RESEARCH ARTICLE

Expression of Neuronal Markers, NFP and GFAP, in Malignant Astrocytoma

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Abstract

 Background: Immunohistochemical markers are considered as important factors in diagnosis of malignant astrocytomas. The aim of the current study was to investigate the frequency of the immunohistochemical markers neurofilament protein (NFP) and glial fibrillary acidic protein (GFAP) in malignant astrocytoma tumors in Firoozgar and Rasool-Akram hospitals from 2005 to 2010. Materials and Methods: In this cross-sectional study, immunohistochemical analysis of NFP and GFAP was performed on 79 tissue samples of patients with the diagnosis of anaplastic and glioblastoma multiform (GBM) astrocytomas. Results: The obtained results demonstrated that all patients were positive for GFAP and only 3.8% were positive for NFP. There was no significant association between these markers and clinical, demographic, and prognostic features of patients (p>0.05). Conclusions: NFP was expressed only in GBMs and not in anaplastic astrocytomas. It would be crucial to confirm the present findings in a larger number of tumors, especially in high grade gliomas.

Keywords: Malignant astrocytoma - GFAP - NFP

Introduction

 Gliomas are a broad range of brain tumors with the origin of sustentacular neuroglial cells and astrocytomas are the main group of them (Efird, 2011). The worldwide incidence of brain tumors are 7 per 100,000 population each year. According to world health organization (WHO) classification, four types are considered for astrocytomas. Grade I or Pilocytic astrocytoma is benign and curable, grade II or Low-grade (fibrillary) astrocytoma is a slow-growing astrosytoma with a survival time of about 4 years. Grade III or anaplastic shows increased proliferation and anaplasia with the survival of around 18 months. Most common symptoms include headaches, seizures, neurologic deficits and changes in mental status. Grade IV or glioblastoma multiform (GBM) is the most prevalent and malignant primary brain tumors with the symptoms such as headache, seizure, nausea and vomiting, etc. (Louis et al., 2007; Doroudchi et al., 2013; Zeybek et al., 2013). GBMs have been divided to 2 groups with regards to their clinical presentations, primary and secondary. Primary is more common in older patients and not derives from lower-grade astrocytomas but secondary is more prevalent in patients under age of 45, originates from low-grade and anaplastic astrocytomas and shows lower degree of necrosis (Furnari et al., 2007; Ohgaki and Kleihues, 2013).

 Immunohistochemical markers are considered as one of the prominent factors in diagnosis of malignant astrocytomas. Two of these markers which were accessed in some studies for astrocytomas diagnosis are including glial fibrillary acidic protein (GFAP) and neurofilament protein (NFP). NFP is a class four intermediate filament protein which consists of 3 heteropolymeric polypeptide units with the molecular weights of 200 kilodalton (kDa), 160 kDa and 68/70 kDa and which presents in almost all neurons. NFPs are unphosphorilated in perikaria and after phosphorilation they move along the axon. NFP can be detected in astrocytomas, meningiom, ganglioneuroma, ganglioneuroblastoma and etc. (Gotow, 2000; Morrison and Prayson, 2000). GFAB is an intermediate filament that is expressed by some central nervous system cells including glial cells. It is believed to stabilize the cytoskeleton of normal astrocytes. GFAP is frequently used as a trustworthy marker of glial astrocytes and tumors (Restrepo et al., 2011).

 An important factor in the incidence of malignant glioma tumors is neuronal differentiation. Therefore, the proof of this issue and the comparison of the tumors behavior in those with neuronal differentiation and those without can be helpful in patient management. In this study we used NFP marker to determine the neuronal differentiation. Previous studies showed controversial results. In a study performed by Varlet et

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al neuronal differentiation was evaluated in 40 patients with astrocytomas and found that all of the tumors were NFP and GFAP positive (Varlet et al., 2004). Pallud et al revealed that NFP could be helpful in determination of a special subgroup of GBMs and that NFP can be a reliable marker for prediction of survival (Pallud et al., 2012). In another study the class III b-tubulin isotype (bIII) which its expression in neuronal tumors is differentiation dependent was significantly higher in malignant astrocytomas (Katsetos et al., 2001).

It seems that the previous studies were not enough and were controversial. Therefore, this study was aimed to evaluate the frequency of immunohistochemical markers of NFP and GFAP in malignant astrocytoma tumors in Firoozgar and Rasool-Akram hospitals from 2005 to 2010 and whether it can be a good marker in diagnosis and prognosis of high-grade astrocyromas.

