Neuropathology 2007; **27**, 36–42 doi:10.1111/j.1440-1789.2006.00737.x

Original Article

NF2 gene expression in sporadic meningiomas: Relation to grades or histotypes real time-PCR study

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One of the most common regions involved in the meningiomas tumorigenesis is chromosome 22q where the *NF2* gene resides. The deficiency or loss of the *NF2* gene product, merlin/schwannomin, plays a role in tumor development and metastatization. Conflicting results have been reported on the prognostic value of merlin in meningiomas. Several studies have indicated *NF2* gene inactivation as an early tumorigenic event unrelated to the histological grade or clinical behavior. On the contrary, the *NF2* gene alteration rate differs between the different histotypes. A pathogenesis independent from the *NF2* gene has been suggested in meningothelial meningiomas. In the present work, we studied the *NF2* gene expression through real time-PCR (RT-PCR) in 30 meningiomas. The average of the *NF2* gene expression of all meningiomas was considered as reference value. The average of expression of WHO grade I and II meningiomas was higher than the average of all meningiomas, whereas that of WHO grade III meningiomas was lower. When we compared the *NF2* gene expression in the different meningioma grades we did not note a significant difference ($P = 0.698$) despite the tendency to decrease from grade I to grade III. The average expression of meningothelial meningiomas was higher than the reference value, and that of non-meningothelial meningiomas was lower. The difference in *NF2* gene expression between meningothelial and non-meningothelial meningiomas was statistically significant ($P = 0.013$). Our data supports the finding that alterations in *NF2* gene alteration are histotype related but not grade related.

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Received 11 February 2006; revised and accepted 3 April 2006.

Key words: meningioma, meningothelial, merlin, *NF2*, RT-PCR.

INTRODUCTION

Meningiomas are frequent intracranial neoplasms (a fourth of all primary tumors in this site) arising from the leptomeningeal covering of the central nervous system (CNS). They preferentially affect middle aged or elderly women. Radiation exposure, hormonal and genetic factors, particularly neurofibromatosis 2 syndrome (NF2), have been implicated in their development and growth. Meningiomas initiation, both in *NF2* associated and in sporadic cases, is linked to the inactivation of the members of the protein 4.1 superfamily, that is, *NF2* gene product merlin/schwannomin. Approximately 60% of sporadic meningiomas are caused by the LOH on chromosome 22q12 where the *NF2* tumor suppressor gene is localized; no causative gene is known for the remaining 40%.[1–8]

Within the 4.1 superfamily, merlin shares the highest degree of homology with a subgroup of proteins including ezrin, radixin, and moesin (ERM proteins) that link the actin cytoskeleton (by an actin-binding region in the COOH-terminus) to cell membrane glycoproteins such as CD44 (by its NH_2-terminal residues) in polarized cells. The NH_2- and COOH- terminal halves of ERM proteins mutually interact intramolecularly to suppress their binding activities. The COOH- terminal threonine phosphorylation maintains ERM proteins in the active state by suppressing the intramolecular interaction.[5,7,9,10] The region of the merlin with the greatest structural similarity to the ERM proteins correspond to the its NH_2-terminal two-thirds. The merlin COOH-terminus lacks the conventional actin-binding region of the ERM proteins. Contrary to ERM proteins, merlin interacts with F-actin through the

NH_2-terminus.[5,9,11–14] The similarity of merlin with the ERM proteins indicates that its functions may be related to those of the ERM proteins: organization of membrane extensions and cell adhesion, membrane traffic, and cell signaling. Indeed, merlin interacts, either directly or indirectly, with components of cell junctions and with a number of proteins influencing cell-growth regulation, including paxillin, erbB2, p21-activate kinase, and p53 (through MDM2 degradation). Biochemical studies showed that in the CNS merlin also forms a complex with integrin β1 and with Caspr/paranodin, an essential neuronal component of paranodal axoglial junctions.[5,7,9,10,13,15–17]

Several experimental data demonstrated that merlin overexpression results in a significant decrease in cell proliferation, reversion of Ras-induced transformation, and reduction in tumor formation in nude mice. In contrast, the inactivation of the *NF2* gene leads to the development of cancer especially through loss of the contact-dependent inhibition of the growth.[13,14,18,19] The majority of the mutations identified in the *NF2* gene, result in a truncation of the protein and are clinically associated with a severe phenotype; occasionally missense mutations associated with a mild phenotype may occur.

