Relationship between Survival and Edema in Malignant Gliomas: Role of Vascular Endothelial Growth Factor and Neuronal Pentraxin 2

Marc R.J. Carlson,[1] Whitney B. Pope,[2] Steve Horvath,[1,3] Jerome G. Braunstein,[1] Phioanh Nghiemphu,[4] Cho-Lea Tso,[1] Ingo Mellinghoff,[5] Albert Lai,[4] Linda M. Liau,[6] Paul S. Mischel,[7] Jun Dong,[1] Stanley F. Nelson,[1] and Timothy F. Cloughesy[4]

Abstract

Purpose: Vascular endothelial growth factor (VEGF) is a potent mediator of vascular permeability. VEGF inhibition reduces edema and tumor burden in some patients with malignant glioma, whereas others show no response. The role of VEGF expression in edema production and the relationship to survival is not well understood.

Experimental Design: Using DNA microarray analysis, we examined VEGF and related gene expression in 71 newly diagnosed malignant gliomas and analyzed the relationship to edema and survival.

Results and Conclusions: VEGF expression was predictive of survival in tumors with little or no edema [Cox proportional hazard model, 6.88; 95% confidence interval (95% CI), 2.61-18.1; $P < 0.0001$], but not in tumors with extensive edema. The expression of several proangiogenic genes, including *adrenomedullin* (correlation coefficient, 0.80), *hypoxia-inducible factor-1A* (0.51), and *angiopoietin-2* (0.44), was correlated with VEGF expression (all with $P < 0.0001$), whereas that of several antiangiogenic genes was inversely correlated. The expression of six genes was increased greater than 3-fold in edematous versus nonedematous tumors in the absence of increased VEGF expression. The most increased, *neuronal pentraxin 2* (NPTX2, 7-fold change), was predictive of survival in tumors with the highest levels of edema, in contrast to VEGF (hazard ratio, 2.73; 95% CI, 1.49-5.02; $P = 0.049$). NPTX2 was tightly correlated with expression of the water channel *aquaporin-3* (0.74, $P < 0.0001$). These results suggest that there are both VEGF-dependent and VEGF-independent pathways of edema production in gliomas and may explain why edema is not reduced in some patients following anti-VEGF treatment.

Malignant (grade III and grade IV) gliomas are heterogeneous tumors in both appearance and in gene expression (1, 2). Prognosis for these tumors remains poor. Survival of patients with newly diagnosed glioblastoma multiforme (GBM) has been reported to be 86% at 6 months, 61% at 1 year, and 26% at 2 years (3).

Several imaging features of GBM, including edema, negatively correlate with survival (4). Edema is also associated with shortened survival in grade III gliomas. The vascular endothelial growth factor (VEGF) is a key mediator of tumor angiogenesis and edema. Multiple VEGF isoforms are generated by alternative splicing, although the first five exons are conserved across all variants (5). Through the activation of receptor tyrosine kinases, VEGF impacts endothelial cell permeability, activation, survival, proliferation, invasion, and migration, all of which play a significant role in tumor progression. VEGF is also a potent mediator of vascular permeability, being 50 times more effective than histamine (6). Malignant gliomas express both VEGF and its receptors (7), and glioblastoma cell lines have been shown to secrete VEGF (8). Anti-VEGF therapy has been shown to improve survival in patients with metastatic colorectal cancer, breast cancer, and lung cancer (6).

Microarray analysis offers the ability to assess gene expression and to correlate these data with imaging features, including edema. Although many glioma patients show dramatic reduction in tumor and edema following anti-VEGF therapy, other patients show no response (9, 10). Therefore, we sought to use microarray analysis to investigate the possibility of VEGF-independent pathways linked to edema and survival in patients with malignant glioma.

Materials and Methods

Patient database. A total of 71 patients with newly diagnosed malignant gliomas were selected from the University of California at

Authors' Affiliations: Departments of [1]Human Genetics, [2]Radiological Sciences, [3]Biostatistics, [4]Neurology, [5]Molecular and Medical Pharmacology, [6]Neurological Surgery, and [7]Pharmacology and Laboratory Medicine, University of California at Los Angeles and David Geffen School of Medicine at the University of California at Los Angeles, Los Angeles, California

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Note: M.R.J. Carlson and W.B. Pope contributed equally to this work.

