

Prognostic analysis of astrocytic gliomas correlating histological parameters with the proliferating cell nuclear antigen labelling index (PCNA-LI)

F.F. Cruz-Sánchez^{1,2}, J.C. Ferreres³, J. Figols³, A. Palacin², A. Cardesa², M.L. Rossi¹ and J.F. Val-Bernal³

¹Neurological Tissue Bank and ²Department of Pathology, Hospital Clínico y Provincial, School of Medicine, University of Barcelona and ³Department of Pathology, Marqués de Valdecilla University Hospital, Faculty of Medicine, Santander, Spain

Summary. Eighty out of 250 cases of astrocytic glioma collected from a practice served by a single clinical team over a 15-year period were studied using a full complement of clinical, follow up, histopathological analysis and proliferating cell nuclear antigen (PCNA) immunostaining for the obtention of the PCNA-labelling index (LI). A statistical evaluation and discriminant analysis were carried out with the aim of clarifying the importance of various parameters as predictors of tumor behaviour. Data are correlated with survival (with a 10-year follow up).

A significant correlation with survival was found when histological grouping and the PCNA-LI were studied with the Cox test. Most significant features were histological as detected using classical techniques including histological grading. The utilization of objective values (mitosis, cellular density and necrosis) appears to be useful in grading astrocytic tumors. Our results emphasize the importance of cytological, histological and PCNA-LI parameters as predictors of tumor behaviour.

Key words: Astrocytomas, PCNA, Prognosis

Introduction

Histological diagnosis of a brain tumor helps not only in its classification but also in taking a clinical decision as to its treatment and in predicting its further biological behaviour (Burger and Vollmert, 1980; Nelson et al., 1983; Burger et al., 1985; Fuhling and García, 1985; Cruz-Sánchez et al., 1988a, 1992; Alvord, 1992). However, in many cases evolution and histological diagnosis are at variance. In a «special» sense, some astrocytic gliomas are difficult to classify and to

prognosticate (Nelson et al., 1983; Burger et al., 1985).

For a long time, pathologists, clinicians and scientists have tried to find signposts to predict the prognosis of a brain tumor (Burger and Vollmer, 1980; Cruz-Sánchez et al., 1988a,b, 1991, 1993). Tumor proliferating activity has been assessed by immunohistochemical methods using antibodies for bromodeoxyuridine (BrdU) (Nagashima et al., 1985) or polymerase alpha (Kunishio et al., 1990). However, they are not routinely used due to technical difficulties (Sasaki et al., 1992). The proliferating cell nuclear antigen (PCNA) has been shown to be a cell-cycle-related nuclear protein which has been used for the determination of tumor proliferating activity (Sasaki et al., 1992; Zhao et al., 1994).

In the study of tumor behaviour, long-term follow up is necessary to obtain credible and well-correlated results (Kernohan et al., 1949; Nelson et al., 1983; Fulling and García, 1985; Kleihues et al., 1993). On the other hand, retrospective studies require a large number of cases, because the number of participants will almost inevitably be reduced due to insufficient or unavailable material (Winger et al., 1989). Follow up is also difficult, as most patients die at home and little or no information is available.

We have had the opportunity to study a large series of astrocytic gliomas using different pathological approaches and have followed up the same cases for 10 years. Out of a large group, we selected cases in which a whole battery of techniques could be applied and for which a full clinical history was available. A group of cerebellar pilocytic astrocytomas was also included in spite of their different and well-recognized biological behaviour (Kleihues et al., 1993). All cases were treated by the same surgical team with a standardized approach. We were therefore in an optimal position to perform a pathological study encompassing a statistical evaluation with a view to clarifying the importance of various cytological and histological parameters and the tumor proliferating activity as predictors of tumor behaviour.

Offprint requests to: Dr. F.F. Cruz-Sánchez, M.D., Ph.D., Banco de Tejidos Neurológicos, Hospital Clínico, Villarroel 170, 08036 Barcelona, Spain

Materials and methods

Clinical material

Two hundred and fifty astrocytic glioma cases were collected in a circumscribed region of Northern Spain served by a single clinical team over a 15-year period.

All cases underwent surgery which consisted in either a lobectomy, «total» or subtotal removal.

