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Proliferating cell nuclear antigen expression in central nervous system neoplasms

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Summary. Proliferating cell nuclear antigen (PCNA) is a cell-cycle-regulated protein, which can be demonstrated in routinely fixed specimens. Studies on various tissues, cell cultures and neoplasms have shown that PCNA labelling index (LI) correlates with flow cytometry, tritiated thymidine LI, bromodeoxyuridine (BrdU) incorporation and Ki67 LI. PCNA LI may have prognostic value in various neoplasms. The present study concerns PCNA immunostaining in a series of neuroglial tumours. We demonstrate that there is a relation between PCNA LI and histological grade, and between PCNA LI and reported thymidine LI, BrdU LI and Ki67 LI. Pleomorphic xanthoastrocytomas and low-grade astrocytomas had the lowest LI, whereas metastases of small cell lung cancer and medulloblastomas had the highest LI. Glioblastomas sometimes showed a certain degree of intratumoral heterogeneity of distribution of immunostained cells. Intratumoral heterogeneity underscores the critical importance of representative sampling of central nervous system neoplasms for kinetic studies. As expected, PCNA LI are somewhat higher than tritiated thymidine LI, BrdU LI and Ki67 LI because PCNA is a marker of G1, S, G2 and M-phases of the cell cycle and not of S-phase only. In addition, because of its long half-life, PCNA may be detected immunohistochemically in cells that have recently left the cell cycle. The immunohistochemical evaluation of PCNA LI is easy to perform on routinely processed material, allowing retrospective studies. PCNA LI may be a useful tool in grading gliomas. However, its prognostic value must be validated by comparing PCNA LI with the follow-up of the neoplasms, and possibly with the responsiveness to anti-proliferative therapy.

Key words: Proliferating cell nuclear antigen – Glial neoplasms – Astrocytoma – Glioblastoma

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Introduction

The proliferative activity of neoplasms is an exciting field for histopathological investigation because of the suggested correlation between proliferation rate and tumour aggressiveness (Tubiana and Courdi 1989). Monoclonal antibodies (mAbs) directed against antigens related to proliferative activity of cells are being applied to the study of most kinds of human tumours. These mAbs include the Ki67 mAb (Brown and Gatter 1990) and the mAbs against proliferating cell nuclear antigen (PCNA) (Ogata et al. 1987; Waseem and Lane 1990).

PCNA is an auxiliary protein of DNA polymerase delta (Tan et al. 1986), which appears to be a cell-cycle-regulated protein, co-ordinating leading and lagging strand synthesis and rendering the polymerase more processive (Prelich and Stillman 1988). PCNA is expressed in late G1, S, G2 and M phases of the cell cycle. Studies on various tissues, cell cultures and neoplasms have shown that PCNA labelling index (LI) correlates with data obtained with flow cytometry (Garcia et al. 1989; Dawson et al. 1990; Woods et al. 1990), tritiated thymidine LI (Battersby and Anderson 1990; Hall et al. 1990), bromodeoxyuridine (BrdU) incorporation (Hall et al. 1990; Coltrera and Gown 1991) and Ki67 LI (Levison 1990).

Proliferative activity of central nervous system (CNS) neoplasms has been evaluated with tritiated thymidine incorporation (Hoshino and Wilson 1979; Hoshino et al. 1985), BrdU incorporation (Hoshino et al. 1986, 1989; Nakashima et al. 1985; Nishizaki et al. 1989) and with Ki67 mAb (Burger et al. 1986; Giangaspero et al. 1987; Ostertag et al. 1987; Landolt et al. 1987; Roggendorf et al. 1987; Shibata and Burger 1987; Boecker and Stark 1988; Plate et al. 1989, 1990; Hara et al. 1990; Zuber et al. 1988; Patsouris et al. 1988).

