Mitosin, a novel marker of cell proliferation and early recurrence in intracranial meningiomas

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Summary. The expression of mitosin, a novel proliferation-associated molecule was evaluated immunohistochemically in a consecutive series of 47 patients with primary intracranial benign and atypical meningiomas. Mitosin expression was correlated with proliferation markers Ki-67 (MIB-1), proliferating cell nuclear antigen (PCNA), topoisomerase IIα (TopoIIα) and mitotic index, as well as with standard clinicopathological parameters and patient outcome. Seven tumors recurred (14.8%) following gross total resection, within a follow-up period ranging from 21 to 108 months (median 60 months). The higher proliferation indices were obtained with mitosin and PCNA and the lower ones with TopoIIα. Mitosin labeling index (LI) ranged from 0.1 to 57% (median 3%), with a significant overlapping of values between grades. A significant positive correlation was shown between mitosin LI on the one hand and Ki-67 LI (p<0.001), or the mitotic index (p=0.027) on the other. The incidence of recurrence was higher in cases with a mitosin LI higher than 3% (p=0.048). Univariate analysis disclosed mitosin LI (p=0.033) along with the mitotic index (p=0.024) and tumor size (p=0.028) as significant predictors of shortened recurrence-free survival. In multivariate analysis, the labeling indices of mitosin (p=0.035) and Ki-67 (p=0.032), along with tumor size, were shown to provide independent prognostic information, beyond that obtained by standard clinical and pathological parameters. However, as indicated by factor analysis, the prognostic information yielded by mitosin was superior to that provided by the remaining proliferation markers (p=0.041). We conclude that mitosin immunohisto-chemical expression, although failing to discriminate between benign and atypical meningiomas, may be of use as a novel cell proliferation marker and as a predictor of tumor recurrence.

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Introduction

Meningiomas constitute one of the most intriguing and challenging groups of tumors of the central nervous system, for the definition of malignant potential is beset by the frequent discordance between histology and biology. Thus, although some histological features connote more aggressive behavior (Louis et al., 2000), recurrence has been documented inasmuch as 30% of histologically benign, totally resected tumors (Christensen et al., 1983; Mirimanoff et al., 1985).

The imprecision and controversy surrounding the histopathological predictors of aggressiveness and recurrence has prompted numerous attempts to identify additional quantitative prognostic parameters, such as those reflecting the proliferative potential of meningiomas. Cell kinetic investigations in intracranial neoplasms have further been driven by the presumption that the proliferative capacity may be linked to the tempo of tumor recurrence, given that these tumors grow within the confines of the skull and become symptomatic as they enlarge (Hoshino et al., 1988). Most meningiomas manifest a slow growth in situ, contrasting with their vigorous growth in vitro (Kersting and Lennartz, 1957; Lumsden, 1971) and their high clonogenicity as determined by colony formation assay (Rosenblum et al., 1978). These puzzling observations may indicate retarded cellular proliferation *in situ* or unusual cell kinetics that cancel out cellular increase within the tumor (Hoshino and Wilson, 1975).

Several methods have been employed to measure the proliferative potential of meningiomas, including the quantitative evaluation of the mitotic rate, nucleolar organizer regions (AgNOR), bromodeooxyuridine (BrdU) incorporation, expression of cell cycle-related antigens, such as proliferating cell nuclear antigen (PCNA) and Ki-67, as well as data obtained from flow cytometric analyses (reviewed by Hsu et al., 1994).

Numerous studies dealing with these parameters have shown their controversial role in predicting meningioma recurrence (Hsu et al., 1994; Nakasu et al. 1995).

Mitosin is a 350 KD nuclear phosphoprotein involved in cell division and has therefore been considered a suitable target for evaluating proliferation by immunohistochemistry (Zhu et al., 1995a). It was identified through its binding to purified retinoblastoma protein (Zhu et al., 1995a). It is expressed in the late G1, S, G2 and M phases of the cell cycle but is absent in G0. It associates with the centromere, spindle, and midbody of the mitotic apparatus during mitosis and completely degrades following cytokinesis (Zhu et al., 1995a). The carboxy-terminus of the molecule has been shown to be essential for the localization of mitosin in the nucleus (Zhu et al., 1995b).

