Central Asian Journal of Medical Sciences
CAJMS

Original Article

Cent Asian J Med Sci. 2018 Sep;4(3):211-219. https://doi.org/10.24079/CAJMS.2018.09.007

Prognostic Value of Immunohistochemical Biomarkers in Patients with Astrocytic Brain Tumor

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Submitted: September 14, 2018
Revised: September 21, 2018
Accepted: September 25, 2018

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Objectives: Astrocytoma is a common primary malignant brain tumor and it is typically associated with poor outcome. In this study, we aimed to determine role of various immunohistochemical (IHC) biomarkers and their relationship with patient prognosis. **Methods:** Patients who underwent surgery for astrocytic brain tumor were selected. Tissue specimens were collected from surgically removed tumors and stained by various IHC stains. All-cause mortality was chosen for study endpoint. **Results:** A total of 133 patients with who underwent surgery for astrocytic brain tumor were included (mean age 38±19 and 49.6% male). During follow-up, 84 patients (63%) died and the median time of death was 8 months (IQR 4; 16) after hospital discharge. Patients who died had higher tumor grade (60.7% vs. 34.7%, $p<0.01$). After adjustment of possible predictors, surgical type (HR=0.51, 95% CI 0.31-0.82, $p<0.01$), expression level of Ki67 (HR=1.02, 95% CI 1.00-1.04, $p<0.05$) and p53 (HR=1.01, 95% CI 1.00-1.02, $p<0.05$) were independently associated with long term mortality. Kaplan-Meier estimation showed overexpression of WT1 (42% vs. 52%, $p<0.05$) and Ki67 (27% vs. 58%, $p<0.001$) are associated with poor survival. **Conclusion:** IHC biomarkers are independently associated with long term prognosis in patients with astrocytic brain tumor. Overexpression of WT1 and Ki67 marker are associated with poor survival.

Keywords: Glioma, Primary Brain Tumor, Prognosis, Survival

Introduction

Astrocytoma is common type of primary brain tumor that originates from glial cells called astrocytes located in the cerebrum [1]. Astrocytes have important role in maintaining normal brain function by providing structural and metabolic support to the neurons, regulating homeostasis of extracellular matrix, maintaining blood-brain barrier and modulation of synaptic signal transmission [2, 3].

Prognostic prediction of astrocytic brain tumor is mainly based on World Health Organization Classification of Tumors of the Central Nervous System which classifies brain tumors based on their histological appearance and molecular parameters. Long term prognosis of astrocytic brain tumor decreases with increasing tumor grade and high-grade tumors often require a combined rather than single treatment approach [4, 5]. Therefore,

identification of the tumor grade in patients with astrocytic brain tumors is essential to choose the appropriate therapeutic strategy such as surgical removal, chemotherapy, radiotherapy or combined approach [6, 7]. However, an ongoing debate exists because the differences in routine histologic grading of these brain tumors does not adequately explain the differences in their biologic behavior [8-10].

Immunohistochemical (IHC) staining is powerful method which can reveal the biologic behavior of the neoplasms and thus will give useful prognostic information such as survival association and tumor recurrence for patients with astrocytic brain tumor [11]. Previous biomarker studies have investigated only one or two potential biomarkers in tumor samples from patients with glioblastoma and identified their relationship with prognosis and correlation with other biomarkers [12-15]. Nonetheless, the development and pathogenesis of glioma tumor is complex process which is regulated by interactions of various intracellular and nuclear biological markers.

In this study, we used several cytoplasmic and nuclear IHC staining, including WT1, Ki67, p53, EGFR, GFAP, Vimentin, IDH1, PTEN, in tumor samples from patients with astrocytic brain tumors. We sought to determine the independent predictive effect of IHC biomarkers on patient prognosis.

