Molecular alterations in the *neurofibromatosis Type 2* gene and its protein rarely occurring in meningotheelial meningiomas

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**Object.** The *neurofibromatosis Type 2* (NF2) gene is the only tumor suppressor gene that has been clearly implicated in the development of benign meningiomas. Interestingly, previous data obtained by the authors indicate that reduced NF2 protein expression seldom occurs in meningotheelial meningiomas, the most common histological type of meningioma. The goal of the current study was to explore further the hypothesis of NF2 gene–independent tumorigenesis of meningotheelial meningiomas.

**Methods.** The authors performed a mutational analysis of all 17 exons of the NF2 gene by using single-stranded conformational polymorphism (SSCP). In addition, expression levels of the NF2 protein and μ-calpain, a protease suggested to inactivate the NF2 protein, were determined by immunoblotting analysis of 27 meningiomas (20 meningotheelial and seven nonmeningotheelial). Mutations of the NF2 gene were found in only one (5%) of 20 meningotheelial meningiomas and three (43%) of seven nonmeningotheelial tumors (Fisher’s exact test, *p* = 0.042). The levels of NF2 protein were severely reduced in six (28.5%) of 21 meningotheelial meningiomas, in contrast to six (86%) of seven nonmeningotheelial meningiomas (Fisher’s exact test, *p* = 0.023). Activation of μ-calpain did not correlate with the status of NF2 protein expression in the meningiomas analyzed, demonstrating that μ-calpain activation does not account for the loss of NF2 protein in meningiomas with apparently normal NF2 genes.

**Conclusions.** These results clearly demonstrate that NF2 gene mutations and decreased NF2 protein expression rarely occur in meningotheelial meningiomas compared with other histological types of meningiomas. The clinical behavior of meningotheelial meningiomas, however, is similar to that of other benign meningiomas. It is likely, therefore, that the tumorigenesis of meningotheelial meningiomas is the result of deleterious alterations of genes that have final phenotypical effects similar to inactivation of the NF2 gene.

**Key Words • meningotheelial meningioma • neurofibromatosis • tumorigenesis • calpain**

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**MENINGIOMAS** are a heterogeneous group of tumors that occur in the central nervous system of adults and are classified by the World Health Organization into three histological grades according to clinical prognosis: benign (Grade I), atypical (Grade II), and malignant (Grade III). Benign meningiomas can display a meningotheelial, fibroblastic, or transitional histological appearance. Meningotheelial meningiomas are the most common histological variety and account for approximately half of all meningiomas. Although the majority of meningiomas are histologically benign and can be cured by complete resection, up to 20% of meningiomas recur after what was thought to be complete removal. Moreover, surgical cure is often not possible for meningiomas involving the skull base or vital neurovascular structures. Even with great advances in microsurgery and approaches to the skull base, complete removal of some meningiomas may not be possible safely. Although currently available adjunctive therapies, such as radiation therapy and radiosurgery, often provide excellent control of subtotally resected meningiomas, the efficient long-term control of these tumors is not possible in approximately 10% of patients. To offer these patients some additional options for treatment, it is important to characterize molecular genetic events that give rise to meningiomas. Such efforts will enable the discovery of novel targets for therapy not only for meningiomas but, possibly, also for other tumors in the central nervous system.

Results of cytogenetic studies and loss-of-heterozygosity analyses have revealed genetic changes involving chromosome 22 in approximately 60% of meningiomas, suggesting the presence of one or more tumor suppressor genes on this chromosome. Meningiomas are frequently observed in patients with NF2 as part of the disease...
spectrum. The NF2 gene was mapped by linkage analysis to chromosome 22q12 and isolated by positional cloning. Analysis of mutational studies data subsequently revealed abnormalities of the NF2 gene in approximately one third of sporadic (that is, nonfamilial) meningiomas, although allelic loss on chromosome 22q and dramatically reduced levels of NF2 protein expression are consisently observed in approximately 60% of meningiomas. In the 40% of meningiomas that have no allelic loss on chromosome 22q, gene mutations and reduced NF2 protein expression levels have not been observed. These findings indicate that genes other than NF2 are probably involved in the development of meningiomas, and several candidate genes have been proposed.

Despite several reports in which similar incidence rates of NF2 gene mutations have been demonstrated in different histological types of meningiomas, we and others have provided data that indicate meningothelial meningiomas develop independently of alterations in the NF2 gene and the NF2 protein. Wellenreuther and colleagues were the first to report that mutations of the NF2 gene and lower levels of NF2 messenger RNA occur significantly more frequently in fibroblastic, transitional, atypical, and anaplastic meningiomas than in meningothelial meningiomas. Analyses of NF2 protein expression in sporadic meningiomas supported this observation. Using immunoblotting experiments, we have shown that expression levels of the NF2 protein are drastically reduced in all meningiomas of fibroblastic, transitional, and malignant histological types, but not in 86% of meningothelial meningiomas. These findings have been confirmed by an immunohistochemical analysis of NF2 protein in meningiomas conducted by Hitotsumatsu and associates.

