Relationship between Gene Expression and Enhancement in Glioblastoma Multiforme: Exploratory DNA Microarray Analysis¹

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Purpose:

To determine the difference in gene expression between completely versus incompletely enhancing glioblastoma multiforme (GBM).

Materials and Methods:

Gene expression was determined for 52 newly diagnosed GBMs by using DNA microarrays, and the relationship to enhancement pattern and survival was analyzed. This study was approved by the institutional review board and was HIPAA compliant; informed consent was obtained.

Results:

Thirty-eight percent (20 of 52) of GBMs were incompletely enhancing (IE). The expression of eight genes was increased more than twofold in IE GBM when compared with completely enhancing (CE) GBM. Among these were tight junction protein-2 (2.2-fold increase, $P = .019$), and the oligodendrogloma markers oligodendrocyte lineage transcription factor 2 (2.4-fold increase, $P = .029$) and Achaete-scute complex-like 1 (ASCL1; 2.7-fold increase, $P = .023$). The expression of 71 genes showed relative overexpression in CE when compared with IE GBM. These included several proangiogenic and edema-related genes, including vascular endothelial growth factor (2.1-fold, $P = .005$) and neuronal pentraxin-2 (3.0-fold, $P = .029$). Several genes associated with primary GBM were overexpressed in CE tumors, whereas ASCL1, which is associated with secondary GBM, was overexpressed in IE tumors. Many genes overexpressed in IE GBM were associated with longer survival, whereas several genes overexpressed in CE GBM correlated with shortened survival.

Conclusion:

The enhancement pattern divides GBM in two groups with differing prognoses. By comparing gene expression between IE and CE GBMs, it was possible to identify genes that may affect magnetic resonance imaging features of edema and enhancement, and genes whose expression levels are predictive of both improved and shortened survival.

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Glioblastoma multiforme (GBM) has a highly varied appearance at magnetic resonance (MR) imaging. Typically, GBMs are avidly enhancing, with central areas of necrosis and extensive surrounding vasogenic edema (1). In addition to areas of necrosis, which lack enhancement, GBMs may contain regions of nonnecrotic tumor, which also lack enhancement (2). This is not surprising since individual GBM exhibits histologic heterogeneity, and can arise from lower-grade nonenhancing tumors (secondary GBM [3]). Glioma grading is determined on the basis of the most malignant portion of the tumor, and thus, GBM often contains some areas that are histopathologic grade IV, when other regions of the same tumor may show lower-grade histologic results (1). The development of focal areas of contrast material enhancement and necrosis in previously nonenhancing tumors may indicate degeneration to secondary GBM, even though tumor areas without enhancement remain (4). Protein expression profiles have been shown as distinct between enhancing and nonenhancing components of GBM, suggesting a fundamental difference between these tumoral components (5).

Initially, it can be difficult to distinguish nonenhancing tumors from edema at MR imaging, since both are characterized by increased T2-weighted signal intensity. However, there are subtle and sometimes less

subtle differences (2). For instance, edema tends to be brighter on T2-weighted images than does a nonenhancing tumor. Often, the T2-weighted signal intensity of edema approaches that of cerebrospinal fluid. Conversely, a nonenhancing tumor is much less bright than cerebrospinal fluid, and typically is only slightly brighter than gray matter. Furthermore, edema is mostly confined to the white matter, resulting in increased conspicuity of the gray matter–white matter junction. The opposite is true for a nonenhancing tumor, which blurs the gray matter–white matter junction as this interface becomes infiltrated by the tumor. Additionally, a nonenhancing tumor may cause the cortex to be focally thickened, which is not a finding seen with peritumoral edema. Since GBMs are highly infiltrative, regions of edema may contain small numbers of tumor cells that can only be detected histologically (1). However, these are not of a concentration that results in mass effect and architectural distortion that are visible at MR imaging. Thus, while the sensitivity for a microscopic nonenhancing tumor by using MR imaging is limited, there are specific imaging features that allow bulk noncontrast-enhancing tumors to be confidently distinguished from edema with a high interobserver reliability (2).

Evidence suggests that the enhancement pattern of GBM affects prognosis (2). Therefore, in this preliminary study, we sought to determine the difference in gene expression between GBM with complete versus incomplete enhancement.