Materials and Methods

Study design and population

This retrospective study was approved by the Ethics Committee of Mongolian National University of Medical Sciences (2017/3-201702), and informed consent was obtained from all participants. A retrospective review identified 135 patients with formalin fixed paraffin embedded tissue blocks who had surgically removed tumors in the Department of Neurosurgery, State Third Central Hospital. The tissue specimens which were kept in the National Center for Pathology archive. All tumors were graded and classified histologically from low grade to high grade according to World Health Organization (WHO) classification. We enrolled 133 patients with astrocytic brain tumor after tissue preparation for immunohistochemical evaluation. Two tissue preparations were excluded due to unsatisfactory quantity of tissue.

Immunohistochemical staining

Immunohistochemical staining for WT1 was performed using formalin-fixed paraffin-embedded tumor tissue microarray (TMA) blocks. Briefly, 4-μm-thick tissue sections were deparaffinized in xylene and hydrated by immersing in a series of graded ethanol. Antigen retrieval was performed in a microwave by placing the sections in epitope retrieval solution (0.01 M citrate buffer, pH 6.0) for 20 minutes; endogenous peroxidase was inhibited by immersing the sections in 0.3% hydrogen peroxide for 10 minutes. Sections were then incubated with anti-WT1 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), EGFR (1:150, Dako, Camarillo, CA, USA), p53 (1:1,000, Dako, Glostrup, Denmark), IDH-1 (1:100, DIANOVA, Hamburg, Germany), Ki67 (1:50, Dako, Camarillo, CA, USA) antibodies. Next, an Ultra View universal DAB kit (Ventana Medical Systems, Inc., Arizona, USA) was used following the manufacturer's recommendations in conjunction with an automated staining procedure. Finally, the samples were counterstained with hematoxylin, dehydrated, mounted, evaluated and photographed (20× and 40× objective) under a light microscope equipped with an Olympus Cx21 camera.

Immunohistochemical stains for WT1 and EGFR were graded as follows: 0 (no cell stained), 1+ (<5% tumor cells stained), 2+ (5-50% cells stained), and 3+ (>50% cells stained). Nuclear staining of p53 and Ki67 were scored semi-quantitatively in the most prominently stained area of the tissue slides. The percentage of positive cells was counted as follows: cases in which greater than 10% cells were stained were considered positive (overexpression of p53 and Ki67), and cases in which less than 10% of the cells of were stained were considered low expression. Cytoplasmic immunoreactivity to the IDH-1 antibody was considered as positive immunostaining. Examples of IHC-stained histological sections are shown in Figure 1.

Study endpoint

In this study, we choose long term all-cause mortality after hospital discharge for primary endpoint. Data on the occurrence of endpoint was collected from the database of General Authority for State Registration.

Statistical analysis

Continuous data were presented as the mean ± standard deviation (SD) and categorical data were presented as frequencies and percentages. Differences in baseline characteristics between

