The Expression of PAX6, PTEN, Vascular Endothelial Growth Factor, and Epidermal Growth Factor Receptor in Gliomas: Relationship to Tumor Grade and Survival

Yi-Hong Zhou, Fang Tan, Kenneth R. Hess, and W. K. Alfred Yung

ABSTRACT

Purpose: Malignant astrocytic gliomas, including anaplastic astrocytoma (AA) and glioblastoma multiforme (GBM), result from various genetic perturbations and dysregulated gene expression. Identifying genetic prognostic markers could be more useful for stratifying glioma patients than gross pathology alone.

Experimental Design: cDNAs were generated by reverse transcriptase using mRNAs from gliomas and adjacent normal tissues from 86 patients. The tissues used for analysis were 45 AAs, 42 GBMs, and 7 samples of adjacent normal tissue. The levels of PAX6, PTEN, vascular endothelial growth factor (VEGF), and epidermal growth factor receptor (EGFR) gene expression were quantified using real-time quantitative reverse transcription-PCR and normalized to β-actin. All statistical tests used were two-sided.

Results: PAX6 expression was significantly reduced in GBM compared with AA (P < 0.0001). The relative levels of PTEN, EGFR, and VEGF expression also differed significantly among glioma grades. Multivariate Cox analysis of glioma samples, adjusting for patient age, histology, recurrent status, and levels of PTEN, EGFR, VEGF, and PAX6 (7 variables) showed a correlation between a low level of PAX6 expression in malignant astrocytic gliomas and unfavorable patient outcomes (hazard ratio, 0.34; 95% confidence interval, 0.18–0.63). Recursive partitioning analysis showed a favorable outcome for patients with high expression values of PTEN and PAX6 compared with low expression values of one or both genes (P < 0.0001).

Conclusion: The expression levels of PAX6, PTEN, and VEGF but not EGFR were independent prognostic markers, and the model including 7 variables was able to account for 55% of the variation in survival times for malignant astrocytic glioma patients.

INTRODUCTION

More than one-third of primary brain and central nervous system tumors are astrocytic gliomas. The WHO classification scheme divides these tumors into four stages, or grades (1). Grades I and II are the least malignant phenotypes, whereas grade III, AA,3 and grade IV, GBM, are highly malignant and are also the most frequently reported glioma histologies (2). The survival of patients with astrocytic gliomas is closely related to WHO tumor grade. However, within a tumor grade, especially the higher grades, clinical outcomes are variable (3, 4). Identifying genetic markers that are associated with outcomes for glioma patients undergoing specific therapeutic procedures may assist in pinpointing the variation and prognostic significance within each tumor grade, and could be useful when analyzing and comparing large data sets.

Malignant astrocytic gliomas either arise de novo (primary) or from the progression of lower-grade astrocytic gliomas (secondary). The most frequent genetic anomalies found in primary GBM are a gain of chromosome 7 and amplification of the EGFR gene, LOH of chromosome 10, and mutation or deletion of the PTEN and p16 tumor suppressor genes (5–9). Frequent occurrences of mutations of the p53 tumor suppressor gene and alteration of genes involved at the G1–S checkpoint have been observed in secondary GBM and AA patients (10, 11).

Of particular importance is that in addition to mutagenesis, the expression of specific genes correlates with gliomagenesis and with patient outcomes. The expression of PTEN, for one, is inversely correlated with the progression of GBM and a poor patient outcome (12). VEGF is well known as an important angiogenic factor and as a prognosticator of glioma progression (13), as demonstrated by reports of its overexpression in HT glioma cell lines and high-grade gliomas (14, 15). EGF gene amplification has also been reported in many cases of GBM and has been implicated as playing an important role in tumor progression (16, 17). Less well known is the role of the PAX6 gene. Located on 11p13, this gene encodes a transcription factor composed of two DNA-binding domains: a paired domain and a paired-like homeodomain (18). PAX6 is critical for the development of the eye, brain, and pancreas, and, in normal adults, it is continuously expressed in these organs (19–22). We observed

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3 The abbreviations used are: AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; RT-PCR, reverse transcription-PCR; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; LOH, loss of heterozygosity; UTMDACC, University of Texas M. D. Anderson Cancer Center; LC, LightCycler; HT, highly tumorigenic; NT, nontumorigenic; CI, confidence interval.
differential PAX6 levels (protein and RNA) in different grades of glioma, with greater expression in HT than in NT glioma cell lines (23).

