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Caspase-3 immunohistochemical expression is a marker of apoptosis, increased grade and early recurrence in intracranial meningiomas

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Abstract Caspase-3 is the ultimate executioner caspase that is essential for the nuclear changes associated with apoptosis. We investigated caspase-3 immunohistochemical expression in 58 primary intracranial meningiomas, using one monoclonal antibody detecting both precursor and cleaved caspase-3 (CPP32) and a second recognizing only the cleaved activated form (ASP175). Caspase-3 expression was analyzed in relation to baseline apoptosis—as illustrated by the expression of anti-single stranded DNA (ss-DNA), the antiapoptotic protein bcl-2, proliferation indices (Ki-67, PCNA, topoisomerase IIa, mitosin C), hormonal status (estrogen, progesterone, androgen receptors), standard clinicopathological parameters and patients' disease-free survival. Caspase-3 immunostaining was observed in 62% of cases for CPP32 and in 24% for ASP175. In both instances, the labeling index (LI) was significantly correlated with ss-DNA LI ($p = 0.038$ and $p = 0.018$). CPP32 but not ASP175 LI positively correlated with the mitotic index ($p = 0.001$) and PCNA LI ($p = 0.004$). Both CPP32 and ASP175 LIs were increased in nonbenign meningiomas ($p < 0.0001$ and $p = 0.0035$ respectively). In univariate and multivariate survival analyses, caspase-3 predicted meningioma recurrence, independently affecting disease-free survival ($p = 0.011$ and $p = 0.047$ respectively for CPP32; $p < 0.0001$ and $p = 0.012$ respectively for ASP175). Caspase-3 may prove to be a useful predictor of early recurrence in a group of neoplasms characterized by the frequent discordance between histology and clinical behavior.

Keywords Meningioma · Active caspase-3 · CPP32 · ASP175 · ss-DNA · bcl-2 · Ki-67 · PCNA · Topoisomerase IIa · Mitosin C · Apoptosis · Proliferation · Recurrence · Prognosis · Disease-free survival

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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 [1, 2]. Although the classical form of necrosis is a major histological factor influencing grading and prognosis in meningiomas [1–3], the occurrence and significance of apoptosis has been the subject of limited reports [4–7].

Abnormal apoptosis is a hallmark of human tumors and deregulation of the genes controlling the apoptotic cascade is closely related to tumor development, progression and recurrence [8, 9]. Among the many known regulators and effectors of apoptosis, the family of caspases play an important role in the execution phases of apoptosis [10–12]. Caspases are cysteine proteases synthesized as inactive proenzymes (zymogens) and activated in a cascade-like fashion [10–12]. Once activated, they are able to cleave a broad range of cellular

targets and ultimately cause apoptosis in diverse cell types. From a functional percpective, the caspase gene family has been classified into either initiator or effector caspases [10, 11]. Intrinsic as well as extrinsic pathways for activating caspases have been demonstrated in detail [13]. Both ways, active initiator caspases have been shown to directly cleave and activate the effector protease caspase-3 [14, 15]. Caspase-3 is a member of the interleukin-1β-converting enzyme (ICE) family, which specifically cleaves substrates at the C-terminal of aspartic acid residues [12, 13]. It is synthesized as an inactive 32 kd proenzyme and processed during apoptosis into its active form that is composed of two subunits, p17–20 and p10–12 [10]. Activated caspase-3 is responsible for the cleavage of polyADP-ribose polymerase (PARP), actin and steroid regulatory element binding protein (SREBP) which relates to apoptosis [14, 15]. In this study, caspase-3 was selected because it represents the point of convergence of multiple "executioner" apoptotic pathways [14]. Moreover, experimental evidence on animal models suggest that caspase-3 is the most abundant among the known caspases in the brain with important implications in brain development [16, 17].

This is the first study in the published literature to investigate caspase-3 expression in meningiomas and to relate it with apoptosis, apoptosis regulatory proteins, proliferation indices, clinicopathological parameters and patients' disease free survival.

Materials and methods

Patients, clinical data and histopathological classification

A consecutive series of 58 meningiomas was selected from archival material of 172 patients with brain tumors diagnosed at the Department of Pathology of the National University of Athens, between 1990 and 2001. The cases entering this study referred to intracranial meningiomas, totally resected at the initial operation. Total resection was based on the surgeon's assessment at the time of surgery and on postoperative computer tomography (CT). For those tumors arising at the base of the skull, CT was followed by contrast administration. The tumors arose in the anterior half of the cranial cavity, in relation to the cerebral convexities (81%), in the posterior fossa (13.8%), the base of the skull (3.5%), and the lateral ventricles (1.7%). The latter two groups were classified as Simpson grade 2 (5.2%), all the remaining tumors falling into Simpson grade 1 category (94.8%) [18]. On the basis of the most recent criteria established by the 2000 WHO classification [1], tumors fell into 3 groups: grade I (43 cases, 74%) including variants of histologically benign meningiomas, grade II (13 cases, 22.5%) including atypical meningiomas, of which one chordoid subtype, and anaplastic grade III tumors (2 cases, 3.5%). Microscopic brain invasion was noted in 7 cases (1 "otherwise benign", 4 atypical and 2 anaplastic). Grade I meningiomas included 17 of meningotheliomatous type, 8 fibroblastic, 12 transitional, 2 psammomatous, 1 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 10–108 months (median 52 months). Within this period, eight tumors recurred (14.28%), the interoperative interval ranging from 8 to 60 months (median 21.25 m). Of the recurring tumors, 5 were benign (11.62% of the benign tumors) and 3 atypical (23% of the atypical tumors). Follow-up data were not available for the 2 patients with anaplastic meningiomas. Clinicopathological data regarding the cases of recurrence are shown in Table 1. Only pre-treatment biopsies from the initial tumor at first operation were evaluated.