The present study concerns PCNA immunostaining in a series of CNS neoplasms. The study aims at finding

Table 1. Proliferating cell nuclear antigen labelling index (PCNA LI) in central nervous system neoplasms

Histology	PCNA LI	Mean ± SD	Median
PXA			
	0.1		
	0.2		
	0.6		
	0.7		
	1.3		
	1.5		
		0.73 ± 0.57	0.65
Recurrence of PXA with features of GBL			
	26.7		
Astrocytomas (low grade)[a, b]			
	0.1*		
	0.2		
	0.4		
	0.4		
	0.6		
	0.8*		
	0.8		
	1.2*		
	1.2		
	1.3*		
	1.3		
	1.4*		
	1.4		
	1.5*		
	1.9		
	2.6*		
	3.6		
	4.0*		
	4.3		
	4.9		
	6.0		
	7.5		
	13.5		
		2.64 ± 3.08	1.35
Oligodendrogliomas[b]			
	0.2		
	0.8		
	5.2		
	7.5		
	13.5		
		5.44 ± 4.43	5.2
Mixed glioma			
	1.8		
	4.0		
		2.9 ± 1.56	
Anaplastic astrocytoma[b]			
	2.1		
	9.6		
	13.9		
	17.7		
	18.3		
	28		
		14.93 ± 8.76	15.8
Anaplastic mixed glioma			
	10.5		
	29		
		19.75 ± 13.08	
Glioblastoma[b]			
	17.5		
	20		
	21		
	24.5		
	25.3		
	26		
	26.7		
	29.8		
	31.2		
	32		
	34		
	37.8		
	39		
	43.9		
	50		
		30.58 ± 9.13	29.8
Gliosarcoma			
	80		
Ependymoma			
	0.2		
	24.5		
		12.35 ± 17.18	
Ganglioglioma			
	2.5		
	2		
		2.25 ± 0.35	
Medulloblastoma			
	77		
	82		
	85		
		81.3 ± 4.04	
Meningioma			
	0.1		
	1.5		
	1.6		
	1.7		
	4.5		
	5		
		2.4 ± 1.92	1.65
Metastasis (carcinoma)			
Small cell lung carcinoma	72.3		
SCLC	82		
SCLC	89		
		81.1 ± 8.38	

GBL, Glioblastoma; PXA, pleomorphic xanthoastrocytoma; SCLC, small cell lung carcinoma

[a] The group of low-grade astrocytomas includes neoplasms of WHO grade I and II; grade I astrocytomas are marked with *. Mean and median vàlues for grade I astrocytomas are: 1.61 and 1.35; and for grade II are: 3.2 and 1.35. The differences between mean PCNA LI values in grade I and grade II tumours did not reach significance level

[b] The differences between mean PCNA LI in low-grade astrocytomas, anaplastic astrocytomas and glioblastomas were highly significant ($P < 0.05$). The differences between PCNA LI of low-grade astrocytomas and oligodendrogliomas were significant ($P < 0.05$)

out if there is a relation between PCNA LI and histological grade, and between PCNA LI and reported thymidine LI, BrdU LI and Ki67 LI. We also investigated whether PCNA was useful in differential diagnosis between pleomorphic xanthoastrocytomas (PXA) and other more aggressive, but sometimes histologically similar, neoplasms. Moreover, as one of our cases of PXA showed two recurrences, the last with features of glio-

blastoma, we investigated whether PCNA LI was different in the primary tumour when compared with other non-recurrent PXA, and whether there were differences between the primary tumour and its recurrences.

Materials and methods

We investigated 23 low-grade astrocytomas, 5 oligodendrogliomas, 2 mixed gliomas, 6 anaplastic astrocytomas, 2 anaplastic mixed gliomas, 15 Glioblastomas (GBLs), 1 gliosarcoma, 5 PXA, 2 ependymomas, 2 gangliogliomas, 3 medulloblastomas, 6 meningiomas and 3 CNS metastases of small cell lung carcinoma. One case of PXA showed three recurrences, the last with features of a giant cell GBL. The clinico-pathological features of the cases of PXA have been described elsewhere (Allegranza et al. 1991). Neoplasms were classified according to Burger et al. (1985, 1991). All specimens were obtained at open surgery and were routinely fixed and paraffin-embedded. No biopsy specimen was included in the present series. One to three different paraffin blocks were investigated for each case. Normal brain samples obtained at autopsy, performed within 4 h of death, were concurrently immunostained.

Sections were submitted to immunohistochemistry, using the peroxidase-labelled streptavidin technique. PCNA mAb (PC10 – murine IgG2, Novocastra, Newcastle upon Tyne, UK) (Waseem and Lane 1990) was commercially obtained. Tritration experiments were performed on routinely fixed specimens of our routine files, including normal tonsils, lymph nodes, large bowel and gastric mucosa, skin and various neoplasms (breast carcinomas, lymphomas). The optimal dilution was 1:700, with overnight incubation at room temperature. Biotinylated secondary antibody (Vector, Burlingame, Calif., 1:200) and peroxidase-labelled streptavidin (Vector, 1:100) were added in sequence. Diaminobenzidine was used as chromogen, and nuclei were lightly counterstained with haematoxylin.