As reported here, our use of real-time quantitative RT-PCR on cDNA samples confirmed the differential expression of the PAX6 gene in glioma cell lines. Furthermore, we quantified PAX6, PTEN, VEGF, and EGFR gene expression in cDNA samples from 87 malignant gliomas (45 AAs and 42 GBMs). This article reports our assessment of the prognostic values of these four genes, adjusted for the clinical variables of age, histology, and recurrent status, and compares the use of a three-variable model with the prognostic acuity of a seven-variable model.

MATERIALS AND METHODS

Cell Lines and cDNA Materials. NHA, U118, U373, and Hs683 cell lines were obtained from American Type Culture Collection (Manassas, VA). The LN229 cell line was obtained from the Department of Neurosurgery at the UTMDACC. U251-chromosomal 10 hybrid clones were from Per- shouse et al. (24). All of the other cell lines are described in Ke et al. (14). Cells were cultured in DMEM-F12 supplemented with 10% FCS. The cDNA of each cell line was reverse transcribed from 3 μg of total RNA by Superscript II reverse transcriptase (Life Technologies, Inc., Carlsbad, CA) with 20 pmol of polydeoxythymidylic acid primer in a 20-μl reaction according to the manufacturer’s protocol.

Glioma cDNA Samples. cDNA samples of gliomas and some adjacent normal tissues were created and used previously by Shouse et al. Survival times were calculated from the date of initial surgery. All of the patients were treated with similar regimens, which included surgery followed by radiation therapy and/or chemotherapy. Ratios of PAX6, PTEN, EGF, and VEGF expression were correlated with survival, and were analyzed using a Cox proportional hazards regression analysis. The Kaplan-Meier method was used to estimate, and the Cox-Mantel log-rank test was used to compare survival distributions. All of the statistical analyses were performed using S-PLUS 2000 computer software (MathSoft Inc., Seattle, WA).

RESULTS

PAX6 Gene Expression in Glioma Cell Lines. On the basis of our initial observations using semiquantitative RT-PCR on different levels of PAX6 gene expression in glioma cell lines, we used a quantitative approach (real-time quantitative RT-PCR) to determine relative PAX6 gene expression levels in a series of glioma cell lines for which tumorigenicity information was available.

To select a good internal control for quantifying PAX6 expression, we measured the expression of GAPDH, β-actin, enolase-α, and RPS9, genes that have been used frequently as internal controls in semiquantitative or quantitative RT-PCR experiments. We determined which control gene(s) were expressed most stably in these glioma cell lines and found that the expression of GAPDH and enolase-α correlated best ($r = 0.85$). PAX6:GAPDH and PAX6:enolase-α ratios were then calculated and used to represent the relative levels of PAX6 expression. We included a normal astrocytic cell line, NHA, for comparing the relative expression of the PAX6 gene in different glioma cell lines. As shown in Fig. 1A, a marked difference existed between the normalized ratios of PAX6:GAPDH and PAX6:enolase-α in...
the NHA cell line compared with the ratios found in the glioma cell lines, suggesting that GAPDH and enolase-α gene expression levels are strikingly different between normal astrocytic and glioma cell lines. Because reintroducing chromosome 10 into the U251 GBM cell line completely suppressed tumor formation in nude mice (24), we included three U251 chromosome 10 hybrid clones (N10) in this analysis. As shown in Fig. 1A, PAX6 was expressed at a relatively low level in HT cells and at a relatively high level in NT glioma cell lines, and these differences were significant (P < 0.001; Fig. 1B).

**Differential Expression of PAX6, PTEN, EGFR, and VEGF Genes in Malignant Astrocytic Gliomas.** The differential expression of the PAX6 gene in diverse glioma cell lines suggested that the degree of PAX6 expression was likely related to glioma grade. To test this hypothesis, we quantified the relative levels of PAX6 gene expression in cDNA samples from 87 gliomas (45 AAAs and 42 GBMs) and in 7 adjacent normal tissue samples using real-time PCR. For comparison, we also measured PTEN, EGFR, and VEGF gene expression in these cDNA samples. The expression of the PTEN gene was measured previously using a semiquantitative RT-PCR technique with the same cDNA samples used in this study (12).

