

## **Different Activation of Mitogen-Activated Protein Kinase and Akt Signaling Is Associated with Aggressive Phenotype of Human Meningiomas**

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**Abstract Purpose:** Activation of intracellular signaling cascades has been implicated in the growth control of benign meningiomas, but their role for meningioma progression and outcome is unknown. Here we determined the expression and function of proteins involved in mitogen-activated protein kinase (MAPK) and phosphoinositol-3 kinase (PI3K)/Akt signaling in benign, atypical, and malignant meningiomas and studied their association with clinicopathologic data including meningioma recurrence.

**Experimental Design:** Expression of various MAPK and PI3K signaling proteins was determined in 70 primary meningiomas and, if present, in recurrent tumors by immunohistochemistry and Western blotting. The expression patterns in primary and recurrent tumors were related to clinical data. The effect of MAPK and PI3K pathway inhibition on cell proliferation and apoptosis was determined using a primary malignant meningioma cell culture.

**Results:** Atypical and malignant meningiomas showed higher levels of phospho-Akt compared with benign tumors, and their proliferation could be inhibited by PI3K blocking using wortmannin. PI3K inhibition did not induce apoptosis in malignant meningioma cells. In contrast, expression of phospho-Raf and phospho-MAPK was decreased in aggressive meningiomas compared with benign tumors, but MAPK inhibition by PD98059 resulted in tumor cell apoptosis and decreased proliferation. Reduced MAPK activation was associated with meningioma recurrence, and PI3K activation was associated with poor preclinical condition and brain invasion of malignant meningiomas.

**Conclusions:** Both MAPK and PI3K/Akt pathways are activated at different levels in benign and malignant meningiomas. Activation of PI3K/Akt signaling contributes to the aggressive behavior of malignant meningiomas, whereas MAPK activation is involved in both proliferation and apoptosis of malignant meningiomas.

Meningiomas are among the most frequent tumors of the brain and spinal cord, accounting for 15% to 20% of all central nervous system tumors (1). According to the recent WHO classification of brain tumors, they are graded as benign meningiomas, covering 13 histologic variants, as well as atypical or anaplastic meningiomas (2). Usually, the benign (WHO grade 1) forms of these neoplasms are associated with a relatively good prognosis, but the recurrence rate, 15% to 25% within 10 years, is rather high (3).

About 20% of the cases are graded as atypical (WHO grade 2) or anaplastic (WHO grade 3) meningiomas (4). These tumors are histologically characterized by frequent mitoses, high nuclear to cytoplasmic ratio, a patternless or sheet-like growth pattern, and foci of necrosis (5). The atypical and anaplastic tumors exhibit a more aggressive clinical behavior and recurrence rate than the benign meningiomas. Atypical meningiomas recur in about 29% to 40%, and the recurrence rate of anaplastic meningiomas is raised up to 50% to 78% (6–8). It has been shown that the prognosis of meningioma patients is significantly affected by the extent of surgical resection. In addition, the proliferation rate of the tumors as determined by MIB-1, DNA topoisomerase II, or cyclin A immunostaining has been shown to predict recurrence probability and recurrence-free survival (9). Other predictive markers that have been proven to be useful are proliferating cell nuclear antigen and bromodeoxyuridine labeling (10, 11).

However, even within the benign meningiomas, there is a wide heterogeneity in the outcomes of the patients which cannot be accounted for by clinical or pathologic variables. To overcome this shortcoming, recent studies have used gene expression profiling to identify genes that are differentially expressed between benign and malignant meningiomas (12).

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However, it would be useful to determine additional prognostic markers that can be easily applied to routine surgical meningioma samples.

The response of tumor cells to growth factors and other mitogens is mediated by specific receptors, including protein tyrosine kinase- and G protein-coupled receptors. In response to stimulation, these receptors are activated and initiate intracellular signaling events. Growth factor receptors such as epidermal growth factor receptor (13) are known to be overexpressed in human meningiomas. Coexpression of platelet-derived growth factor (PDGF) and PDGF receptor in meningiomas indicates an autocrine or paracrine stimulation of meningioma growth (14). Following receptor binding, the signals are transduced intracellularly via phosphorylation of members of the mitogen-activated protein kinase (MAPK) cascade (13, 15), resulting in enhanced meningioma cell proliferation. Recent studies have shown that beside the MAPK signaling pathway, another route involving the phosphoinositol-3 kinase (PI3K)/Akt pathway seems involved in the control of meningioma cell proliferation in response to transforming growth factor  $\beta$  (16).

However, thus far, the detailed contribution of these signaling pathways to the biology of atypical and malignant meningiomas has not been studied. Moreover, it is unknown if the activation of a certain signaling pathway contributes to the tendency for tumor recurrence in meningiomas. In the present study, we analyzed benign, atypical, and anaplastic meningiomas for the activation of the MAPK and PI3K/Akt pathways and determined their contribution to meningioma cell proliferation in cell culture studies. We also correlated the expression of signaling proteins with clinical features of the patients, including tumor recurrence.

## Materials and Methods

**Tumor material.** A total of 116 paraffin blocks from 70 adult patients with meningioma were retrieved from the files of the Neuropathology Department at the Otto-von-Guericke University, Magdeburg, Germany. The patients included 52 women and 18 men. The mean age at diagnosis was  $61.1 \pm 14.4$  and  $53.3 \pm 18.4$  years, respectively. Specimens were collected between March 1990 and July 2002. Cases with neurofibromatosis and embolized tumors were not included. All patients gave written consent to use tumor material and additional clinical data for research purpose.

Tumors were classified according to the WHO criteria (2) into the various subtypes of benign meningiomas and atypical or anaplastic meningiomas by evaluation of H&E-stained tissue sections. The clinical data of patients from each meningioma subgroup are given in Table 1.

