Comparative Proteomic Profiles of Meningioma Subtypes

Hiroaki Okamoto,1,3 Jie Li,1 Alexander O. Vortmeyer,1 Howard Jaffe,2 Youn-Soo Lee,4 Sven Gläsker,1 Tae-Sung Sohn,1 Weißen Zeng,3 Barbara Ikejiri,1 Martin A. Proescholdt,6 Christina Mayer,6 Robert J. Weil,1,5 Edward H. Oldfield,1 and Zhengping Zhuang1

1Surgical Neurology Branch and Protein/Peptide Sequencing Facility, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, Maryland; 2Department of Neurosurgery, Faculty of Medicine, Saga University, Saga, Japan; 3Department of Neurosurgery, Saga University, Saga, Japan; 4Department of Clinical Pathology, Cleveland Clinic Foundation, Cleveland, Ohio; 5Department of Neurosurgery, Regensburg University Medical Center, Regensburg, Germany

Abstract

Meningiomas are classified into three groups (benign, atypical, and anaplastic) based on morphologic characteristics. Atypical meningiomas, which are WHO grade 2 tumors, and anaplastic meningiomas, which are WHO grade 3 tumors, exhibit an increased risk of recurrence and premature death compared with benign WHO grade 1 tumors. Although atypical and anaplastic meningiomas account for <10% of all of meningiomas, it can be difficult to distinguish them from benign meningiomas by morphologic criteria alone. We used selective tissue microdissection to examine 24 human meningiomas and did two-dimensional gel electrophoresis to determine protein expression patterns. Proteins expressed differentially by meningiomas of each WHO grade were identified and sequenced. Proteomic analysis revealed protein expression patterns unique to WHO grade 1, 2, and 3 meningiomas and identified 24 proteins that distinguish each subtype. Fifteen proteins showed significant changes in expression level between benign and atypical meningiomas, whereas nine distinguished atypical from anaplastic meningiomas. Differential protein expression was confirmed by Western blotting and immunohistochemistry. We established a genetic model of progression in meningiomas by accumulated alterations (de novo and secondary tumors) in which low-grade gliomas develop through a multistep process (6, 7). Generally, meningiomas are also thought to have the potential to progress from low grade to high grade, as colorectal carcinomas or secondary glioblastomas (1, 8, 9). Weber et al. documented a genetic model of progression in meningiomas by accumulated alterations (8). However, it is still unclear whether high-grade meningiomas progress from benign meningiomas, or arise primarily as more aggressive de novo tumors (9). Thus, specific markers that would reliably coincide with meningiomas of a particular grade and clinical behavior, and with pathogenetic mechanisms, would be useful.

Proteomics is a broad protein profile screening approach that permits a direct analysis of proteins differentially or uniquely expressed by a cell or a tissue type. Proteomic research has been developed recently in the post-genome era. Using a combination of two-dimensional gel electrophoresis, mass spectrometry, and bioinformatics, one can now readily scan a particular cell type and establish protein expression patterns. Recent studies have shown that specific proteomic patterns and proteins can distinguish subtypes or grades of human brain tumors (10, 11). We set out to use selective tissue microdissection to obtain a pure population of meningioma tumor cells to determine whether we could identify proteins and protein patterns that distinguish WHO grade 1, 2, and 3 meningiomas.

Materials and Methods

Patients and clinical data. Twenty-four meningiomas were collected at surgery, immediately snap-frozen in liquid nitrogen, and stored at −80°C until analyzed. Tumors were classified according to the 2000 WHO Histologic Classification (1): 10 benign meningiomas (grade 1), 9 atypical meningiomas (grade 2), and 5 anaplastic meningiomas (grade 3). Tissues and clinical information were obtained as part of an Institutional Review Board–approved study at the Cleveland Clinic Foundation, Cleveland, OH and the University of Regensburg, Regensburg, Germany. Additional independent 30 paraffin slides were obtained from the Cleveland Clinic Foundation for immunohistochemical studies, including benign (n = 10), atypical (n = 10), and anaplastic (n = 10) tumors.

Introduction

Meningiomas are common central nervous system (CNS) tumors that arise from the coverings of the brain and the spinal cord, with an incidence of between 13% and 26% of all primary intracranial tumors (1). Most meningiomas (>90%) are slowly growing benign tumors and correspond histologically to grade 1 according to the WHO classification of tumors of the CNS (1). Anaplastic meningiomas are WHO grade 3 neoplasms and are associated with a high risk for recurrence and a less favorable prognosis; fortunately, they are rare (1-2.8% of all meningiomas; refs. 1–3). An intermediate group, the atypical meningiomas (WHO grade 2), also exhibit an increased risk of recurrence and death compared with WHO grade 1 tumors (4.7-7.2% of meningiomas; refs. 1–3). However, sampling issues and imperfect histopathologic features of these tumors can make exact grading difficult. Some markers, such as vimentin, are frequently present in meningiomas (1, 4) but do not correlate specifically with grade.