Because there is no reliable way to know the precise amount of cDNA used, an internal control gene was used to normalize the results. Because we would not expect levels of housekeeping genes to vary between two groups of tumors with different histologies if they were expressed stably, we used each gene to normalize the others to determine through test analysis which one yielded the least significant differences between grade III and IV histologies. RPS9 gene expression differed significantly among the AA and GBM groups (P < 0.05), so its expression was eliminated as a useful internal control. Although the levels of expression of the other three housekeeping genes did not differ significantly between the two groups (P > 0.4), the β-actin gene had the largest P (data not shown) and was selected for calculating and statistically analyzing gene ratios.

**Table 1 Median expression levels normalized by β-actin**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Normal (n = 7)</th>
<th>AA (n = 45)</th>
<th>GBM (n = 42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAX6</td>
<td>0.018</td>
<td>0.019</td>
<td>0.006</td>
</tr>
<tr>
<td>PTEN</td>
<td>0.012</td>
<td>0.010</td>
<td>0.004</td>
</tr>
<tr>
<td>EGFR</td>
<td>0.007</td>
<td>0.013</td>
<td>0.027</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.029</td>
<td>0.021</td>
<td>0.086</td>
</tr>
</tbody>
</table>

As shown in Table 1, the median relative expression of PAX6 and PTEN genes in AA is greater than that in GBM by >3-fold and 2-fold, respectively. The median relative expression levels of the EGFR and VEGF genes in GBM is greater than in AA by >2-fold and 4-fold, respectively. No marked change in gene expression was found in the AA and normal samples for the PAX6, PTEN, and VEGF genes, except for a 1.7-fold increase in EGFR gene expression in AA compared with normal tissue samples.

We compared the distribution of β-actin-normalized log10-transformed expression values of PAX6, PTEN, EGFR, and VEGF genes between the AA and GBM groups using the nonparametric Wilcoxon rank-sum test, essentially a t test performed on the established ranks. Fig. 2 shows that all of the genes of interest were expressed heterogeneously in various tissues. Overall, however, PAX6 gene expression in GBM was significantly reduced compared with that in AA (P < 0.0001; Fig. 2A). The Wilcoxon Ps were each <0.0001 for PAX6 normalized by enolase-α and GAPDH among GBM and AA specimens (data not shown).

We also analyzed seven specific instances when tumor and surrounding normal tissues were available. The PAX6 gene was expressed at a lower level in all of the patients with GBM (n = 5) and in 1 AA patient (#6) than in the normal tissues surrounding the tumor (Table 2). For AA patient #7, no marked differences were apparent between values from tumor and surround-
Gene Expression and Prognosis Markers in Gliomas

We found a significantly higher expression of the PTEN gene in GBM than in normal tissue. For patient #8, who was initially diagnosed as having AA with a later recurrence as GBM, PAX6 gene expression was 31-fold higher in the AA than in the GBM specimen. A Wilcoxon rank-sum test showed that the difference in PAX6 expression was significant (P = 0.0014) when GBM PAX6 gene expression (n = 42) was compared with its expression in normal specimens (n = 7), but was not significant (P = 0.67) when AA samples (n = 45) were compared with normal samples. The combined results suggest that decreased PAX6 expression correlates with GBM development.

It was reported previously that PTEN gene expression was significantly lower in GBM than in normal or non-GBM samples using semiquantitative RT-PCR (12). The real-time quantitative RT-PCR technique used here to measure PTEN gene expression in identical tumor cDNA samples produced the same conclusion: the expression of the PTEN gene in GBM was less than in AA (P = 0.0002; Fig. 2C). Although the difference is not as striking as for the other three genes studied, the expression of EGFR gene in GBMs is significantly higher than in AAs (P = 0.046; Fig. 2D).

Additional analysis of GBM versus AA comparing the expression of all four of the genes using a logistic regression analysis suggested that expression of the PAX6 and VEGF genes is diversely distributed among GBM and AA histologies (P = 0.0004 and P = 0.0005, respectively). GBM tends to have high levels of VEGF expression and low levels of PAX6 expression, whereas the opposite is true in AA, with a tendency toward high PAX6 and low VEGF expression.