The aim of our study was to explore the usefulness of mitosin expression as a proliferation marker in meningiomas, in relation to PCNA, Ki-67 and TopoIIα expression, mitotic counts, established clinical/pathological parameters and tumor recurrence.

Material and methods

Clinical data and histopathological classification

A series of 47 meningiomas was selected from archival material of 113 cases operated on at the K.A.T. General Hospital of Athens, between 1988 and 1995. The cases entering this study referred to intracranial nonmalignant meningiomas, totally resected at the initial operation. Total resection was based on the surgeon's assessment at the time of surgery and on post-operative computerized tomography with contrast, when this was considered necessary (i.e. for tumors of the skull base). The tumors arose in the anterior half of the cranial cavity in relation to the cerebral convexities (63.8%), in the posterior fossa (17 %), in the base of the skull (14.9%), and in the lateral ventricles (4.3%). The latter two groups were classified as Simpson grade 2 (19.2%), all the remaining tumors falling into Simpson grade 1 category (80.8%) (Simpson, 1957). All available histological slides routinely stained with haematoxylin and eosin were reviewed and evaluated for tumor grade, and selected cases were immunocytochemically stained for vimentin, glial fibrillary acid protein (GFAP), epithelial membrane antigen, S-100 protein, peptide 19 and α_1-antichymothrypsin, in order to confirm the diagnosis. On the basis of the most recent criteria established by the 2000 WHO classification (Louis et al., 2000), tumors fell into two groups: grade I (36 cases), including variants of histologically benign meningiomas, and grade II (11 cases), including atypical meningiomas. Two cases of anaplastic grade III neoplasms were excluded from the study. Microscopic brain invasion was noted in 3 cases (1 "otherwise benign" and 2 atypical). Grade I tumors included 12 meningotheliomatous meningiomas, 8 fibroblastic, 9 transitional, 2 psammomatous, 2 angiomatous, 2 metaplastic and 1

microcystic. The presence of recurrence was based on data obtained by clinical and histological records, office notes on follow-up visits and telephone contact following informed consent of the patients. The range of follow-up time was 21-108 months (median 60 months). Within this period, seven tumors recurred (14.8%), the interoperative interval ranging from 8 to 60 months (median 17.5 months). Of the recurring tumors, 5 were benign and 2 atypical, 4 were located at the anterior cranial cavity (two of them being parasagittal), 2 at the posterior fossa and 1 at the base of the skull, while 1 demonstrated brain invasion and 2 invaded bone. All but one belonged to Simpson grade 1 group. Only pre-treatment biopsies from the initial tumor at first operation were evaluated.

Immunohistochemistry

Sections (4 μm) of paraffin-embedded tissue were stained with mouse monoclonal antibodies anti-mitosin/14C10 (Genetex, San Antonio, TX), anti-topoIIα/3F6 (Novocastra, Burlingame, CA), anti-Ki-67/MIB1 (DAKO, Carpinteria CA) and anti-PCNA/PC10 (Oncogene Science, Uniondale, NY), diluted 1:40, 1:75, 1:50 and 1:100 respectively in BSA/TBS. The incubation time was 18h (4 °C) for mitosin and TopoIIα and 1 h (room temperature) for Ki-67 and PCNA. Before staining for mitosin, TopoIIα and Ki-67, slides were incubated four times for 5 minutes in citrate buffer, pH 6 and at 750 W, in a microwave oven (Taniguchi et al., 1999). Immunostaining was then performed with the three-stage immunoperoxidase method, using streptABComplex/HRP, Duet (DAKO) and diaminobenzidine (DAB) as chromogen. Control sections for endogenous peroxidase were processed by substituting the primary antibody for non-immune mouse serum. Known positive controls consisting of normal tonsil were also stained in each run.

Assessment of labeling indices (LI) and mitotic index (MI)

For the determination of proliferative indices, an automatic computer-assisted quantification of the immunostaining was used (Sigma Scan Pro 5.0, Science, Erkrath, Germany). All four markers were selectively assessed in areas presenting a highest density of immunoreactive cells and LIs were expressed as the percentage of labeled tumor nuclei out of the total number of cells counted. The denominator in the calculations varied from 1500 to 3000 cells, counted in 10 high power optical fields (HPF), x400.

