between normal human adult brain (temporal lobe) and primary GBMs. In addition, we applied molecular biological, immunohistochemical, and electrophysiological methods to confirm independently selected findings. Our results show that several ion and solute transport-related genes [e.g., N-methyl-D-aspartate (NMDA) receptors, \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)-2 receptors, glutamate transporters] and cytokines [e.g., macrophage inhibitory factor (MIF)] are either downregulated or upregulated in primary GBMs compared with normal neuroglial cells. The findings from this study establish that alterations in expression of genes encoding proteins important for cell cycle control and membrane ion and solute transport exist between normal and glioblastoma tumor cells. This information will be useful for the ultimate identification of patterns of genes that have altered expression in GBMs and thus to define specific disease within a given category of tumor.

MATERIALS AND METHODS

Oligonucleotide array analysis. The Affymetrix human oligonucleotide array (HU Gene Fl Array) is complementary to 7,000 human genes and expressed sequenced tags (ESTs) (9). Each gene or EST is represented on the array by 20 feature pairs. Each feature pair contains a 25-bp oligonucleotide sequence, which is either a perfect match to the gene or a single central-base homomorphic mismatch control. Affymetrix GeneChip analysis was performed using standard procedures (33, 57). Briefly, total RNA was extracted from snap-frozen, freshly dissected tissue samples, after dissecting away necrotic and hemorrhagic portions. Three independent samples of whole brain tissue (temporal lobe, white matter) and four independent samples of primary GBM obtained from newly diagnosed patients who had not received chemotherapy or radiation therapy were used in these experiments. Biotinylated mRNA was prepared according to manufacturer’s suggestions (see Refs. 33 and 35 for details) as follows. Poly(A)\(^+\) RNA was prepared from 100 \(\mu\)g of total RNA and converted into doubled-stranded cDNA using a cDNA synthesis kit from Life Technologies. Biotinylated cRNA was then generated from the cDNA by an in vitro transcription reaction in which biotin-11-CTP and biotin-16-UTP were included. The labeled cRNA was purified using RNaseasy spin columns (Qiagen). The cRNA was fragmented by alkaline treatment and hybridized to a GeneChip probe array. The total intensity of the hybridization was given by the mean filtered perfect match minus mismatch intensities corrected for variations between individual segments on the arrays. The new intensity of each gene sequence on an array was divided by the mean intensity of all the genes represented on that array and multiplied by a nominal average intensity of 50 to compensate for variations between different arrays. The expression level of any particular transcript was calculated by subtracting the difference between the fluorescence intensities of the perfect match and mismatch feature pairs and then averaging over the entire probe set. A negative expression value (i.e., green in Figs. 2–5) indicates that there was more unmatched signal than perfect match signal, whereas a positive expression value (i.e., red in Figs. 2–5) represents the reverse. Data were analyzed by the Cluster and TreeView software programs developed by Michael Eisen and colleagues (16) and available on the world wide web (http://rana.lbl.gov/). The results are presented as normalized intensities.

RNA extraction for RT-PCR. Freshly excised human brain tissue (GBM or white matter from temporal lobe) was frozen and stored in liquid nitrogen until ready for processing. The frozen tissue was ground into a fine powder using a mortar and pestle, under liquid nitrogen, after which 1 ml of Trizol (Life Technologies) containing 250 \(\mu\)g of glycerol was added. The Trizol/powder mixture was transferred to a chilled glass/Teflon homogenizer and ground for 10 strokes while on ice. The homogenate was sequentially passed through 25- and 26-gauge needles, to shear genomic DNA, and then transferred to a 1.5-ml microcentrifuge tube. A volume of 200 \(\mu\)l of cold Trizol was added to the homogenate, vortexed for 30 s to mix, and centrifuged at maximum speed (14,000 rpm) for 5 min using a tabletop centrifuge at room temperature. The aqueous phase was transferred to a fresh 1.5-ml tube, 500 \(\mu\)l of ice-cold isopropanol were added, and the RNA was allowed to precipitate overnight at \(-20^\circ\text{C}\). The precipitated RNA was pelleted by centrifugation at maximal speed for 15 min at room temperature, washed with 1 ml of 70% ethanol, and centrifuged for 5 min. The pellet was dissolved in 100 \(\mu\)l of RNase-free \(\text{H}_2\text{O}\), re-extracted with phenol/chloroform (1:1), and vortexed, and the aqueous phase was precipitated with 1/10 volume of 7.5 M ammonium acetate and 2.5 vol of 100% ethanol overnight at \(-20^\circ\text{C}\). The precipitated RNA was pelleted by centrifugation at maximal speed for 15 min at room temperature, washed with 1 ml of 70% ethanol, and resuspended in RNase-free \(\text{H}_2\text{O}\). The integrity of the RNA was verified following electrophoresis through 1% agarose-formaldehyde gels. All equipment (e.g., homogenizers, mortar, pestle, etc.) were pretreated with RNase-Zap (Ambion) and rinsed with diethyl pyrocarbonate (DEPC)-treated \(\text{H}_2\text{O}\) prior to use.