The preoperative clinical condition was assessed according to Kallio et al. (17): class I, patients without neurologic symptoms; class II, patients with slight neurologic deficits; class III, patients with major neurologic deficits requiring assistance in daily living activities; and class IV, patients requiring acute hospital care because of impaired consciousness and/or placement to satellite health facilities in the postoperative stage.

**Immunohistochemistry.** The tumor specimens were routinely formalin-fixed and paraffin-embedded. For immunohistochemistry, 4- $\mu$ m-thick sections were deparaffinized with xylene for 15 minutes and dehydrated through a series of graded alcohols. Sections were pretreated in a microwave oven using 0.01 mol/L sodium acetate buffer (pH 6.0) for  $3 \times 10$  minutes. Endogenous peroxidase activity was blocked by incubation (30 minutes) in 0.3%  $H_2O_2$  in methanol. The sections were gently rinsed with TBS buffer and incubated with bovine

serum albumin for 30 minutes to reduce nonspecific antibody binding. Sections were incubated with monoclonal antibodies against MIB-1 (clone Ki-S5; DAKO, Hamburg, Germany; dilution 1:50), Ras (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100), phospholipase  $C\gamma 1$  (PLC $\gamma$ ; Santa Cruz Biotechnology; 1:100), phospho-MAPK (pMAPK, clone E10; Cell Signaling, Beverly, MA; 1:100), and polyclonal antibodies against the PDGF (Calbiochem, La Jolla, CA; 1:50), phospho-Raf (pRaf; 1:100), and phosphorylated Akt (pAkt; both from Cell Signaling; 1:50) for 60 minutes at 37°C in a humidified chamber. Negative controls included omission of the primary antibody and its substitution by an irrelevant mouse monoclonal antibody. The signal was detected using the streptavidin-biotin-peroxidase complex method according to the manufacturer's recommendation (DAKO). 3,3'-Diaminobenzidine hydrochloride containing 0.08% hydrogen peroxide was used as a chromogen to visualize the peroxidase activity. Finally, the sections were counterstained with hematoxylin.

**Evaluation of immunostaining.** The immunoreaction of the antibodies was evaluated by two independent observers (C.M. and T.S.) and tumors were grouped as immunopositive or immunonegative, with separation into cytoplasmic and/or nuclear staining patterns. To assess the proliferation activity, the Mib-1 labeling index (%) was calculated by determining the number of immunopositive nuclei among 100 tumor cells per high power field ( $\times 400$ ) in a total of 10 high power fields.

**Apoptosis detection by the terminal deoxynucleotidyl transferase-mediated nick end labeling method.** Apoptosis was detected using the *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 4- $\mu$ m-thick paraffin sections were mounted on glass slides and treated with xylene for 5 minutes and in 100%, 95%, and 75% ethanol. The deparaffinized tissue sections were incubated with proteinase K (2 mg/mL) at room temperature for 15 minutes. After PBS washing, endogenous peroxidase was blocked by the addition of 3%  $H_2O_2$ . Sections were then treated with terminal deoxynucleotidyl transferase and biotinylated dUTP. This step was followed by incubation with anti-digoxigenin-peroxidase for 30 minutes, and color development with  $H_2O_2$  and diaminobenzidine for 3 to 6 minutes. Then the slides were counterstained with hematoxylin and coverslipped. For positive control, thyroid tissue was used which typically shows chromatin fragmentation in the epithelium labeled by terminal deoxynucleotidyl transferase-mediated nick end labeling. Negative controls were done by omission of terminal deoxynucleotidyl transferase from the incubation buffer.

**Western blot analysis.** Fresh samples from surgically removed meningiomas were snap-frozen in liquid nitrogen and stored at  $-70^\circ C$  until further processing. The tumor tissue was homogenized 2 mL lysis buffer [20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1  $\mu$ g/mL leupeptin, and 10  $\mu$ g/mL aprotinin], homogenized, and incubated on ice for 30 minutes. After centrifugation at 13,000 rpm for 15 minutes, the supernatant was collected to measure the total protein content. The amount of protein was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Twenty micrograms of protein were loaded on 10% SDS-polyacrylamide gels for electrophoresis. After separation, proteins were transferred to a nitrocellulose membrane (Hybond C, Amersham Pharmacia Biotech, Freiburg, Germany) at 150 mA for 2 hours. Western blot analysis was done after blocking of the membrane with 5% skim milk in TBST buffer for 1 hour. The membrane was incubated with the specific antibody at 4°C overnight. The membrane was washed four times in TBST buffer. Secondary detection was done using horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G (1:2,000; Amersham Pharmacia Biotech). After four times washing with TBST, horseradish peroxidase activity was visualized by applying enhanced chemiluminescent substrate (Amersham Pharmacia Biotech) followed by exposure

**Table 1.** Clinicopathologic data of 70 primary meningioma samples

Meningioma subtype*	No. tumors (%)	Mean age (y)	No. men (%)	Preoperative condition <sup>†</sup> [n (%)]	Mib-1 LI (%)	No. recurrences (%)	Brain invasion no. (%)
Benign (WHO 1)	47 (67)	58.7	7 (15)	I: 1 (2)	5.7 ± 4.4	9 (19)	0
Meningothelial	10 (14)			II: 21 (45)			
Transitional	16 (23)			III: 17 (36)			
Fibroblastic	8 (11)			IV: 8 (17)			
Psammomatous	7 (10)						
Angiomatous	3 (4)						
Metaplastic	1 (1)						
Secretoric	1 (1)						
Microcystic	1 (1)						
Atypical (WHO 2) <sup>‡</sup>	12 (17)	67.0	5 (42)	I: 2 (17)	13.6 ± 13.2	8 (67)	2 (17)
				II: 4 (33)			
				III: 2 (17)			
				IV: 4 (33)			
Anaplastic (WHO 3)	11 (16)	51.7	6 (55)	I: 1 (9)	24.9 ± 15.7	9 (82)	7 (64)
				II: 1 (9)			
				III: 5 (46)			
				IV: 4 (36)			

\*Graded according to the recent WHO classification of brain tumors (2); No. = number of cases.

