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Original Article

Immunohistochemical assessment of cannabinoid type-1 receptor (CB1R) and its correlation with clinicopathological parameters in glioma

Nader Choucair[1*], Zahraa Saker[1*], Hassane Kheir Eddine[1*], Hisham F. Bahmad[2*], Youssef Fares[1,3], Mariana Zaarour[4], Hayat Harati[1], Sanaa Nabha[1]

[1] Neuroscience Research Center, Faculty of Medical Sciences, Lebanese University, Beirut, Lebanon; [2] Arkadi M. Rywlin M.D. Department of Pathology and Laboratory Medicine, Mount Sinai Medical Center, Miami Beach, FL, USA; [3] Department of Neurosurgery, Faculty of Medical Sciences, Lebanese University, Beirut, Lebanon; [4] Department of Pathology, Faculty of Medical Sciences, Lebanese University, Beirut, Lebanon
**These authors contributed equally to this work as co-first authors*

Summary

Background. Glioma is the most frequent primary brain tumor and one of the most aggressive forms of cancer. Recently, numerous studies have focused on cannabinoids as a new therapeutic approach due to their antineoplastic effects through activation of the cannabinoid receptors. This study aimed to investigate the immunohistochemical expression level of cannabinoid type-1 receptors (CB1R) in human glioma samples and evaluate its clinicopathologic significance.

Materials and methods. We analyzed the expression of CB1R in 61 paraffin-embedded glioma and 4 normal brain tissues using automated immunohistochemical assay. CB1R expression was categorized into high versus low expression levels. Statistical analyses were performed to evaluate the association between CB1R and phosphorylated extracellular signal-related kinase (p-ERK) expression levels and the clinicopathologic features of glioma.

Results. Our results showed that CB1R immunopositivity was seen in 59 of 61 cases (96.7%). CB1R was down-expressed in glioma compared to normal brain tissues. However, CB1R expression was not correlated with clinicopathological parameters except for p-ERK.

Conclusion. Our findings indicate the down-expression of CB1R in glioma tissues when compared to non-cancerous brain tissues. This change in CB1R expression in gliomas should be further tested regardless of the clinicopathological findings to provide a therapeutic advantage in glioma patients.

Key words: CB1R, p-ERK, expression level, glioma, immunohistochemistry

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Correspondence
Sanaa Nabha
Neuroscience Research Center, Faculty of Medical Sciences, Lebanese University, Hadath, Beirut, Lebanon
E-mail: snabha@ul.edu.lb

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Introduction

Glioma represents the majority of primary brain tumors in adults, accounting for nearly 70% of these tumors [1]. It is among the most lethal cancers with an increasing incidence over the years [2]. Despite advancements in cancer treatment, glioma is still characterized by low survival rates compared to other malignancies. This instigates a dire need for identifying novel target-directed therapies based on the increased knowledge of the molecular and cellular biology of glioma [3].

In this regard, intense research is ongoing to develop novel therapeutic approaches to overcome treatment resistance, among which

cannabinoids represent a potential means in anti-cancer management. Cannabinoids are chemical substances produced by cannabis plants referred to as resins. They 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 [4,5]. In addition, a number of clinical trials involving cannabinoids have found a significant reduction in tumor size and prolonged survival time among patients with brain tumors [6]. The antineoplastic effects of cannabinoids involve different signaling pathways such as the mitogen-activated protein kinase (MAPK)/(ERK1/2) and PI3K/Akt pathways [7,8]. The effects of cannabinoids are mediated through specific G-protein coupled receptors (GPCR), CB1R, and CB2R [9]. On one hand, CB1Rs are highly expressed in the brain and mediate many neuronal effects produced by endocannabinoids and cannabinoid drugs [10]. On the other hand, 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 [11].

CB1Rs are also expressed in many cancers, and have been found to be overexpressed at the protein level in prostate cancer [12], pancreatic cancer [13], hepatocellular carcinoma [14], melanoma [15], non-small cell lung cancer [16], lymphoma [17] and ovarian cancer [18]. However, downregulation of CB1R has been reported in other tumors including renal cell carcinomas [19], while no change in the expression of CB1R was noted in bone tumors [20]. The expression of CB1R in human glioma remains controversial wherein different results have been reported [21-23]. The aim of the present study is to examine the immunohistochemical expression of CB1R in human glioma tissues compared to normal brain tissues and assess its clinicopathological and prognostic significance.