Advances in Knowledge

- In this preliminary study, several genes appear to be differentially expressed between incompletely versus completely enhancing glioblastoma multiforme (GBM).
- Some genes found to be overexpressed in incompletely enhancing (IE) GBM are associated with secondary GBM, oligodendroglioma differentiation and longer survival, whereas some genes overexpressed in completely enhancing (CE) GBM are associated with shortened survival.

Implications for Patient Care

- Patient prognosis may be affected by the enhancement pattern of GBM, and by the expression levels of particular genes.
- The gene expression of some therapeutic targets currently being developed is substantially different between CE and IE GBM; therefore, the enhancement pattern may predict a better or worse response to these new therapies.

Materials and Methods

Patient Database

A total of 52 patients was selected from our institution's Neuro-oncology Clinic database. All patients participating in this study signed institutional review board consent, and data acquisition was performed in compliance with all applicable Health Insurance Portability and Accountability Act regulations. Inclusion criteria for patients were newly diagnosed pathologically confirmed GBM, MR imaging performed prior to tumor resection, and tissue available for microarray analysis. All patients that met these three criteria were included in the study. No patients were lost to follow-up. Other than not meeting the inclusion criteria, there were no exclusion criteria. A higher percentage of completely enhancing (CE) tumors (15 of 32, 47%) underwent gross total resection, compared with five (25%) of 20 for incompletely enhancing (IE) tumors, whereas IE tumors were more often treated with adjuvant chemotherapy

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Abbreviations:

ASCL1 = Achaete-scute complex-like 1
 CE = completely enhancing
 GBM = glioblastoma multiforme
 IE = incompletely enhancing
 MMP = matrix metalloproteinase
 OLIG2 = oligodendrocyte lineage transcription factor 2
 VEGF = vascular endothelial growth factor

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(16 [80%] of 20 for IE tumors, 15 [47%] of 32 for CE tumors). Roughly equal percentages of patients in the IE and CE groups were treated with steroids at the time of tumor resection (15 [47%] of 32 for CE tumors, nine [45%] of 20 for IE tumors). All but six patients were treated with radiation therapy (28 [88%] of 32 for CE tumors, 19 or [95%] of 20 for IE tumors). All deceased patients showed neurologic signs of progression and there were no patients who died from disease or other causes not related to their brain tumor. Survival assessment was last done in March 2007. All patients received a histopathologic diagnosis of GBM by a neuropathologist (P.S.M.) on the basis of the modified World Health Organization classification system. Forty-four of 52 patients have died. The median follow-up time for the eight surviving patients is 2097 days (range, 903–2689 days); the median time to survival for the 44 nonsurviving patients is 334 days (range, 56–2305 days).

MR imaging sequences were performed with a 1.5-T imager and included, in most cases, sagittal T1-weighted (repetition time msec/echo time msec, 400–550/14; section thickness, 5 mm), axial T1-weighted (400/15; section thickness, 3 mm), T2-weighted fast spin-echo (4000/126–130; section thickness, 3 mm), proton density (4000/13–15; section thickness, 3 mm), and gadodiamide (Omniscan, 10–20 mL; Nycomed Amersham, Princeton, NJ)-enhanced axial and coronal T1-weighted (400/15; section thickness, 3 mm) images with a field of view of 24 cm² and a matrix size of 256 × 256. All images contained at least T1-weighted nonenhanced and contrast-enhanced and T2-weighted images.

An IE tumor was defined as a GBM that contained a clearly defined region of T2-weighted hyperintensity, less than the intensity of cerebrospinal fluid, corresponding to a region of T1-weighted hypointensity which was associated with mass effect and architectural distortion, including blurring of the gray matter–white matter junction, and/or expansion of the deep nuclei, and which showed no obvious enhancement. GBMs that lacked

any such regions were scored as CE. Images were scored as either IE or CE by a board-certified, trained neuroradiologist (W.P.). To confirm that IE tumors could be reliably distinguished from CE tumors, two additional reviewers independently scored 45 of 52 MR images (the other seven were no longer available). The two additional reviewers (N.S. and J.P.V., with 8 and 14 years experience with neuroradiology, respectively) both have interpreted hundreds of brain tumor MR images yearly. All readers were blinded to all clinical data, including outcome. Cases with disagreement (three of 45) were resolved by consensus.