Materials and Methods

Tumor samples

Seventy nine patients who were diagnosed with the anaplastic and GBM astrocytomas at the Firoozgar and Hazrat-e-Rasool hospitals, Tehran, Iran between January 2006 and August 2011 were selected for the study. Patient's characteristics and symptoms were collected from their records.

Immunohistochemistery analysis

The immunohistochemical staining of the GFAB and NFP antigen was performed on sections prepared from formalin-fixed paraffin-embedded tissues (FFPE) as described previously (Madjd et al., 2011; Sotoudeh et al., 2012; Gheytanchi et al., 2014; Keymoosi et al., 2014). Polyclonal rabit anti-human glial fibrillary acidic protein (GFAP) (Code No.Z0334, Dako, Denmark); and monoclonal mouse anti-human neurofilament NE14 (anti-NF, clone NE14, BioGenex, The Netherlands), which reacts with the 200-kilodalton component of this protein (NFP), was used as a primary antibodies. Tissue slides were deparaffinized, rehydrated using decreasing graded of ethanol and the endogenous peroxidase were blocked by hydrogen peroxide. Then, the slides were transferred to microwave for 20 minutes at 98°C (manufacturer recommendations) in citrate buffer to retrieve the antigen. To exclude any microwave induced nonspecific immunostaining, we repeated NFP immunostaining without antigen retrieval in all cases. Tissues were incubated overnight at 4°C in primary antibody NFP and GFAB with the optimal dilutions of 1:100 and 1:500, respectively. Then, to visualize the antigen they were incubated with the secondary antibody including HRP labeled polymer anti rabit/mouse (Dako EnVisionTM+/HRP, Dual link Rabbit/Mouse) for 40 min at room temperature. After 3 washes in TBS Tween 0.05%, the sections were developed with diaminobenzidine (DAB) (DAKO, Denmark) for 4 min. Finally, the sections were counterstained with haematoxylin (DAKO, Denmark), dehydrated and mounted with Entellan (Merck KGaA, Darmstadt, Germany). Human astrocytoma specimen was used as a positive control and to provide the negative

control, a slide was incubated without primary antibody. The results of the immunohistochemistry were evaluated by two pathologists with a careful evaluation of all parts of the tumor tissue section to ensure the absence of a false-positive reaction of normal entrapped neurons. A minimal threshold at 1% of the total stained tumor cells considered as a cutoff for defining NFP-positive staining.

Statistical analysis

Data were analyzed using SPSS version 16 (Chicago-ILUSA). Frequency and frequency percentage were used for categorical variables and mean and standard deviation was used for quantitative variables. T-test, chi-square and Fisher exact tests were used to compare the results and find a correlation between them. Data were significant at $p<0.05$.

Results

Study population and clinicopathological features of patient

In this cross-sectional study, a series of 79 cases which diagnosed with anaplastic astrocytomas (5 cases) and glioblastoma multiform (GBM) (74) were studied. The patients were 52 males and 27 females and the median age of patients at the time of diagnosis was 57 ± 18.6 with ages ranging from 6 to 82 years old. Forty one (52%) patients were less than 57 years of age, whereas 38 (48%) patients were older than median age. The initial symptoms included headaches (14 cases), seizures (11 cases), focal neurological deficits (18 cases), increased intracranial pressure (ICP) (20 cases) and multiple symptoms (16 cases). Duration of symptoms before diagnosis varied from 1 to 12 months (median, 3 months). Of this series of patients, 72 (91%) showed necrosis and 63 (80%) of them showed vascular proliferation. Most patients, 78 (99%), experienced recurrence and 1 (1%) had no recurrence. During the follow-up of the patients, 77 cases died due to the cancer related causes and only 2 cases are still alive. The clinical and demographic parameters are summarized in Table 1.
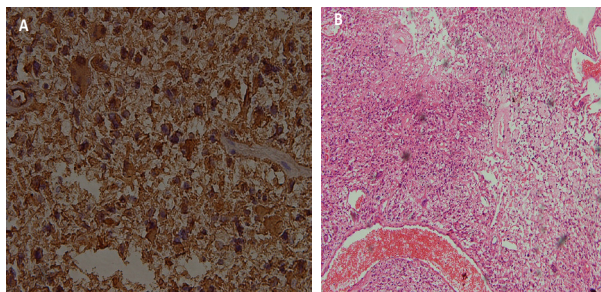
Immunohistochemistry