Materials and methods

Patient 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 the Institute National de Pathologie (INP). All the experimental protocols herein were carried out in accordance with the Declaration of Helsinki. Ethics Committee approval was obtained from the Faculty of Medical Sciences of the Lebanese University (LU) and INP prior to conduction of this study. No informed consent was obtained since most of the patients are deceased.

Based on the World Health Organization (WHO) 2016 classification system [24], special types of glioma were identified: 29 glioblastoma multiforme (grade IV), 17 oligodendroglioma (14 cases of grade II and 3 cases of grade III) and 15 astrocytoma (6 cases of grade I, 4 cases of grade II and 5 cases of grade III). These tumors were further categorized according to grade as follows: 24 cases with low-grade glioma (grade I and II) and 37 cases with high-grade glioma grade III and IV). 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 10 glioma specimens. The mean age of patients was 48.84 years (range, 12-80) and the 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 epileptic characteristics or inflammation. 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 selected from areas adjacent to the glioma tumors (internal controls) and those areas were chosen, by two independent pathologists, based on the absence of any cellular or architectural atypia.

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 pathology 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 95 °C. Prior to starting our experiment, 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 clear-

ly 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 damage. 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 Ki-67 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) [25,26] and shown to produce an appropriate pattern of staining in paraffin-embedded formalin-fixed sections. Anti-CB1R antibody specificity has been verified using IHC on normal human cerebral cortex tissue slides purchased from Abcam, Cambridge, UK (ab4296) and to verify the validity of the normal brain tissues in our study. Normal brain tissues from our cohort of patients were also stained in order to compare the staining of tumor tissues with that in normal ones. In addition, positive staining of antibodies was compared to an appropriate negative control to avoid the false results of background staining. The visualization system was OptiView DAB. The counterstaining with Hematoxylin II and Bluing reagent followed the immunostaining step.

IHC evaluation

All the immunostained sections were examined 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 (immunoreactivity). The CB1R and p-ERK immunoreactivity was scored from 0 to 3 (tissues with no staining were scored as 0; weak staining intensity as 1, moderate staining intensity as 2, and strong staining intensity as 3). For statistical analysis, 0 and 1 scores were counted as low expression, whereas 2 and 3 scores were counted as high expression [26].

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 (Supplementary Fig. 1). Data from the different datasets are presented as box and whiskers plots indicating median and interquartile range, and *p*-values were obtained using *t*-tests.

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 the Pearson χ^2 test. Fisher's exact test was used when necessary. 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 $P < 0.05$.

Results

CB1R and p-ERK expressions in normal brain samples

The expression of CB1R was 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 and 32/38 (84.2%) normal tissues adjacent to tumors. The immunoreactivity of CB1R did not vary between normal brain tissues ($P = 0.692$, χ^2). 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. 1A).

With respect to the expression 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 immunoreactivity of p-ERK in normal tissues compared to normal tissues adjacent tumors ($P = 0.219$; χ^2). Neurons and glial cells exhibited weak positive cytoplasmic and nuclear staining for p-ERK (Fig. 1B).

Expressions of CB1R and p-ERK in glioma tissues

CB1R expression level was evaluated in glioma tissues. Positive IHC staining of CB1R was detected in 59 patients (96.7%) out of 61. The expression of CB1R