Table 1 Clinicopathological data and caspase-3 labeling indices for the 8 cases of meningioma recurrence

Case no.	1	2	3	4	5	6	7	8
Gender[a]	F	M	F	M	M	F	F	M
Age	66	67	66	58	72	66	61	65
Location[b]	CC	CC	PF	B	PF	CC	CC	CC
Size (cm)	5.5	5	2.5	6.7	4.2	3.5	6	8.5
Simpson grade	1	1	1	2	1	1	1	1
Histologic grade	I	I	I	I	I	II	II	II
Bone/dural invasion	−	+	−	−	−	−	−	+
Brain invasion	−	+	−	−	−	−	+	+
Time to relapse (months)	12	15	60	25	36	8	17.5	28
CPP32 LI								
Total score (% × intensity)	2.70	5.36	2.98	0.39	3.56	–[c]	12.05	4.42
ASP175 LI								
Total score (% × intensity)	0	4.25	2.46	0	1.50	–[c]	8.62	3.54

[a]M: male; F: female

[b]CC: cerebral convexities; PF: posterior fossa; B: base of skull

[c]Tissue not available for caspase-3 assessment

Immunohistochemistry

Sections (4 μm) of paraffin-embedded tissue were stained with mouse monoclonal antibodies: anti-caspase-3/CPP32 (Chemicon Int.), anti-cleaved-caspase-3/ASP175 (Cell Signaling), anti-ssDNA/F-7-26 (Alexis), anti-bcl-2/124 (DAKO), anti-p53/DO-7 (DAKO), anti-Ki-67/MIB1 (DAKO), anti-PCNA/PC10 (Oncogene Science), anti-mitosin/14C10 (Genetex, San Antonio, TX), anti-topoIIa/3F6 (Novocastra), anti-ER/1D5 (DAKO), anti-PR/1A6 (DAKO) and anti-AR/AR27 (Novocastra), diluted 1:250, 1:100, 1:10, 1:50, 1:50, 1:50, 1:100, 1:40, 1:75, 1:50, 1:10 and 1:50 respectively in bovine serum albumin/Tris-buffered saline (BSA/TBS). CPP32 recognizes the full length 32 kDa caspase-3 protein of human origin and detects both precursor and cleaved forms of caspase-3. ASP175 recognizes the large fragment (17/19 kDa) of activated caspase-3, resulting from cleavage adjacent to ASP175. Both antibodies do not cross-react with other caspases. The staining protocol with F-7-26 was performed as previously described [5]. A high temperature antigen retrieval method was applied for antigens CPP32, bcl-2, Ki-67 and p53. For ASP175 and hormonal receptors a microwave antigen retrieval method was applied. For hormonal receptors immunostaining was performed with the catalysed signal amplification (CSA) system (DAKO). For the rest of immunomarkers, staining was performed with the three-stage immunoperoxidase method, using streptABComplex/HRP, Duet (DAKO) and diaminobenzidine (DAB) as chromogen. Control sections for endogenous peroxidase were processed substituting the primary antibody by non-immune mouse serum. Known positive controls (embryonic kidney for caspase-3, a Burkitt's lymphoma for ssDNA, a follicular lymphoma for bcl-2, a normal tonsil for Ki-67, topoIIa and PCNA, a colorectal carcinoma for p53, breast carcinoma for ER and PR, prostate carcinoma for AR) were also stained in each run.

Caspase-3 scoring and assessment of labeling indices (LI)

For the assessment of immunohistochemical staining, any number above zero of immunolabeled cells was considered as positive caspase-3 expression. An overall score was calculated in which the percentage positive score was multiplied by the intensity of the staining (overall score = percentage positive tumor cells multiplied by intensity), as previously described [19]. Staining intensity was graded as follows: 1: weak; 2: moderate; and 3: intense (Fig. 1).

For the determination of all labeling indices an automated computer-assisted quantification of the immunostaining was used (Sigma Scan Pro 5.0; Science). All markers were assessed with reference to the total number of immunolabeled neoplastic cells in several high power (400 $\times$) microscopic fields and LIs were expressed as the percentage of labeled tumor nuclei out of the total number of cells counted [20]. Likewise, the apoptotic index (AI) was calculated as the ratio of neoplastic cells stained with the anti-ssDNA antibody to the total number of cells counted [5]. All markers were selectively assessed in areas presenting the highest density of immunoreactive cells, as is the practice in tumors with low proliferative and apoptotic potential and heterogeneous staining. For PCNA assessment, only intense immunostaining was recorded as positive. The denominator in the calculations varied from 2500 to 3000 cells. Non-neoplastic cells (e.g. endothelial cells, lymphocytes) were excluded from counting. Cell counting was performed by two independent observers.

