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Tumor associated seizures in glioblastomas are influenced by survival gene expression in a region-specific manner: a gene expression imaging study

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Abstract

Tumor associated seizures (TAS) are common and cause significant morbidity. Both imaging and gene expression features play significant roles in determining TAS, with strong interactions between them. We describe gene expression imaging tools which allow mapping of brain regions where gene expression has significant influence on TAS, and apply these methods to study 77 patients who underwent surgical evaluation for supratentorial glioblastomas. Tumor size and location were measured from MRI scans. A 9-set gene expression profile predicting long-term

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Conflict of interest:

Kristen Oelschlager and Derek Maetzold are employees of Castle Biosciences. None of the other authors have conflicts of interest.

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survivors was obtained from RNA derived from formalin-fixed paraffin embedded tissue. A total of 32 patients (42%) experienced preoperative TAS. Tumor volume was smaller (31.1 vs 58.8 cubic cm, $p < 0.001$) and there was a trend toward median survival being higher (48.4 vs 32.7 months, $p = 0.055$) in patients with TAS. Although the expression of only OLIG2 was significantly lower in patients with TAS in a groupwise analysis, gene expression imaging analysis revealed regions with significantly lower expression of OLIG2 and RTN1 in patients with TAS. Gene expression imaging is a powerful technique that demonstrates that the influence of gene expression on TAS is highly region specific. Regional variability should be evaluated with any genomic or molecular markers of solid brain lesions.

1. Introduction

Tumor associated seizures (TAS) are both common and debilitating (Hildebrand et al., 2005; Ruda et al., 2012). They are experienced by approximately half of the patients at some point, and about half of those will experience generalized tonic-clonic (GTC) seizures (Chang et al., 2008; van Breemen et al., 2007). TAS as well as the side effects from the use of antiepileptic drugs (AEDs) substantially contribute to morbidity (Hildebrand et al., 2005) and cause a marked decrease in the quality of life in patients with brain tumors (Klein et al., 2003; Moots et al., 1995; van Breemen et al., 2007). As advances in brain tumor treatments increase life expectancy, morbidity from TAS is becoming increasingly burdensome to the patient.

The mechanisms of TAS are incompletely understood and are likely multifactorial (Englot et al., 2012; Ruda et al., 2012; van Breemen et al., 2007). Previous neuroimaging studies demonstrated that tumor size and location play crucial roles in determining seizures related to tumors, and that these neuroimaging characteristics depend on the grade of the tumor. TAS are more likely to occur with smaller tumors in high grade tumors, and vice versa in low grade tumors (Lee et al., 2010). Tumors located in the cortex and in the temporal lobe are more likely to be associated with TAS than deeply seated tumors (Chang et al., 2008; Glantz et al., 1996; Liigant et al., 2001; Lynam et al., 2007; You et al., 2012a; Zaatreh et al., 2003).

Recently, dysregulation of glutamate control through the system x_c^- transporter (Buckingham et al., 2011) resulting in an increase of extracellular glutamate has been demonstrated to play a critical role in TAS in a mouse model. In human gliomas, increased system x_c^- expression and decreased expression of the membrane glutamate transporter protein EAAT2, also resulting in increased extracellular glutamate, has been demonstrated in patients with TAS (Yuen et al.). In low grade tumors, abnormal expressions of several genes have been demonstrated from tumor and peritumoral tissue in patients with TAS (Niesen et al.; You et al., 2012b).

Given that both gene expression and neuroimaging features are key characteristics in TAS, there is likely significant interaction between these critical features. In this study, we examine the expressions of 9 genes that are associated with long term survival (Colman et al., 2010). These genes may be of particular interest in TAS as the presence of seizures has been found to be a good prognostic factor in human gliomas (Okumus et al.; Stark et al.). We hypothesize that the influence of gene expression on TAS is regional, e.g. gene

expression plays a significant role in determining epileptogenicity in certain regions of the brain whereas it plays little role in other regions where the location of tumor may predominate the determination of epileptogenicity. In regions where TAS is primarily determined by tumor location, we expect little influence of gene expression on TAS, whereas in other regions, gene expression may significantly affect TAS.