RT-PCR. RT-PCR was performed using a OneStep RT-PCR kit (Qiagen) according to manufacturer’s instructions, using 0.2 \(\mu\)g of total RNA as template. Custom primers specific to the genes of interest were synthesized by Life Technologies and used at a final concentration of 0.6 \(\mu\)M. The forward and reverse primers for the human NMDA receptor subtype 2A (GRIN 2A, GenBank accession no. U09002) were 5\'-GACTATCCCCACCTTTTG-3' and 5\'-CTCCATCTCCAATCACCC-3' and corresponded to bases 2023–2042 and 2349–2367 of the coding region, respectively. The forward and reverse primers for the human NMDA receptor subtype 2B (GRIN 2B, GenBank accession no. U28758) were 5\'-CCAAAGAGCATCATCACACC-3' and 5\'-CTGTAACATAGCGATCAGTCA-3' and corresponded to bases 2180–2199 and 2573–2590 of the coding region, respectively. The forward and reverse primers for the human NMDA receptor subtype 2C (GRIN 2C, GenBank accession no. L76224) were 5\'-TCTATGATGCTGCTGTCCTC-3' and 5\'-AGATGCCCTGCTGAA-AG-3' and corresponded to bases 2180–2199 and 2573–2590 of the coding region, respectively. The forward and reverse primers for the human brain Na\(^+\) channel 1 (BNaC1 or ASIC2, GenBank accession no. U57352) were 5\'-GCCAATCTACCCACCC-3' and 5\'-AGCAGGAATCTCCTCCAAG-3' and corresponded to bases 1091–1110 and 1518–1537 of the coding region, respectively. The forward and reverse primers for the human brain Na\(^+\) channel 2 (BNaC2 or ASIC1, GenBank accession no. U78181) were 5\'-CCCCGCTA...
Reverse transcription was performed using a single cycle of 50°C for 30 min. This was followed by a single cycle of 95°C for 15 min, which inactivates the reverse transcriptases while activating the HotStarTaq DNA polymerase, followed by 30 cycles of 94°C for 1 min, 56°C for 1 min (60°C for BNac1), and 72°C for 1 min, and finally a single 10-min cycle at 72°C. Aliquots of each reaction mixture were electrophoresed on a 2% NuSieve (FMC) agarose gel using PCR markers (Promega) to determine molecular size. Products of the correct molecular size were isolated from the gel using the QIAquick Gel Extraction kit (Qiagen) and subcloned into the pCR-2.1 vector using the TOPO-TA cloning kit (Invitrogen) following manufacturer's instructions. Recombinants were selected by blue/white screening and restricted with EcoRI (Promega) to verify incorporation of insert of correct size. Sequences were verified by further restriction enzyme digest analysis and automated DNA sequencing (DNA Sequencing Facility, Iowa State University).

Whole cell patch-clamp studies. Fresh normal temporal lobe specimens (obtained from patients undergoing surgery for intractable epilepsy) consisting primarily of white matter or GBM tumor specimens were obtained from patients undergoing surgical resection. Samples were immediately transported in sterile culture medium on ice from the operating room to the laboratory. As described above, after dissecting away any visible necrotic and/or hemorrhagic regions, a portion of the remaining tissue was minced, pipetted repeatedly to dissociate cells, and transferred directly to the patch-clamp chamber. Once the cells had adhered to the bottom of the glass perfusion chamber, they were patch-clamp studies.

![Fig. 1. Log-log plot of the expression level of a given transcript in a single glioblastoma multiforme (GBM) vs. the expression level of the same transcripts in a single sample of normal brain tissue obtained from temporal lobe. The Affymetrix HuFL human GeneChip probe array was used. Each point represents an individual gene. The solid line represents identical expression levels, and the dotted lines indicate the range of 5-fold up-expression or down-expression.](image1)

