

Neurofibromatosis 2: Genetic Analysis of Mild Disease, and Biology of the Gene Product, Merlin

Markku Sainio

From the Department of Neurology and the Department of Pathology,
Haartman Institute, University of Helsinki, Finland

Academic Dissertation

Helsinki 2000

To be publicly discussed with the permission of the Medical Faculty of the
University of Helsinki, in Large Lecture Hall of Haartman Institute,
Haartmaninkatu 3, Helsinki, on January 28th, 2000, at 12 noon.

SUPERVISED BY:

Docent Olli Carpén

Department of Pathology
Haartman Institute, University of Helsinki
Helsinki, Finland

Docent Juha Jääskeläinen

Department of Neurosurgery
University of Helsinki
Helsinki, Finland

REVIEWED BY:

Docent Helena Pihko

Department of Pediatric Neurology
Helsinki University Central Hospital
Helsinki, Finland

Professor Juha Peltonen

Department of Anatomy
University Oulu
Oulu, Finland

OPPONENT:

Dr. D. Gareth R. Evans, MB BS, MD, FRCP

Department of Medical Genetics
St. Mary's Hospital
Manchester, United Kingdom

To Irene, Tatu, Samuli and Aino

Enthusiasm keeps us going

CONTENTS

LIST OF ORIGINAL PUBLICATIONS	7
ABBREVIATIONS	8
1. INTRODUCTION	9
2. REVIEW OF THE LITERATURE	10
2.1. Neurofibromatosis 2	10
2.1.1. Neurofibromatosis 1 (NF1) and 2 (NF2) - two different diseases	10
2.1.2. NF2 genetics	11
2.1.3. Epidemiology	11
2.1.4. Clinical manifestations	11
2.1.5. Clinical course	13
2.1.6. Treatment of NF2 tumours	13
2.2. Schwannoma and meningioma	14
2.2.1. Schwannoma	14
2.2.2. Schwannomatosis	15
2.2.3. Meningioma	15
2.3. NF2 gene	16
2.3.1. Structure	17
2.3.2. Tissue expression and alternative splicing	17
2.3.3. Germ-line mutations	18
2.3.4. Genotype-phenotype correlation	19
2.3.5. Somatic mutations	19
2.4. NF2 gene product, merlin	20
2.4.1. Homology with ERM proteins	20
2.4.2. Distribution of merlin and ERM proteins	21
2.4.3. Function of ERM proteins	22
2.4.4. Tumour suppressor function of merlin	23
3. AIMS OF THE STUDY	24
4. PATIENTS AND METHODS	24
4.1. Clinical investigation (I, III)	24
4.2. Linkage and LOH analysis (I, II)	25
4.3. Mutation analyses (I-III)	25
4.4. Cell expression studies (IV)	26
4.5. Antibodies (II, IV)	26
4.6. Immunoblotting (II, IV)	27
4.7. Immunofluorescence and confocal microscopy (IV).....	27
4.8. CD44 binding assay (IV)	27

5. RESULTS	28
5.1. Characterization of a large Finnish pedigree with very mild and uniform NF2 (I, II)	28
5.2. Splice site NF2 gene mutation, aberrant transcript and aberrant merlin associated with mild phenotype (II)	28
5.3. Characterization of patients with multiple schwannomas (III)	29
5.4. Cell biology of merlin compared with the ERM protein ezrin (IV)	29
5.4.1. Subcellular distribution	29
5.4.2. Cytoskeletal association	30
5.4.3. Association with CD44	30
5.4.4. Morphogenic effects	31
6. DISCUSSION	31
6.1. Large pedigree with very mild and uniform NF2 (I).....	31
6.2. Splice site mutation causes mild NF2 (II)	32
6.3. Sporadic schwannomatosis is not classical NF2 (III)	33
6.4. Merlin is a functional member of the ERM protein family (IV)	34
7. SUMMARY AND CONCLUSIONS	37
ACKNOWLEDGEMENTS	39
REFERENCES	41
ORIGINAL PAPERS I-IV	53

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, referred to in the text by their Roman numerals:

- I Sainio M, Strachan T, Blomstedt G, Salonen O, Setälä K, Palotie A, Palo J, Pyykkö I, Peltonen L and Jääskeläinen J. Presymptomatic DNA and MRI diagnosis of neurofibromatosis 2 with mild clinical course in an extended pedigree. *Neurology* 1995;45:1314-1322.
- II Sainio M, Jääskeläinen J, Pihlaja H, Carpén O. Mild familial neurofibromatosis 2 associates with expression of merlin with the C-terminus of isoform III. *Neurology*, in press.
- III Seppälä MT*, Sainio MA*, Haltia MJ, Kinnunen JJ, Setälä KH, Jääskeläinen JE. Multiple schwannomas: schwannomatosis or neurofibromatosis type 2? *J Neurosurg* 1998;89:36-41.
- IV Sainio M, Zhao F, Heiska L, Turunen O, den Bakker M, Zwarthoff E, Lutchman M, Rouleau GA, Jääskeläinen J, Vaheri A and Carpén O. Neurofibromatosis 2 tumour suppressor protein colocalizes with ezrin and CD44 and associates with actin-containing cytoskeleton. *J Cell Sci* 1997;110:2249-2260.

* These authors contributed equally to the work.

Article III is also in the thesis by M. Seppälä 1998, University of Helsinki.

ABBREVIATIONS

NF1	neurofibromatosis 1
NF2	neurofibromatosis 2
BVS	bilateral vestibular schwannomas
WHO	World Health Organization
SSCP	single strand conformation polymorphism
DGGE	denaturing gradient gel electrophoresis
LOH	loss of heterozygosity
PCR	polymerase chain reaction
RT	reverse transcription
cDNA	complementary deoxyribonucleic acid
ERM	ezrin/radixin/moesin
α -domain	α -helically predicted domain
N-domain	aminoterminal domain
FERM domain	4.1 protein/ezrin/radixin/moesin
N-terminus	aminotermminus
C-domain	carboxyterminal domain
C-terminus	carboxyterminus
ERMAD	ERM association domain
F-actin	filamentous actin
GST	glutathione-S-transferase
PIP ₂	phosphatidylinositol 4,5-bisphosphate
SDS	sodium dodecyl sulfate
kDa	kilodalton
M _r	relative molecular mass
PAGE	polyacrylamide gel electrophoresis

Names of genes are in *italics*.

Electronic-database information:

OMIM = Online Mendelian Inheritance in Man, <http://www.ncbi.nlm.nih.gov/Omim/>

GDB= Genome Data Base, <http://www.gdb.org>

NNFF Online=National Neurofibromatosis Foundation, <http://www.nf.org>

NF2 mutation map is at <http://neuro-trials1.mgh.harvard.edu/nf2/>

1. INTRODUCTION

Approximately 1% of all tumours arise in individuals with hereditary neoplastic syndromes. More than 20 of these hereditary syndromes have been defined, and eight of them predispose to tumours in the nervous system⁶¹. Most of the syndromes demonstrate a dominant pattern of inheritance and loss-of-function mutations in specific tumour suppressor genes. According to Knudson's "two-hit" hypothesis, the inherited first "hit" of a suppressor gene is recessive and the inactivation of the normal allele by somatic mutations initiates tumorigenesis. Typically, the affected individuals develop at young age multiple tumours of amazingly few histologic types at restricted target tissues.

Neurofibromatosis 2 (NF2) is a tumour suppressor syndrome that predisposes to benign but multiple tumours of the nervous system, *i.e.* schwannomas, characteristically at both vestibular nerves, meningiomas, and spinal ependymomas. Consistent with the two-hit model, a germ-line *NF2* gene mutation is present in every cell of the body, and inactivating mutations are found in both alleles of the *NF2* gene in the NF2-associated tumours. Two-hit inactivation of the *NF2* gene is found also in their much more common sporadic counterparts. Thus, multiple early-onset tumours in NF2 are explained by the need of only one additional mutation instead of two at the somatic level. The clinical course of NF2 ranges from mild and late-onset disease to devastating forms leading to early death from multitumour disease. There are also individuals with schwannomatosis, a condition with multiple schwannomas, but no other manifestations of NF2. The association of this disorder with NF2 is not clear.

The *NF2* gene protein product is a novel protein with yet unidentified functions. It was named merlin (for moesin-ezrin-radixin-like protein) because of structural similarity with the ERM family of proteins, ezrin, radixin and moesin. ERM proteins function as linkers between the cytoskeleton and plasma membrane, and may be involved in cellular adhesion and in signal transduction. It is crucial to reveal merlin's functional mechanism for the development of a specific antitumour therapy for this single-gene disease.

The relevance of studying rare NF2 arises from the fact that defects in *NF2* tumour suppressor gene are the initial steps also in the pathogenesis of the much more common sporadic tumours of the nervous system. This thesis on NF2 aims to analyze the genotype-phenotype correlation in a large pedigree with extremely mild and uniform disease, and the molecular background of schwannomatosis. Furthermore, in order to understand the tumour suppressor mechanism of merlin, the normal function of merlin was studied, and compared to ezrin, the prototype member of the ERM family.

2. REVIEW OF THE LITERATURE

2.1. Neurofibromatosis 2

2.1.1. Neurofibromatosis 1 (NF1) and 2 (NF2) - two different diseases

Neurofibromatosis 1 (NF1), previously known as peripheral or von Recklinghausen's neurofibromatosis, and neurofibromatosis 2 (NF2), previously known as central neurofibromatosis or bilateral acoustic neurofibromatosis, are both inherited tumour suppressor syndromes. NF2 predisposes to multiple schwannomas, meningiomas and spinal ependymomas of the central nervous system, with bilateral vestibular nerve schwannomas as the classic diagnostic hallmark, and few non-tumour manifestations⁷⁰. The more common NF1 (insidence 1:4000) predisposes to multiple skin neurofibromas, iris hamartomas (Lisch nodules), optic pathway gliomas and astrocytomas, pheochromocytomas, leukemia and malignant peripheral nerve sheath tumours, but not to NF2-associated tumours²⁰⁷.

The first description of NF2 was probably by Wishart in 1822³. The patient had multiple intracranial tumours arising from the dura and cranial nerves and also from both internal auditory meati, with no cutaneous features. When the German neuropathologist Friedrich von Recklinghausen reported cases of NF1 with neurofibromas in 1882²¹³, NF1 and NF2 cases became collectively referred to as von Recklinhausen's disease¹⁸². The final separation in to two distinct diseases came in 1987, when the *NF1* gene was mapped to chromosome 17¹⁶ and the *NF2* gene to chromosome 22¹⁵⁴. This led to the establishment of the following diagnostic criteria by the National Institute of Health (NIH) Consensus Group clearly separating NF1 and NF2¹.

A clinical diagnosis of NF1 can be made if two or more of the following conditions are met:

1. Six or more café-au-lait macules, over 15 mm in diameter.
2. Two or more neurofibromas or one plexiform neurofibroma.
3. Axillary or inguinal freckling.
4. Optic glioma.
5. Two or more iris hamartomas, "Lisch nodules".
6. Typical bony changes like sphenoid dysplasia or thinning of the cortex of long bones.
7. A first degree relative with NF1.

The NIH diagnostic criteria for NF2:

1. Bilateral eighth nerve masses; or
2. A first-degree relative with NF2 and either a unilateral eighth nerve mass, or two of the following: neurofibroma, meningioma, glioma, schwannoma, or juvenile posterior subcapsular lenticular opacity.

2.1.2. NF2 Genetics

The inheritance of NF2 is autosomally dominant. About half of the NF2 cases represent new mutations, which gives a high calculated mutation frequency for the *NF2* gene, 7.5×10^{-6} , partly reflecting the low reproductive fitness of severe sporadic cases⁵⁶. The penetrance of clinical NF2 is age-dependent and considered complete by age 60 years^{55,57}.

When the gene for NF2 was mapped to chromosome 22, in 1987,¹⁵⁴ predictive testing by linkage analysis became possible in families (≥ 2 affected)^{138,159}. All familial NF2 cases have been linked to this region, suggesting that NF2 is a single-gene disease^{59,138}. The cloning of the *NF2* gene^{155,197}, in 1993, made direct mutation detection possible. However, the detection of mutations is very laborious due to a large variation in the type and location of *NF2* gene mutations, almost always different in unrelated individuals¹²¹. Somatic mosaicism, apparently rather common in NF2^{60,100}, leads to a lower transmission risk and can cause misleading linkage results²⁴.

2.1.3. Epidemiology

Only two epidemiological studies on NF2 have been published. Evans and associates found 19 NF2 cases in a population of 4 016 100, a diagnostic prevalence of 1 in 210 000, and estimated the birth incidence of 1 in 33 000-40 562⁵⁶. Antinheimo and associates reported a clearly lower incidence, 1 in 87 410, in southern Finland¹¹, probably because the study was based on medical records, while in England also the relatives at risk were screened by CT or MR imaging.

2.1.4. Clinical manifestations

Bilateral schwannomas of the vestibular (8th cranial) nerve, the hallmark of NF2, develop in virtually all cases⁵⁴, and multiple schwannomas of other cranial nerves occur in more

than one third^{54,131,141}. Schwannomas affect primarily the sensory nerves and spinal dorsal roots^{54,113}. Meningiomas, often multiple, are seen intracranially in half of the patients^{54,131,141}. Characteristic of NF2 are also the multiple spinal tumours seen in MRI scans in up to 90% of patients^{51,54,130,131}. The spinal tumours are typically schwannomas, meningiomas, or mainly asymptomatic intramedullary ependymomas^{54,73,110,130,131,141}.

Skin tumours arising from peripheral nerves seen in over half of NF2 patients are predominantly schwannomas, but also some neurofibromas occur^{54,73,132,141}. One third of NF2 patients have skin café-au-lait macules, but only in less than 2% their number reaches six, one of the diagnostic criteria for NF1^{54,132,141}.

Another diagnostic hallmark of NF2 are ocular abnormalities, cortical and posterior subcapsular or capsular cataract, retinal hamartomas and epiretinal membranes, encountered in over 90% of cases^{92,131,142,150}.

