

Evaluation of the Expression Levels of Cannabinoid Type-1 (CB1) Receptor and Phosphorylated Extracellular Signal-Regulated Kinase (p-ERK) in Gliomas

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Research

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Abstract

Background

Gliomas are the most frequent primary brain tumors and one of the most aggressive forms of cancer. Recently, numerous studies have focused on cannabinoids as a new cancer-therapeutic approach due to their antineoplastic effects through activation of the cannabinoid receptors, CB1 and CB2. The aim of this study was to investigate the expression levels of cannabinoid type-1 (CB1) receptors and phosphorylated extracellular signal-regulated kinase (p-ERK) in human glioma samples and evaluate their clinicopathologic significance.

Materials and Methods

We analyzed the expressions of CB1 receptors (CB1R) and p-ERK in 61 paraffin-embedded gliomas and 4 normal brain tissues using automated immunohistochemical assay. CB1R and p-ERK expressions were categorized into high versus low expression levels. Statistical analyses were performed to evaluate the association between CB1R and p-ERK expression levels and the clinicopathologic features and overall survival.

Results

Our results showed that CB1R is down-expressed in glioma tissues compared to normal brain tissues. However, the low expression of CB1R was found to be not related to the malignancy grade of gliomas. Conversely, higher expression of p-ERK was noted in gliomas compared to normal brain tissues, with no association observed with tumor grades. In addition, CB1R expression was not observed to be associated with clinicopathologic features of gliomas, except for p-ERK expression.

Conclusion

Our findings indicate a down-expression of CB1R and overexpression of p-ERK in glioma tissues when compared to non-cancerous brain tissues. This change in CB1R expression in gliomas may contribute to further research on the therapeutic effects of cannabinoids in human gliomas.

1. Introduction

Gliomas or glial tumors represent the majority of primary brain tumors in adults accounting for nearly 70% of these tumors [1]. They are amongst the most lethal cancers with an increasing incidence rate over the years [2]. Despite advancements in cancer treatment, gliomas are still characterized by low survival rates compared to other tumors. This instigates a dire need for identifying novel target-directed therapies based on the increased knowledge of the molecular and cellular biology of gliomas [3].

From the hemp plant, *Cannabis sativa*, more than 400 chemical entities can be extracted, of which more than 60 of them are cannabinoid compounds. The effects of cannabis administration involve impairment of the motor coordination, impairment of short-term memory, hypothermia, catalepsy, analgesia, vasodilation, tachycardia, and bronchodilation among others [4]. The most abundant psychotomimetic plant-derived cannabinoid is Δ^9 -tetrahydrocannabinol (THC). Other cannabinoid compounds incorporate cannabidiol (CBD) and cannabinol (CBN) and have psychoactive properties similar to THC [5].

Cannabinoids have been recently utilized adjunctively in cancer treatment to mitigate the adverse effects of chemotherapy and improve quality of life. Numerous studies have shown that cannabinoids can inhibit cancer growth [6–7]. A number of clinical trials involving cannabinoids have announced a significant reduction in tumor size and prolonged survival time of patients with brain tumors [8]. The antineoplastic effects of cannabinoids involve different signaling pathways such as the mitogen-activated protein kinase (MAPK)/(ERK1/2) pathway [9–10].

The biological effects of cannabinoids are mediated through specific G-protein coupled receptors (GPCR), CB1 and CB2 receptors [11]. The cloning of the cannabinoid receptors, CB1 receptors (CB1Rs) in 1990 [12] and CB2 receptors (CB2Rs) in 1993 [13], prompted recognizable proof of the presence of endogenous ligands, called endocannabinoids including Anandamide (*N*-arachidonoyl ethanolamine, AEA) and 2-arachidonoyl glycerol (2-AG) [14]. CB1Rs are highly expressed in the brain and mediate many neuronal effects produced by endocannabinoids and cannabinoid drugs [15]. CB2Rs are also distributed in many areas in the brain at lower levels than CB1Rs, yet they have a more pronounced expression in peripheral immune and hematopoietic cells [16].

Cannabinoid CB1Rs are also expressed in many cancers; however, their expression levels vary. De Jesús *et al.* reported a decrease in the expression of CB1R in human glioma, with direct correlation with tumor grades [17]. In contrast, another study showed overexpression of CB1Rs in human glioma, which was positively associated with tumor grade [18–19]. Another study revealed that CB1Rs' overexpression was not associated with tumor grade in glioma [20]. Nonetheless, no difference in the expression level of CB1R was noted between normal brain tissues and glioblastoma multiforme cells as reported by Schley *et al.* [21], and weakly positive expression of CB1R was shown in both normal brain tissues and gliomas by Calatuzzolo *et al.* [22]. In other tumors, CB1R have been found to be overexpressed at the protein level in prostate cancer [23], pancreatic cancer [24], hepatocellular carcinoma [25], melanoma [26], breast cancer [27], lung cancer [28], lymphoma [29] and ovarian cancer [30]. However, CB1 receptors downregulation was observed in renal tumor tissues [31], and no change in the expression of CB1R was reported in bone cancer [32]. To date, expression levels of CB1R in human gliomas remain controversial. Therefore, the aim of the present study was to examine CB1R and p-ERK expression levels in human gliomas compared to that in normal brain tissues and reveal its clinicopathologic and prognostic significance.