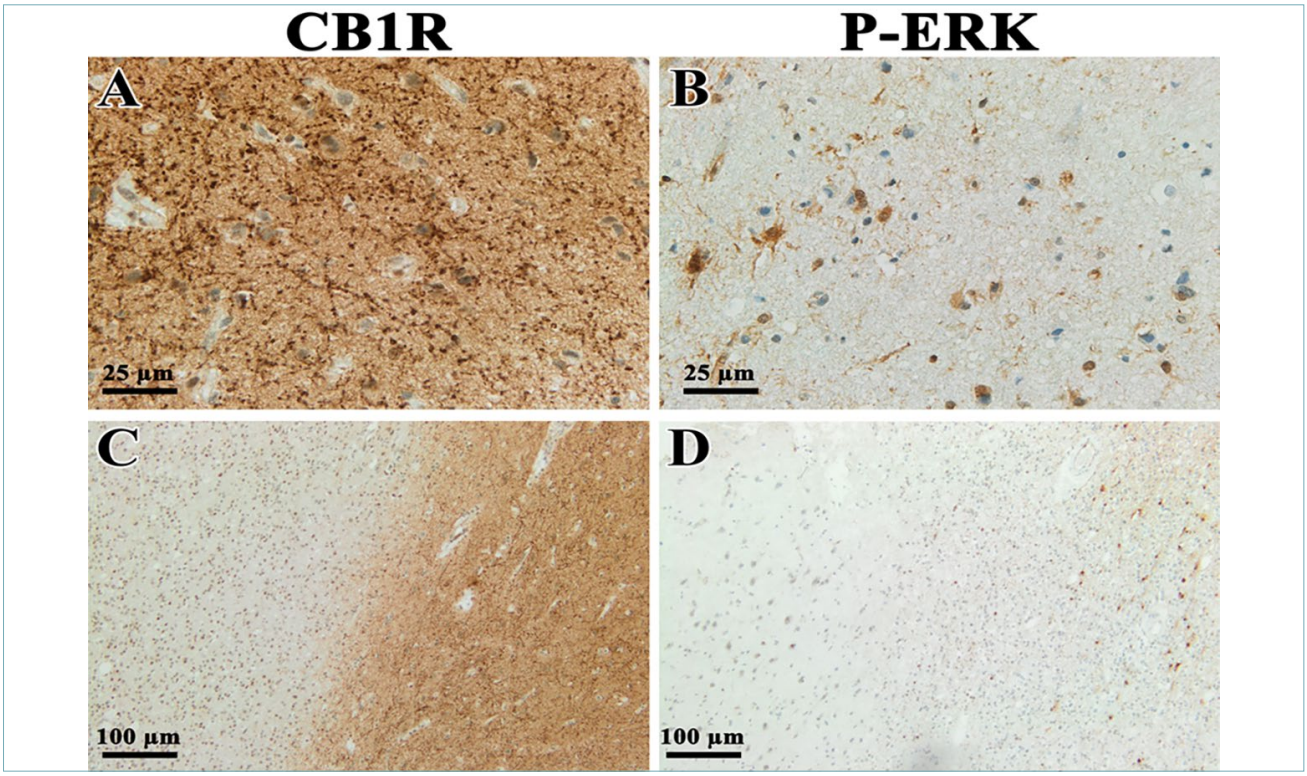

Figure 1. Representative images of CB1R and p-ERK immunohistochemical staining. (A) Positive high immunohistochemical staining of CB1R in normal brain tissue, and (B) positive weak immunohistochemical staining of p-ERK in normal brain tissue. (C) High expression of CB1R in normal tissues compared to 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.

Table I. CB1R expression and clinicopathological parameters of the glioma.

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)	
Vessel density				
Normal	8 (13.1)	4 (6.6)	4 (6.6)	0.055
Increased	53 (86.9)	9 (14.8)	44 (72.1)	
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.

