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ORIGINAL ARTICLE

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BCL2A1 is a Potential Biomarker for Postoperative Seizure Control in Patients with Low-grade Gliomas

Gan You,[1] Lin Feng,[2] Wei Yan,[1] Wei Zhang,[1] Yong-Zhi Wang,[1] Shao-Wu Li,[3] Shou-Wei Li,[4] Gui-Lin Li,[5] Yi-Jun Song,[6] Chun-Sheng Kang,[6] Yong-Ping You[7] & Tao Jiang[1]

1 Department of Neurosurgery, Beijing Tiantan Hospital, Capital Medical University, Beijing, China
2 State Key Laboratory of Molecular Oncology, Cancer Institute Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, China
3 Department of Neuroradiology, Beijing Tiantan Hospital, Capital Medical University, Beijing, China
4 Department of Neurosurgery, Beijing Sanbo Brain Hospital, Capital Medical University, Beijing, China
5 Department of Neuropathology, Beijing Neurosurgical Institute, Beijing, China
6 Department of Neurosurgery, Laboratory of Neuro-oncology, Tianjin Medical University General Hospital, Tianjin Neurological Institute, Tianjin, China
7 Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China

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Correspondence
T. Jiang, M.D., Ph.D., Department of Neurosurgery, Beijing Tiantan Hospital, Capital Medical University, Beijing 100050, China.
Tel.: +86-010-6703-6083;
Fax: +86-010-6703-6038;
E-mail: taojiang1964@yahoo.com.cn
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The first two authors contributed equally to this work.

SUMMARY

Aims: To identify molecular genetic factors that influence preoperative seizure occurrence and postoperative seizure control in patients with low-grade gliomas (LGGs). **Methods:** Fifty-four WHO grade II astrocytomas were used for microarray analysis under strict inclusion criteria. The primary endpoint was seizure control at 12 months after surgery. Biological processes were investigated by gene ontology (GO) analysis. Quantitative RT-PCR and immunohistochemistry were used to validate key genes. **Results:** Differentially expressed genes correlated with seizure occurrence failed to significantly distinguish patients with and without a history of seizures. With respect to postoperative seizure control, a transcript profile of 92 genes was identified, which successfully separated patients with good and poor seizure prognosis. GO analysis revealed that the most striking overrepresentation of genes was found in a category of anti-apoptotic genes and their regulation. Increased expression was also observed for genes involved in immune and inflammatory responses. BCL2A1 was proven to be a novel marker associated with seizure prognosis. **Conclusion:** Increased anti-apoptotic activity of tumor cells appears to contribute to seizure recurrence after surgery in patients with LGGs. These findings provide insights that may lead to the development of effective treatment strategies for prolonging the survival of patients with LGG in the future.

Introduction

Epilepsy is one of the most common neurological disorders. Seizures have been recognized for over a century as a symptom of intracerebral tumors [1], in particular slow-growing primary brain tumors for which seizure incidence may reach 75–100% [2,3]. Glioma, accounting for more than half of brain tumors, is one of the most common causes of tumor-related seizures. The presence of seizures plays an important role in the quality of life after surgery [4], especially for patients with low-grade gliomas (LGGs) because of the long duration of survival [5]. Neurological and neuropsychiatric impairments caused by the disease are often inevitable. Dealing with tumor-related seizures remains a clinically complicated problem.

Despite the importance of this subject to the field of neuro-oncology, the pathophysiological mechanisms underlying these seizures remain poorly understood. It is widely accepted that tumor-related seizures are generally induced by a peritumoral epileptic focus. However, factors inside the tumor could also contribute to epileptogenesis. Recent studies [3,6] have found that patients with the histological subtypes oligodendroglioma and oligoastrocytoma are significantly more likely to present with seizures than patients with astrocytoma. This suggests that the biological properties of gliomas, such as genetic components, may be closely related to seizure occurrence. Furthermore, several clinical studies [6–8] have revealed that seizure recurrence is associated with tumor progression. These findings strongly indicate that recurrent seizures may reflect the underlying molecular genetic activity of tumor cells.