2. Materials And Methods:

2.1. Patients selection and tissue specimens:

A total of 61 formalin-fixed paraffin-embedded glioma samples from patients who underwent surgical resection at different hospitals in Lebanon between June 2005 and October 2018 were provided by INP. Based on the World Health Organization (WHO) classification system, tumors were divided according to pathological grade as follows: 24 cases with low-grade gliomas and 37 cases with high-grade gliomas. Out of the 61 specimens, 19 were resected from the frontal lobe, 18 from the temporal lobes, 13 from the parietal lobes, and one specimen from the occipital lobe. The localization was not documented for the remaining ten glioma specimens. The average age of the patients was 48.84 years (range, 12–80) and male-to-female ratio was 37:24. None of the patients received any type of therapy prior to surgery. As a control, 4 normal cerebral cortex brain tissues were obtained from patients who received epilepsy surgery and verified for the absence of any tumor. Three of these 4 normal brain tissues were from the temporal lobes and the remaining one was from the frontal lobe. In addition, 38 normal brain tissues were found adjacent to tumors (internal controls) and were verified for the absence of any infiltration of tumor cells by a pathologist.

2.2. Immunohistochemical procedure:

Serial sections of 4 μm thickness from each paraffin block were mounted on charged slides and dried in an oven at 60°C for about 30 min. The immunostaining was carried out using an automated immunohistochemical staining system Ventana BenchMark XT autostainer with Ultraview Universal DAB detection kits (Ventana Medical Systems, Tucson, AZ). All the sections were stained in runs following one another. The automated staining technique is widely used in histology laboratories and research studies as it allows monitoring for errors such as inadequate volumes of reagents and unadjusted temperature. The solutions used were from Ventana Medical System, Inc., USA. Antigen retrieval was achieved using heat-induced epitope retrieval (HIER) for 10 min at a temperature of 100 °C. Prior to starting our experiments, we assessed two protocols for antigen retrieval: the first was based on enzymatic retrieval by protease I, and the other was HIER, at different concentrations. Results of HIER were compared with those obtained by protease digestion showing clearly superior influence of heat. The appropriate staining was achieved by HIER at 1:40 dilution. The used protocol in our study was approved by two independent pathologists, taking into consideration that HIER was performed in a convenient buffer and in a short period of time to prevent tissue damaging. The antibodies used were anti-cannabinoid receptor I rabbit polyclonal antibody (diluted 1:40; ab23703; Abcam; Cambridge, UK), anti-ERK1 (phospho Y204) + ERK2 (phospho Y187) rabbit polyclonal antibody (diluted 1:100; ab47339; Abcam; Cambridge, UK) and Ki67 anti-human mouse monoclonal antibody (ready to use dilution; PA0118; Leica Biosystem, UK) with an incubation time of 30 min at 36 °C. Antibodies used were previously validated, such as anti-cannabinoid receptor I rabbit polyclonal antibody (ab23703; Abcam; Cambridge, UK) [33][34], and shown to produce an appropriate pattern of staining in paraffin-embedded formalin-fixed sections. CB1 receptor specificity has been verified using IHC on normal human cerebral cortex brain tissue slides purchased from Abcam, Cambridge, UK (ab4296) (Fig. 1A). Normal cerebral cortex brain tissues from our cohort of patients were also stained in order to compare the staining of tumor tissues with that in normal ones (Fig. 1B). Besides,

positive staining of antibodies was compared to an appropriate negative control to avoid the false results of background staining (Fig. 1C). The omission of the primary antibody served as a negative control by which it was substituted with non-immune serum. The visualization system was OptiView DAB. The counterstaining with Hematoxylin II and Bluing reagent followed the immunostaining step. The prepared slides were dehydrated and covered by coverslips. Normal brain tissues used as negative and positive controls were included in each run.

2.3. IHC evaluation:

All the immunostained sections were examined for CB1R, p-ERK and Ki67 expressions under a light microscope and assessed by two independent pathologists in a blinded manner without any knowledge of the clinicopathologic characteristics of the samples. The evaluation of the immunostained sections was based on the staining intensity. The CB1R and p-ERK staining was scored in normal and tumor tissues from 0 to 3 (tissues with no staining were scored as 0; weak staining as 1, moderate staining as 2, and strong staining as 3). For statistical analysis, 0 and 1 scores were counted as low expression, whereas 2 and 3 scores were counted as high expression [33]. Ki67 scores were determined by assessing the percentage of cells expressing Ki67 immunoreactivity. The percentages ranged from 0–70%. The division of data as $\leq 30\%$ and $> 30\%$ represents a division around the median and considered as low and high Ki67 expression respectively [35].

2.4. Analysis of mRNA expression patterns among the different publicly available online datasets:

We surveyed different publicly available datasets using data retrieved from the online database Oncomine (URL: <http://www.oncomine.org>; RRID:SCR_007834). Those datasets are comprised of human glioma and brain tumor tissues of different stages and types to better understand the expression pattern of *CNR1* (*CB1R*) gene. Expression within tumor tissues was presented by fold-change expression and *p*-values were obtained using *t*-tests. Data from the different datasets is presented as box and whiskers plots indicating median and interquartile range, and *p*-values were obtained using *t*-tests.

2.5. Statistical analyses

Statistical analysis was conducted using SPSS 24.0 statistical analysis software (SPSS, Inc., Chicago, IL, USA). The association between the expression of CB1R and clinicopathological variables was analyzed by Chi-square test. Fisher's exact test was used when necessary. Independent sample *t*-test was used to assess the difference in expression between groups. Pearson's correlation coefficient was performed to quantify CB1R and p-ERK expressions. Survival analysis was performed by the Kaplan-Meier test, and groups were compared by the log-rank test. The factors related to overall survival were determined by the Cox proportional hazard regression model. The differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. CB1R and p-ERK expressions in normal brain samples

The expressions of CB1R and p-ERK were assessed in normal brain tissues including 4 normal tissues and 38 normal tissues adjacent gliomas. CB1R positive staining was detected in all the normal brain tissues. High immunoreactivity was shown in all normal tissues (100%) and in 32/38 (84.2%) normal tissues adjacent to tumors. The staining intensity of CB1R did not vary between normal brain tissues ($p = 0.429$, independent sample t -test). The expression of CB1R was mainly located at the cell membrane and in the cytoplasm of neurons and glial cells and clearly found on the axons (Fig. 2A and 1B).