The MI was determined as the total number of mitotic figures found in 10 HPF containing the highest number of mitoses. Only unequivocal mitotic figures were counted; doubtful structures were excluded.

Statistical analysis

The categorical or categorized variables entered into

the analysis were patient's gender, histological subtype, tumor grade, Simpson grade, localization, presence of meningeal/bone invasion, presence of brain invasion and recurrence. Numerical and continuous parameters were age at initial operation, tumor size, mitosin LI, TopoIIα LI, MI, PCNA LI, and Ki-67 LI.

The normality of distributions was tested with the Kolmogorov-Smirnov test. Logarithmic transformation of mitosin LI, TopoIIα LI, MI, PCNA LI, and Ki-67 LI was necessary to obtain normal distributions. Pearson' s correlation coefficient (r) was used to determine the strength of association between all continuous and numerical variables. Student t-test was used to detect mean differences of numerical variables between subgroups. One-way analysis of variance (ANOVA) was performed to detect the differences among histological subtypes. Significant differences in the incidence of categorical parameters between various subgroups were tested using chi-square test. Progression-free survival curves were made based on Kaplan-Meier's Product-Limit Survival Estimates method. Possible predictors for recurrence were tested by using the log-rank test. Multivariate analysis was performed using the stepwise Cox's regression model, to evaluate the predictive power of each proliferation marker in the presence of classical clinicopathological parameters. Given that there were significant interrelations among the proliferation markers (see Results), factor analysis with principal components as the extraction method was performed.

Thus, the initial values of mitosin, TopoIIα, Ki-67 and PCNA LIs were replaced by the respective principal components, which then could be entered into multivariate analysis, in order to identify whether any of these markers is prognostically more informative than the remaining ones. To avoid any "data-driven" categorization, numerical parameters were entered in the analysis in continuous form. Statistical calculations were performed using the SPSS for Windows software (SPSS, Chicago Il.) on an IBM compatible PC. Statistical significance was attributed to p-values lower than 0.05.

Results

Satisfactory nuclear staining with proliferation markers was obtained in all cases, although some Ki-67-stained sections showed faint nuclear staining throughout. In these cases the immunostaining was repeated, to ensure that variation in staining intensity was not caused by suboptimal staining method. Mitotic figures always displayed a strong signal with all four proliferation markers. Positive nuclei showed diffuse staining, with a granular quality as far as mitosin was concerned (Fig. 1a,b). TopoIIα produced focally a punctate nuclear pattern. However, there was no nucleolar accentuation as seen with Ki-67. Variation in staining intensity was more common with PC-10 or MIB-1, but it was always fairly easy to decide whether a cell was positive or not, especially when assessing

Table 1. Incidence of cell proliferation per histological grade.

HISTOLOGICAL GRADE	No. OF CASES	MITOSIN INDEX % median (range)	TopoIIα INDEX % median (range)	Ki-67 INDEX % median (range)	PCNA INDEX % median (range)	MITOTIC COUNTS median (range)
I	36	2.75 (0.1-57)	0.4 (0.1-10)	1.0 (0.1-9.2)	2.15 (0.1-20.6)	0 (0-3}
II	11	8.5 (0.1-55)	3.0 (0.1-8)	3.1 (0.8-15.2)	10.0 (4.5-78.8)	10 (4-15)
Total	47	3.0 (0.1-57)	0.5 (0.1-10)	1.0 (0.1-15.2)	3.1 (0.1-78.8)	0 (0-15)

Table 2. Univariate and multivariate analysis following factor analysis of recurrence-free survival.

PARAMETER	UNIVARIATE ANALYSIS (log-rank test)	MULTIVARIATE ANALYSIS FOLLOWING FACTOR ANALYSIS (Cox's model)		
	p-value	p-value	Hazard ratio (HR)	95% confidence limits for HR
Age	0.117	0.256		
Gender	0.713	0.935		
Location	0.731	0.779		
Size	0.028	0.066		
Brain invasion	0.421	0.666		
Grade	0.456	0.155		
Simpson grade	0.661	0.863		
Mitotic activity	0.024	0.169 (factor)*		
PCNA LI	0.659	0.525 (factor)		
Ki-67 LI	0.460	0.107 (factor)		
TopoIIα LI	0.083	0.112 (factor)		
Mitosin LI	0.033	0.041 (factor)	2.367	1.036 – 5.411

*: p-values obtained following replacement of initial values with principal components.