The alterations in merlin functions did not show an incontrovertible prognostic value in meningiomas. They occurred in transitional and fibroblastic meningiomas more often than in meningothelial variant.

In the present work, we studied the *NF2* gene expression through real time-PCR (RT-PCR) in a group of 30 meningiomas and we evaluated its possible correlation with tumor grade and histotype.

MATERIALS AND METHODS

Patients

Tissue specimens were obtained from 30 patients affected by meningiomas surgically treated at the Neurosurgical Service (Careggi Hospital, Florence, Italy) in which fresh tumoral tissue was available for the RT-PCR. Seven (23%) were from men and 23 (77%) were from women. Average age at the time of the surgery was 54 years (range 29–76). Twenty-nine (97%) meningiomas were intracranial (one of which was intraventricular; case no. 22 Table 1), the remaining (3%) case was spinal meningioma (case no. 3; Table 1). Two meningiomas (7%) were relapsed tumors (case no.s 27, 28 Table 1); two meningiomas (7%) had multiple localizations (case no.s 1, 6 Table 1).

Real-time PCR

From each fresh surgical specimen we selected a fragment macroscopically representative of the tumor. Successively, we cut it in half: from one half several 5-μm frozen sections stained with hematoxylin–eosin (HE) were obtained to verify the adequacy of the specimens selected for RT-PCR (presence of pathological tissue only); the other half was immersed in RNAlater (QIAGEN, Valencia, CA, USA) and kept overnight at +4°C and finally stored at –80°C until it was analyzed. The thawed specimens were cut into small pieces and homogenized. After proteinase K digestion (250 μg/mL for 1 h at 37°C), total RNA was isolated with 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA). The RNA concentration and purity in preparations was assessed spectrophotometrically by measuring their absorbance at 260 nm and 280 nm. The RNA fragmentation state was estimated with ethidium bromide-stained 1% agarose gel. The fragment length of RNA was >650 bp in all cases. Total RNA (500 ng) was subject to reverse transcription to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems). The quantitative RT-PCR was performed on an ABI PRISM 7000 Sequence Detector System (Applied Biosystems). PCR products for *NF2* were detected using gene-specific primers and probes labeled with reporter day FAM (Assay on Demand, Applied Biosystems). GAPDH was used as endogenous control gene for normalization. PCR reactions were carried out in 96-well plate with 20 μL per well using 1× Taq-

Table 1 Histopathology and RT-PCR results

Case	Histopathology		RT-PCR
	Who grade	Histotype	
1	I	Fibrous	–0.174
2	I	Fibrous	–0.896
3	I	Fibrous	0.461
4	I	Fibrous	–0.198
5	I	Fibrous	–0.365
6	I	Fibrous	0.501
7	I	Fibrous	–0.037
8	I	Fibrous	–0.219
9	I	Meningothelial	0.361
11	I	Meningothelial	0.385
12	I	Meningothelial	0.432
13	I	Meningothelial	0.376
14	I	Meningothelial	0.339
15	I	Meningothelial	0.09
16	I	Secretory	0.277
17	I	Transitional	–0.054
18	I	Transitional	–0.372
19	I	Transitional	–0.051
20	I	Angiomatous	0.08
21	II	Atypical	0.457
22	II	Atypical	–0.337
22	II	Atypical	0.07
23	II	Atypical	–0.326
24	II	Chordoid	0.265
25	III	Anaplastic	–0.298
26	III	Anaplastic	–0.275
27	III	Anaplastic	0.226
28	III	Anaplastic	–1.277
29	III	Anaplastic	0.487
30	–	Oncocytic	0.055

Man Universal PCR MasterMix. After an incubation for 2 min at 50°C and 10 min at 95°C, the reaction continued for 50 cycles at 95°C for 15 s and 60°C for 1 min.