Requests for reprints: Whitney B. Pope, Department of Radiological Sciences, University of California at Los Angeles, 10833 LeConte Avenue, BL-153/CHS, Los Angeles, CA 90095-1271. Phone: 310-794-7923; Fax: 310-206-5958; E-mail: wpope@mednet.ucla.edu.

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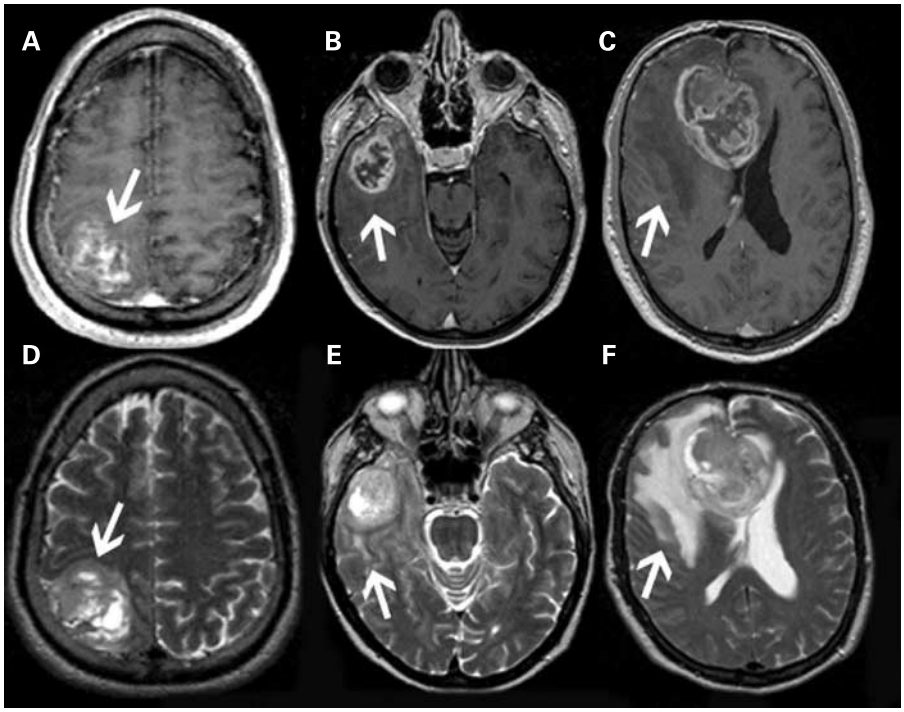

Fig. 1. Grading edema in patients with GBM. T1 postcontrast (*A-C*) and T2-weighted (*D-F*) images show tumors with edema grade 0 (*A* and *D*; no detectable edema), edema grade 1 (*B* and *E*; edema extending up to 2 cm beyond the tumor margin) and edema grade 2 (*C* and *F*; edema extending more than 2 cm beyond the tumor margin). Arrows mark the edge of edema.

Los Angeles Neuro-oncology Clinic database. All patients participating in this database have signed institutional review board consent. Patients with both magnetic resonance imaging (MRI) scans and tissue available for microarray analysis were used. All GBM patients received radiation therapy. The majority were also treated with chemotherapy. No patients were treated with anti-VEGF therapy. Most grade III gliomas were treated with radiation therapy. Survival assessment was last done in November 2005. Histologic diagnosis and MRI data acquisition for this data set have been published previously (4). Briefly, for grading of edema, no detectable edema is assigned grade 0, edema extending up to 2 cm beyond the tumor margin is assigned grade 1, and edema extending more than 2 cm beyond the tumor margin is assigned grade 2. Of the 71 patients, 52 were diagnosed with GBM, and 19 with grade III gliomas. Grade III gliomas consisted of seven anaplastic astrocytomas, six anaplastic mixed gliomas, and six anaplastic oligodendrogliomas. Of the patients with grade III tumors, 6 out of 19 have died. For the GBM patients, 39 out of 52 have died. For the grade III tumor survivors, the average follow-up time is 1,436 days, with a range of 175 to 2,949 days. For GBM survivors, the average follow-up time is 888 days, with a range 116 to 1,670 days.

Microarray data. Using the TRIzol reagent (Invitrogen Life Technologies), total RNA was extracted from the tumor samples, and processed using an RNeasy column (Qiagen). cDNA and cRNA were generated using standard protocols (11). All samples were processed, scanned, and quality checked as previously described (12).