Results of our previous studies and those of others described earlier indicate that meningothelial meningiomas may differ from other types of meningiomas not only histologically, but also genetically. To explore the hypothesis of NF2 gene–independent tumorigenesis of meningothelial meningiomas further, we conducted the simultaneous examination of NF2 gene mutation status and NF2 protein expression levels in 20 meningothelial meningiomas. We found that abnormalities in the NF2 gene and its protein product rarely occur in meningothelial meningiomas, establishing that the molecular characteristics of these tumors are distinct from other meningiomas.

Materials and Methods

Tissue Samples

Human brain and tumor tissue specimens were supplied directly from the operating room with the patients’ consent and the approval of the Institutional Review Board at Cleveland Clinic Foundation. The diagnoses of meningiomas and their histopathological classifications were determined by a neuropathologist. The normal brain-tissue specimen was excised from a patient with epilepsy who had undergone temporal lobectomy.

Single-Stranded Conformational Polymorphism Analysis

For mutation analysis of the NF2 gene, a set of 17 pairs of primers was used for PCR amplification of the entire coding region of the NF2 gene. The primer sequences for exons of the NF2 gene were as those reported by Rutledge, et al. and Mérel, et al. Genomic DNA was isolated from 21 tumor samples in the manner described by Sambrook and associates. The SSCP analysis was performed essentially in the same manner as that described by Hogg, et al. Each exon of the NF2 gene was amplified from 250 ng of genomic DNA by using 2 μM of exon-specific primers in the presence of 2 μM of deoxycytidine triphosphate, 2-deoxyguanosine-5’-triphosphate, and deoxythymidine triphosphate, and 0.1 μM of α-32P deoxycytidine triphosphate (3000 Ci mmol−1). The products of PCR reactions were placed on agarose gel and analyzed to ensure that only a single product of the correct size had been amplified. The PCR products were denatured by boiling for 10 minutes, chilled on ice, and loaded onto a 1 × mutation detection enhancement gel. Electrophoresis was performed for 14 hours at 6 W. The gels were dried and exposed to film. Exons displaying a migration shift on SSCP were subjected to the tumor DNA and were gel purified and used as a template for a direct sequencing. The DNA sequencing was performed by the Cleveland Clinic Foundation Biotechnology Core Sequencing Service by using big-dye termination methods on a DNA sequencer.

Preparation of Tissue Extracts and Immunoblotting Analysis

Immediately on excision, tumor tissue from 21 patients with meningothelial meningiomas as well as normal human brain tissue from nonneoplastic donors were homogenized in the laboratory in which the tumor tissue was homogenized and soluble and Triton X-100–insoluble protein fractions were prepared exactly as previously described. Immunoblotting analysis was performed using the commercially available polyclonal anti-NF2 protein antibody designated anti–NF2(A-19) IgG. This antibody recognizes an epitope localized at the amino terminus of the human NF2 protein (amino acid residues 2–21). Immunoblotting with anti–actin antibody was used to confirm that an equal amount of protein for each sample was loaded onto the gel. The integrity of the actin bands also served as an internal control for nonspecific protein degradation. To reprobe Western blots with anti–actin antibody, the anti–NF2 antibody was stripped from the membranes, which were then immunoblotted with a mouse monoclonal antibody to actin. Full-length and proteolytically cleaved μ-calpain were detected by immunoblotting, which was performed using a commercially available antibody. Human μ-calpain isolated from erythrocytes was used as a positive control in the immunoblotting analysis.

Sources of Supplies and Equipment

The mutation detection enhancement gel was purchased from BioWhittaker Molecular Applications (Rockland, ME). The anti–NF2 IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and the mouse monoclonal antibody to actin from Roche Molecular Biochemicals (Indianapolis, IN). Human μ-calpain was acquired from Calbiochem–Novabiochem Corp. (San Diego, CA), and the antibody used to detect the μ-calpain was purchased from Alexis Corp. (San Diego, CA). The 377XL DNA sequencer was obtained from Applied Biosystems (Foster City, CA).