The $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen was used to calculate fold expression levels relative to the average value of the all meningiomas RNA specimens (the calibrator).[20] We chose the average expression of all meningiomas as reference value to especially stress the stronger difference of expression.

Histopathology

After the drawing of the small samples for RT-PCR, the remaining tissues were routinely fixed in 10% buffered formalin and embedded in paraffin. Five-μm thick sections were stained with HE for morphological evaluation. The diagnostic criteria used were those indicated by the most recently revised World Health Organization (WHO) classification of tumors of the nervous system.[21]

Statistical analysis

The shift of expression level of *NF2* gene as estimated through relative RT-PCR was calculated according to the Kruskal–Wallis test. Data analysis was performed using Primit Version 3.03 (McGraw-Hill, Milan, Italy, 1994) statistical package. A P-value ≤ 0.05 was considered to be statistically significant.

RESULTS

Cases and RNA extraction

Nineteen (63%) lesions were WHO grade I meningiomas (eight fibrous, six meningothelial, three transitional, one secretory and one angiomatous) (Fig. 1), five (17%) were WHO grade II meningiomas (four atypical and one chordoid) (Figs 2,3), five (17%) were WHO grade III meningiomas (anaplastic) (Fig. 4) and one (3%) was an oncocytic meningioma, a novel uncategorized rare variant showing oncocytic differentiation (wide granular cytoplasm full of numerous swollen mitochondria) and uncertain prognosis (Fig. 5).[22]

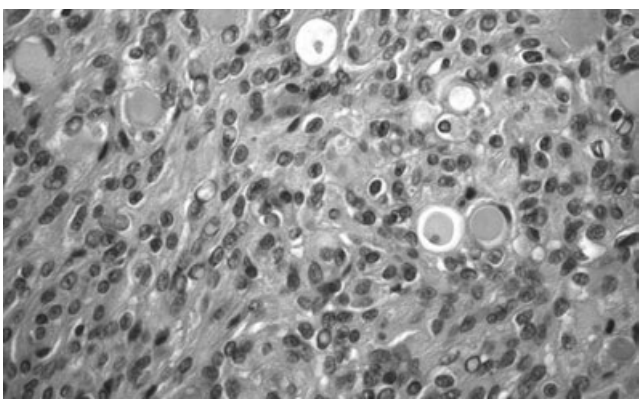

Fig. 1 Secretory meningioma showing intra or extracytoplasmic round hyaline and eosinophilic bodies in a lesion otherwise classifiable as meningothelial meningioma. HE. Original magnification 400×.

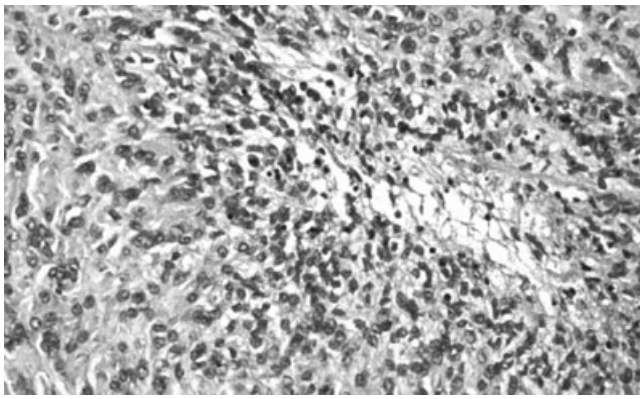

Fig. 2 WHO II meningioma. Atypical meningioma showing foci of necrosis. HE. Original magnification 200×.

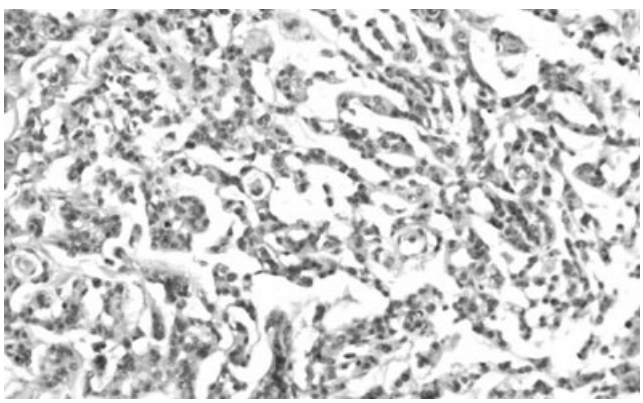

Fig. 3 WHO II meningioma. Chordoid meningioma. HE. Original magnification 200×.