For analysis of gene expression measures, affymetrix data were normalized using the justRMA method provided by the Bioconductor group (13). Most samples were processed on the HG-U133A arrays. Recent samples were processed on the HG-U133 2.0 arrays. These two array pools were normalized as above. It was observed that the average brightness of these two pools was similar but not identical. Thus, the smaller pool was uniformly scaled to match the average brightness of the median brightness array from the larger group. Because the HG-U133A platform is a subset of the HG-U133 2.0 platform, only data from probe sets that were shared between the two platforms were used.

Probe sets designed to detect VEGF expression were assessed for their ability to represent VEGF expression. All four representative probe sets behaved similarly for all tests. The probe set with the greatest sensitivity as evidenced by the largest coefficient of variation was selected to represent the results presented here.

To confirm VEGF expression levels, 25 tumor samples spanning the range of VEGF expression values were analyzed with real-time PCR. The ratio of VEGF to actin was calculated, and the Pearson correlation coefficient was determined.

Statistical analysis. The Kaplan-Meier method was used to estimate the survival distributions (14). Log-rank tests were used to test the difference between stratified survival groups. To assess which covariates affect survival, we used multivariable Cox proportional hazard models (15). Hazard ratios correspond to risk of death, and thus, an increased hazard ratio implies a worse prognosis. The proportional hazard assumption was tested using scaled Schoenfeld residuals (16). For each covariate, the relative hazard rate and the associated P value were examined. For all analyses, a P value of <0.05 was accepted as significant. Statistical analyses were carried out with the freely available software packages R.[8] The relationship between edema and VEGF expression levels was cross-validated with the use of a Kruskal-Wallis test. Univariate differences in covariates were tested across categorical groupings by using the Kruskal-Wallis test (17). Distributions of covariates across categorical groupings were visualized with box plots.

Results

Verification of VEGF expression levels. Real-time PCR was used to confirm VEGF expression levels on representative tumor samples. The VEGF-to-actin expression ratios were determined

[8] http://cran.r-project.org/

for 25 representative samples (out of 71 total). There was a good correlation between the two methods, with a Pearson correlation coefficient of 0.92.

Expression of VEGF in grade III and IV gliomas. VEGF expression was highly variable in GBM. The range of VEGF expression was 78 to 5,584 (n = 52), with a mean of 1,288 and SD of 1,206. For grade III gliomas (n = 19), the mean VEGF expression was 313 with a SD of 541. Thus, VEGF expression was ~4.1 times higher in GBM than grade III gliomas (P < 0.0001), in agreement with a previous report (18). For all malignant glioma, the median expression of VEGF was 508. Of the tumors with greater than this level of VEGF expression (n = 35), 33 were GBM.

Correlation of VEGF expression with edema. Figure 1 illustrates grading of edema, with examples of edema grades 0, 1, and 2 categorized per Materials and Methods. For grade III tumors, 79% were edema grade 0, 16% were edema grade 1, and 5% were edema grade 2. For GBM, those percentages were 23%, 23%, and 54%, respectively. For all malignant gliomas, VEGF expression was correlated with edema (Kruskal-Wallis test, P = 0.0011). When subdivided into edema grades, the mean expression of VEGF in grade 0 (n = 27) was 479 (SD, 582; range, 64-1,873), mean expression of VEGF in grade 1 (n = 15) was 1,373 (SD, 1182; range, 84-3,605), and mean expression of VEGF in grade 2 (n = 29) was 1,359 (SD, 1,353; range 94-5,584). Note that some tumors had high levels of edema, without elevated VEGF expression. There was a large range of VEGF expression within the different edema subgroupings, particularly for tumors with grade 2 edema (Fig. 2).

VEGF expression and survival by edema grade. Next, we looked at survival curves using the Kaplan-Meier method for the different grades of edema (Fig. 3). We found that VEGF expression predicted time to survival in malignant gliomas when the edema grade 2 group was excluded (P < 0.001), that is, when there was little or no edema. This also was the case for GBM (P = 0.04; Fig. 4). For edema grade <2, the Cox proportional hazard model was 6.88 [95% confidence interval (95% CI), 2.61-18.1; P < 0.0001]. For edema grade = 2, the Cox proportional hazard ratio was not significant. For all gliomas, irrespective of edema status, the Cox proportional hazard ratio was 4.34 (95% CI, 2.24-8.43; P = 0.0007). For edema grade <2, both VEGF (P = 0.014) and edema (P = 0.017) were independent predictors of survival in a Cox multivariable proportional hazard model using both as covariates.

