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Original Research

Immunohistochemical study of prognostic relevance of nestin and survivin expression in astrocytic glioma

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ABSTRACT

Background: Identification of the cellular origin of astrocytic gliomas is a step for improving the treatment strategies. Tumor stem cells have been detected in different neoplasms and have a major role in tumor initiation, progression, and therapy resistance. **Objective:** The study aimed to investigate the expression of nestin and survivin in different grades of astrocytic glioma as well as evaluation of their prognostic role in relation to other prognostic parameters. **Materials and Methods:** Immunohistochemical expressions of nestin and survivin were evaluated in 40 paraffin blocks of different grades of astrocytic glioma and correlated with other prognostic parameters. The obtained data were statistically analyzed. **Results:** The cases included 10 low grade, 12 anaplastic, and 18 glioblastoma multiforme. There was a significant correlation between each of nestin and survivin with the histological grade of astrocytoma and tumor size ($P < 0.001$). Nestin was strongly correlated to survivin index ($P < 0.001$). The univariate analysis showed that high histological grade, high nestin score, and high survivin index were significantly correlated with overall survival (OS). Multivariate analysis for all investigated cases proved the independent prognostic significance of the nestin expression ($P = 0.011$). **Conclusion:** Nestin and survivin are adverse prognostic markers for astrocytic glioma that increase significantly with tumor progression and associated with poor OS. Therefore, nestin and survivin could be used to predict high-risk group of astrocytoma with unfavorable outcome.

KEY WORDS: Astrocytoma, immunohistochemistry, nestin, prognosis, survivin

INTRODUCTION

Gliomas are the most common primary tumors of the central nervous system. In Egypt, astrocytic gliomas are the most common glial tumors (79.4% of all gliomas) [1]. Histopathological diagnosis is important for definitive prognosis and treatment. According to WHO classification (2007), astrocytomas are classified into four histopathological grades as follow: Low-grade astrocytoma (GI-II), anaplastic astrocytoma (GIII), and glioblastoma multiforme (GBM) (GIV) [2].

The tumor cells vigorously invade the surrounding brain tissue, which renders complete surgical removal of the tumor more difficult leading to the high incidence of recurrence. The patient clinical history reflects the aggressiveness of his tumor; and despite the great advances in therapeutic modalities, the median survival time of GBM cases is not more than 12 months [3]. However, some patients with similar grades have clear differences in survival. This assures that certain tumor biomarkers are needed for prognosis assessment of tumors other than the pathological grading system [4].

Identification of the cellular origin of gliomas is a tool for improving the treatment strategies. The neural stem cell (NSC) and progenitor cells, in addition to differentiated adult glia, represent the origin for neoplastic transformation [5]. Evaluation of the NSC in a particular tumor might be an important parameter in detecting the clinical course of disease [6].

Recent research suggests that the tumor biology and the resistance to treatment are correlated to the presence of cancer stem cells (CSCs). The importance of CSCs for estimating the prognosis of patients with gliomas has been commonly evaluated using several markers intimately related to the presence of these cells [7].

Sanai *et al*. [5] have suggested a theory that NSC found in the subventricular zone of the mature brain tissue infiltrate and grow into the surrounding area, then differentiate into glioma progenitor cells, and extend into the brain forming glioma.

Nestin is an intermediate filament protein implicated in the organization of the cytoskeleton, cell signaling, organogenesis, and cell metabolism. It is intensely expressed during early embryogenesis in neuroepithelial stem cells and absent in most mature cells [8]. However, it may be expressed in adult astrocytes in response to cellular stress such as neoplastic transformation. It has been detected in primary central nervous system (CNS) tumors but not in metastatic carcinomas [9-12].

Since the proliferative activity is a reliable method to assess tumor biology, there have been continuous researches to assess such biological markers [13]. Survivin is a member of the apoptosis inhibitors, which promotes survival of tumor cells through suppression of apoptotic cell death and regulation of cell division [14]. It is commonly expressed in embryonic and neoplastic tissues and rarely expressed in normal cells [15]. There is limited data about survivin immunostaining and prognosis in cases of anaplastic astrocytoma, and conflicting results regarding glioblastoma [16,17]. Furthermore, survivin may promote radiation resistance in glioblastoma [18].