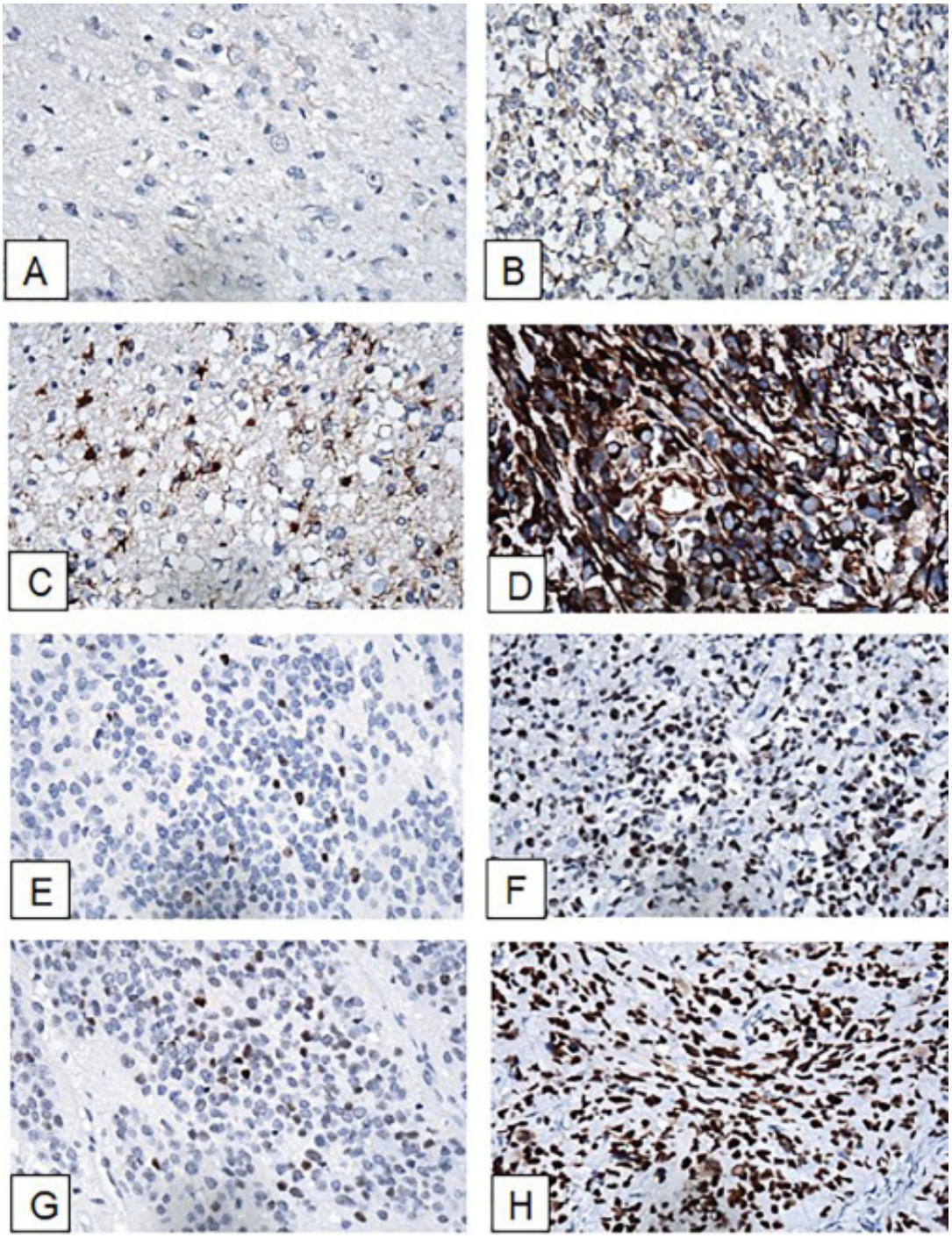

Figure 1. The level of Wilms' tumor 1 (WT1) expression was classified. 0 (A, Immunohistochemistry, x100), no staining of glioblastoma tissues, up to 5% (B, Immunohistochemistry, x100), slightly increased staining WT1 protein was detected (brown color) in some tumor cells compared with that in normal glial cells; 5-50% (C, Immunohistochemistry, x100), staining at intermediate intensity in some tumor cells; above 50% of WT1 high expression (D, Immunohistochemistry, x100), strong staining in almost all glioblastoma tumor cells. Low (E, Immunohistochemistry, x100) and high expression (F, Immunohistochemistry, x100) of Ki-67 proliferative index was detected in tumor cells positive nuclei. No over expression (G) and over expression of P53 (H, Immunohistochemistry, x100) were detected in positive tumor cells nuclei.

patients' survival and mortality were evaluated by using the independent sample t-test and chi-squared test. Continuous variables that were not normally distributed (as evaluated by Kolmogorov–Smirnov tests) were presented as medians and 25th and 75th percentiles and were compared using the Wilcoxon's rank-sum test.

Univariable Cox proportional hazard regression analysis used to determine the relationship between individual parameters and endpoint. After univariable analysis, multivariable Cox proportional hazard regression analysis was conducted to evaluate independent association between immunohistochemical biomarkers and endpoint. Multivariable analysis consisted WHO tumor grade, surgery type, high expression level of WT1, Ki67 and p53.