In this study, we performed gene expression profiling of a series of 54 WHO grade II astrocytomas, with respect to preoperative seizure occurrence and postoperative seizure control. We found that BCL2A1, an apoptosis-related gene, was a robust marker of seizure prognosis in patients with LGGs.

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Patients and Methods

Patients and Tissue Samples

This study was approved by the Ethics Committee of Beijing Tiantan Hospital, and written informed consent was obtained from all the patients included. It also received institutional approval and that experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC). Between September 2005 and June 2009, 1134 patients with LGGs were treated in the Glioma Treatment Center of Tiantan Hospital (Beijing, China), and 508 of them (>16 years of age) underwent primary resection of supratentorial LGGs. Tissue samples were recovered immediately after surgery, snap-frozen with liquid nitrogen, and stored at −80°C until use. For the purpose of creating a more homogeneous sample, only patients that strictly met the following criteria were selected: (1) age <65 years, (2) no treatment with antiepileptic drugs (AEDs) before admission, (3) single lesion mainly involving frontal or temporal lobe on MRI, and (4) pure WHO grade II astrocytomas in histopathology. To make a meaningful comparison between seizure tissue and nonseizure tissue, we classified patients into two groups: (1) seizure group (containing patients who only had one or two secondarily generalized seizures before surgery), and (2) nonseizure group (duration of disease ≥3 months). Fifty-four samples were included in the microarray analysis (Figure S1). Thirty-two independent samples meeting the criteria above were obtained from our center and another two hospitals in China for further RT-PCR analysis. One hundred LGG samples were randomly selected for immunohistochemical analysis. Normal brain tissue from three trauma patients and epileptic tissue from patients with idiopathic temporal lobe epilepsy (ITLE) were collected as negative and positive controls, respectively. The clinical features and seizure prognosis information of the included 54 cases are summarized in Table 1.

Surgical Procedure and Assessment

The goal of surgery was gross total removal of tumor while protecting functional brain tissue as much as possible. Subtotal resection was performed mainly in cases of tumor involvement in eloquent brain areas, as verified by preoperative MRI or intraoperative cortical direct electrical stimulation mapping.

The extent of resection was retrospectively classified from reports on MR images performed either 3 months postoperatively or less than 72 h after resection. The classifications were as follows: (1) gross total resection (complete resection of the preoperative FLAIR or T2 signal abnormality, as seen from axial, coronal, or sagittal images), and (2) subtotal resection (nodular or thin residual FLAIR or T2 signal abnormality as seen from axial, coronal, or sagittal images). The extent of resection was assessed by an independent neuroradiologist who was blinded to patient outcomes. For patients without pre- or postoperative MR images, the extent of resection was determined by the surgeon's intraoperative impression.

Seizure Management and Outcome Measures

There are no universally accepted guidelines for the use of AEDs, and the choice of a specific AED is based on the clinician's preference. Among the 54 cases, patients without seizures were routinely administered valproic acid (VPA) for seizure prophylaxis prior to surgery and were then weaned off the medication at discharge. Patients with preoperative seizures typically continued VPA treatment for 3–6 months after surgery and were then weaned off gradually. If a patient had any evidence of seizures, AEDs treatment was continued.

The primary outcome variable was seizure status, which was evaluated at 6 and 12 months after surgery using the Engel Classification of Seizures (Class I, seizure free; Class II, rare seizures; Class III, meaningful seizure improvement; and Class IV, no sei-

Table 1 Clinical characteristics and seizure control at 12 months after surgery in 54 patients with WHO grade II astrocytoma[a]

		Seizure group			
		Seizure prognosis			
Variable	Total	Engel I	Engel II–IV	Total	No-seizure group
No. of patients	54	19	11	30	24
Male sex	34 (63.0)	9 (47.4)	8 (72.7)	17 (56.7)	17 (70.8)
Median age in yrs (range)	36.6 (18–61)	34.2 (20–48)	39.1 (24–61)	36.0 (20–61)	37.3 (18–54)
Duration in days (range)	178.5 (1–2100)	33.1 (1–90)	46.8 (7–240)	38.2 (1–240)	353.9 (90–2100)
Tumor location (involved)					
Frontal	28 (51.9)	10 (52.6)	8 (72.7)	18 (60.0)	10 (41.7)
Temporal	26 (48.1)	9 (47.4)	3 (27.3)	12 (40.0)	14 (58.3)
Extent of resection[b]					
Gross total	20 (37.0)	9 (47.4)	1 (9.1)	10 (33.3)	10 (41.7)
Subtotal	34 (63.0)	10 (52.6)[c]	10 (90.9)[c]	20 (66.7)	14 (58.3)