2. Materials and Methods

2.1 Cohort Selection

This retrospective study examined patients who underwent surgical evaluation of glioblastomas at the Brigham and Women's Hospital between January 2005 and September 2007. Inclusion criteria are as follows: age ≥ 18 , new diagnosis of brain tumor, supratentorial location, pathologically proven glioblastomas, preoperative acquisition of high quality volumetric MRI scan, and the availability of a gene expression data on the tumor. Because gliomas with an oligodendroglial component are more prone to TAS (Wang et al., 2012), we excluded tumors with oligodendroglial components. Electronic medical records were reviewed by a board certified epileptologist (JWL) and patients were determined to have TAS if seizures were clinically present preoperatively. The AED regimen administered postoperative, chemotherapeutic regimen, and use of radiation therapy were recorded. Carbamazepine and phenytoin were considered enzyme inducing AEDs; none of the patients received phenobarbital. Survival duration was determined by the date of diagnosis by biopsy until date of death. Approval for this study was obtained from the local human research institutional review board and was performed using a combination of consent and waiver of consent where appropriate for minimal risk studies (Dana Farber Cancer Institute and Partners Healthcare Institutional Review Boards).

2.2 Image acquisition

Patients underwent preoperative imaging with one of two MRI scanners from which T2 and volumetric T1 images were obtained: 1.5T General Electric Signa Excite scanner; 3T General Electric Signa scanner.

2.3 Image processing

Tumors were manually segmented from the MRI by a blinded rater (JWL) using standard image processing software to create a lesion mask (3D Slicer, www.slicer.com; MNI Display, www.bic.mni.mcgill.ca). Tumor margin was delineated by the area of contrast enhancement on the volumetric T1-weighted image (Lacroix et al., 2001).

MR images were transformed into a standardized coordinate space based on the Talairach atlas (Talairach and Tournoux, 1988) to account for differences in brain orientation and differences in intracranial volume. Automatic registration using linear affine transformation was performed from the T1-weighted images (Collins et al., 1994). Because distortions of the anatomy caused the registration procedure to fail at times, registration validity was checked by selecting 6 points on both the template and the target brains (maximal anterior and posterior cortical extent along the anterior-posterior commissure (AC-PC) line, upper and lower extent along the perpendicular line through the AC; left and right extent along the

third axis formed by the two previous lines); if the root mean square is greater than 5mm, registration was re-performed using the manually selected coordinates (MNI Register, www.bic.mni.mcgill.ca). Tumor volumes were calculated after registration (MATLAB, Mathworks, Natick, MA). Tumor masks were then used to determine the regions where patients were more likely to present with or without TAS using a previously described method (Lee et al., 2010). Briefly, a chi-square statistic map was calculated at each voxel to determine the deviation from the expected number of tumors at that voxel presenting with TAS. The significance of the chi-square statistic was then determined by a clustering and nonparametric resampling method. A complementary analysis was performed to determine where tumors were more likely to present in patients without TAS.

2.4 Gene expression

Blocks or regions of tumor were selected for study by a neuropathologist (KLL) for >50% tumor nuclei and <10% necrosis. Total RNA was extracted from formalin-fixed, paraffin-embedded tumor tissue (6 × 5um standard sections) and the absolute gene expression values for 9 genes were measured: [AQP1 (Aquaporin 1), CHI3L1 (Chitinase-3-like protein 1), EMP3 (Epithelial membrane protein 3), GPNMB (Glycoprotein NMB), IGFBP2 (Insulin-like growth factor binding protein 2), LGALS3 (Galectin 3), PDPN (Podoplanin), OLIG2 (Oligodendrocyte lineage transcription factor 2), RTN1 (Reticulon 1)] using the commercially available QRT-PCR based assay, DecisionDX-GBM (Castle Biosciences Inc, Phoenix, AZ). The identification of the 9 genes has been detailed previously to represent a multigene predictor with prognostic value in glioblastomas (Allingham-Hawkins et al., 2010; Colman et al., 2010) and found to be associated with long-term survival >2 years. Expression levels of OLIG2 and RTN1 were lower in patients who are long-term survivors; expressions of the other 7 genes were higher in long-term survivors.