NF2 patients display also other lesions of the nervous system including asymptomatic schwannosis (proliferation of Schwann cells), glial hamartias, meningioangiomas and intracranial calcification, and polyneuropathy^{115,157,212}.

Several large clinical studies have confirmed the clinical picture of NF2^{54,93,131,141}. Based on the clinical picture, additional diagnostic criteria were proposed⁵⁵, because the original NIH criteria seemed to be too rigid¹.

Current diagnostic criteria for NF2⁵⁵:

Bilateral vestibular schwannomas (VS) OR family history of NF2 *plus*

1. Unilateral VS *or*
2. Any two of: meningioma, glioma, neurofibroma, schwannoma, posterior subcapsular lenticular opacities (SLO).

or (additional criteria)

1. Unilateral VS + Any two of: meningioma, glioma, neurofibroma, schwannoma, SLO *or*
2. Multiple meningiomas (2 or more) + unilateral VS *or* any two of: glioma, neurofibroma, schwannoma, cataract, cerebral calcification.

The best diagnostic examination is the magnetic resonance imaging (MRI) with gadolinium contrast enhancement of the entire neuraxis, complemented with eye and skin examination. To exclude NF2, repeated investigations should be considered if the individual has a family history of NF2, unilateral vestibular schwannoma or meningioma

at age < 30 years, multiple spinal tumours, or café-au-lait macules and skin tumours insufficient for a diagnosis of NF1.

2.1.5. Clinical course

The severity of NF2 ranges from mild (late onset at ≥ 30 years; slowly growing vestibular schwannomas; few other manifestations) to aggressive (early onset at ≤ 20 years; multiple rapidly growing tumours causing early death in the 3rd or 4th decade)⁵⁶. The onset of symptoms and manifestations has varied between age 2 years and the 5th decade^{54,57,131,141}. The mean age at onset of symptoms is about 20 years, the diagnosis is made 8 years later, and mean life span is about 40 years^{54,141}. In most cases, the initial symptoms are due to vestibular schwannomas, hearing loss, tinnitus, balance problems or vertigo^{54,131,141}. Young patients are likely to present symptoms from skin and spinal tumours or ocular manifestations^{54,129,141}.

NF2 generally demonstrates intrafamilial similarity in its course^{52,54,141}, but some families present with both mild and severe phenotypes^{17,98,141,171}, and monozygotic twins are not identical¹⁸.

2.1.6. Treatment of NF2 tumours

In NF2, the majority of tumours grow slowly, and gradually cause symptoms by compressing the adjacent structures. Complete microsurgical removal is the treatment of choice, but the tumours are also amenable to stereotactic radiotherapy. The management of bilateral vestibular schwannomas is especially demanding because of the high probability of hearing loss in both ears, facial nerve paresis and disequilibrium. The treatment of tumours must take into account tumours elsewhere in the nervous system or the need of repeated interventions due to later arousal of other tumours. Thus, the timing of the intervention and the treatment option should be evaluated individually for each NF2 patient.

Available treatment for bilateral vestibular schwannomas includes: 1) observation without surgical intervention; 2) bony decompression of the internal auditory canal; 3) partial tumour removal; 4) complete removal with auditory brainstem implant; 5) total tumour removal with attempted hearing preservation; and 6) stereotactic radiation³⁵. However, in NF2-associated tumours hearing preservation and the recovery of facial nerve has been less successful than in non-NF2 vestibular schwannomas^{26,27,166}. A less favourable

outcome is also seen in NF2-associated spinal schwannomas compared with sporadic tumours⁹⁶. Recently at centres of expertise, there are promising reports of a fair chance (up to 65%) of hearing preservation with excellent facial nerve recovery in small completely resected NF2-associated vestibular schwannomas^{166,185} and similar success rates for stereotactic radiation (tumours with diameter < 30 mm)¹⁹⁰. However, in severe NF2, multiple meningiomas with aggressive growth may be incurable by surgery or radiotherapy. Other treatment options are limited, although some results have been obtained with hydroxyurea, antiestrogen and antiangiogenic compounds^{126,170,193}. The usefulness of targeted treatments, such as vector-mediated *NF2* gene transfer with response in primary meningioma cultures⁸⁶, remains to be seen.

2.2. Schwannoma and meningioma

2.2.1. Schwannoma

A schwannoma (previously neuroma or neurilemmoma) is almost always a benign (grade I, WHO), slowly growing and sharply demarcated tumour of the cranial, spinal, and peripheral nerves, with sensory nerves as the preferred site²¹⁶. Schwannomas account for an estimated 8% of all intracranial and one fourth of spinal tumours in adults, and the approximated annual incidence of vestibular schwannomas is 1.2 tumours/100 000¹⁹⁶ and spinal schwannomas 0.3-0.4/100,000¹⁷⁷. The incidence of peripheral nerve schwannomas is not known. For an unknown reason, most intracranial schwannomas originate from the vestibular part of the eighth cranial nerve in the cerebello-pontine angle¹⁴. The peak incidence of sporadic schwannomas is in the fourth to sixth decades of life, and they occur approximately equally in both sexes at all ages²¹⁶.

Morphologically, schwannomas are usually encapsulated tumours composed of spindle-shaped Schwann cells with elongated nuclei. The growth pattern consists of Antoni A areas, represented by closely packed tumour cells, and Antoni B areas, where the tumour cells are loosely arranged. Sometimes the cells are arranged in a palisade fashion with their nuclei aligned, occasionally forming roundish Verocay bodies. Schwannomas differ from neurofibromas, the hallmark tumours in NF1, which are non-encapsulated tumours composed mainly of the cell types forming the fine ordered supportive compartments of peripheral nerves, but organized in a haphazard way.

Bilateral vestibular schwannomas, the indicators of NF2, represent 8.5% of all vestibular schwannomas¹⁶⁵. In general, NF2-associated schwannomas are similar to their sporadic counterparts, but there are also differences. NF2 tumours present at an earlier age, are multiple, and show a lobular "grape-like" growth pattern at both macroscopic and microscopic examination, extremely uncommon in sporadic schwannomas¹⁸⁶. NF2 schwannomas demonstrate higher proliferation potential (MIB-1)^{2,8} and more foci of high cellularity¹⁸⁶, suggesting more aggressive behaviour. NF2-associated vestibular schwannomas more often contain embedded nerve fibres of the adjacent facial nerve than sporadic schwannomas^{74,91,123}. Consequently, preservation of the continuity and function of the facial and cochlear nerves during surgery is more difficult in NF2¹⁶⁶.

Cytogenetic and molecular studies show that deletions on 22q occur in the majority of sporadic schwannomas, and chromosome 22q seems to be the only constant chromosomal region involved in the pathogenesis of schwannomas^{10,42,173}.

2.2.2. Schwannomatosis

Multiple schwannomas (≥ 2) without other manifestations of NF2, a condition termed as schwannomatosis (neurilemmomatosis) may be a separate entity from NF2^{120,148,181}. In the earlier reports of schwannomatosis, many cases actually fulfill the diagnostic criteria of NF2^{1,120}. Convincing evidence of a distinct disease entity came from the patient series of MacCollin et al.¹²⁰, who carefully excluded NF2, confirmed the tumour histology, and demonstrated the lack of family history suggesting a non-inherited condition. Some patients had a highly localized disease, suggesting a possible segmental mutation of the NF2 gene, whereas other individuals had a more general disease possibly due to a tumour suppressor gene syndrome¹²⁰. The candidates for the genetic syndrome could be (1) NF2, at the mildest end of the clinical spectrum, which is supported by the identification of a germ-line *NF2* mutation in a schwannomatosis patient⁸²; (2) another locus, predisposing to the development of schwannomas; or (3) NF1, although unlikely, since tumours in NF1 are almost exclusively neurofibromas not schwannomas⁵³.

2.2.3. Meningioma

Meningiomas are usually slowly growing tumours attached to the dura, composed of neoplastic meningotheelial (arachnoidal) cells. Meningiomas are graded according to signs

of anaplasia into three categories (WHO): Grade I (90%), Grade II (atypical) or Grade III (anaplastic)¹¹⁴. The histological subtypes in Grade I meningiomas are meningothelial (53%), transitional (28%), and fibrous (8%)¹⁰⁵. Meningiomas constitute 20% of all primary intracranial tumours, they occur in 2.3/100 000 population and show a male-to-female ratio of 1:2²⁸. Meningiomas are rare in childhood and adolescence, and their incidence increases with age with a peak in the 7th decade²⁸. Most meningiomas are asymptomatic and go unnoticed if not found incidentally on neuroradiological examination or autopsy (found in 1.4% of autopsies)¹⁵¹.

In some sporadic cases (4-10%), meningiomas are multiple^{11,114}. Interestingly, multiple tumours from patients with more than two lesions have shown to be clonal in origin, suggesting a subarachnoidal spread from a single progenitor cell^{109,187,206,220}. In extremely rare cases meningiomas may be familial without any signs of NF2^{133,183}, and at least in one such family the *NF2* gene locus was excluded, suggesting a different meningioma predisposition gene¹⁴⁶. No cases of familial meningiomatosis were found in a population of 1.6 million in Finland¹¹.

In general, multiple meningiomas or their occurrence at young age suggests NF2. Most NF2 meningiomas are also of WHO grade I, but more frequently of fibroblastic type¹¹⁵, and demonstrate more aggressive growth (higher MIB-1 indices) than sporadic meningiomas⁹.

Cytogenetic and molecular studies show that deletions on 22q occur in the majority of sporadic meningiomas of all malignancy grades, suggesting that inactivation of this chromosomal region represents an early genetic event in the pathogenesis of meningiomas¹⁶¹. In atypical and malignant meningiomas, other regions than 22q seems to be associated with malignant progression, as suggested by frequent losses on chromosomes 1p, 6q, 9q, 10q, 14q, 17p and 18q, and chromosomal gains, most commonly on 20q, 12q, 15q, 1q, 9q and 17q^{95,108,209}.

2.3. NF2 gene

Frequent loss of alleles on chromosome 22 in NF2-associated schwannomas and meningiomas, and genetic linkage analyses of large NF2 families located the defect, in 1987, to 22q11.1-22q13.1^{154,173-175,211}. In 1993, the *NF2* gene, with germ-line

mutations in NF2 patients, was cloned by two separate groups^{155,197}. Mutations in both alleles of the *NF2* gene in tumor tissue confirmed the two-hit pathogenetic mechanism of tumour suppressor genes, earlier proposed by Knudson for retinoblastoma¹⁰¹.

2.3.1 Structure

The *NF2* gene at 22q12.2 contains 17 exons and an open reading frame of 1785 nucleotides encoding a 595 amino acid protein^{155,197}. The first intron contains a highly polymorphic CA-repeat marker, NF2CA3, and four biallelic DNA variants immediately flank the *NF2* gene^{31,221}. The *NF2* gene is phylogenetically conserved and is found in *C. elegans* and *Drosophila*²⁰². The cloned mouse gene shows 90% cDNA sequence identity with the human *NF2* gene^{41,72}.

2.3.2. Tissue expression and alternative splicing

The *NF2* gene is expressed widely despite the restricted phenotype of the NF2 disease. Two major *NF2* RNA transcripts of 2.6 and 7 kb, and a weaker one at 4.4 kb, are detected in adult human kidney, lung, breast, ovary, brain, liver, and pancreas²¹. In rat and mouse the wide foetal expression is restricted to the adult distribution pattern during the embryonic development^{68,84}. In adult rats and mice, *NF2* expression is detected by RT-PCR in the central nervous system (cerebral cortex, brainstem, cerebellum, spinal cord), dorsal root ganglia, testis, ovary, adrenal gland tissues and cultured Schwann cells. By in situ hybridization, the expression in the nervous system is restricted to neurons of the hippocampus, brainstem (pons, mid-brain, medulla), facial and trigeminal motoneurons, neurons in vestibular and coclear nuclei, cerebellum (Purkinje and granule cells, locus coeruleus, reticular nuclei), spinal cord (neurons in the gray matter) and primary sensory neurons in the dorsal root ganglia⁶⁸.

Two major mRNA isoforms, I and II, result from alternative splicing of the last two exons (16/17)²¹. They are expressed at about equal levels, and have a tissue-specific and a development-specific expression level in human, mouse and rat cells⁶⁸. In the human nervous system, cultured Schwann cells and pure glial cultures express relatively more isoform I than II⁶⁸. Several other alternatively spliced *NF2* transcription products have been identified in normal human tissues involving exons 1a (within intron 1), 2, 3, 15^{13,149}, and also exon 8⁸¹ and exon 10¹⁴⁹. The isoform III (also termed E2 or D208),

splice variant without exon 15 and 16, is detected at low levels in various human tissues^{13,149} but not at all in mouse or rat⁶⁸. Recently three new splice variants have been shown to be expressed in fibroblasts, lymphoblasts and adult retina but not in other adult tissues: Mer151 with exons 1/4/8/9/10/13/14/17 and Mer150 with exons 9-13 and Mer162 with exons 5-16 skipped¹⁶⁹. The biological significance of the isoforms and the mechanisms that regulate their expression is not known.

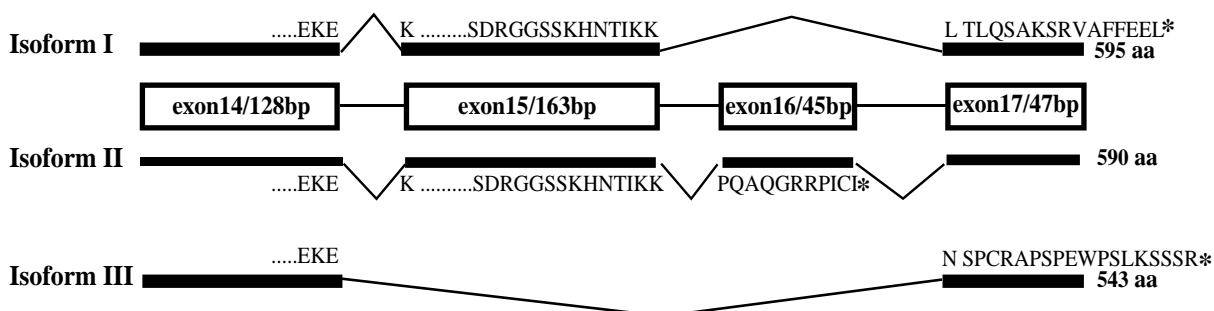


Figure 1. The COOH-terminal *NF2* gene isoforms I, II, and III, respectively, result from alternative splicing of exons 15, 16 and 17. The number of residues contained in each polypeptide is indicated. An asterisk denotes the merlin COOH-terminus.