With respect to the intensity of p-ERK, positive staining of p-ERK was also detected in all the normal brain tissues. However, weak p-ERK immunoreactivity was observed in all normal tissues (100%) and in 17/38 (44.7%) normal tissues adjacent to tumors. Statistical analysis showed no difference in the intensity of p-ERK expression in normal tissues compared to normal tissues adjacent tumors ($p = 0.083$; independent sample t -test). Neurons and glial cells exhibited weak positive cytoplasmic and nuclear staining for p-ERK (Fig. 2B).

3.2. Comparison of CB1R and p-ERK expressions in glioma and normal brain tissues

CB1R and p-ERK expression levels in glioma were also evaluated. Positive IHC staining of CB1R was detected in 59 patients (96.7%) out of 61. The expression of CB1R was found at the cell membranes, in the cytoplasm and nuclei of tumor cells. The tumors exhibited distinct degrees of CB1R immunoreactivity. Zero immunoreactivity was observed in 2 specimens (3.3%), weak immunoreactivity in 11 specimens (18.0%), moderate immunoreactivity in 21 specimens (34.4%), and high immunoreactivity in 27 specimens (44.3%). A significant difference in the staining intensity of CB1R was noted between glioma and normal tissues ($p < 0.001$; independent sample t -test) and between glioma and the normal tissues adjacent tumors ($p < 0.001$; independent sample t -test) with more pronounced expression in normal tissues in both cases (Fig. 2C).

With respect to the intensity of p-ERK, positive staining of p-ERK was also found in 59 (96.7%) glioma cases out of 61 in which p-ERK expression was observed in the cytoplasm and nuclei of tumor cells. High p-ERK immunoreactivity was detected in 49 (80.3%) of glioma samples. Statistical analysis revealed a significant difference between glioma and normal tissues ($p < 0.001$; independent sample t -test) and between glioma and normal areas adjacent tumors ($p < 0.001$; independent sample t -test) with p-ERK higher expression in glioma in both cases. Representative images are presented in Fig. 2D.

3.3. Clinicopathologic significance of the expression of CB1R in glioma

Investigation of the clinicopathologic significance of CB1R expression in glioma is summarized in Table 1. Samples with scores 0–1 and 2–3 were categorized as having low and high expression of CB1R and p-ERK (Fig. 3). No obvious relationships were noted between the expression of CB1R and

clinicopathologic parameters, including patient gender ($P = 0.539$), age ($P = 0.063$), tumor grade ($P = 0.941$), resection size ($P = 0.433$), necrosis ($P = 0.571$), vessel density ($P = 0.055$) and Ki67 expression ($P = 0.910$). Yet, the expression of CB1R was significantly associated with p-ERK expression ($p = 0.028$). In addition, Pearson's correlation coefficient indicated that there was a weak positive correlation between CB1R and p-ERK expressions ($r = 0.210$, $P < 0.01$).

Table 1
 Characteristics of glioma tissues with high and low expression of CB1R.

Clinicopathologic characteristics	Total = 61 No. (%)	Expression of CB1R		P-value
		Low No. (%)	High No. (%)	
Gender				
Male	37 (60.7)	9 (14.8)	28 (45.9)	0.539
Female	24 (39.3)	4 (6.6)	20 (32.7)	
Age				
≤ 49	29 (47.5)	3 (4.9)	26 (42.6)	0.063
> 49	32 (52.5)	10 (16.4)	22 (36.1)	
Tumor Grade				
Low grade	24 (39.3)	5 (8.2)	19 (31.1)	0.941
High grade	37 (60.7)	8 (13.1)	29 (47.6)	
Resection size (cm)				
≤ 2	34 (55.8)	6 (9.9)	28 (45.9)	0.433
> 2	27 (44.2)	7 (11.5)	20 (32.7)	
Necrosis				
Absence	24 (39.3)	6 (9.9)	18 (29.4)	0.571
Presence	37 (60.7)	7 (11.5)	30 (49.2)	
Vessels density				
Normal	8 (13.1)	4 (6.6)	4 (6.6)	0.055
Increased	53 (86.9)	9 (14.8)	44 (72.1)	
Ki67				
Low expression	32 (52.5)	7 (11.5)	25 (40.0)	0.910
High expression	29 (47.6)	6 (9.9)	23 (37.7)	
p-ERK				
Low expression	4 (6.6)	3 (4.9)	1 (1.7)	0.028
High expression	57 (93.4)	10 (16.4)	47 (77.0)	
P-value < 0.05 was considered significant.				

3.4. Factors affecting overall survival

Kaplan-Meier analysis showed that there was no significant evidence of a statistical difference in the survival times for patients with low and high expression of CB1R ($P = 0.554$) and with low and high p-ERK expression ($P = 0.883$) (Fig. 4). Moreover, multivariate analysis using Cox's proportional hazards model revealed that there was no evidence of a greater risk of death in association with age, histological grade, CB1R expression, p-ERK expression and Ki67 expression (Table 2).

Table 2

Contribution of many prognostic factors to the survival by Cox regression analysis in glioma specimens.

	Hazard ratio	95% confidence interval	P-value
Age	1.006	0.990–1.022	0.467
Histological grade	0.773	0.444–1.341	0.358
Expression of CB1R	1.213	0.636–2.315	0.557
p-ERK expression	1.357	0.916–2.010	0.067
Ki67 expression	1.009	0.599-1.700	0.974

Statistical analysis was performed by the Cox regression analysis. P-value < 0.05 was considered significant.