Results

The SSCP analysis was used to screen for mutations in all 17 exons of the NF2 gene in 21 meningothelial and seven nonmeningothelial meningiomas (three fibroblastic, two transitional, and two atypical). Aberrant band mobility on SSCP was detected in three meningothelial tumors, and direct DNA sequencing of these samples was performed. Two samples contained a wild-type DNA sequence, whereas one sample was found to have an NF2 gene mutation during direct sequencing. Thus, only one (5%) of the 20 meningothelial meningioma samples studied was found to have a mutated NF2 gene (Table 1). This mutation consisted of a 24-bp in-frame deletion in exon 8, which is predicted to result in the synthesis of a truncated NF2 protein that lacks amino acids 232 through 239. In contrast with the meningothelial tumors, aberrant SSCP
band motility and NF2 gene mutations were found in three (43%) of the seven nonmeningothelial meningiomas analyzed, as follows: one (33%) of three fibroblastic, one (50%) of two transitional, and one (50%) of two atypical meningiomas. These aberrations consisted of a 7-bp frameshift deletion in exon 13 in sample No. 224, a 1-bp deletion in the acceptor site of exon 4 in fibroblastic meningioma sample No. 180, and an alteration in the intron upstream of exon 3 in atypical meningioma sample No. 222 (Table 1). In spite of the small number of nonmeningothelial meningiomas analyzed, the difference in the frequency of NF2 gene mutations between meningothelial (5%) and nonmeningothelial (43%) meningiomas was statistically significant (Fisher’s exact test, p = 0.042; Fig. 1A).

To establish the relationship between the mutational status of the NF2 gene and the expression of its protein product, an immunoblotting analysis of the NF2 protein in the same group of meningiomas was performed. The results of this survey suggested a higher occurrence rate of NF2 protein inactivation than that of NF2 gene mutations detected by SSCP analysis and DNA sequencing. As shown in Fig. 2, levels of NF2 protein were severely reduced (that is, the NF2 protein band intensity was less than 20% of that observed in normal human brain) in six (29%) of 21 meningothelial meningiomas analyzed. In contrast, barely detectable levels of NF2 protein were found in six (86%) of seven nonmeningothelial meningiomas: all three fibroblastic, both atypical, and one of the two transitional (Fig. 2 and Table 1). In two meningothelial meningiomas (Nos. 151 and 230) we observed only an approximately 50% reduction in the intensity of the NF2 protein.

### Table 1

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<tr>
<th>Tumor Sample No.</th>
<th>Patient Age (yrs), Sex</th>
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<th>Histological Finding</th>
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<th>Sequence Altered</th>
<th>Codon Altered</th>
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* A = atypical meningioma; Ala = alanine; Asp = aspartic acid; F = fibroblastic meningioma; Gln = glutamine; Glu = glutamic acid; Gly = glycine; Leu = leucine; Leu454fs = Leu454 frameshift mutation; Lys = lysine; M = meningothelial meningioma; NP = nuclear pleomorphism; PB = psammoma bodies; recur = recurrent meningioma; T = transitional meningioma; Val = valine.

† Notations: + = the intensity of the NF2 protein 66-kD band is comparable to that of normal brain tissue; ± = the intensity of the NF2 protein 66-kD band is 30 to 70% that of normal brain tissue; – = the intensity of the NF2 protein 66-kD band is less than 20% that of normal brain tissue.

‡ codon 231

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§ codon 452

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113
Discussion

Our results clearly demonstrate that \( NF2 \) gene mutations and decreased \( NF2 \) protein expression rarely occur in meningothelial meningiomas compared with other histological types of meningioma. In this study, we found that \( NF2 \) gene mutations occur in only 5% of meningothelial meningiomas. This low incidence of \( NF2 \) gene mutations in meningothelial meningiomas is supported by the findings of Wellenreuther and colleagues\(^29\) and Ueki, et al.\(^28\) (25% and 10%, respectively).

Clear discrepancies in the incidence of \( NF2 \) gene mutations observed in meningiomas of different histological subtypes have been reported.\(^3,13,17,22,28,29\) Some authors report no difference in the mutation rate of the \( NF2 \) gene in meningothelial compared with nonmeningothelial meningiomas,\(^3,13,17,22\) whereas others report rates that vary considerably between these tumor types.\(^8,28,29\) The variation in mutation frequency occurring between studies could be due to a number of factors, including both the small numbers of tumors examined in some studies and the methods used to identify the \( NF2 \) gene mutations. In some studies the ability to detect mutations was improved by changing the temperature and denaturing conditions of electrophoresis. It is still possible that a few mutations might have been missed, but this would not account for the significant difference between the meningothelial and other histological subtypes of meningiomas.