The aim of this study was to investigate the expression of nestin and survivin in different grades of astrocytoma and to evaluate its possible prognostic role in correlations to other prognostic parameters.

MATERIALS AND METHODS

Tissue Specimens' Collection

This retrospective study was conducted on a 40 formalin-fixed paraffin-embedded primary astrocytoma specimens with different grades, which were surgically resected at the Department of Neurosurgery, diagnosed at Pathology Department and treated at Clinical Oncology Department, Zagazig University Hospitals, Egypt during the period from January 2008 to December 2011. The clinical data were obtained from patients' clinical records including age, sex, treatment strategies, details of the follow-up, and post-operative survival data. None of the patients were under chemotherapy or radiotherapy before surgery. Patients were excluded if they had cancer other than astrocytoma, recurrent brain tumor, incomplete clinical information, or incomplete follow-up data in their files. In addition, patients without post-operative radiotherapy were excluded from the prognostic study. The follow-up period was the interval from the date of initial diagnosis to the date of death or last follow-up. Median follow-up time was 21.5 (4-48) months. The pathological diagnosis and grading of the resected astrocytic tumors were confirmed independently by two pathologists (AE, HR) using the established criteria of the WHO classification of the central nervous system tumors [2]. In addition, five normal brain tissue samples were taken and served as control cases.

According to the protocol, patients with low-grade astrocytoma (GII) received three-dimensional conformal radiotherapy 45-54 Gy in 1.8-2 Gy fractions [19]. As regard to high grade, GIII (anaplastic astrocytoma) received three-dimensional conformal radiotherapy 60 Gy in 2 Gy fractions while GIV (GBM) received chemoradiotherapy temozolomide 75 mg/m^2 daily with three-dimensional conformal radiotherapy 60 Gy in 2 Gy fraction, followed by 6 cycles of chemotherapy temozolomide 150 mg/m^2 for 5 days every 28 days [20]. The study complied with the guidelines of the Local Ethics Committee.

Immunohistochemical Procedure

Immunohistochemical staining was performed on 4 μm thick sections using the streptavidin–biotin–peroxidase technique. The sections were deparaffinized in xylene and rehydrated through graded alcohol. Antigen retrieval was performed by treating the tissue sections with 0.1 mol/L citrate buffer, pH 6.0 for 10 min in a microwave at 100°C for 20 min, then left to cool at room temperature. The endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide for 10 min. After washing with phosphate buffer saline (PBS), blocking serum was applied for 10 min. At room temperature, the sections were incubated overnight with a rabbit polyclonal anti-human antibody raised against nestin (Catalog E18610; Spring Bioscience, Pleasanton, California, USA), diluted 1:100, and with monoclonal anti-survivin antibody (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, California, USA). After rinsing in PBS, the tissues were incubated with a biotin-conjugated secondary antibody and then incubated by using the streptavidin–biotin system for an hour at room temperature. The peroxidase reaction was visualized by incubating the sections with DAB. The sections were counterstained with Mayer's hematoxylin followed by dehydration, clearing, and mounting. Sections from the kidney were used as positive control for nestin while colonic adenocarcinoma was the positive control for survivin. Negative controls were done by substitution of primary antibodies with a non-immune serum.

Interpretation of Immunohistochemistry

Nestin immunostaining

Nestin was expressed in the cytoplasm of tumor cells. The expression of nestin in astrocytoma was evaluated by the percentage and staining intensity of immunoreactive cells in the entire slide or a large representative area of the tumor specimen under low magnification (×100) and was then confirmed by determining the percentage of stained cells in several randomly selected areas of each specimen at high magnification (×200 and ×400). The frequency of nestin immunoreactivity was scored as follows: "0" when no positive cells were observed within the tumor; "1" if less than 30% of tumor cells are positive; "2" if 30-60% of tumor cells are positive and "3" if more than 60% of tumor cells are positive. The intensity of staining was evaluated as 0, 1, 2, and 3 for no staining, weak staining, moderate staining, and strong staining, respectively. The immunoreactivity score (IRS) for nestin was

calculated by multiplying the frequency score and intensity score. The staining scores were stratified as low IRS (scored 0-2) and high IRS (scored 3-9) [12].