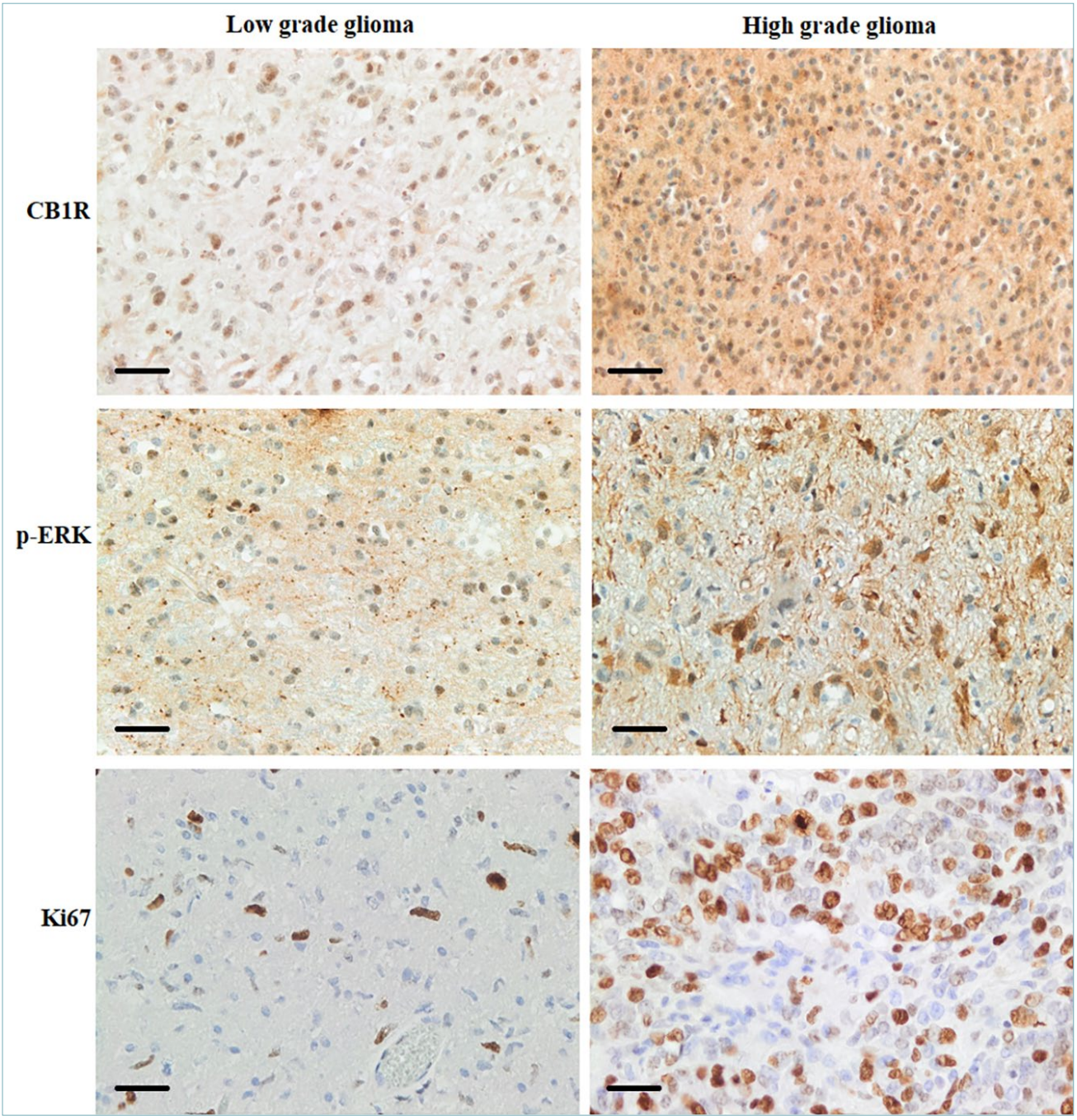

Figure 2. Immunohistochemical staining of CB1R, and p-ERK and Ki-67 in glioma. Sections from low and high-grade glioma were assessed for the expressions of CB1R, p-ERK, and Ki-67. No significant change in the immunoreactivity of both CB1R and p-ERK was observed with respect to the tumor grade. Images were obtained at magnification x400. Scale bar = 25 µm.

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 immunoreactivity of CB1R was noticed between glioma and normal tissues ($P < 0.001$; χ^2) and between glioma and the normal