<sup>†</sup>Preoperative clinical condition as determined after Kallio et al (17); LI = labeling index.

<sup>‡</sup>Including one clear cell meningioma.

of the membrane to X-ray film. Equal protein loading was confirmed by reprobing the membranes with anti-actin antibody (Sigma, St. Louis, MO) following antibody stripping using the Restore Western Blot Stripping Buffer (Pierce).

**Cell culture.** A sample of a malignant meningioma was placed immediately after surgical tumor removal into DMEM (high glucose, PAA, Austria), supplemented with the antibiotics penicillin, streptomycin and fungizone and pressed through a sterile 200-µm steel mesh. The resulting material was incubated 24 hours at 37°C and 5% CO<sub>2</sub> in a T75-cell culture flask together with 20 mL of DMEM supplemented with penicillin, streptomycin, and 10% heat inactivated FCS. After removal of debris and nonadhering cells, the remaining adherent cells were further cultivated under the same conditions until 80% confluency was reached. Following trypsinization and PBS washing, they were seeded into 96-well microtiter plates in a density of 3,000 cells per well for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays or in 16-well chamber slides in a density of 20,000 cells per well for the <sup>3</sup>H-thymidine assays. All freshly seeded cells were initially grown for 24 hours in the original cell culture medium before the start of the experiments.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.** The microtiter plates were subdivided into two sections, which were designated "serum" and "serum-free". Cells of the first section were all treated for 6 days with normal cell culture medium containing either 10 µmol/L of the MAPKK-inhibitor PD 98059, 3.5 µmol/L of the PI3K inhibitor wortmannin (both from Sigma), the corresponding concentrations of the solvent DMSO or no treatment at all. All treatments were represented 8-fold. Cells of the serum-free section were treated in the same way with one exception: the medium in all wells initially contained no serum, which was added after 48 hours of serum-free culture to a final concentration of 10% FCS. After a total incubation time of 6 days, all media were replaced by DMEM containing 0.75 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (Sigma) and the plates were further incubated at 37°C to allow tetrazolium reduction to a blue formazane dye. After 2 hours, the medium was replaced by DMSO, which leads to cell lysis and solubilization of the

formazane dye. The absorbance at 562 nm (reference wavelength, 620 nm) was read using an Anthos-2010-ELISA-Reader (Anthos, Krefeld, Germany). After subtraction of a background control (cell-free medium), the number of viable cells was compared with the completely untreated controls, which were set to 100%. This was done to get an overview about general toxicity of the treatments, including solvent toxicity. To determine the specific antiproliferative effects of the various inhibitor treatments (as shown in the figures), the values were normalized to the corresponding DMSO controls.

**<sup>3</sup>H-thymidine proliferation assay.** We used the measurement of the DNA synthesis rate by determining the <sup>3</sup>H-thymidine incorporation as a variable reflecting mitotic activity as described previously (18). Cells were seeded in 96-well plates at a density of 20,000 cells per 200 µL per well in the presence or absence of 10 µmol/L PD98059, 3.5 µmol/L wortmannin, or solvent (DMSO) only. After 42 hours of culture at 37°C, 5% (v/v) CO<sub>2</sub>, cell cultures were pulsed for an additional 6 hours with <sup>3</sup>H-methyl-thymidine (0.2 µCi per well, GE Healthcare, Braunschweig, Germany). Cells were harvested onto glass fiber membranes, and the incorporated radioactivity was measured by scintillation counting. In each case, DNA synthesis was assessed six times in parallel.

**Cytotoxicity assay.** The LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR) was used to study cytotoxic activity of the tyrosine kinase inhibitor imatinib (STI571, Glivec) which primarily inhibits Bcr-Abl, PDGF and c-Kit tyrosine kinase receptors, against malignant meningioma cells by fluorescence microscopy. Cells were cultured in 4-well Lab-Tek Chamber-Slide (Nunc, Naperville, IL) at a density of 20,000 cells per 500 µL per chamber in presence or absence of 10<sup>-7</sup> mol/L Glivec. After 24 hours, the cells were washed twice in PBS (pH 7.4) and suspended in 200 µL of the same buffer. Then 0.2 µL ethidium homodimer-1 [2 mmol/L in 25% (v/v) DMSO and 0.1 µL calcein AM (4 mmol/L) in DMSO] was added to each well and cells were incubated for 30 minutes at 37°C. Cells were examined by fluorescence microscopy using an Axiovert 135 TV (Carl Zeiss Jena, Jena, Germany) at 20× magnification and the optical filter set No. 23 (Carl Zeiss Jena).