[a]Unless otherwise indicated, values represent numbers of patients with percentages in brackets. [b]Predictor of seizure prognosis in patients with preoperative epilepsy, $P = 0.032$, χ^2 test. [c]Used for analysis of seizure prognosis by mRNA expression profiling.

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zure improvement or worsening) [9]. For statistical analysis, the Engel classification was dichotomized as Class I (completely seizure free) versus Class II–IV (not seizure free) [6,9]. Early postoperative seizures (within 1 week of surgery) were not considered in the evaluation of seizure prognosis. Seizure control at 12 months after surgery was the primary endpoint.

RNA Preparation

Total RNA was extracted from frozen tumor tissues with the mirVana miRNA Isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA was quantified using a ND-1000 UV-VIS spectrophotometer (NanoDrop Technologies, DE, USA). The integrity of RNA was assessed with the RNA 6000 LabChip kit in combination with the Agilent 2100 Bioanalyzer (Agilent, CA, USA) according to the manufacturer's instructions. The RNA samples used in this study all had a 260/280 ratio of >1.9 and an RNA integrity number of >7.0.

Microarray Expression Profiling

The expression profiling of all the samples was carried out with the Agilent Whole Human Genome Oligo Microarray kit, which has a 4 × 44K slide format, with each block representing more than 41,000 unique human genes and transcripts. All the sample labeling, hybridization, washing, and scanning steps were conducted according to the manufacturer's specifications at a laboratory in the Cancer Institute (Hospital) of Peking Union Medical College. In brief, Cy3-labeled cRNA was generated from 500 ng input total RNA by *in vitro* transcription, using Agilent's Low RNA Input Linear Amplification Kit PLUS. Then, 1.65 μg of cRNA from each labeling reaction was hybridized to one block of the microarray. After hybridization, slides were washed and then scanned with the Agilent G2565BA Microarray Scanner System. Fluorescence intensities of the scanned images were extracted and preprocessed with the Agilent Feature Extraction Software (v9.1). Data normalization and filtering were performed using the GeneSpring GX 11.0 (Agilent Technologies). Only genes with expression levels marked as "Present" or "Marginal" in all of the chips (blocks) passed through the quality filtering. The data were normalized using the default settings of the GeneSpring software: set measurements less than 0.01–0.01, and chip data normalized to 50th percentile of per chip intensity.

Quantitative RT-PCR

Real-time RT-PCR (qPCR) was used to investigate mRNA expression. PCR primers were designed using Primer Premier 5.0 based on the reported cDNA sequences (Table 2). PCR reactions were carried out in a total of 25-μL reaction mixture (2 μL of cDNA, 12.5 μL of 2 × SYBR Green PCR Master Mix, 1.5 μL each of the 5 μM forward and reverse primers, and 7.5 μL of H_2O) in the Mx3000P™ real-time PCR system (Stratagene, USA). The PCR program was initiated for 5 min at 95°C before 40 thermal cycles, each for 15 second at 95°C, and 1 min at 60°C. Data were analyzed according to the comparative Ct method and were normalized using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression in each sample.

Immunohistochemistry

Immunoperoxidase staining for the BCL2A1 protein (polyclonal rabbit, Abcam, USA, 1:100) was performed on formalin-fixed, paraffin-embedded tissue sections following the standard protocol recommended by the manufacturer. Approximately 10–15 fields at ×200 magnification were analyzed per specimen. For purposes of statistical analysis, the percentage of positive cells for each specimen and the cutoffs for dichotomization analysis were determined as described previously [10,11]. High BCL2A1 expression was defined as strong cytoplasmic staining in at least 30% of the tumor cells (Figure 1).