PCNA immunostaining was scored counting at least 500 cells in more than 10 high power representative fields. All stained cells were recorded as positive independently of staining intensity. In cases where intratumoral heterogeneity of staining was seen, the fields examined included those with the highest and those with the lowest percentage of stained cells. Tumours were independently and blindly scored by two observers. The percentage of positively stained cells was calculated and recorded as LI.

Analysis of variance (Fisher test) was used to evaluate the statistical significance of the differences of mean PCNA LI in the different groups of tumours.

Results

PCNA-immunolabelled nuclei were clearly and easily identified. Nuclear staining was always granular in methacarn-fixed specimens and was both diffuse and granular in formalin-fixed specimens. Staining intensity was sometimes variable and only cells that were undoubtedly stained were considered. PCNA LI are reported in Table 1. Positive cells in low-grade astrocytomas were usually homogeneously dispersed throughout the tumour, with minor variation from field to field (Fig. 1). In GBLs, strong PCNA positivity was found both in small and large/giant multinucleated cells (Fig. 2). Intratumoral heterogeneity of PCNA labelling was sometimes seen, particularly in anaplastic astrocytomas and in GBLs,

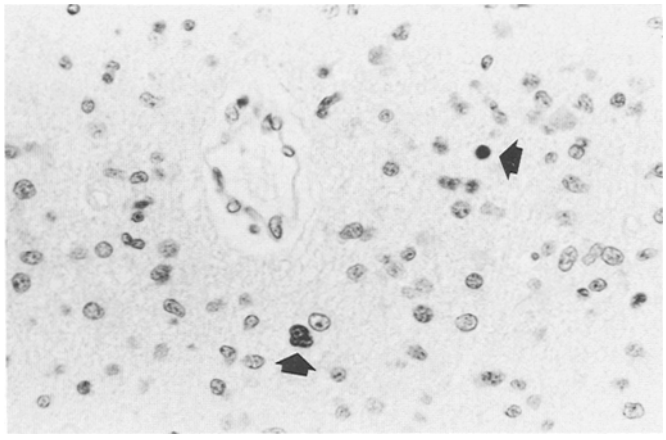

Fig. 1. Proliferating cell nuclear antigen (PCNA) immunostaining in low-grade astrocytoma (grade II). Immunolabelled cells are very few (*arrows*) and correspond to a labelling index (LI) value of 2%. ABC with haematoxylin counterstain, ×400

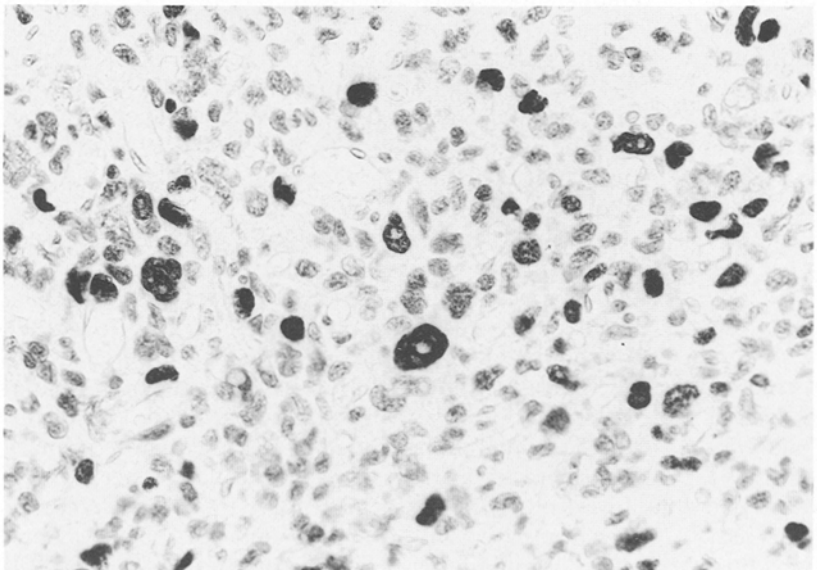

Fig. 2. PCNA immunostaining in glioblastoma. Labelled tumour cells include the ones with giant nuclei. This case had a LI value of 31%. ABC with haematoxylin counterstain, ×400