NFP Immunostaining: In the tumor tissues of 79 patients, only 3 (3.8%) cases showed positive NFP expression. It was found to be expressed strongly only in glioblastomas. The extent of expression varied from case to case and was cytologically indistinct from other tumor
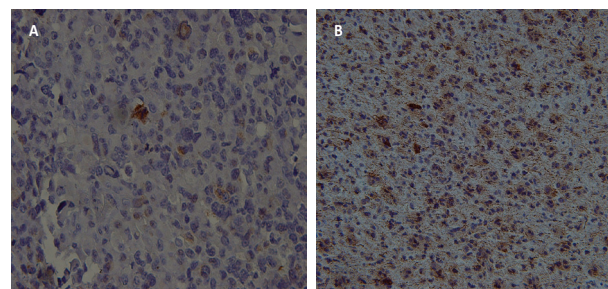
Figure 1. NFP Expression in Human Astrocytoma. (A) NFP negative; and **(B)** NFP positive IHC staining on sections from patient specimens in the high magnification (40×)

Table 1. Summary of Patient Characteristics of the Study and Clinico-Pathological Parameters

Patients and variables	All cases (n=79) Number (%)	NFP-Positive (n = 3)	NFP-Negative (n = 76)	p-value	GFAP-Positive (n = 79)	GFAP-Negative (n = 0)	p-value
Median age at diagnosis (yrs) ± SD	57±18.6						
Range	6-82						
≤57	41 (52)	2	39	0.602	41	0	0
>57	38 (48)	1	37		38	0	
Sex							
Male	52 (66)	2	50	0.975	52	0	0
Female	27 (34)	1	26		27	0	
Initial symptom(s)							
Headache	14 (18)	0	14	0.549	14	0	0
Seizures	11 (14)	1	10		11	0	
Focal deficit	18 (23)	1	17		18	0	
Increased intracranial pressure (ICP)	20 (25)	0	20		20	0	
Multiple symptoms	16 (20)	1	15		16	0	
Duration of symptoms before diagnosis (median)							
≤3 mo	40 (51)	2	38	0.468	40	0	0
>3mo	39 (49)	1	38		39	0	
Pathological diagnosis (WHO histological subtype)							
GBM	74 (94)	3	71	0.646	74	0	0
Anaplastic astrocytoma	5 (6)	0	5		5	0	
Necrosis							
Yes	72 (91)	3	69	0.582	72	0	0
No	7 (9)	0	7		7	0	
Vascular Proliferation							
Yes	63 (80)	3	60	0.374	63	0	0
No	16 (20)	0	16		16	0	
Recurrence							
Yes	78 (99)	3	75	0.842	78	0	0
No	1 (1)	0	1		1	0	

Figure 2. GFAP Expression in Human Astrocytoma. (A) GFAP positive IHC staining; and **(B)** H&E staining of GBM on sections from patient specimens in the high magnification (40×)

cells (Figure 1). But, low grade astrocytomas showed no NFP expression. In our series, tumors with NFP expression showed necrosis, vascular proliferation and recurrence. There was no relation between NFP expression and the other variables including age (p-value=0.602), gender (p-value=0.975), initial symptoms (p-value=0.549), duration of symptoms (p-value=0.468), pathological diagnosis (p-value=0.646), necrosis (0.582), vascular proliferation (p-value= 0.374) and tumor recurrence (p-value= 0.842) (Table 1).

GFAP Immunostaining

GFAP was expressed in all tumors (79 out of 79) which was extremely variable from one case to another, but was generally strong in both low grade astrocytomas and glioblastomas (Figure 2). Necrosis was observed in 72 (91%) cases and 80% (63/79) of patients showed

vascular proliferation. However, many of patients (99%) were experienced a relapse (Table 1). Since the GFAP was positive in all patients, it was impossible to evaluate the correlation between this marker and other clinico-pathological variables.

Discussion

Neuronal markers including NFP and GFAP express in various glial tumors and glioblastomas (GBMs) with neuronal differentiation patterns. Previous studies revealed that NFP is a therapeutically prognostic factor in primary supratentorial GBMs (Pallud et al., 2012). It can also help to predict the overall patient survival and progression-free survival (PFS) in GBMs (Pallud et al., 2012). Therefore, we decided to evaluate NFP and GFAP expression in collected cases. We found that GFAP marker was positive in GBMs and anaplastic astrocytomas, but NFP expression was only positive in 3.8% of GBMs. Interestingly, no significant association was found between NFP expression and the clinical, demographic and prognostic features of patients.