For 80 of these cases, the full complement of clinical, follow-up, histopathological study and PCNA immunostaining were available. Patients with low and high grade astrocytomas and glioblastomas were uniformly treated with radiotherapy after surgery (45 Gy whole cranial irradiation and 15 Gy local irradiation).

Tumor grouping

The pathological diagnosis was made blindly by three independent neuropathologists (FFC-S, JF, MLR). There were 42 astrocytomas, 7 of which were pilocytic, 12 low grade and 23 anaplastic, and 38 glioblastomas.

Mean ages and location of the tumors according to histological subgrouping are represented in Table 1.

Histology and quantification of histological parameters

Cellular density

This was assessed quantitatively and semi-quantitatively. The former by counting the number of cells in 1 mm² in the most representative area of the tumor with a x1000 final magnification under oil immersion; the latter by grading the cellular density as + (low), ++ (moderate) and +++ (high).

Mitosis

They were quantified after counting the number of mitoses/mm² and according to the following formula:

$$\frac{\text{No. mitosis} / \text{mm}^2}{\text{No. of cells} / \text{mm}^2} \times 1000 = \text{No. mitosis} / 10^3 \text{ cells}$$

Mitosis were differentiated from pyknotic nuclei on the basis of the criteria of Baak and Oort (1983).

Vascular abnormalities

We sought the presence of vascular abnormalities (increased number of vessels and/or endothelial changes) which included endothelial proliferation and we identified it simply as yes or no.

Necrosis

The presence of necrosis whether or not in

pseudopalisade was assessed as present or absent. Necrosis was also observed in some cases of anaplastic astrocytoma (19 cases) consisting of 1) scattered necrotic cells, 2) several necrotic cells or microcystic foci, and/or 3) small areas of necrosis without pseudopalisading or large areas of microcystic degeneration with loosening of cell texture. When large necrotic foci and/or necrosis with pseudopalisading were observed, tumors were classified as glioblastoma. Some glioblastomas did not show pseudopalisading but they displayed a high mitotic index and large necrotic foci.

Nuclear pleomorphism

This feature was graded as: + (minimal variation in shape or size of nuclei); ++ (moderate variation in shape or size of nuclei); +++ (severe variation in shape or size of nuclei or presence of bizarre cells).

Mononuclear cell infiltrate

The parenchymal and perivascular infiltrate was assessed on 3 μm-thick paraffin sections stained with hematoxylin and eosin (H&E).

Parenchymal and perivascular mononuclear cell infiltrates were graded as present or absent.

For the perivascular infiltrate the maximum number of layers of lymphocytes around the vessels, the percentage of vessels with the perivascular infiltrate and number of layers and the percentage of vessels with «layering» were also assessed.

PCNA immunohistochemistry

Tissue samples were handled in a single laboratory in a standardized fashion. The avidin biotin complex technique was applied to paraffin-embedded 5 μm-thick sections of tumors for the PCNA clone PC10 from Dako, diluted 1:200. The counting of positive cells was done by counting 300 cells from the more representative area of the tumor using the immersion objective (x1000 total magnification). We considered as positive any degree of positivity.

Results were expressed as a percentage of positive nuclei (PCNA-labeling index (PCNA-LI)).

Statistical analysis

Statistical analysis was performed using the SPSS, (SPSS Inc.) and PRESTA programs (Fondo de Investigaciones Sanitarias de la Seguridad Social, Spanish health Ministry).

General principles for qualitative variables (chi-square, Yates correction, Fisher test), quantitative (t student and Pearson lineal correlation), variance analysis, Kaplan-Meier test (Kaplan and Meier, 1958), multivariate analysis (Cox test) (Cox, 1972) and discriminant analysis were also carried out.

PCNA-LI vs. survival in astrocytomas

Results

Clinical material

A strong correlation was established between age of patients and histological grading ($p < 0.0001$) (Table 1). Young patients survived longer than older ones. No significant correlation was obtained for other clinical data such as sex and location.