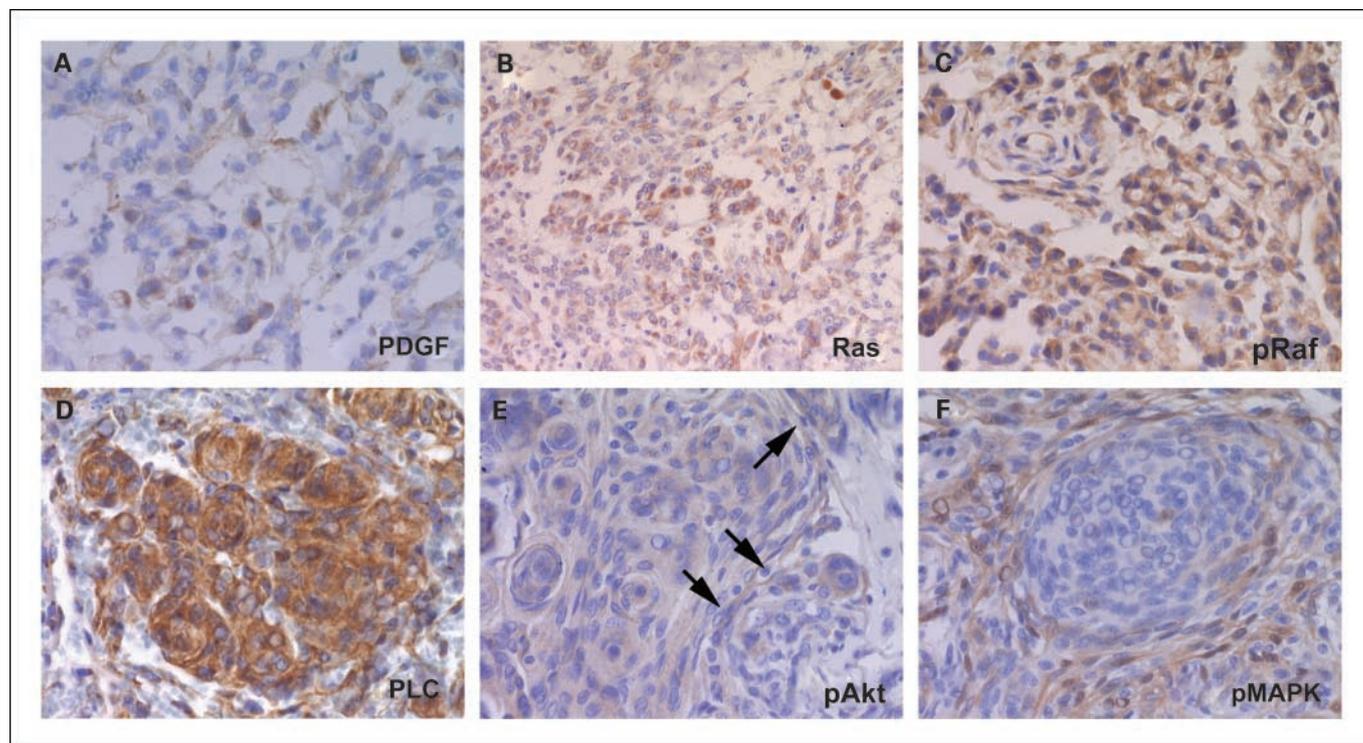
**Statistical analysis.** For the statistical analysis, we coded the immunostainings of signaling proteins as positive or negative. Then the rate of positive findings was compared between subgroups of patients defined by patient's sex, presence or absence of tumor recurrence, preoperative clinical symptoms according to Kallio et al. (17), and presence or absence of brain infiltration. For this purpose, a  $\chi^2$  test was used with the option of using the finite sample distribution. For those subgroups corresponding to an ordinal scale (i.e., preoperative clinical symptoms), a  $\chi^2$  test for linear association was used. For tumor grading, the global tests with all three WHO grades were completed by pairwise comparisons (also  $\chi^2$  tests). In this special case with three subgroups, the closure test principle ensures the family-wise error rate  $\alpha$  for all pairwise comparisons, if these pairwise comparisons are carried out only after the global test gave significant results. Analyses for an association between immunoreexpression and time to tumor recurrence were carried out using the Mann-Whitney *U* test. Significance level in all tests was  $\alpha = 0.05$  (two sided). All analyses are exploratory and were carried out with SPSS, version 11.0.1.

## Results

**Expression of intracellular signaling proteins in primary meningiomas.** At first, we screened 70 primary meningioma samples of different histologic subtype and malignancy (for clinicopathologic data, see Table 1) for the expression of intracellular signaling proteins known to be involved in the transduction of PDGF receptor signaling (19, 20). Representative immunostainings are shown in Fig. 1. As summarized in Table 2, nearly all benign meningiomas WHO grade 1 showed strong immunoreexpression of PDGF, Ras, pRaf, and pMAPK. These findings of a general activation of the MAPK signaling

pathway in benign meningiomas were confirmed by Western blot studies (Fig. 2). However, compared with benign meningiomas, the frequency of tumors immunopositive for Ras, pRaf, and pMAPK was decreased in atypical (WHO grade 2) and anaplastic (WHO grade 3) meningiomas (Table 2; Fig. 2). This suggests that these meningioma subtypes might activate other signaling pathways in addition to the MAPK pathway. The percentage of tumors immunopositive for PLC $\gamma$ , a protein which is proposed to transmit PDGF receptor mediated cell proliferation via the PI3K signaling pathway (19), was not significantly different between benign and malignant meningiomas (Table 2). However, we observed that the immunoreexpression of the pAkt, which is an essential factor for the PI3K-mediated cell proliferation, as well as for the inhibition of apoptosis (21), was significantly more frequent among malignant meningiomas compared with benign and atypical meningiomas (Table 2; Fig. 2).

**Inhibition of mitogen-activated protein kinase and Akt has different effects on survival and proliferation of malignant meningioma cells.** Based on our findings of an increased immunoreexpression of pAkt in malignant meningiomas compared with benign meningiomas (and vice versa for pMAPK), we hypothesized that the influence of PI3K and MAPK pathway activation on tumor cell proliferation and fate differs between benign and malignant meningiomas. To further study the contribution of both signaling pathways to malignant meningioma proliferation, we administered specific MAPK and PI3K inhibitors to primary malignant meningioma cells cultured under serum starvation or with media containing 10% FCS. As shown in Fig. 3A, the administration of the



**Fig. 1.** Immunoreexpression of intracellular signaling proteins involved in signal transduction via the MAPK and PI3K pathways in human meningiomas. *A*, expression of PDGF in a malignant meningioma, showing predominant membranous staining. *B*, strong cytoplasmic Ras expression in a malignant meningioma. *C*, benign meningioma with pRaf expression. *D*, strong PLC $\gamma$  expression within whorls of a benign meningioma. *E*, pAkt expression in benign meningothelial meningioma showing both diffuse cytoplasmic staining and additional increased immunoreactivity of plasma membranes (arrows). *F*, benign meningothelial meningioma with both nuclear and cytoplasmic expression of pMAPK. Note that the center of the meningothelial whorl is devoid of staining (*A* and *C-F*; original magnification  $\times 400$ ; *B*,  $\times 200$ ).