TopoIIα reactivity. The higher proliferation indices were obtained with mitosin and PCNA and the lower ones with TopoIIα (Table 1).

Association of proliferation markers with clinical-pathological variables

TopoIIα LI, Ki-67 LI and PCNA LI tended to significantly increase with increasing grade (p=0.046, p=0.049 and p<0.001 respectively), but mitosin did not (p=0.278). Moreover, there was significant overlapping between benign and atypical meningiomas for all four LI ranges (Table 1). The MI was significantly related to the grade of malignancy as expected (p<0.001). A marginal positive correlation emerged between mitosin LI and

tumor size (r=0.247, p=0.095), while there was no significant relationship between mitosin LI on the one hand and Simpson grade, histological type, age, sex and meningeal or bone involvement on the other. Meningiomas with mitosin LI over 3% displayed a higher incidence of relapse (Fisher's exact test, p=0.048).

Correlation of mitosin with Ki-67, PCNA, TopoIIα and MI

We observed a significant positive correlation between mitosin LI and the MI (r=0.323, p=0.027; Fig. 2). Likewise, a positive correlation was documented between mitosin LI and Ki-67 LI (r=0.544, p<0.001; Fig. 3), which was stronger in grade I tumors (r=0.647,
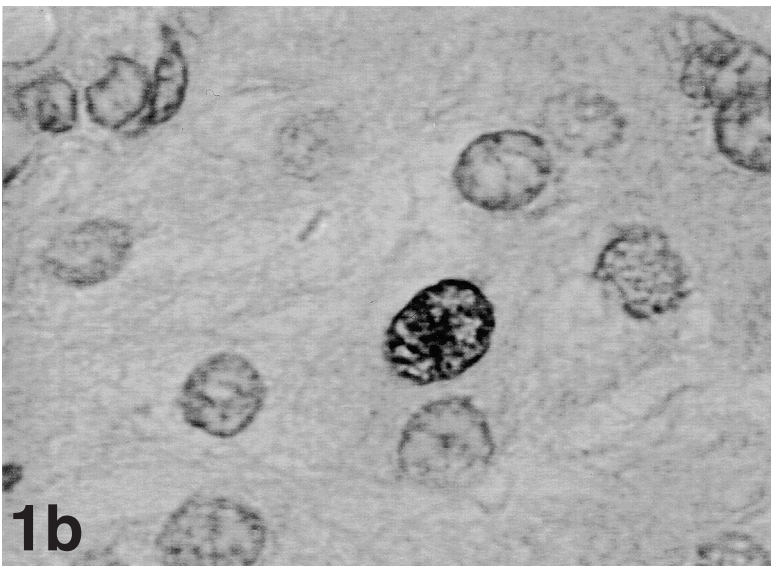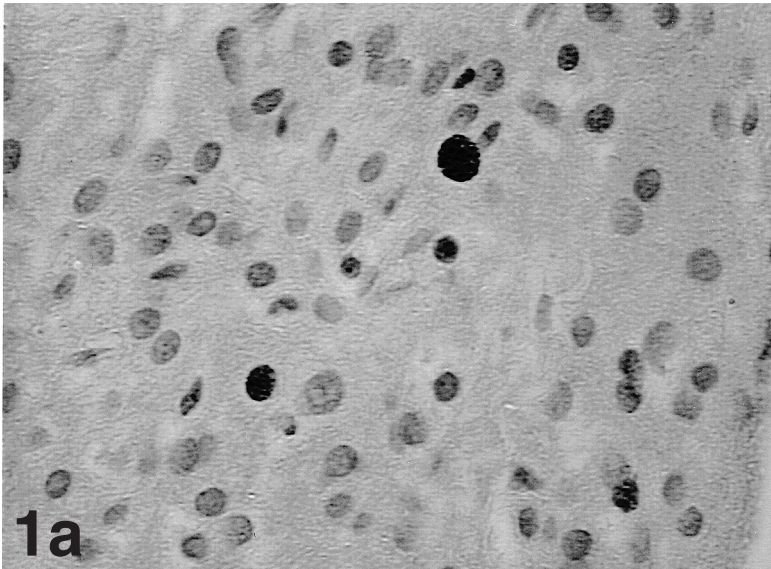

1a

1b

Fig. 1. a. Mitosin immunostaining in a grade I meningioma. x 400. Labeling index is 2.3%. **b.** Granular pattern of staining in a positive nucleus. x 1,000

p<0.001). No relation was seen between mitosin and TopoIIα or PCNA.