3.5. CNR1 (CB1R) mRNA expression patterns in human glioma tissues

In the present study, we aimed at better understanding the expression pattern of *CNR1 (CB1R)* gene in human glioma tumor tissues, so we surveyed different publicly available datasets (data retrieved from the online database Oncomine.org) comprised of human glioma tumor tissues of different stages and types. Interestingly, the analysis revealed that *CNR1 (CB1R)* gene was down-expressed in glioma tissues among the different datasets (fold change ranged between -1.521 and -9.886) (Figs. 5 and 6).

4. Discussion

Cannabinoids, the active component of cannabis, have been widely used for medical purposes for years [36], and proposed for treatments of numerous diseases such as multiple sclerosis, neurodegenerative disorders, epilepsy, schizophrenia and cancer [37]. Many studies have considered cannabinoids as a promising drug for glioma due to their antiproliferative effect [20], apoptotic potential and inhibition of angiogenesis [38]. So far, cannabinoids exert their effects by activation of specific receptors, CB1 and CB2.

The present study was motivated by the studies done before on human gliomas that indicated contradictory results regarding the expression level of CB1R [10–11]. We have found that CB1Rs are

mainly located at the cell membrane and in the cytoplasm of normal neurons and in glial cells. The same localization of CB1Rs was found in tumor cells including some mitotic cells, in addition to the nucleus. Our result is consistent with previous findings that reported the presence of CB1R at the cell membrane [39], in the mitochondria, ER and nucleus of neurons [40], as well as in astrocytes [41] and in vascular endothelial cells [21] [42]. In cancer, CB1Rs were also detected in the cytoplasm of pancreatic tumor cells [24], and at the cell membrane, in the cytoplasm, and nuclei of colorectal cancer cells [33].

In our study, we found a down expression of CB1R in glioma tissues compared to normal tissues and normal areas adjacent tumors. Previous studies on CB1Rs in different types of cancers showed discrepancy results, specifically in glioma. Our results are consistent with that of De Jesús *et al.* where CB1Rs were reported to be down expressed in gliomas [17]. However, overexpression of CB1Rs in gliomas was stated by Ciaglia *et al.* and Wu *et al.* [11–12]. Whereas, Held-Feind *et al.* showed a little increase in CB1R expression in glioma compared to normal brain tissue [20]. Schley *et al.* found no difference in CB1R expression between normal brain tissues and gliomas [21], and Calatozzolo *et al.* reported weak positive expression of CB1R in both normal brain tissues and gliomas [22]. Regarding other types of cancer, down-expression of CB1R was reported in renal and colorectal tumors [19–21]. In contrast, overexpression of CB1R was stated in prostate cancer [23], pancreatic cancer [24], hepatocellular carcinoma [25], melanoma [26], breast cancer [27], lung cancer [28], lymphoma [29] and ovarian cancer [30]. While the expression of CB1R showed no change in bone cancer [32].

The contradictory results regarding the expression of CB1R in gliomas in different studies could be explained by the variation in the source of control tissues, where CB1R normally show distinct distribution in brain areas [10, 31]. However, in our study, the evaluation of the expression of CB1R was assessed in gliomas based on the comparison with normal brain tissues in one hand, and normal tissues adjacent tumors on the other hand (double control). Both revealed down-expression of CB1R in glioma. The different results in IHC assay could be sometimes due to the variation in the used antibodies and antigen retrieval techniques. In our study, the down-expression of CB1R in gliomas could be explained by receptor phosphorylation and endocytosis [44]. Endocytosis is a major mechanism for signal attenuation via the degradation of signaling receptors [45]. It is considered one of the impaired processes in cancer and plays a critical role in cancer progression [46].

We further analyzed the association between the expression of CB1R and the clinicopathologic characteristics of patients. The analysis showed no significant association between the expression of CB1R and many clinicopathologic characteristics including gender, age, tumor grade, resection size, vessel density, necrosis, and Ki67 expression. However, the expression of CB1R was associated with p-ERK expression. Several studies have reported the activation of ERK by CB1R [27]. Even though we did not see an overexpression in CB1R in tumors, the activation of ERK by phosphorylation can be due to the activation of several other signaling pathways [47]. Previous studies have described the activation of MAPK/ERK pathway and its involvement in the development of many types of cancers including breast cancer [48], gastric cancer [49], non-small cell lung cancer [50], gallbladder tumors [51] and gliomas [52]. Future studies on glioma could tackle the activation of ERK and role of CB1R in this regard.

5. Limitations

We believe that our study has some limitations. First, although our aim in this research paper was to evaluate the expression patterns of CB1R and p-ERK in human glioma tumor tissues, the sample size is relatively small and hence, the results obtained require conducting subsequent studies on a larger cohort. In accordance, we believe that more data and follow up is required to assess the correlation of CB1R and p-ERK expression with clinical outcomes and to compare this expression among the different types of glioma tumors as well. Total ERK expression could also be determined in future studies to assess the activation status of ERK (by comparing total ERK with p-ERK expressions). Second, we used in our study a small number of normal brain specimens as a control. In fact, only 4 specimens were acquired since obtaining normal cerebral cortex brain tissue is indeed challenging and brain resection is usually done only in limited cases as in epilepsy patients or patients with brain tumors who need surgical resection. So, we collected in our study 4 brain specimens from epilepsy patients, and these tissues were verified for the absence of any tumor by a pathologist. Third, although the cannabinoid field lacks reliable antibodies to precisely detect expression of CB1R, we used in our study anti-cannabinoid receptor I rabbit polyclonal antibody (diluted 1:40; ab23703; Abcam; Cambridge, UK) that is validated in Western blotting, immunohistochemistry, immunocytochemistry, and immunofluorescence, cited in more than 35 publications, and independently reviewed in 17 reviews (Source: <https://www.abcam.com/cannabinoid-receptor-i-antibody-ab23703.html>). Immunohistochemistry has been previously performed and results have been published using this antibody on human gastric carcinoma tissues [53] heart tissues[54], and articular cartilage samples [55] among others. In future studies, results shall be confirmed using an additional CB1 receptor antibody other than Abcam. Lastly, double immunofluorescence analyses could be also used to stain for neural cells (NeuN) versus glial cells (GFAP) to improve the quality of the study.

