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THE STATUS OF RETINOBLASTOMA GENE EXPRESSION IN BRAIN TUMORS

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Abstract – *Objective:* *Malignant brain tumors, including Glioblastoma Multiforme (GBM), are among the deadliest brain tumors. Given the fact that the expression of the retinoblastoma (RB) gene in malignant tumors can change the tumor behavior, we seek to investigate the alterations of RB expression in brain tumors.*

Materials and Methods: *The archives of the Pathology Department of Yazd Hospitals were examined, and all the brain tumors diagnosed between 2013 and 2017 were extracted. All paraffin embedded blocks underwent immunohistochemical staining for RB gene expression. Based on a pre-set checklist, demographics data, tumor type, location, and survival status were entered into and analyzed by SPSS version 25.* p-*value less than 0.05 was considered statistically significant.*

Results: *Out of 90 blocks studied, 64.4% belonged to male patients and 35.5% to female patients. The frequencies of brain tumors subtypes were non-glioma (45.6%), low grade astrocytoma (14.4%), anaplastic astrocytoma (18.9%) and GBM (21.1%), respectively. The intensity of RB expression was significantly different between men and women (p-value=0.008), and in different subtypes of the tumors (*p*=0.04). Multivariate analysis revealed that GBM (HR: 9.933, 95% CI 1.888-52.254,* p-*value=0.007), age >50 (HR: 8.648, 95% CI 5.116-16.406,* p-*value= 0.0001), female sex (HR:2.139, 95% CI 1.212-3. 775, p-value= 0.09), RB negative tumors (HR:2.502, 95% CI 1.061-5.896, p-value= 0.036) significantly affect patient survival.*

Conclusions: *There was a significant difference between men and women, and among different subtypes of the brain tumors in terms of RB gene expression. RB expression had a significant effect on patient survival independent from patient's age, sex, and tumor subtypes.*

KEYWORDS: *Retinoblastoma gene, Glial tumors, Brain tumors, Glioblastoma multiforme, Brain tumors survival.*

INTRODUCTION

The role of proto-oncogenes and tumor suppressor genes in pathogeneses and progression of many human cancers are now well studied[1]. Retinoblastoma (RB) gene (located at chromosome 13ql4) encodes RB protein, which is one of the vital controllers of cell proliferation. Usually, when a cell is not in the proliferative phase, RB protein is not phosphorylated and binds to a transcription factor called E2F. This binding inhibits cells from entering the S phase of the cell cycle. Whenever cells reach the end of mitoses phases of cell division and DNA synthesis, D-cyclins bind to cyclin-dependent kinases (CDKs) and initiate phosphorylation of RB protein. When RB protein is phosphorylated, E2F is released and cell proliferation occurs[2]. RB gene is the prototype of the tumor suppressor genes whose mutation was discovered in inherited retinoblastoma (germ cell

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mutation)[3]. RB gene mutation was also observed in other human cancers such as bladder cancer, lung cancer, osteosarcoma, and glioblastoma multiforme (GBM), but they were somatic mutations[4]. RB protein is a negative cell cycle regulator, so mutation in the RB gene leads to unlimited cell proliferation[3,5].

Brain tumors account for only 3% of all human malignancies. Surgery to resect tumors in combination with chemotherapy and radiotherapy are the main treatment modalities for brain tumors[6]. Despite the progression of these treatment methods, survival of the patients with primary malignant tumors has not improved[7], so it appears necessary to develop and use new treatment modalities. Mutation of RB gene in brain tumors was first reported by Tsuzuki et al[8] in three astrocytoma. Several other studies investigated the changes in expression of RB protein in the development and prognosis of different subtypes of brain tumors[9-12]. Gene therapy is one of the new treatment options used for many human cancers including brain tumors[13,14]. These new treatment modalities may explain a better survival rate reported recently for brain tumors in high-income countries[6,15] . Therefore, investigating genetic alterations in brain tumors is an important issue. The present study aimed at demonstrating differences of RB gene expression in brain tumor subtypes, anatomical location, patients' age and gender, and their contribution to patient survival.