2.5 Statistical analysis

Fischer's exact and chi-square tests were used to determine differences in categorical data. Student's t-test was used to determine differences in continuous normally distributed data. The Mann-Whitney U test was used to determine the difference in median survival between patients with and without TAS. Logistic regression was used to estimate odds ratios and 95% confidence intervals for patient characteristics associated with TAS. SAS version 9.1 was used (SAS Institute, Cary, NC).

To determine whether the expression of any of the 9 survival genes was significantly different between patients with and without TAS, t-tests were calculated and significance determined by permutation testing with 10,000 iterations (ComparativeMarkerSelection module from the GenePattern software (Reich et al., 2006), www.broadinstitute.org/cancer/software/genepattern). Effect of multiple hypothesis testing was estimated using the False discovery rate (FDR) (Benjamini and Hochberg, 1995).

Gene expression imaging analysis

For each candidate gene, to assess the localizing value of the tumors causing seizures, a T-statistic map was calculated. For each patient, the entire tumor mask was assigned the gene expression value associated with that patient after linearly normalizing the expression to a

baseline of zero and a common mean. At each voxel, the T-statistic of the gene expression difference between patients with and without TAS was calculated. To assess the areas of significance of the resulting T-statistic map, a non-parametric mapping method based on permutation testing was employed (Nichols and Holmes, 2002) which requires making minimal assumptions regarding the distribution of our data. Smoothing was performed with a Gaussian filter, $\sigma=1.7$ voxels (FWHM 4). Signal clusters were obtained through a 6-connectivity model (Bullmore et al., 1999). To determine the significant clusters without making arbitrary assumptions regarding cluster strength threshold, the threshold-free cluster enhancement (TFCE) technique was first applied, resulting in a map of TFCE scores (Smith and Nichols, 2009). Thereafter, the labeling of the each patient as “TAS” versus “no TAS” was randomly reassigned, with the constraint of preserving the original ratio of the number of “TAS” and “no TAS” patients. From the relabeled group, a T-statistic map and TFCE scores were calculated, and the maximum TFCE score across all voxels was recorded. This was repeated 5000 times to obtain a null hypothesis distribution of maximal TFCE (Bullmore et al., 1999; Manly, 1991). Clusters of the original image whose TFCE scores exceeded significance of $p<0.05$ were considered significant. Analysis was performed using MATLAB Version 7.6 (R2008a) (Mathworks, Natick, MA).

3. Results

3.1 Patient population

Seventy-seven patients with glioblastomas were included in this study. Their main clinical characteristics are listed (Table). There was no difference in patient age or sex between the two groups. Of patients with TAS, 13 had simple partial seizures, 4 had complex partial seizures, and 15 had generalized seizures.

3.2 Tumor size and location

Patients with TAS presented with smaller tumor volumes than patients without TAS (31.1 vs 58.8 cubic cm, $p<0.001$). In patients with TAS, there was a greater number of parietal lobe tumors (34.4% vs 13.3%) and nonsignificant trends towards fewer frontal lobe (28.1% vs 48.9%) and predominantly deep (12.5% vs 26.7%) tumors. Temporal lobe tumors affected patients with and without TAS in nearly equal proportions. Tumors frequently occupied greater than one lobe.

3.3 TAS and survival genes

There was a trend towards patients with TAS surviving significantly longer (median 48.4 months) as compared to patients without TAS (32.7 months, $p=0.055$). Nearly all patients (28 of 32 patients with TAS, 42 of 45 patients without TAS) underwent initial treatment with temozolomide and radiation. There was no difference in the 3 most common treatment modalities (temozolomide, bevacizumab, radiation). Nearly all patients were exposed to levetiracetam postoperatively. Patients with TAS were more likely to have used an enzyme inducing antiepileptic drug (phenytoin, carbamazepine) during treatment. Two patients from each group were still alive at the last follow-up: three of these patients were last seen at our institution in 2013, and one patient in July 2012, with survival between 48 and 199 months.