Histology

Table 2 summarizes quantitative and semi-quantitative results of some anaplastic features in 80 cases of astrocytic gliomas. Malignancy correlated positively with high cellular density, high mitotic rate and with the semiquantitative observations on cellular density, vascular abnormality, necrosis and pseudopalisading. The latter was the most important feature separating glioblastomas from anaplastic astrocytomas. Poor prognosis was observed in anaplastic astrocytomas with high mitotic index. Therefore, necrosis,

pseudopalisading and mitotic index are important features in grading astrocytomas. Poor prognosis was significantly correlated with high cellular density (Fig. 1) and mitotic index (Fig. 2), but not with mononuclear cell infiltrate in the perivascular space and in the actual tumor.

PCNA

The PCNA-LI correlated significantly ($p < 0.05$) with survival. Anaplastic astrocytomas and glioblastomas showed a high percentage of PCNA-positive nuclei (Table 3). These results were statistically significant according to the variance analysis ($p < 0.01$).

Discriminant and multivariate analysis

The discriminant analysis for the group of anaplastic astrocytomas which showed a short survival compared with the remaining tumors demonstrated that in this group, the discriminant percentage correlated in 84% for mitosis, 69% for age and 30% for number of PCNA-

Table 1. Clinical features of 80 cases of astrocytic glioma

HISTOLOGICAL TYPE (n)	MEAN AGE±SD	SEX		LOCATION												SURVIVAL (years)							MEAN±SD (months)*				
		m	f	F	T	P	O	FP	PT	PO	TO	FT	POT	ML	CRB	<1	1-2	2-3	3-4	4-5	5-6	6-7		>7			
Pilocytic (7)	13±9	5	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	-
Low grade (12)	30±13	6	6	R3	R3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	60.8±49.4
Anaplastic (23)	51±15	14	9	R6	R5	R1	0	R2	R1	R1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15.6±14.5
Glioblastoma (38)	54±13	19	19	R8	R6	R5	R0	R1	R1	R2	R1	R1	R0	1	0	0	0	0	0	0	0	0	0	0	0	0	8.2±3.9
TOTAL (80)		44	36	R17	R14	R6	R0	R3	R3	R3	R1	R1	R0	2	6	47	11	3	5	3	1	0	10			50.5±14.4	

n: number of cases; m: male; f: female; SD: standard deviation; F: frontal; T: temporal; P: parietal; O: occipital; FP: fronto-parietal; PT: parieto-temporal; PO: parieto-temporal; TO: temporo-occipital; FT: fronto-temporal; POT: parieto-occipito-temporal; ML: middle line; CRB: cerebellum; R: right; L: left; *: only patients who died.

Table 2. Quantitative and semiquantitative results of some anaplastic features in 80 cases of astrocytic glioma.

HISTOLOGICAL TYPE (n)	QUANTITATIVE FEATURES (mean±SD)			CELLULAR DENSITY	NUCLEAR PLEOMORPHISM	VASCULAR ABNORMALITY	NECROSIS *	PSEUDO-PALISADING **	HISTOLOGICAL SCORING (mean±SD)				
	Cellular density (cell/mm ²)	Mitosis (nr/mm ²)	Mitosis (nr/10 ³ cells)	+ ++ +++	+ ++ +++								
Pilocytic (7)	2689±745	0	0	2	5	0	1	0	4±1				
Low grade (12)	2363±726	0.44±0.95	0.17±0.34	5	7	0	0	0	3.67±0.89				
Anaplastic (23)	4864±1428	8.18±5.5	1.82±1.28	0	4	19	19	0	11.32±1.76				
Glioblastoma (38)	5540±2160	13.69±9.01	2.56±1.49	0	4	34	10	28	15.28±2.37				
TOTAL	$p < 0.01$	$p < 0.01$	$p < 0.01$	7	20	53	20	47	13	60	30	28	$p < 0.01$

Cellular density: +, low; ++, moderate; +++, high. Nuclear pleomorphism: +, minimal variation in shape or size of nuclei; ++, moderate variation in shape or size of nuclei; +++, severe variation in shape or size of nuclei or the presence of bizarre cells. Vascular abnormality: see text. *Necrosis: cases with necrosis without pseudopalisading. **Pseudopalisading: cases with necrosis with pseudopalisading. nr: number; n: number of cases; SD: standard deviation.

PCNA-LI vs. survival in astrocytomas

positive nuclei. The other anaplastic astrocytomas with a long survival period had a percentage of 57, 66 and 71 respectively.