MATERIALS AND METHODS

Study Population and Sample Collection

This study was conducted on brain tumor samples at the Pathology Departments of Yazd Province Hospitals from 2013 to 2017. Metastatic tumors were excluded, leaving 90 samples for the study. Histological grade and tumor subtypes were reestablished by an expert pathologist and classified based on the 2016 WHO Classification of Tumors of the Central Nervous System. The brain tumors then were divided into non-glioma and glioma tumors. Glioma tumors were subdivided into lower grade glioma, anaplastic astrocytoma and glioblastoma multiforme (GBM). Previously immunohistochemistry (IHC) was used to identify isocitrate dehydrogenase (IDH) mutation in glioma. IDH mutant low grade and anaplastic astrocytoma and IDH wild type GBM were included to the study. Alterations in the production of RB gene were studied using IHC staining method. Demographic and clinical information including age, sex, survival status, and anatomical location of the tumor was obtained from hospital records. The follow-up period was defined as the time of diagnosis of brain tumor till the end of the study. The study was approved by Ethics Committee of Shahid Sadoughi General Hospital Research Center.

Immunohistochemistry Method

Tissue sections of 3 microns were prepared for IHC staining from formalin-fixed paraffin embedded tissue blocks. Glass slides were used for picking up the sections, which were air-dried overnight and dried in the oven for 20 minutes at 60°C. Then the slides were deparaffinized and rehydrated using xylene and washed with decreasing concentration series of ethanol. The slides were then water-washed.

Hydrogen peroxide 0.3% (H2O2) in methanol was used to block endogenous peroxidase activity followed by a 30-minute incubation and washing with distilled water. For antigen retrieval, the slides were implanted in the boiling sodium citrate buffer (pH: 6) and placed in a microwave oven at 600 Watt for 8 minutes followed by 200 Watt for 30 minutes.

After cooling down for 20 minutes, the slides were washed with distilled water and then with Tris-Buffered Saline (TBS). Subsequently, positive control slides were incubated with primary and polyclonal antibodies at room temperature for 60 minutes while negative control slides were incubated under the same conditions without primary antibodies.

After washing with TBS again, the slides were incubated with biotinylated linking antibody at room temperature for 15 minutes. Then, the slides were washed with TBS several times. Labelled Streptavidin Biotin Complex (LSAB) was used to detect the primary antibody, subsequently, the slides were incubated at room temperature for 15 minutes and washed with TBS.

The slides were then incubated with diaminobenzidine tetrahydrochloride (DAB) for 10 minutes for localizing pRB. Finally, all the slides were dehydrated and transparent using increasing concentration series of ethanol and xylene, respectively.

The primary antibody was the mouse monoclonal anti-human retinoblastoma gene product RB1 (Diagnostic Biosystem, Emergo Europe, The Netherland, E 181, clone 1f8) prediluted and ready. Labelled streptavidin biotin method was used to detect the primary antibody. A well-differentiated adenocarcinoma of colon known to have pRB positivity was the positive control.

Evaluation of Immunohistochemistry

Samples were analyzed using a high-power microscope by an experienced pathologist (ST). Five non-overlapping fields with at least 100 cells per field were chosen randomly for each slide to define intensity and percentage of IHC staining. The score was defined based on the percentage of stained nuclei (score 0 for negative, 1 for <10%, 2 for 10–50%, 3 for 51–80%, 4 for >80%). In addition to the percentage of staining, the staining intensity was described as 1 for weak, 2 for moderate and 3 for strong staining.

Statistical Analysis

All of the data were analyzed in SPSS ver.25 (IBM Corp. SPSS Statistics for Windows, Version 25; Armonk, NY, USA). *p*-value <0.05 was considered statistically significant.

Cross-tabulation was used to describe the number and percentage of brain tumors in different anatomical locations, age groups, and subtypes. Chi-square and Fisher's exact test were used to compare categorical variables. Kaplan-Meier test was used to estimate overall survival and log rank test was used to compare survival curves. Univariate and multivariate Cox proportional hazard model were used to find variables associated with patient survival. For survival analysis, tumors that scored 3 or 4 for percentage of staining and 3 for intensity of staining were considered as RB positive tumors.