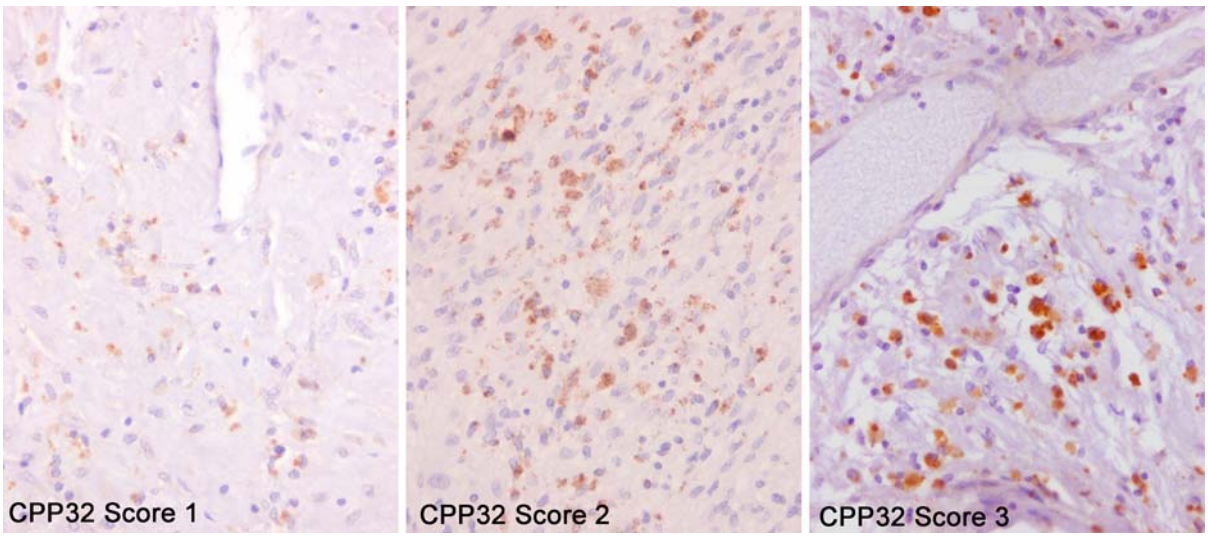

Fig. 1 Examples of caspase-3 scoring according to staining intensity

Mitotic index (MI)

The MI was determined by totaling the numbers of mitotic figures found in 10 HPF (400 ×) containing the highest number of mitoses. These values were also used for grading of tumors, according to the WHO criteria [1]. 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, location, presence of meningeal/bone or brain invasion, recurrence and caspase-3 expression. Numerical and continuous parameters were age at initial operation, tumor size, AI, MI and the labeling indices of CPP32, ASP175, bcl-2, p53, Ki-67, PCNA, topo-IIa, mitosin, ER, PR and AR.

The normality of distributions was tested with the Kolmogorov-Smirnov test. Spearman rank correlation coefficient (ρ) was used to determine the strength of association between all continuous and numerical variables. Mann-Whitney U-test and Kruskal-Wallis analysis of variance were used to detect differences in the distribution of numerical variables between subgroups. Significant differences in the incidence of categorical parameters between various subgroups were tested using Pearson's 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 variable independently of the others. 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

Caspase-3 immunohistochemical expression

Caspase-3 immunohistochemical expression was observed in 36 of 58 (62%) meningiomas using CPP32 and in 14 of 58 (24%) using ASP175. Both antibodies demonstrated a heterogeneous pattern of staining with a tendency of immunopositive cells towards cluster formation, as previously observed [19, 21]. Staining localization was mostly cytoplasmic with a granular pattern of expression consisting of coarse granules in the majority of cases (Fig. 2). A fine granular, almost dusty, staining pattern was, however, seen in some cases with perinuclear localization. Moreover, in a few cases a nuclear immunostaining was also observed in addition to the cytoplasmic one (Fig. 2(B)). Most ASP175-positive cells were morphologically apoptotic, which was not always the case with CPP32-positive cells (Fig. 2(A, C)). There was a statistical correlation between CPP32 and ASP175 LIs (Spearman's ρ test, $p < 0.0001$, $\rho = 0.558$). Labeling indices for CPP32 and ASP175 are demonstrated in Table 2.

Caspase-3 immunohistochemical expression in relation to histological subtype and grade of meningiomas

There was no significant association between benign histological subtypes and caspase-3 expression. Nonbenign meningiomas (grade II and III) demonstrated a significantly