2.3.3. Germ-line Mutations

Over 200 germ-line *NF2* gene mutations have been reported, including all different types of mutations^{12,23,30,32,43,59,82,87-89,98-100,117-119,135,142,155,162-164,167,171,197,203,208,221}. The mutations span the entire gene, except the variantly spliced last exon 16/17, without mutational hot spots¹²¹. Nonsense mutations due to C to T transitions in five of the six CGA codons in exons 2, 6, 8, 11 and 13, are especially frequent¹⁶⁴. The nonsense, frameshifting and splice site mutations predicted to cause premature translation termination and protein truncation account for a vast majority of all *NF2* mutations¹²¹ (Table 1).

Table 1. Germ-line *NF2* mutations^a

<u>Exons:</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>
<u>Mutations (no):</u>	13	26	15	6	10	19	11	22	1
<u>Exon size (bp)^b:</u>	114	126	123	84	69	83	76	135	75
<u>Exons:</u>	<u>10</u>	<u>11</u>	<u>12</u>	<u>13</u>	<u>14</u>	<u>15</u>	<u>16</u>	<u>17</u>	
<u>Mutations (no)</u>	11	26	18	10	10	17	0	0	
<u>Exon size (bp):</u>	114	123	218	106	128	163	45	47	

^aReported *NF2* mutations; a mutation map is accessible at <http://neuro-trials1.mgh.harvard.edu/nf2/>

^bExon size and numbering according to Jacoby et al.⁸⁷

In NF2, the mutation detection rate by exon screening (SSCP and DGGE) has been low (34 to 66%)^{59,98,118,135,142,162}. This is seen especially in mild cases, possibly due to the inability to detect large deletions or low level somatic mosaicism, both associated with mild disease^{59,60,142,221}. Recently, mutation search by combining different molecular methods has revealed a significant proportion of large deletions (6 out of 16 mutations) increasing the mutation detection rate to about 85%²²¹.

2.3.4. Genotype-phenotype correlation

There is some correlation between the type of mutation and the severity of NF2 (age of onset, hearing loss and diagnosis, and number of tumours)^{59,98,121,135,142,162}. No effect of the position of the mutation has been noted in NF2. Truncating mutations typically associate with a severe disease, whereas missense and splice site mutations, and also large deletions associate with milder NF2^{59,135,142,162}. In severe cases, truncating mutations are seen in 86%, splice mutations in 49%, and missense in only 33%¹²¹. Splice donor site mutations associate with milder disease than splice acceptor mutations, although exceptions occur^{59,98,99,135,142,162}. The unpredicted outcome, also within families, is associated especially with splice site mutations⁹⁹. Also, truncating mutations usually associated with severe NF2, may cause mild disease in a somatic mosaic^{29,89,100}. Furthermore, phenotypic variability (tumour burden) seen in monozygotic twins suggests that the outcome depends also on stochastic events, such as loss of the second *NF2* allele¹⁸. Altogether, in individual cases, cautiousness is suggested in the prediction of outcome based on the mutation type⁵⁹.

2.3.5. Somatic mutations

In two-thirds of NF2-associated tumours and their sporadic counterparts, two different *NF2* gene mutations have been found in the same tumour. Often seen as a single mutation combined with a loss of the normal allele, detected as loss of heterozygosity of polymorphic markers at *NF2* region or entire chromosome 22^{50,88,160}. Inactivation of at least one allele of the *NF2* gene is seen in most sporadic counterpart tumors, i.e. in over 90% of sporadic schwannomas⁸⁸. This provides strong evidence for a *NF2* gene tumour suppressor mechanism in NF2-associated and sporadic schwannomas, meningiomas and ependymomas.

In tumours, the vast majority of somatic *NF2* gene mutations are predicted to cause a truncation of the protein product, similar to germ-line mutations. However, somatic mutations are more often out-of-frame deletions or insertions than base substitutions¹²¹. The mutations in schwannomas span throughout the *NF2* gene⁸⁸, while mutations in meningiomas may be more unevenly distributed, affecting mainly exons 1 to 13 (majority in exons 1-8)⁴⁴. In sporadic meningiomas, *NF2* gene mutations are found mainly in the tumours of the fibroblastic and transitional type (70 to 80%) and less in the meningothelial type (25%)^{115,210}. In some meningiomas, other genes on chromosome 22q have been shown to be disrupted, for example the β -adaplin (BAM22)¹⁴⁴ and the *MNI* gene¹¹². Sporadic benign meningiomas without allelic losses on 22q have displayed deletions in 1p and 3p, suggesting that these regions may contribute to meningioma tumorigenesis in a subset of cases³⁸. Most of the WHO grade II sporadic spinal ependymomas harbour *NF2* gene mutations^{25,50}.

Cytogenetic aberrations and DNA sequence copy number losses in 22q involving the *NF2* gene region have been detected in some tumour types not associated with *NF2*¹⁰². *NF2* gene mutations have been demonstrated frequently in malignant mesotheliomas (MM), especially in MM cell lines^{22,39,45,176}, and occasionally in melanomas, colon and breast carcinomas^{13,21}. The presence of *NF2* mutations in non-*NF2* tumours with an array of other genetic aberrations suggests a role for the *NF2* gene in the progression of other malignancies¹¹¹.

2.4. *NF2* gene product, merlin

2.4.1. Homology with ERM proteins

The *NF2* gene product, merlin¹⁹⁷ or schwannomin¹⁵⁵ or *NF2* protein, is a 595-amino acid protein with a predicted molecular weight of 66 kDa. Merlin demonstrates sequence similarity with the ERM proteins, ezrin, radixin and moesin, three highly homologous membrane-cytoskeleton linker molecules^{15,122,199,204}. ERM proteins belong to a larger superfamily of band 4.1 proteins, which includes as its prototype the erythrocyte band 4.1 protein with its many homologues, several protein tyrosine phosphatases and talin¹²². Merlin shares the predicted overall domain structure of ERM proteins, which consists of a globular N-terminal domain (~1-310 amino acids in merlin), an α -helical region (~300-470 aa), a proline rich stretch between the α - and C-domain, and a charged C-terminal domain (~470-595). Merlin

demonstrates highest homology to the ERM proteins in its N-terminal domain with over 60% identity, while the homology in α -helical and C-terminal domains is only 20-30%²⁰² (Figure 2). The homologous ~300-amino-acid FERM (F for 4.1 protein, E ezrin, M moesin, R radixin) domain is present in all band 4.1 superfamily members and in some other molecules such as myosin VIIA⁴⁰.

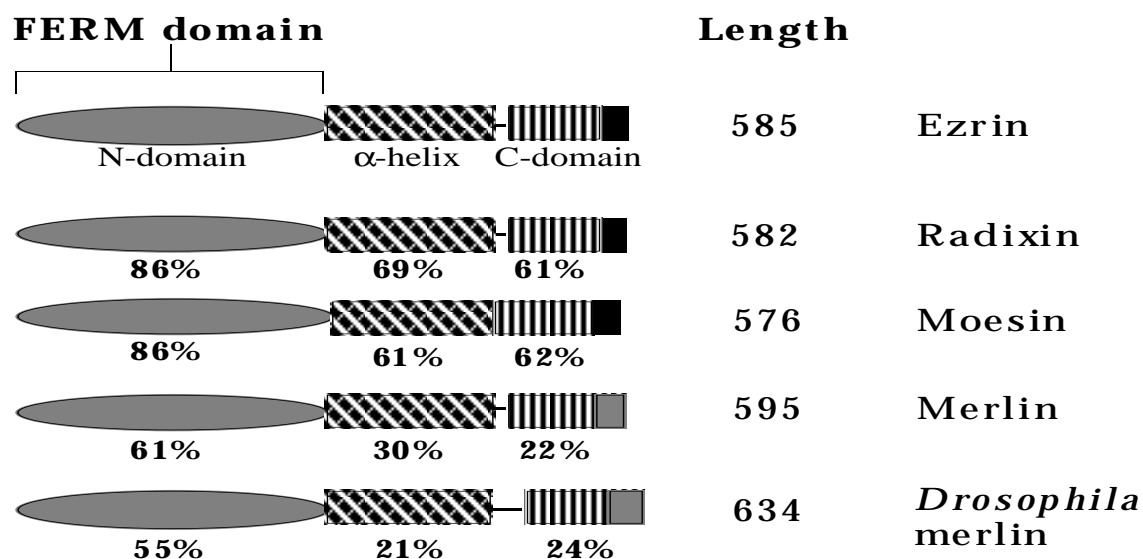


Figure 2. Overall domain organization of ERM proteins, human and *Drosophila* merlin. The globular N-domain is the most homologous region termed as FERM, followed by an α -helix. α - and C-domains are likely to have an extended conformation and are separated by a polyproline-strech. The C-terminal actin-binding site (n) is shown. Length is in amino acids.

2.4.2. Distribution of merlin and ERM proteins

Ezrin, radixin and moesin are concentrated in actin-enriched specialized plasma membrane structures, such as microvilli, filopodia and membrane ruffles³³. Cultured cells usually express all ERM proteins, and all cell types within the body express one or more of the ERM proteins, but their expression pattern in tissues is cell-type specific²⁰. Although the subcellular distribution of all ERM proteins seems to be very similar, this is not always the case. In cytokine-stimulated motile T lymphocytes, ezrin is distributed homogeneously in the cytoplasm, whereas moesin is located preferentially at the tip, and radixin in the neck of uropods¹⁷⁸.

Although *NF2* RNA expression indicated wide distribution, merlin is detected mainly in human epithelia, mesothelium, smooth muscle, endothelial cells, neurons of the central nervous system, Schwann cells, arachnoidal cells and faintly in glial and ependymal cells^{47,48,85,163,168,188,189}. Some discrepancy between immunohistochemistry and mRNA-in-situ-hybridization results may be due to the low expression of merlin in some tissues.

2.4.3. Function of ERM proteins

ERM proteins are membrane-cytoskeleton linkers involved in the organization of the cortical cytoskeleton, formation and maintenance of membrane structures, cell migration and adhesion. The overlapping functions of ERM members are highlighted by the finding that all membrane projections disappear and cellular adhesions are disrupted when expression of all three ERM proteins is blocked by antisense oligonucleotides¹⁹⁴. The linker mechanism of ERM proteins is based on their ability to bind the cytoplasmic domains of transmembrane proteins through the N-terminal FERM domain and to anchor actin microfilaments. ERM proteins interact directly with the cytoplasmic domain of cell surface adhesion molecules such as CD44 (hyaluronate receptor), CD43 (sialophorin), and intercellular adhesion molecules, ICAM-1, -2 and -3^{76,77,198,218,219}. ERM proteins bind also indirectly to plasma membrane through interaction with EBP50 (for ezrin-binding-protein) and E3KARP molecules which have PDZ domains, known to bind to cytoplasmic tails of specific membrane proteins¹⁵³. F-actin interaction sites have been mapped to the last 34 C-terminal amino acids and to the N-terminal domain of ERM family members^{143,156,201}.

A characteristic feature of ERM proteins is their ability to self-associate, oligomerize and bind to each other by head-to-tail joining^{20,63}. The two interacting domains, defined as N- and C-ERMADs (for ERM association domains) regulate the functional activity of ERM proteins by masking binding sites for other molecules such as actin or transmembrane proteins. Thus, soluble full length molecules are in a “closed” or “dormant” state in which the biologically relevant binding sites are masked. Activation of the ERM proteins is proposed to occur through a conformational change, possibly by phosphorylation and by interaction with phosphoinositide phosphatidylinositol 4,5-bisphosphate (PIP₂), which relieves the interaction between N- and C-domains^{128,137}. The open form of the molecule allows phosphorylation of the conserved Thr residue within the F-actin binding region by Rho-kinase and protein kinase C- θ ^{34,127,128,137,145}. Activation un masks the F-actin and EBP50 binding sites, increases binding, and causes a

translocation of the ERM proteins from the soluble pool to the membrane-skeleton while dephosphorylation reverses the effect^{122,184}. Also the secondary messenger phospholipid, PIP₂, known to conformationally regulate several cytoskeletal components, binds the N-terminal part of ezrin and enhances the interaction between ezrin and transmembrane proteins⁷⁹.

An apparently important function of ERM proteins is their yet incompletely understood role in the Rho and cAMP-dependent protein kinase A (PKA) signalling pathways. ERM proteins serve as anchoring sites for proteins of intracellular signalling cascades such as Rho-GDP dissociation inhibitor (GDI), Rho-GDP/GTP exchange factor Dbp1^{191,192} and a regulatory subunit of PKA⁴⁹. Also, ezrin associates with the E-cadherin/ β -catenin complex, which has a role in the control of cell proliferation^{19,80}. ERM proteins may serve as major coordinators of cell signalling at very relevant subcellular localizations, *i.e.* sites of intercellular connections, membrane protrusions of migrating cells. Although ERM proteins are not known to associate with tumour suppression, in a recent study, inhibition of ezrin expression by antisense oligonucleotides caused increased invasion in colorectal cancer cells⁸⁰. Other reports have suggested that ezrin may be linked to increased proliferation and cell survival. Abnormal or high expression of ezrin is associated with transformation and increased proliferation⁹⁴ and ezrin is an integral component in the cell survival machinery by conveying antiapoptotic signals^{64,106}.