Prognostic capacity of immunohistochemical biomarkers were assessed using area under receiver-operating characteristic curve analysis (c-statistics). The c-statistic reflects the concordance of predictions with actual outcomes in rank order, with a c-statistic of 1.0 indicating perfect discrimination. Additionally, the incremental prognostic values of immunohistochemical biomarkers over WHO tumor grade and surgery type were tested. The incremental improvement in model performance was tested by estimation of the model chi-square.

Finally, Kaplan-Meier curve estimation was used to compare survival rate according to the different expression level of immunohistochemical biomarkers.

All statistical tests were two-sided, and a p-value of <0.05 indicated statistical significance. SPSS software (version 22.0, SPSS Inc., Chicago, IL, USA) was used to perform all statistical analyses.

Results

Baseline characteristics

In this study, the prevalence of astrocytic brain tumor was equal in both genders (male 49.6% and female 50.4%). During long term follow-up all-cause mortality occurred in 84 patients (63%) and the median time of death was 8 months (interquartile range [IQR] 4; 16) after hospital discharge. Patients with all-cause mortality were more likely have had high tumor grade (60.7% vs. 34.7%, p<0.01), received partial tumor removal surgery (63.1% vs. 40.8%, p<0.05), higher expression of WT1 (37.5% vs. 19.1%, p<0.05) and higher cellular proliferation rate as assessed by expression of Ki67 (7.5% vs. 5%, p<0.05). Characteristics of study population are shown in Table 1.

Predictors of long-term mortality

All patients were followed until death or until December of 2017. The univariate and multivariate Cox proportional hazard regression was used to estimate relationship between individual variables and primary endpoint (all-cause mortality). Results were shown in Table 2. Univariate Cox regression analysis showed that long-term mortality rate was increased 1.66 times in patients with high expression level of WT1 (HR=1.66, 95% CI 1.05-2.63, p<0.05), increased 1.03 times for every 1 percent increase of Ki67 expression level (HR=1.03, 95% CI 1.01-1.04, p<0.001) and increased 1.01 times for every 1 percent increase of p53 expression level (HR=1.01, 95% CI 1.00-1.02, p<0.01), respectively. Also, WHO tumor grade was associated with increased risk of long-term mortality (HR=1.42, 95% CI 1.12-1.82, p<0.01) but complete surgical removal of tumor was associated with decreased risk of long-term mortality (HR=0.5, 95% CI 0.32-0.79, p<0.01) in univariate analysis.

After univariate Cox proportional hazard regression, multivariate analysis was performed to determine the independent relationship between individual variables which had significant P values in univariate analysis and long-term mortality. Therefore, multivariate analysis consisted of WHO tumor grade, surgical type, high expression of WT1, Ki67 expression level and p53 expression level. After adjustment of the above-mentioned variables, surgical type (HR=0.51, 95% CI 0.31-0.82, p<0.01), Ki67 expression level (HR=1.02, 95% CI 1.00-1.04, p<0.05) and p53 (HR=1.01, 95% CI 1.00-1.02, p<0.05) were independently associated with long term mortality. Furthermore, Ki67 expression level was the only significant biomarker to predict long term mortality in ROC curve estimation (c-statistic 0.63, 95% CI 0.54-0.73, p<0.05).

The incremental prognostic value of immunohistochemical biomarkers over WHO tumor grade and surgery type was evaluated using three step model, as described in Figure 2. Adding immunohistochemical biomarkers into the model which consisted of WHO tumor grade and surgery type significantly increased the model chi-square (p<0.05).

Survival analysis

All the patients were followed-up to occurrence of endpoint or December 2017. Median follow-up time was 8 months (IQR 4;

Table 1. Characteristics of study patients who had surgery for astrocytoma.