In some studies, no differences in the incidence of \( NF2 \) gene mutations were found between the histological types of meningiomas. For example, Rutledge and associates\(^22\) found 24 inactivating \( NF2 \) gene mutations in 151 meningo-

protein band (Table 1 and Fig. 2), whereas all other meningothelial tumors displayed \( NF2 \) protein band intensities comparable with that of the normal human brain control sample. The difference in \( NF2 \) protein expression between meningothelial and fibroblastic meningiomas and between meningothelial and all other nonmeningothelial meningiomas taken as a group was statistically significant (Fisher’s exact test, \( p = 0.041 \) and \( p = 0.023 \), respectively; Fig. 1B). The low-intensity \( NF2 \) protein band demonstrated in tumors with poor \( NF2 \) protein expression, including those with \( NF2 \) gene mutations, is probably due to the signal derived from nontumorous cells, as we and others have reported previously.\(^12,28\)

Interestingly, among the six meningothelial tumors found to have significantly reduced \( NF2 \) protein levels, three tumors displayed additional characteristics: one was recurrent, one contained mild nuclear pleomorphism and focally prominent nucleoli, and one had psammoma bodies (Table 1). In our previous study, a meningothelial meningioma with psammoma bodies was the only meningothelial meningioma with a reduced \( NF2 \) protein level.\(^12\) Taken together, our results provide support for the idea that loss of the \( NF2 \) protein occurs rarely in meningothelial meningiomas.

It is not clear why tumors with an apparently normal \( NF2 \) gene lack the \( NF2 \) protein. It has been shown that the \( Ca^{++}\)-dependent cysteine protease, \( \mu\)-calpain, can cleave the \( NF2 \) protein,\(^9\) which indicates that \( \mu\)-calpain–dependent proteolytic inactivation of the \( NF2 \) protein could be involved in development of meningiomas that have an apparently normally functioning \( NF2 \) gene. In a recent report, however, it was shown that activation of \( \mu\)-calpain did not correlate with the loss of \( NF2 \) protein expression in meningiomas without \( NF2 \) gene mutations.\(^28\) In response to these contradicting results and because of the potential clinical application of \( \mu\)-calpain inhibitors and \( Ca^{++} \) channel blockers for treatment of meningioma, we examined the expression levels and activation status of \( \mu\)-calpain in our set of meningiomas. The cytosolic protein fraction of 15 meningiomas with normal, intermediate, and reduced \( NF2 \) protein expression levels were investigated by immunoblotting with an anti–\( \mu\)-calpain antibody. The presence of activated (76-kD) and intermediate (78-kD) forms of \( \mu\)-calpain were detected in most tumors at various levels but did not correlate with the \( NF2 \) protein expression status (Fig. 3). These results show that activation of \( \mu\)-calpain in most meningiomas without detectable \( NF2 \) gene mutations does not account for the loss of \( NF2 \) protein.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Bar graphs demonstrating that meningothelial meningiomas rarely have \( NF2 \) gene and \( NF2 \) protein alterations compared with nonmeningothelial tumors based on results obtained by SSCP analysis (A) and immunoblotting analysis (B), as described in Materials and Methods and Table 1. The frequency of \( NF2 \) gene mutations and the levels of \( NF2 \) protein expression were significantly lower in meningothelial meningiomas than in the remaining tumors, taken as a group (\( p = 0.042 \) and \( p = 0.023 \), respectively, Fisher’s exact test).}
\end{figure}
Meningothelial meningioma NF2 gene mutations

Fig. 2. Results of immunoblotting analysis demonstrating that the NF2 protein is expressed at normal levels in most meningothelial meningiomas. A 100-μg aliquot of the Triton X-100-insoluble (cytoskeleton-associated) protein fraction of meningiomas and human brain (HB) was separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted using anti–NF2(A-19) IgG, as previously described.12 Immunoblotting with anti–NF2(A-19) IgG revealed the 66-kD NF2 protein (upper panels). As a control, after the anti–NF2 IgG was stripped off, the same membrane was immunoblotted with anti–actin IgG. Immunoblotting with the anti–actin antibody revealed a 42-kD actin band (lower panels) of comparable intensity in all samples, indicating that equal amounts of protein were loaded on the gel and that no nonspecific degradation of proteins occurred in any of the samples analyzed. Meningioma specimen numbers are indicated above the panels and described in Table 1.