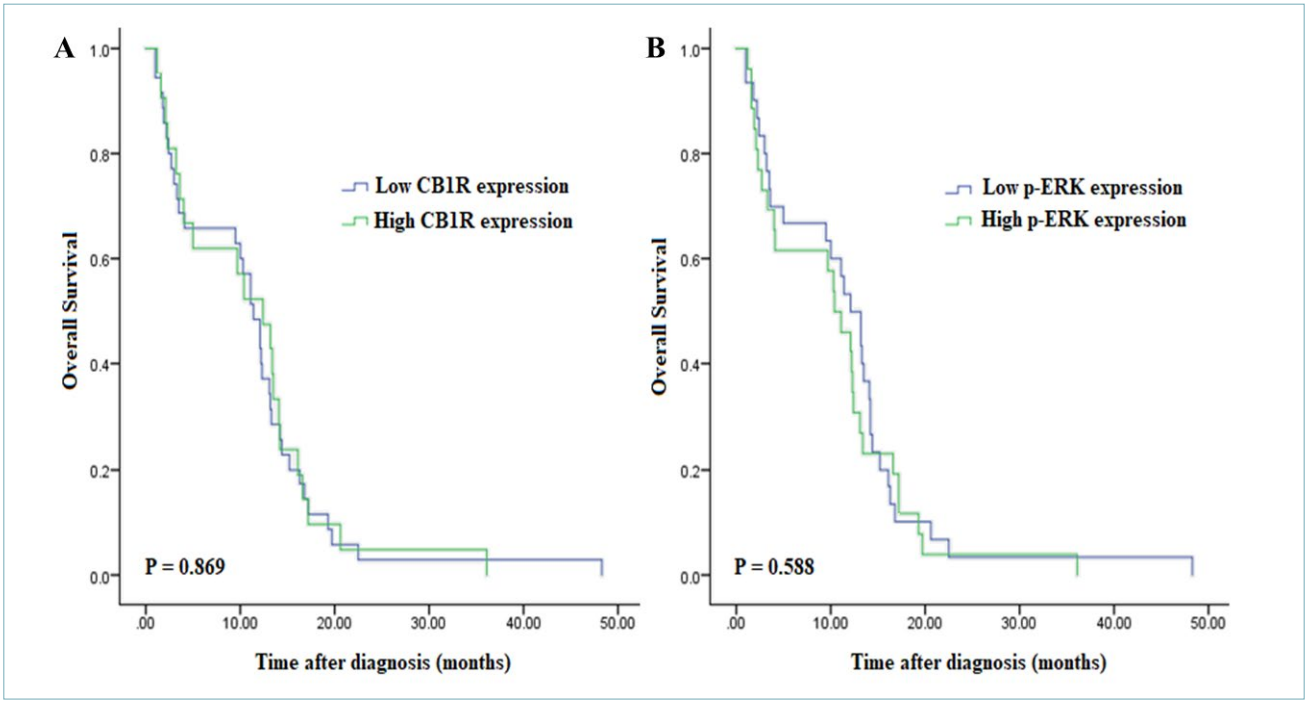

Figure 3. Influence of the expression of CB1R and p-ERK on overall survival by Kaplan-Meier analysis. This analysis demonstrated that neither the expression of CB1R (A) nor p-ERK (B) had an influence on the survival time.

tissues adjacent tumors ($P < 0.001$; χ^2) with more pronounced expression in normal tissues in both cases (Fig. 1C). However, no significant difference in the percentage of positive cells was observed in glioma considering tumor grade ($P = 0.556$; Independent sample *t*-test) or glioma subtype ($P = 0.318$; Independent sample *t*-test).

The expression of p-ERK was also assessed in glioma tissues. Positive IHC staining of p-ERK was 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 ($P < 0.001$; χ^2) and between glioma and normal areas adjacent to tumors ($P < 0.001$; χ^2) with higher p-ERK expression in glioma in both cases. Representative images are presented in Figure 1D.

Clinicopathologic significance of the expression of CB1R in glioma

Investigation of the clinicopathologic significance of CB1R expression in glioma is summarized in Table I. Samples with scores 0-1 and 2-3 were categorized as having low and high expression of CB1R and p-ERK (Fig. 2). No obvious relationships were observed 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 ($P = 0.571$) and vessel density ($P = 0.055$). However, 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 ($r = 0.210$, $P < 0.01$).

Factors affecting overall survival

Kaplan-Meier analysis showed that there was no evidence of a significant difference in the survival times for patients with low and high expression of CB1R ($P = 0.869$) and with low and high p-ERK expression ($P = 0.588$) (Fig. 3). Moreover, multivariate analysis using Cox's proportional hazards model revealed no evidence of a greater risk of death in association with age, tumor grade, and CB1R or p-ERK expression (Tab. II).

CNR1 (CB1R) mRNA expression patterns in human glioma tissues

In the present study, we also aimed to better under-

Table II. 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
Tumor 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

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

stand 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) (Supplementary Fig. 1).