RESULTS

General Characteristics

Among 90 patients with brain tumors, 58 (64.5%) were male, and 32 (35.5%) were female. The number (%) of patients in each age group was as follows: 13 (14.4%) in 0-20 years, 29 (32.2%) in 21-40, 30 (33.3%) in 41-60, 16 (17.8%) in 61-80, 2 (2.2%) over 80 years. Brain tumor subtypes included non-glial tumors (n=41, 45.6%), low grade astrocytoma (n=13, 14.4%), anaplastic astrocytoma (n=17, 18.9%) and GBM (n= 19, 21.1%). As per anatomical location, tumors were divided into cerebral 68 (76.5%) and non-cerebral 22 (24.5%) part of intracranial space. Among cerebral tumors, 26 were in the temporal lobe, 19 in the parietal lobe, 12 in the frontal lobe and 11 in the occipital lobe. In non-cerebral tumors, five were in the pineal gland, 12 in the spinal cord and five in the cerebellum. Brain tumor subtypes were not significantly different in terms of patients' age, sex, and anatomical location (Table 1).

TABLE 1. Demographic characteristics and tumor locations based on tumor subtypes.

	Non-glioma	*Low grade Astrocytoms*	*Anaplastic Astrocytoma*	*GBM*	*p-value*
Sex					0.821
Male	28 (68.3%)	7 (53.8%)	11 (64.7%)	12 (63.2%)	
Female	13 (31.7%)	6 (46.2%)	6 (35.3%)	7 (36.8%)	
Total	41 (100%)	13 (100%)	17 (100%)	19 (100%)	
Age					0.741
<20	6 (14.6%)	0	4 (23.5%)	3 (15.8%)	
21-40	15 (36.6%)	6 (46.2%)	3 (17.6%)	5 (26.3%)	
41-60	11 (26.8%)	5 (38.5%)	7 (41.2%)	7 (36.8%)	
61-80	8 (19.5%)	2 (15.4%)	2 (11.8%)	4 (21.1%)	
>80	1 (2.4%)	0	1 (5.9%)	0	
Total	41 (100%)	13 (100%)	17 (100%)	19 (100%)	
Anatomical location					0.768
Frontal	5 (12.2%)	1 (7.7%)	2 (11.8%)	4 (21.1%)	
Parietal	7 (17.1%)	3 (23.1%)	5 (29.4%)	4 (21.1%)	
Occipital	7 (17.1%)	2 (15.4%)	0	2 (10.5%)	
Temporal	13 (31.7%)	4 (30.8%)	3 (17.6%)	6 (31.6%)	
Pineal	2 (4.9%)	1 (7.7%)	1 (5.9%)	1 (5.3%)	
Cerebellum	1 (2.4%)	0	3 (17.6%)	1 (5.3%)	
Spinal cord	6 (14.6%)	2 (15.4%)	3 (17.6%)	1 (5.3%)	
Total	41 (100%)	13 (100%)	17 (100%)	19 (100%)	