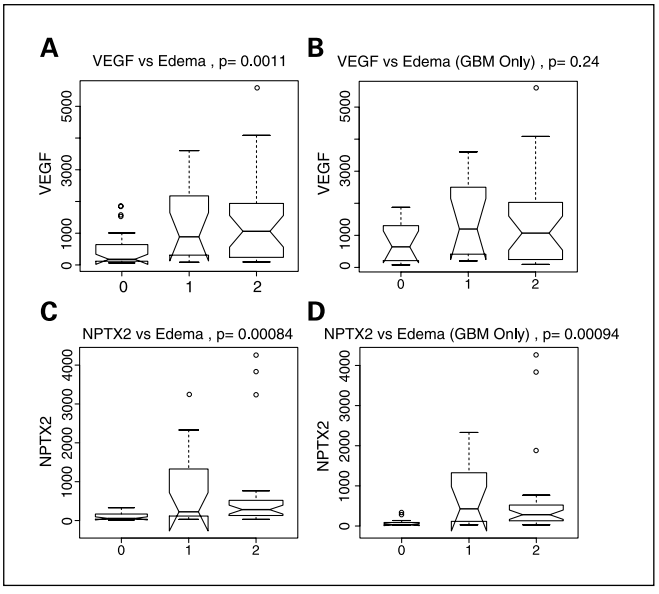

Fig. 2. Box plots by edema grade show increased VEGF and NPTX2 expression in edema-producing tumors versus tumors without edema. VEGF is increased in tumors with edema (grades 1 and 2) compared with no edema (grade 0) in all gliomas (*A*). A similar pattern is shown for the GBM subset (*B*), but this was not statistically significant, potentially related to smaller sample size. NPX2 is increased in tumors with edema, both for all gliomas (*C*), and for the GBM subset (*D*).