![Fig. 2. Cluster diagram of genes that displayed >5-fold increase in GBM samples compared with normal brain tissue. Colors represent relative levels of gene expression (as described in MATERIALS AND METHODS), with the brightest red indicating the highest level of expression and green depicting low levels or absence of expression. Fold differences were determined by taking the ratio of the mean of the four tumor samples (samples T1, T2, T3, and T4) divided by the mean expression value for the three normal samples (N1, N2, and N3). Diagram displays only those genes that showed a greater than 5-fold difference and were statistically significant (P < 0.05) as determined by Students t-test.](image2)
clamped. Micropipettes were fashioned using a Narishige PP-83 two-stage micropipette puller (internal tip diameter of 0.3–0.5 mM). The pipettes were filled with a solution containing (in mM) 100 potassium gluconate, 30 KCl, 20 HEPES, 0.5 EGTA, and 4 ATP. This solution had less than 1 nM free Ca$^{2+}$ and had a pH of 7.2. The electrical resistance of the filled pipettes was 1–3 MΩ. The bath solution was serum-free RPMI 1640 cell culture medium. These solutions approximate the normal ionic gradients across a mammalian cell membrane. Pipettes were mounted in a holder and connected to the head stage of an Axon model 200A patch-clamp amplifier fixed to a three-dimensional micromanipulator system attached to the microscope. The pipette was pressed to the cell and slight suction was applied. Seal resistance was continuously monitored on a Nicolet model 300 oscilloscope, using 0.1-mV electrical pulses from a pulse generator. After formation of seals with greater than 1-GΩ seals, a negative pressure pulse was applied to the pipette to enter the whole cell configuration. Successful completion of this procedure was assessed by the sudden increase in capacitance with no change in seal resistance. Typically, this capacitance was between 5 and 10 pF. Subsequently, any capacitative transients were compensated by the use of the capacitative and resistance circuits of the amplifier.

Once the whole cell configuration was obtained, the pipette solution and the cytoplasmic compartment equilibrated within 30 s. The cells were held at a membrane potential of 0 mV for 1 s between each test voltage. This procedure produced inward sodium (at more depolarized potentials) and outward potassium (at more hyperpolarized potentials) currents to flow across the membrane. The currents were recorded digitally and filed in real time. The entire procedure was controlled using a computer modified for analog-to-digital signals with pCLAMP 6 software (Axon Instruments, Sunnydale, CA). For any given sample, 3–12 individual cells were examined.

**Immunostaining of tissues.** Normal temporal lobe specimens were obtained from excess brain tissues surgically removed from patients with intractable epilepsy. GBM specimens were obtained from excess tissues obtained from patients undergoing surgical resection of their tumors. The diagnosis of GBM was confirmed by a neuropathologist. Tissue procurement was approved by the UAB Institutional Review Board for Human Experimentation. Tissues were transported on ice to the lab, debrided of areas of hemorrhage and necrosis, and divided into portions ~4–6 mm on a side. Portions of these tissues were snap-frozen in cryovials for later processing for analysis of mRNA expression, and other aliquots of the same tissues were placed in OCT embedding medium and frozen on dry ice. For immunostaining, sections 8–12 μm thick were cut on a Zeiss H505E cryotome, mounted on triethoxy-3-aminopropylsilane (TEPSA)-coated slides and air dried. Representative sections were stained with hematoxylin and eosin and examined to determine the quality and suitability of the tissues for immunostaining. Sections were fixed in acetone at −20°C for 10 min and rehydrated in blocking medium. Antibodies against MIF (R&D Systems, Minneapolis, MN), aquaporin-1 (Alomone Laboratories, Jerusalem, Israel), NMDA receptor 2A/B (Chemicon International, Temecula, CA), GABA_A receptor-α (Boehringer, Mannheim, Germany), GABA_A receptor-β (Chemicon), and glutamate receptors 2 and 3 (Chemicon) were used.

Dilutions of commercially obtained polyclonal or monoclonal antibodies were prepared as recommended by the manufacturers, and 100–200 μl of each or nonimmune IgG were applied to adjacent serial sections of the tissues for 30–60 min. Sections were rinsed and incubated with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) specific to the primary antibody. Slides were rinsed and incubated with avidin-biotin-peroxidase (ABP) reagent. Binding of the primary antibody was detected by rinsing unbound ABP reagent from the slides and adding diamino-

---

**Table:**

<table>
<thead>
<tr>
<th>Normal</th>
<th>GBM</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td></td>
</tr>
<tr>
<td>N3</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td></td>
</tr>
</tbody>
</table>