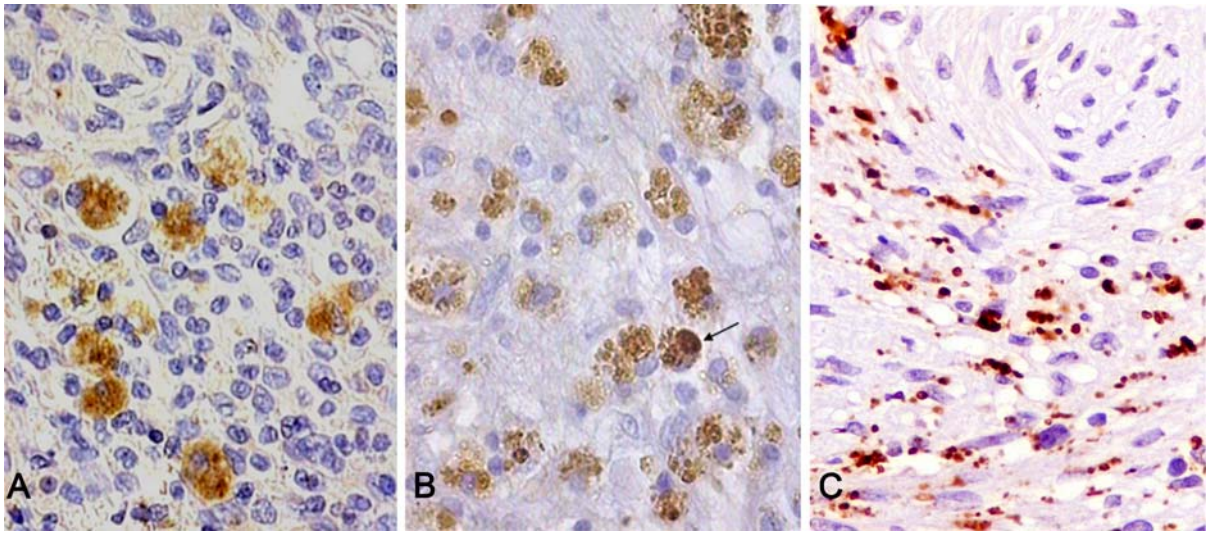

Fig. 2 (A, B) CPP32 expression with granular cytoplasmic pattern of staining, without prominent apoptotic morphology. (B) CPP32 with nuclear reaction (*arrow*). (C) ASP175 expression with prominent apoptotic morphology (A, B, C: × 400)

Table 2 Caspase-3 expression according to histologic grade of meningiomas

		CPP32		ASP175	
Histologic grade	No. of cases	Percentage (%) *median* (*range*)	Total score (% × intensity) *median* (*range*)	Percentage (%) *median* (*range*)	Total score (% × intensity) *median* (*range*)
I	43 (74%)	0 (0–25.75)	0 (0–51.52)	0 (0–20.64)	0 (0–45)
II + III	15 (26%)	4.42 (0.53–28.54)	8.20 (1.06–85.62)	2.83 (0–25)	6.64 (0–75)
		$p < 0.0001$	$p < 0.0001$	$p = 0.0035$	$p = 0.0029$
Total	58 (100%)	0.95 (0–28.54)	1.49 (0–85.62)	0 (0–25)	0 (0–75)

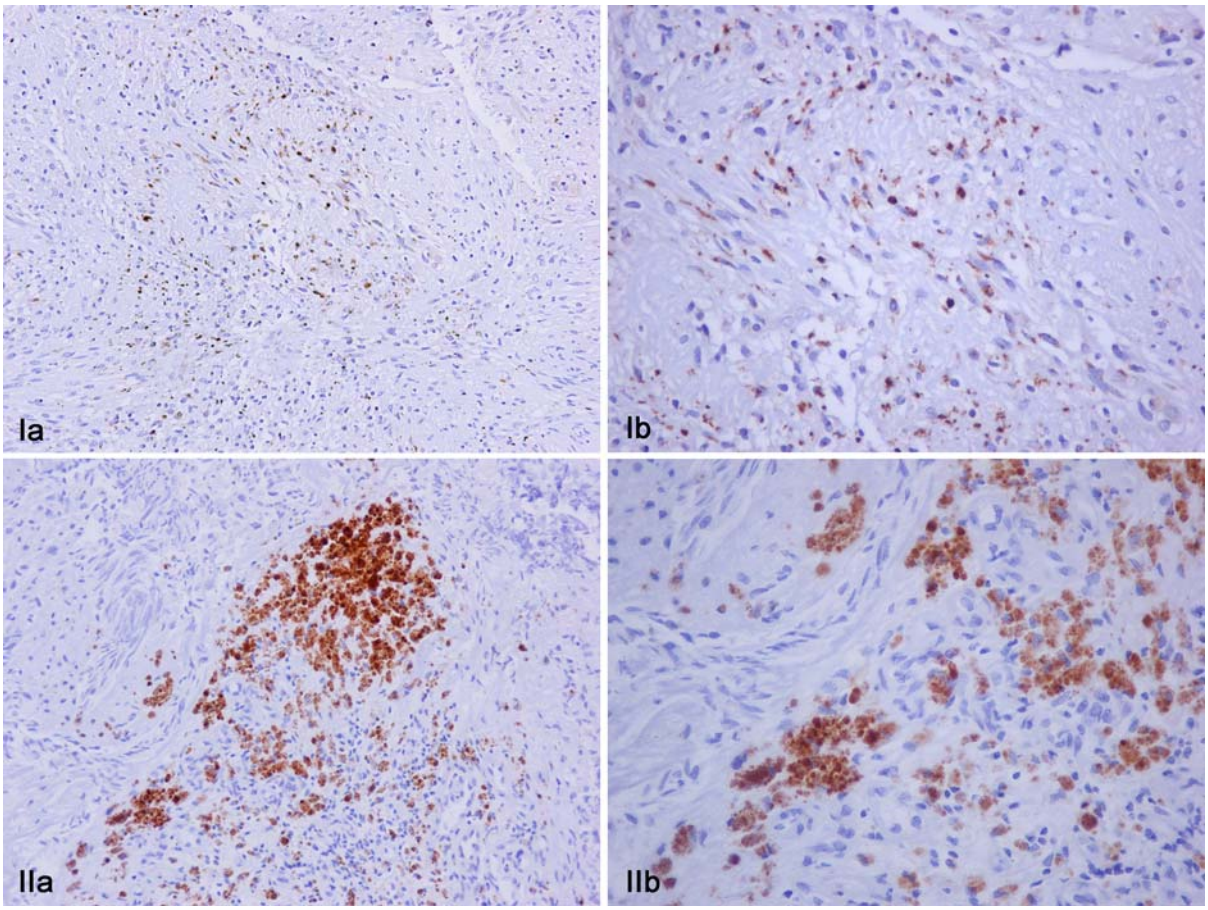

Fig. 3 I(a, b) Grade I meningioma with ASP175 LI 1.5%. II(a, b) Grade II meningioma with ASP175 LI 8.6%. Both tumors recurred. (Left: × 200; Right: Detail × 400)

increased labeling index, compared to grade I tumors ($p < 0.0001$ for CPP32; $p = 0.0035$ for ASP175 Mann-Whitney U test; Table 2; Figs. 3 and 4).