2.4.4. Tumour suppressor function of merlin

Several lines of evidence indicate a direct growth suppressor function for merlin. Most schwannomas, meningiomas and malignant mesothelioma cell lines demonstrate inactivation of the *NF2* gene and absence of merlin^{39,45,71,113}. Knock-out heterozygous (*NF2*^{+/-}) mice develop a variety of highly metastatic tumours which have lost both *NF2* alleles¹³⁴, and in *Drosophila*, loss of the respective *NF2* homologue (Dmerlin) results in cellular overproliferation¹⁰⁷. *In vitro*, merlin suppresses the growth rate of NIH-3T3 cells¹¹⁶ and reverses the malignant phenotype, *i.e.* anchorage-independent growth, of Ha-ras-transformed cells¹⁹⁵. Also, an increase in the expression of merlin due to increased confluency suggests a role in the contact inhibition of cell growth¹⁷⁹. This is in line with the cell detachment and increased proliferation seen in response to antisense oligonucleotide inhibition of merlin expression⁸³. In experimental models, the potential to

inhibit cell growth is associated with isoform I, whereas neither isoform II, nor constructs truncated at amino acid 547 of the C-terminal domain, possess such a function^{116,180}.

3. AIMS OF THE STUDY

The specific aims of this study were:

1. to characterize the clinical course of NF2 in a large Finnish pedigree.
2. to identify the mutation causing very mild NF2 and to study its consequences at the RNA and protein level.
3. to determine whether the *NF2* gene is involved in the etiology of schwannomatosis.
4. to determine whether the *NF2* gene product, merlin, is functionally a member of the ERM protein family.

4. PATIENTS AND METHODS

4.1. Clinical investigation (Studies I and III)

Patient ascertainment: A large Finnish NF2 pedigree included 65 descendants in four generations (I). Patients with two or more schwannomas were identified from a cohort of 243 patients operated on for spinal schwannoma at the Department of Neurosurgery, Helsinki University Hospital (III). A **clinical** examination was done to detect any neurological abnormalities, skin manifestations or peripheral tumours. The patients were questioned for family history suggestive of NF2 or NF1: any tumours, hearing loss, ocular changes or skin pigmentation. The **otological** examination included audiometry, brainstem auditory evoked responses, and posturography (I). The **ophthalmological** examination included biomicroscopy of the iris and the lens, measurement of the density of the lens, and dilated fundus examination with an indirect ophthalmoscope (I, III). **MRI** (Study I/1.0 T and Study III/ 0,23 T) of the head and spine was performed with a gadolinium-contrast enhancement. Review of the **histological** specimens was performed for the operated schwannomas (I, III) and 20 sporadic controls (III). **Death certificates** for the deceased patients were obtained from Statistics Finland (I, III).

The written informed consent of the family members was obtained prior to the examinations. Data were given to family members only if requested. The results of the

presymptomatic molecular testing were given, if requested by a clinical geneticist counselling a family member. The study protocol was approved by the ethical committees of the Departments of Otolaryngology, Neurosurgery, Neurology and Radiology, Helsinki University Central Hospital.

4.2. Linkage and LOH analysis (I, II)

DNA was extracted from peripheral blood leukocytes of 73 members, or skin fibroblasts from three family members, and five tumours (stored at -80° C). Three of the tumours were surgically removed vestibular schwannomas and two were obtained at autopsy.

Four polymorphic flanking markers in the vicinity of the NF2 gene were used: a HincII polymorphism at the human heavy neurofilament subunit gene (NEFH); and three CA-repeat polymorphisms, CRYB2 at the beta-crystalline 2 gene, D22S268 and D22S280 (Genome Data Base). Two-point lod scores and the risk analyses were calculated by the MLINK program (5.10) with the following assumptions: a female to male recombination ratio of 1.4; an age-dependent penetrance of 0.05 (0-19 years), 0.25 (20-29 years), 0.50 (30-39 years), 0.75 (40-49 years), or 0.90 (50 years or over)²¹¹; a disease gene frequency of 2.9×10^{-5} and a mutation rate of 6.5×10^{-6} for the NF2 gene⁵⁶. The probability of a double-recombination to occur with two nested pairs of flanking markers was estimated at < 1%. Therefore, if the flanking markers of the NF2 gene are informative in an individual, the risk of being affected can be given with either < 1% or > 99%, depending on which haplotype is inherited.

In the LOH analysis (II), DNA from tumour and patient fibroblasts was analyzed for the DNA copy number with an intragenic NF2 microsatellite marker CA3 and with two flanking markers, CRYB22 and D22S280 (Genome Data Base). LOH was considered to be present, if the signal was less than half of the strength of the remaining allele.

4.3. Mutation analyses (I-III)

The molecular genetic DNA analysis included all 17 exons of the NF2 gene, which were either studied by Single Stranded Conformation Polymorphism (SSCP) (II) or sequenced using an automatic sequencer (II-III). Fibroblast RNA was converted by reverse transcriptase and amplified (RT-PCR) with 8 overlapping pairs of oligonucleotides covering the NF2 gene coding region and studied by gel electrophoresis and SSCP analysis (I, II). Southern blot analysis was performed with three restriction enzymes and

the complete [³²P]CTP-labeled NF2 cDNA, kindly provided by Dr. James Gusella, was used as a probe (I).

Identification of the *NF2* gene mutation allowed us to adopt the solid-phase minisequencing method for direct mutation detection and presymptomatic diagnosis (II). In the minisequencing reaction with genomic DNA a detection primer hybridizing immediately adjacent to the 1737+3 a → t mutation was elongated with radiolabeled nucleotides, TTP to detect the mutant t₁₇₃₇₊₃ and subsequently ATP to detect the wild-type a₁₇₃₇₊₃.

4.4. Cell expression studies (IV)

NF2 cDNA was subcloned into the eukaryotic expression vector pcDNA3 and pREP7 (hygromycin selection marker). Monkey COS-1, Chinese hamster ovary (CHO), human embryonic kidney-derived 293-EBNA and U251 malignant glioma cells were grown on untreated or fibronectin coated coverslips. Transfections of COS-1 and CHO cells were performed using the DEAE dextran and LipofectAMINE method, respectively. 293-cells were transfected by the calcium phosphate coprecipitation method and were studied after four weeks in hygromycin selection.

Association of merlin with the cytoskeleton was studied using two approaches, extraction of transfected COS-1 cells with a non-ionic detergent (0.5% Triton X-100) and treatment of cells with cytochalasin B. The comparison of the detergent-soluble and detergent-insoluble fractions was done by the densitometric analysis option of the NIH Image program. Detergent-extracted cells on coverslips were labeled for merlin and ezrin and with phalloidin, and analyzed by confocal microscopy. The actin-containing cytoskeleton of COS-1 cells was disrupted with cytochalasin B.

4.5. Antibodies (II, IV)

Four antibodies raised against NF2 cDNA-derived peptides were used: (1) 1398NF2⁴⁶ against residues 508-533 and (2) mAb HB7⁴⁶ against residues 192-209 (both kindly provided by Drs. den Bakker and Zwarthoff), (3) anti-SCH¹¹⁶ against residues 566-595 (kindly provided by Dr. Rouleau), (4) anti-GST-M/S rabbit antiserum against residues 131-253. Ezrin was detected with mAb 3C12³⁷ and CD44 with mAb Hermes-3⁹⁰ (kindly provided by Dr. S. Jalkanen).

4.6. Immunoblotting (II, IV)

Cultured cells or tumour lysates, in Laemmli buffer, were separated by SDS-PAGE, transferred to nitrocellulose sheets and used for immunoblotting. Merlin antisera, ezrin mAb 3C12 and commercial peroxidase-conjugated secondary antibodies were used, and the peroxidase was detected by enhanced chemiluminescence. Molecular weights were evaluated by the NIH Image program (version 1.60).

4.7. Immunofluorescence and confocal microscopy (IV)

Transfected cells and U251 cells were stained for merlin with anti-SCH, anti-GST-M/S and 1398NF2 antiserum followed by TRITC-conjugated secondary antibody. For double staining of merlin and ezrin, fixed cells were incubated simultaneously with a merlin-antiserum and mAb 3C12, followed by TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG. Double staining of merlin and CD44 was carried out as above, but the primary antibodies used were 1398NF2 and mAb Hermes-3. To visualize merlin and F-actin, COS-1 cells grown on fibronectin-coated coverslips were incubated with anti-SCH and TRITC-labeled phalloidin. The specimens were viewed with a confocal 410 Invert Laser Scan Microscope. In some experiments, cell contours were visualized with FITC-conjugated wheat germ agglutinin (WGA) and using the differential interference contrast (DIC) option of the confocal microscope. Cell apoptosis was analyzed by direct fluorescence detection of genomic DNA fragmentation.

Merlin and control pSV β -Galactosidase-transfected CHO cells, stained with FITC-WGA and anti-SCH or anti- β -galactosidase mAb, were studied by confocal microscopy using immunofluorescence and DIC options. Transfected cells from three experiments were measured for the largest diameter (length, d) and surface attachment area (SA) using the morphometry software of the microscope. The significance of differences between merlin and β -galactosidase expressing CHO cells was calculated by the Mann-Whitney U-test.

4.8. CD44 binding assay (IV)

Recombinant merlin was produced by expressing merlin as a GST-fusion protein, using the baculovirus expression system and Sf9 insect cells. Interaction of merlin and CD44 was studied with lysates of Namalwa human B lymphoma cells without CD44, Namalwa

transfectants stably expressing CD44⁴, and recombinant GST-merlin. The CD44 that was bound to GST-merlin was detected with iodinated anti-CD44 mAb Hermes-3.

5. RESULTS

5.1. Characterization of a large pedigree with very mild and uniform NF2 (I, II)

Linkage analysis of all family members at risk identified six manifestation-free individuals with NF2. Clinical examination of 22 family members and gathering all follow-up data disclosed eleven members with NF2. Nine imaged tumours, three of them asymptomatic, have not been operated on due to slow growth and retained hearing. MRI disclosed 5 occult tumours in four affected individuals: 2 convexity meningiomas, 2 spinal schwannomas, and one with appearance of intraspinal glioma. Eight tumours of six members were operated between 1969 and 1992 without tumour recurrence. The eight operated vestibular tumours, and the intraspinal tumour (radiologically a glioma) analyzed at autopsy, were schwannomas (I, II). Characteristic for the mild disease was the late onset of hearing deterioration at ages 40 to 50 years with slow progression at few dB per year. The late-onset tumorigenesis is highlighted by the absence of tumours in three asymptomatic adult members with NF2 at age 29 to 41 years or before age 21 years. The tumour progression was slow and only one vestibular schwannoma grew large enough to occlude the fourth ventricle and cause hydrocephalus, and none of the affected members have died of the disease. Furthermore, the tumours demonstrated lower proliferation indices than a series of unrelated NF2-associated vestibular schwannomas (II). Minimal other manifestations of NF2 were present. One member had minor posterior lens opacity and a Lisch nodule, and had presented at age 11 with an apparent paraganglioma (glomus tumour).

5.2. Splice site NF2 gene mutation, aberrant transcript and aberrant merlin associated with mild phenotype (II)

The mild and uniform disease in this large pedigree was shown to be caused by a novel mutation in the *NF2* gene at intron 15 splice donor site (1737 + 3 a -> t). The mutation was identified by the minisequencing method in all NF2 family members previously identified with linkage analysis. The mutation resulted in splicing out of exon 15 and production of two RNA transcripts: a novel mutant transcript with exon 16 and 17, and

the overexpression of isoform III, normally detected at a low level. Both transcripts encode for the C-terminus of isoform III. Immunoblotting of the patient fibroblasts and tumour tissue demonstrated variant merlin with altered C-terminus at an intensity comparable to the wild-type protein. Loss of heterozygosity, a common somatic event in schwannomas, was studied in three rather quiescent tumours of this family, and all showed loss of the wild-type *NF2* allele.

5.3. Characterization of patients with multiple schwannomas (III)

Thirteen patients with at least one spinal and an additional spinal or peripheral schwannoma were identified from a cohort of 243 patients operated on for spinal schwannoma. Five of the thirteen patients appeared to have classical NF2. The remaining eight patients with multiple schwannomas, and one with no spinal but four peripheral schwannomas, were further studied to reveal any other manifestations of NF2. In the histological reanalysis, all tumours were schwannomas. The tumours demonstrated, more often than sporadic control schwannomas, a lobular appearance and frequent Verocay bodies, signs suggestive of NF2. The patients were middle-aged at first surgery (median 43.5 years) and all were sporadic cases, *i.e.* no positive family history of schwannomas, NF2, NF1 or associated features. In a thorough clinical study, including MRI of the neuraxis after a median follow-up time of 9.9 years, none had any cutaneous, ocular or tumour manifestations suggestive of NF1 or NF2. In the follow-up, the peripheral tumours did not increase in number, and the number of spinal tumours increased only in one case from 3 to 8 during 21 years, and no intracranial tumours developed despite advanced age (five cases between 55 and 68 years). Two of the nine schwannomatosis patients had died, both from unrelated causes at 50 and 65 years. No germ-line mutations could be detected by sequencing all 17 exons of the *NF2* gene from lymphoblast-derived DNA of the seven schwannomatosis patients who were alive.

5.4. Cell biology of merlin compared with the ERM protein ezrin (IV)

5.4.1. Subcellular distribution

Transfected merlin and endogenous ezrin in COS-1 cells, and the endogenous merlin and ezrin in U251 glioma cells revealed a highly overlapping distribution underneath the plasma membrane and in cell surface projections and blebs. Merlin and ezrin showed an almost

identical staining pattern in COS-1 cells grown on fibronectin. The localization was quite similar at intracellular structures which sometimes stained in a network-like or filamentous pattern, and at regions near the cell membrane. In COS-1 cells grown on glass coverslips, filopodia and the ruffling membrane which contained merlin often lacked ezrin, although in untransfected and mock-transfected COS-1 cells these structures contained ezrin. This suggests that transfected merlin may relocate ezrin from its normal subcellular regions under certain growth conditions.

