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Tight Association of Loss of Merlin Expression with Loss of Heterozygosity at Chromosome 22q in Sporadic Meningiomas[1]

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ABSTRACT

Mutations of *NF2*, the gene for neurofibromatosis 2, are detected in 20–30% of sporadic meningiomas, and almost all mutations lead to loss of merlin expression. However, loss of heterozygosity (LOH) at chromosome 22q is found at a much higher frequency, up to 50–70%, and the possibility of another tumor suppressor gene in this region has not been excluded. Furthermore, a recent report proposed that abnormal activation of a protease μ-calpain can be an alternative pathway for merlin loss in meningiomas and schwannomas. To determine the correlation of merlin loss with *NF2* genetic alteration or μ-calpain activation, we performed a molecular genetic analysis of 50 sporadic meningiomas and also examined the expression status of merlin and active form μ-calpain. LOH assay of five microsatellite markers franking *NF2* revealed LOH in 22 cases, and single-strand conformation polymorphism assay detected six frameshift mutations, two splicing mutations, one nonsense mutation, and one missense mutation, all accompanied by 22q LOH. In addition, a multiplex PCR assay indicated homozygous deletion of *NF2* in two cases. Interestingly, a marked decrease of merlin expression was seen exclusively in the 22 cases with 22q LOH. Activated μ-calpain expression was observed in 28 cases at various levels but showed no correlation with merlin status. These data strongly support the notion that *NF2* is the sole target of 22q LOH in meningiomas and that loss of merlin expression is always caused by genetic alteration of *NF2*, following the classic "two hit" theory.

INTRODUCTION

Meningiomas are one of the neoplasms associated with neurofibromatosis 2, and mutations of *NF2*, the gene for neurofibromatosis 2, are found also in sporadic meningiomas (1–4). The frequency of mutations ranges from 20 to 30% in most reports and is significantly different among subtypes, with 10–20% in meningothelial type and 60–80% in fibroblastic and transitional type meningiomas (5). The vast majority of the mutations are null mutations, *i.e.*, nonsense mutations, frameshift mutations, or splice donor site mutations, which all result in a nonfunctional, truncated protein. Such mutations are always accompanied by loss of a wild-type allele in tumors, indicating that complete loss of merlin is a major mechanism leading to meningioma formation (1, 3, 4). On the other hand, loss of heterozygosity at chromosome 22q on which *NF2* is located is found at a higher frequency, around 50–70% (1, 2), and there still is controversy over whether or not there exist other tumor suppressor genes at this chromosomal region (6, 7).

Neither NF2 mutation nor LOH at chromosome 22q is detected in the remaining 40–50%, but a recent report suggested that abnormal activation of μ-calpain, a protease mainly targeting various cytoskeleton proteins, may cause merlin loss in some meningiomas without *NF2* mutation, thereby constituting an alternative pathway for merlin inactivation (8). Because such meningiomas could be good candidates for calpain-inhibiting reagents for tumor growth suppression, it is important to determine what portion of meningiomas falls into that category. To address these issues, we performed an extensive molecular genetic analysis of *NF2* on 50 sporadic meningiomas and compared the genetic results with the merlin and calpain expression status.

MATERIALS AND METHODS

All human samples were obtained with full informed consent after approval by the intramural committee. The tumor tissues were obtained at surgery. None of the patients had neurofibromatosis 2: none of the patients had acoustic schwannoma or other accompanying intracranial tumors on magnetic resonance imaging nor had family history suggestive of neurofibromatosis 2. After a portion was processed for routine histological examinations, samples were snap-frozen in liquid nitrogen and then stored at −80°C until use. Peripheral blood was drawn from the patient and was subjected to immediate DNA extraction as previously described (9). DNA from the tumor tissue was extracted using the QIAmp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol.