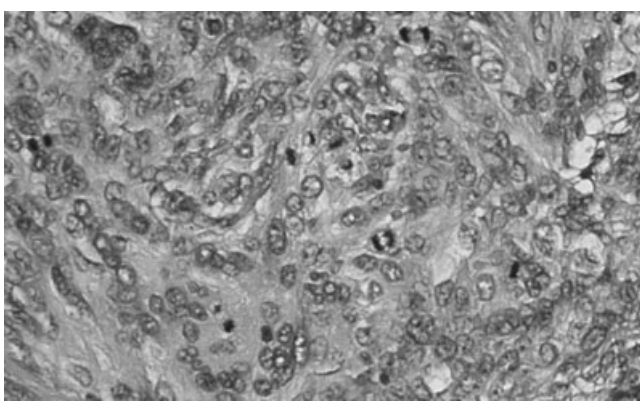

Fig. 4 WHO III meningiomas. Anaplastic meningiomas showing numerous mitoses. HE. Original magnification 400×.

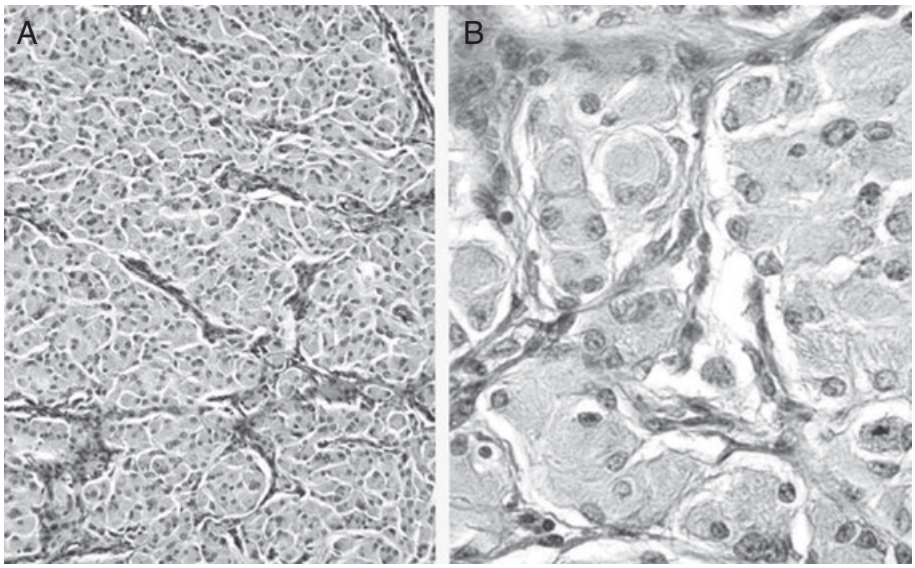

Fig. 5 Oncocytic meningioma: the lesion is composed of sheets (A) of rounded cells with wide granular eosinophilic cytoplasm (B). HE. Original magnification 100× (left), 400× (right).

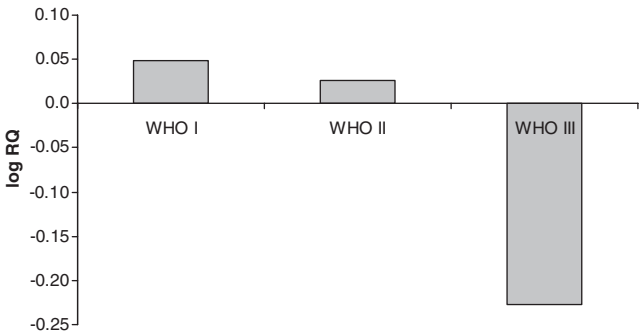

Fig. 6 *NF2* relative expression in WHO grade I, II and III meningiomas as compared with the average expression of all meningiomas.