Table 2 List for primers used for RT-PCR

Gene	Reference sequence	Forward primer (5′-3′)	Reverse primer (5′-3′)	Tm
CCL8	NM_005623	CAGCCACTTTCAGCCCTCA	TGGAATCCCTGACCCATCTC	60°C
HES5	NM_001010926	ACATCCTGGAGATGGCTGTCA	GCCTTCGCTGTAGTCCTGGT	60°C
BCL2A1	NM_004049	GTCCGTAGACACTGCCAGAACA	AAAGTCATCCAGCCAGATTTAGG	60°C
GAPDH	NM_017008	AGTGCCAGCCTCGTCTCATAG	CCT TGACTGTGCCGTTGAACT	60°C

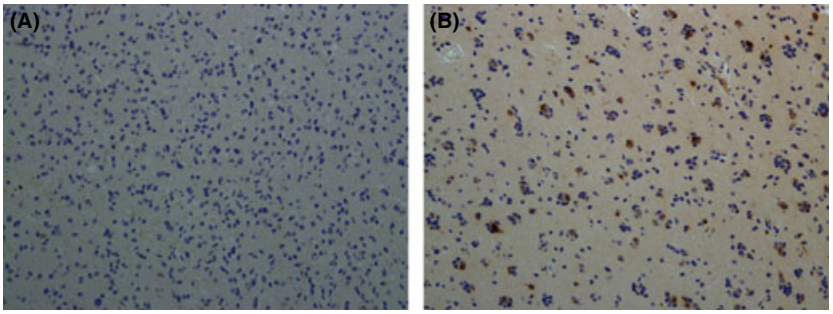

Figure 1 Photomicrographs of tumor tissue stained for BCL2A1, showing different expression levels. (**A**) Low BCL2A1 expression. (**B**) High BCL2A1 expression. Original magnification ×200.

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Statistical Analysis

Statistical analysis was performed using SPSS (13.0) and was designed to address factors influencing postoperative seizure controls at 12 months of follow-up. Univariate analyses were carried out using the chi-squared (χ^2) test for dichotomous variables, and the Mann–Whitney *U*-test was used for continuous nonparametric data. To identify significantly differentially expressed genes, two-tailed Student's *t*-tests were used. Principal component analysis (PCA) and unsupervised clustering were carried out using R, a language and environment for statistical computing (http://www.r-project.org/). Gene ontology (GO) analysis was performed using David Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/home.jsp). Independent samples *t*-tests were used for qPCR-related analyses. $P < 0.05$ was considered statistically significant.

Results

Microarray Gene Expression Analysis

To identify genes that were differentially expressed between the seizure group and the nonseizure group, we performed PCA and cluster analysis. The results revealed thousands of differentially expressed genes, but PCA and clustering did not reveal any signature (data not shown).

Regarding postoperative seizure control, we divided the patients with preoperative seizures (n = 30) into two groups (good vs. poor seizure prognosis) using the Engel Classification. Because the extent of tumor resection was the only factor influencing seizure prognosis ($P = 0.032$, χ^2 test), we subsequently stratified these 30 patients into a gross total group (10 patients) and a subtotal group (20 patients). Two-tailed Student's *t*-tests were used to identify genes associated with postoperative seizure control in the subtotal group. The algorithm for patient selection is shown in Figure S2. In total, 3006 significantly differentially expressed transcripts (1143 overexpressed and 1863 underexpressed) were identified using a criterion of $P < 0.05$. PCA analysis revealed regional distribution of these 20 samples (Figure 2A). After defining the fold change to more than 1.8, 92 transcripts (Table S1) were identified. Table 3 only shows the 10 most affected genes (mRNA with known gene symbol). Then, we performed hierarchical clustering, which showed a clear separation of the patients with good seizure control from those with poor seizure control (Figure 2B). These results show