Microarray Data

Complete RNA samples were extracted from the tumor samples, and processed by using a kit (RNeasy mini-kit; Qiagen, Valencia, Calif). Complementary DNA and complementary RNA were generated by using standard protocols (6). All samples were processed, scanned, and quality-checked as previously described by using gene array equipment (Affymetrix, Santa Clara, Calif) (7). A total of 22 215 probe sets for approximately 14 500 genes were analyzed for differences in expression levels between IE and CE GBMs. Real-time polymerase chain reaction was used to confirm vascular endothelial growth factor

Figure 1

Figure 1: (a, c) T1-weighted gadolinium-enhanced axial images with (b, d) corresponding T2-weighted images of CE GBM show medial margin of enhancing tumor (arrowhead). Note surrounding hyperintense T2-weighted signal abnormality is nearly as bright as cerebrospinal fluid and respects cortical margin (arrows), consistent with vasogenic edema.

(VEGF) expression levels on tumor samples. The VEGF-to-actin expression ratios were determined for 25 representative samples. There was a good correlation between the two methods, with a Pearson correlation coefficient of 0.92.

Statistical Analysis

The Fleiss generalized κ statistic was used to assess interobserver agreement for interpretation of MR images across multiple raters (8). The Kaplan-Meier

method was used to estimate the survival distributions (9). To assess how each covariate affects survival, we used univariate Cox proportional hazard models (10). Hazard ratios correspond to risk of death compared with baseline level, and thus, an increased hazard ratio implies an unfavorable prognosis. For each covariate, the transformed hazard ratio (Z score) and the associated P value were examined. For all analyses, a P value of less than .05 was

considered significant. Statistical analyses were carried out with freely available online software packages (R, Vienna University of Economics and Business Administration, Vienna, Austria, <http://www.r-project.org>; and dChip, Harvard School of Public Health, Boston, Mass, <http://www.dchip.org>). Univariate differences in covariates were quantitatively studied across categorical groupings by using a combination of fold change and two-sample unpaired t tests. All reported correlation coefficients are Pearson product-moment correlation coefficients. The associated P values are from a t distribution. The false discovery rate was determined by using a permutation-based algorithm with software (dChip) with 300 permutations. The Fisher exact test was used to test gene expression data in which multiple probe sets showed a greater than twofold change at the 95% confidence level.

Results

IE Gene Expression in GBM

Of 52 tumors, 20 (38%) were classified as IE tumors at contrast-enhanced MR imaging. There was excellent interobserver agreement in the scoring of IE and CE tumors, with a κ statistic of 0.908 ($P < .000001$) (8). Examples of CE (Fig 1) and IE (Figs 2, 3) GBMs are shown. Note that nonenhancing areas of tumor are mildly T2-weighted hyperintense, and blur the gray matter–white matter boundary, in contrast to vasogenic edema, which has a T2-weighted signal intensity approaching that of cerebrospinal fluid and respects the cortical ribbon (“fingers of edema”) (1). Median patient survival for IE tumors was 663 days (mean, 955 days $\pm$ 187 [standard error of the mean]) versus 325 days (mean, 501 days $\pm$ 92) for CE tumors ($P = .053$), in agreement with a previous report (2). The higher P value of our report is consistent with fewer subjects. The Kaplan-Meier plot of survival for patients with IE versus CE tumor is shown in Figure 4.

Differential Gene Expression

A total of 104 probe sets for 79 genes were differentially expressed between

Figure 2

Figure 2: (a, c) T1-weighted gadolinium-enhanced axial images and (b, d) corresponding T2-weighted images of IE GBM show area of hyperintensity (arrow). (b) T2-weighted hyperintensity (arrow) outside region of enhancement extends to brain surface. gray matter–white matter junction is obliterated and T2-weighted signal intensity is much less than that of cerebrospinal fluid. In contrast, there is higher T2-weighted signal abnormality along medial margin of tumor (arrowhead), consistent with edema. (d) Entire right subfrontal region affected by tumor, which lacks contrast enhancement, as seen on c. Note complete lack of visible gray matter–white matter differentiation and mass effect with leftward bowing of midline associated with mild T2-weighted hyperintensity (arrow). Regions of higher T2-weighted signal abnormality consistent with edema are present in left inferior frontal lobe (arrowhead).