LOH[3] Assay and SSCP Assay. LOH and SSCP assay were performed using the Genetic Analyzer 310 (PE Biosystems, Norwalk, CT) capillary electrophoresis system. For the LOH assay, five microsatellite polymorphic markers franking *NF2* were selected from the GDB: centromeric to telomeric, *D22S268*, *D22S1163*, *D22S929*, *D22S280*, and *D22S282*. Primer sequences for those markers are available from the Genome Database.[4] Of those, *D22S929* is an intragenic marker located within the 32.2-kb-long intron 1 of the *NF2* gene. For each marker, the sense primer was labeled by a fluorescent dye, and PCR was performed for 25–30 cycles with 58°C-60°C annealing temperature on the Gene Amp 9700 Thermal Cycler (PE Biosystems). PCR products were separated by the capillary electrophoresis of the Genetic Analyzer 310, and the analysis was performed using the Gene Scan Program (PE Biosystems) following the manufacturer's protocol. For SSCP analysis, previously reported primer pairs and PCR conditions amplifying all 17 exons of *NF2* with splice donor sites were used (10). For each primer set, the sense and antisense primers were labeled with a different fluorescent dye to allow specific detection of the sense or antisense strand. PCR products were separated in a capillary electrophoresis and analyzed with the Gene Scan program to detect tumor-specific migration shifts. Any exon showing a migration shift was reamplified from the tumor DNA with nonlabeled primers, gel-purified, and was used as a template for direct sequencing. The sequencing reactions were done with the BigDye Terminator Sequencing Kit (PE Biosystems), and the products were separated and analyzed by the Genetic Analyzer 310 following the manufacturer's protocol.

For the multiplex PCR, previously described primers for exon 8 of the p53 gene, which is known not to be altered in meningiomas, was used as a control (11). PCR was performed in a 20-μl reaction containing 5 pmol each of control primers, 10 pmol each of D22S929 primers, 50 ng of template DNA, 1.5 mM MgCl2, and 5% DMSO. The annealing temperature was gradually decreased by 0.3°C at each step from 65.0°C to 58.1°C, followed by 15 more cycles at 58°C annealing temperature. PCR products were separated on a 2% agarose gel and were visualized by ethidium bromide staining.

Western Blot Analysis. Tumor tissue was minced and sonicated in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 2% SDS, 1 mM EDTA (pH 8.0), 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 50

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[3] The abbreviations used are: LOH, loss of heterozygosity; SSCP, single-strand conformation polymorphism; FISH, fluorescent *in situ* hybridization.

[4] http://www.gdb.org/.

μg/ml leptin. After heating for 5 min at 95°C, debris was removed by centrifuging at 15,000 rpm for 30 min. Fifty micrograms of total protein were separated on 7.5% SDS-PAGE and electrically transferred to Immobilon-P Membrane (Millipore, Bedford, MA). The membrane was incubated with 5% nonfat dry milk in 0.05 M Tris-buffered saline and then incubated with a primary antibody. For merlin, a rabbit polyclonal antibody recognizing the COOH-terminus of merlin C-18 (Santa Cruz Biotechnology, Santa Cruz, CA) was used. Extract from normal brain tissue obtained at autopsy was used as a positive control. Anti-β-actin monoclonal antibody AC-15 (Sigma, United Kingdom) was used for the detection of β-actin. The two polyclonal antibodies for μ-calpain were kind gifts from Dr. T. C. Saido (RIKEN, Wako, Japan). One antibody specifically recognizes the preactivated μ-calpain of 80 kDa (12), and the other raised against the D-rich segment recognizes three forms of activated μ-calpain: 80 kDa, 78 kDa intermediate, and 76 kDa activated form (13). After washes with Tris-buffered saline-0.05% Tween 20, the membrane was incubated with an appropriate secondary antibody conjugated with horseradish peroxidase and was detected by the chemiluminescence method using the enhanced chemiluminescence kit (Amersham, Uppsala, Sweden) following the manufacturer's protocol. Membranes were stripped and reprobed with another antibody each time. The amount of each sample loaded was adjusted to obtain the equivalent intensity for β-actin. With regard to merlin expression status, all cases were evaluated by two independent investigators in a blinded fashion; *i.e.*, without the knowledge of 22q LOH status. Cases with obviously lower intensity of merlin signal compared to the normal control were considered to have "reduced merlin," whereas the cases showing merlin expression equivalent to or stronger than the normal control were assigned as merlin-positive cases.