Analysis performed to determine whether the expression of any of the 9 survival genes was significantly different between patients with and without TAS revealed that OLIG2 expression was significantly lower in patients with TAS ($p=0.037$, $FDR=0.33$). (Figure 1). Univariate testing revealed that tumor volume, expression of OLIG2, and total survival duration were significantly different between patients with and without TAS. Of these, multivariate logistic regression revealed that OLIG2 expression ($p=0.0498$) and tumor volume ($p=0.0052$) remained significant predictors of TAS.

3.4 Regional effect of survival genes to TAS

The aggregate map of tumors generated from the sum of the binary tumor masks for all patients (Figure 2a) reveals highest density of tumors over the midline frontal callosal and bilateral posterior temporal/parietal regions. Regions where patients were more likely to present without TAS were determined (Figure 2b) (Lee et al., 2010). There were no regions where patients were more likely to present with TAS. The average expression map for each of the 9 tested genes is shown in Figure 3.

Gene expression imaging analysis revealed significant regions in OLIG2 and RTN1. The average expression values of these genes for patients with and without TAS are shown in Figure 4. The T-statistic map is shown for the regions for OLIG2 and RTN1 were significantly lower in patients with TAS (Figure 5). The cluster for RTN1 was 26.2 cm³ was located in the region of the right insula and temporal lobe. The cluster for OLIG2 was 4.6 cm³ in size and was located in the right posterior parietal region. There were no regions where OLIG2 and RTN1 were significantly higher in patients with TAS. None of the other genes had regions of statistical significance.

4. Discussion

We examined the effects of glioblastoma neuroimaging and the expression of 9 genes associated with long-term survival in TAS. Expression of OLIG2 was significantly lower in patients with TAS. However, in examining the regional variability, we found that there were significant effects of gene expression on TAS for 2 of the 9 genes.

None of the 9 genes have any previously described seizure-specific mechanisms of action. GPNMB (glycoprotein nonmetastatic melanoma protein B) encodes for a transmembrane protein implicated in a variety of biological process including cell differentiation, inflammation, tissue regeneration, and invasion and metastasis of malignant tumors (Huang et al., 2012). IGFBP2 encodes insulin-like growth factor-binding protein 2 which inhibits insulin-like growth factor and is overexpressed in glioblastomas (Fukushima and Kataoka, 2007); no definite seizure-related mechanism has been described. Insulin-like growth factor regulation is altered in brains after ketogenic diets although IGFBP2 does not appear involved (Cheng et al., 2003). LGALS3 encodes for the protein galectin-3, which may mediate the inflammatory response in the epileptogenic brain injury induced by the neurotoxin trimethyltin (Yang et al., 2012). It was also noted to be elevated in pilocarpine-induced seizures, though its functional role is unclear (Bischoff et al., 2012). OLIG2 encodes the oligodendrocyte transcription factor 2 protein, a helix-loop-helix transcription factor that is a universal marker of diffuse gliomas (Meijer et al.), and has higher average

expression in oligodendrogliomas relative to glioblastomas (Marie et al., 2001). We found that lower OLIG2 expression was associated with TAS, although one may have expected higher OLIG2 expression in TAS, perhaps due to unrecognized oligodendroglial components. The etiology of this discrepancy is unclear. RTN1 encodes for protein reticulon-1 involved in the neuroendocrine secretion; expression of RTN1 is decreased in mice prone to ethanol withdrawal seizures (Schafer et al., 2001). AQP1, encoding an osmotic water channel, has a higher expression in the anterior temporal neocortex of patients with intractable epilepsy as compared to control subjects (Zhou et al., 2008), though it is unclear whether this is a cause or result of seizures. Though yet unknown mechanisms of epileptogenicity is a possibility, it is more likely that these genes have strong influence in determining the location and rate of growth of tumors. Both of these factors may significantly influence both survival as well as epileptogenicity. Tumors that are more cortically located would be more amenable for more complete surgical resection as well as more likely to cause TAS. Slow growing tumors may also be more likely to be associated with longer survival as well as increased risk for TAS. Please refer to Colman et al. (Colman et al., 2010) for further details regarding the function of these 9 genes.