Analysis of Survival. Of the 86 glioma patients whose tumor specimens were used (1 AA and 1 GBM are from 1 patient), 70 had died by the time of analysis. The two main clinical variables that correlated with survival were tumor grade and tumor recurrence status. For the 45 AA patients, median survival was 117 weeks, whereas for the 42 GBM patients, median survival was only 44 weeks. In 63 newly diagnosed patients, median survival was 80 weeks, whereas the median survival was 32 weeks for 23 patients after tumor recurrence. In a Cox proportional hazards regression analysis model taking into account the variables of age, histology (GBM versus AA), and recurrence status, the adjusted hazard ratio for GBM versus AA was 3.0, and the adjusted hazard ratio for recurrence was 2.2. However, this model accounted for only ~30% of the variation in survival times for GBM and AA.

To improve the survival model, we included the relative values of PAX6, PTEN, VEGF, and EGFR gene expression through a series of Cox proportional hazards regression analyses. The approach we used was to select cutoff points for the level of expression of each gene, and by dichotomizing, groups were formed above and below the cutoff point. The best cutoff for each gene was selected using recursive partitioning analysis of residuals adjusted for age, histology, and disease recurrence status. As shown in Fig. 3, a lower value of VEGF expression and low levels of PAX6 expression, whereas the opposite is true in AA, with a tendency toward high PAX6 and low VEGF expression.

![Image](https://example.com/image.png)

**Fig. 2** Analysis of the relative levels of PAX6, PTEN, VEGF, and EGFR gene expression by real-time quantitative RT-PCR in 45 AA, 42 GBM, and 7 samples of adjacent normal tissue, and normalized by β-actin. The Y axis is a log 10-transformed PAX6/β-actin ratio. The position of a bar and a vertical line for each class indicate the mean and SD, respectively. The statistical significance between AAs and GBMs is shown by P.<0.0001; P = 0.0002; P = 0.0002; P = 0.046; Fig. 2D). Additional analysis of GBM versus AA comparing the expression of all four of the genes using a logistic regression analysis suggested that expression of the PAX6 and VEGF genes is diversely distributed among GBM and AA histologies (P = 0.0004 and P = 0.0005, respectively). GBM tends to have high levels of VEGF expression and low levels of PAX6 expression, whereas the opposite is true in AA, with a tendency toward high PAX6 and low VEGF expression.

### Table 2 Comparison of relative PAX6 expression levels in the same patients

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Tissue</th>
<th>PAX6/β-actin</th>
<th>Normal/tumor (AA/GBM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>0.071</td>
<td>11.2</td>
</tr>
<tr>
<td>2</td>
<td>Normal</td>
<td>0.027</td>
<td>9.0</td>
</tr>
<tr>
<td>3</td>
<td>Normal</td>
<td>0.012</td>
<td>1.9</td>
</tr>
<tr>
<td>4</td>
<td>Normal</td>
<td>0.010</td>
<td>4.0</td>
</tr>
<tr>
<td>5</td>
<td>Normal</td>
<td>0.013</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>Normal</td>
<td>0.051</td>
<td>1.9</td>
</tr>
<tr>
<td>7</td>
<td>Normal</td>
<td>0.018</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>Normal</td>
<td>0.032</td>
<td>31.1</td>
</tr>
<tr>
<td>9</td>
<td>Normal</td>
<td>0.019</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Table 2: Comparison of relative PAX6 expression levels in the same patients.
theing effect exists between gene expression and clinical variables, and survival correlate with tumor grade. Although a confound is consistent with the observation that gene expression values increased values over a simple univariate approach. This finding adjusted for age, histology, and recurrence, yielded greatly The four of the genes were significant (P/H11021

Thus, the relative expression values of all three of the genes dichotomized at their expression value derived from optimal dichotomization.

Importantly, the Ps derived from an analysis adjusting for seven covariates (age, histology, recurrence status) and the four genes dichotomized at their “optimal” cutpoints of expression retained significance for PAX6, PTEN, and VEGF (Table 3). Thus, the relative expression values of all three of the genes are clearly independent prognostic markers of tumor grade. Consistent with this conclusion and as shown in Table 4, the proportion of variation in survival explained by the model with three clinical variables (30%) increased when the relative value of the expression for each gene was included, except for EGFR (36%–39%). In contrast, when seven variables were included in the model, 55% of the variation in survival times was accounted for.