RESULTS

Of the 50 patients, 14 were male and 36 were female. Histological subtype of the 50 examined meningiomas was meningothelial in 28, fibroblastic in 11, transitional in 8, atypical in 2, and angioblastic in 1. Of those, 22 cases showed loss of heterozygosity in at least one of the five microsatellite markers examined (Table 1). The frequency of LOH at 22q within each subtype was significantly different: 5 of 28 (18%) meningothelial, 9 of 11 (82%) fibroblastic, and 6 of 8 (75%) transitional subtype had LOH at 22q. The intragenic marker D22S929 showed LOH in 14 of the 22 cases and was noninformative in 6 cases, but in two cases, cases 29 and 30, it maintained heterozygosity, whereas other markers showed LOH (Fig. 1). Because homozygous deletion, usually franked by hemizygous deletion, occur in other tumor suppressor genes (14), we set up a comparative multiplex PCR to evaluate for homozygous deletion of the *NF2* gene. Compared to

Table 1 *Molecular genetic analysis of 50 sporadic meningiomas*

Case	Sex	Subtype	*D22S268*	*D22S1163*	*D22S929 (NF2)*	*D22S280*	*D22S282*	*NF2*	Mutation
1	F[a]	Fbl	ni	LOH	ni	ni	LOH	Exon 5, codon 155	CCC → CC
2	M	Mth	1,2	ni	ni	ni	1,2		
3	F	Fbl	ni	LOH	ni	LOH	LOH		
4	M	Ts	ni	LOH	LOH	LOH	LOH		
5	F	Atp	ni	1,2	ni	1,2	1,2		
6	F	Ts	?	LOH	LOH	LOH	LOH		
7	F	Mth	ni	LOH	LOH	ni	ni	Exon 4, codon 126	ATT → TT
8	M	Mth	ni	1,2	1,2	1,2	ni		
9	F	Mth	ni	1,2	ni	ni	ni		
10	F	Mth	ni	ni	1,2	1,2	1,2		
11	F	Fbl	ni	LOH	LOH	LOH	LOH	Exon 12, codon 428	CAG → AG
12	F	Mth	ni	1,2	1,2	1,2	1,2		
13	F	Mth	1,2	1,2	ni	ni	1,2		
14	F	Mth	ni	1,2	ni	ni	?		
15	M	Mth	ni	ni	1,2	ni	1,2		
16	M	Mth	ni	1,2	1,2	ni	1,2		
17	F	Mth	1,2	1,2	1,2	1,2	1,2		
18	F	Mth	1,2	ni	1,2	1,2	ni		
19	F	Mth	1,2	1,2	1,2	1,2	1,2		
20	F	Fbl	LOH	LOH	LOH	ni	LOH	Exon 7, codon 207	TAT → TAG
21	M	Mth	ni	ni	1,2	ni	1,2		
22	M	Abl	1,2	ni	LOH	LOH	ni	Exon 12, codon 392	GAG → GTG
23	F	Fbl	1,2	1,2	1,2	ni	1,2		
24	F	Ts	ni	LOH	LOH	LOH	LOH		
25	F	Mth	1,2	1,2	ni	ni	1,2		
26	F	Mth	ni	LOH	ni	LOH	LOH	Exon 4, codon 126	ATT → TT
27	F	Fbl	LOH	LOH	LOH	LOH	LOH		
28	F	Mth	1,2	1,2	1,2	1,2	1,2		
29	F	Atp	LOH	LOH	1,2	LOH	LOH	Homozygous deletion ?	
30	F	Ts	ni	LOH	1,2	1,2	ni	Homozygous deletion ?	
31	F	Mth	ni	1,2	1,2	ni	1,2		
32	F	Mth	ni	LOH	LOH	ni	LOH		
33	F	Mth	ni	1,2	ni	ni	1,2		
34	M	Fbl	ni	ni	LOH	LOH	LOH		
35	F	Fbl	ni	1,2	1,2	1,2	1,2		
36	F	Mth	1,2	ni	1,2	1,2	1,2		
37	F	Mth	ni	1,2	ni	1,2	1,2		
38	M	Ts	ni	1,2	ni	ni	1,2		
39	M	Fbl	ni	LOH	LOH	LOH	LOH		
40	F	Ts	ni	ni	ni	1,2	1,2		
41	F	Ts	LOH	LOH	ni	LOH	LOH		
42	M	Mth	1,2	1,2	1,2	1,2	1,2		
43	F	Ts	LOH	LOH	LOH	LOH	LOH	Exon 5, splice donor site	15-bp deletion
44	M	Fbl	LOH	ni	LOH	LOH	LOH	Exon 10, codon 325	GCC → GC
45	F	Mth	ni	1,2	1,2	1,2	1,2		
46	F	Fbl	LOH	ni	LOH	LOH	LOH	Exon 13, splice donor site	AG → AT
47	M	Mth	1,2	1,2	1,2	1,2	1,2		
48	F	Mth	ni	ni	ni	LOH	LOH	Exon 4, codon 126	ATT → AT
49	M	Mth	ni	1,2	1,2	1,2	1,2		
50	F	Mth	ni	ni	ni	LOH	LOH		