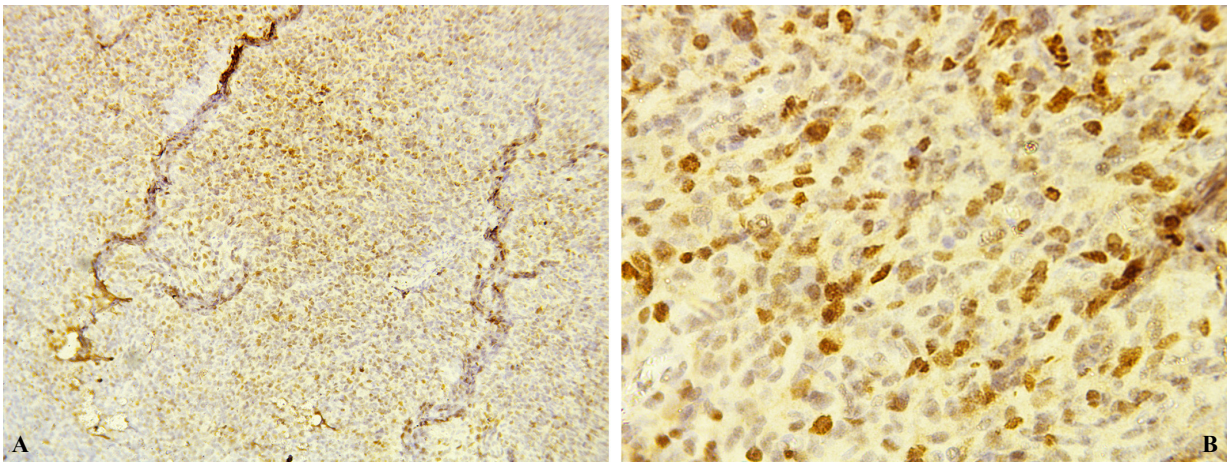

Fig. 1. *A*, IHC staining of GBM x10. *B*, IHC staining of GBM x40.

IHC Analysis

Among non-glioma tumors, 48.8% had a score of four, whereas only 5.2% of GBM had a score of four. However, the percentage of staining was not significantly different among tumor subtypes. There was a significant difference in the intensity of staining among tumor subtypes, 56.1% of non-glioma tumors had strong staining, whereas only 5.3% of GBM and 11.5% of anaplastic astrocytoma had strong staining (p-value=0.04). IHC staining of GBM is shown in Figure 1A-B.

In 34.5% of men and 31.3% of women, the percentage of staining score was 4, and there was no significant difference among the percentage of staining based on gender (p-value=0.58). There was a statistically significant difference between men and women in terms of the intensity of tumor staining (p-value=0.008). Strong intensity was found in 36.2% of men and 18.7% of women.

There were not significant differences in percentage and intensity of staining regarding patients' age group and anatomical location of the tumors (Table 2).

Survival Analysis

The mean overall survival time of patients with brain tumors was 31.226±2.282 months (95%CI 28.441-42.019, Figure 2). Overall survival time was significantly different between glioma and non-glioma tumors (17.490 95% CI 13.055-21.925 *vs.* 41.290 95% CI 36.209-46.371, p-value=0.03, Figure 3). The median survival time was not significantly different between Rb positive and negative tumors (35.230 95% CI 3.567-66.893 *vs.* 34.060 95% CI 25.220-42.90, p-value=0.879, Figure 4). In multivariate Cox regression model, RB negative tumors, GBM, age >50 and female sex were significantly associated with shorter survival duration (Table 3).

DISCUSSION

Brain tumors are heterogeneous neoplasms with a wide range of genetic alterations that play roles in their pathogenesis[9,16]. Since brain tumor treatment is going toward gene-targeted therapy, it is imperative to know which genetic alteration occurred in tumors based on their clinical and pathological characteristics. In this study, we investigated the differences in RB expression in brain tumors. Data analysis showed significant differences in RB expression between different groups in terms of gender and tumor subtypes. Changes in RB expression can modify normal cell proliferation and lead to formation of neoplasms[3,5,9,17-19]. Goldhoff et al[20] used different genomic methods to detect RB expression in brain tumors and compare the results with IHC analysis, and reported IHC analysis as a reliable method. In the present study, IHC was used for detecting RB expression in brain tumors.

All tumors originating from intracranial space are called brain tumors[21]; however, their histologic patterns and cellular origins are diverse. The major subtypes of brain tumors based on histologic patterns include neuroepithelial tumors (gliomas), meningeal tumors (meningioma and hemangioblastoma), sellar region tumors (pituitary tumors and craniopharyngioma). Alterations in RB gene expression may not only cause gliomas, but also play a role in the progression of gliomas from lower grades to higher grades[9]. In addition to gliomas, hyperphosphorylated (inactivate) form of RB protein is also found in other brain tumor subtypes including meningioma, pituitary adenoma, and

TABLE 2. IHC analysis regarding clinical-pathological characteristics.