Interestingly, when recursive partitioning analysis was performed on all seven of the covariates, PAX6 was the first variable selected (i.e., it yields the most significant cutoff among all seven of the covariates). No secondary significant splits were identified for patients with lower PAX6 values, whereas a second partition was found only for PTEN and for patients with higher PAX6 values. The median survival for the 14 patients with elevated PAX6 and PTEN expression was 341 weeks. The median survival for the 33 patients with low PAX6 and PTEN expression values was 33 weeks. The median survival for the 39 patients with mixed values (patients had high values for one gene and low values for the other) was 62 weeks. Fig. 4 is a Kaplan-Meier survival curve based on the values of PAX6 and PTEN gene expression. The hazard ratio for patients with higher values for both PAX6 and PTEN gene expression compared with those with lower values for both is 0.1 (95% CI, 0.005–0.3). The hazard ratio for patients with mixed values compared with those with lower values for both is 0.5 (95% CI, 0.3–0.8). When all three groups of patients were segregated by the values of expression of the PAX6 and PTEN genes, significant differences in survival were found (P < 0.0001). These results were unchanged after adjusting for age, histology, and tumor recurrence status.

DISCUSSION

To date there has not been a good prognostic model for stratifying glioma patients, probably because of the heterogeneity and genomic instability inherent in malignant astrocytic
gliomas. By using the quantitative values of PTEN, VEGF, EGFR and PAX6 gene expression, we were able to greatly improve a prognostic model based on conventional clinical variables. Importantly, we identified a new prognostic marker, the expression value of the PAX6 gene, of which the role in gliomas has not yet been delineated.

Although EGFR gene amplification and overexpression were seen in some gliomas, its expression has no prognostic value according to our analysis. In the case of EGFR gene amplification, there are frequent and various mutations of different regions of the gene that could lead to the increased or decreased oncogenic activity of EGFR (25). In some cases, however, the total number of EGFR transcripts cannot account for the functional level of EGFR. Quantifying the levels of alternatively spliced EGFR transcripts that have enhanced oncogenic activity, such as EGFRvIII, may improve its prognostic value.

Turning our attention to PAX6 gene expression, we wished to determine if its down-regulation in GBMs was affected by the LOH of chromosome 10, which occurred predominantly in the specimens studied here (26), or if it was merely a side effect of the LOH of chromosome 10. After we screened nine chromosome 10 U251-hybrid clones that did not produce tumors in nude mice (24), we found markedly increased RNA and PAX6 protein compared with the parental U251 cell line and with U251 chromosome 2 hybrid cells (Fig. 1A). Interestingly, some of the chromosome 10 hybrid clones retained only a partially exogenous chromosome 10, the region marked as 10pter-q11 (27). This finding suggests that gene(s) on 10q do not, including the PTEN gene, located on 10q23.3. This result is in accord with the expression level of PAX6 not being up-regulated in U251 cells overexpressing PTEN. Although PAX6 and PTEN expression in the glioma specimens from this study are minimally correlated (r = 0.31), this correlation likely occurs because of LOH of the entire, or of a large portion of, chromosome 10. Significantly, however, the relative values of PAX6 and PTEN gene expression are two independent and powerful prognostic markers of outcome for patients with astrocytic malignant gliomas.

Overall, our real-time quantitative RT-PCR approach of quantifying the relative values of PAX6 gene expression in gliomas, together with PTEN and VEGF gene expression from the small amount of cDNA available, has clinical importance for its ability to prognosticate survival in individual cases and within tumor grades. We observed overlaps between two groups of AAs and GBMs (Fig. 2), which could be because of the heterogeneity and genomic instability inherent in malignant gliomas. Because clinical outcomes are highly variable for patients with these gliomas, we anticipate a better classification of these patients by identifying certain prognostic gene expression variables in the tumors. Although our model that included seven variables was greatly improved from the earlier model that included only three clinical variables, increasing the prognostic ability of the model from 30% to 55%, we anticipate addition-ally improving the power of the survival model for glioma patients by including additional independent prognostic variables in future studies.

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REFERENCES


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