**Legend:**

- Positive
- Zero
- Negative

**Glutamate receptor 2 (HGR2) [L20814]
Cytochrome c oxidase subunit VIa (COX7A) [M83186]
Clone CCG-B7 sequence [L10372]
Myelin basic protein (MBP) [M15777]
Delta-catenin [U53820]
KIAA0193 [D83777]
Clone CTTG-A4 sequence [L10374]
UNG-18 homolog [D63506]
PDGF receptor alpha (PDGFR) [M21574]
MXII [L07648]
Nonerythroid alpha-spectrin [SPTAN1] [J00243]
EF-1delta (elongation factor-1-delta) [Z15097]
Zipper protein kinase (zpk) [U807358]
N-methyl-d-aspartate receptor (NR1-1) [L12246]
Amy [D82343]
Lanosterol 14-demethylase cytochrome P450 (CYP51) [U23942]
KIAA0025 [D07467]
apoipoprotein D [J00611]
N-ethylmaleimide-sensitive factor [HG4102-HT4372]
Guanine nucleotide regulatory protein (ABR) [L01147]
Myelin oligodendrocyte glycoprotein (MOG) [Z48651]
Myelin proteolipid protein [N54927]

---

Fig. 3. Cluster diagram of genes that displayed >5-fold decrease in GBM samples compared with normal brain tissue. Colors represent relative levels of gene expression (as described in MATERIALS AND METHODS), with the brightest red indicating the highest level of expression and green depicting low levels or absence of expression. Fold differences were determined by taking the ratio of the mean of the three normal samples (samples N1, N2, and N3) divided by the mean expression value for the four tumor samples (T1, T2, T3, and T4). Diagram displays only those genes that showed a greater than 5-fold difference and were statistically significant (P < 0.05) as determined by Student’s t-test.

http://physiolgenomics.physiology.org
benzidine (0.1 mg/ml) in the presence of 0.03% H2O2. After 5 min, slides were rinsed, counterstained with 1% methyl green, dehydrated, coverslipped with Cytoseal (Stephens Scientific, Riverdale, NJ), and examined microscopically.

RESULTS

Oligonucleotide microarray analysis. Global gene expression profiles were compared in human brain tissue samples obtained from adult individuals undergoing surgery for intractable epilepsy (normal tissue) or for removal of primary GBM. Tissue was obtained from three patients in the former group and from four patients in the GBM group. Because 7,000 separate genes are represented on a single Affymetrix GeneChip, we examined our data in one of two ways: 1) by identifying genes either overexpressed or underexpressed in the glioblastomas vs. the normal tissue or 2) by classifying them into specific functional categories, such as neurotransmitters, angiogenesis factors, ion channels, etc. Prior to analysis, all genes identified as “absent” in all six samples (both normal and tumor) were first eliminated. Moreover, an expression sensitivity threshold (at least 2× background) against which average difference values were screened was applied. Figure 1 presents a log-log representation of the differential gene expression of the 7,000 transcripts analyzed in one GBM sample plotted against the expression level of the same genes measured in a single normal sample obtained from the temporal lobe. This pattern of expression was typical for all the other combinations of tumor vs. normal tissues. While the expression levels of most of the transcripts fall within a fivefold range (higher or lower) of the identity line, there are a number of genes whose expression levels are either fivefold greater or fivefold smaller than that seen in so-called normal temporal lobe. Using a color-coded scheme derived from the TreeView program, we created Fig. 2, which shows all of the genes that were significantly (at the 0.05 level) expressed to greater than fivefold in all of the glioblastomas compared with normal tissue samples. While only 34 of the 7,000 transcripts appear in this category, some important genes did emerge, including MDM2 p53-associated protein, α1-antitrypsin, metalloproteinase stromelysin-2 (MMP-10), and caveolin. Moreover, several genes, including 1) certain transcription factors (e.g., FUSE binding protein 2 and HLH IR21/heir1), 2) immune-related genes (e.g., lactoferrin, osteopontin, and PTX3 long pentraxin), 3) genes encoding cytoskeletal proteins [e.g., SNAP25 (synaptosomal-associated protein of 25 kDa), filamin, emerin, and lamin B], and 4) miscellaneous genes [e.g., aquaporin-1 (AQP-1), a nuclear Cl− channel protein, epithelin (granulin), MDM2, MDMX, (a p53-binding protein with homology to MDM2), nicotine β-N-methyltransferase, and the cellular ligand of annexin II], are all expressed in GBMs to a greater extent than in the normal tissue samples (see Supplemental Table 1;

http://physiolgenomics.physiology.org
please refer to the Supplementary Material for this article, published online at the Physiological Genomics web site). The expression of many of these genes or proteins has already been reported to be upregulated in GBM compared with normal brain or cultured human astrocytes (17, 30, 32, 45). However, we found that the message for several secreted peptides, including insulin-like growth factor binding peptides 5 and 6, interferon-γ, and macrophage migration inhibitory factor (MIF), appear to be upregulated in the GBMs vs. the normal samples. To our knowledge, none of these genes has been associated previously with brain tumors.