	Percentage of staining				*p-value*	*Intensity of staining*			*p-value*
	1	*2*	*3*	*4*		*weak*	*moderate*	*strong*	
Sex					0.58				0.008
Male	14 (24.1%)	8 (13.8%)	16 (27.6%)	20(34.5%)		17 (29.3%)	20(34.5%)	21 (36.2%)	
Female	5 (15.6%)	9 (28.1%)	8 (25%)	10(31.3%)		19 (59.4%)	7 (21.9%)	6 (18.7%)	
Age groups					0.28				0.26
0-20	2 (15.4%)	6 (46.1%)	5 (38.5%)	0		8 (61.5%)	3 (23.1%)	2 (15.4%)	
21-40	7 (24.1%)	9 (31.1%)	5 (17.2%)	8 (27.6%)		10(43.5%)	10(34.5%)	9 (31%)	
41-60	5 (16.7%)	1 (3.3%)	10(33.3%)	14 (46.7%)		10(33.3%)	6 (20%)	14 (46.7%)	
61-80	5 (31.3%)	1 (6.2%)	3 (18.7%)	7 (43.8%)		7 (43.7%)	7 (43.7%)	2 (12.6%)	
Anatomical location					0.12				0.04
Cerebellar									
Temporal	6 (23.1%)	1 (3.8%)	8 (30.8%)	11 (42.3%)		13 (50%)	4 (15.4)	9 (34.6%)	
Parietal	3 (15.8%)	9 (47.4%)	2 (10.5%)	5 (26.3%)		8 (42.1%)	7 (36.8%)	4 (21.1%)	
Occipital	2 (18.2%)	0	5 (45.4%)	4 (36.4%)		6 (54.5%)	4 (36.4%)	1 (9.1%)	
Frontal	4 (33.3%)	2 (16.7%)	4 (33.3%)	2 (16.7%)		5 (41.7%)	7 (58.3%)	0	
Non-Cerebellar									
Pineal	1 (20%)	1 (20%)	1 (20%)	2 (40%)		2 (40%)	3 (60%)	0	
Cerebellum	1 (20%)	0	2 (40%)	2 (40%)		2 (40%)	2 (40%)	1 (20%)	
Spinal Cord	2 (16.7%)	4 (33.3%)	2 (16.7%)	4 (33.3%)		0	0	12 (100%)	
Tumor subtypes					0.15				0.01
Non-glioma	7 (17.1%)	1 (2.4%)	13 (31.7%)	20(48.8%)		3 (7.3%)	15 (36.6%)	23 (56.1%)	
Low grade Astrocytoma	2 (15.4%)	2 (15.4%)	3 (23.1%)	6 (46.1%)		10(76.9%)	2 (15.4%)	1 (7.7%)	
Anaplastic Astrocytoma	4 (23.5%)	5 (29.4%)	5 (29.4%)	3 (17.7%)		10(58.8%)	5 (29.4%)	2 (11.8%)	
GBM	6 (31.6%)	9 (47.4%)	3 (15.8%)	1 (5.2%)		13 (68.4%)	5 (26.3%)	1 (5.3%)	

schwannoma[11]. Compared to meningioma, gliomas have a higher percentage of hyperphosphorylated Rb protein[11]. This may explain the more aggressive behavior of gliomas including metastases, higher proliferation, and higher progression rates. In this study, the intensity of IHC staining was significantly different among subtypes of the brain tumor. Most of the GBM had weak staining whereas most non-glioma tumors had strong staining. In the study of Kim et al[22] RB gene alterations were significantly associated with lower survival time even after adjusting for age, sex and histology. In line with previous reports, the result of this study showed that RB expression besides tumor subtype, age and sex independently affect patient survival.