[a] F, female; M, male; Mth, meningotheliomatous; Fbl, fibroblastic; Ts, transitional; Atp, atypical; Abl, angioblastic; LOH, loss of heterozygosity; ni, noninformative.

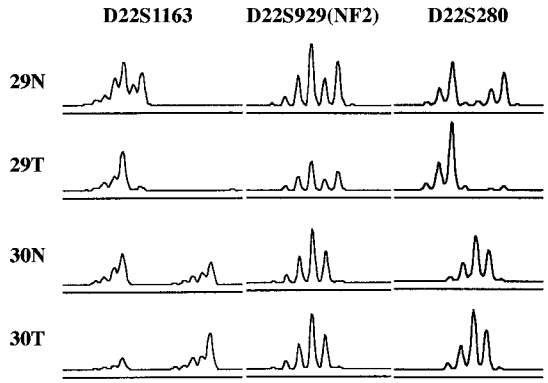

Fig. 1. Microsatellite analysis on blood (*N*) and tumor (*T*) DNA from cases 29 and 30. In both cases, an intragenic marker *D22S929* demonstrated heterozygosity, whereas a centromeric marker *D22S1163* (cases 29 and 30) or a telomeric marker (case 29) showed loss of heterozygosity indicated by a marked decrease of amplicon from one of the two alleles.

the p53 exon 8 control, *D22S929* amplicon was significantly less amplified from the tumor DNAs of cases 29 and 30. In both cases, blood DNA showed equivalent amplifications from two primer sets (Fig. 2). Because *D22S929* amplicon from tumor DNAs in these cases maintained heterozygosity in the LOH analysis (Table 1), the most likely explanation for the decrease of *D22S929* amplicon from tumor DNA compared to that from blood DNA was that this portion of the *NF2* gene is homozygously deleted in the tumor. This experiment was repeated three times to confirm its reproducibility.

SSCP analysis was performed on all 50 cases, and we detected a tumor-specific migration shift in 10 cases. The direct sequencing revealed a 1-bp deletion causing a frameshift in 6 cases, nonsense mutation in 1 case, splice donor site mutation in 2 cases, and missense mutation in 1 case (Table 1). All of the mutations were accompanied by LOH at 22q.