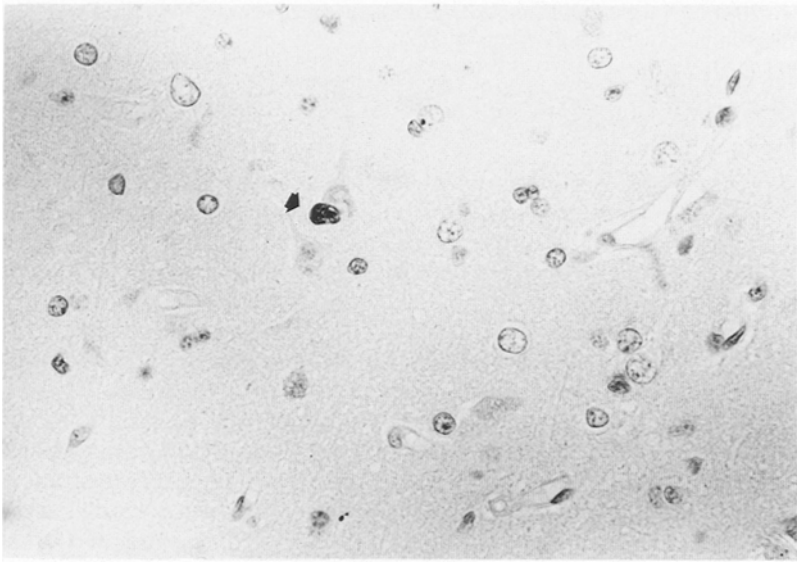

Fig. 3. PCNA immunostaining in an area adjacent to glioblastoma. A neoplastic cell showing perineuronal satellitosis is strongly immunolabelled. ABC with haematoxylin counterstain, ×400

with notable differences from field to field. The highest ranges of field to field heterogeneity were 5–21% (mean 17.7%) for anaplastic astrocytomas and 3–28% (mean 17.5%) for GBLs. In a few cases, rare endothelial cells were PCNA positive. One case with histological features of gliosarcoma showed a very high PCNA LI of glial cells, whereas mesenchymal cells were usually negative. Cells in mitosis were usually positive. One case showed occasional cytoplasmic positivity. Tumour cells infiltrating adjacent normal parenchyma were also PCNA positive and were clearly seen among normal neurons and glial cells (Fig. 3). Primary PXA had PCNA LI in the range of low-grade astrocytomas. The first recurrence of one case of PXA had a very low PCNA LI, whereas its second recurrence had PCNA LI in the range of GBLs. Normal nervous tissue was always unstained. Statistical analysis showed that mean PCNA LI of low-grade astrocytomas, anaplastic astrocytomas and GBLs were significantly different ($P<0.001$). Subdividing the group of low-grade astrocytomas into two groups of WHO grade I and II tumours, two different mean PCNA LI were found but their difference did not reach significance level. Mean PCNA LI of oligodendrogliomas was significantly different from that of low-grade astrocytomas ($P<0.05$).

A correlation between PCNA LI and survival from time of operation has not been attempted because of the insufficient follow-up period and heterogeneous distribution among the groups.

Discussion

Cell kinetics, or estimate of the rate of cell turnover, has been shown to bear on the prognosis of various neoplasms (Tubiana and Courdi 1989; Brown and Gatter 1990), including CNS tumours (Hoshino et al. 1989). Among parameters to evaluate cell kinetics are tritiated thymidine LI, flow cytometry determinations of ploidy and of S-phase, BrdU incorporation, silver stained nucleolar organizer regions (AgNOR) counts and immunostaining with antibodies against cell cycle related antigens. Most of these antibodies are suitable only for frozen section immunostaining because of the poor preservation of the corresponding antigens during fixation procedures. The advent of antibodies against PCNA which work on routinely fixed and paraffin-embedded specimens seems to open a promising field for kinetics evaluation on large series of neoplasms.