The origins of atypical and anaplastic meningiomas are still unclear. It is well known that some high-grade tumors arise from low-grade tumors. For instance, Vogelstein et al. showed accumulating molecular alterations that paralleled clinical progression in colorectal tumors (5). It has also been reported that glioblastomas consist of two different types of tumors with different pathogenesis (de novo and secondary tumors) in which low-grade gliomas develop through a multistep process (6, 7). Generally, meningiomas are also thought to have the potential to progress from low grade to high grade, as colorectal carcinomas or secondary glioblastomas (1, 8, 9). Weber et al. documented a genetic model of progression in meningiomas by accumulated alterations (8). However, it is still unclear whether high-grade meningiomas progress from benign meningiomas, or arise primarily as more aggressive de novo tumors (9). Thus, specific markers that would reliably coincide with meningiomas of a particular grade and clinical behavior, and with pathogenetic mechanisms, would be useful.

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Tissue and sample preparation. Selective tissue microdissection was done, as previously described, to avoid normal brain or areas of necrosis, hemorrhage, and inflammation (10). Microdissected tissues were dissolved in extraction buffer II containing 8 mol/L urea, 4% (w/v) Bio-Lyte 4-7, and 2 mol/L tributyl phosphate (Bio-Rad, Hercules, CA); vigorously vortexed; and centrifuged in a microcentrifuge. The supernatant was combined with a rehydration buffer containing rehydration buffer [8 mol/L urea, 2% CHAPS, 50 mol/L DTT, and 0.2% (w/v) Bio-Lyte 4-7 ampholytes; Bio-Rad], IPG buffer (Amer sham Biosciences, Piscataway, NJ), and bromophenol blue and subsequently rehydrated overnight with Immobiline Drystrips (pH 4-7, 11 cm; Amer sham Biosciences) on a Reswelling Tray (Amer sham Biosciences).

Two-dimensional gel electrophoresis. Isometric focusing for the first dimensional electrophoresis was done with a Multiphor II Electrophoresis System (Amer sham Biosciences). The strips were subjected to high voltages at 300 to 3,500 V. Immobilized pH gradient (IPG) strips were equilibrated with equilibration buffer I containing 6 mol/L urea, 2% SDS, 375 mol/L Tris-HCl (pH 8.8), 20% glycerol, and 2% (w/v) DTT and buffer II containing 6 mol/L urea, 2% SDS, 375 mol/L Tris-HCl (pH 8.8), 20% glycerol, and 2.5% (w/v) iodoacetamide (Bio-Rad). Precast gels (ExcelGel SDS gradient gel; pH 4-7 12-14%, 245 × 180 × 0.3 mm; Amer sham Biosciences) were used for the second dimension of protein separation by a Multiphor II Flated System (Amer sham Biosciences) under a constant voltage of 700 V. A silver staining kit (Amer sham Biosciences) was used to detect protein spots according to the manufacturer’s instructions. All samples were run in duplicate.

Image analysis and in-gel digestion. The intensities of protein spots on two-dimensional gels were analyzed with Proteome Weaver according to the manufacturer’s protocol (Definiens, Munich, Germany). Statistically significant (P < 0.05) protein spots of interest were excised from the gel and subjected to in-gel digestion with trypsin.

Mass spectrometry. Peptides from in-gel digest were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) on a ProteomeX LC/MS system (ThermoElectron, San Jose, CA) operated in the high-throughput mode. Reversed-phase high-performance liquid chromatography was carried out using a BioBasic-18 column (0.18×150 mm; ThermoElectron) eluted at 1 to 2 μL/min with a gradient of 2% to 50% B over 30 minutes. Mobile phase A was H2O (0.1% FA), and mobile phase B was CH3CN (0.1% FA). Column effluent was analyzed on the LCQ Deca XP Plus (ThermoElectron) operating in the “Top Five” mode.

Protein identification. Uninterpreted MS/MS spectra were searched against a human database using the BioWorks and SEQUEST programs (ThermoElectron). Protein identification was accepted when the MS/MS spectra of at least two peptides from the same protein exhibited at a minimum the default Xcorr versus charge values set by the program (for Z = 1, 1.50; for Z = 2, 2.00; for Z = 3, 2.50).