On Western blot analysis, decrease of merlin expression was observed in 22 cases and showed a completely identical pattern with the status of LOH at chromosome 22q (Table 1; Fig. 3). The degree of the reduction was obvious in all such cases, with no equivocal cases. The preactive form of μ-calpain of 80 kDa was expressed at various degrees, and there was no apparent association in merlin expression (Fig. 3). In most of the cases with a significant level of preactivated form, the 76-kDa fully autolysed μ-calpain was evident, indicating full activation of μ-calpain in those cases. Specifically, of the 28 cases with positive merlin expression, 13 showed relatively high μ-calpain expression, whereas 15 showed no or very low expression. Of the 22 cases with merlin loss, 15 showed high-level μ-calpain expression, but 7 showed no or very low expression.

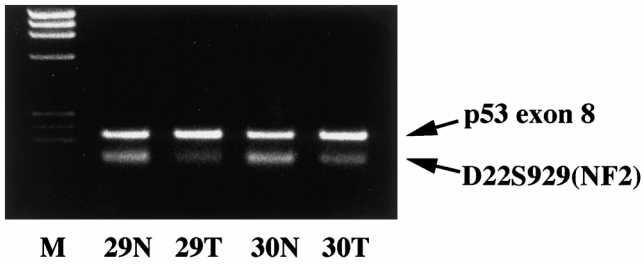

Fig. 2. Multiplex PCR demonstrating probable homozygous deletion of *NF2* in cases 29 and 30. Relative amplification of *D22S929* at intron 1 (134 bp) compared to p53 exon 8 (204 bp) is significantly less in PCR from tumor DNA (*29T* and *30T*) than in PCR from blood DNA (*29N* and *30N*).

DISCUSSION

Our data showing the complete concordance of LOH at 22q and merlin loss strongly suggests that *NF2* is the sole target of LOH in meningiomas whether or not the mutation can be pinpointed by the conventional methods for mutation detection. In addition, the data also confirm that merlin loss occurs through genetic alterations following the classic "two hit" theory of Knudson (15) and argue against the alternative pathway through abnormal μ-calpain activation (8).

On SSCP analysis, we did not detect mutation in 12 of 22 cases with 22q LOH. Although we cannot exclude the possibility that SSCP assay missed some mutations, the overall rate of detected mutation (20%) was similar to previous reports (1, 4, 16). Therefore, a more likely explanation, we believe, would be that at least some of those do harbor mutations in *NF2* undetectable by SSCP, leading to loss of merlin expression. As one of possible mechanisms, we demonstrated probable homozygous deletions of *NF2* in cases 29 and 30. We do not know the exact extent of the deletion in these cases, but the D22S929 marker lies within the 32.2-kb intron 1 of *NF2*, and deletion of this portion most likely abolishes merlin production. In neurofibromatosis 2 patients, detailed mutation analysis on the whole genomic sequence using FISH also showed that deletion of a large fragment of *NF2* is not a rare event (17).

Homozygous deletion of *NF2* in meningiomas has previously been reported but only as a very rare event (18). Because of the normal tissue contamination, showing direct evidence of homozygous deletion requires more laborious assays such as FISH or quantitative Southern hybridization, which have not been applied in screening a large series. Therefore, it may be possible that homozygous deletion of *NF2* may be more frequent than previously recognized. Another well-known mechanism to silence a tumor suppressor gene is the DNA methylation. Although methylation is not known to be involved in NF2 silencing in any type of tumor, it is a frequent event for some tumor suppressor genes like *CDKN2A/p16* (14, 19), and we still cannot exclude the possibility.

Mutations leading to loss of transcription of the gene, such as homozygous deletion of whole or a large portion of the gene and