Likewise, several genes were found to be absent (Fig. 3 and Supplemental Table 1) or underexpressed by at least fivefold in the tumor samples compared with the normals. Several of these genes code for ion channels, solute transporters, or neurotransmitter-related proteins. For example, MDR1 and 3, UNC-18 homolog, potassium channels hKvβ3 and hKv2.1, glutamate/aspartate transporter II, the glutamate transporters, EAAT1 and 3, GLUT 5, NHE-1, the ionotropic glutamate receptors 2 (HBGR2) and 4 (Glur4), GABA_A receptor β1, β2, and β3 subunits, and the NMDA receptors (GRIN 2A and NR1-1) were all underexpressed compared with control samples. Figures 4 and 5 display cluster analyses showing the differential expression profiles of the ion channels and all of the solute and neurotransmitters, respectively, represented on the GeneChip.

Supplemental Table 1 presents a summary of the GeneChip expression data in selected categories for all of the normal and GBM samples analyzed. It is important to note that there was considerable variation in expression levels among samples, both normals and tumors. This observation undoubtedly reflects genetic variations among individuals as well as heterogeneity of GBMs themselves (28). These gene alterations may be important in implicating various pathways that will define a particular type of disease.

Whole cell patch clamp. Based on the results of the GeneChip analysis, we wanted to provide some functional verification of the predictors indicated above. We investigated by electrophysiological means two hypotheses generated by microarray analyses, namely, that a voltage-gated K⁺ channel β3 subunit (hKvβ3) and NMDA receptor-activated currents are downregulated in GBMs compared with normal brain. Potassium channels are crucial for the proper activity of all excitable cells, including neurons and astrocytes (29). The pore-forming K⁺ channels constitute a large family of
proteins with diverse properties. These channels in native cells undergo a variable time course of inactivation presumably because of the presence of different associated β-subunits. There are at least three Kvβ subunits that affect Kv inactivation (21). Kvβ3 has been isolated and cloned from rat brain (21). This subunit, in heterologous expression studies in oocytes, was shown to associate with hKv1.5 and to convert this channel from one acting as a delayed rectifier (i.e., one with very slow inactivation properties) to a channel with a rapid and well-defined inactivation characteristic (18). We therefore hypothesized that if hKvβ3 was downregulated in GBM cells, then the relaxation characteristics at hyperpolarizing clamp voltages would be different than those of normal cells. Figure 6 shows typical current traces recorded from freshly isolated, normal human astrocytes, glial GBM tumor cells (from two of the studied specimens, T1 and T3), and SKMG cells (a continuous glial cell line originating from a GBM). Two cells from each group are shown, but these same results were seen in a minimum of 10 cells in each group. A pronounced relaxation of the outward current traces at the two most positive voltage clamp potentials (+80 and +100 mV) could be seen in the normal astrocytes, but this was absent in the tumor cells. These electrophysiological characteristics are consistent with the microarray findings, namely, that the hKvβ3 gene is downregulated in the tumor cells, thus altering the biophysical characteristics of the K+ conductance.

We next examined the functionality of NMDA receptor expression in normal human astrocytes compared with that in glial cells obtained from GBM tumors. Currents elicited in the absence or presence of 1 mM NMDA chloride at different voltage clamp potentials are shown for normal astrocytes in Fig. 7A. It should be noted that Mg2+ was not present in the pipette solution, and, for these experiments, the extracellular solution was supplemented with 1 μM glycine to saturate the glycine recognition site on the NMDA receptor (25). As shown in the associated current-voltage curves, the NMDA-induced currents were significantly increased, particularly in the inward direction, and reversed around 0 mV. These findings are in agreement with those previously reported (13, 44, 48). In contrast, 1 mM NMDA superfusion of GBM cells did not affect the whole cell currents whatsoever (Fig. 7B). Although there are a multitude of different NMDA receptor subtypes in human brain (41), these functional observations are in good agreement with the prediction of the gene profiling experiments, demonstrating that the NMDA receptor (NR1-1 and GRIN 2A) gene expression were downregulated in GBMs compared with normal brain tissue. RT-PCR confirmed the presence of NMDA R subtypes 2A, B, and C in normal brain and the decreased abundance of these receptor subtypes in GBM samples T1, T2, and T4 (Fig. 9).