Western blot analysis. Meningioma samples were homogenized in T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) with protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) and centrifuged; 40 μg of each lysate were loaded to 4%–20% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA). Proteins were electrophoretically transferred to nitrocellulose membranes (Invitrogen), blocked with Starting Block Blocking Buffer (Pierce), washed, and incubated with polyclonal antibodies against galectin-1 (1:500; R&D Systems, Minneapolis, MN), α-enolase, and RP/EB family, member 1 (EB1; 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and monoclonal antibodies against calponin, smooth muscle actin (1:200; DakoCytomation, Glostrup, Denmark), tropomyosin, and β-actin (1:500; Sigma, Saint Louis, MO). These membranes were exposed to the secondary anti-goat antibody for galectin-1, and α-enolase, anti-rabbit antibody for EB1, and anti-mouse antibody for calponin, smooth muscle actin, tropomyosin, and β-actin (1:20,000; Santa Cruz Biotechnology). Signals were detected by enhanced chemiluminescence Substrate (Pierce).

Immunohistochemical analysis. Calponin-1 was detected immunohistoch imically on paraffin-embedded tumor sections using antigen retrieval. The streptavidin-biotin complex method was applied. Primary antibody incubation was done with monoclonal anti-calponin antibody (1:50; DakoCytomation).

Results

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis of the 10 benign, nine atypical, and five anaplastic meningiomas was done over a pH range of 4 to 7 (Fig. 1). We compared proteomic patterns between the benign and atypical meningiomas and between the atypical and anaplastic meningiomas. Consistently expressed protein patterns and individual proteins on two-dimensional gels within each group were selected with statistically significant values by t-test (P < 0.05) using two-dimensional gel analysis software (Proteome Weaver, Definiens, Munich). Twenty-four proteins that were differentially expressed in all members of each tumor grade were isolated and identified by LC-MS/MS.

Comparison between benign and atypical meningiomas. First, we compared proteomic patterns and identified proteins that differentiated the 10 benign and 9 atypical meningiomas (Table 1A and B). Fifteen distinguishing proteins that were highly expressed in either the benign or atypical group were identified. Six of the 15 distinguishing proteins were differentially overexpressed in benign meningiomas (Table 1A). Meanwhile, 9 of the 15 identified proteins were differentially up-regulated in atypical meningiomas (Table 1B). These proteins reliably and reproducibly distinguished benign from atypical meningiomas.

Comparison between atypical and anaplastic meningiomas. Next, we isolated and identified nine proteins that differentiated the nine atypical and five anaplastic meningiomas (Table 2A and B).
Six of the nine identified proteins were significantly up-regulated in atypical meningiomas compared with anaplastic meningiomas, whereas three proteins were differentially overexpressed in anaplastic meningiomas (Table 2A and B).

### Table 1.

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<th>Name</th>
<th>Swiss-Prot ID</th>
<th>Location</th>
<th>kDa</th>
<th>Function</th>
<th>P</th>
<th>Intensity in two-dimensional gels</th>
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<td>A. Up-regulated proteins in benign meningiomas compared with atypical meningiomas</td>
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<td>ATP synthase</td>
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### Western blot and immunohistochemical analysis for verification of identified proteins.

To validate the two-dimensional gel electrophoresis results, we did Western blot analysis and immunohistochemistry (Figs. 2 and 3). Subject to the availability of

### Table 2.

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<th>Name</th>
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<td></td>
<td>Benign</td>
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<td>A. Up-regulated proteins in atypical meningiomas compared with anaplastic meningiomas</td>
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<td>Binding to actin filaments</td>
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<td>B. Up-regulated proteins in anaplastic meningiomas compared with atypical meningiomas</td>
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<td>4q31.3</td>
<td>51.5</td>
<td>Platelet aggregation</td>
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antibodies, we did Western blot analysis on selected proteins that were differentially expressed in the comparisons between benign and atypical meningiomas, with antibodies against galectin-1, calponin-1, and smooth muscle actin as well as to several proteins that were significantly expressed in the comparison between atypical and anaplastic meningiomas with antibodies against enolase, EB1, and tropomyosin (Fig. 2). Western blot for β-actin was used as an internal control. Western blotting consistently confirmed the differential protein expression determined by two-dimensional gel electrophoresis. Finally, we did immunohistochemical staining for the expression of calponin on independent paraffin sections of 30 meningiomas, which further validated the differential proteomic findings in this diverse series of meningiomas (Fig. 3).