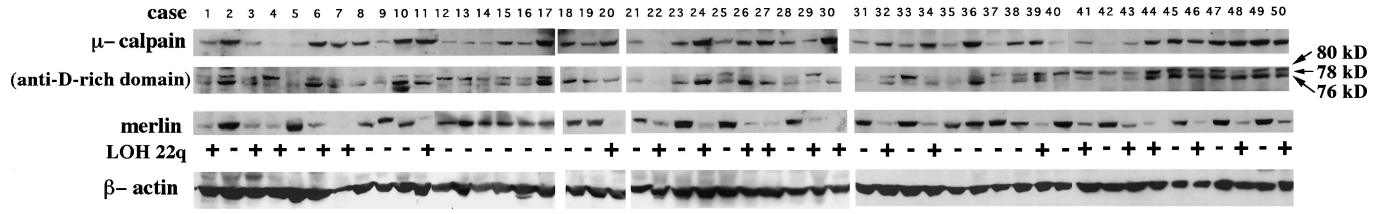

Fig. 3. Western blot analysis on merlin and activated form of μ-calpain in 50 sporadic meningiomas. Marked decrease of merlin (66 kDa) expression was observed in 22 cases, showing complete concordance with LOH status at chromosome 22q. The preactive form of μ-calpain (80 kDa) was expressed in various degrees without any correlation to the merlin expression status. The anti-D-rich domain antibody detected an intermediate (78 kDa) and fully autolysed (76 kDa) form as well, and in most cases with a 80-kDa preactivated form, the μ-calpain was fully activated. Cases 7, 20, and 26 had *NF2* mutations resulting in truncation of merlin before the cleavage sites by calpain while expressing significant levels of μ-calpain.

methylation, cannot be detected by the protein truncation assay that was used in the Kimura *et al.* (8) study. In tumors with such mutations, the initial reverse transcription step of protein truncation assay would only amplify normal mRNAs from contaminating normal tissues, which are then translated *in vitro* to full-length proteins giving false negative results. On the other hand, homozygous deletion and methylation generally accompany loss of one allele, which is detected by the LOH analysis. Therefore, the protein truncation assay is not a sufficient method to rule out mutations if it is not supplemented with more detailed genetic analysis such as LOH analysis or FISH analysis. We speculate that some of the cases in the study by Kimura *et al.* (8) designated as having wild-type *NF2* based solely on protein truncation assay might have harbored undetected mutations, which led to the suggestion that loss of merlin occurred without *NF2* mutation.

Our data demonstrate no correlation between μ-calpain activation and merlin loss, and indicate that abnormal activation of μ-calpain probably is not functioning as an alternative pathway for merlin loss in meningiomas (8). Although some of the meningiomas expressed high levels of active form μ-calpain, its expression did not show any correlation with merlin expression status, and loss of merlin never occurred without LOH at 22q. μ-calpain has been shown to be involved in the degradation of a wide range of proteins, and it would be difficult to determine which proteins are the native targets of activated μ-calpain found in some meningiomas. Furthermore, calpain activation was also observed in cases (cases 7, 20, and 26) carrying a mutation causing premature truncation before codon 295 and 299 (Table 1), the proposed cleavage sites (8). Therefore, the primary target of activated calpain should not be merlin at least in these cases. We cannot exclude the possibility of rare cases in which calpain activation is involved in meningioma formation, but theoretical candidates for calpain inhibitor administration to suppress meningioma growth would most likely be limited to such rare cases, if any, and it would not obtain in general.

The importance of genetic markers in clinical oncology are increasingly recognized. In anaplastic oligodendrogliomas, for instance, LOH at chromosome 1p has recently been shown to predict marked sensitivity to chemotherapy and better prognosis (20). Similarly, the p53 mutation status of an individual tumor will be key genetic information in the currently ongoing gene therapy trial using adenovirus-mediated induction of wild-type p53 (21). Our current study showed that LOH at chromosome 22q apparently defines two genetic subsets of meningiomas: one with LOH and loss of merlin expression and the other with merlin expression and both copies of 22q. Interestingly, such dichotomy is not observed in the other NF2-related tumor schwannomas. Merlin expression has been shown to be universally lost in schwannomas, indicating that complete inactivation of merlin may be an absolute requirement for schwannoma formation (22). Because surgical cure cannot be achieved in many patients with meningiomas, a novel strategy to control recurrence by suppressing tumor growth is awaited. In evaluating such a new modality in the future, LOH at 22q may be an important marker to be considered.

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