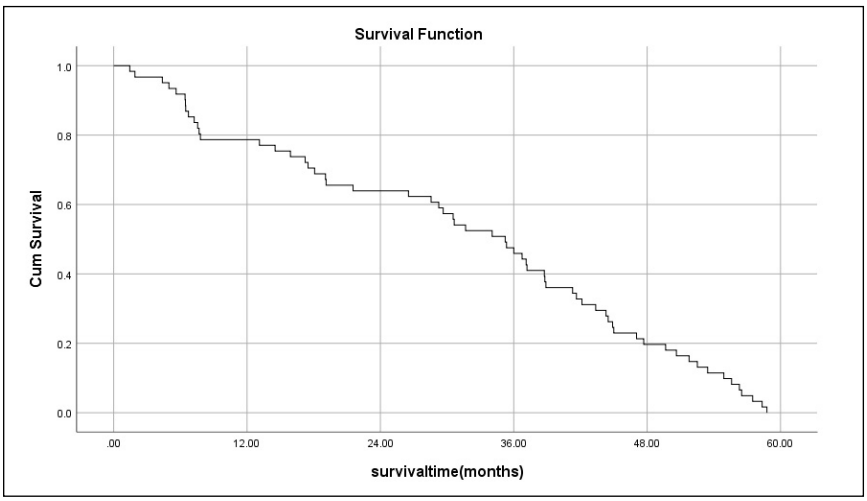

Fig. 2. Patients' overall survival.

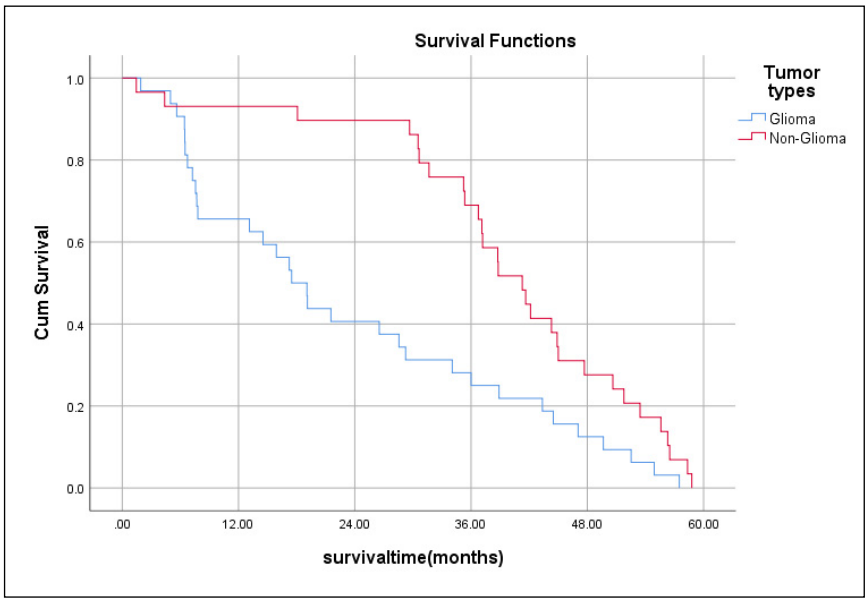

Fig. 3. Glioma *vs*. non glioma survival curves.

Primary malignant tumors are more common in men whereas benign tumors are more common in women[6, 23]. In the study of McKinely et al[24], women in all age groups (except infants) had a lower risk for gliomas, and female sex was determined as a protective factor against developing gliomas. In the present study, expression of RB gene was significantly higher in men, and survival time was significantly lower in women. The effect of sex on patient survival was still true after adjusting for RB expression, age, and tumor subtype. In the study of Clause et al[25], female sex was associated with a better prognosis in patients with astrocytoma, but not in those with oligodendroglioma or mixed glioma. This difference may be explained by the small sample size in this study. Accordingly, future studies should be performed with a larger sample size to determine the effect of sex on survival of patients with brain tumors in each subtype.

Patients diagnosed with primary brain tumors had a median age of 57 year[23]. Age is a prognostic factor in all types of brain tumors. Patients in older age groups had lower survival time, but when compared with patients in the same age group, GBM had the lowest survival[6,23]. In this study, most patients were 20-60 years old. There was no significant difference among age groups in the expression of RB protein. Also, according to the results of this study, older age was associated with lower survival time. In line with this study, Hong et al[26] found that patient's age was independently associated with overall survival of patients with anaplastic gliomas.