Survivin immunostaining

The tumor cells were considered positive for survivin when immunoreactivity was observed in their nuclei and cytoplasm. The survivin index was classified as "low" if less than 50% of tumor cells were stained and "high" if more than 50% of tumor cells were stained [21].

Statistical Analysis

Continuous variables were expressed as the mean ± standard deviation for normally distributed data and median (range) for non-normally distributed data, and the categorical variables were expressed as a number (percentage). Nestin (IRS) and survivin index were analyzed using the Mann–Whitney *U*-test for two groups and Kruskal–Wallis *H*-test for more than two groups. Percent of categorical variables were compared using the Pearson's Chi-square (χ^2) test. Stratification of overall survival (OS) was done according to grade, survivin index and nestin expression. These time-to-death distributions were estimated using the method of Kaplan–Meier curves, and compared using two-sided exact log-rank test. The Cox proportional-hazards regression model was used to identify the independent prognostic factors as well as to estimate their effects on OS. All tests were two-sided. A *P* value was considered significant if <0.05. All statistics were performed using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) and MedCalc 13 for windows (MedCalc Software bvba).

RESULTS

Clinicopathological Features

The age of the patients (n = 40) at surgery time ranged from 18 to 60 years; the mean age was 44.7 ± 13.7 years. The mean age of patients with low-grade astrocytoma (GII), anaplastic astrocytoma (GIII), and GBM (GIV) was 33.1 ±15, 42.7 ±13, and 52.5 ± 7.6 years, respectively. The patients were 22 men and 18 women, diagnosed with low-grade astrocytoma (n = 10), anaplastic astrocytoma (n = 12) and glioblastoma (n = 18). During the follow-up period, 25 patients (62.5%) died, while 15 patients (37.5%) were still alive by the end of this study. The median OS was 22 months after initial surgery. Clinicopathological characteristics were demonstrated in Table 1.

Nestin Expression and Association with Clinicopathological Parameters

All sections of different WHO grades were immunoreactive to nestin [Figure 1], whereas normal brain tissue was negative (P < 0.001). The distribution pattern of nestin was variable with perivascular and/or diffuse pattern. In some sections, nestin-positive cells were condensed in the tumor infiltration zone at interface of normal brain tissue. There was statistically significant up-regulation of median nestin IRS (P < 0.001) from GII to GIV (2 [0-4], 4 [2-9], and 6 [4-9], respectively).

Table 1: Clinicopathological characteristics of the studied cases of astrocytoma (n=40)

Characteristics	Number (%)
Age (year)	
Mean±SD	44.7±13.7
Median (range)	50 (18-60)
≤50 years	23 (57.5)
>50 years	17 (42.5)
Sex	
Male	22 (55)
Female	18 (45)
Astrocytoma grade	
GII	10 (25)
GIII	12 (30)
GIV	18 (45)
Resection extent	
Total	13 (32.5)
Subtotal	27 (67.5)
Chemotherapy	
No	25 (62.5)
Yes	15 (37.5)
Radiotherapy dose (Gy)	
45	3 (7.5)
50	4 (10)
54	3 (7.5)
60	30 (75)
Nestin (IRS)	
Median (range)	5 (0-9)
Low	10 (25)
High	30 (75)
Survivin index	
Median (range)	60 (5-95)
Low	11 (27.5)
High	29 (72.5)

Continuous variables were expressed as the mean±SD for normally distributed data and median (range) for non-normally distributed data; categorical variables were expressed as a number (percentage). SD: Standard deviation, IRS: Immunoreactivity score

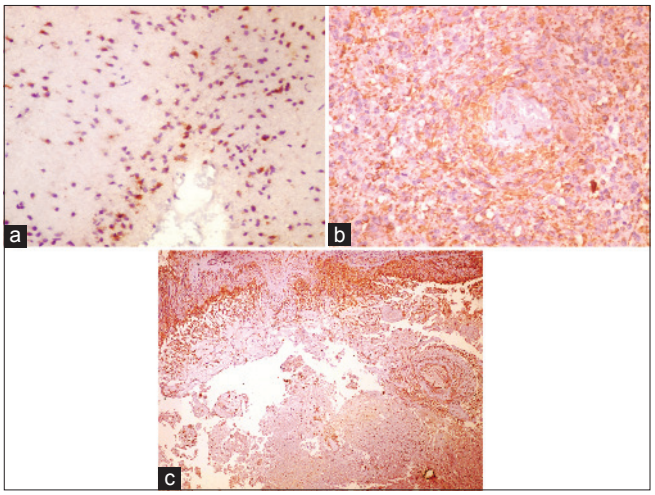

Figure 1: (a) Low-grade astrocytoma showing low cytoplasmic nestin expression (IHC, ×400); (b) anaplastic astrocytoma showing high cytoplasmic nestin expression (IHC, ×400); (c) glioblastoma multiforme showing high cytoplasmic nestin expression (IHC, ×100)