the two groups by a factor of two or greater. The false discovery rate, made on the basis of a permutation-based algorithm, was 16.3%. Eight genes demonstrated an increase in expression by more than a factor of two ($P < .05$) in at least one probe set comparing IE and CE GBMs (Table 1). One of these was tight junction protein-2 (also known as zonula occluden-2), which is a component of the brain endothelial tight junction (11), and acts to maintain the blood-brain barrier. Other tight junction-related proteins such as claudin-1, vinculin, occludin, plakoglobin, and various catenins did not correlate with the IE group. The oligodendrogloma markers oligodendrocyte lineage transcription factor 2 (OLIG2) and Achaete-scute complex-like 1 (ASCL1) were at increased levels in the IE group. ASCL1 showed multiple probe sets with a greater than twofold change at the 95% confidence interval. Combining these data by using the Fisher exact test resulted in a P value of less than 2.63×10^{-10} for ASCL1. Since oligodendrogloma-associated gene expression was increased in the IE group when compared with the CE group, we analyzed the pathologic specimens for substantial oligodendrogloma histologic evidence. We found four of 20 IE tumors and three of 32 CE tumors had a substantial oligodendrogloma component. Although the percentage of IE tumors with oligodendrogloma features was higher than in the CE tumors, many IE tumors lacked such a component. However, mean OLIG2 expression was significantly increased in tumors with oligodendrogloma features ($n = 1560$) versus those without ($n = 322$, $P = .006$).

Seventy-one genes were overexpressed by more than a factor of two ($P < .05$) in CE GBM when compared with IE (Table 2). Several of these genes are associated with the hypoxia-angiogenesis-edema pathway in GBM, most notably VEGF (12,13). Matrix metalloproteinase (MMP) 7, which is expressed by gliomas and is associated with increased invasion in an in vitro stomach cancer model (14,15), also was enriched in CE tumors when compared with IE tumors. Other extracel-

Figure 3

Figure 3: (a, c) Contrast-enhanced T1-weighted images with (b, d) corresponding T2-weighted images of IE GBM. (a) Biopsy site (arrowhead) is clearly visible in region of nonenhancing, mild hyperintensity (arrow). This unusual case of GBM showed no substantial contrast enhancement. (c) Intraventricular mass with central enhancement. Enhancing component (curved arrow) is surrounded by nonenhancing T2-weighted signal change (arrow), which is less intense than adjacent cerebrospinal fluid (arrowhead).

lular matrix modifiers in previously proposed tumor invasion mechanisms, such as A Disintegrin And Metalloproteinase metalloproteinase with thrombospondin (ADAMTS) type 1 motif-like-4 ($P = .82$) and ADAMTS 5 ($P = .18$), and other MMPs, did not significantly differ between IE and CE groups (16–18). Decorin showed multiple probe sets with a greater than twofold change at the 95% confidence interval resulting in a P value of less than 2.92×10^{-7} by using the Fisher exact test. Adrenomedullin, interleu-

kin-8, neuritin-1, tenascin C, caveolin 1, caveolin 2, transgelin, and thrombospondin-1 also were seen at elevated levels in CE tumors when compared with IE tumors (Table 2).

Gene Expression and Survival

Cox regression analyses of gene expression and survival were conducted for those probe sets with significantly increased or decreased expression in GBM listed in Tables 1 and 2 ($n = 104$). Those with significant correlation with survival are listed in Table 3. Of the

eight genes overexpressed in IE GBM when compared with CE GBM, four had at least one probe set that was significantly correlated with longer survival: astrotactin-2, Usher Syndrome 1C, inhibitor of DNA binding 4, dominant negative helix-loop-helix protein, and brevican. Three genes (OLIG2, ASCL1, and tight junction protein-2) had probe sets that showed a trend of longer survival (all $P < .1$). Many of the genes overexpressed in IE tumors correlated with longer survival; conversely, a few of the genes overexpressed in CE tumors correlated with or trended to shortened survival. For instance, brevican, whose gene expression was increased in IE tumors, was significantly correlated with increased survival in all three probe sets ($Z = -2.37$ to -2.71 ,