**Table 2.** Summary of immunohistochemical results from primary meningioma samples

Meningioma subtype	PDGF (%)	Ras (%)	PRaf (%)	pMAPK (N), (%)	pMAPK (C), (%)	PLC $\gamma$ (%)	pAkt (N), (%)	pAkt (C), (%)
Benign ( <i>n</i> = 47)	46 (97)	34 (72)	46 (97)	40 (85)	41 (87)	36 (77)	5 (11)	23 (49)
Atypical ( <i>n</i> = 12)	11 (92)	12 (100)	9 (75)*	11 (92)	10 (83)	9 (75)	1 (8)	4 (33)
Anaplastic ( <i>n</i> = 11)	10 (91)	9 (81)	8 (73)*	6 (55)*,†	6 (55)*	10 (91)	5 (45)*	7 (64)

NOTE: The number and percentage of immunopositive tumors are given. Comparisons between meningioma subtypes were performed based on the calculation of a significant overall difference for the factor WHO grade (see Materials and Methods). The  $\chi^2$  test was used for all comparisons.

Abbreviations: N, nucleus; C, cytoplasm.

\*Significant difference to the benign meningioma group with *P* < 0.05.

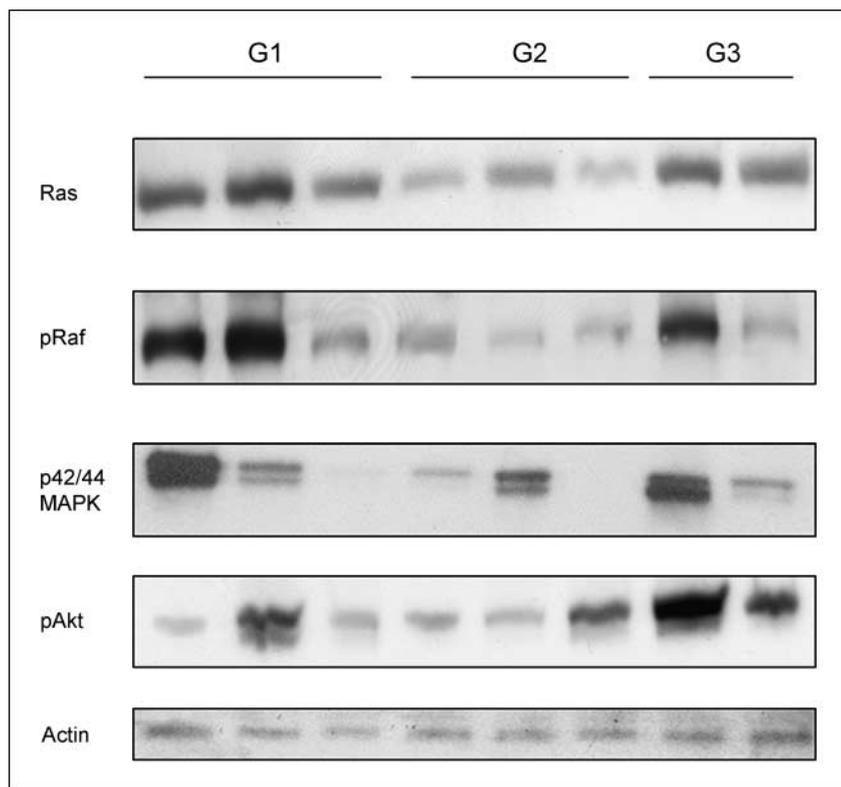
†Significant difference to the atypical meningioma group with *P* < 0.05.

MAPK inhibitor PD98059 reduced the cell viability to about 35% to 40%, independently of serum conditions. Interestingly, administration of wortmannin, a specific PI3K inhibitor, reduced the cell viability to about 70% only if the cells were grown with serum. In a parallel experiment, cells were used which had been grown in the absence of serum for 48 hours with or without wortmannin and were then serum stimulated for the rest of the culture period. In these "serum-starved" cells, no major difference was observed between wortmannin-treated and untreated cells. This result indicates that the slowly proliferating serum-starved cells do not depend on PI3K signaling to the same extend as actively proliferating cells, suggesting that highly proliferating malignant meningioma cells substantially depend on the activation of the PI3K signaling pathway. Additional analyses of the DNA synthesis rate (using fully proliferating meningioma cells in standard medium with serum) showed that both PD98059 and