Individual statistical analysis demonstrated that there was significant statistical correlation between survival and histological grouping ($p < 0.0005$), mitosis ($p < 0.0005$), necrosis ($p < 0.0005$), cellularity ($p < 0.0001$) and the PCNA-LI ($p < 0.0005$). PCNA-LI values correlated significantly with survival when analysed independently with necrosis ($p < 0.01$), histological grouping ($p < 0.001$), high cellularity ($p < 0.01$) and high mitotic index ($p < 0.01$). However, only histological grouping ($p < 0.05$) and the number of PCNA-positive nuclei obtained by immunohistochemistry showed significant p values ($p < 0.001$) vs. survival according to the multivariate analysis.

Discussion

Several aspects related to the cytogenesis and molecular biology of astrocytomas have recently been assimilated by the new WHO classification (Kleihues et al., 1993). Biological features which could predict the prognosis of these tumors are continuously studied.

Clinical features, such as patient's age and the type of treatment used, are important factors in forecasting prognosis (Alvord, 1992). In our series, tumor aggressiveness correlated significantly with age of

patients. The relationship between old age and poor prognosis could be due to diminishing resistance of the host (Burger et al., 1985). Thus, variation in histological features such as cellular density or the mitotic rate may be related to the increasing age and to phenomena occurring in aging which may add to or affect the growth potential of the tumor.

Astrocytic gliomas have been classified into 3 or 4 grades of malignancy according to histological features (Kernohan et al., 1949; Ringertz, 1950; Russell and Rubinstein, 1971; Burger and Vogel, 1982; Rubinstein, 1972; Nelson et al., 1983). Difficulties in grading arise because of the ill-defined histological criteria and/or subjectivity. An appropriate weight value for an individual histological factor may be useful to predict aggressiveness. Several authors have studied the prognostic value of some histological criteria (Burger et al., 1985; Cohadon et al., 1985; Fulling and Garcia, 1985; Nelson et al., 1983; Schiffer et al., 1988). Our study demonstrates that cellular density and mitotic rate are the most significant histological features apt to separate low and high grade astrocytomas. Endothelial proliferations is a feature present in other gliomas (Burger and Vogel, 1982; Cruz-Sánchez et al., 1988a,b) which may indicate a high tumor growth potential (Cruz-Sánchez et al., 1991) but in some cases it does not indicate a worse prognosis.

Necrosis with pseudopalisading is a significant feature which better defines glioblastomas and allows prognosis (Nelson et al., 1983). Alvord (1992) concluded that necrosis is helpful in the grading of gliomas and that its presence in astrocytomas defines a subset with worse prognosis. On the basis of our results we conclude that necrosis is an important factor in the grading of astrocytomas and that its morphological characteristics (pseudopalisading) are also important to further define a histological type of tumor and that it is of prognostic value. Identification of histological «subtypes» of necrosis has been proposed in order to grade malignant astrocytomas (Rubinstein, 1972; Zülch, 1979). However, this approach did not take into

Table 3. Summary of PCNA-LI results obtained by immunohistochemistry in 80 astrocytic gliomas.

HISTOLOGICAL TYPE	No. OF CASES	PCNA-LI %of positive nuclei (mean±SD)
Pilocytic	7	8.65±9.72
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Glioblastoma	38	21.41±9.97
		$p < 0.01$

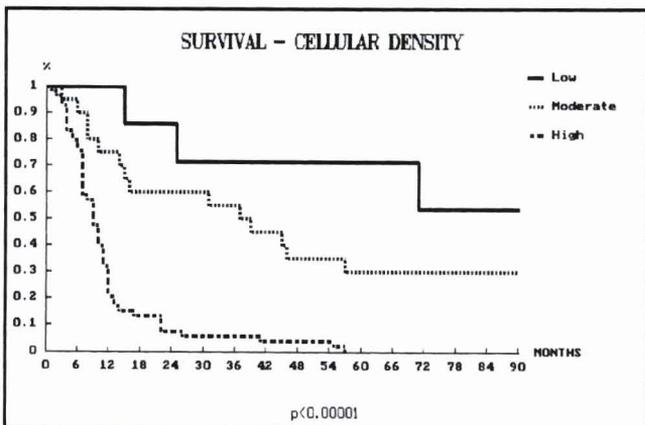


Fig. 1. Survival curves based upon the semiquantitative estimation of the cellular density.