**Fig. 6.** Whole cell patch-clamp recordings from two normal adult brain astrocytes (top), two GBM cells (middle), and two SKMG cells (bottom). Cells were voltage clamped between −100 and +100 mV in 20-mV increments from a holding potential of 0 mV. Cells were superfused with RPMI 1640 medium, and the pipette contained (in mM) 100 potassium gluconate, 30 KCl, 10 NaCl, 20 HEPES, 0.5 EGTA, and 4 ATP, as well as <10 nM free Ca2+ (pH 7.2).
experiments, i.e., tumor samples T1 and T3, showed strong cell-associated staining for AQP-1 and MIF (Fig. 8, A and C). Sections from normal brain tissue (samples N2 and N3) did not show any specific staining for either AQP-1 or MIF (Fig. 9). These same tumor tissues were stained at weaker and more disperse levels for NMDA receptor 2A/B, GABA receptor-α, GABA receptor-β, and the glutamate receptor 2α (Fig. 8B). Human GBMs were consistently positive for only AQP-1 and MIF proteins. Sections from normal temporal cortex tissues (i.e., samples N2 and N3) were also probed with these antibodies but showed very little positive staining. One sample showed low levels of positive staining over 50% of the specimen for NMDA receptor 2A/B (Fig. 8E). A summary of the immunohistochemical results is presented in Table 2.

Brain voltage-independent Na\(^+\) channels. Because of our previous observations that high-grade astrocyte-derived brain tumors like GBMs functionally express a constitutively activated amiloride-sensitive Na\(^+\) conductance (10), we analyzed our data for the presence of any transcripts of the degenerin/epithelial Na\(^+\) channel supergene family that may contribute to this conductance (53). The human orthologs of the brain Na\(^+\) channel (BNaC1 and BNaC2, alternatively spliced isoform) and the α- and β-subunits of the epithelial Na\(^+\) channel (α-hENaC and β-hENaC) were all identified as absent by the microarray analysis software in each of the normal and tumor samples analyzed (Supplemental Table 1). However, γ-hENaC was positive in each of the samples, with no clear distinction of expression intensity between the normal and GBM samples. The physiological significance of these findings remains to be determined, but variable expression of ENaC subunits has been documented previously (19). Moreover, other subunits belonging to this gene family, such as the acid-sensing ion channel ASIC3 (note: ASIC2a is the same as BNaC1) and δ-hENaC, are not represented on the HuFL GeneChip probe array. Several of these subunits have been shown to co-assemble and form heteromeric cation channels (e.g., Ref. 5).

Although GeneChip technology is highly reliable and sensitive, the transcript amount is not necessarily amplified to the same level as obtained with RT-PCR. Therefore, mRNA present within a cell at very low levels may go undetected. To this end, we wanted to verify the presence of BNaC message in normal and GBM by RT-PCR as was done previously (10). Figure 10 shows that BNaC1 and BNaC2 message was present in both normal samples and in most of the tumor samples, at least for BNaC2. Only tumor sample T2 showed the presence of BNaC1. Direct sequencing of the PCR products confirmed their identity. The results are in contrast to the results of the microarray analyses, at least for BNaC1. The RT-PCR results for BNaC2 are not directly comparable to those of the microarray, because the microarray only has the alternatively spliced version of BNaC2 represented. However, the primers utilized to amplify BNaC2 were complementary to both the parental isoform (accession no. U78181) and the alternatively spliced isoform (accession no. U78180) of this gene, which contains a 38-nucleotide insertion between bp 1296 and 1297. Only a single band was detected for the BNaC2 PCR product, at the appropriate weight for the parental BNaC2, indicating absence of the alternatively spliced isoform in these samples.

DISCUSSION

Many different genes and intracellular signaling pathways that control important aspects of a cell’s life
cycle, including proliferation, differentiation, maintenance of ion and solute homeostasis, and apoptosis, have been implicated in cancer. New techniques, such as cDNA and oligonucleotide microarray analysis, have now been brought to bear on the problems of cancer initiation and progression (2, 3, 20, 27, 30, 38, 46, 49). Using this technology, broad patterns of gene expression and even new individual genes of potential import can be identified in tumor cells vs. normal tissue. Although several reports have appeared that apply DNA microarray analysis to human brain tumors (30, 38), no consistent genetic blueprint defining a given tumor type has emerged. This, undoubtedly, is due to the complex and variable nature of the tumor itself, particularly GBMs, which are notorious in their heterogeneity. Standard neuropathological examination of brain tumors, while useful for classification and staging, has limitations in terms of helping define an appropriate and efficacious treatment regimen. A problem that has not been overcome is that tumors with similar histopathological features often display divergent clinical outcomes despite their stereotypical appearance. In oligodendrogliomas, however, recent data have shown that despite the identical histological ap-
pearence of these tumors, a subset exists that harbors a specific loss at chromosome 1p. Patients with this subset have a significantly better survival after chemotherapy (52). Thus it is reasonable to predict that similar advances will be made by correlating genomic differences in GBMs with patient outcomes using the more precise genetic information provided by GeneChip studies such as reported here.