There are several case report studies on NFP expression in malignant supratentorial ganglioglioma, ganglioglioma and glioblastoma multiforme, but controversy exists regarding NFP expression and its relation with nonspecific immunostaining (Wacker et al., 1992; Jay et al., 1994; Sasaki et al., 1996; Dash et al., 1999; Hayashi et al., 2001).

It has been shown that low NFP expression may be falsely negative in formalin fixed and paraffin embedded

tissues (Gould et al., 1990).

Other studies found that the antigen retrieval process may induce nonspecific immunostaning (Varlet et al., 2004). In a study performed by Valret et al on malignant glioneuronal tumors (MGNTs), all tumors coexpressed GFAP and NFP, in which NFP immunostaining was performed without antigen retrieval. The staining result was poor (10/12) in comparison with tumors stained with antigen retrieval (Varlet et al., 2004). In our study, to decrease false negative results, once the sections were stained without antigen retrieval and again were stained with antigen retrieval. However, no difference observed in the expression level of NFP using two staining methods. Our finding regarding the expression of GFAP is consistent with the findings of their study. However, there is a significant difference regarding the NFP expression (3.8%) between 2 studies.

Powel et al showed that the expression of glial and neuronal polypeptides including GFAP, synaptophysin (SYN), and NFP in microwave-enhanced single- and double-immunolabelling experiments could be responsible for pleomorphic cell morphology in pleomorphic xanthoastrocytomas (PXAs) (Powell et al., 1996).

In other study NFP immunostaining of primitive neuroectodermal tumors showed different results depending on the NFP subunits (Kleinert, 1991; Kleinert, 1991). In this study, we applied only one subunit of the NFP (200-kilodalton); therefore it was impossible to examine the expression patterns of NFP in different subunits. It is recommended that future studies should be designed to evaluate the different subunits in astrocytomas.

In a study performed by Fiks et al on neuronal and glial markers (GFAP,SYN,NFP) using imunohistochemistry in pilocytic astrocytomas (58 cases) and gangliogliomas (11cases), all tumors showed immunopositive reaction for NFP and SYN (Fiks et al., 1999; Fiks et al., 2001).

Immunocytochemical in vitro studies on pediatric brain tumor cell lines showed that the cell lines were weakly positive for NFP but were strongly positive for GFAP (Rooprai et al., 1997).

Wharton et al showed that NFP was expressed in 50% of cases, but was generally weak and focal (Wharton et al., 2002).

Pallud et al found 66.1% (117 of 177) NFP positive GBMs (Pallud et al., 2012) which was related to disease prognosis in adults. Therefore, NFP could be considered as a strong and prognostic marker for diagnosis and treatment of primary supratentorial GBMs (Pallud et al., 2012).

In contrast to some previous studies, due to the small number of NFP positive cases, we could not found the prognostic role of this marker to apply for diagnosis of GBMs.

Other studies observed the neuronal differentiation in gelial tumors (particularly in Oligodendrogliomas) and demonstrated that many of the tumor cells represent GFAP (Wolf et al., 1997; Dehghani et al., 2000). In agreement with the previous studies, we found the GFAP expression and neuronal differentiation in all cases.

In other study, markers such as class III beta-tubulin, microtubule-associated protein 2 (MAP2), neuron-specific enolase (NSE) and NFP markers were evaluated using immunocytochemistry and western blotting, results showed that all markers except NFP were expressed both in GBM cell lines and biopsies (Yan et al., 2011).

Results of studies on low-grade and high-grade gliomas using tissue microarray and immunohistochemistry showed that the expression of NFP and GFAP did not relate to the grade of tumors (Rushing et al., 2010). We found that the expression of these markers in GBMs and anaplastic astrocytomas did not correlate with the grade of tumors. Our study is consistent with the Rushinhg study, but we studied on the small number of GBMs and particularly anaplastic astrocytomas. Thus, it is suggested that these findings should be confirmed in a large number of cases.

Findings of reviewed studies showed that there are some conflicting attitudes in this respect. This suggests that the expression pattern of NFP is different in various gliomas and it depend to the grade of tumors.

In conclusion, findings of the current study showed that NFP expressed only in GBMs, but anaplastic astrocytomas showed no NFP expression. On the other hand, due to the limited number of samples, further studies are needed to investigate whether NFP expression is related to the grade of tumors and whether it can be applied as a valuable marker to distinguish low grad tumors from high grades. Finally, it would be crucial to confirm these findings in a larger number of tumors especially in high grade gliomas.

Acknowledgements

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