Discussion

Most meningiomas are benign, WHO grade 1, and tend to follow indolent clinical course. However, atypical and anaplastic meningiomas, WHO grade 2 and 3 tumors, respectively, exhibit more aggressive behavior and are associated with poorer outcome. Atypical meningiomas are characterized histologically by several of the following features: increased cellularity, small cells with nuclear pleomorphism, increased mitotic activity (MIB-1 staining of nearly 5% compared with <0.1% for grade 1 tumors), an uninterrupted pattern of sheet-like growth, and occasional foci of necrosis (1, 12). Anaplastic, WHO grade 3 meningiomas exhibit morphologically more aggressive histologic features with higher mitotic activity (mean MIB-1 index is ~15%; ref. 1) than atypical meningiomas. The median survival of patients with this subgroup is <2 years due to aggressive, invasive, and recurrent tumors (1, 13). Although there has been keen interest in identifying adjuvant markers that will be of use in meningiomas grading, none have been identified that have consistent use. Therefore, we applied proteomic tools to identify proteins that distinguish meningiomas of varying grades.

We identified 15 proteins that are differentially expressed between benign and atypical meningiomas (Table 1A and B). Six of these proteins were up-regulated in benign meningiomas, some of which have previously been shown to have potential tumor suppression activity. Vitamin D–binding protein (DBP), significantly up-regulated in WHO grade 1 tumors, is a multifunctional protein that binds and transports vitamin D, acts as an actin scavenger, and is a precursor of macrophage-activating factor (MAF), which is a tumoricidal agent in various types of tumors (14–17). DBP possesses antiangiogenic activity, and cancer cells can inactivate DBP function by deglycosylation (14–17). Photodynamic therapy with DBP-derived MAF has been shown to have synergistic antitumor effect in squamous cell carcinoma tumor models (18). DBP is inactivated in several other cancers as well (14–17). Another protein that was identified, calponin-1, is an actin-associated protein originally isolated from bovine aorta and chicken gizzard (19, 20). Horiiuchi et al. showed that calponin may function as a tumor suppressor gene in human uterine leiomyosarcomas (19). Thus, loss of calponin-1 may potentiate or accelerate progression from a low-grade meningioma into a higher-grade tumor.

Several of the nine proteins that were significantly overexpressed in WHO grade 2 meningiomas have potential cancer-related functions. Galectin-1 mediates adhesions between cells and with the extracellular matrix and has been shown to play a role in tumorigenesis and to enhance metastatic potential (21). It was also reported to play a role in synaptogenesis, axon guidance, and cell process fasciculation in the brain (22, 23). Expression of galectin-1 enhances the activity of cell migration in human gliomas (23, 24). Gliomas with overexpression of galectin-1 behave in a more malignant fashion. Antisense against galectin-1 inhibits growth of 9L glioma cells (25). Paz et al. found that interaction of galectin-1 and Ras, which is associated with cell proliferation in meningiomas, was necessary for membrane fixation of Ras and subsequent cell transformation (26). This suggests that galectin-1 could be a
potential therapeutic target in these meningiomas, either indirectly, through interactions with Ras (e.g., using farnesyltransferase inhibitors) or directly.

We also identified two antioxidant proteins, peroxiredoxin 2 and 6, in grade 2 tumors. Resistance to oxidation is a feature of many tumors, which results in improved tolerance for hypoxia and resistance to chemotherapy. Antisense of peroxiredoxin 2 enhances cisplatin-induced or radiation-induced cell death in cancer cells (27, 28). Finally, two more identified proteins (voltage-dependent anion-selective channel protein 2 and annexin A1) also may play roles in the genesis or progression to atypical meningiomas (29, 30).

When comparing the proteomic profiles of atypical and anaplastic meningiomas, we identified nine proteins that were expressed differentially in one grade of tumor or the other. Six were up-regulated in atypical meningiomas compared with anaplastic meningiomas (Table 2A). Selenium-binding protein 1, which was significantly up-regulated in WHO grade 2 meningiomas, is one of the three mammalian selenium-containing proteins (31, 32). Supplementation of selenium has been proposed to reduce total cancer incidence and cancer mortality with prostate, lung, and colorectal cancers (33). Reduced expression of selenium-binding protein 1 was observed in poorly differentiated lung adenocarcinomas and is associated with poor prognosis (32).