$P = .0068$ – $.018$). For below-median expression of brevican, mean survival was 430 days (median, 308 days $\pm$ 75) and for above-median expression of brevican, mean survival was 924 days (median, 619 days $\pm$ 167). Therefore, above-median levels of brevican expression corresponded to approximately a doubling of the survival time. Tight junction protein-2 showed a trend of being positively correlated with survival but did not reach significance ($P = .067$). Similarly, there was a trend for OLIG2 ($P = .075$). For genes enriched in CE tumors, decorin showed a correlation with shorter survival for all three probe sets. Above-median levels of decorin expression was associated with a mean survival of 508 days (median, 352 days $\pm$ 506) and below-median decorin expres-

sion corresponded to a mean survival of 847 days (median, 504 days $\pm$ 164). MMP7 expression showed no significant relationship with survival ($P = .45$), even though its gene product has been linked to tumor invasiveness (14,15). We also performed a survival analysis in an unsupervised manner. We assessed genes associated with adverse versus favorable outcomes (by using the Cox proportional hazard model) and determined whether these genes were associated with IE or CE groups. We found that brevican ($Z = -2.71$, $P = .0068$) and astrotactin-2 ($Z = -2.19$, $P = .028$) were associated with IE tumor and longer survival, whereas decorin ($Z = 3.28$, $P = .002$) was associated with CE tumor and shorter survival, confirming the supervised analysis.

To further characterize the relationship between survival and gene expression, Kaplan-Meier survival curves for all GBMs ($n = 52$) with above- and below-median gene expression levels were calculated (Fig 4). As expected, the P values in the Kaplan-Meier curves appear less significant than do the P values in Table 3, where continuous instead of categorical variables are used in the Cox regression analysis. There was a significant association between increased brevican expression and longer survival ($P = .0084$). Similar curves were generated for ASCL1 ($P = .088$). In contrast to genes overexpressed in IE, increased levels of the gene for decorin were associated with shorter survival ($P = .081$).

Discussion

The presence of regions of nonenhancing tumor in GBM is associated with improved survival and can be determined from standard MR imaging with high interobserver reliability (2). Previous work has shown differences in protein expression patterns between enhancing and nonenhancing portions of individual GBMs on the basis of mass spectrometry, although differences in individual proteins were not identified in that study (5). In the current report, we have compared gene expression in IE and CE GBMs to screen for molecular

Figure 4

Figure 4: Kaplan-Meier survival curves for (a) IE versus CE GBM ($P = .053$), (b) brevican ($P = .0084$), (c) ASCL1 ($P = .088$), and (d) decorin ($P = .081$). Cross-hatches represent censored data. (a) Survival for patients with IE GBM ($n = 20$, solid line) and patients with CE GBM ($n = 32$, dashed line). (b–d) Patients with above-median expression (solid line) of corresponding gene are compared with below-median expression (dashed line). Note that patients with IE GBM have better survival curve. High levels of brevican and ASCL1 expression are associated with better survival rate; high levels of decorin expression are associated with shortened survival rate.

Table 1

Probe Sets Enriched in IE GBM

Gene	Probe Set*	Fold Change	P Value
Brevican	1	2.6	.004
	2	1.8	.044
	3	1.8	.057
ASCL1	1	2.7	.023
	2	2.7	.039
	3	2.3	.025
OLIG2	1	2.4	.029
	2	1.7	.039
Tight junction protein-2	1	2.2	.019
	2	2.0	.0071
	3	1.6	.10
Patched homolog	1	2.1	.047
	2	1.2	.2
	3	1.1	.39
Usher syndrome 1C	1	2.1	.043
	2	1.6	.039
	3	1.4	.01
Inhibitor of DNA binding 4	1	2.1	.023
	2	1.9	.018
	3	1.3	.24

* Genes with at least one probe set with a greater than twofold increase and a *P* value of less than .05 are shown.

candidates that may underlie this important difference. We found that probe sets for eight genes were enriched in IE GBM and probe sets for 71 genes were enriched in CE GBM, with a false discovery rate of 16.7% at this level of differential expression (two-fold).

Histopathologically defined oligodendrogloma component correlates with IE GBM and is associated with improved survival (2). The OLIG2 gene product is abundant in oligodendroglial foci of GBM (19) and both OLIG2 and ASCL1 are known to be associated with oligodendrogloma differentiation (19–21). We found that the genetic signature of

Table 2

Selected Probe Sets Enriched in CE GBM

Gene	Probe Set	Fold Change	P Value
Neuronal pentraxin II	1	3.0	.029
Tenascin C	1	2.9	.037
	2	1.6	.078
Matrix Gla protein	1	2.4	.013
Adrenomedullin	1	2.3	.0078
MMP7	1	2.1	.019
VEGF	1	2.1	.005
	2	1.8	.058
	3	1.7	.07
	4	1.5	.25
Neuronal pentraxin receptor	1	2.0	.048
	2	1.0	.368
Neuritin-1	1	2.1	.00024
Decorin	1	3.6	.022
	2	2.4	.03
	3	2.4	.033
Caveolin 1	1	2.6	.0057
	2	2.1	.0088
Caveolin 2	1	2.2	.026
	2	2.1	.013
Interleukin-8	1	5.0	.035
	2	3.4	.016
Neuronal pentraxin receptor	1	2.0	.049
Thrombospondin-1	1	3.6	.0084
	2	3.3	.02
	3	1.8	.02
	4	1.2	.12
	5	1.1	.7
Transgelin	1	2.8	.012

Note.—For a complete list of all genes, please refer to the Web site.