5.4.2. Cytoskeletal association

In transfected COS-1 cells, merlin colocalized with some of the F-actin filaments in the cell body and with membrane-associated blebs, but most of the F-actin-containing microfilaments showed no reactivity for merlin. Cytochalasin B treatment resulted in disruption of the actin-containing filaments and clustering of F-actin, and major reorganization of merlin and ezrin. In peripheral areas of the cells, especially in retraction fibres, merlin was accumulated in clusters which always contained F-actin and in most cases ezrin. On the other hand, many F-actin clusters, particularly in the cell body, were devoid of merlin. Also unclustered merlin, which presumably represents a detergent-soluble fraction, could be detected.

In transfected COS-1 cells, extracted with Triton X-100, the proportion of both proteins in the non-extractable cytoskeleton-associated fraction varied between 20 and 25%. Confocal microscopy of COS-1 cells, briefly treated with detergent before fixation, revealed that merlin and ezrin were retained in the cell ghosts and their distribution was overlapping. A faint network-like staining pattern of merlin and ezrin was more evident in detergent-treated cells than in untreated cells, presumably due to removal of the soluble cytoplasmic protein.

5.4.3. Association with CD44

The reported linkage between CD44 and the ERM proteins^{79,198} prompted us to study whether endogenous CD44 in COS-1 cells would colocalize with merlin, and whether merlin expression would affect CD44 distribution. In untransfected or mock-transfected cells, CD44 was distributed diffusely on the cell surface or colocalized with ezrin in delicate microvillar structures. In contrast, in cells transfected with merlin, CD44 and merlin were accumulated at structures resembling thick microspikes and blebs at the dorsal membrane. The CD44/merlin clusters were retained in cells subjected to detergent prior to fixation,

suggesting that CD44 in these structures is associated with the cytoskeleton via merlin. When lysates of CD44 Namalwa-transfectants were reacted with recombinant GST-merlin immobilized on glutathione beads, a binding of CD44 was detected.

5.4.4. Morphogenic effects

Many of COS-1, 293 and CHO cell transfectants were elongated and demonstrated merlin-positive cell membrane blebs, not seen in β -galactosidase transfectants. CHO cells, least variable in morphology, were most suitable for a quantitative morphogenic analysis of the maximum diameter, surface attachment area and cell elongation after merlin or β -galactosidase transfection. The transfected CHO cells were much more elongated ($p < 0.0001$) when expressing merlin than β -galactosidase, but the cell surface attachment area was not affected.

6. DISCUSSION

6.1. Large pedigree with very mild and uniform NF2 (I)

The family described here presents a very mild course of NF2 with slowly growing and late-onset vestibular schwannomas, no marked hearing deficit before 40 to 50 years of age, and few other tumours. NF2 is arbitrarily subdivided into mild, intermediate and severe forms, and in the mild form NF2 presents at age ≥ 30 years⁵⁹. Compared to the severe form, the milder disease associates with later mean age of onset of hearing loss (33 years vs. 22 years) and mean age at death (61 years vs. 42 years)¹⁴¹. The course of NF2 in the members of this family demonstrated remarkable clinical uniformity, which has been noted in NF2 families^{54,55,142}. However, reports of families with both mild and severe phenotypes^{17,98,141,171}, and monozygotic twins with a non-identical disease¹⁸ suggest that epigenetic, stochastic or environmental factors may modify the phenotype. However, in this large NF2 pedigree with eleven affected and six as yet manifestation-free individuals, it is unlikely that the mild phenotype would be due to genetic factors other than NF2. Furthermore, there is no evidence of modifying genes or environmental factors in NF2.

6.2. Splice site mutation causes mild NF2 (II)

In general, NF2 provides an ideal model for the genotype-phenotype analysis of a tumour suppressor gene. Since somatic inactivation of the *NF2* gene is typically a large loss in 22q, the inherited germ-line *NF2* mutation should primarily dictate the clinical outcome. The penetrance of NF2 is virtually complete, and limited variation of the phenotype is seen within a single family. The family in this study is one of the few large and well-characterized NF2 pedigrees and thus especially suitable for genotype-phenotype correlation. This mild NF2 disease was caused by a splice donor site mutation at intron 15 in the *NF2* gene. In accordance, mild NF2 has been associated with exon 15 splice donor mutations and skipping of exon 15^{97,162}. In these families the disease course is similar, although clinical variability frequently associates with splice site mutations⁹⁹. The mutation gave rise to the expression of two transcripts in fibroblasts, a novel mutant transcript with exon 16 and 17, and the overexpression of isoform III. Also previously, overexpression of isoform III has been seen to occur with intron 15 splice donor mutations (1737 AGgt to ATgt and 1737+12 ins 220 bp), but no novel transcripts were detected^{88,97}. This might be due to different splicing mechanisms in the tissues analyzed (fibroblasts vs. lymphoblasts and schwannoma), or due to different transcriptional consequences.

The expression of the aberrant transcripts and merlin in the patient fibroblasts at levels comparable to wild-type protein and in a schwannoma shown in this study, agrees with the concept that certain mutations, theoretically missense or in-frame changes, may result in protein products with partially retained tumour suppressor function⁶⁹. This is supported by the presence of truncated and full-length merlin in a subset of schwannomas^{71,75,85}. This highlights the importance of studying the mutational consequences at the protein level, which may aid in the prediction of the disease course and reveal domains important for the tumour suppressor function.

In the presented family, both transcripts from the mutant allele encode the C-terminus of isoform III. The decreased proliferation rate of tumours in association with the C-terminus of isoform III suggests that this isoform has partially retained the tumour suppressor function. So far, there is no information on the function of isoform III. Interestingly, transgenic mice expressing merlin truncated at the C-terminus demonstrated no tumorigenesis, and also the growth suppressive functions of *Drosophila* merlin are contained within the N-terminal domain^{65,107}.

6.3. Sporadic schwannomatosis is not classical NF2 (III)

Multiple schwannomas have been suggested to represent a third form of NF, schwannomatosis, although some authors have considered it to be an attenuated form of NF2^{58,147}. In agreement with previous reports on sporadic schwannomatosis^{58,120,139,215}, the patients in this study had multiple schwannomas but no other manifestations of NF2 including meningiomas and ocular abnormalities, which are found in >50% and >90% of NF2 cases, respectively^{54,131,141,150}. The favourable prognosis, presentation in middle-age and no intracranial tumours has been also recognized by others^{58,82,89,120,139,215}. This does not distinctively rule out NF2 because, in NF2, tumours may initially appear elsewhere than on the vestibular nerves, and in late-onset families, members with an *NF2* gene mutation without tumour manifestations are occasionally found in their seventies^{55,57,162}. Although the clinical aspects in this study were not supportive of classical NF2, the schwannomas displayed histological features associated with NF2: more lobular "grape-like" growth pattern, and more Verocay bodies, uncommon in sporadic schwannomas¹⁸⁶. Patients with two schwannomas (three of the nine cases in our series) may represent chance occurrence against the annual incidence of 0.3 - 0.4/100 000 of symptomatic spinal schwannomas¹⁷⁷ (the incidence of occult spinal or peripheral schwannomas is unknown). None of our patients had a positive family history, although a subset of schwannomatosis is known to be familial^{58,89,215} as also shown by Antinheimo in a population-based study in Finland. Schwannomatosis families have been shown to associate with the *NF2* gene in genetic linkage analyses⁵⁸.

Recently, the molecular analysis of tumours from schwannomatosis patients suggested four alternative molecular backgrounds in schwannomatosis⁸⁹. (1) The disorder may not be due to the inactivation of the *NF2* gene, as three tumours of one individual with a highly aggressive disease did not display any *NF2* gene abnormality, seen in > 90% of schwannomas⁸⁸. This is supported by the detection of schwannomas with no alterations in the *NF2* gene but deletions elsewhere on 22q³⁶. (2) It may be due to somatic mosaicism, as multiple tumours at anatomically different locations displayed the same *NF2* mutations, and in one case the mutation was detected at an extremely low level in the lymphocytic lineage⁸⁹. Indeed, somatic mosaicism has been recently proposed to be a fairly common (15% of cases) phenomenon in NF2⁶⁰. (3) Multiple tumours may be a noncontinuous spread of a single tumour, when tumours with the same *NF2* mutations are restricted to a confined anatomical region. This has not been shown for schwannomas, but may explain

multiple meningiomas in sporadic cases^{187,206,220}. (4) Schwannomatosis may be due to an inherited predisposition to schwannomas, because in some familial cases tumors display different somatic mutations but LOH at always the same allele⁸⁹.

No germ-line *NF2* gene mutations were found in the present series, in line with other reports of sporadic or familial schwannomatosis^{58,89}. Thus, classical *NF2* mutations are unlikely, although the current mutation detection methods miss one-third of the mutations¹⁴². Occasional *NF2* mutations have been identified in schwannomatosis^{82,147}, but because some of the cases have later turned to be NF2, there is a high probability of late-onset classical NF2 in these cases⁹⁷⁻⁹⁹. Somatic mosaicism, shown to occur occasionally in sporadic schwannomatosis⁸⁹, could not be excluded in this study because mutation search by exon sequencing does not detect mutations at a very low level. Also, lower mutation detection rates in mild than in severe NF2¹⁴² suggest that other mutational mechanisms not detectable by the current methods, such as large deletions of the *NF2* gene, may be involved in schwannomatosis. Altogether, sporadic schwannomatosis seems to be distinct from the classic NF2, with a more favourable prognosis.

Based on the current knowledge and our observations, slight modifications can be suggested to the clinical diagnostic criteria for schwannomatosis, as proposed by Jacoby et al.⁸⁹ to strengthen the discrimination to mild NF2, segmental NF2 and chance tumour occurrence. Furthermore, evidence of any intracranial tumours, especially meningiomas at young age, or other NF2 manifestations should raise suspicion of classic NF2.

The modified criteria of schwannomatosis:

1. three or more pathologically diagnosed schwannomas without an anatomically limited distribution *and*
2. lack of radiographic evidence of vestibular schwannoma, at age >18 years, and meningioma at age < 30 years

6.4. Merlin is a functional member of the ERM protein family (IV)

The *NF2* tumour suppressor protein, merlin, shares structural characteristics with the ERM family of membrane-organizing proteins. Thus far, limited information has been available on its cellular distribution and functions. This study shows that merlin has a subcellular distribution beneath the cell membrane and at specialized cellular protrusions, similar to that of ERM-family members^{6,62,140,214}. The observed distribution is in accordance with the preferential membrane-bound localization of the transfected merlin at

the dorsal surface of COS-1 cells⁴⁶ and at the ruffling edges in primary meningioma cells⁶⁶. Furthermore, our experiments demonstrated that merlin in most subcellular regions colocalized with ezrin. The result is not surprising as ezrin, radixin and moesin are able to dimerize with each other^{7,63,143}. Furthermore, an interaction between merlin and ezrin and the ability of head-to-tail dimerization *in vitro* and *in vivo* has been demonstrated⁶⁷. The apparent discrepancy in staining results with Gonzalez-Agosti et al.⁶⁶, who found a non-overlapping distribution for merlin, ezrin and moesin in a primary meningioma cell line, probably reflects cell type or stimulation specific variation in ERM protein distribution. We also observed a difference in the distribution in response to different growth substrata. When COS-1 cells were grown under conditions which result in poor development of the F-actin network, merlin appeared to redistribute ezrin from sites where it is located in untransfected cells. This is of interest as ERM proteins demonstrate interchangeability and functional redundancy^{78,194}. For instance, overexpressed radixin displaces moesin from microvilli and filopodia⁷⁸. In this respect, merlin shows functional similarity to other members of the ERM family.

The association of merlin with the cytoskeleton was indicated by its resistance to detergent extraction and by coclustering with F-actin after cytochalasin B treatment. The partial detergent-insolubility was alike the behavior of ezrin and in line with previous reports^{5,46}. The presence of merlin in the soluble and cytoskeleton-associated fraction, like other ERM proteins, suggests that also merlin undergoes a change in conformation that could coincide with the translocation between the soluble pool and the membrane-skeleton. The ERM proteins are in their phosphorylated form when associated with the membrane-skeleton and revert to the soluble pool following dephosphorylation^{33,122,200,204}. Interestingly, merlin has been shown to change rapidly between phosphorylated and unphosphorylated state in response to growth inhibition stimuli by serum starvation and loss of cell adhesion¹⁷⁹. The association between merlin and F-actin-containing cytoskeleton is evident, but whether a direct interaction exists between merlin and actin remains to be determined. The C-terminal F-actin-binding site of ERM proteins (ezrin residues 552-585) is only partially retained in merlin, but the villin/ERM actin-binding motif at this site is conserved^{201,202}. Evidence against a functional actin-binding site is suggested when C-domain expressed as GST-fusion proteins or by *in vitro* transcription/translation does not bind polymerized actin²¹⁷ (Turunen, unpublished). Recently, we have detected that merlin interacts with globular β -actin and filamentous α -actin by the N-domain residues 1-339 (Zhao, Sainio, Grönholm, Carpén, unpublished), in line with Xu et al., who detected an actin-binding domain at residues 178-367²¹⁷. It is

tempting to propose that the homologous RKKK cluster (residues 299-328 in merlin), critical for the actin-binding of ERM proteins, is the likely actin binding site also in merlin^{124,156}. Furthermore, merlin (residues 519-590 of isoform II) binds also to another component of the cytoskeleton, β II-spectrin¹⁷².