6. Conclusions

The present study demonstrated that CB1R is down expressed in glioma. This decrease in the expression of CB1R was not related to malignancy grades and other clinicopathologic features of glioma. Yet, our results showed a weak positive correlation between the expression of the CB1R and p-ERK. However, this study utilized a small number of samples and the results were only obtained from IHC assay. Therefore, a larger scale tumor sample size of well-characterized patients is indeed needed to confirm the obtained results. Future studies are required to clarify the molecular mechanisms of CB1R and other endocannabinoid components, and to identify whether the CB1/endocannabinoid system might serve as a promising therapeutic target for brain tumors.

Declarations

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Authors' contributions: N.C., H. K., and S.N. worked on study conception and design and contributed to the writing of the hypothesis, data collection, and data analysis. Z. S., and M. Z., H. B., and S. N. worked on the pathological slides review, data analysis, and histology figures. N. C., Z. S. performed the statistical analyses. Z.S., M. Z., H. B., and S. N. worked on the figures illustrations. H. B. assessed mRNA. N. C., Z. S., H. K., H. B., and S. N. contributed to the drafting of the manuscript, and critically revised and edited the manuscript prior to approving the final draft. H.H. and Y.F. revised the final draft of the manuscript. H. B. and S. N. critically revised the manuscript with input from the entire team. S. N. was responsible for the study supervision and conduction of the whole project. All authors have read and approved the final draft.

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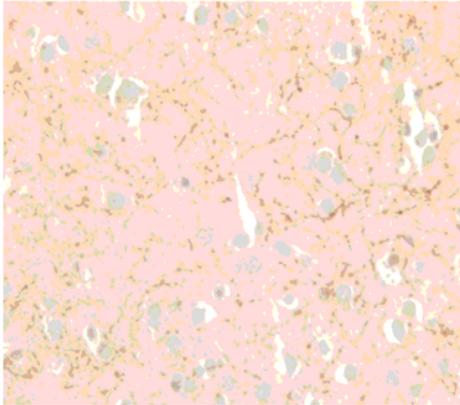
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Figures

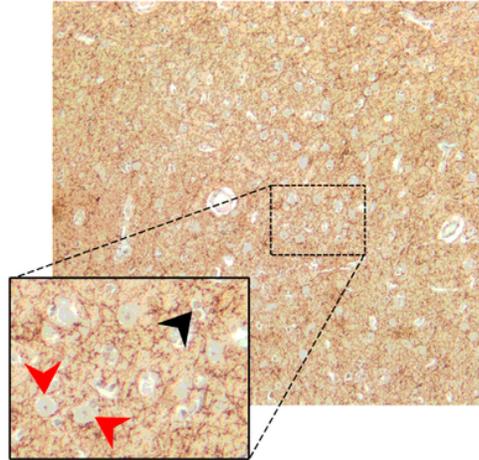
CB1R

Negative Control

A. Normal human cerebral cortex brain tissue from Abcam



B. Normal cerebral cortex brain tissue from our cohort of patients



C. Normal cerebral cortex brain tissue from our cohort of patients



Figure 1

Representative images of positive and negative controls used in our study. (A) CB1 receptors (CB1R) immunohistochemical staining in normal human cerebral cortex brain tissue obtained from Abcam (original magnification x 400) and (B) a normal cerebral brain tissue from our cohort of patients (black arrow refers to glial cell and red arrow refers to neuron; original magnification x 100). (C) Normal cerebral brain tissue from our cohort of patients in which the primary CB1R antibody was substituted with normal rabbit IgG of the same concentration (negative control; original magnification x 100).