Table 2: Correlation between nestin (IRS)/survivin index and clinicopathological parameters in the studied cases of astrocytoma

Characteristics	*N* (%)	Nestin (IRS)		Survivin index	
		Median (range)	*P*§	Median (range)	*P*§
Age (years)					
≤50	23 (57.5)	3 (0-9)	0.010	50 (5-90)	0.033
>50	17 (42.5)	6 (3-9)		60 (50-95)	
Sex					
Male	22 (55)	6 (0-9)	0.758	60 (5-95)	0.662
Female	18 (45)	4 (1-9)		55 (20-90)	
Astrocytoma grade					
GII	10 (25)	2 (0-4)	<0.001	20 (5-50)	<0.001
GIII	12 (30)	4 (2-9)		60 (40-80)	
GIV	18 (45)	6 (4-9)		70 (50-95)	
Tumor size					
≤4 cm	12 (30)	2 (0-9)	<0.001	25 (5-90)	<0.001
>4 cm	28 (70)	6 (2-9)		65 (40-95)	
Nestin (IRS)					
Low	10 (25)			20 (5-50)	<0.001
High	30 (75)			70 (45-95)	
Survivin index					
Low	11 (27.5)	2 (0-4)	<0.001		
High	29 (72.5)	6 (3-9)			

Continuous variables were expressed as the median (range); §Mann–Whitney *U*-test for two groups, Kruskal–Walls *H*-test for more than two groups; $P<0.05$ is significant. IRS: Immunoreactivity score

A highly significant association of median value of nestin scores with grade, age, tumor size, and survivin index was found ($P < 0.001$). Non-significant association of nestin IRS with gender ($P = 0.758$) was observed [Table 2].

Survivin Expression and Association with Clinicopathological Parameters

All sections from studied astrocytoma showed positive cells, but the survivin index was variable among grades, whereas normal brain tissue was negative [Figure 2]. The median percentage of immunoreactive cells in each specimen was 20% (5-50%) in low-grade astrocytoma, 60% (40-80%) in anaplastic astrocytoma, and 80% (50-95%) in GBM. The antiserum detected both cytoplasmic and nuclear forms of survivin. A significant association of survivin index with the grade, age, tumor size, and nestin score was observed. However, non-significant association between survivin index and gender ($P = 0.662$) was observed [Table 2].

Prognostic Relevance

Follow-up varied from 4 to 48 months (median, 21.5 months). During this time, 62.5% (25/40) of the patients were died. The mortality was lower in low-grade tumors (2/10, 20%) than in high-grade tumors (III, IV) (23/30, 76.7%). Kaplan–Meier analysis showed that grade of astrocytoma was significantly associated with OS ($P < 0.001$) [Figure 3].

Twenty-five cases (83.3%) with high nestin-expression were died during follow-up period while none of low nestin-expression cases was died. Survival analysis using Kaplan–Meier analysis

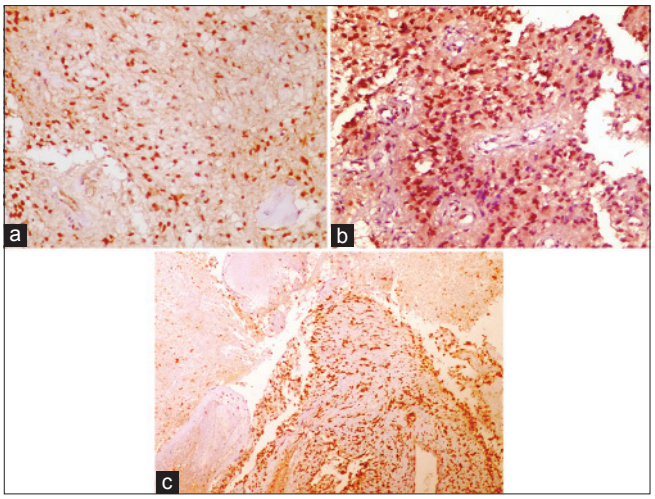

Figure 2: (a) Low-grade astrocytoma showing low cytoplasmic and nuclear survivin expression (IHC, ×400); (b) anaplastic astrocytoma showing high cytoplasmic and nuclear survivin expression (IHC, ×400); (c) glioblastoma multiforme showing high cytoplasmic and nuclear survivin expression (IHC, ×400)