No associations were established between caspase-3 expression and the following parameters: age, gender, tumor location, tumor size and brain invasion.

Correlation of caspase-3 expression with apoptosis related indices

Labeling indices of all markers are demonstrated in Table 3. A statistically significant though not strong correlation emerged between caspase-3 expression and the ssDNA LI (Table 4). (Spearman's ρ test, $p = 0.038$, $\rho = 0.280$ for CPP32; $p = 0.018$, $\rho = 0.303$ for ASP175.) On the other hand, no significant association was seen between caspase-3 expression and bcl-2 apoptosis suppressor protein or p53 apoptosis regulatory protein (Table 4).

Correlation of caspase-3 expression with proliferation indices

A significant positive correlation emerged between the mitotic index and CPP32 LI (Spearman's ρ test, $p = 0.001$,

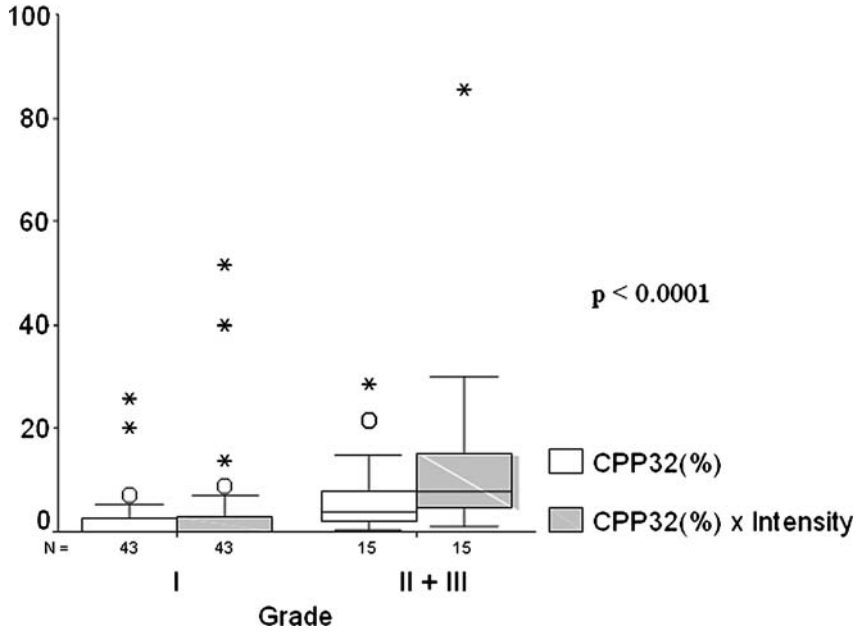

Fig. 4 Non-benign meningiomas display a significantly increased caspase-3 labeling index, compared to benign tumors (Mann-Whitney *U* test). The association remains significant when the labeling score is corrected for staining intensity

$\rho = 0.433$) (Table 4). ASP175 LI tended to increase with increasing mitotic index, however this trend did not reach statistical significance ($p = 0.069$). When the mitotic activity was categorized on the basis of the presence or absence of mitoses, a significant association with the CPP32 LI was also established (Mann–Whitney *U* test, $p = 0.002$). Among the remaining proliferation indices, only PCNA LI demonstrated a positive correlation with CPP32 LI (Table 4), evaluated either as a percentage positive score (Spearman's ρ test, $p = 0.006$, $\rho = 0.410$) or as an overall score (percentage × intensity) (Spearman's ρ test, $p = 0.004$, $\rho = 0.430$). No significant correlation could be established between caspase-3 and Ki-67, topoisomerase IIa or mitosin labeling indices (Table 4).

Table 3 Labeling indices of apoptotic, proliferation and hormonal markers

	Median %	Range %
CPP-32		
Percentage (%)	0.95	0–28.54
Total score (% × intensity)	1.49	0–85.62
ASP-175		
Percentage (%)	0	0–25
Total Score (% × intensity)	0	0–75
ssDNA	0.57	0–2.90
Bcl-2	1.20	0–14.3
PCNA	3.05	0–28
Ki-67	4.30	0.1–30
Mitosin	3	0.1–57
Topoisomerase IIa	0.50	0.1–10
PR	3.60	0–85
ER	0	0–30
AR	0	0–45

Correlation of caspase-3 expression with hormonal receptors

No associations could be demonstrated between hormonal receptors (ER, PR, AR) on the one hand and caspase-3 expression on the other, according to our results (Table 4).