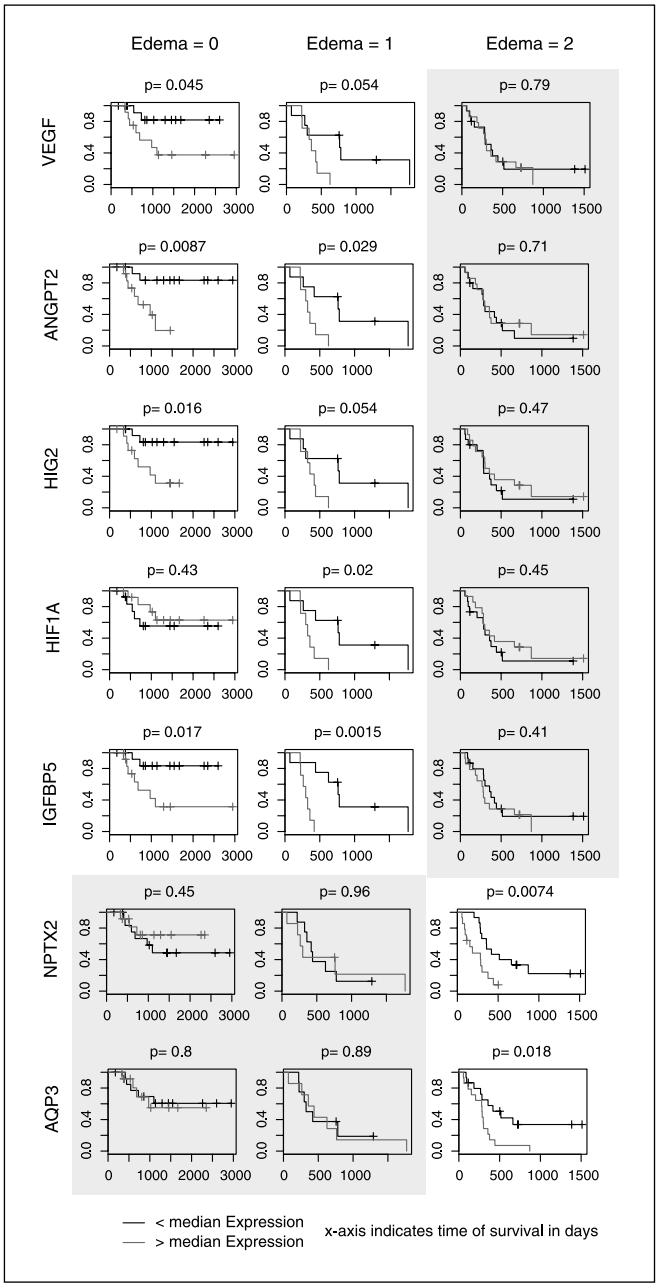

Fig. 3. VEGF and related angiogenic gene expression is predictive of survival in gliomas with little or no edema, whereas NPTX2 and aquaporin 3 are more predictive of survival in patients with highly edematous tumors. VEGF is an independent predictor of survival when edema is absent or grade 1, but not when edema is grade 2. Several angiogenic genes that correlate with VEGF show a similar relationship. This relationship is reversed for NPTX2 and aquaporin 3.

Relationship between VEGF and pro- and antiangiogenic genes. Microarray expression data were sorted according to correlation with VEGF expression. A total of 339 probe sets with >0.5 Pearson correlation coefficient, with *P* value <0.0001, were identified. This list was searched for proangiogenic genes based on a recent review (19). This led to the identification of (correlation coefficients and *P* values in parentheses), *adrenomedullin* (0.80, $P < 0.0001$), *hypoxia inducible protein-2* (HIG-2; 0.66, $P < 0.0001$), *insulin-like growth factor binding protein 5* (IGFBP-5; 0.64, $P < 0.0001$), IGFBP-7 (0.59, $P < 0.0001$), *retinoblastoma binding protein 8* (RBBP-8; 0.55, $P < 0.0001$), IGFBP-5 (0.52, $P < 0.0001$), and *hypoxia-inducible factor-1A* (HIF-1A; 0.51, $P < 0.0001$). Another putative angiogenesis-related gene, *angiopoetin-2*, was less well correlated with VEGF expression (0.44, $P < 0.0001$).

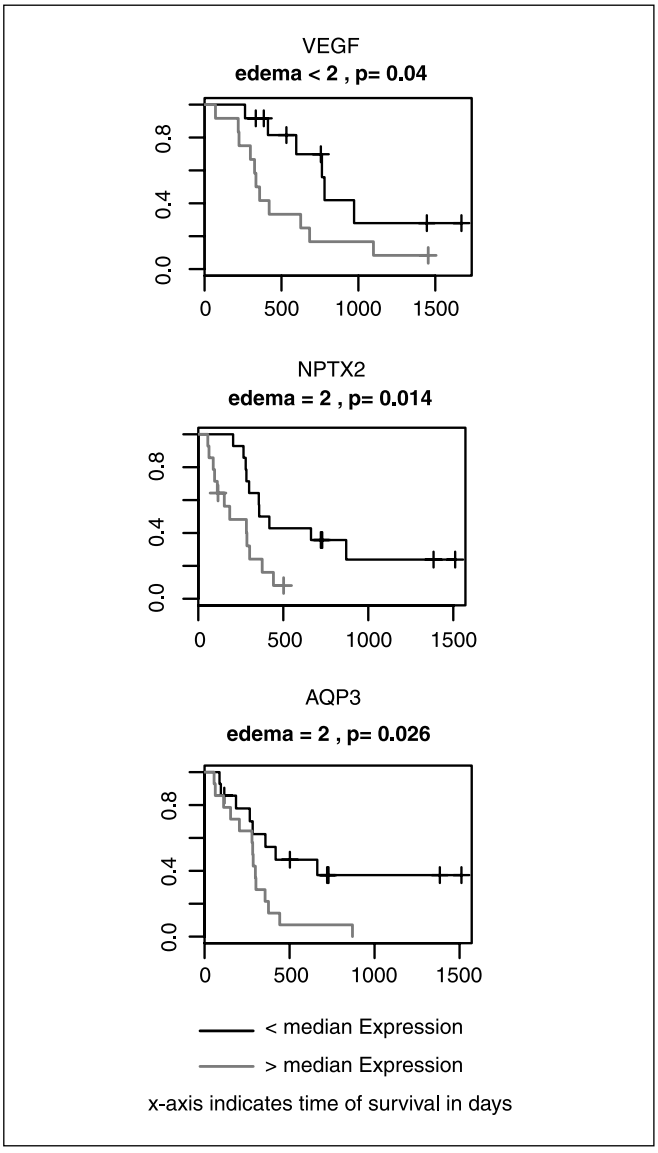

Fig. 4. Correlation with survival based on edema grade between VEGF, NPTX2, and aquaporin 3 is maintained in the subset of GBM patients ($n = 52$). VEGF expression levels correlate with survival in tumors with little or no edema (edema grades 0 and 1). NPTX2 and aquaporin 3 expression correlate with survival in tumors with the extensive edema (edema grade 2). This is the same relationship to survival based on edema grade found for all malignant gliomas.