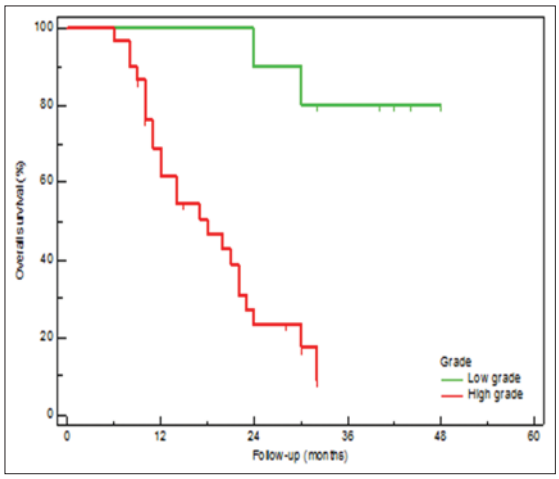

Figure 3: Kaplan–Meier overall survival curve in all the studied cases of astrocytoma ($N = 40$) stratified according to grade ($P < 0.001$)

demonstrated that nestin expression was associated with OS ($P < 0.001$) [Figure 4].

Twenty-four cases (82.8%) with high survivin index were died during follow-up period while only one case (9.1%) of low survivin index was died. Survival analysis using Kaplan–Meier analysis demonstrated that survivin expression was associated with OS ($P < 0.001$) [Figure 5]. Effect of grade/nestin (IRS)/survivin index on OS was presented in Table 3.

Poor predictors of survival on univariate analysis using log-rank testing were the age group >50 years, tumor size >4 cm, subtotal resection, high tumor grade, high survivin index and high nestin expression. However, multivariate analysis revealed the independent prognostic significance of nestin IRS in tumor cells (Hazard ratio = 77.367, 95% confidence interval [2.675-2237.8], $P = 0.011$) but other clinicopathological factors lost their significance [Table 4].

Table 3: Effect of grade/nestin (IRS)/survivin index on overall survival in the studied cases of astrocytoma

Characteristics	All (*N*=40)	Grade			Nestin (IRS)			Survivin index		
		Low (*N*=10)	High (*N*=30)	*P*	Low (*N*=10)	High (*N*=30)	*P*	Low (*N*=11)	High (*N*=29)	*P*
Number of deaths (%)	25 (62.5)	2 (20)	23 (76.7)	0.001*	0 (0)	25 (83.3)	<0.001*	1 (9.1)	24 (82.8)	<0.001*
Median follow-up (months)	21.5	42	14.5		42	14.5		42	14	
Median overall survival (months)	22	NR	18	<0.001§	NR	18	<0.001§	NR	17	<0.001§
1 year overall survival (%)	71.7	100	61.7		100	61.7		100	60.3	
2 years overall survival (%)	41	90	23.3		100	19.5		90.9	20.3	
3 years overall survival (%)	31.6	80	8.7		100	0		90.9	0	
4 years overall survival (%)	31.6	80	8.7		100	0		90.9	0	
HR	-	8.555			-			20.818		
95% CI of HR	-	3.87-18.89			-			9.486-45.687		

Qualitative data are presented as number (%); NR denote not reached yet. *Chi-square test, §Log rank test, CI: Confidence interval, HR: Hazard ratio, *P*<0.05 is significant

Table 4: Predictive value of clinicopathological parameters for mortality in the studied cases of astrocytoma