Variables	All patient (n=133)	Patients who died (n=84)	Patients who survived (n=49)	p-value
Age	38±19	37±20	38±16	0.722[a]
Gender				0.056[b]
Male	49.6% (n=66)	56% (n=47)	38.8% (n=19)	
Female	50.4% (n=67)	44% (n=37)	61.2% (n=30)	
WHO tumor grade				0.002[b]
Grade I	6.8% (n=9)	8% (n=9.5%)	2% (n=1)	
Grade II	42.1% (n=56)	29.8% (n=25)	63.3% (n=31)	
Grade III	24.8% (n=33)	31% (n=26)	14.3% (n=7)	
Grade IV	26.3% (n=35)	29.8% (n=25)	20.4% (n=10)	
High grade tumor	51.1% (n=68)	60.7% (n=51)	34.7% (n=17)	0.004[b]
Tumor location				0.178[b]
Temporal	13.5% (n=18)	11.9% (n=10)	16.3% (n=8)	
Multiple	27.8% (n=37)	29.8% (n=25)	24.5% (n=12)	
Frontal	25.6% (n=34)	19% (n=16)	36.7% (n=18)	
Thalamus	12.8% (n=17)	14.3% (n=12)	10.2% (n=5)	
Cerebellum	8.3% (n=11)	9.5% (n=8)	6.1% (n=3)	
Parietal	12% (n=16)	15.5% (n=13)	6.1% (n=3)	
Surgery type				0.013[b]
Complete removal	45.1% (n=60)	36.9% (n=31)	59.2% (n=29)	
Partial removal	54.9% (n=73)	63.1% (n=53)	40.8% (n=20)	
Biomarker				
WT1				0.030[b]
No expression	4.5% (n=6)	NA	NA	
High expression	29.3% (n=39)	37.5% (n=30)	19.1% (n=9)	
Low expression	66.2% (n=88)	62.5% (n=50)	80.9% (n=38)	
Ki67 (%)	6 (2; 12)	7.5 (2; 16)	5 (1; 7)	0.008[c]
p53 (%)	4 (0; 24)	5 (0; 25)	4 (0; 22)	0.512[c]
EGFR score	0 (0; 1)	0 (0; 1)	0 (0; 1)	0.705[c]
GFAP score	3 (2; 3)	3 (2; 3)	3 (2; 3)	0.250[c]
Vimentin score	2 (1; 3)	2 (1; 3)	1 (0; 3)	0.068[c]
IDH1				0.684[b]
Negative	77.4% (n=103)	21.4% (n=18)	24.5% (n=12)	
Positive	22.6% (n=30)	78.6% (n=66)	75.5% (n=37)	
PTEN				0.262[c]
Loss	54.9% (n=73)	51.2% (n=43)	61.2% (n=30)	
No loss	45.1% (n=60)	48.8% (n=41)	38.8% (n=19)	

[a] independent sample t-test, [b] chi-square test, [c] Wilcoxon's rank-sum test. WHO=World Health Organization, WT1=Wilms Tumor 1, EGFR=Endothelial Growth Factor Receptor GFAP=Glial Fibrillary Acidic Protein, IDH1=Isocitrate Dehydrogenase 1, PTEN=Phosphatase and Tensin Homolog.

16). During follow-up, all-cause mortality occurred in 84 patients (63%). The survival rates for tumor grade, surgery type, WT1 expression level and Ki67 proliferation index are described in Figure 3.

At 5 years, the survival rate was 34% (95% CI 22-46%) for high grade tumor and 64% (95% CI 52-76%) for low grade tumor

Table 2. Univariate and multivariate Cox proportional hazard regression.

Variables	Univariate analysis			Multivariate analysis[a]		
	HR	95% CI	p-value	HR	95% CI	p-value
Age	0.99	0.98-1.00	0.214			
Gender	0.79	0.52-1.23	0.301			
WHO tumor grade	1.42	1.12-1.82	0.004			
Tumor location	1.07	0.93-1.23	0.355			
Surgery type	0.50	0.32-0.79	0.003	0.51	0.31-0.82	<0.01
WT1 high expression	1.66	1.05-2.63	0.030			
Ki67	1.03	1.01-1.04	0.001	1.02	1.00-1.04	<0.05
p53	1.01	1.00-1.02	0.006	1.01	1.00-1.02	<0.05
EGFR	1.01	0.80-1.28	0.898			
GFAP	0.99	0.80-1.24	0.966			
Vimentin	1.17	0.97-1.41	0.094			
IDH1	0.81	0.48-1.38	0.443			
PTEN	0.66	0.43-1.03	0.066			

[a] WHO tumor grade, surgery type, WT1 high expression, Ki67 and p53 were included in multivariate analysis. HR=Hazard Ratio, WT1=Wilms Tumor 1, EGFR=Endothelial Growth Factor Receptor, GFAP=Glial Fibrillary Acidic Protein, IDH1=Isocitrate Dehydrogenase 1, PTEN=Phosphatase and Tensin Homolog.