Expression of several antiangiogenic genes was anticorrelated with VEGF expression: *HIF-1α subunit inhibitor* (HIF-1AN; -0.54, $P < 0.0001$), *brain-specific angiogenesis inhibitor 1* (BAI1; -0.45, $P < 0.0001$), *phosphatase and tensin homologue* (PTEN; -0.36, $P < 0.0001$), BAI3 (-0.34, $P < 0.003$), *somatostatin* (-0.31, $P < 0.008$), and BAI2 (-0.25, $P = 0.034$).

Survival analysis showed that for tumors with edema <2, HIG-2, *angiopoetin-2*, IGFBP-5, IGFBP-7, *neuropilin*, and RBBP-8 were all significant predictors of survival, whereas HIF-1A was not. HIF-1A was significant only for the edema grade 1 group. Kaplan-Meier analysis segregated by edema grades shows the similarities between the survival curves of several of the proangiogenic genes and that of VEGF (Fig. 3).

Genes associated with edema in low VEGF tumors. Some tumors with low VEGF expression were found to have large edema volumes, suggesting VEGF-independent edema pathways in malignant gliomas. To generate a list of potential genes involved in producing edema without VEGF involvement, we analyzed the subset of tumors with low VEGF (<400), comparing expression levels in tumors with edema versus tumors without edema (see Table 1). *Neuronal pentraxin 2* (NPTX2) was identified as the gene in which expression was most increased (7-fold) in the edematous versus non-edematous tumors. NPTX2 was positively correlated with edema in all malignant gliomas (0.24, $P = 0.04$; Kruskall-Wallis $P = 0.0008$; Fig. 2) and within the GBM subset (Kruskall-Wallis $P = 0.0009$; Fig. 2). NPTX2 was not significantly correlated with VEGF expression. To assess the effects of NPTX2 expression on survival, we again used the Kaplan-Meier method, sorting tumors by edema grade. Unlike VEGF, NPTX2 was shown to be a negative predictor of survival in the highest edema grade (edema grade 2, Fig. 3). NPTX2 was also significant for survival in all gliomas, as well as the GBM subset. The hazard ratio for NPTX2 using the Cox model was 2.73 ($P = 0.049$; 95% CI, 1.49-5.02). Both NPTX2 ($P = 0.024$) and edema ($P < 0.0001$) were independent predictors of survival when used together in a Cox multivariable proportional hazard model. These results suggest that NPTX2 may mediate edema independent of VEGF expression. The other genes with greater than 3-fold increase in the edematous tumors were the *tissue inhibitor of metalloproteinase 1*, *matrix Gla protein*, *CD163 antigen*, *cyclic AMP-regulated phosphoprotein 21 kDa*, and *neurofilament 3*. The expression of these genes did not show a significant correlation with survival in the high-edema tumors.

Microarray expression data were sorted according to correlation with NPTX2 expression. *Aquaporin 3* was the most tightly correlated with NPTX2 expression (0.74, $P < 0.0001$). The Cox proportional model hazard ratio was 3.61 ($P = 0.0004$; 95% CI, 1.36-9.6). The relationship between survival and the next four gene products most correlated with NPTX2 expression was not significant in the high-edema tumors. These genes were *popeye domain containing 3*, *hepatocyte growth factor*, *prodynorphin*, and *phospholipase D* family, member 3. Interestingly, hepatocyte growth factor (correlation coefficient 0.65, $P < 0.0001$) inhibition has been reported to prevent glioma growth in xenografts (20).