Survival analysis

In univariate analysis, the parameters with a significant impact on recurrence-free survival were the size of the tumor, the mitotic index and mitosin LI (Table 2). Tumors larger than 6 cm (n=8) or those displaying more than 10 mitoses per 10 HPF (n=6) or mitosin LI greater than 3% (n=23) (Fig. 4) were more

Fig. 2. Mitosin LI increases with increasing mitotic counts (MI). The upper and lower curves represent the 95% confidence limits of the regression line (middle curve).

Fig. 3. Positive correlation between mitosin LI and Ki-67 LI. The upper and lower curves represent the 95% confidence limits of the regression line (middle curve).

often associated with early recurrence. Tumor size and mitosin LI were significant predictors of early recurrence also in multivariate analysis, along with Ki-67 LI, and retained their significance when the remaining clinicopathological parameters were entered into the model (p=0.035 and p=0.032 for mitosin LI and Ki-67 LI respectively). Tumor size emerged as an independent predictor of early recurrence in all five multivariate analyses testing each proliferation marker separately (all five p-values <0.02). Multivariate analysis following factor analysis indicated that the principal component of mitosin LI was the single significant predictor of early recurrence (Table 2).

Discussion

The prognostic significance of various parameters in the clinical outcome of meningiomas has been the subject of intense scrutiny in recent years. It is to be noted that in the majority of recent investigations aiming to predict meningioma recurrence, discrimination between completely and incompletely excised tumors has not been taken into account. Our material selectively consisted of meningiomas with macroscopically and radiologically total extirpation, in order to isolate the factors leading to recurrence from the influence of partial resection.

Although there are several techniques for estimating or measuring cell proliferation, immunohistochemical detection of PCNA and Ki-67 gained wide usage because it is better suited for routine archival tissue. In meningiomas, PCNA LI and Ki-67 LI have been shown to be positively associated to histological grade (Hsu et al., 1998; Karamitopoulou et al., 1998; Konstantinidou et al., 1998). However, the significant overlap in the PCNA values among the various grades, as well as its
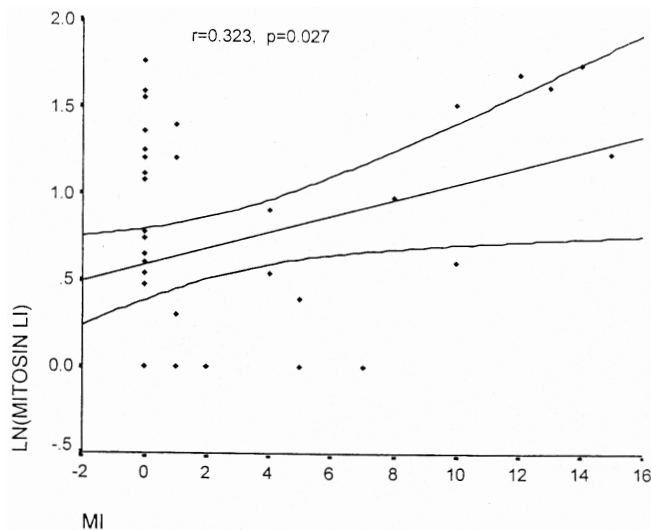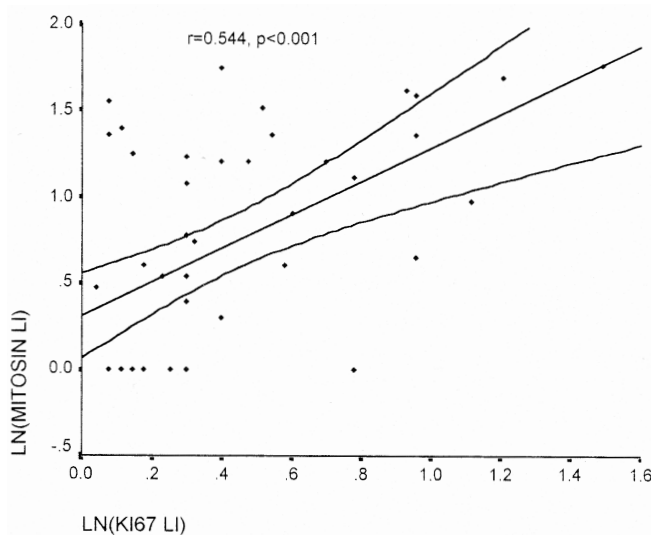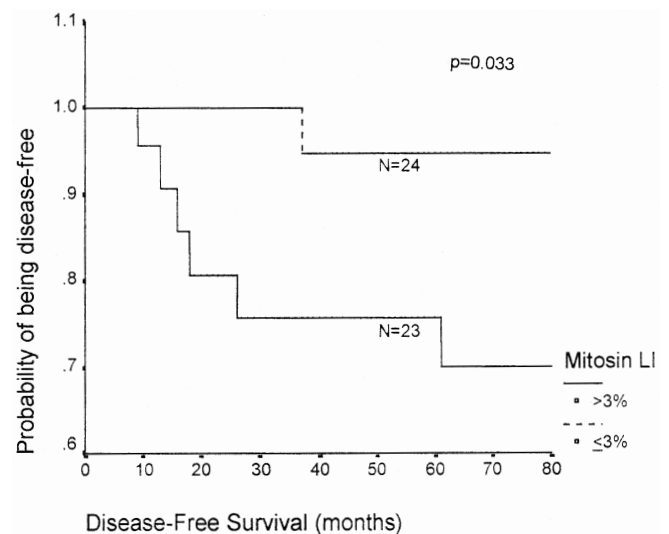
Fig. 4. Disease-free survival in relation to mitosin LI (Kaplan-Meier curves).