CB1R

P-ERK

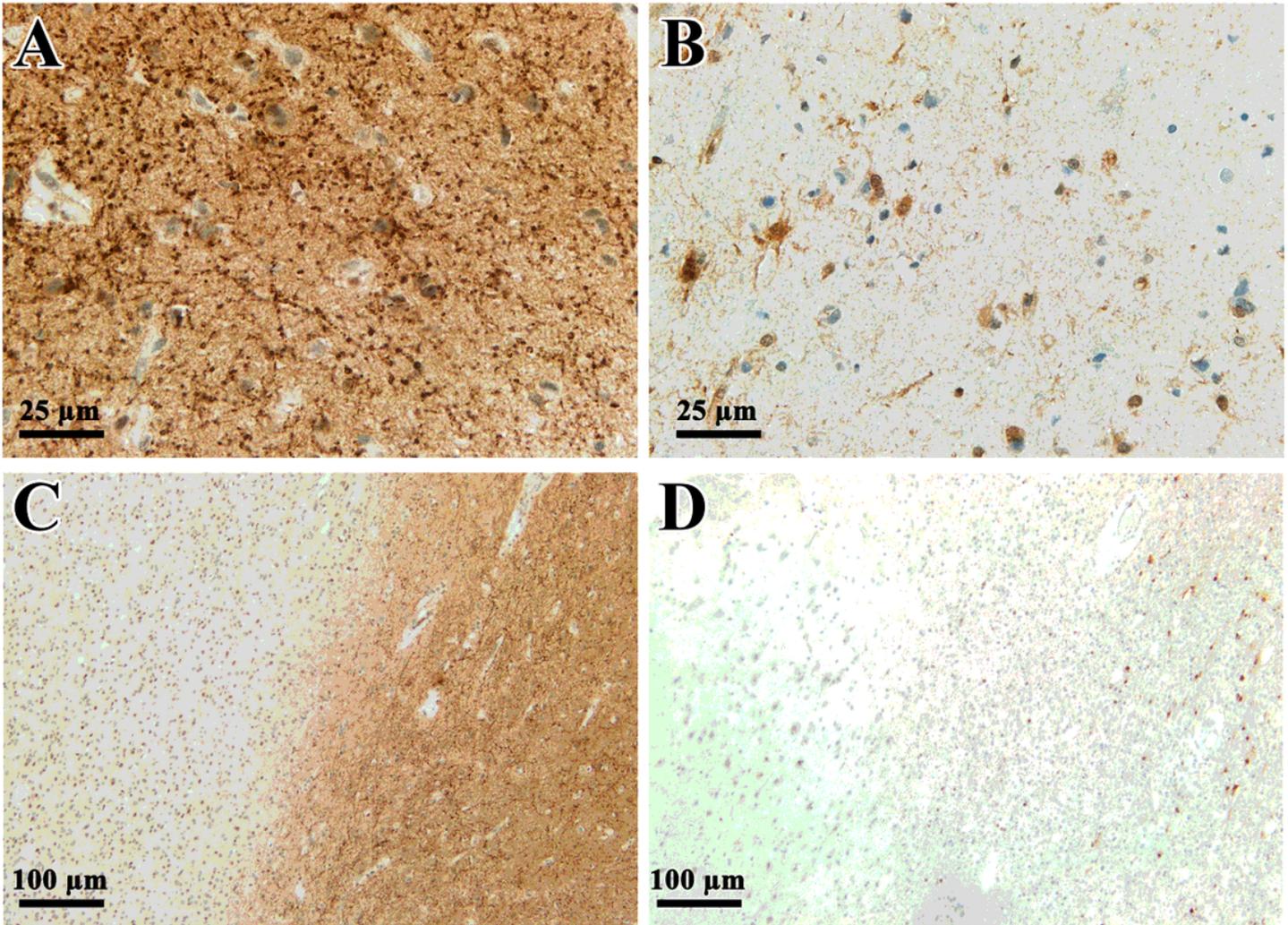


Figure 2

Representative images of CB1 receptors (CB1R) and p-ERK immunohistochemical staining. (A) Positive high immunohistochemical staining of CB1 receptors (CB1R) in normal brain tissue, and (B) positive weak immunohistochemical staining of p-ERK in normal brain tissue. (C) High expression of CB1R in normal tissues compared to the tumor. (D) Low p-ERK expression in normal tissues compared to glioma. A and B were obtained at magnification x400. C and D were obtained at magnification x100.