Survival analysis

On univariate analysis, the parameters showing a significant impact on recurrence-free survival were the apoptotic index (ssDNA LI) ($p = 0.015$), caspase-3 expression ($p = 0.011$ for CPP32 and $p < 0.0001$ for ASP175; Fig. 5) and mitosin expression ($p = 0.045$). Patients, whose tumors demonstrated an apoptotic rate $> 0.6\%$, CPP32 or ASP175 LI $> 0\%$ and mitosin LI $> 3\%$ were more often associated with shorter disease-free survival. Larger meningiomas (> 6 cm) showed a trend towards early recurrence, although this failed to reach statistical significance ($p = 0.080$). On multivariate analysis, caspase-3 LI emerged as an independent predictor of recurrence ($p = 0.047$ for CPP32 and $p = 0.012$ for ASP175), whereas the predictive value of ssDNA was of suggestive statistical significance ($p = 0.061$).

Discussion

In the present study, we investigated caspase-3 immunoreactivity in a series of intracranial, totally resected meningiomas, as a continuum of previous studies of our group, aiming to determine prognosticators of meningioma recurrence [5, 22–24]. Despite the importance of apoptosis in the development of central and peripheral nervous system, there are rather few

Table 4 Associations of Caspase-3 expression with apoptotic index, apoptotic proteins, proliferation indices and hormone receptors

	CPP32		ASP175	
Apoptotic index	Percentage %	Total score (% × intensity)	Percentage %	Total score (% × intensity)
Apoptotic proteins				
ssDNA	*P = 0.048*	*P = 0.038*	*P = 0.025*	*P = 0.018*
bcl-2	N.S.	N.S.	N.S.	N.S.
p53	N.S.	N.S.	N.S.	N.S.
Proliferation indices				
Mitotic Index	*P = 0.001*	*P = 0.001*	P = 0.073	P = 0.069
Ki-67	N.S.	N.S.	N.S.	N.S.
PCNA	*P = 0.006*	*P = 0.004*	N.S	N.S
Topoisomerase-IIa	N.S.	N.S.	N.S.	N.S.
Mitosin	P = 0.065	P = 0.072	N.S.	N.S.
Hormone receptors				
ER	N.S.	N.S.	N.S.	N.S.
PR	N.S.	N.S.	N.S.	N.S.
AR	N.S.	N.S	N.S.	N.S

reports concerning the magnitude of cell deletion in meningiomas [4–7].

Interrelation between CPP32 and ASP175

As expected, there was a strong correlation between immunohistochemical expression of CPP32 and ASP175, the former detecting cells expressing both pro- and active caspase-3 and the latter reflecting only the cleaved activated caspase-3. Among CPP32-positive cases no more than 39% were positive for active caspase-3, the remaining apparently representing tumors in which CPP32 detection corresponded solely to procaspase-3 expression. There were no cases positive for active caspase-3 (ASP175) without expressing pro-and-active caspase-3 (CPP32).

Relation between caspase-3 expression and apoptosis

In a recent study, we have pointed out that the apoptotic rate, evaluated by means of a novel monoclonal antibody recognizing exposed single-stranded (ss) regions in the DNA of apoptotic cells during heating, is implicated in meningioma growth and recurrence [5]. In the present study, caspase-3 expression was significantly associated with the levels of ss-DNA verifying the association between the result—i.e. the apoptotic fractions—and the underlying mechanism—i.e. caspase-3 upregulation. Despite evidence that a caspase-independent pathway of programmed cell death also exists [25], our finding suggests that in meningiomas apoptosis follows a caspase-dependent pathway. However, the weak correlation between active caspase-3 expression and ss-DNA, as well as the broader range of ASP-175 (0–25%) values when compared to ss-DNA range (0–2.9%), indicate that caspase-3 activation may occur in the absence of cell death and apoptosis. This possibility has been suggested by previous investigators, reporting caspase-3 activation without commitment to apoptotic cell death in T-lymphocytes [26] and in neurons of hypoxic rat brains [16]. These observations have led the authors to speculate on the possibility that caspases may have additional functions and participate in other than the apoptotic signaling pathways. We should also keep in mind that endogenous inhibitors of caspases exist and are also known as inhibitors of apoptosis or IAPs. An endogenous mammalian caspase inhibitor XIAP contains one domain (BIR2) that specifically inhibits the effector caspases 3 and 7 [27]. These data seem to cast doubt on the reliability of caspase-3 as the "no return point" to apoptosis and suggest that its activation may not invariably result in cell death.

Relation between caspase-3 expression and apoptosis regulatory proteins

A plausible association between caspase-3 and bcl-2 expression could be expected, given that bcl-2 overexpression inhibits cytocolic accumulation of cytochrome c and caspase-3 activation [28]. It has also been reported that bcl-2 at the endoplasmic reticulum is able to interact with the endoplasmic protein βap31, thus inhibiting caspase activation at the endoplasmic reticulum [29]. On the other hand, caspase-3 is able to modulate the function of bcl-2 by cleaving it to a shorter truncated form which, in contrast to the longer anti-apoptotic form, is pro-apoptotic [30]. Furthermore, bcl-xl, another anti-apoptotic protein, is cleaved by caspases to a similar pro-apoptotic fragment [31]. The complexity of these interactions may account for the absence of any clearcut association between caspase-3 and bcl-2. Whatever the causes at a molecular level, our finding is in keeping with the results of Arai et al, reporting no significant association between bcl-2 expression and caspase-3 activity in diffuse large B-cell lymphomas of the CNS [32]. Furthermore, Ng and Chen