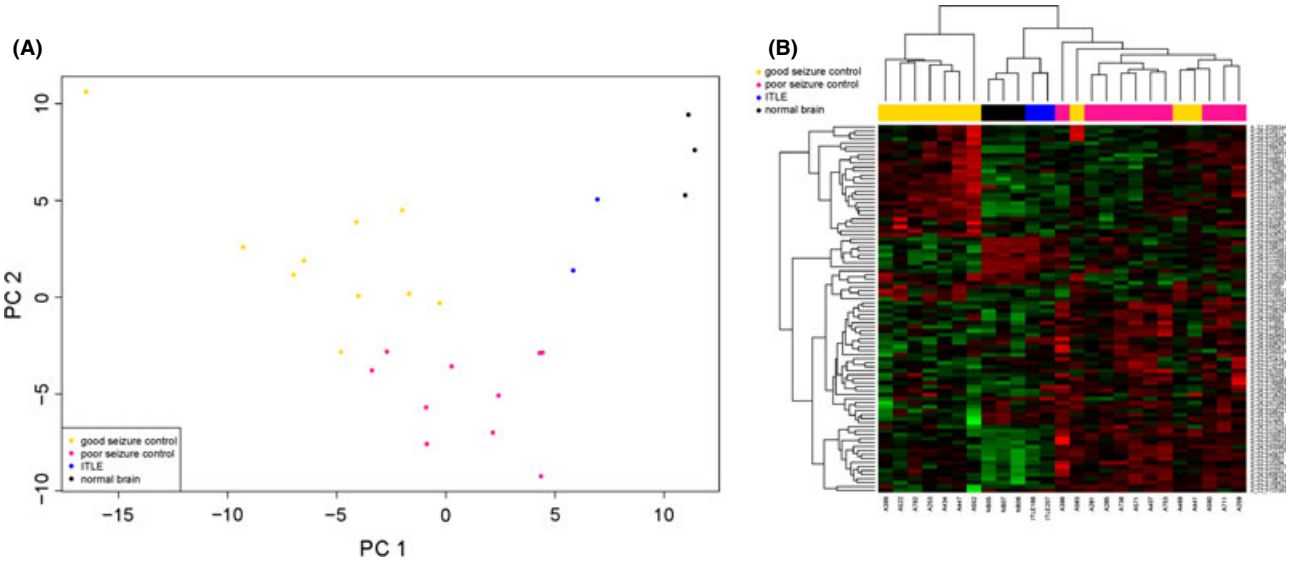

Figure 2 Principal component analysis (PCA) and cluster analysis of the differentially expressed genes associated with postoperative seizure control. (**A**) PCA results showing regional distribution of the 20 samples. (**B**) Heat map showing a clear separation of them.

Table 3 Top ten genes dysregulated in patients with poor seizure prognosis

Probe Name	GeneSymbol	Regulation	Fold change	*P*-value	Description
A_23_P207456	CCL8	↑	3.739250	0.034445	Homo sapiens chemokine (C-C motif) ligand 8
A_32_P234184	HES5	↓	3.506132	0.037387	Homo sapiens hairy and enhancer of split 5 (Drosophila)
A_23_P152002	BCL2A1	↑	3.008584	0.038437	Homo sapiens BCL2-related protein A1
A_24_P932435	ANGPT2	↑	2.684968	0.039039	Homo sapiens angiopoietin 2
A_23_P257043	GEM	↑	2.571687	0.000163	Homo sapiens GTP-binding protein overexpressed in skeletal muscle
A_23_P86470	CH25H	↑	2.550651	0.049221	Homo sapiens cholesterol 25-hydroxylase
A_23_P85693	GBP2	↑	2.501403	0.029115	Homo sapiens guanylate-binding protein 2, interferon-inducible
A_24_P926783	ZNF880	↑	2.495428	0.006305	Homo sapiens zinc finger protein 880
A_23_P47058	CUZD1	↓	2.274426	0.006830	Homo sapiens CUB and zona pellucida-like domains 1
A_23_P133474	GPX3	↑	2.249667	0.048845	Homo sapiens glutathione peroxidase 3 (plasma)

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that specific expression profiles can be found that are characteristic for the respective seizure prognosis.