Gene expression and survival in GBM. As stated above, we found a significant relationship between survival and VEGF expression in low-edema tumors, whereas high-edema tumors showed a relationship between survival and both NPTX2 and

aquaporin 3 expression. To determine if these relationships were affected by tumor grade, we also analyzed these data for the subset of GBM patients ($n = 52$; Fig. 4). Kaplan-Meier analysis showed a relationship between survival and VEGF in GBM patients with little or no edema ($P = 0.04$). GBM patients with grade 2 edema also showed a significant relationship between survival and both NPTX2 ($P = 0.014$) and *aquaporin 3* ($P = 0.026$) expression as shown for all malignant glioma patients. Lastly, in a Cox multivariable proportional hazard model using VEGF and NPTX2, both were independent predictors of survival (VEGF, $P = 0.011$; NPTX2, $P = 0.016$). For all gliomas, these values are $P < 0.0001$ for VEGF and $P = 0.004$ for NPTX2.

Relationship between steroid dose, edema, and gene expression. Corticosteroids are used to diminish edema and mass effect in brain tumor patients. Steroid dose was determined for patients at the time of the MRI used for edema grading by reviewing the medical records. Of the 71 patients, 34 were not on steroids, and 34 were on steroids (22 on decadron, doses from 4 to 60 mg/day, 10 on decadron with unknown dose, 1 on prednisone, and 1 on dexamethasone), and no data on steroids were available for 3 patients. Of the patients on steroids, 16 had edema grade 2 and, thus, did not have edema levels diminished by steroid administration. There was no significant difference in steroid use by edema grade for the patients with known steroid dose ($n = 56$; Kruskal-Wallis test, $P = 0.37$). There was no significant relationship between steroid use and VEGF ($P = 0.16$), NPTX2 ($P = 0.22$), or *aquaporin 3* ($P = 0.91$) expression. We also analyzed Kaplan-Meier survival data in patients who were not on steroids ($n = 34$). For edema grade 2, the relationship between survival and NPTX2 maintained significance ($P = 0.0049$), although *aquaporin 3* did not ($P = 0.15$), most likely because of the reduced statistical power. When edema grade 2 patients were added to the no-steroid group (i.e., the subset of patients in which steroids were not given or had no effect on edema grade), *aquaporin 3* expression maintained a significant relationship to survival ($P = 0.018$). For VEGF, the relationship with survival for the edema <2 groups showed a low P value ($P = 0.055$) and showed a similar survival curve for all gliomas with edema <2. Therefore, we conclude that differences in steroid treatment do not account for the relationship between VEGF, NPTX2, and *aquaporin 3* expression and survival.

Table 1. Increase in gene expression between edematous and nonedematous tumors with low VEGF

Gene	Fold change	*P*
Neuronal pentraxin II	7.68	0.0299
Tissue inhibitor of metalloproteinase 1	5.69	0.0232
Matrix Gla protein	4.04	0.0364
CD163 antigen	3.80	0.0426
Cyclic AMP–regulated phosphoprotein, 21 kDa	3.47	0.0246
Neurofilament 3 (150 kDa medium)	3.02	0.0156

NOTE: Less than median VEGF expression.

Discussion

VEGF was originally described in the setting of brain tumors as a vascular permeability factor (21). The authors of that report hypothesized that VEGF may cause vasogenic edema in gliomas. Recently, we reported that inhibition of VEGF does indeed markedly reduce edema in patients with malignant gliomas (9). Edema causes mass effect and is associated with poor prognosis in these patients. In the same study, we found that not all patients responded to VEGF inhibition, however, suggesting that in some gliomas, edema is a result of VEGF-independent pathways.

In the current study, we used microarray data to show a large range of VEGF expression in malignant gliomas. We found that VEGF expression was correlated with edema. However, some tumors with a large amount of peritumoral edema had low levels of VEGF expression. This is consistent with our hypothesis that some gliomas cause edema through VEGF-independent pathways, potentially accounting for failure of these tumors to respond to anti-VEGF therapy.

In addition to being a potent stimulator of vascular permeability, VEGF is thought to play a pivotal role in angiogenesis in malignant gliomas (19, 22). We found that VEGF expression levels were predictive of survival independent of edema in tumors with little or no edema. This was true both for malignant gliomas and for only GBM. This supports the conclusion that whereas part of the deleterious effect of VEGF expression may be due to the promotion of edema and mass effect, other properties of VEGF, presumably its role in angiogenesis, may also contribute to shortened survival.