Discussion

Cannabinoids, the active component of cannabis, have been widely used for medical purposes for years [27], and proposed for the treatment of numerous diseases such as multiple sclerosis, neurodegenerative disorders, epilepsy, schizophrenia, and cancer [28]. Many studies have considered cannabinoids as a promising drug for glioma due to their antiproliferative effect [29], apoptotic potential, and inhibition of angiogenesis [30]. Cannabinoids exert their effects by activation of specific receptors, CB1R and CB2R. The present study was based on results from studies previously done on human gliomas that indicated contradictory results regarding the expression level of CB1R [9,8]. 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 results are consistent with previous findings that reported the presence of CB1R at the cell membrane [31], in the mitochondria, endoplasmic reticulum (ER) and nucleus of neurons [32], as well as in astrocytes [33] and in vascular endothelial cells [34]. In cancer, CB1Rs have been also detected in the cytoplasm of pancreatic tumor cells [13], and at the cell membrane, in the cytoplasm, and nuclei of colorectal cancer cells [26].

In our study, we demonstrated the down-expression of CB1R in glioma tissues compared to normal tissues and normal areas adjacent to tumors. Previous studies on CB1Rs in different types of cancers showed different results, specifically in glioma. Our results are consistent with those of De Jesús et al. where CB1Rs were reported to be down-expressed in glioma [21]. However, overexpression of CB1Rs in glioma was seen by Ciaglia et al. and Wu et al. [9,35]. Held-Feind et al. showed a small increase in CB1R expression in glioma compared to normal brain tissue [29]. Schley et al. found no difference in CB1R expression between normal brain tissues and glioma [36], and Calatozzolo et al. reported weak positive expression of CB1R in both normal brain tissues and glioma [37].

The contradictory results regarding the expression of CB1R in glioma in different studies could be explained by the variation in the source of control tissues, where CB1R normally shows a distinct distribution within brain areas [10]. However, in our study, the evaluation of the expression of CB1R was assessed in glioma based on the comparison with normal brain tissues on one hand, and normal tissues adjacent to tumors on the other hand (double control). Both revealed down-expression of CB1R in glioma. The different results of IHC can be sometimes due to the variation in the used antibodies and antigen retrieval techniques. The down-expression of CB1R in glioma could further be explained by receptor phosphorylation and endocytosis [38] where endocytosis is a major mechanism for signal attenuation via the degradation of signaling receptors [39]. It is considered one of the impaired processes in cancer and plays a critical role in cancer progression [40].

We also 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 clinicopathologic parameters including gender, age, tumor grade, resection size, vessel density, or necrosis. However, the expression of CB1R was associated with p-ERK expression. Several studies have reported the activation of ERK by CB1R [41]. Even though we did not see overexpression in CB1R in tumors, the activation of ERK by phosphorylation can be due to the activation of several other signaling pathways [42]. Previous studies have described the activation of MAPK/ERK pathway and its involvement in the development of many types of cancers including breast cancer [43], gastric cancer [44], non-small cell lung cancer [45], gallbladder tumors [46] and glioma [47]. Future studies on glioma could tackle the activation of ERK and role of CB1R in this regard.

Limitations

We believe that our study has some limitations. First, although our aim was to evaluate the expression patterns of CB1R 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, 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 a small number of normal brain specimens as a control. In fact, only 4 specimens were acquired since obtaining normal 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. 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 [48], heart tissues [49] and osteoarthritic cartilage samples [50] among others. In future studies, the results will be confirmed using an additional CB1 receptor antibody other than Abcam. Lastly, double immunofluorescence analyses could also be used to stain for neural cells (NeuN) versus glial cells (GFAP) to improve the quality of the study.

Conclusion

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. However, 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 suggested 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 CB1R/endocannabinoid system might serve as a promising therapeutic target for brain tumors.

Conflict of interest

The Authors declare no conflict of interest.

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Ethical consideration

Ethics Committee approval was obtained from the Faculty of Medical Sciences of the Lebanese University (LU) and the Institute National de Pathologie (INP). Ethical clearance was accomplished as per the norms and in accordance with relevant guidelines and regulations of INP and LU. The need for informed consent from patients was waived.

Authors' contributions

NC, HK, and SN worked on study conception and design and contributed to the writing of the hypothesis, data collection, and data analysis. ZS, and MZ, HB, and SN. worked on the pathological slides review, data analysis, and histology figures. NC, ZS performed the statistical analyses. ZS, MZ, HB, and SN worked on the figures illustrations. HB assessed mRNA. NC, ZS, HK, HB, and SN contributed to the drafting of the manuscript, and critically revised and edited the manuscript prior to approving the final draft. HH and YF revised the final draft of the manuscript. HB and SN critically revised the manuscript with input from the entire team. SN 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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