Gene Ontology Analysis

To identify functionally relevant gene clusters, we performed GO enrichment analysis of the 92 transcripts mentioned above, using the David Bioinformatics Resources. Although a *P*-value cutoff of 0.05 is often used for exploratory research, we used a cutoff of 0.01 to select the most relevant functions and processes (Table 4). The most striking overrepresentation of genes was found in a category of anti-apoptotic genes and their regulation.

Quantitative RT-PCR Analysis

To independently validate our results, we used qPCR to quantify mRNA levels. Three top genes (CCL8, HES5, and BCL2A1) were selected based upon the fold change (>3.0). First, to analyze the reliability of the microarray, five samples of RNA were randomly selected from the 54 samples and used for qPCR. The correlation between ΔCt of the qPCR and the gene signal of the microarray was analyzed. As shown in Figure 3, the qPCR analysis for CCL8 and BCL2A1 replicates the microarray data well, but the difference in HES5 expression was not significant. Therefore, HES5 was excluded from further analysis. Second, for the purpose of validating the roles of CCL8 and BCL2A1 in predicting seizure control, qPCR of these two genes was performed in an independent multicentric cohort of 32 samples. We found that patients with higher BCL2A1 expression were more likely to have poor seizure control after surgery ($P = 0.009$, *t*-test) (Figure 4). We found no significant association for CCL8 ($P = 0.109$, *t*-test).

Immunohistochemical Analysis

Based on the above findings, we performed immunohistochemical validation for BCL2A1 as a marker for predicting seizure control. We used 100 LGG samples, including 13 oligodendrogliomas, 55 astrocytomas, and 32 oligoastrocytomas. All the patients had a history of seizures before surgery and available follow-up information at 12 months after surgery. For the patients with astrocytomas and oligoastrocytomas, high BCL2A1 expression was associated with poor seizure control ($P = 0.018$ and 0.049, respectively, χ^2 test). This further confirmed the results of the qPCR and microarray analysis, strongly suggesting that BCL2A1 is a potential biomarker for seizure prognosis in patients with LGGs. No significant correlation was found for oligodendrogliomas (Table 5).

Table 4 Gene ontology results of 92 dysregulated transcripts

GO Accession	GO terms	Count	*P*-value
GO:0045768	Positive regulation of anti-apoptotic	3	5.80E-05
GO:0005576	Extracellular region	14	8.74E-05
GO:0045767	Regulation of anti-apoptotic	3	9.27E-05
GO:0002011	Morphogenesis of an epithelial sheet	2	1.30E-04
GO:0004630	Phospholipase D activity	2	1.30E-04
GO:0042542	Response to hydrogen peroxide	3	3.22E-04
GO:0007165	Signal transduction	17	4.36E-04
GO:0034381	Lipoprotein particle clearance	2	7.31E-04
GO:0000302	Response to reactive oxygen species	3	7.92E-04
GO:0001817	Regulation of cytokine production	4	9.10E-04

Discussion

Tumor-related epilepsy is one of the most common types of symptomatic epilepsy, but the underlying pathogenesis remains unclear. Various studies [12,13] have implicated changes in amino acid neurotransmitters and their receptors, ion channels, pH, reactive astrocytosis, microglial proliferation, and gap junctions, as well as derangements in the blood brain barrier and inflammatory changes. A recent study [14] indicated that glutamate released by the primary brain tumor could induce epileptic activity, suggesting that an important mechanism of seizure genesis originates from the tumor itself. Consistent with this hypothesis, previous microarray studies [15,16] have demonstrated that aberrant development of neuronal precursors and immune and inflammatory responses were the most prominent processes expressed in epilepsy-associated gangliogliomas.

Our study reports an array-based comparison of glioma specimens from patients with and without epilepsy. Unfortunately, our results did not clearly indicate any relationships between gene expression patterns and seizure occurrence. This may be owing to shortcomings in the inclusion criteria. More unique and strict conditions may need to be used to define the two group, such as the inclusion only of patients with a single tumor with a frontal or temporal lobe location or with a longer duration of preoperative seizures.