As tumor location has strong influence on epileptogenicity, it is reasonable to expect the influence of gene expression to be regional. In brain regions where tumors almost never or almost always cause TAS, gene expression likely has little influence in determining TAS, aside from potentially determining the location of the tumor, because location has overriding influence on TAS. The regions where gene expression is of greatest interest are in areas where tumor location has minimal upon influence TAS. In glioblastomas, location has relatively little predictive value in determining epileptogenicity in temporal lobe tumors. Our results indicate that RTN1 has particularly high correlation in this region, whereas none of the genes have correlation over frontal corpus callosum, even though the tumor density was similar in both these locations. This is consistent with our reasoning because the frontal callosal tumors are infrequently associated with TAS, and as such, it would be very unlikely that any of the gene expression values would have significant correlation in this region.

It is possible that the influence of gene expression on TAS is spatially uniform, and that our results merely indicate regions where locational influences are particularly strong. However, our maps indicate that gene expression itself is spatially non-uniform; and as such, we can expect the influence of gene expression to remain regional, even after accounting for locational differences. Further studies are required to assess these possibilities.

Our results also demonstrate the potential increase in power in detecting significant differences in gene expression when tumor location is taken into account. Only OLIG2 revealed significant difference in expression between patients with and without TAS. For RTN1, there was no significant group-wise difference in expression, even though there were specific regions in which the differences were significant. The overall groupwise test is likely diluted by regions in which gene expression does not play a significant role.

Although we have demonstrated regional variability in the influence of a gene that does not have a specific mechanism for seizures, this finding has implications for molecular mechanisms that are specific to seizures as well. In particular, targeted therapies against

such mechanisms of TAS may provide suboptimal without incorporating tumor location. This concept may be generalized to any neurological dysfunction caused by discrete brain lesions in which potential genomic or molecular markers are sought. Further investigation also needs to be performed to determine whether currently used antiepileptic drugs have differential regional effectiveness in TAS.

Patients with TAS were exposed to enzyme inducing antiepileptic drugs more than patients without TAS. There is considerable uncertainty whether this has a bearing on survival (Jaeckle et al., 2009; Oberndorfer et al., 2005). However, in this instance, the significant difference in antiepileptic drug exposure is due to the fact that patients without TAS were virtually never exposed to any other drug aside from levetiracetam, whereas patients with TAS were exposed to other drugs, presumably due to difficulties in controlling postoperative TAS. As such, we do not believe any conclusions should be drawn regarding survival and antiepileptic drugs from this study.

We have also found that patients who present with TAS have a trend towards longer survival than patient who present with other symptoms. The presence of TAS has been found to be a good prognostic factor in multiple other studies (Okumus et al.; Stark et al.). It has been postulated that TAS may result in early tumor diagnosis and that this represents a lead time bias (Stark et al.). Although our results demonstrating that patients who present with TAS have smaller tumors would seem to support that hypothesis, it is possible that tumors that cause seizures are inherently slower growing. Rapidly growing tumors are likely to cause poorly tolerated mass effect, resulting in other neurological dysfunction, rather than TAS, which likely is a more prolonged process during which the peritumoral tissue becomes epileptogenic. This hypothesis is further supported by the fact that in low grade gliomas, in which mass effect is well tolerated, large tumors are more likely to indicate longer presence of tumor, and thus produce more frequent TAS (Lee et al., 2010).

4.1 Limitations

Although the distribution of our tumor map is similar to previously published, larger tumor maps (Ellingson et al., 2012), the interpretation of this study is limited by the relatively small sample size. Some voxels were completely noninformative; for example, there were no TAS in a large portion of the right frontal lobe in our population. In addition, the border morphologies of the significant clusters are likely affected due to the abrupt change in tumor density despite smoothing. Furthermore, because the distribution of tumors is nonuniform across the brain, the power at each voxel is therefore nonuniform as well. This may limit the predictive power for an individual patient's tumor.

Because of the retrospective nature of this study, we were unable to control the MR imaging scanning parameters and as a result, some patients received scans on a 1.5T magnet while others received 3T scans. However, all patients obtained volumetric T1-weighted scans; we do not believe this difference significantly influenced our measurements.