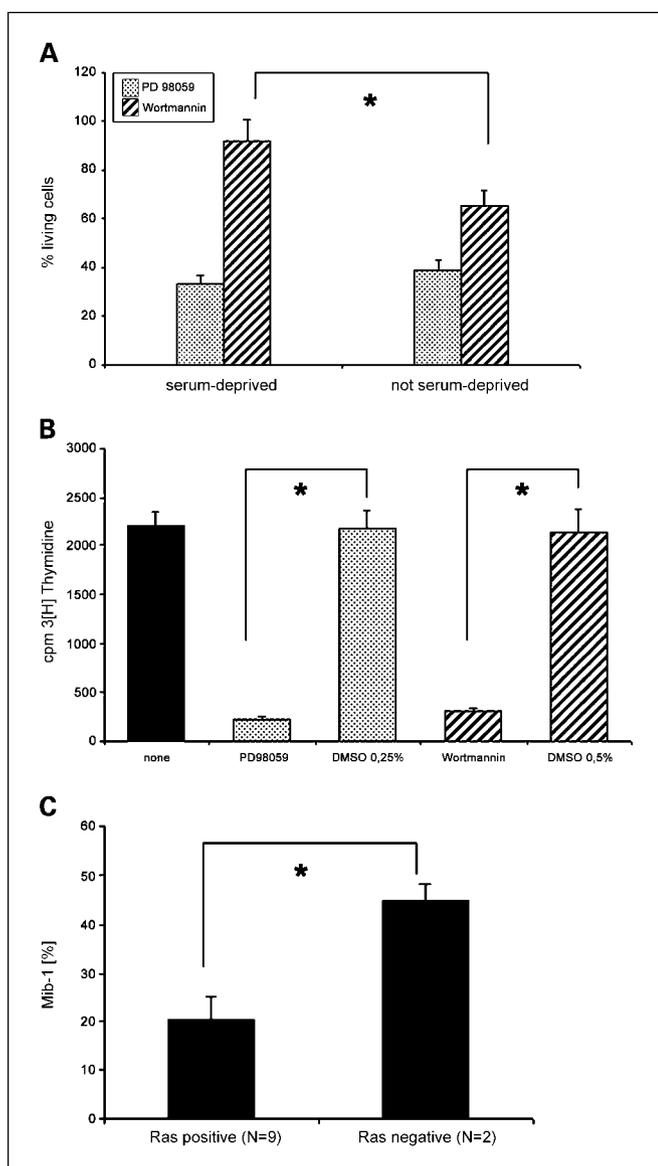
wortmannin substantially reduced the proliferation of meningioma cells (Fig. 3B).

Moreover, whereas no clear association was found between the proliferation rate (as determined by Mib-1 immunostaining) and the expression of the majority of MAPK-associated signaling proteins, we observed that malignant meningiomas lacking Ras expression had a significantly higher proliferation activity compared with Ras-positive malignant meningiomas (Fig. 3C).

Besides the control of cell proliferation, the PI3K pathway is also significantly involved in a negative regulation of apoptotic cell death. The increase of pAkt protein might thus contribute to a low apoptotic rate in malignant meningioma. Screening of 5 benign, 5 atypical, and 5 anaplastic meningiomas for apoptotic cell death using terminal deoxynucleotidyl transferase-mediated nick end labeling staining revealed that none of the benign and atypical meningiomas showed terminal



**Fig. 2.** Western blot analyses of primary meningioma samples from benign meningiomas WHO grade 1 (G1), atypical meningiomas WHO grade 2 (G2), and anaplastic meningiomas WHO grade 3 (G3) for expression of signaling proteins involved in the MAPK and PI3K pathway. Twenty micrograms of protein were loaded, and equal loading was confirmed by reprobing with actin antibody after stripping of the membrane.



**Fig. 3.** A, reduction of malignant meningioma cell number in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after treatment with 10  $\mu\text{mol/L}$  of the MAPK inhibitor PD98059 or 3.5  $\mu\text{mol/L}$  of the PI3K inhibitor Wortmannin. Cells were grown with or without serum starvation (for details, see Materials and Methods). B, inhibition of DNA synthesis after treatment with 10  $\mu\text{mol/L}$  PD98059 or 3.5  $\mu\text{mol/L}$  Wortmannin in malignant meningioma cells cultured in standard medium with serum supplementation. All experiments were done in triplicate. C, association between the proliferation activity in malignant meningiomas as determined by the Mib-1 labeling index, and expression of Ras protein. \*,  $P < 0.05$ .

deoxynucleotidyl transferase-mediated nick end labeling-positive tumor cell nuclei. In malignant meningiomas, only two of five tumors occasionally showed apoptotic tumor cells (Fig. 4A). Malignant meningiomas have a high proliferation activity as evident from Mib-1 immunostaining (Fig. 4B). Analysis of malignant meningioma cell death using the LiveDead assay (Fig. 4C) showed an increased number of propidium iodide-positive cells after PD98059 administration (bottom left) compared with untreated controls (top left). In contrast, inhibition of the PI3K pathway by wortmannin (bottom right) did not result in the induction of apoptotic cell death.

**Influence of mitogen-activated protein kinase and Akt activation on clinical course and meningioma recurrence.** To evaluate the effect of signaling protein expression on the clinical course of the tumor disease, we correlated the immunohistochemical data with basic clinical features of patients with primary meningiomas. The presence or absence of immunorexpression for PDGF, Ras, pRaf, pMAPK, PLC $\gamma$ , and pAkt was related to the patient's sex, presence of tumor recurrence, preoperative clinical condition according to Kallio et al. (17), and the presence of brain infiltration.

The rate of tumor recurrence was significantly higher, in cases where pRaf expression was not detected ( $P = 0.001$ ;  $\chi^2$  analysis), because all seven tumors immunonegative for pRaf had tumor recurrence (100%). In contrast, among 63 meningiomas immunopositive for pRaf, only 19 cases (30%) had recurrent tumors, whereas the remaining 44 cases (70%) had no recurrent tumor. The same association was observed for the downstream target of pRaf, pMAPK. Among 13 cases with absent nuclear pMAPK expression, nine cases (69%) had a recurrent tumor. In contrast, the recurrence frequency among the 57 pMAPK (N)-positive cases was only 20% (17 tumors). This difference was statistically significant ( $P = 0.012$ ). No significant associations were calculated between tumor recurrence and expression of the remaining signaling proteins.