Real-Time PCR

RNA extraction was successful in all samples. The *NF2* gene expression in all types of meningiomas was compared to the average value of expression (reference value) of the all meningiomas (Table 1).

The mean of *NF2* relative gene expression of all WHO grade I and II meningiomas showed an expression higher (0.049 and 0.025, respectively) than the average of all meningiomas used as calibrator. The mean of *NF2* relative gene expression of WHO grade III meningiomas showed an expression lower (–0.227) than the calibrator (Fig. 6). The only oncocytic meningioma had an expression higher than the calibrator (0.055). When we compared the *NF2* gene expression between the different meningioma grades we did not note a significant difference ($P = 0.698$) despite the tendency to decrease from grade I to grade III (Fig. 6). Analogously, there were not differences between the two relapses and the primary meningiomas ($P = 0.360$). The mean of *NF2* relative gene expression of non-meningothelial meningiomas was lower (–0.100) than the reference value, that of meningothelial meningiomas (we considered together meningothelial meningiomas and the only one secretory meningioma because secretory meningioma is an infrequent meningioma subtype characterized by an advanced epithelial differentiation in a lesion frequently otherwise classifiable as meningothelial meningiomas,[23] Fig. 1) was higher (0.306). Meningothelial meningiomas (meningothelial meningiomas and secretory meningioma) exhibited significantly higher *NF2* gene expression compared with those of non-meningothelial meningiomas ($P = 0.013$ when all meningiomas were considered; $P = 0.018$ when WHO grade I meningiomas only were considered). Within each group of meningiomas, there is a high variability of relative expression of each single case (Table 1; Fig. 7).

DISCUSSION

In previous years, the genetic events involved in the molecular pathogenesis of meningiomas begun to be defined. One of the most common regions involved in the meningiomas tumorigenesis is chromosome 22q where the *NF2* gene resides.[24,25] Inactivating mutations of both alleles are required in the tumorigenesis.[26,27] In the familial setting (NF2 syndrome), a germline mutation inactivates the first allele; the second allele is inactivated by loss of chromosome or part of chromosome 22 on which the gene is located in target cells. Somatic biallelic *NF2* gene mutations are implied for sporadic meningiomas. In some cases the merlin expression has been demonstrated to be reduced or absent also in absence of biallelic *NF2* gene inactivation suggesting that a post-translational regulation of merlin may be involved in the loss of its expression.[28–30]

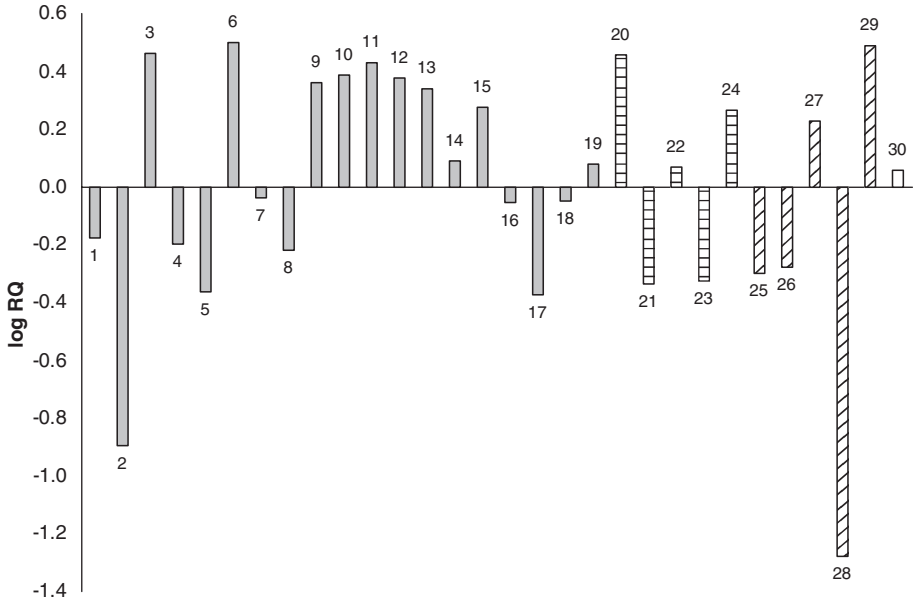

Fig. 7 *NF2* relative expression case per case in WHO grade I (▩), grade II (▤) and grade III meningiomas (▨) and in oncocytic meningioma (case n° 30).