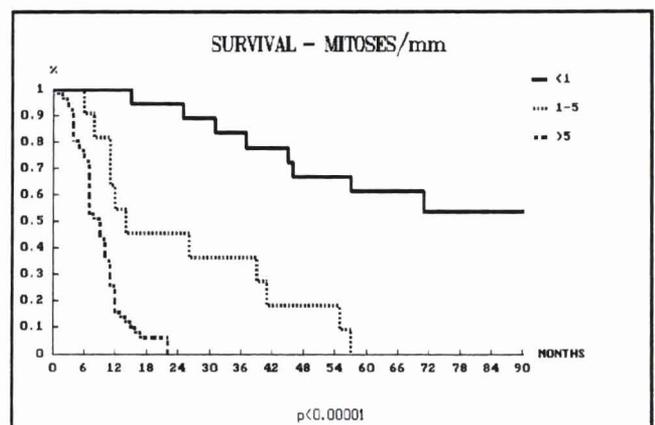


Fig. 2. Survival curves based upon the mitotic index.

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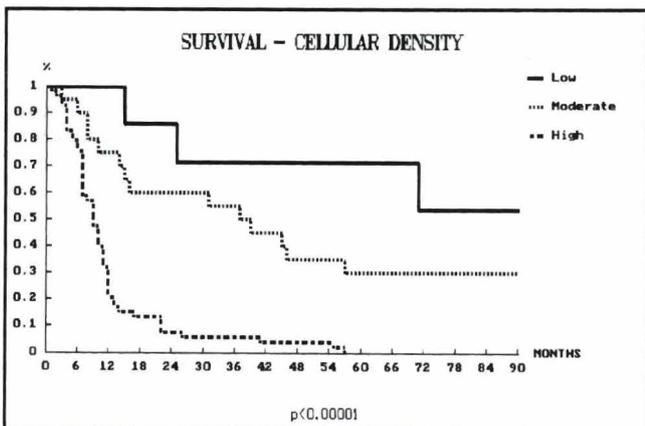


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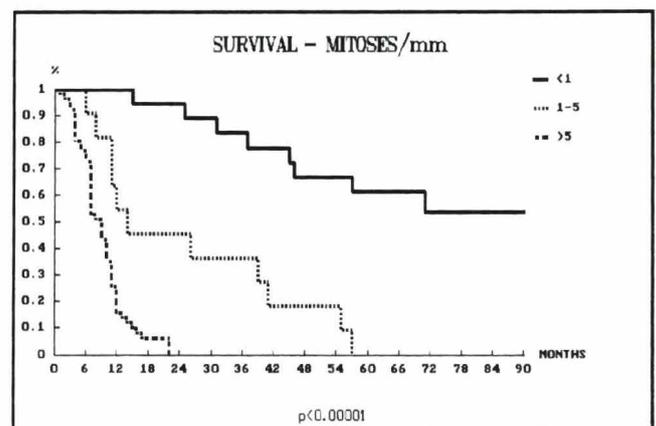


Fig. 2. Survival curves based upon the mitotic index.

consideration the current classification of brain tumors (Kleihues et al., 1993). Ependymomas and oligodendrogliomas also show necrosis, but pseudopalisading is rare in these tumors. In spite of necrosis and/or pseudopalisading, ependymomas and oligodendrogliomas have a better prognosis than glioblastomas (Alvord, 1992; Cruz-Sánchez et al., 1988a,b, 1991). Thus, this may indicate that a different growth potential exists in gliomas, and that pseudopalisading could be a distinctive feature useful in the establishment of prognosis in an individual case.

Other histological features, such as nuclear pleomorphism (Gullota, 1981; Schiffer et al., 1983) and the presence of mononuclear cells do not appear to influence survival in astrocytic gliomas (Burger et al., 1985; Rossi et al., 1989; Schiffer et al., 1988) and should be interpreted cautiously (Gullota, 1981).

Other techniques can be carried out to try to find correlation with survival: our group has recently performed a parallel study with the same cases mentioned in the present paper. We have compared the number of argyrophilic nucleolar organizer regions (AgNOR's) with survival, findings a significant linear correlation ($p < 0.05$) between both parameters (Ferreris et al., 1996).