Parameters	Univariate analysis			Multivariate analysis		
	β	HR (95% CI)	*P*	β	HR (95% CI)	*P*
Age >50 years	1.279	3.593 (1.515-8.519)	0.003			
Sex (female)	−0.178	0.836 (0.376-1.858)	0.663			
High grade	2.328	10.265 (2.355-44.735)	0.002			
Tumor size >4 cm	2.090	8.087 (2.297-28.472)	0.001			
Subtotal resection	2.698	14.858 (3.340-66.090)	<0.001			
RT dose >50 Gy	3.783	43.942 (1.136-1700.4)	0.043			
Chemotherapy	3.693	40.167 (8.420-191.61)	<0.001			
High nestin	4.349	77.367 (2.675-2237.8)	0.011	4.349	77.367 (2.675-2237.8)	0.011
High survivin	3.358	28.568 (3.699-220.60)	0.001			

Null model-2 log likelihood=159.834; Full model-2 log likelihood=129.060; SE of nestin=1.717. Overall model Chi-square=30.774, d.f=1, *P*<0.001. β: Regression coefficient, 95% CI: 95% confidence interval, HR: Hazard ratio, *P*<0.05 is significant

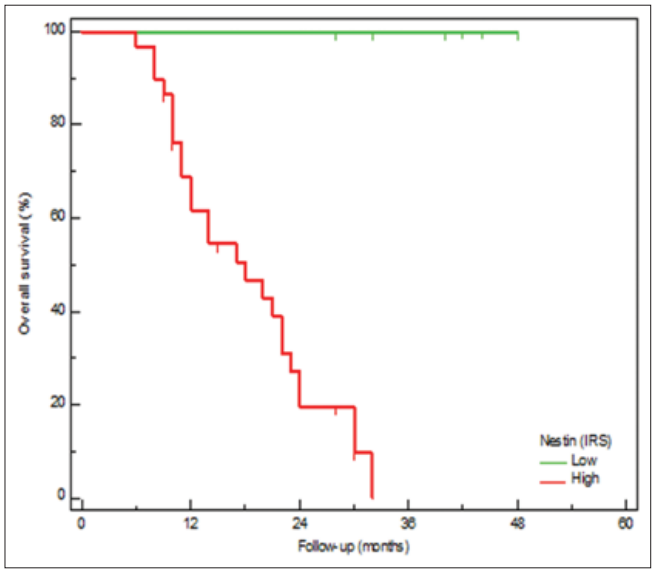

Figure 4: Kaplan–Meier overall survival curve in all the studied cases of astrocytoma (*N* = 40) stratified according to nestin expression (*P* < 0.001)

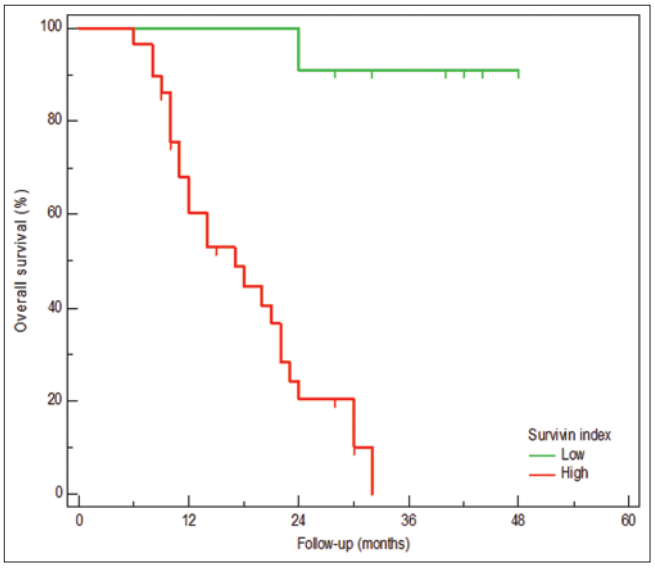

Figure 5: Kaplan–Meier overall survival curve in all the studied cases of astrocytoma (*N* = 40) stratified according to survivin index (*P* < 0.001)

DISCUSSION

It is important to characterize the tumor-driving cells and to understand how these cells influence the clinical behavior of a given tumor. Hence, we studied the expression of the NSC-associated marker "nestin" in a set of 40 primary astrocytic gliomas. In agreement with other studies [10,12,22], we showed that nestin was abundantly expressed in the most of tumor biopsies and IRS increased in a tumor grade-dependent manner. This result confirms that nestin may be associated with the tumor malignancy and the important role of stem cell in carcinogenesis of astrocytic gliomas.