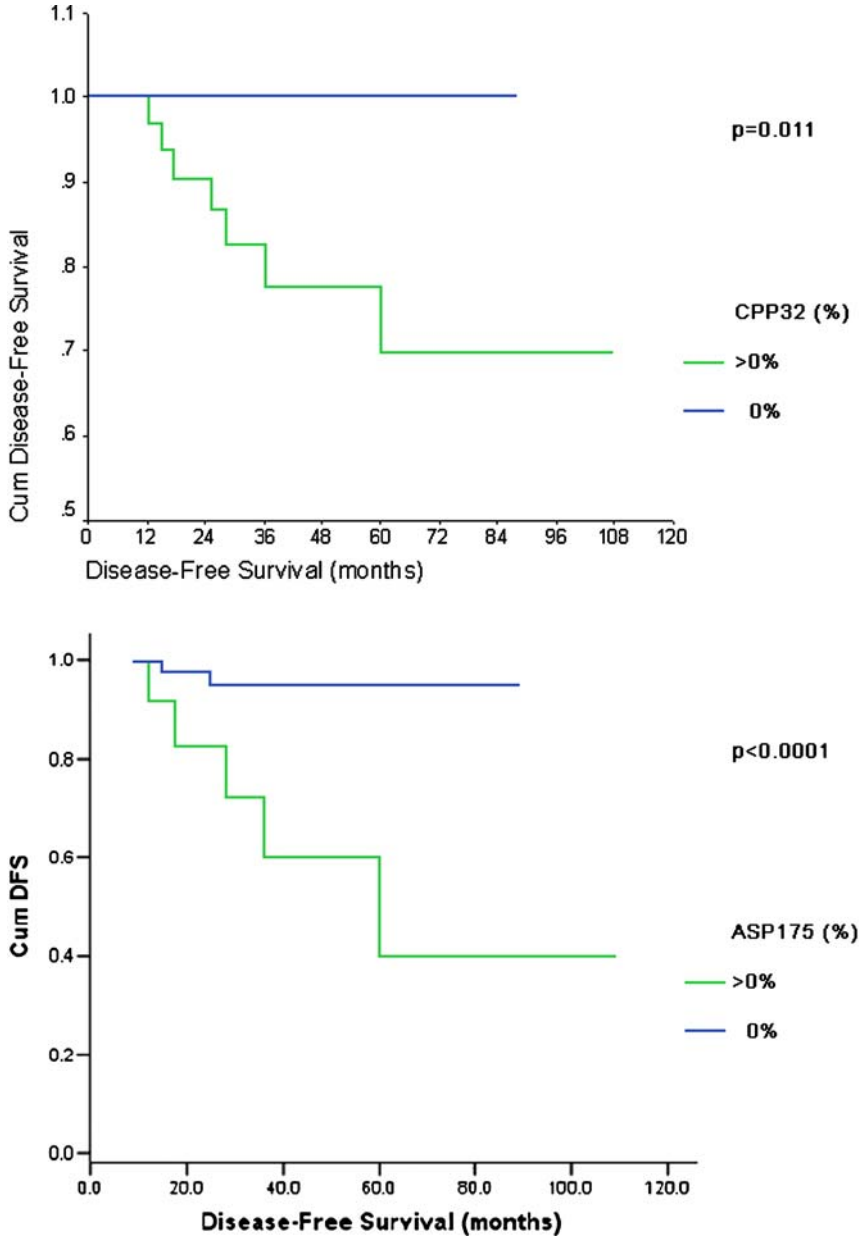

Fig. 5 Kaplan-Meier curves for recurrence-free survival in relation to caspase-3 labeling index (*Upper*: CPP32 LI; *Lower*: ASP175 LI). The time to recurrence is shorter in positive caspase-3 expressors

[4] also failed to establish in meningiomas a clearcut correlation between apoptosis labeling and the immunopositivity rates of bcl-2 and other apoptosis related genes, like p53 and c-myc. This is also in line with the results of Ellison et al. [33] concerning bcl-2 immunostaining in astrocytic tumors. The repeated documentation of such negative findings may suggest that the control of apoptosis in CNS tumors involves genes other than bcl-2, p53 and c-myc.

Association between caspase-3 expression and histological grade

In the present study, atypical meningiomas showed higher scores of caspase-3 expression than benign ones. The direct relation of apoptosis with increasing grade is no longer a surprising finding, as it has been observed in various subgroups of tumors [9]. In meningiomas, this relation may suggest a role of caspase-3 in the progression from benign to atypical tumors. Alternatively, caspase-3 upregulation as a marker of increased apoptosis may reflect increasing anaplasia and cell proliferation associated with increasing grade [7]. In this context, it is also possible that genes that are activated in higher grade lesions may also be responsible for the induction of a higher extent of apoptosis in them, as is the case of caspase recruitment by Fas activation shown to be upregulated in atypical and malignant meningiomas [7]. Our finding of increasing apoptosis by means of caspase-3 expression in higher grade meningiomas is in accordance with the results

of Ng and Chen [4] reporting increasing apoptotic rate by means of in-situ end-labeling of DNA fragments, along with the histological grade of these tumors. The authors suggest that similarly to classical necrosis, apoptosis is a biological process associated with atypical or malignant change in meningiomas [4]. Interestingly, in meningiomas presenting necrosis, an increased concentration of caspase-3 positive cells in the border zones between necrotic core and vital neoplastic tissue was noted, in keeping with the observed in our previous study increased occurrence of fragmented DNA near necrotic foci [5]. Our finding of increased caspase-3 immunoreactivity near necrotic areas supports recent data underscoring that caspase activation, especially caspase-3 activation, is implicated in the ischemic cell death [16, 34].