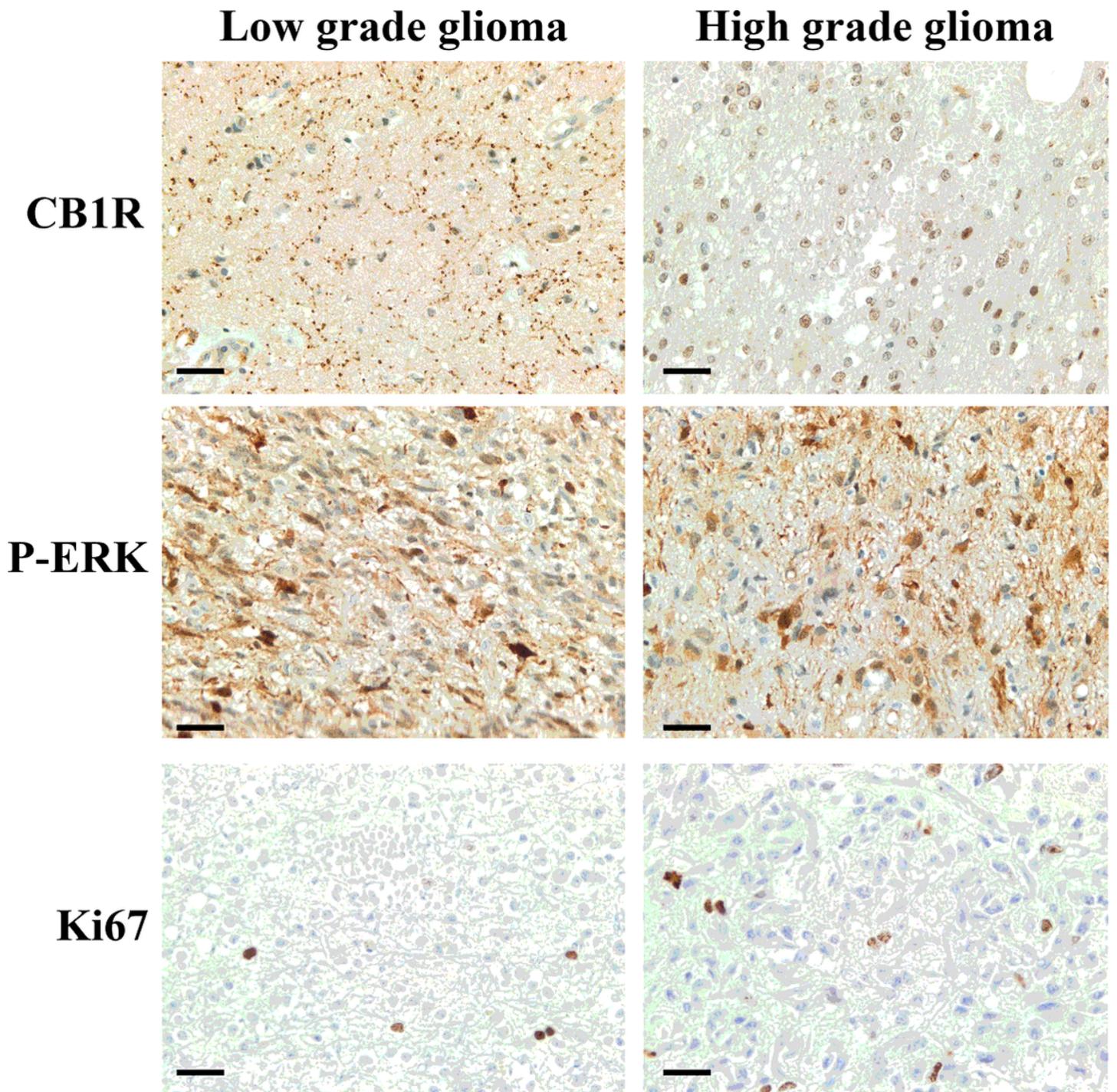


Figure 3

Immunohistochemical staining of CB1 receptors (CB1R), and p-ERK and Ki67 in glioma. Sections from low and high-grade glioma were assessed for the expressions of CB1R, p-ERK, and Ki67. No significant change in the immunoreactivity of both CB1R and p-ERK was noted with respect to the histological grade. Images were obtained at magnification x400. Scale bar = 25 μ m.

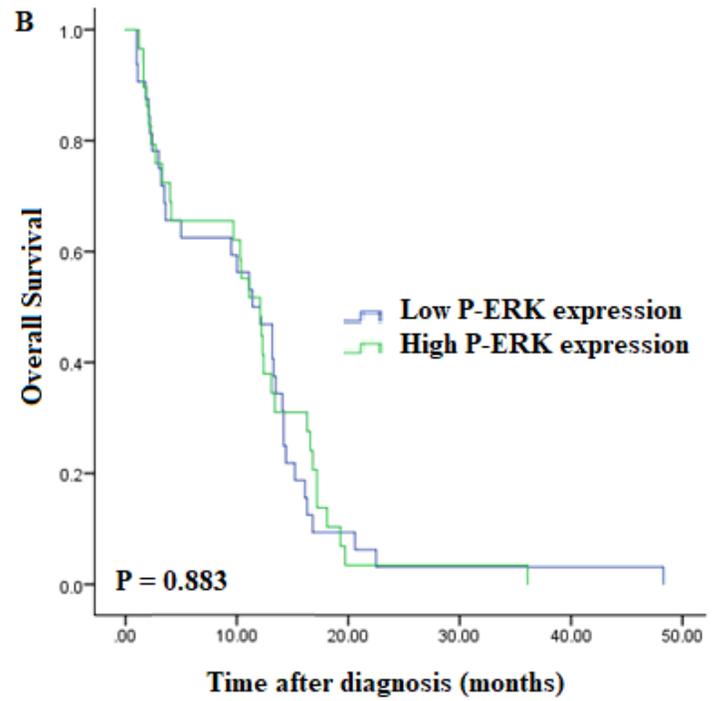
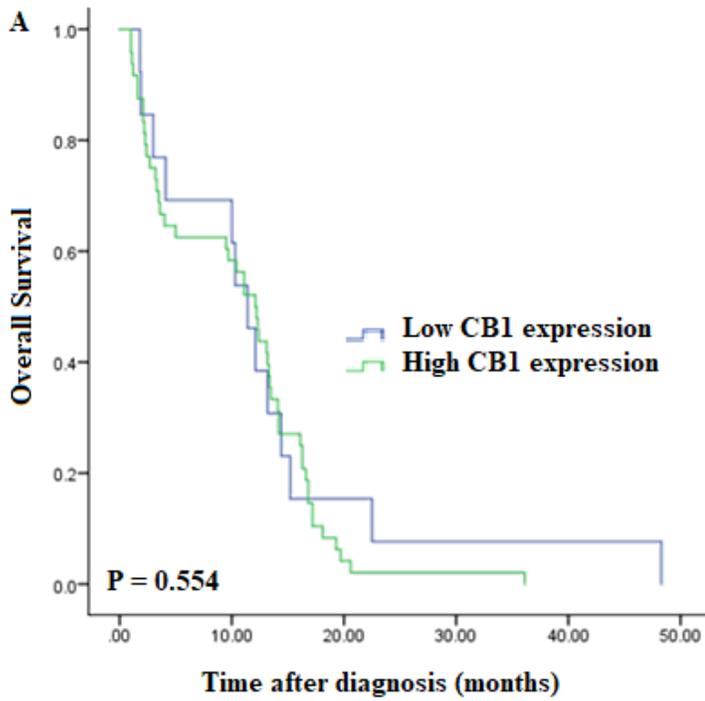
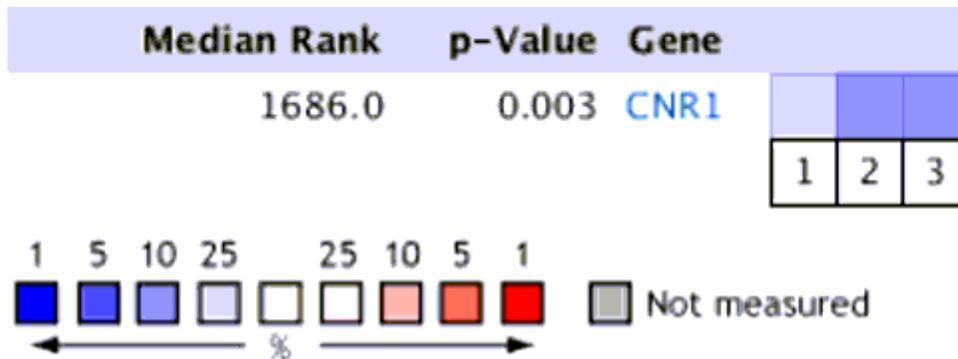


Figure 4

Influence of the expression of CB1 receptors (CB1R) and p-ERK on the overall survival by Kaplan-Meier analysis. This analysis demonstrated that neither the expression CB1R (A) nor p-ERK expression (B) had an influence on the survival time.