The preoperative clinical condition was found to be significantly correlated to PLC $\gamma$  and pAkt. For PLC $\gamma$ , among 15 immunonegative cases, six (40%) were evaluated as having poor preoperative clinical condition (class III and IV), whereas among the 55 PLC $\gamma$ -positive tumors, 34 (62%) had poor clinical condition. However, the frequency of malignant (WHO grades 2 and 3) meningiomas was higher in the latter group compared with the PLC $\gamma$ -negative tumors (49% versus 27%). This difference is most likely the cause for the significant relation between preoperative clinical condition and PLC $\gamma$  expression ( $P = 0.020$ ). For cytoplasmic pAkt expression, the significant influence ( $P = 0.004$ ) was caused by the fact that patients grouped as having good preoperative clinical condition (class I-II) were characterized by the lack of pAkt (C) expression (20 tumors: 65% grade 1, 30% grade 2, 5% grade 3); among pAkt (C)-positive tumors, all patients were grouped in class II (10 tumors: 90% grade 1, 10% grade 3). In contrast, among cases grouped as having poor clinical condition (class III-IV), there were no differences regarding the distribution of WHO grading between pAkt (C)-negative and -positive tumors (pAkt (C)-negative tumors [ $n = 16$ ]: 69% grade 1, 13% grade 2, 19% grade 3; pAkt (C)-positive tumors [ $n = 24$ ]: 58% grade 1, 17% grade 2; 25% grade 3). Other signaling proteins examined showed no statistically significant association with the preoperative clinical condition.

A significant association between pAkt expression and the presence of brain infiltration ( $P = 0.029$ ) was based on the fact that the latter was only noted in high-grade meningiomas (two grade 2 and seven grade 3). Among these tumors, no grade 2 meningioma was pAkt positive, whereas four malignant meningiomas with brain infiltration (57%) were pAkt positive. The expression of the remaining proteins were not significantly associated with meningioma brain infiltration. In addition, the patient's sex was not found significantly related to the expression of any of the signaling proteins examined.

Finally, we asked if the presence or absence of MAPK protein immunorexpression does affect the time to meningioma tumor

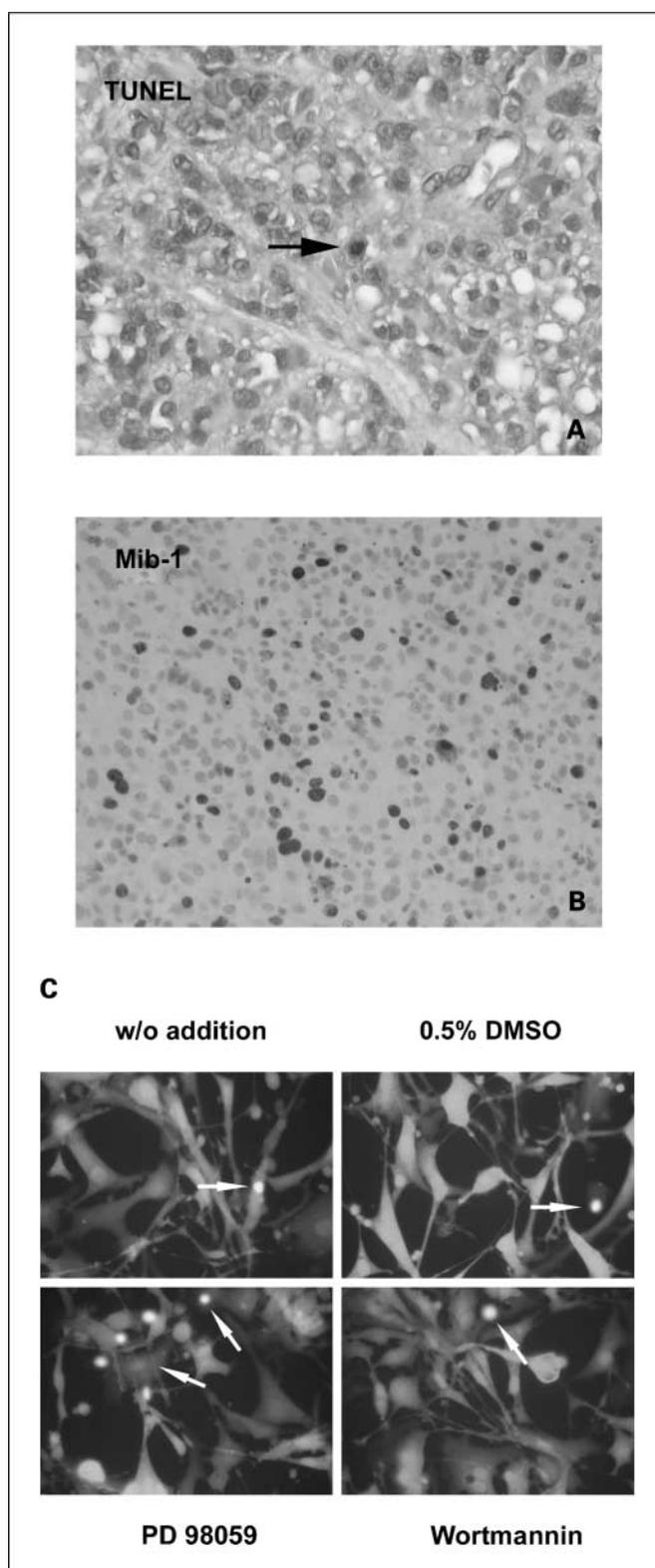
recurrence. However, we could not find associations between the time period until tumor recurrence and expression of any protein examined in this study.

### Discussion

In the present study, we have found that the activation of factors involved in MAPK- and Akt-associated intracellular signaling differs between benign and malignant meningiomas. We also showed that Akt activation is prominent in malignant meningiomas and it affects tumor cell growth in these tumors. Furthermore, the activation of MAPK and Akt signaling seems to have different influences on clinicopathologic factors like tumor recurrence and brain invasion of meningiomas.