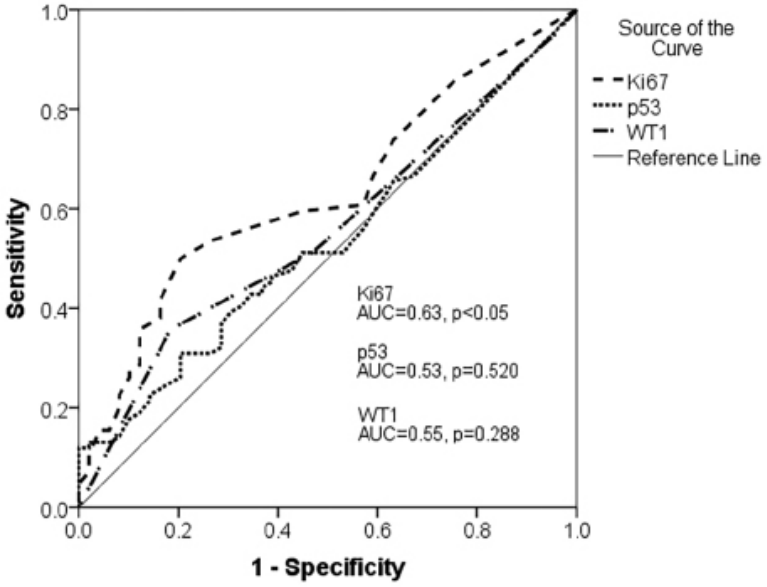

Figure 2. Receiver operating characteristics (ROC) curve estimation for predictive capacities of immunohistochemical biomarkers. AUC; area under curve.

(p<0.001), 41% (95% CI 29-53%) for partial surgery group and 57% (95% CI 43-71%) for complete surgery group (p<0.01), 42% (95% CI 26-58%) for WT1 high expression group and 52% (95% CI 40-64%) for WT1 low expression group (p<0.05), 27% (95% CI 13-41%) for Ki67 high proliferation index group and 58% (95% CI 48-68%) for Ki67 low proliferation index group (p<0.001), respectively.

Over expression of WT1 and Ki67 were significantly associated with poor long-term prognosis in study population. In patients with high expression of WT1 (≥3 score in immunohistochemical staining), 5-year survival rate was significantly lower than patients with low expression of WT1 (42% vs. 52%, p<0.05). Also, a high proliferation index of Ki67 (>10% on immunohistochemical staining) was significantly

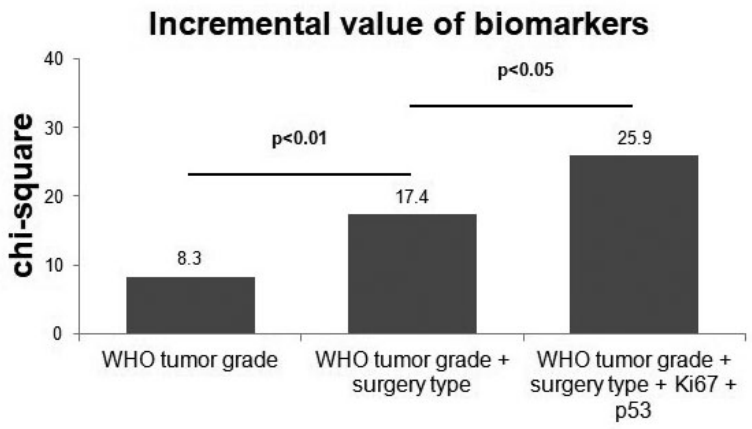

Figure 3. Incremental predictive value of immunohistochemical biomarkers by model chi-square value. WHO=World Health Organization.

associated with low survival compared with low proliferation index of Ki67 (27% vs. 58%, p<0.001) at the end of follow-up.