Comparison of CNR1 Across 3 Analyses

Under-expression



The rank for a gene is the median rank for that gene across each of the analyses.
 The p-Value for a gene is its p-Value for the median-ranked analysis.

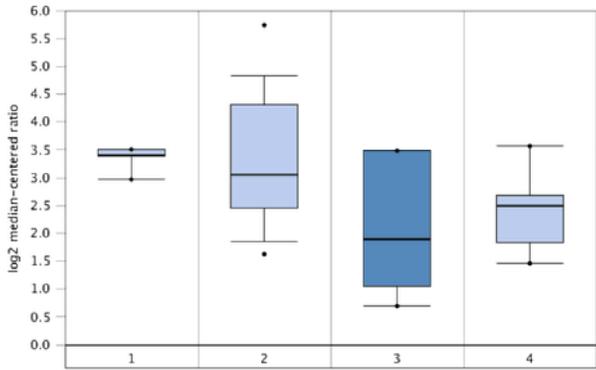
Legend:

1. **Bredel Brain 2, Cancer Res, 2005** (54 samples)
 Brain and CNS Cancer Type: Mixed Glioma
 Fold change = -2.128; p = 0.962; Reporter IMAGE:26295
2. **Pomeroy Brain, Nature, 2002** (85 samples):
 Malignant Glioma, NOS vs. Normal
 Fold change = -9.886; p = 0.990; Reporter U73304_ma1_at
3. **Sun Brain, Cancer Cell, 2006** (180 samples):
 Diffuse Astrocytoma vs. Normal
 Fold change = -1.521; p = 0.854; Reporter 208243_s_at

Figure 5

Expression levels of CNR1 (CB1R) mRNA were assessed in three sets comprised of human glioma tumor samples. Expression within tumor tissues was presented by fold-change expression of CNR1 (CB1R) and p-values were obtained using t-tests (Data retrieved from the online Oncomine database Research Platform; URL: <http://www.oncomine.org>; RRID:SCR_007834; Figure 6).

CNR1 Expression in Bredel Brain 2
Brain and CNS Cancer Type: Mixed Glioma



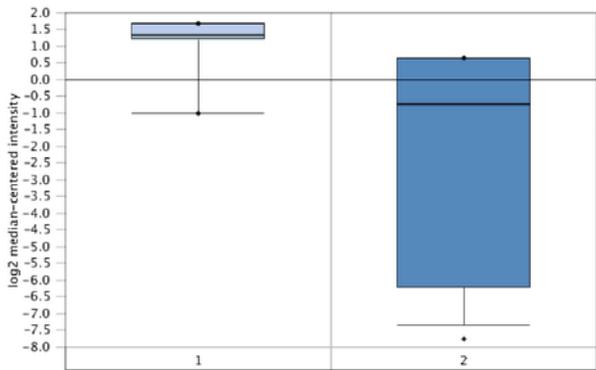
Legend:

- 1. Astrocytoma (4)
- 2. Glioblastoma (31)
- 3. Mixed Glioma (6)
- 4. Oligodendroglial Tumor (8)

P-value: 0.038
t-Test: -2.121
Fold change: -2.128

Cancer Res 2005/10/01	54 samples
mRNA	14,836 measured genes

CNR1 Expression in Pomeroy Brain
Malignant Glioma, NOS vs. Normal



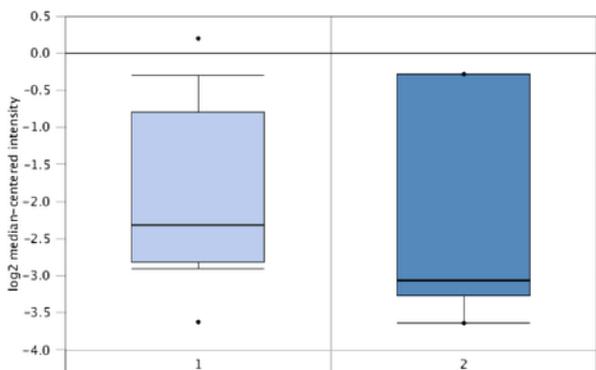
Legend:

- 1. Cerebellum (4)
- 2. Malignant Glioma, NOS (10)

P-value: 0.010
t-Test: -2.675
Fold change: -9.886

Nature 2002/01/24	85 samples
mRNA	5,338 measured genes

CNR1 Expression in Sun Brain
Diffuse Astrocytoma vs. Normal



Legend:

- 1. Brain (23)
- 2. Diffuse Astrocytoma (7)

P-value: 0.146
t-Test: -1.119
Fold change: -1.521

Cancer Cell 2006/04/01	180 samples
mRNA	19,574 measured genes

Figure 6

Expression levels of CNR1 (CB1R) mRNA were assessed in three sets comprised of human glioma tumor samples. Expression was presented by log (base 2) median-centered expression of CNR1 (CB1R). Box and whiskers plots indicate median and interquartile range. p-values were obtained using t-tests (Data retrieved from the online Oncomine database Research Platform; URL: <http://www.oncomine.org>; RRID:SCR_007834).