Studies on PCNA immunoreactivity are few to date. Our data in a series of CNS neoplasms show that PCNA LI reflect histological grading. Low-grade astrocytomas have usually low PCNA LI, whereas high-grade tumours show higher values, and metastases of small cell lung cancer and medulloblastomas had the highest LI. The extremely high values of PCNA LI in small cell lung cancer metastases and medulloblastomas, may not only reflect a very high percentage of cycling cells, but also a deregulated synthesis of the protein. In fact, PCNA seems to be down regulated by the product of the *p53* tumour suppressor gene (Mercer et al. 1991), which is in turn frequently mutated in small cell lung cancer (Takahashi et al. 1989; Iggo et al. 1990; Barbareschi et al. 1991) and in medulloblastomas (Loda et al. 1991). However, this might not be the only explanation, since some low-grade astrocytomas, with low PCNA LI, may show immunohistochemical patterns consistent with *p53* gene mutations.

A certain degree of variability of PCNA LI was seen in each tumour group, but mean values showed a constant increase which paralleled histological grading. PCNA immunostaining clearly separates low-grade from more aggressive neoplasms. The differences between mean PCNA LI of low-grade astrocytomas, anaplastic astrocytomas, and GBLs are highly significant. PCNA immunolabelling also differentiates primary PXA from its aggressive recurrence. However, PCNA LI were not different in the primary PXA which recurred when compared with those that did not. Lastly, PCNA immunostaining was clearly seen in neoplastic cells infil-

Table 2. Comparison of some of the reported Ki67 LI, thymidine LI, bromodeoxyuridine (BrdU) LI and present proliferating cell nuclear antigen (PCNA) LI in central nervous system neoplasms

Authors Method Histological type	1 Ki67 LI	2 Ki67 LI	3 Ki67 LI	4 Ki67 LI	5 Ki67 LI	6 Ki67 LI	7 BrdU LI	8, 9 Thymidine LI	10 BrdU LI	11 PCNA LI
Pilocytic astrocytoma (WHO GI)	1	0.5	1.1		0.1	0.03				1.61
Astrocytoma (WHO GII)	–	4.2	2.1	0.5	1.27	2.01	1.6	0.75	1.2	3.2
Anaplastic astrocytoma (WHO GIII)	30	9.5	1.6	3	4.2	1.04	4.28	3.96	4.6	14.93
Glioblastoma (WHO GIV)	28	8.0	6.1	5.2	11.1	8.51	9.92	9.3	8.6	30.58
Oligodendrogliomas (WHO GII)		6	1.6							5.44
Anaplastic oligodendroglioma			1.7			1.73				
Mixed glioma (WHO GII)		1.36	1.3							2.9
Anaplastic mixed glioma (WHO GIII)			11.2			1.7				19.75
Ependymoma	7	12.5	1.4			5.72				12.35
Ganglioglioma	1									2.25
Medulloblastoma	43	11.2						13.2		81.3
Meningioma (WHO GI)	3.2	2.8	0.5			1.56	1.82			2.4
Anaplastic meningioma (WHO GII/III)						6.72				
Metastasis		29.5	33.6			10.23				81.1

Authors: (1) Giangaspero et al. 1987; (2) Ostertag et al. 1987; (3) Burger et al. 1986; (4) Hara et al. 1990; (5) Zuber et al. 1988; (6) Plate et al. 1990; (7) Nishizaki et al. 1989; (8) Hoshino and Wilson 1979; (9) Hoshino et al. 1985; (10) Hoshino et al. 1989; (11) Present data on PCNA LI

trating adjacent brain tissue, a fact which could allow a more accurate detection of these infiltrating cells, which might be lost on haematoxylin and eosin stained slides. This was quite a constant finding in the neoplastic specimens where normal brain tissue adjacent to neoplasia was included in the sample. Nuclei of normal glial and neuronal cells were always negative, both in the normal tissue adjacent to neoplasia and in the samples of autopsy material, as described also by Hall et al. (1990). This fact may have some diagnostic utility when examining biopsies, which may not be representative of the neoplasms, but may contain only adjacent nervous tissue. We suggest that in these cases, PCNA immunostaining might be a useful tool to detect single immunoreactive neoplastic cells.

GBLs and some anaplastic astrocytomas sometimes showed a certain degree of intratumour heterogeneity of distribution of immunostained cells. This heterogeneity usually correlated with the different histological aspects of the same neoplasm. In fact in a tumour where GBL features co-existed with areas of low-grade astrocytoma, the former had much higher PCNA LI than the latter. Moreover, the areas of GBLs composed of small anaplastic cells had PCNA LI higher than the areas composed of large bizarre cells. This observation is consistent with previous studies indicating that the small anaplastic cells are the most aggressive population in GBLs. In this view, heterogeneous PCNA immunolabelling highlights the biological heterogeneity of different areas of the same tumour. Intratumoral heterogeneity underscores the critical importance of representative sampling of CNS neoplasms for kinetic studies. This was the reason why we did not include in the present series the small biopsy samples, which actually make up the greater part of our everyday histological material.