The ERM family members bind to the cytoplasmic domain of transmembrane glycoprotein CD44^{79,198}, and in this study such an association was also shown for merlin. Furthermore, we have recently verified the direct interaction between CD44 and the N-terminal domain of merlin by the yeast two-hybrid method (Sainio, Grönholm, unpublished). The interaction of merlin and CD44 and merlin-dependent redistribution of CD44 suggests that merlin could regulate the adhesive functions of transmembrane molecules by regulating their distribution. The aberrant expression of CD44 is associated with tumour progression and metastasis¹⁵⁸. The intracellular interactions of the cytoplasmic domain of CD44 are known to regulate the ultrastructural localization of CD44 on the cell surface, and to affect cell motility and cell-matrix adhesion²⁰⁵. Thus, the tumour suppressor mechanism of merlin could be comparable to that of the APC protein, which in association with the E-cadherin/catenin complex, is associated with colon carcinogenesis¹⁹. This would provide an explanation to the impairment of cell adhesion subsequent to antisense oligonucleotide inhibition of merlin expression or expression of mutant merlin^{83,103}. In an analogous model, transfection of ezrin to thymoma cells leads to clustering of ICAM-2 and an increase in ICAM-2-dependent adhesion⁷⁷. Another mechanism may be that merlin and ERM proteins via their interaction with molecules containing PDZ domains (Na⁺-H⁺ exchanger regulatory cofactor, hNE-RF or EBP50) regulate the outside-in signalling events by clustering transmembrane proteins and key components of downstream transduction pathways^{34,136,152}.

The membrane-cytoskeleton linker mechanism of merlin is thus similar to that of ERM proteins (Figure 3). Interestingly, NF1 tumour suppressor protein, neurofibromin, has recently been shown to associate with epithelial cell adhesion site components, CK 14, desmoplakin and β 4-integrin in differentiating keratinocytes¹⁰⁴. Thus, neurofibromin may also have a role in connecting cytoskeletal components and cell adhesion molecules.

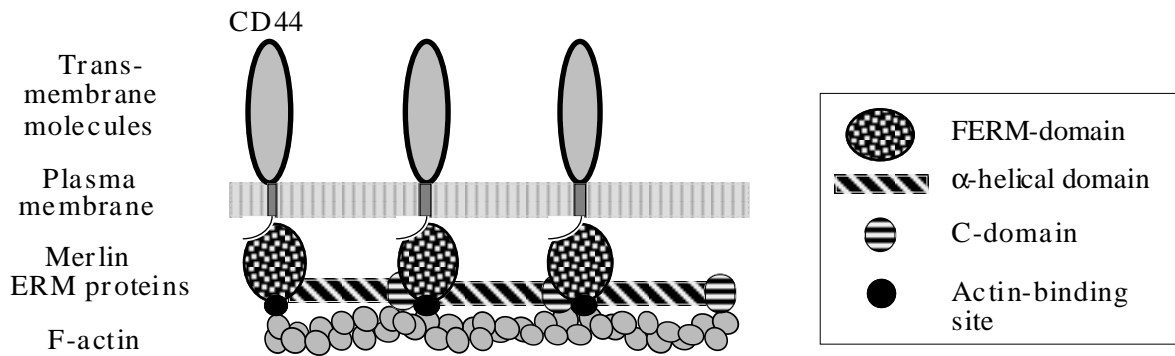


Figure 3. Membrane-cytoskeleton linker model for merlin. In the cortical cytoskeleton, merlin and ERM proteins form head-to-tail oligomers. They interact with cytoplasmic tails of transmembrane adhesion molecules (CD44) with the N-terminal FERM domains. An F-actin binding site in FERM-domain anchors actin microfilaments.

A major function of ERM proteins is the regulation of dynamic cell surface processes. In present experiments, transfected merlin induced morphological changes which do not occur with wild-type ezrin or radixin^{78,124}. However, in non-adherent thymoma cells, transfected wild-type ezrin induces cells to form uropod-like extensions⁷⁷. Transfected cells contained membrane blebs and their cell body was significantly elongated. Such changes in cell length must be associated with reorganization of the cytoskeleton, apparently regulated by merlin. The morphogenic effect of merlin is also seen in NIH-3T3 transfectants¹¹⁶. In further characterization of the merlin domains responsible for the morphogenic effects, such as long cell surface projections or blebbing (Zhao, Sainio, Carpén, unpublished), revealed striking similarities with the morphogenic effects induced by expressed domains of ezrin^{124,125}.

7. SUMMARY AND CONCLUSIONS

Inactivation of the neurofibromatosis 2 (NF2) tumour suppressor gene leads to the development of multiple nervous system tumours, accompanied by loss of the NF2 protein, merlin or schwannomin. The severity of the disease varies between patients, but the biological basis of this variation is poorly understood. We studied the genotype-phenotype correlation in a large pedigree with an extremely mild and uniform disease, the molecular background of schwannomatosis, and the normal function of *NF2* gene protein product, merlin.

In a large NF2 pedigree, the disease course was very mild and uniform, manifesting as late-onset slowly growing bilateral vestibular nerve schwannomas and minimal other

manifestations. This phenotype is caused by a novel mutation in the *NF2* gene at intron 15 splice donor site (1737 + 3 a → t). The mutation resulted in splicing out of exon 15 and production of two transcripts encoding for the C-terminus of isoform III and expression of variant merlin with altered C-terminus. In association with exceptionally mild NF2, this suggests retained tumour suppressor activity for the “mutant” protein and isoform III. The detection of expressed mutant proteins in fibroblasts may provide useful information in the prediction of the clinical outcome of individual mutations.

Further, we studied whether germ-line *NF2* gene mutations were involved in the etiology of schwannomatosis, which manifests as multiple schwannomas but not at vestibular nerves. In this study, nine cases of sporadic schwannomatosis presented tumours at middle-age, lacked other clinical manifestations of NF2, and had a favourable prognosis. There were no detectable germ-line mutations in the *NF2* gene. Thus, schwannomatosis seems to represent a separate clinical entity from NF2, although the molecular background may associate with the *NF2* gene.

To understand the tumour suppressor mechanism of the *NF2* gene protein product, merlin, we studied its normal function in comparison to ezrin, the prototype member of the ERM family. In cultured cells, merlin was codistributed with ezrin and localized subcellularly underneath the plasma membrane at the cortical cytoskeleton in a pattern typical of ERM proteins. Merlin colocalized with the transmembrane adhesion molecule, CD44, and bound to CD44 *in vitro*. Merlin was, by several criteria, associated with the cytoskeleton, particularly with F-actin. The interaction was indicated by partial colocalization, cytochalasin B-induced coclustering, and retention of merlin in the detergent-insoluble fraction. Merlin overexpression induced morphogenic changes such as membrane extensions and blebs, and cell elongation. Thus, merlin is not only homologous with ERM proteins, but possesses membrane-organizing properties characteristic of ERM-family members, and also affects cell morphology. Based on these results, merlin appears to act as a dynamic anchor protein between the cell surface and the cytoskeleton, and possesses functional properties of ERM family members, linkers between plasma membrane molecules and the cytoskeleton. Therefore, merlin is a unique type of tumour suppressor protein. Its mechanism of action may extend from being a structural protein to the coordination of adhesion and cell signalling pathways, functions recently demonstrated for ERM proteins.

ACKNOWLEDGEMENTS

This study was carried out between 1994 and 1999 in the Department of Neurology, the research laboratory at Meilahti Hospital, and the Department of Pathology, Haartman Institute, University of Helsinki. I warmly thank Professors Jorma Palo and Eero Saksela for the excellent research environment and advanced facilities. I extend my thanks to the Department of Otolaryngology, where Professor Ilmari Pyykkö created a researcher-friendly atmosphere.

I thank Jorma Palo, Professor of Neurology, a scientist and writer, for convincing me that neurology was my field. During my residency, Jorma aroused my interest in neurofibromatosis and led me to the world of genetics of neurological diseases.

I am grateful to Professor Leena Palotie and Docent Aarno Palotie for initiating me into molecular genetics (far from squash courts) and their high spirit in scientific work. Leena Palotie's '24-hour open lab' with always someone there is an unforgettable experience. I heartily thank all the members of the Palotie lab at the National Institute of Public Health. Aarno, always kind and encouraging, kept me going even through difficult times. Thanks to Aarno and Dr. Pekka Nokelainen who got me to 'understand' linkage analysis.

Then came along neurosurgeon Juha Jääskeläinen and others in the acoustic neuroma research group, Professor Ilmari Pyykkö and Docent Göran Blomstedt. I thank all of you for giving me the opportunity to plunge into neurofibromatosis 2. I thank you, Juha, for guiding me with this thesis, impulsively, but with focus. I thank also neuroradiologist Oili Salonen and neuro-ophthalmologist Kirsi Setälä, who taught me how scientific clinical studies are carried out.

I learned to appreciate the neurosurgical perspective to research through my dear collaborator Dr. Matti Seppälä, as well as Dr. Juha-Pekka Antinheimo and Dr. Mika Niemelä.

After clinical studies, it was merlin's turn. In this work, I am most grateful to Docent Olli Carpén, the second supervisor of this thesis, for his guidance into the cell biology of merlin. I have always enjoyed your company, as well as Juha's; your friendship has made the long work worthwhile. I thank Dr. Fang Zhao for his never-failing support and help with the merlin project. I never forget the dark-room techniques. I thank Leena 'scrambled peptide' Heiska and Mikaela 'yeast-two-hybrid' Grönholm for their collaboration and great spirit in the 'Olli lab'. I am also grateful to other Olli's crew members on this voyage: Nina von Numers, Olli-Matti 'night-shift' Mykkänen, Paula 'myotilin' Salmikangas, Anu 'smile' Taivainen, Emma Lindell, Heli Suila, Maciej Lalowski, and Luca Mologni. Olli does indeed need a bigger ship.

Apart from Olli's gang, I want to express my gratitude also to the other members of the ezrin-merlin group. This group was gathered together by the Virology Professor Antti 'father ezrin' Vaheri, and included Ossi 'departed son' Turunen, Tuomo Timonen, Päivi Majander-Nordenswan, Tom Böhling, Antti Salminen, Toni Järvelä, Eeva Auvinen and Torsten Wahlström. Special thanks go to Ossi and Tuula Helander for the profound conversations on all topics. Thank you all for creating an inspiring research atmosphere, where also humour played a role.

I thank Professor Matti Haltia for his collaboration in the nervous system tumour studies. I appreciate his accurate work and his wonderful personality. Also, I thank Dr. Minna Pöyhönen for her great interest and expertise on neurofibromatoses.

I thank my collaborators abroad, Professor Tom Strachan from the University of Newcastle-upon-Tyne, and his lab members, especially Dr. David Bourn, for providing

research facilities and help in an intensive short-term NF2 mutation study. I remember your hospitality with warmth, and recall the beautiful scenery at the mouth of Tyne. I appreciate the contribution of Professor Ellen Zwarthoff, as well as Drs. Michael den Bakker and Peter Riegman from Rotterdam, the Netherlands. I am thankful to my collaborators, Guy Rouleau and Mohini Lutchman, in Montreal, Canada, for providing valuable merlin antiserum.

During this long period of hard work, I received help from the technical staff of the Neurology research lab, especially Kaija Lindberg and Kati, among others on the first floor, and Aarno's lab at Meilahti Hospital. I am indebted to Ms. Hannele Pihlaja, the soul and the super 'teacher' technician of the Neurology lab. She was always eager to tackle innovations. I am also grateful for the invaluable help of expert technicians Tuula Halmesvaara, Mona Mäntylä, Tuula Niilola and Leena Harju at the Pathology Department. I appreciate the help of Lea Pylkkänen, Dr. Sebsebe 'ethiopian' Woldesenbet, and I am grateful to Virpi Tiilikainen for her skills in organizing practical things. Mr. Ben 'hardware' Roimola and Mr. Antti 'software' Huittinen of the Haartman Institute also deserve my warmest thanks.

I want to thank all my colleagues and other co-workers for the many good times at the Department of Neurology and Pathology. Especially, I am grateful to Pentti Tienari for his humane perspective to science and for his encouragement. Also, I have enjoyed the humour of neurologist Lauri Soinne. Our dilemma "why does our scientific work progress so slowly?" is left for the next generation to solve.

Docent Helena Pihko and Professor Juha Peltonen are gratefully acknowledged for reviewing and improving the manuscript of this thesis. I am also grateful to Ms. Terttu Kaustia for revising the English of this thesis.

I thank the head of the Department of Occupational Medicine at the Finnish Institute of Occupational Health, Professor Kaj Husman, for providing me the opportunity to finish this work. I am especially grateful to Dr. Kiti Müller, who for the last squeeze, harnessed me with blinkers and provided the isolation room. Also, I appreciate the support of my colleagues, Helena Huhmar and Hannu Laaksovirta, and all the members of the Neuroscience section. I thank Janne Muhonen and Aira Lindeman for library assistance.

I want to express my ultimate and most heartfelt thanks to my family, Irene, Tatu, Samuli and Aino, for their support and patience during the long work process. Though physically present, sometimes I must have been spiritually at work. I am deeply grateful to my mother-in-law, Meerri, and Stig for the innumerable times they have offered us their help.

I wish to thank my mother, Liisa, for her high values in life and her gentle encouragement toward academic achievements, and my father, Pertti, for his persistence and 'civil courage' to fight for justified values.

This study was supported by the Finnish Cancer Organizations, the Paulo Foundation, the Neurology Foundation, the Maire Taponen Foundation and the Academy of Finland.