Nestin immunoreactivity could also be located within tumor infiltration zones, and might characterize stem-like tumor cells with particular migratory activity [23]. These observations agree with our results as we observed condensation of nestin-positive cells at interface between tumor and normal brain tissue. This observation supported the fundamental role of nestin positive stem cell in invasion with consequent poor prognosis.

A highly significant association of the median value IRS with grade, age, tumor size, and survivin index was observed ($P < 0.001$). This confirmed that nestin expression is correlated with unfavorable clinical course in patients with astrocytoma.

It seems that the effect of nestin on clinical malignancy is mediated through a direct effect on proliferation. This suggests the possibility that nestin could potentially increase net proliferation by inhibiting apoptosis [24]. In this study, we observed that nestin expression in tumor cells was significantly associated with tumor proliferation marker "survivin." This result confirms that both indexes have a combined role in tumor progression through proliferation and apoptosis inhibition of malignant astrocytes. However, the abundant expression of nestin, which was present in low-grade tumors and its correlation with tumor proliferation, suggests that nestin-positive stem cells might be involved in initial stages of tumor development. Therefore, stem cells are not only involved in early stages of tumorigenesis but also share in tumor progression.

Strojnik and co-workers further identified nestin as a strong prognostic marker for decreased OS in a study comprising 87 CNS tumors [3]. This typically agreed with our results obtained by univariate survival analysis and confirmed in the multivariate analysis where we excluded known prognostic confounders such as age at time of diagnosis, extent of tumor resection, tumor size, and WHO grade.

In this study, OS was significantly different for cases with high and low nestin expression. This result is in accordance with previous studies [3,12,25]. However, there were other reports of nestin expression not correlated with prognosis of patients with glioblastoma [26,27]. Where Chinnaiyan *et al*. [26] showed that nestin is not a prognostic factor when investigating 143 GBMs; and Kim *et al*. [27] reported that only gross total resection and combined radiotherapy and chemotherapy are prognostic factors. This difference in the results might be due to the inclusion of different histological subtypes, different scorings systems, and antibody clones that have been used.

Survivin (member of the apoptosis-inhibitors family), is a bifunctional protein that acts as a suppressor of apoptosis, plays a central role as cytoprotective factor in cell division and it is expressed in many malignant tumors. Survivin overexpression has been reported to be a poorer prognostic factor in various malignancies [16]. In this study, we observed the accumulation of survivin both in the nucleus and the cytoplasm in all astrocytoma cases as described by others [27,28]. Nuclear and cytoplasmic survivin immunoexpression denoted cell proliferation and anti-apoptotic activity, respectively.

Survivin has been reported to be abundantly overexpressed in malignant gliomas [29]. Our study showed that survivin immune-expression was different significantly among various grades of astrocytoma ($P < 0.001$). These results agreed with those of Kogiku *et al*. [21], who reported that survivin expression was up-regulated in high-grade gliomas. The distribution patterns of survivin in gliomas cells were highly variable and might be due to the phenotype of gliomas.

A significant association of survivin index with the grade, age, tumor size, and nestin score was observed ($P < 0.001$). In addition, it was observed that the median survival of patients with a high survivin index was significantly shorter than that of patients with a low survivin index (2 years OS was 20.3% vs. 90.9%, $P < 0.001$). These results are similar to that of Kogiku *et al*. [21], who observed that survivin expression was strongly associated with the prognosis of glioma patients. They reported that the median survival of patients with a high survivin index was significantly shorter than that of patients with a low survivin index (322 vs. 1084 days, $P < 0.001$). However, the results of Habberstad *et al*. [13] observed no correlation between survivin expression and survival. This difference could be due to the difference in the assessment method of survivin expression where only nuclear survivin positivity was estimated in Habberstad *et al*. study.

CONCLUSION

In conclusion, nestin is a promising stem cell marker. Nestin and survivin are adverse prognostic markers for astrocytic glioma that increase significantly with tumor progression and associated with poor overall survival. Therefore, nestin and survivin could be used to predict high-risk group of astrocytoma with unfavorable outcome. The significant association between nestin and survivin denoted that the effect of nestin on tumor progression could be mediated through a direct effect on proliferation, and additional studies are required to examine the possibility that nestin inhibits apoptosis in astrocytic gliomas.

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