IE tumors also suggested an oligodendrogloma component, as OLIG2 and ASCL1 were enriched in IE tumors. Conversely, the gene expression for caveolin 1, which is found in higher levels in astrocytoma when compared with oligodendrogloma (22), was more highly expressed in CE tumors. Our results also agree with previous work that found a close association between OLIG2 and ASCL1 expression in a hierarchic clustering analysis of GBM (23).

Of the eight genes overexpressed in IE versus CE GBMs, many were correlated with longer survival. The correlation between increased brevican expression and survival was surprising, because others have suggested that brevican expression is linked to shorter survival (24). One potential important difference between these

studies is that ours is restricted to GBM and does not include lower-grade tumors. Thus, it could be the association of brevican with higher tumor grades that led to effect on survival, whereas this trend may be reversed in a particular tumor grade.

Whereas only eight genes were increased in expression by more than twofold in IE GBM, there were 71 genes overexpressed in CE GBM. None of the genes enriched in CE GBM were correlated with better survival. Several trended to or significantly correlated with shorter survival, including decorin. Although it has been suggested that decorin inhibits glioma progression (25), a more recent report has shown that decorin promotes survival of human glioblastoma cells in culture following oxygen and glucose deprivation

(26). Thus, decorin levels may promote tumor growth in hypoxic conditions in vivo as well.

The function of several of CE-enriched gene products are known to be interrelated, as they are associated with the hypoxia-edema-angiogenesis pathway. For instance, VEGF is known to be a key component of the angiogenic pathway induced by hypoxia and is also a potent permeability factor that causes edema (12,13). It has also been shown that inhibiting VEGF reduces edema and tumor burden in GBM patients (27,28). VEGF has been shown to be predictive of survival in GBM on the basis of edema grade (29). The association between enhancement and increased VEGF levels may help explain two previous observations: (a) regions of highly necrotic and enhancing tumor appear more susceptible to anti-VEGF therapy than nonenhancing tumor, even in the same patient; and (b) progressive disease in GBM patients treated with anti-VEGF therapy tends to be less enhancing and less necrotic than tumor prior to therapy (27,30).

Adrenomedullin and interleukin-8 are other molecules linked to hypoxia-induced angiogenesis that were also higher in the CE group. Other genes

associated with hypoxia that were increased in the CE group include neurtin-1, which is induced by hypoxia (31), and decorin (as discussed above). Hypoxia leads to necrosis, with breakdown of the blood-brain barrier, which results in enhancement and edema. Therefore, the presence of hypoxic conditions may be a fundamental difference between IE and CE tumors.

We found that genes for interleukin-8 and VEGF were overexpressed in CE tumors when compared with IE tumors. Interleukin-8 and VEGF have been shown to be attractants for marrow stromal cells, which show tropism for gliomas (32). Since anti-GBM therapies that make use of marrow stromal cells are currently being investigated, upregulation of VEGF and interleukin-8 in CE compared with IE tumors may have important implications for response to this therapeutic approach. The same is true for tenascin C, which has successfully been used as a target for glioma therapies in a mouse model (33). Therefore, IE tumors may be less responsive to these therapies when compared with CE tumors, potentially another important distinction between the two groups.

MMPs are a family of enzymes that

degrade the extracellular matrix and disrupt the blood-brain-barrier. Tight junction proteins, occludin, and claudin-5, which form the endothelial barrier of the blood-brain barrier, are vulnerable to attack by MMPs. MMP7 has been shown to be expressed in GBM and has been produced by glioma tumor cells in vitro (14). In addition, evidence implicating MMP7 in tumor invasiveness has been reported for stomach cancer cells in vitro (34). We found that expression of MMP7 was elevated in CE GBM. Conversely, tight junction protein-2 expression was increased in IE GBM. This suggests the possibility that the balance between MMPs and tight junction proteins may be responsible for maintaining the blood-brain-barrier in GBM. A shift in this balance may promote a more aggressive phenotype associated with edema, invasiveness, and contrast enhancement.