The heterogeneity of GBMs is undoubtedly a function of their genomic variation and manifests as intertumoral differences with respect to specific markers (e.g., Ref. 7); indeed, there is a high degree of intratumor variability as well (23). Thus, to identify distinct subclasses of any given specific tumor type, the greater the number of samples whose gene expression can be determined, the greater our confidence in predicting outcomes of specific tumor treatment. To that end, we provide new data on gene expression profiling of four unique primary GBM tumors.

Gliomas are primarily derived from astrocytes (>80%), which take a variety of forms and subserve many different functions. Thus, while brain tissue is complex with respect to cell type and specific gene expression, the cells in the tissues used for these studies were primarily astrocytic in origin. It is true that an even more uniform cell population could be obtained from primary astrocytic tumor cell culture. However, cultured cells inevitably alter gene expression as a consequence of the selection pressures of the in vitro environment, thereby adding an artificial complication to attempts to delineate specific tumor-associated genes. To avoid this complication, we chose to restrict our analyses to cells from freshly resected tumors.

Since the objective of this study was to identify genes that play a role in tumorigenesis and altered physiology of tumor cells compared with normal cells, we reasoned that the most relevant findings could be obtained from tissues and cells most closely related to the in vivo situation.

Although only the NMDAR1 (NR1-1), NMDAR2A, NMDAR2B, and NMDAR2C genes were represented on the microarray, there are numerous (note: several NMDAR1 variants, including GRIN 1, NR1-1, NR1-2, etc.) NMDAR human subunit cDNAs that have been cloned, with at least four isoforms of the NMDAR2C gene identified (14). There was measurable gene expression of NMDAR2A and NMDAR2C in all of the normal samples, but the expression levels appeared reduced or absent in the tumor samples (see Fig. 9). Based upon our gene expression, RT-PCR, and immunohistochemical results, the functional NMDA receptor activation measured in normal astrocytes (Fig. 7) was probably due to the combination of 1, 2A, 2B, and 2C and to the presence of other receptor subtypes not detected in our assays. The NMDA receptor subtype of glutamate-gated ion channels possesses high Ca<sup>2+</sup> permeability and can form heteromeric receptors (14, 39, 41). Wang et al. (56) showed that upregulation of NMDA receptors in neurons led to increased NMDA-induced apoptosis. There was no measurable NMDA-activated current in our samples of freshly excised GBM cells. Likewise, the expression levels of the voltage-gated K<sup>+</sup> channel β3-subunit (hKV<sub>β3</sub>) and a number of glutamate transporters, including EAAT2, EAAT3, and GLAST, were depressed in the GBMs compared with normal brain. These observations conform to measured changes in relaxation of outward K<sup>+</sup> currents in GBM cells (Fig. 6) and the reduced ability of human glioma cells to take up glutamate (60). Thus it is tempting to speculate that an apparent lack of these functional receptors may confer some selective advantage for tumor cell survival by failing to recognize apoptotic-inducing signals. Whether a similar apoptotic mechanism is expressed by normal glial cells remains to be determined.

In most of the four primary GBM, two genes, namely, AQP-1 and MIF, appeared overexpressed compared with normal, although there was wide scatter in the results. This finding was confirmed independently by immunohistochemistry, which demonstrated increased

<table>
<thead>
<tr>
<th>Tissue ID</th>
<th>Diagnosis</th>
<th>Aquaporin-1</th>
<th>MIF</th>
<th>NMDAR2A&lt;sup&gt;AR&lt;/sup&gt;</th>
<th>GABA&lt;sub&gt;A&lt;/sub&gt; R&lt;sub&gt;α&lt;/sub&gt;</th>
<th>GABA&lt;sub&gt;B&lt;/sub&gt; R&lt;sub&gt;β&lt;/sub&gt;</th>
<th>Glut R&lt;sub&gt;α3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>GBM</td>
<td>3+/85%</td>
<td>2+/40%</td>
<td>2+/25%</td>
<td>1+/10%</td>
<td>0</td>
<td>2+/15%</td>
</tr>
<tr>
<td>T2</td>
<td>GBM</td>
<td>1+/20%</td>
<td>2+/50%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N2</td>
<td>Normal brain</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N3</td>
<td>Normal brain</td>
<td>0</td>
<td>0</td>
<td>1+/50%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Score is arbitrarily based on intensity/extent of staining. Intensity was graded as 0 = none; 1+ = weak; 2+ = moderate; 3+ = strong, with extent noted by approximate percentage of stained tissue area (0–100%). GBM, glioblastoma multiforme.