Histological scoring based upon the recognition of the presence or absence of particular morphological criteria has been proposed (Daumas-Duport et al., 1988). According to our results, quantification of histological features may give statistical significance to certain parameters, such as cellular density, mitosis and necrosis and excludes less objective ones such as endothelial proliferation or pleomorphism which could be present in distinct tumor types. Based on the estimation of tumor growth potential, technical approaches have been developed in an attempt to define prognosis. The tumor proliferating activity has been assessed by immunohistological techniques using antibodies for BrdU (Nagashima et al., 1985; Kunishio et al., 1990; Labrouse et al., 1992; Sasaki et al., 1992) and polymerase alfa (Kunishio, 1990). A key event in cycling cells is DNA synthesis (S-phase) (Woosley, 1991). Several methods have been developed to study the S-phase including immunohistochemistry. Two cell cycle-associated nuclear proteins have been most widely exploited for immunocytological techniques (Garcia et al., 1989; VanderBerg, 1992). Ki67 and PCNA. The latter is a nuclear protein synthesized at a faster rate during early S-phase. Epitopes recognized by diverse antibodies are affected differently by tissue processing techniques; hence conflicting results have been obtained (Galand and Degref, 1989; Allegranza et al., 1991; Louis et al., 1991; Karamitopoulou et al., 1993). PCNA-LI has also been used in evaluating the proliferative activity of pre-malignant and malignant lesions of the liver with good results (Zhao et al., 1994).

Our results in this series of astrocytic gliomas demonstrate that the PCNA-LI could be suitable for recognizing actively proliferating tumor cells. These

findings were significantly correlated with survival and with other factors when they were analysed with the Cox test. Controversial results have been found in different tumor types including astrocytomas; most of these results may be attributed to different technical conditions. The role of tissue fixation has also to be considered (Hall et al., 1990; Golik and Rice, 1992; Karamitopoulou et al., 1993). For Karamitopoulou and co-workers (1993), it appears that low PCNA-LI in high-grade tumors should not be considered as indicating a better prognosis because of possible technical variations in its evaluation.

Theunissen and Blaauw (1993) found a significant correlation between malignancy and high PCNA-LI. However, when results were correlated with other clinical or histological factors, they were not statistically significant. In our studies, PCNA-LI values correlated significantly with survival when analysed independently with necrosis, histological grouping, cellular density and mitotic index according to the statistical analysis. However, when two factors were considered individually with the Cox test, only the histological grouping and the number of PCNA-positive nuclei correlated significantly with survival. Correlation between high mitotic rate and PCNA-LI indexes vs. poor prognosis suggests a high proliferating activity of these tumors which is correlated statistically with histological grading. According to these features, the PCNA-LI may be useful in the cases of small diagnostic samples such as stereostatic biopsies.

Different results were obtained in the evaluation of anaplastic astrocytomas with a survival similar to glioblastomas using the discriminant analysis. It has been demonstrated that in only 30% of cases, value obtained corresponded to the group of anaplastic astrocytomas and the remaining (70%) showed values similar to the glioblastoma group. On the other hand, anaplastic astrocytomas with a long survival showed that in 71% of cases PCNA-LI values correlated with histological diagnosis, but that in 29% values corresponded to low grades astrocytomas. Positive correlations between PCNA-LI values with other factors studied and survival were not found in either group.

Although the PCNA-LI and histological grading are based on different principles, these two methods appear to be complementary. Despite the statistically significant correlation of grade with survival and PCNA-LI, it is probable that some tumors may have an intermediate malignancy between two grades (Labrouse et al., 1992). This may be the case of tumors with histological aggressiveness but long survival.

In summary, we have studied retrospectively a large number of astrocytic gliomas using classical histological parameters and PCNA immunostaining with the aim to recognize predictive prognostic values. We have demonstrated that histological characterization of tumors using classical techniques, including the histological grading and PCNA-LI, are important factors in prognostic significant value. The development of techniques such as PCNA immunohistochemistry

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applied to current knowledge promises a productive future in the diagnosis and management of patients suffering from brain tumors. Further studies are necessary for the prospective valuation and the analysis of astrocytic tumor behaviour by PCNA-LI.

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