Fig. 3. Results of immunoblotting analysis showing that activated forms of μ-calpain are detected in most meningiomas and do not correlate with the NF2 protein expression status. A 20-μg aliquot of the cytosolic (soluble) fraction of meningiomas and rabbit brain (R.B.) was separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted using the anti–μ-calpain antibody. A pure human μ-calpain, autolyzed in vitro by the addition of CaCl2, was used as a positive control for calpain activation (Lane C). Sizes of the full-length (80 kD), intermediate (78 kD), and activated (76 kD) μ-calpain are shown on the left of the figure. Meningioma specimen numbers are indicated above the panels, and the NF2 protein status of these samples is shown below the panels (see Table 1 legend for definitions of plus and minus signs).
some 22q in meningothelial compared with fibroblastic meningiomas (18% compared with 82%). The authors did not comment on whether the difference in the incidence of NF2 gene mutations also was significant between meningothelial and fibroblastic meningiomas (10% compared with 45%). Detailed analysis of their report, however, revealed that this difference was, in fact, statistically significant (Fisher’s exact test, p = 0.028).

The results of the immunoblotting analysis reported in our current study indicate that reduced levels of NF2 protein occur much less frequently in meningothelial meningiomas than in nonmeningothelial meningiomas (Table 1 and Figs. 1 and 2). The clear distinction between meningothelial tumors and other meningiomas is even more compelling when one combines the results of NF2 protein analysis of our earlier study and our current results.12 In all, only seven (25%) of 28 meningothelial meningiomas had reduced NF2 protein expression levels, whereas 13 (93%) of 14 nonmeningothelial meningiomas analyzed expressed barely detectable levels of NF2 protein (Fisher’s exact test, p = 0.0001). None of four fibroblastic meningiomas analyzed in the combined studies expressed NF2 protein, thus making this benign variant clearly distinctive from meningothelial meningiomas (Fisher’s exact test, p = 0.001). These immunoblotting data are strongly supported by the results of an immunohistochemical analysis of NF2 protein reported by Hitotsumatsu and associates13 in which the NF2 protein was observed to be present in 80% of meningothelial meningiomas, but absent in all other histological types of meningiomas.

Additional support for the hypothesis of NF2 gene–independent tumorigenesis of meningothelial meningiomas comes from studies of NF2- and non–NF2-associated familial meningiomas. These data indicate that meningothelial meningiomas seldom occur in patients with NF2 and that most meningiomas in these patients are fibroblastic.21 Moreover, in a number of families in which there are multiple meningiomas and no clinical signs of NF2, the types of meningiomas have been reported to be almost exclusively meningothelial.3,8,23,25,26

Examination of NF2 protein expression suggested a higher rate of NF2 gene inactivation than was expected from the results of the SSCP analysis. The reasons for such a discrepancy are not clear. There are many ways that a gene can be inactivated, of which not all involve the coding region. Typically, the SSCP procedure is used to examine individual exons and flanking intron regions of a gene. It is now well established that mutations in the introns or the promoter region can also result in gene inactivation.5 It is, therefore, possible that such alterations could account for the reduced NF2 protein expression in meningiomas that have no mutations in the coding region of the NF2 gene. In addition, nongenetic changes, such as activation of μ-calpain protease, have recently been suggested.9

Most of the meningiomas we examined displayed variable, but high levels of μ-calpain activation (Fig. 3). Recently, Ueki, et al.18 reported that activated μ-calpain was present in approximately one half of the sporadic meningiomas they examined. It is important to note, however, that our immunoblotting analysis was performed with a different anti–μ-calpain antibody, which could account for a more frequent detection of activated μ-calpain in our study. Nevertheless, the expression of activated μ-calpain did not correlate with NF2 protein expression status in either study. These results indicate that elevated activation of μ-calpain is probably not responsible for the reduction in NF2 protein expression observed in tumors without NF2 gene mutations. Thus it is unlikely that the use of μ-calpain inhibitors could play a role in restoring the functional integrity of the NF2 protein and serve as a treatment for meningiomas.

When all the data reported here and elsewhere are taken into consideration, it is clear that different mechanisms of tumorigenesis, independent of the loss of function of the NF2 gene, are responsible for the development of meningothelial meningiomas, compared with other types of meningiomas. Despite this significant genetic difference, meningothelial meningiomas exhibit clinical behavior that is similar to that of other benign meningiomas. It is likely, therefore, that the development of meningothelial meningiomas is the result of deleterious alterations in genes that have final phenotypical effects similar to inactivation of the NF2 gene. One possibility includes alterations of one or more proteins that interact functionally with the NF2 protein or function downstream of it. It is also very likely, however, that genes that are unrelated to the NF2 gene–controlled tumorigenic pathway are altered in meningothelial meningiomas. A comparison of global gene expression between meningothelial meningiomas with normal NF2 gene expression and the rare meningothelial meningiomas with nonfunctional NF2 genes will be necessary to identify novel genes involved in meningioma tumorigenesis.

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References


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