presence in non-cycling cells due to its long half life, have tempered initial optimism regarding its use in the assessment of proliferation. Furthermore, in some tumor types, the relationship between PCNA expression and proliferation is lost, because it is deregulated by autocrine growth factor secretion (McCormick and Hall, 1992; Khoshyomn et al., 1993). The immunoreactivity of PCNA and MIB-1 may be weakened by prolonged fixation and storage (McCormick and Hall, 1992; Munakata and Hendricks, 1993). In addition, the staining patterns of these markers are variable, necessitating use of high magnification for cell counting to avoid missing lightly stained nuclei, whereas the wide spectrum of variably labeled nuclei produced particularly by PCNA may result in inter-observer and inter-laboratory variability in the determination of the LI, further limiting its informational value (Louis et al., 1991).

In recent years, interest in cell cycle research has been rejuvenated owing to the discovery of the newer generation of proliferation markers, namely TopoIIα and mitosin, which appear to be more promising in terms of specificity for dividing cells and staining performances in archival tissue. Most recently, the authors and others have shown that TopoIIα correlates with Ki-67, PCNA and marginally with mitotic counts (Tanaka et al., 1999; Konstantinidou et al., 2001) and significantly rises with increasing grade, though displaying a significant overlap between benign and atypical meningiomas, which limits its value as a grading tool (Konstantinidou et al., 2001).

Mitosin, because of its direct participation in mitotic phase progression (Zhu et al., 1995a,b), is an attractive potential target for the quantitative assessment of proliferation by immunohistochemistry in routine archival tumor tissue. We are aware of only two published studies evaluating mitosin expression in breast carcinomas (Clark et al., 1997) and diffuse astrocytomas (Korkolopoulou et al., 2001). Unlike PCNA, Ki-67 and TopoIIα, mitosin expression was not related to grade, arguing against its utility as an additional marker of histological malignancy. However, its positive correlation with Ki-67 and mitotic activity denotes that it might be added to those molecules that are used to measure cell proliferation in meningiomas. Interestingly, mitosin indices were much higher than Ki-67 indices in our cases, in alignment with the observations in diffuse astrocytomas (Korkolopoulou et al., 2001). Given that Ki-67 is present in G_1, S, G_2 and M phases, while mitosin is undetectable during early and mid-G1 phase, one would expect Ki-67-positive cells to outnumber mitosin-positive cells. A plausible explanation for this discrepancy is that prolonged storage causes Ki-67 antigen to degrade, hence making it unreliable for determining proliferation in retrospective studies (Munakata and Hendricks, 1993; Holt et al., 1997). The trend of elevated mitosin counts to correlate with large tumor size suggests that mitosin may have a role in the evaluation of tumor growth. However, the lack of any significant strong correlation between tumor size and proliferative activity, as expressed by any of the five

markers, suggests that, in these cases, tumor growth may be also influenced by other parameters, notably the rate of cell loss.