The maximum extent of contrast enhancement was selected as the tumor boundary. It is likely that the tumor extends beyond this margin (Ramakrishna et al., 2010). However, this method results in boundaries that are easily identified, well validated, and provides

reasonable approximation of the extent of tumors. The process of tumor delineation and measurement may be questioned as TAS arise from the peritumoral tissue rather than the tumor proper (Haglund et al., 1992). However, as tumor resection often results in good seizure control (Englot et al., 2012; Rosati et al.), the intrinsic nature of the tumor likely plays a vital role in TAS.

Only 9-gene expression datasets were available for the patients in this study, limiting our ability to find the most significant genes involved in TAS. As such, we were unable to assess other genes that may have specific seizure-related mechanisms. The RNA was also obtained from paraffin embedded tissue is likely of lower quality than RNA obtained from frozen tissue. However, experiments performed during development of the genetic assay suggest that the expression pattern of the GBM genetic signature is not compromised when analyzing paraffin embedded tissue (Colman et al., 2010). Further studies systemically evaluating a complete set of gene expressions are essential to determine molecular targets of TAS. In addition, IDH mutational status was not available in this dataset; although IDH1/2 mutations predispose patients to TAS (Stockhammer et al., 2012), they are found in only a small percentage of patients with glioblastomas.

Although surgical excision occurred soon after discovery of the tumor, frequently within days, it is possible that treatment in the intervening period could have influenced gene expression. For example, corticosteroid usage, treatment with antiepileptic drugs, or stress response may all affect gene expression. In addition, selective alteration in gene expression as a result of preoperative TAS may also affect gene expression. We were unable to assay these potential confounders in this retrospective study.

The study makes two key assumptions. By assigning single scalar gene expression values to a solid tumor, the assumption of gene expression uniformity throughout a tumor is made. A higher than expected amount of intratumoral genetic heterogeneity has been demonstrated, including gliomas (Gerlinger et al., 2012; Jung et al., 1999). In this study, samples are only obtained from one region of the tumor, potentially introducing a bias. Secondly, if a tumor is associated with seizures, we are making the assumption that the entire solid lesion is epileptic. Particularly for a large lesion, there are likely regions of the tumor that do not promote epileptogenicity and other regions that do.

Despite these limitations, we used gene expression imaging analysis to demonstrate that the influence of gene expression on TAS is regional. As molecular and genomic markers are found for an increasing number of neurological diseases, particularly for discrete lesions, regional variability of these markers should be analyzed.

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Highlights

- Both imaging and gene expression play key roles in tumor associated seizures
- We developed a powerful technique called gene expression imaging
- OLIG2 and RTN1 have regional influence on tumor associated seizures
- Regional variability of any genomic or molecular markers should be considered

Figure 1.

Expression of 9 genes associated with long term survival. The difference in expression of only OLIG2 (*) is statistically significant ($p<0.05$).

Figure 2.
(A) Aggregate image of glioblastomas generated from the sum of binary tumor masks; (B)
Region in where patients were significantly more likely to present without TAS ($p < 0.05$)

Figure 3.
Relative average expression maps of the 9 genes, normalized to zero baseline

Figure 4.
Relative average expression in patients with and without TAS in genes with regional significance

Figure 5. T-statistic showing regions of significance ($p < 0.05$) in the expression difference between patients with and without TAS. Gene expression is lower in patients with TAS in OLIG2 and RTN1.

Table**Demographics**

	Seizure (32)	No seizure (45)	p value
Median age (range)	59 (26–87)	57 (24–82)	NS
Sex (M/F)	23/9	23/22	NS
Tumor volume (mean cubic cm)	31.1	58.8	<0.001
Location (%):			NS
Frontal	9 (28.1)	22 (48.9)	
Temporal	13 (40.6)	15 (33.3)	
Insular	13 (40.6)	18 (40.0)	
Parietal	11 (34.4)	6 (13.3)	0.0491
Occipital	1 (3.1)	5 (11.1)	
Predominantly deep	4 (12.5)	12 (26.7)	
Median survival months (range)	48.4 (1.9–165)	32.7 (6–199)	0.055
Bevacizunab	24	25	NS
Temozolomide	28	42	NS
Radiation	29	43	NS
Levetiracetam	31	43	NS
Enzyme inducing AED	7	1	0.008
Valproic acid	2	2	NS
Not followed until death	2	6	NS
Alive at time of study	2	2	NS