Seizures play an important role in the patients' quality of life after surgery. Although more than half of the patients with LGGs may have favorable seizure prognosis after surgery, about 30% suffer from uncontrolled seizures despite treatment with different AEDs [6,17]. de Groot et al. [18] indicated that expression of synaptic vesicle protein 2A in glioma and peritumoral tissue predicts seizure prognosis, because it is correlated with the clinical response to levetiracetam. This strongly suggests that some genetic factors of tumor cells may contribute to seizure control after surgery.

In the present study, we first used large-scale expression profiling to identify genes associated with postoperative seizure control in patients with glioma. Analysis using 92 differentially expressed features perfectly separated patients with good and poor seizure prognosis. By cluster analysis of the gene expression profiles, we found a few characteristic biomarkers correlated with seizure prognosis. Throughout the study, we placed emphasis on patient selection, reliability, and reproducibility. Eventually, BCL2A1 was proved to be an extremely important gene by both qPCR and immunohistochemical analysis in an independent patient population. These findings were consistent with the results of GO analysis, which suggested that anti-apoptotic activity of tumor cells may be correlated with seizure recurrence after subtotal tumor resection.

B-cell lymphoma 2 (BCL2) proteins are important cell death regulators, whose main function is to control the release of cytochrome c from mitochondria in the intrinsic apoptotic pathway. A homologous protein, BCL2-related protein A1, is one of the less

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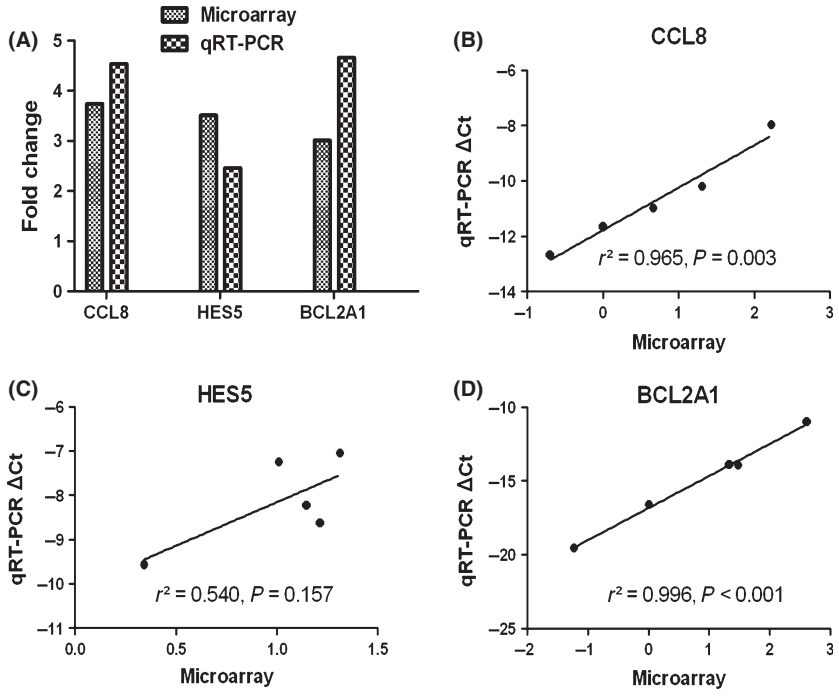

Figure 3 Validation of microarray data with qPCR. (**A**) The fold change in expression of the three genes identified with microarray analysis was confirmed by qPCR. (**B**, **D**) The qPCR analysis for CCL8 and BCL2A1 supported the microarray data. (**C**) No significant correlation was observed for HES5.

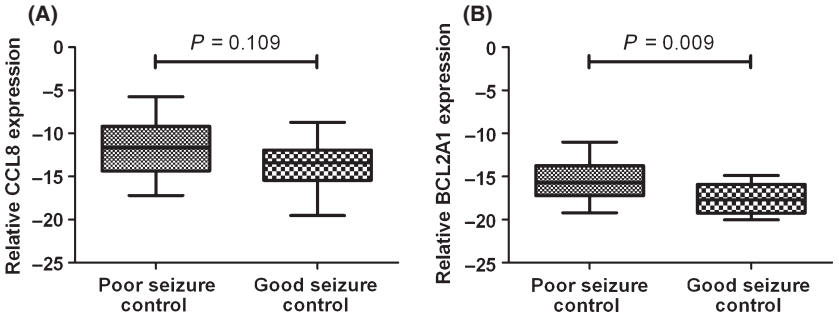

Figure 4 Comparison of qPCR results in patients with good versus poor seizure control (values represent $-\Delta$Ct). (**A**) No significant differences in transcript abundance were observed for CCL8. (**B**) BCL2A1 expression was significantly up-regulated in patients with poor seizure control.