When analyzing the changes in gene expression between IE and CE tumors, we noted that several genes associated with primary GBM are higher in CE tumors, whereas at least one gene associated with secondary GBM is higher in IE tumors. Thus, ASCL1, which is upregulated in 88% of secondary GBM and only 33% of primary GBM (35), was higher in IE tumors. Conversely, probe sets for caveolin 1, caveolin 2, interleukin-8, transgelin, and thrombospondin-1, which are all upregulated in primary GBM (36), were higher in CE tumors. Furthermore, none of the eight genes higher in IE tumors were overexpressed in primary GBM, and none of the 71 genes higher in CE tumors were overexpressed in secondary GBM. These data suggest that the presence of a non-enhancing tumor may help in distinguishing primary from secondary GBM. Distinguishing between the two cannot be done reliably by using histopathologic analysis alone. Thus, this finding, if verified, may have important implications for GBM therapy because it has been suggested that angiogenic pathways are markedly different in primary and secondary GBMs and may require different antiangiogenic treatment strategies (37).

Our report was an exploratory anal-

Table 3

Cox Regression Analysis of Selected Genes and Survival in GBMs

Gene	Probe Set	Survival Rate*	Z Score	P Value
Astrotactin-2	1	Increased	-2.19	.028
Usher syndrome 1C	1	Increased	-2.14	.033
Inhibitor of DNA binding 4	1	Increased	-2.73	.0063
	2	Increased	-2.13	.033
Brevican	1	Increased	-2.71	.0068
	2	Increased	-2.52	.012
	3	Increased	-2.37	.018
OLIG2	1	Increased	-1.78	.075
Tight junction protein-2	1	Increased	-1.83	.067
ASCL1	1	Increased	-1.75	.081
	2	Increased	-1.67	.095
	3	Increased	-1.64	.1
Decorin	1	Decreased	3.28	.001
	2	Decreased	3.25	.0011
	3	Decreased	2.96	.0031
Matrix Gla protein	1	Decreased	1.92	.055
Neurtin-1	1	Decreased	1.84	.066

* Indicates the effect of increasing the expression level of this gene on patient survival.

ysis, and therefore had several important limitations. For instance, being able to assign every tissue sample from the IE group as taken from regions of nonenhancement by using MR imaging would be necessary to optimize the signal-to-noise ratio for the samples. Comparing regions of enhancing and nonenhancing tumor from the same patient also would help confirm the genetic differences between the two regions in a single tumor. This would complement work showing that protein expression profiles are varied between these regions (5). Additional comparisons between enhancing regions from CE and IE tumors would help determine if there are differences in gene expression even between regions of tumors with similar appearance on MR images. There are more sophisticated measures of enhancement available now, compared with when we began our data acquisition. For instance, the transfer constant of contrast material into the extracellular space could be used as a more objective measure of blood-brain barrier breakdown. Additionally, cerebral blood volume may be a better predictor of outcome than contrast enhancement (38), and thus, the relationship between gene expression and cerebral blood volume would be of great interest.

There also are many limitations inherent with the use of DNA arrays. DNA arrays often have multiple probe sets for a particular gene, which may yield varying results, owing to differences in sensitivity and specificity for the particular gene by the different probe sets. Another limitation of DNA arrays is the "multiple hypothesis testing" caveat: Given the large number of tests (eg, in our study we analyzed 22 215 probe sets for approximately 14 500 genes for changes in expression between IE and CE tumors), it is possible that some tests will show significance with a *P* value of less than .05 on the basis of chance alone. Some statistical adjustments, such as the Bonferroni method that reduce type I error (false positive), also dramatically increase type II error (false negative). Therefore, the goal is to balance type I and type II errors. Previous studies have

demonstrated that increasing the magnitude of change accepted as significant is more effective than using increased stringency for the *t* statistic (39). The false discovery rate is an estimation of the chance that a positive test will result from a type I error. The use of a larger sample size would help to reduce the false discovery rate of our study.

In summary, our results suggest that DNA microarrays may be used to identify changes in gene expression that correlate with specific MR imaging features, and which might affect patient survival. The pattern of enhancement may help distinguish secondary from primary GBM, and may be important in the future for predicting response to therapies tailored to specific GBM subtypes.

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