, January 12, 2000

REFERENCES

1. Neurofibromatosis. Conference statement. National Institutes of Health Consensus Development Conference. *Arch Neurol* 1988;45:575-578.
2. Aguiar PH, Tatagiba M, Samii M, Dankoweit-Timpe E, Ostertag H. The comparison between the growth fraction of bilateral vestibular schwannomas in neurofibromatosis 2 (NF2) and unilateral vestibular schwannomas using the monoclonal antibody MIB 1. *Acta Neurochir* 1995;134:40-45.
3. Ahn MS, Jackler RK, Lustig LR. The early history of the neurofibromatosis. Evolution of the concept of neurofibromatosis type 2. *Arch Otolaryngol Head Neck Surg* 1996;122:1240-1249.
4. Aho R, Kalimo H, Salmi M, Smith D, Jalkanen S. Binding of malignant lymphoid cells to the white matter of the human central nervous system: role of different CD44 isoforms, beta 1, beta 2 and beta 7 integrins, and L-selectin. *J Neuropathol Exp Neurol* 1997;56:557-568.
5. Algrain M, Turunen O, Vaheri A, Louvard D, Arpin M. Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. *J Cell Biol* 1993;120:129-139.
6. Amieva MR, Furthmayr H. Subcellular localization of moesin in dynamic filopodia, retraction fibers, and other structures involved in substrate exploration, attachment, and cell-cell contacts. *Exp Cell Res* 1995;219:180-196.
7. Andreoli C, Martin M, Le Borgne R, Reggio H, Mangeat P. Ezrin has properties to self-associate at the plasma membrane. *J Cell Sci* 1994;107:2509-2521.
8. Antinheimo J, Haapasalo H, Seppälä M, Sainio M, Carpén O, Jääskeläinen J. Proliferative potential of sporadic and neurofibromatosis 2-associated schwannomas as studied by MIB-1 (Ki-67) and PCNA labeling. *J Neuropathol Exp Neurol* 1995;54:776-782.
9. Antinheimo J, Haapasalo H, Haltia M, Tatagiba M, Thomas S, Brandis A, Sainio M, Carpén O, Samii M, Jääskeläinen J. Proliferation potential and histological features in neurofibromatosis 2-associated and sporadic meningiomas. *J Neurosurg* 1997;87:610-614.
10. Antinheimo J. Meningiomas and schwannomas in neurofibromatosis 2. Thesis. University of Helsinki, 1999.
11. Antinheimo J, Sankila J, Carpén O, Pukkala E, Sainio M, Jääskeläinen J. Population-based analysis of sporadic and NF2-associated meningiomas and schwannomas. *Neurology* (in press).
12. Arai E, Ikeuchi T, Nakamura Y. Characterization of the translocation breakpoint on chromosome 22q12.2 in a patient with neurofibromatosis type 2 (NF2). *Hum Mol Genet* 1994;3:937-939.
13. Arakawa H, Hayashi N, Nagase H, Ogawa M, Nakamura Y. Alternative splicing of the NF2 gene and its mutation analysis of breast and colorectal cancers. *Hum Mol Genet* 1994;3:565-568.
14. Arnold E. Pathology of Tumours of the Nervous System. In: Russell DS RL, ed. *Tumours of the cranial, spinal and peripheral nerves sheaths*. 5th ed. London, 1989:533-589.
15. Arpin M, Algrain M, Louvard D. Membrane-actin microfilament connections: an increasing diversity of players related to band 4.1. *Curr Opin Cell Biol* 1994;6:136-141.
16. Barker D, Wright E, Nguyen K, Cannon L, Fain P, Goldgar D, Bishop DT, Carey J, Baty B, Kivlin J, Willard H, Waye JS, Greig G, Leinwand L, Nakamura Y, O'Connell P, Leppert M, Lalouel J-M, White R, Skolnick M. Gene for von Recklinghausen neurofibromatosis is in the pericentromeric region of chromosome 17. *Science* 1987;236:1100-1102.
17. Baser ME, Mautner VF, Ragge NK, Nechiporuk A, Riccardi VM, Klein J, Sainz J, Pulst SM. Presymptomatic diagnosis of neurofibromatosis 2 using linked

- genetic markers, neuroimaging, and ocular examinations. *Neurology* 1996;47:1269-1277.
18. Baser ME, Ragge NK, Riccardi VM, Janus T, Gantz B, Pulst SM. Phenotypic variability in monozygotic twins with neurofibromatosis 2. *Am J Med Genet* 1996;64:563-567.
 19. Behrens J. Cadherins and catenins: Role in signal transduction and tumor progression. *Cancer Metast Rev* 1999;18:15-30.
 20. Berryman M, Franck Z, Bretscher A. Ezrin is concentrated in the apical microvilli of a wide variety of epithelial cells whereas moesin is found primarily in endothelial cells. *J Cell Sci* 1993;105:1025-1043.
 21. Bianchi AB, Hara T, Ramesh V, Gao J, Klein-Szanto AJ, Morin F, Menon AG, Trofatter JA, Gusella JF, Seizinger BR, Kley N. Mutations in transcript isoforms of the neurofibromatosis 2 gene in multiple human tumour types. *Nature Genet* 1994;6:185-192.
 22. Bianchi AB, Mitsunaga SI, Cheng JQ, Klein WM, Jhanwar SC, Seizinger B, Kley N, Klein-Szanto AJ, Testa JR. High frequency of inactivating mutations in the neurofibromatosis type 2 gene (NF2) in primary malignant mesotheliomas. *Proc Natl Acad Sci USA* 1995;92:10854-10858.
 23. Bijlsma EK, Merel P, Fleury P, van Asperen CJ, Westerveld A, Delattre O, Thomas G, Hulsebos TJ. Family with neurofibromatosis type 2 and autosomal dominant hearing loss: identification of carriers of the mutated NF2 gene. *Hum Genet* 1995;96:1-5.
 24. Bijlsma EK, Wallace AJ, Evans DG. Misleading linkage results in an NF2 presymptomatic test owing to mosaicism. *J Med Genet* 1997;34:934-936.
 25. Birch BD, Johnson JP, Parsa A, Desai RD, Yoon JT, Lycette CA, Li YM, Bruce JN. Frequent type 2 neurofibromatosis gene transcript mutations in sporadic intramedullary spinal cord ependymomas. *Neurosurg* 1996;39:135-140.
 26. Black FO, Brackmann DE, Hitselberger WE, Purdy J. Preservation of auditory and vestibular function after surgical removal of bilateral vestibular schwannomas in a patient with neurofibromatosis type 2. *Am J Otol* 1995;16:431-443.
 27. Blomstedt GC, Jääskeläinen JE, Pyykkö I, Ishizaki H, Troupp H, Palva T. Recovery of the sutured facial nerve after removal of acoustic neuroma in patients with neurofibromatosis-2. *Neurosurg* 1994;35:364-368.
 28. Bondy M, Ligon BL. Epidemiology and etiology of intracranial meningiomas: a review. *J Neuro Oncol* 1996;29:197-205.
 29. Bourn D, Carter SA, Evans DG, Goodship J, Coakham H, Strachan T. A mutation in the neurofibromatosis type 2 tumor-suppressor gene, giving rise to widely different clinical phenotypes in two unrelated individuals. *Am J Hum Genet* 1994;55:69-73.
 30. Bourn D, Carter SA, Mason S, Gareth D, Evans R, Strachan T. Germline mutations in the neurofibromatosis type 2 tumour suppressor gene. *Hum Mol Genet* 1994;3:813-816.
 31. Bourn D, Strachan T. Dinucleotide repeat polymorphism at the NF2 gene. *Hum Mol Genet* 1994;3:813-816.
 32. Bourn D, Evans G, Mason S, Tekes S, Trueman L, Strachan T. Eleven novel mutations in the NF2 tumour suppressor gene. *Hum Genet* 1995;95:572-574.
 33. Bretscher A, Reczek D, Berryman M. Ezrin: a protein requiring conformational activation to link microfilaments to the plasma membrane in the assembly of cell surface structures. *J Cell Sci* 1997;110:3011-3018.
 34. Bretscher A. Regulation of cortical structure by the ezrin-radixin-moesin protein family. *Curr Opin Cell Biol* 1999;11:109-116.
 35. Briggs RJ, Brackmann DE, Baser ME, Hitselberger WE. Comprehensive management of bilateral acoustic neuromas. Current perspectives. *Arch Otolaryngol Head Neck Surg* 1994;120:1307-1314.
 36. Bruder CE, Ichimura K, Tingby O, Hirakawa K, Komatsuzaki A, Tamura A, Yuasa Y, Collins VP, Dumanski JP. A group of schwannomas with interstitial deletions on 22q located outside the NF2 locus shows no detectable mutations in the NF2 gene. *Hum Genet* 1999;104:418-424.

37. Böhling T, Turunen O, Jääskeläinen J, Carpén O, Sainio M, Wahlström T, Vaheri A, Haltia M. Ezrin expression in stromal cells of capillary hemangioblastoma. An immunohistochemical survey of brain tumors. *Am J Pathol* 1996;148:367-373.
38. Carlson KM, Bruder C, Nordenskjöld M, Dumanski JP. 1p and 3p deletions in meningiomas without detectable aberrations of chromosome 22 identified by comparative genomic hybridization. *Genes Chromosom Cancer* 1997;20:419-424.
39. Cheng JQ, Lee WC, Klein MA, Cheng GZ, Jhanwar SC, Testa JR. Frequent mutations of NF2 and allelic loss from chromosome band 22q12 in malignant mesothelioma: Evidence for a two-hit mechanism of NF2 inactivation. *Genes Chromosom Cancer* 1999;24:238-242.
40. Chishti AH, Kim AC, Marfatia SM, et al. The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. *Trends Biochem Sci* 1998;23:281-282.
41. Claudio JO, Marineau C, Rouleau GA. The mouse homologue of the neurofibromatosis type 2 gene is highly conserved. *Hum Mol Genet* 1994;3:185-190.
42. Couturier J, Delattre O, Kujas M, Philippon J, Peter M, Rouleau G, Aurias A, Thomas G. Assessment of chromosome 22 anomalies in neurinomas by combined karyotype and RFLP analyses. *Cancer Genet Cytogenet* 1990;45:55-62.
43. De Klein A, Riegman PH, Bijlsma EK, Helldoorn A, Muijtjens M, den Bakker MA, Avezaat CJ, Zwarthoff EC. A G-->A transition creates a branch point sequence and activation of a cryptic exon, resulting in the hereditary disorder neurofibromatosis 2. *Hum Mol Genet* 1998;7:393-398.
44. De Vitis LR, Tedde A, Vitelli F, Ammannati F, Mennonna P, Bigozzi U, Montali E, Papi L. Screening for mutations in the neurofibromatosis type 2 (NF2) gene in sporadic meningiomas. *Hum Genet* 1996;97:632-637.
45. Deguen B, Goutebroze L, Giovannini M, Boisson C, van der Neut R, Jaurand MC, Thomas G. Heterogeneity of mesothelioma cell lines as defined by altered genomic structure and expression of the NF2 gene. *Int J Cancer* 1998;77:554-560.
46. den Bakker MA, Riegman PH, Hekman RA, Boersma W, Janssen PJ, van der Kwast TH, Zwarthoff EC. The product of the NF2 tumour suppressor gene localizes near the plasma membrane and is highly expressed in muscle cells. *Oncogene* 1995;10:757-763.
47. den Bakker MA, Tascilar M, Riegman PH, Hekman AC, Boersma W, Janssen PJ, de Jong TA, Hendriks W, van der Kwast TH, Zwarthoff EC. Neurofibromatosis type 2 protein co-localizes with elements of the cytoskeleton. *Am J Pathol* 1995;147:1339-1349.
48. den Bakker MA, Vissers KJ, Molijn AC, Kros JM, Zwarthoff EC, van der Kwast TH. Expression of the neurofibromatosis Type 2 gene in human tissues. *J Histochem Cytochem* 1999;47:1471-1479.
49. Dransfield DT, Bradford AJ, Smith J, Martin M, Roy C, Mangeat PH, Goldenring JR. Ezrin is a cyclic AMP-dependent protein kinase anchoring protein. *EMBO J* 1997;16:35-43.
50. Ebert C, von Haken M, Meyer-Puttlitz B, Wiestler OD, Reifenberger G, Pietsch T, von Deimling A. Molecular genetic analysis of ependymal tumors - NF2 mutations and chromosome 22q loss occur preferentially in intramedullary spinal ependymomas. *Am J Pathol* 1999;155:627-632.
51. Egelhoff JC, Bates DJ, Ross JS, Rothner AD, Cohen BH. Spinal MR findings in neurofibromatosis types 1 and 2. *Am J Neuroradiol* 1992;13:1071-1077.
52. Eldridge R, Parry D. Neurofibromatosis 2: Evidence for clinical heterogeneity based on 54 affected individuals studied by MRI with gadolinium, 1987-1991. In: Tos M, Thomsen J, eds. *Proceedings of the First International Acoustic Neuroma Conference, Copenhagen, Denmark, 1991*. Amsterdam, New York: Kugler Publications, 1992.
53. Enzinger FM, Weiss SW. Soft tissue tumors. In: Enzinger FM, Weiss SW, eds. *Benign tumors of peripheral nerves*. 3rd ed. St Louis: Mosby, 1995:821-863.