The prognostic utility of proliferation markers in meningioma recurrence has been a nidus of ongoing controversy. The mitotic index has traditionally been considered to roughly correlate with the growth rate and frequency of recurrence, but its value in predicting tumor doubling time is only suggestive and it has been proven unable to predict the likelihood of recurrence in individual cases (Jääskeläinen et al., 1985). Furthermore, the mitotic rate of nonmalignant meningiomas is too low to measure properly, and the distribution of mitotic figures is too inconsistent to provide the basis for a reliable estimate of prognosis. In our study, the mitotic index, although of prognostic utility, failed to provide independent information in multivariate analysis. Several investigators have correlated the rate of recurrence to high Ki-67 (MIB-1) values (Kakinuma et al., 1998; Karamitopoulou et al., 1998; Tanaka et al., 1999; du Plessis et al., 2000) and very few to high PCNA values (Cobb et al., 1996), whereas others have failed to substantiate the prognostic effect of isolated proliferation indices in meningiomas (Khoshyomn et al., 1993; Konstantinidou et al., 1998). TopoIIα, on the contrary, does not seem to profoundly affect recurrence-free survival (Konstantinidou et al., 2001).

There is no published literature on the clinical relevance of mitosin in meningiomas and there are surprisingly few survival analyses assessing the prognostic value of common proliferation markers in meningioma recurrence (Hsu et al., 1998; Konstantinidou et al., 1998). The interval from resection to recurrence in our study was 1 to 5 years, which correlates with previously reported latency periods after gross total removal (Christensen et al., 1983; Jääskeläinen et al., 1985; Mirimanoff et al., 1985). Admittedly, the small number of recurrences is a drawback of the present series, which, however, reflects the unselected nature of our cohort. Among the various proliferation markers examined in this study, mitosin and Ki-67 were shown to provide independent prognostic information, beyond that obtained by standard clinical and pathological parameters. However, as indicated by factor analysis, the prognostic information yielded by mitosin was superior to that provided by the other proliferation markers. Our findings lend support to the concept advanced by Hoshino and Wilson (1975) that brain tumor cell kinetic investigation may facilitate prognostication of patient survival. Still, mitosin labeling should probably await demonstration that it significantly contributes to the physician's ability accurately to predict patient outcome, due to the lack of prospective studies validating the preliminary findings of the present retrospective investigation. More importantly, it is generally thought that tumor growth involves more than just cell proliferation, depending not only on the percentage of cells undergoing division at any given time, but rather on the overall result of division, the

variant cell types produced and the number of cells surviving the division process (Hoshino and Wilson, 1975; Bookwalter et al., 1986). Hence, however potentially predictive it might appear, a single kinetic determination such as the labeling index should not alone be adequate to prognosticate a phenomenon as complex as a patient's survival, inasmuch as it does not reflect the evolutionary process that selects for cellular and genetic instability as well as therapeutic resistance.

Another parameter of prognostic significance in our series was tumor size. Meningiomas which recurred were significantly larger than those which did not recur, this difference in size being accompanied by a corresponding yet marginal increase in mitosin counts. Tumor size appears not to be included among the usual parameters participating in previous studies of clinicopathological outcome in meningiomas. An increased probability of cell dissemination and seeding during surgical procedures, recently implicated in meningioma recurrence (von Deimling et al., 1999), might be related to this trend of larger tumors to recur.

In conclusion, this comparative study of proliferation markers in meningiomas demonstrates that mitosin immunohistochemical expression may be of use as a novel cell proliferation marker and as a marker of early recurrence, yet failing to assist in the discrimination between benign and atypical tumors. Controlled prospective investigations in a larger number of patients with recurrent tumors are warranted to validate this assumption.

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