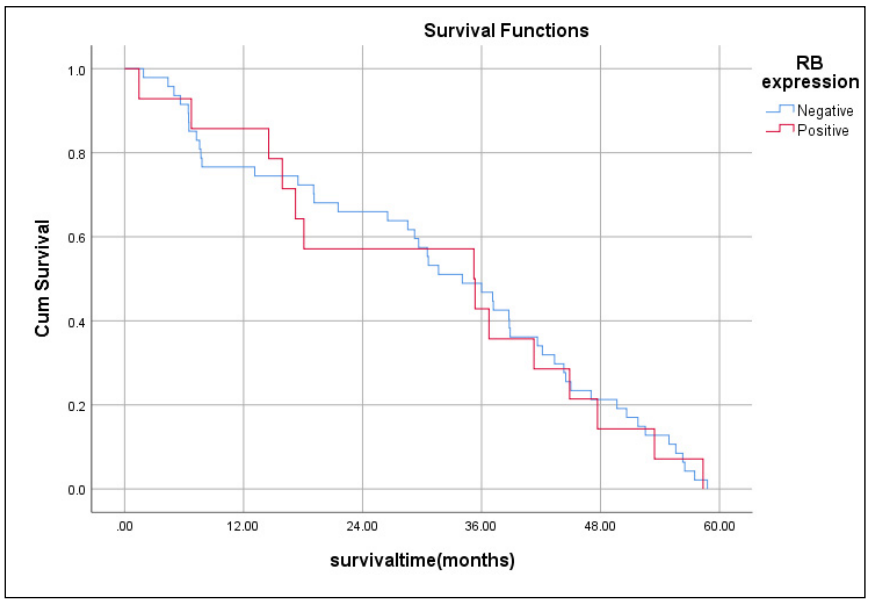

Fig. 4. RB positive vs. negative survival curves.

TABLE 3. Univariate and multivariate cox regression model.

	Univariate analysis		*Multivariate analysis*	
	HR (95%CI)	*p-value*	*HR (95%CI*	*P-value*
Sex				
Male	Ref			
Female	1.230(0.721-2.098)	0.447	2.271 (1.143-4.514)	0.019
Age				
<50	Ref			
>50	22.884 (5.771-90.738)	0.001	28.648 (5.116-160.405)	0.0001
Tumor type				
Non glioma	Ref			
Low grade astrocytoma	0.098 (0.09-1.083)	0.58	0.189 (0.214-1.356)	0.189
Anaplastic astrocytoma	0.967 (0.390-2.402)	0.943	1.403 (0.474-4.154)	0.541
GBM	10.085 (2.636-38.586)	0.001	9.993 (1.888-12.254)	0.007
RB expression				
Positive	Ref			
Negative	1.393 (0.772-1.898)	0.027	2.502 (1.061-5.896)	0.048
Anatomical location				
Cerebral	Ref			
Noncerebral	1.798 (0.836-3.865)	0.133	1.697 (0.747-3.856)	0.207

Anatomical location of brain tumors is an important issue because it affects treatment option, hence, tumor prognosis. In previous reports, different mutations in oligodendroglioma tumors were determined based on anatomical location[27,28]. RB expression based on anatomical location of tumors was not previously evaluated. In the present study, different tumor locations did not significantly affect RB expression, and were not significantly associated with patient survival. In the study of Simpson et al[29], GBM tumors found in frontal lobes had longer survival compared to tumors in temporal or parietal lobes.

LIMITATIONS

Due to small sample size of the present study, it is not possible to compare the effect of anatomical location in each brain tumor subtype. Further studies are needed to evaluate genetic alterations of brain tumors based on their anatomical locations. Another limitation is that IHC staining was done without genomic analysis of RB in brain tumor samples. While IHC is a reliable method for detecting RB expression, its combination with genomic methods can reduce the possible bias of IHC staining. Furthermore, due to different behaviors of brain tumor subtypes, it is helpful to investigate each subtype separately in future studies.

CONCLUSIONS

The results of the present study demonstrated a significant difference in RB expression between male and female patients and brain tumor subtypes. RB expression of tumors had a significant effect on survival independently. Unlike previous studies, expression of RB was higher in men compared to women, that is, being female was a risk factor for lower survival. Although age could affect prognosis and survival of patients with brain tumors, there was no significant difference among age groups in terms of RB expression.

Conflict of Interests:
Authors have no conflict of interest to disclose.

Informed Consent:
Not Required

Funding:
No funding was received for this study.

Ethical Approval:
The study was approved by Ethics Committee of Shahid Sadoughi General Hospital Research Center.

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