Discussion

The results of the present study indicate that: 1) after adjustment of previously determined clinical predictors (tumor grade and surgical type), immunohistochemical biomarkers were independent and strong predictor for long term prognosis in patients with a astrocytic brain tumor; 2) over expression of WT1 and Ki67 markers were associated with poor survival in those patients.

Astrocytic brain tumor is common type of primary malignant brain tumor and prognosis is mainly associated with tumor grade [7, 10]. Overall mortality of astrocytic brain tumor is increases with tumor grade and mortality rate is 5.86% for grade I, 51.18% for grade II, 67.40% for grade III and 85.85% for grade IV tumors [16]. Therefore, precise tumor grading is cornerstone of management and prognostication for this type of tumor. The traditional approach to prediction of prognosis for patients with glioma tumor is broadly based on tumor grade which derived from its histological pattern in simple hematoxylin and eosin (H&E) staining [3, 9]. The disadvantages of H&E staining are that cytoplasmic and nuclear biomarkers cannot determined by routine H&E stains. Thus, key cellular processes which play crucial roles in pathogenesis of the neoplasm remain unknown leading to over or underestimation of aggressiveness of the neoplasm.

In recent decades, IHC staining has become important part of diagnosis of various type of undifferentiated neoplasms [11]. Several IHC biomarkers, such as Wilms' tumor protein (WT1), proliferation marker (Ki67), p53 protein, epidermal growth factor receptor (EGFR), glial fibrillary acidic protein (GFAP), vimentin, have been investigated for different types of brain tumors [12, 15, 17-19]. High grade gliomas are characterized by relatively high frequencies of EGFR amplification, phosphatase and tensin homolog (PTEN) deletion, and IDH1 wild type [21]. GFAP is the most frequently used marker in diagnostic neuro-oncology. Positive reaction to GFAP has been demonstrated in astrocytoma and astrocytic cells of mixed gliomas [22]. However, previous researchers selected only potential biomarkers for their studies to identify their role in brain tumor pathogenesis. Therefore, data about cumulative effect of various potential biomarkers on prognosis of patients with astrocytic brain tumor are scarce.

In this study, we aimed to determine the independent relationship of different IHC biomarkers in same patient population. Multivariable Cox proportional hazard regression analysis revealed that Ki67 and p53 protein expression are independently associated with long term prognosis in study populations. Additional ROC curve estimation showed that Ki67 biomarker had greatest c-statistic value to predict mortality. The incremental value of each of 3 IHC biomarkers was tested by using three modeling steps in which tumor grade, surgery type, and then IHC biomarkers were added significantly increasing the chi-square value. All these data confirm that combined use of IHC biomarkers for prognostication of astrocytic brain tumor is associated with increased prognostic capacity.

Kaplan-Meier estimation of survival analysis determined that high expression of WT1 and Ki67 biomarkers were associated with significantly lower survival compared with low expression of those biomarkers. Therefore, patients who had high expression of WT1 and Ki67 biomarkers might benefit from combined therapeutic approach rather than surgery only. Also, complete surgical removal of tumor mass was related to increased survival in study populations. So that identification of precise tumor location, volume and suitable surgical approach should be considered for imaging of tumor before surgery. Camacho et al, found that almost 65% of high grade glioma, especially glioblastoma samples, showed over expression of WT1 which correlated with significantly reduced survival rates [23].

Finally, total study population was relatively small compared with other similar studies [20]. This could be a significant limitation of our study. There were a relatively small number of patients (n=133) for a multivariate analysis. Therefore, we selected covariates which were had significant p-value in univariate analysis for multivariate analysis. Further studies with larger numbers of patients are needed to confirm these findings.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgements

This study was conducted by National Center for Pathology, Department of Pathology, MNUMS, on tissue obtained by the Department of Neurosurgery, The Third State Central Hospital. We acknowledge our sincerest gratitude for them.

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