To investigate the mechanism underlying VEGF-induced angiogenesis, we assessed the correlation between VEGF expression and several putative pro- and antiangiogenic genes. Of these, *adrenomedullin* was the most tightly correlated with VEGF expression. This was not unexpected because both *adrenomedullin* and VEGF are thought to be induced by HIF (19), and HIF was also correlated with VEGF expression. Hypoxia seems crucial in the transformation of glial tumors into more aggressive phenotypes (reviewed in ref. 19). We found that another gene product associated with hypoxia, HIG-2, was also well correlated with VEGF expression. Others have shown a link between HIG-2 and renal cell carcinoma (23). No reports implicating HIG-2 in glioma progression have been published.

Several antiangiogenesis genes were inversely correlated with VEGF expression, including HIF-1AN, BAI1-3, PTEN, and *somatostatin*. Interestingly, PTEN has been shown to inhibit adrenomedullin expression in glioma cell lines (24) and to increase VEGF expression in a mouse model for asthma (25). PTEN is also associated with chromosome 10q deletion and a poor prognosis (26). We analyzed the relationship between edema, survival, and the expression of these angiogenesis-related genes as we had done for VEGF. Several were significant predictors of survival, with many showing the same pattern as VEGF, that is, associated with survival in tumors with little or no edema. Correlation between VEGF expression and the expression of these angiogenesis-related genes, as well as their similar survival curves, suggests a functional relationship that merits further investigation. These findings also support the idea that angiogenesis is a result of

the balance between pro- and antiangiogenic gene products and identifies a subset of genes that may impact survival and be more closely linked to VEGF-mediated angiogenesis in human gliomas.

Given our hypothesis that some malignant gliomas have VEGF-independent pathways leading to edema, we looked for gene expression enriched in edematous versus nonedematous tumors with low VEGF expression. This led to the identification of NPTX2, which was increased 7-fold in the edematous tumor group. This was the greatest percentage increase of all genes analyzed. NPTX2 is normally expressed in the central nervous system and is a member of a family of proteins related to C reactive protein and other acute-phase inflammatory mediators (27, 28) and, thus, is a good candidate for mediation of tumoral edema. NPTX2 was found to be correlated with edema in all gliomas and in only GBM patients. NPTX2 was not correlated with VEGF expression, supporting a VEGF-independent role for this gene product. Most importantly, increased NPTX2 was associated with poorer survival in tumors with the highest levels of edema. This is the reverse of VEGF expression, which was predictive of survival only for tumors with little or no edema. It will be of interest to determine if NPTX2 is elevated in tumors that do not respond to anti-VEGF therapy.

We also searched for genes that correlate with NPTX2 expression, looking for candidates that could be involved in edema production. This led to the identification of *aquaporin 3*, which was the gene with expression levels that most highly correlated (0.74, $P < 0.0001$) with those of NPTX2. Aquaporin 3 is a member of a group of transmembrane proteins that act as water and solute channels and, thus, could potentially be involved in edema regulation (reviewed in ref. 29). Others have suggested a relationship between the expression of other aquaporin isoforms and edema in traumatic brain injury (30). Additionally, aquaporin-1 has been implicated in increased metastatic potential in a mouse tumor model (31) and increased in astrocytomas (32). More investigation will be required to understand the possible role of aquaporin 3 in brain edema in tumor patients, as well as its functional relationship to NPTX2. It is notable, however, that aquaporin 3 is induced by epidermal growth factor in fibroblast culture (33), and amplification of the epidermal growth factor receptor has been implicated in glioma progression (34, 35).

In conclusion, although VEGF expression is correlated with edema, it is an independent predictor of survival, presumably due to its angiogenic properties. Of the many putative pro- and antiangiogenic genes, we have found several that seem closely related to VEGF expression, suggesting these are genes are involved in VEGF-mediated angiogenesis. Because VEGF expression has a greater impact on survival in tumors with little or no edema, our data support the hypothesis that VEGF may be crucial in the "angiogenic switch" that occurs when tumors degenerate into more aggressive, edema-producing phenotypes. How these genetic factors interplay with prognostically important clinical data, including age, treatment, and Karnofsky performance scale, remains to be determined, but this is a focus of ongoing investigation. Our data also suggest that VEGF-independent edema pathways may be important in limiting survival. In this exploratory study, with its limitation of small sample size, we found evidence that NPTX2 and aquaporin 3 are two gene products that merit further investigation as potential mediators of continued high-edema states.

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