Another protein, EB1, a microtubule-associated protein, interacts with the tumor suppressor gene adenomatous polyposis coli (APC) to contribute to microtubule stability (34). Loss of EB1’s interaction with APC could result in genetic instability and trigger tumorigenesis (34). Tropomyosin-1 is an actin-binding protein that is associated with stability of actin filaments and cell motility (35). Expression of tropomyosin-1 is decreased in some types of tumors such as breast cancers compared with normal tissues (36). Hughes et al. reported an inverse relationship between expression of tropomyosin and grades in human brain tumors (mainly astrocytic tumors), and that tropomyosin could be a potential marker for distinguishing the grade of human brain tumors (36). Hughes et al. reported an inverse relationship between expression of tropomyosin and grades in human brain tumors (mainly astrocytic tumors), and that tropomyosin could be a potential marker for distinguishing the grade of human brain tumors (37). In contrast, α-enolase, a potent glycolytic enzyme, was one of the three proteins that were up-regulated in anaplastic meningiomas. Expression of α-enolase has a reverse correlation with tumor differentiation in human hepatocellular carcinomas and is also associated with tumor size and invasiveness (38).

In getting to meningiomas, α-enolase may serve as a similar marker of differentiation status in WHO grade 3 tumors.

Previous investigators have identified a series of genetic alterations that characterize nearly all meningiomas. Loss of heterozygosity (LOH) on chromosome 22, which contains the neurofibromatosis type 2 (NF2) tumor suppressor gene, is the most common genetic abnormality in sporadic meningiomas (1, 8, 39). Mutation, deletion, and hypermethylation of NF 2 leads to depression, or complete loss, of expression of the protein merlin (40). In atypical meningiomas, additional genetic alterations include losses on chromosomes 1p, 6q, 10, 14q, and 18q and gains on 1q, 9q, 12q, 15q, 17q, and 20q (1, 8). Anaplastic meningiomas show supplementary changes, which include deletions along 9p and amplification of 17q (1, 8). Muller et al. showed that monosomy 1p and loss of expression of tissue nonspecific alkaline phosphatase (ALPL), located on 1p36, were strongly correlated with WHO grade 4, which suggests that ALPL may be a tumor suppressor gene in meningiomas. Furthermore, cDNA microarray and comparative genomic hybridization analyses have identified gene expression patterns associated with different grades in 30 cases of meningiomas (42). Wrobel et al. found that expression of proteins within the insulin-like growth factor and WNT signaling pathways were increased in atypical and anaplastic meningiomas and were associated with allelic losses on chromosome 10 or 14 (42).

Simon et al. reported that deletion of 1p32-pter, 10q24-qter, and 14q24-q32 are more common in atypical and anaplastic meningiomas than WHO grade 1 tumors (43). Mihaila et al. showed that losses on chromosome 10p15.1 and 10q26.3 were associated with higher-grade meningiomas, and LOH at 10q26.1 were directly correlated with shorter survival (44). Some of the proteins that we identified are located on these chromosomes, such as activator of 90-kDa heat shock protein ATPase homologue 1 and enoyl-CoA hydratase (Tables 1 and 2). These findings confirm and extend previous genetic and expression profiling studies.

Expressions of some identified proteins are consistently up-regulated/down-regulated parallel to the progression of the grade in meningiomas, such as tropomyosin 1 α chain and α-enolase, but most of the proteins are not. Several possibilities may explain this. The development of most tumors is thought to be initiated by the clonal expansion of a single cell that possesses alterations that convey growth advantage. With tumors that possess more than one grade of malignancy (compared with normal histology of the cell or organ of origin), it is unclear if in every case tumors of a high grade arose from a lower-grade tumor or if they began as a higher-grade tumor. Glycogenoses, for example, arise in either fashion (10). The situation with meningiomas is unclear. Although for most grade 1 tumors, alterations in NF2 seem generally to play a necessary, although not sufficient, role in tumorigenesis. The fact that higher-grade meningiomas may resemble sarcomas suggests that this is one possibility. Al-Mefty et al. documented that with benign meningiomas, which recurred as high-grade tumors, the original tumor already possessed cytogenetic alterations, suggesting the potential to progress (9).

Rempel et al. identified a secreted, extracellular matrix-associated protein (SPARC) as a possible marker of invasive behavior. Although SPARC expression was detected in all grade ≥2 meningiomas, it was found only in grade 1 tumors that developed an invasive phenotype (45). This suggests that proteins or protein patterns, such as some of those that we have identified, may serve to identify lower-grade tumors that are likely to be invasive or behave more aggressively.

In summary, we used selective tissue microdissection to obtain a pure population of meningioma cells and submitted it to two-dimensional gel electrophoresis and definitive LC-MS/MS protein sequencing to identify proteomic profiles and proteins that distinguish WHO grade 1, 2, and 3 meningiomas. A relatively small number of proteins were sufficient to differentiate meningiomas of each grade. This method is simple, accurate, and reliable and may prove useful to gain a broader understanding of the genesis and progression of meningiomas.

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