It has been well established that the growth factor-mediated activation of intracellular signaling cascades contributes to meningioma proliferation (13, 15, 16, 22). However, most of these studies investigated benign WHO grade 1 tumors or primary cell cultures derived from such tumors, but the role of signaling cascade activation in atypical or malignant meningiomas remains to be determined. Furthermore, the knowledge about molecular differences between benign and malignant meningiomas is very limited. Besides the known association of NF2 alterations in meningioma development, recent molecular approaches has been shown that the gene expression profile differs between benign and malignant meningiomas (12). Additional differences have been reported for tumor suppressor in lung cancer-1 (23) expression and chromosome 9p21 deletions (24).

The present study revealed that activation of the PI3-kinase/Akt pathway seems a distinct feature of atypical and anaplastic meningiomas. The PI3K/Akt pathway is involved in the regulation of various cellular processes, such as proliferation, growth, and apoptosis (reviewed in ref. 21). Growth factor stimulation and receptor activation results in Akt phosphorylation (pAkt) and subsequent activation of p70<sup>S6K</sup> via mammalian target of rapamycin. Of note, other growth-regulating targets of the mammalian target of rapamycin also include 4EBP1 and eIF4G. PDGF stimulation of DNA synthesis requires sustained activation of PI3K during the G<sub>1</sub> phase of the cell cycle (25). Deregulation of the PI3K/Akt pathway in human malignancies is commonly associated with alterations in the *PTEN* (phosphatase and tensin homologue) tumor suppressor gene. However, *PTEN* mutations are infrequent in human meningiomas (26). A role of the PI3K/Akt pathway for meningioma proliferation in response to PDGF stimulation has been recently suggested for WHO grade 1 meningiomas (16). Inositol phosphates (the substrate of PI3K) accumulate in meningioma cells after epidermal growth factor treatment (27). By detection of pAkt, we found that anaplastic and malignant meningiomas have high levels of this signaling protein. Interestingly, malignant meningiomas showed only rarely apoptotic tumor cells. We also showed that Akt inhibition by wortmannin reduced malignant meningioma cell proliferation and survival. These data implicate that the activation of the PI3K/Akt pathway might substantially contribute to aggressive meningioma features. However, although we did not observe frequent apoptosis of malignant meningioma cells after treatment with the PI3K inhibitor wortmannin, several studies indicate that activation of the PI3K/Akt pathway inhibits apoptosis (28, 29). Thus, further studies are required to elucidate the detailed effects



**Fig. 4.** A, detection of apoptotic cell death (arrow) using terminal deoxynucleotidyl transferase-mediated nick end labeling staining in malignant meningiomas ( $\times 400$ ). B, malignant meningioma characterized by a high percentage of proliferating Mib-1-immunopositive tumor cells ( $\times 200$ ). C, LiveDead assay demonstrating increased apoptosis (bright cells; arrows) after blocking of MAPK signaling by treatment of malignant meningioma cells with 10  $\mu\text{mol/L}$  PD98059 (bottom left) compared with controls. In contrast, blocking of the PI3K pathway by Wortmannin did not result in the induction of apoptosis (right). Pictures are representative for three independent experiments.

of different signaling proteins of the PI3K pathway on apoptotic cell death and proliferation in malignant meningioma cells.

We also observed that the MAPK signaling pathway is activated in meningioma tumor samples and malignant meningioma cells. MAPKs are a family of serine/threonine kinases involved in numerous cell functions including cell proliferation (30). Activation by upstream growth factor tyrosine kinases, such as the PDGF- $\beta$  receptor or the epidermal growth factor receptor (30, 31), activates Ras via an adaptor molecule (Src homology and collagen, growth factor receptor binding protein 2), leads to recruitment and phosphorylation of Raf, and phosphorylation of Erk1/Erk2 MAPK. PDGF-induced MAPK activation has been recently implicated in the growth regulation of benign meningiomas (15, 32). In our study, besides a clear antiproliferative effect of the MAPK inhibitor PD98059, we observed frequent apoptosis of malignant meningioma cells after PD98059. These data suggest that activation of the Ras/Raf/MAPK pathway has both, growth-promoting and antiapoptotic effects in malignant meningiomas. The importance of MAPK activation for meningioma growth is underlined by our finding that the recurrence of a meningioma is associated with the occurrence of pRaf and pMAPK in the nucleus. Interestingly, a simultaneous activation of both the MAPK and PI3K/Akt pathway has been proposed to be present in benign meningiomas (16). This model is supported by our observations but should be expanded to atypical and anaplastic meningiomas based on the present data.

We further observed that PLC $\gamma$  expression does not differ significantly between meningiomas with different malignancy. It has been shown previously that PLC $\gamma$  is involved in

epidermal growth factor receptor-mediated growth stimulation in benign human meningiomas (22). In contrast, the role of PLC $\gamma$  in atypical and anaplastic meningiomas has not been determined. There seems a significant role of PLC $\gamma$  in meningiomas, because it is known to be phosphorylated and activity increased by PDGF receptor (33). However, it has been shown that both the MAPK pathway (34) and the PI3K signaling pathway (19) are downstream targets of PLC $\gamma$ . This might explain the lack of expression differences between the meningioma subgroups in our study but indicates that PLC $\gamma$  activation is a common feature of meningiomas.

Finally, we looked for associations between signaling protein expression and clinicopathologic variables in benign and malignant meningiomas. Our finding that reduced activation of the MAPK pathway is associated with the recurrence of meningioma shows that other signaling pathways including the PI3K pathway are essential for meningioma growth, independent from the grade of malignancy. This might help to establish future chemotherapy for meningiomas that target this pathway. Additionally, the infiltrative and aggressive growth of malignant meningiomas might be controlled at least partly by inhibition of the PI3K/Akt pathway. Especially in malignant meningiomas, larger series need to be investigated to establish potential effects of PI3K/Akt activation and inhibition of this pathway on patient's outcome.

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