Relation between caspase-3 expression and proliferation indices

The positive relation between CPP32 and the mitotic index as well as the PCNA LI is in line with the tight association existing between oncogenic proliferative signals and the induction of host cell apoptosis [8]. This association appears to apply for the initiation of the apoptotic procedure, since no correlation was found between proliferation indices and active caspase-3. It is of note that in this study only intensely labeled nuclei were recorded as PCNA-positive, based on the experience that not all PCNA stained nuclei are acceptable as belonging to proliferating cells. Thus, the absence of any statistically significant association with the remaining proliferation indices examined in this study may support previous observations that in meningiomas, PCNA is a more accurate marker of the S-phase when intensely-labeled nuclei among cycling cells are taken into account [20]. However, the single relation of CPP32 with PCNA and the mitotic index, although a substantial number of proliferation indices were evaluated, does not allow any firm conclusions to be drawn on the interaction between cell proliferation and caspase-3 related apoptosis, inasmuch as no such relations were seen with active caspase-3.

Relation between caspase-3 expression and hormone receptors

In a previous study, we reported an association between high levels of apoptotic death through ssDNA immunohistochemistry and negative PR status, as described in breast carcinomas [24, 35]. This association was not confirmed in the present study based on caspase-3 immunohistochemistry. This negative finding parallels the results of Vakkala et al. [36] in breast carcinomas, reporting no association between the expression of caspases 3, 6 and 8 and the estrogen and progesterone receptor status, suggesting that their synthesis is not influenced by stimulation of these receptors. Moreover, sreroid-hormone stimulation has been shown to inhibit apoptosis through up-regulation of bcl-2 m-RNA [36]. However, this correlation which is typical of breast carcinomas [37] has not been observed either in the meningiomas of our series or in those of other investigators [38]. Besides, as previously mentioned, in this study no association between bcl-2 and caspase-3 was established.

Prognostic significance of caspase-3 expression

Much advance has been made regarding progression and recurrence-related alterations in meningiomas [39, 40]. A number of cytogenetic aberrations are associated with meningioma recurrence and atypical or anaplastic histology, including the presence of dicentric or ring chromosomes, losses of chromosome arms 1p, 6q, 9p, 10, 14q and 18q, as well as gains/amplifications on 1q, 9q, 12q, 15q, 17q and 20q (reviewed in ref. [39, 40]). In particular, -14q has been associated with benign recurring meningiomas [39]. These investigations have thrown light to the biological events leading to recurrence and progression of these tumors [39, 40]. Nevertheless, biologically meaningful immunohistochemical markers, easily applicable to routine laboratory diagnostic work, still need to be identified and eventually provide prognostic information on this intriguing and challenging group of tumors of the CNS, characterized by the frequent discordance between histology and clinical behavior. Indeed, in our series, over 50% of recurrences were observed among meningiomas demonstrating an otherwise benign histology. Of note also that in our material meningiomas were macroscopically and radiologically totally resected, in order to isolate the factors leading to recurrence from the influence of partial removal. Thus, the most interesting finding of this study lies on the results drawn by the survival analysis. Taken into consideration the limitations due to the low number of recurrences in this consecutive cohort, our results indicate a prognostic role of caspase-3 expression in tumor recurrence. In univariate analysis, patients with positive caspase-3 immunoreactivity demonstrated a significantly shorter disease-free survival compared to caspase-3 negative patients. This association was established using a cut off value of 0%, which practically means that even a few positive cells suffice to draw prognostically useful conclusions. This also implies applicability and low interobserver variability in case of future use of caspase-3 immunohistochemistry in the lab routine. Previous investigations of our group [5, 23] had focused on the two other emerging prognosticators, ssDNA and mitosin, the predictive value of which is also confirmed in this study. In multivariate analysis, caspase-3 immunoreactivity emerged as an independent predictor of early recurrence. Our finding of adverse prognostic influence of caspase-3 protein expression is strengthened on one hand by the rare previous studies investigating the impact of apoptosis on meningioma

recurrence [4–6, 41] and, on the other hand, by previous studies investigating the prognostic significance of caspase-3 immunoreactivity in other tumor types [42]. Despite the controversy surrounding the prognostic value of apoptosis in various tumor subgroups, it appears that in meningiomas consensus has been gained on the adverse prognostic impact of the apoptotic activation [4–6]. The latter may contribute to recurrence by facilitating the selection of cells with an increased ability to survive. Alternatively, genetic instability may be a common link between aggressive clinical behavior and enhanced apoptosis. Meningiomas with genetic aberrations, shown to be more prone to recurrence [39, 40], are possibly more likely to produce cells undergoing apoptosis.

Conclusion

This is the first time that caspase-3 expression, detected either as the full protein or as the cleaved active form, is investigated in meningiomas. The results of the present and our previous studies indicate that apoptosis in meningiomas has a prognostic value. The present findings may have clinical relevance by identifying a biologically meaningful value for caspase-3 not only as a marker of recurrence, but also as a possible therapeutic target. Prospective studies including large numbers of patients are warranted to establish the potential use of caspase-3 in the development of therapeutic modalities for meningiomas.

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