extensively studied anti-apoptotic proteins. The human gene BCL2A1 is located on chromosome 15q24.3 [19] and is overexpressed in a variety of cancer cells, including hematological malignancies [20,21] and solid tumors [22,23]. BCL2A1 appears to be predominantly associated with advanced or metastatic disease stages. To date, no studies have reported a relationship between cell apoptosis and epileptogenicity. However, we propose the following hypothesis as a preliminary basis for guiding further investigation. As shown previously [6,7,17], we found a relationship between the extent of resection and seizure prognosis. It is generally accepted that minimizing the residual volume of tumors by performing gross total resection can decrease the risk of tumor-related epileptogenesis. This strongly suggests that tumor cells may create internal epileptogenicity through their intrinsic characteristics—such as anti-apoptotic activity. Based on the fact that tumor progression may contribute to seizure recurrence, it would be reasonable to suggest that tumors with stronger BCL2A1 expression may be more likely to cause tumor progression, leading to poor seizure prognosis.

Table 5 Relationship between expression of BCL2A1 protein[a] and seizure prognosis in different histology of WHO grade II gliomas[b]

	Seizure prognosis			
Histology	Good	Poor	Total	*P*-value
Oligodendroglioma	9	4	13	0.188
BCL2A1 ↑	3 (33.3)	0 (0)		
BCL2A1 ↓	6 (66.7)	4 (100)		
Astrocytoma	38	17	55	0.018
BCL2A1 ↑	16 (42.1)	13 (76.5)		
BCL2A1 ↓	22 (57.9)	4 (23.5)		
Oligoastrocytoma	23	9	32	0.049
BCL2A1 ↑	9 (39.1)	7 (77.8)		
BCL2A1 ↓	14 (60.9)	2 (22.2)		

↑, high expression of BCL2A1; ↓, low expression of BCL2A1. [a]Immunohistochemical analysis. [b]Values represent numbers of patients, with percentages in brackets.

Early and recent studies [24–26] have indicated that BCL2A1 exhibits an important pro-inflammatory function. Evidence for the importance of BCL2A1 expression in endothelial cells was provided by the finding that monocytes can induce BCL2A1 up-regulation in endothelial cells to protect them from cell death

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[27]. It is still unknown whether BCL2A1 also plays an inflammatory role in tumor genesis or glioma-related epilepsy. Nonetheless, according to some studies [16,28] and our findings (CCL8 and the top three unknown immunoglobulins in the Table S1), it seems that immune and inflammatory responses may also be very important biological processes in tumor-related epilepsy.

Interestingly, in the immunohistochemical validation cohort, we found that BCL2A1 expression was correlated with seizure prognosis only in astrocytic tumors. This might be because of the relatively low expression of BCL2A1 in pure oligodendrogliomas. Further studies are required to investigate whether this suggests that BCL2A1 could also function as a marker for distinguishing astrocytomas from oligodendroglial tumors.

Conclusions

This microarray investigation yielded a large number of genes related to tumor-related epilepsy. BCL2A1 was demonstrated to be a marker associated with the prognosis of postoperative seizure control. Increased anti-apoptotic activity of tumor cells, as well as immune and inflammatory responses, appears to be important processes underlying seizures. Our study gives further support to the idea that tumor cells may create internal epileptogenicity through their intrinsic characteristics. These findings may provide insights useful for the development of treatment strategies aimed at prolonging survival of patients.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Figure S1. Procedure for selection of the 54 samples used for gene expression profile analysis.

Figure S2. Algorithm for patient selection regarding seizure prognosis.

Table S1. 92 probes dysregulated in patients with poor seizure prognosis.

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