The majority of the *NF2* gene mutations results in a non-functional, truncated protein.[31] The mutations like frameshift, nonsense and splice site alterations in the NH_2-terminal domain give rise to truncated or abnormal protein in both NH_2- and COOH-terminal domains. Other mutations exclusively occur in the COOH-terminal domain. The consequent loss of this region confers tumorigenicity confirming that COOH-terminus is essential for efficient antioncogenic activity.[32] The inability of merlin with altered COOH-terminus to form intramolecular complexes was associated with a failure to negatively regulate cell growth.[33] The in-frame deletions and missense mutation involving one or few amino acids cause the loss of merlin function.[1] Some cases with *NF2* gene mutations, not showing merlin presence,[31] suggest a possible decrease stability of the mutant protein and consequently a difficult detection; moreover recent analysis of the only *NF2* gene missense mutation demonstrated only a mildly decreased protein half-life.[30] It has been suggested that in some meningiomas an abnormal activation of μ-calpain may cause merlin loss by his proteolytic activity.[34] Oxidative-stress might cause μ-calpain activation. Based on these findings, Kaneko *et al*. proposed an interesting scheme of meningioma tumorigenesis: first, the oxidative stress (e.g., due to aging) induces Ca^{2+} mobilization, and Ca^{2+} activates calpain; successively, the autolyzed and activated calpain translocates from the cytoplasm to the plasma membrane; merlin is then cleaved by the activated calpain, and this cleavage product presumably transfers into the nucleus via the perinuclear cytoplasm; as a consequence of this the signal pathway for cell adhesion and the contact inhibition may be impaired, leading to meningiomas.[35]

Conflicting results have been reported with regard to the possible prognostic value of merlin in meningiomas. Several studies suggest that merlin loss is relatively equally distributed among clinicopathological subsets. Indeed, it is not associated with recurrences or with histopathological features predicting unfavorable outcomes (i.e., brain invasion, cellular proliferation, or anaplasia). Actually, the majority of the previous studies have indicated the NF2 protein inactivation as an early tumorigenic event in sporadic and NF2-syndrome associated meningiomas.[3,5,7,27,36,37]

In contrast, the *NF2* gene mutation rate differs between histological subtypes of meningiomas. In 1995, Wellenreuther *et al*. demonstrated that *NF2* gene mutation occurs in 83% of transitional meningiomas and in 70% of fibroblastic meningiomas but in only 25% of meningothelial meningiomas.[36] More recently the hypothesis of a different molecular route of pathogenesis independent of the *NF2* gene pathway in meningothelial meningiomas has been further supported.[6,36–39]

RT-PCR is an accurate method for the determination of levels of specific DNA and RNA sequences in tissue samples. The amount of a specific RNA sequence consents to evaluate the expression of a specific gene. Nevertheless, the gene expression analyses do not assess the integrity or the functionality of the translated protein. Consequently, in our study we could not evaluate the stability and the functionality of merlin. Nevertheless, our results on *NF2* gene expression in different WHO grade meningiomas are in agreement with the published literature. Although the mean *NF2* gene expression of the cases that we studied seems to be reducing from WHO I to WHO III, there is an extreme variability from case to case in each group.

Furthermore, analogously to previous studies that support an *NF2* gene-independent pathogenesis histotype, we observed a statistical difference in the *NF2* gene expression between meningothelial (meningothelial meningiomas and secretory meningioma) and non-meningothelial meningiomas. Secretory meningioma showed high *NF2* expression, meaning that the similarity between these two histotypes might be both morphological and molecular.

In conclusion our results indicate that *NF2* gene expression alterations are not a mark of malignance in meningiomas and that meningothelial meningioma may arise independently of *NF2* gene alterations.

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