Fig. 10. RT-PCR detection of amiloride-sensitive brain Na<sup>+</sup> channels. RT-PCR was performed using RNA isolated from freshly excised GBM and normal human tissue from temporal lobe using primers specific for human brain Na<sup>+</sup> channel 1 (hBNaC1, bp 1091–1537) (4) and human brain Na<sup>+</sup> channel 2 (hBNaC2, bp 1109–1587, plus 3 bp of 3' UTR); (B). Lane 1, normal brain (sample N2); lane 2, normal brain (sample N3); lane 3, GBM (sample T1); lane 4, GBM (sample T2); lane 5, GBM (sample T4); and lane 6, negative control (no RNA template).
AQP-1 and MIF protein expression. A potential overexpression of AQP-1 by tumor cells is particularly exciting, because this gene encodes a well-characterized plasma membrane protein (1). Any plasma membrane protein expressed exclusively by neoplastic cells in situ is of great interest because extracellular epitopes from such proteins provide specific targets for immunotherapy without necessitating access to the cellular interior or altering nuclear processes.

AQP-1 was the first member of a large family of “water-channel” proteins expressed by mammalian cells (1). Aquaporins are expressed by erythrocytes, choroid plexus epithelium, renal tubular epithelium, and capillary endothelium (4, 43). Expression of aquaporin by neoplasms has not been well characterized, although it has been reported in renal carcinomas, erythroleukemia cell lines, and rodent mammary carcinomas (17, 26, 42). Recently, AQP-1 expression was examined in a single rat glioma and two human glioma cell lines (17). The findings were mixed, with no expression in rat and definite expression in only one of the two human cell lines. These findings illustrate one of the problems encountered when studying gene expression in cell lines, i.e., specific gene expression, or lack thereof, could simply be a function of culture-induced phenotypic alterations.

The finding that AQP-1 was upregulated in all four primary human GBMs appears to be more consistent than the findings from cell cultures. The significance of this apparently abnormal upregulation remains unknown, but others have speculated that such channels are important in allowing glioma cells to extrude water and shrink sufficiently in size to allow invasion of the surrounding brain through the extracellular matrix (17). Another possibility is that glioma cells utilize cGMP-mediated ionic signaling that AQP-1 may facilitate (4).

A second gene found to be upregulated in three of the four GBMs compared with the three normal brain specimens was MIF. MIF is a proinflammatory cytokine that induces the expression of many molecules that are involved in cellular inflammatory and immune responses. Monocytes and macrophages are an important source of MIF and release this substance after exposure to bacterial endo- and exotoxins (11). MIF expression is induced by glucocorticoids and is antagonistic to the immunosuppressive effects of glucocorticoids (40). MIF mRNA and protein overexpression have been reported in septic shock (11) and in various cancers, such as melanoma (51), lymphoma (12), prostatic tumors (36, 37, 54), breast carcinoma (8), and hypophysial and pituitary adenomas (55). It has not been reported previously as being overexpressed in malignant brain tumors. Hudson et al. (24) recently reported that MIF functionally inactivates the p53 tumor suppressor. As indicated earlier, nearly half of all primary GBMs have intact p53 genes; so, in addition to other exogenous suppressors of p53 transcriptional activity, e.g., MDM2 (59), the overexpression of MIF may contribute to the development or proliferation of primary malignant brain tumors via inhibition of p53.

In summary, this work provides new insights into the pathogenesis of human brain tumors, particularly GBMs. We have shown that GBMs overexpress the proinflammatory cytokine, MIF, and AQP-1 and underexpress a variety of ion and solute transport related proteins, including NMDAR2A and 2C, GABAA, and AMPA-2 receptors, as well as the glutamate/aspartate transporter II and NHE-1. The mechanism by which these alterations in RNA and protein expression relate to brain tumor development, proliferation, and invasion remains to be determined.

The ability to identify tumor-specific genes brings up a number of exciting possibilities for improved therapy. First, improvements in classification specificity can be made, which in turn can improve the choice of specific therapeutic regimens. Moreover, as in the case of AQP-1 or BNaC, specific gene products may turn out to be accessible targets for new therapies such as immunotherapy. When precise functions for over- or under-expressed genes are determined, new avenues for intervention strategies may emerge. These studies are in their infancy; however, the improved technology employed here shows reasonable promise as our understanding of these deadly tumors increases.

We thank Cathleen Guy and Isabel Quinones for expert assistance in the preparation of this manuscript. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-56095 and DK-52789, National Cancer Institute Award P01-CA-71933, and by the National Institute for Neurological Disorders and Stroke Mentored Clinical Scientist Development Award 1K08-NS-01942 (J. M. Markert). J. K. Bubien is an Established Investigator of the American Heart Association.

REFERENCES


http://physiolgenomics.physiology.org
Differential Gene Expression Profiling in Brain Tumors


44. Nygaard JT, Haugland HK, Kristoffersen EK, Lund-Johansen M, Laerum OD, and Tysnes OB. Expression of an-


http://physiolgenomics.physiology.org