The trend of PCNA LI for the different groups of tumours is similar to the reported trends of tritiated thymidine LI (Hoshino et al. 1979, 1985), BrdU LI (Hoshino et al. 1986, 1989) and Ki67 LI (Burger et al. 1986; Giangaspero et al. 1987; Ostertag et al. 1987; Patsouris et al. 1988; Zuber et al. 1988; Hara et al. 1990; Plate et al. 1990) (see Table 2).

As expected, PCNA LI are higher than tritiated thymidine LI and BrdU LI because these latter techniques recognize the S-phase only, whereas PCNA is expressed in late G1, S, G2 and M-phases, and, due to its long half-life (20 h), it may be immunohistochemically detected in cells that have recently left the cell cycle (Hall et al. 1990; Coltrera and Gown 1991). This fact is well appreciated with PCNA immunostaining of those tissues known from other studies to be proliferating actively, like the epithelium of gastrointestinal mucosa. Nuclear PCNA immunoreactivity is present in the proliferative compartments of stomach, small intestine and colon, but weak PCNA staining is generally seen above the generally accepted zone of proliferation within the crypts (Hall et al. 1990; Wilson et al. 1990). Moreover, it is possible that the control of PCNA expression might be deregulated, adding a further bias to the evaluation of PCNA-positive cycling cells. The advantage of PCNA LI over the above techniques is that it does not require the incubation of a tumour sample with tritiated thymidine or

BrdU, nor the administration of BrdU to the patients before surgery. Every surgical sample from routine histological files may be suitable for PCNA immunostaining, allowing retrospective studies.

PCNA LI values are also somehow different from most of the reported Ki67 LI, with PCNA LI usually being higher. It is worth noting that the data on Ki67 LI of CNS tumours are somewhat heterogeneous (Table 2). Giangaspero et al. (1987), for example, reported a Ki67 index ranging from 10% to 40% in a series of malignant astrocytomas, whereas Hara et al. (1990) reported a much lower range (between 2.1% and 16.6%). Such differences may be due to different storage and handling procedures and could raise the question of the validity of direct comparison of Ki67 LI obtained by different laboratories (Brown and Gatter 1990). PCNA mAbs have the advantage of working on routinely fixed specimens, overcoming the need for fresh material with its morphological and practical limitations. Even intratumoral heterogeneity of immunostaining should be a less important bias with PCNA than with Ki67, because of the possibility of performing PCNA staining on all available material without the need to select a sample of the tumour to be frozen to perform Ki67 immunostaining.

Few cases of low-grade astrocytomas and oligodendrogliomas had PCNA LI in the range of higher-grade tumours, and one case of anaplastic astrocytoma had a LI in the range of low-grade astrocytomas. Similar findings have been reported also with other methods to evaluate cell kinetics, and Hoshino et al. (1989) suggested that the results of cell kinetic studies may be more accurate than histological grading, because in their experience, fairly benign appearing tumours but with high BrdU LI behaved like more aggressive lesions.

In conclusion, PCNA LI show a relation with histological grading and with reported thymidine, BrdU and Ki67 LI. PCNA LI are easy to perform on routinely processed material and allow retrospective studies, which would not be possible with tritiated thymidine, BrdU and Ki67. Generally speaking, PCNA LI may prove an interesting tool in regard to the present difficulties in grading gliomas, where it is often difficult, particularly in the lesions of the lower grades, to identify those which are prone to rapid recurrence (Burger et al. 1986). However the prognostic value must be validated by comparing PCNA LI with the follow-up of the neoplasms, and possibly with the responsiveness to anti-proliferation therapy, especially radiation (Tsutsumi et al. 1990).

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Note added in proof

Louis et al. (1991) have recently reported that PCNA-LI increases withincreasing tumor grade. However, in their study, PCNA-LI did not seem to distinguish low-grade from grade 3 gliomas. Moreover their data show that, in grade 3 and 4 gliomas, anti-PCNA mAb labeled fewer nuclei than Ki67 mAb. Some of the discrepancies of the study of Louis et al. (1991) with ours may be due to different handling of the samples (frozen versus fixed).

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