54. Evans DG, Huson SM, Donnai D, Neary W, Blair V, Newton V, Harris R. A clinical study of type 2 neurofibromatosis. *Q J Med* 1992;84:603-618.
55. Evans DG, Huson SM, Donnai D, Neary W, Blair V, Newton V, Strachan T, Harris R. A genetic study of type 2 neurofibromatosis in the United Kingdom. II. Guidelines for genetic counselling. *J Med Genet* 1992;29:847-852.
56. Evans DG, Huson SM, Donnai D, Neary W, Blair V, Teare D, Newton V, Strachan T, Ramsden R, Harris R. A genetic study of type 2 neurofibromatosis in the United Kingdom. I. Prevalence, mutation rate, fitness, and confirmation of maternal transmission effect on severity. *J Med Genet* 1992;29:841-846.
57. Evans DG, Bourn D, Wallace A, Ramsden RT, Mitchell JD, Strachan T. Diagnostic issues in a family with late onset type 2 neurofibromatosis. *J Med Genet* 1995;32:470-474.
58. Evans DG, Mason S, Huson SM, Ponder M, Harding AE, Strachan T. Spinal and cutaneous schwannomatosis is a variant form of type 2 neurofibromatosis: a clinical and molecular study. *J Neurol Neurosurg Psychiatry* 1997;62:361-366.
59. Evans DG, Trueman L, Wallace A, Collins S, Strachan T. Genotype/phenotype correlations in type 2 neurofibromatosis (NF2): evidence for more severe disease associated with truncating mutations. *J Med Genet* 1998;35:450-455.
60. Evans DG, Wallace AJ, Wu CL, Trueman L, Ramsden RT, Strachan T. Somatic mosaicism: a common cause of classic disease in tumor-prone syndromes? Lessons from type 2 neurofibromatosis. *Am J Hum Genet* 1998;63:727-736.
61. Fearon ER. Human cancer syndromes: clues to the origin and nature of cancer. *Science* 1997;278:1043-1050.
62. Franck Z, Gary R, Bretscher A. Moesin, like ezrin, colocalizes with actin in the cortical cytoskeleton in cultured cells, but its expression is more variable. *J Cell Sci* 1993;105:219-231.
63. Gary R, Bretscher A. Ezrin self-association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site. *Mol Biol Cell* 1995;6:1061-1075.
64. Gautreau A, Pouillet PR, Louvard D, Arpin M. Ezrin, a plasma membrane-microfilament linker, signals cell survival through the phosphatidylinositol 3-kinase/Akt pathway. *Proc Natl Acad Sci USA* 1999;96:7300-7305.
65. Giovannini M, Robanus-Maandag E, Niwa-Kawakita M, van der Valk M, Woodruff JM, Goutebroze L, Merel P, Berns A, Thomas G. Schwann cell hyperplasia and tumors in transgenic mice expressing a naturally occurring mutant NF2 protein. *Genes Dev* 1999;13:978-986.
66. Gonzalez-Agosti C, Xu L, Pinney D, Beauchamp R, Hobbs W, Gusella J, Ramesh V. The merlin tumor suppressor localizes preferentially in membrane ruffles. *Oncogene* 1996;13:1239-1247.
67. Grönholm M, Sainio M, Zhao F, Heiska L, Vaheri A, Carpén O. Homotypic and heterotypic interaction of the neurofibromatosis 2 tumor suppressor protein merlin and the ERM protein ezrin. *J Cell Sci* 1999;112:895-904.
68. Gutmann DH, Wright DE, Geist RT, Snider WD. Expression of the neurofibromatosis 2 (NF2) gene isoforms during rat embryonic development. *Hum Mol Genet* 1995;4:471-478.
69. Gutmann DH. Molecular insights into neurofibromatosis 2. *Neurobiol Dis* 1997;3:247-261.
70. Gutmann DH, Aylsworth A, Carey JC, Korf B, Marks J, Pyeritz RE, Rubenstein A, Viskochil D. The diagnostic evaluation and multidisciplinary management of neurofibromatosis 1 and neurofibromatosis 2. *JAMA* 1997;278:51-57.
71. Gutmann DH, Giordano MJ, Fishback AS, Guha A. Loss of merlin expression in sporadic meningiomas, ependymomas and schwannomas. *Neurology* 1997;49:267-270.
72. Haase VH, Trofatter JA, MacCollin M, Tarttelin E, Gusella JF, Ramesh V. The murine NF2 homologue encodes a highly conserved merlin protein with alternative forms. *Hum Mol Genet* 1994;3:407-411.

73. Halliday AL, Sobel RA, Martuza RL. Benign spinal nerve sheath tumors: their occurrence sporadically and in neurofibromatosis types 1 and 2. *J Neurosurg* 1991;74:248-253.
74. Hamada Y, Iwaki T, Fukui M, Tateishi J. A comparative study of embedded nerve tissue in six NF2-associated schwannomas and 17 nonassociated NF2 schwannomas. *Surg Neurol* 1997;48:395-400.
75. Harwalkar JA, Lee JH, Hughes G, Kinney SE, Golubic M. Immunoblotting analysis of schwannomin/merlin in human schwannomas. *Am J Otol* 1998;19:654-659.
76. Heiska L, Alfthan K, Grönholm M, Vilja P, Vaheri A, Carpen O. Association of ezrin with intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2). Regulation by phosphatidylinositol 4, 5-bisphosphate. *J Biol Chem* 1998;273:21893-21900.
77. Helander TS, Carpen O, Turunen O, Kovanen PE, Vaheri A, Timonen T. ICAM-2 redistributed by ezrin as a target for killer cells. *Nature* 1996;382:265-268.
78. Henry MD, Gonzalez Agosti C, Solomon F. Molecular dissection of radixin: distinct and interdependent functions of the amino- and carboxy-terminal domains. *J Cell Biol* 1995;129:1007-1022.
79. Hirao M, Sato N, Kondo T, Yonemura S, Monden M, Sasaki T, Takai Y, Tsukita S, Tsukita S. Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. *J Cell Biol* 1996;135:37-51.
80. Hiscox S, Jiang WG. Ezrin regulates cell-cell and cell-matrix adhesion, a possible role with E-cadherin/beta-catenin. *J Cell Sci* 1999;112:3081-3090.
81. Hitotsumatsu T, Kitamoto T, Iwaki T, Fukui M, Tateishi J. An exon 8-spliced out transcript of neurofibromatosis 2 gene is constitutively expressed in various human tissues. *J Biochem* 1994;116:1205-1207.
82. Honda M, Arai E, Sawada S, Ohta A, Niimura M. Neurofibromatosis 2 and neurilemmomatosis gene are identical. *J Invest Dermatol* 1995;104:74-77.
83. Huynh DP, Pulst SM. Neurofibromatosis 2 antisense oligodeoxynucleotides induce reversible inhibition of schwannomin synthesis and cell adhesion in STS26T and T98G cells. *Oncogene* 1996;13:73-84.
84. Huynh DP, Tran TM, Nechiporuk T, Pulst SM. Expression of neurofibromatosis 2 transcript and gene product during mouse fetal development. *Cell Growth Differ* 1996;7:1551-1561.
85. Huynh DP, Mautner V, Baser ME, Stavrou D, Pulst SM. Immunohistochemical detection of schwannomin and neurofibromin in vestibular schwannomas, ependymomas and meningiomas. *J Neuropathol Exp Neurol* 1997;56:382-390.
86. Ikeda K, Saeki Y, Gonzalez-Agosti C, Ramesh V, Chiocca EA. Inhibition of NF2-negative and NF2-positive primary human meningioma cell proliferation by overexpression of merlin due to vector-mediated gene transfer. *J Neurosurg* 1999;91:85-92.
87. Jacoby LB, MacCollin M, Louis DN, Mohny T, Rubio MP, Pulaski K, Trofatter JA, Kley N, Seizinger B, Ramesh V, Gusella JF. Exon scanning for mutation of the NF2 gene in schwannomas. *Hum Mol Genet* 1994;3:413-419.
88. Jacoby LB, MacCollin M, Barone R, Ramesh V, Gusella JF. Frequency and distribution of NF2 mutations in schwannomas. *Genes Chromosom Cancer* 1996;17:45-55.
89. Jacoby LB, Jones D, Davis K, Kronn D, Short MP, Gusella J, MacCollin M. Molecular analysis of the NF2 tumor-suppressor gene in schwannomatosis. *Am J Hum Genet* 1997;61:1293-1302.
90. Jalkanen S, Bargatze RF, de los Toyos J, Butcher EC. Lymphocyte recognition of high endothelium: antibodies to distinct epitopes of an 85-95-kD glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal, or synovial endothelial cells. *J Cell Biol* 1987;105:983-990.
91. Jääskeläinen J, Paetau A, Pyykkö I, Blomstedt G, Palva T, Troupp H. Interface between the facial nerve and large acoustic neurinomas. Immunohistochemical

- study of the cleavage plane in NF2 and non-NF2 cases. *J Neurosurg* 1994;80:541-547.
92. Kaiser-Kupfer MI, Freidlin V, Datiles MB, Edwards PA, Sherman JL, Parry D, McCain LM, Eldridge R. The association of posterior capsular lens opacities with bilateral acoustic neuromas in patients with neurofibromatosis type 2. *Arch Ophthalmol* 1989;107:541-544.
 93. Kanter WR, Eldridge R, Fabricant R, Allen JC, Koerber T. Central neurofibromatosis with bilateral acoustic neuroma: genetic, clinical and biochemical distinctions from peripheral neurofibromatosis. *Neurology* 1980;30:851-859.
 94. Kaul SC, Mitsui Y, Komatsu Y, Reddel RR, Wadhwa R. A highly expressed 81 kDa protein in immortalized mouse fibroblast: its proliferative function and identity with ezrin. *Oncogene* 1996;13:1231-1237.
 95. Khan J, Parsa NZ, Harada T, Meltzer PS, Carter NP. Detection of gains and losses in 18 meningiomas by comparative genomic hybridization. *Cancer Genet Cytogenet* 1998;103:95-100.
 96. Klekamp J, Samii M. Surgery of spinal nerve sheath tumors with special reference to neurofibromatosis. *Neurosurg* 1998;42:279-289.
 97. Kluwe L, Pulst SM, Koppen J, Mautner VF. A 163-bp deletion in the neurofibromatosis 2 (NF2) gene associated with variant phenotypes. *Hum Genet* 1995;95:443-446.
 98. Kluwe L, Bayer S, Baser ME, Hazim W, Haase W, Funsterer C, Mautner VF. Identification of NF2 germ-line mutations and comparison with neurofibromatosis 2 phenotypes. *Hum Genet* 1996;98:534-538.
 99. Kluwe L, MacCollin M, Tatagiba M, Thomas S, Hazim W, Haase W, Mautner VF. Phenotypic variability associated with 14 splice-site mutations in the NF2 gene. *Am J Med Genet* 1998;77:228-233.
 100. Kluwe L, Mautner VF. Mosaicism in sporadic neurofibromatosis 2 patients. *Hum Mol Genet* 1998;7:2051-2055.
 101. Knudson AG. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci* 1971;68:820-823.
 102. Knuutila S, Aalto Y, Autio K, Björkqvist AM, El-Rifai W, Hemmer S, Huhta T, Kettunen E, Kiuru-Kuhlefelt S, Larramendy ML, Lushnikova T, Monni O, Pere H, Tapper J, Tarkkanen M, Varis A, Wasenius VM, Wolf M, Zhu Y. DNA copy number losses in human neoplasms. *Am J Pathol* 1999;155:683-694.
 103. Koga H, Araki N, Takeshima H, Nishi T, Hirota T, Kimura Y, Nakao M, Saya H. Impairment of cell adhesion by expression of the mutant neurofibromatosis type 2 (NF2) genes which lack exons in the ERM-homology domain. *Oncogene* 1998;17:801-810.
 104. Koivunen J, Ylä-Outinen H, Korkiamäki T, Karvonen S-L, Pöyhönen M, Laato M, Karvonen J, Peltonen S, Peltonen J. New function for NF1 tumor suppressor. *J Invest Dermatol* (in press).
 105. Kolles H, Niedermayer I, Schmitt C, Henn W, Feld R, Steudel WI, Zang KD, Feiden W. Triple approach for diagnosis and grading of meningiomas: histology, morphometry of Ki-67/Feulgen stainings, and cytogenetics. *Acta Neurochir* 1995;137:174-181.
 106. Kondo T, Takeuchi K, Doi Y, Yonemura S, Nagata S, Tsukita S. ERM (ezrin/radixin/moesin)-based molecular mechanism of microvillar breakdown at an early stage of apoptosis. *J Cell Biol* 1997;139:749-758.
 107. LaJeunesse DR, McCartney BM, Fehon RG. Structural analysis of *Drosophila* merlin reveals functional domains important for growth control and subcellular localization. *J Cell Biol* 1998;141:1589-1599.
 108. Lamszus K, Kluwe L, Matschke J, Meissner H, Laas R, Westphal M. Allelic losses at 1p, 9q, 10q, 14q, and 22q in the progression of aggressive meningiomas and undifferentiated meningeal sarcomas. *Cancer Genet Cytogenet* 1999;110:103-110.
 109. Larson JJ, Tew J, Jr., Simon M, Menon AG. Evidence for clonal spread in the development of multiple meningiomas. *J Neurosurg* 1995;83:705-709.

110. Lee M, Rezai AR, Freed D, Epstein FJ. Intramedullary spinal cord tumors in neurofibromatosis. *Neurosurg* 1996;38:32-37.
111. Lee WC, Testa JR. Somatic genetic alterations in human malignant mesothelioma. *Int J Oncol* 1999;14:181-188.
112. Lekanne Deprez RH, Riegman PH, Groen NA, Warringa UL, van Biezen NA, Molijn AC, Bootsma D, de Jong PJ, Menon AG, Kley NA, Seizinger BR, Zwarthoff EC. Cloning and characterization of MN1, a gene from chromosome 22q11, which is disrupted by a balanced translocation in a meningioma. *Oncogene* 1995;10:1521-1528.
113. Louis DN, Ramesh V, Gusella JF. Neuropathology and molecular genetics of neurofibromatosis 2 and related tumors. *Brain Pathol* 1995;5:163-172.
114. Louis DN, Budka H, von Deimling A. Meningiomas. In: Kleihues P, Cavenee WK, eds. *Pathology and Genetics of Tumours of the Nervous System*. Lyon: International Agency for Research on Cancer, 1997:134-141.
115. Louis DN, Wiestler OD. Neurofibromatosis type 2. In: Kleihues P, Cavenee WK, eds. *Pathology and Genetics of Tumours of the Nervous System*. Lyon: International Agency for Research on Cancer, 1997:175-178.
116. Lutchman M, Rouleau GA. The neurofibromatosis type 2 gene product, schwannomin, suppresses growth of NIH 3T3 cells. *Cancer Res* 1995;55:2270-2274.
117. MacCollin M, Mohny T, Trofatter J, Wertelecki W, Ramesh V, Gusella J. DNA diagnosis of neurofibromatosis 2. Altered coding sequence of the merlin tumor suppressor in an extended pedigree. *JAMA* 1993;270:2316-2320.
118. MacCollin M, Ramesh V, Jacoby LB, Louis DN, Rubio MP, Pulaski K, Trofatter JA, Short MP, Bove C, Eldridge R, Parry DM, Gusella JF. Mutational analysis of patients with neurofibromatosis 2. *Am J Hum Genet* 1994;55:314-320.
119. MacCollin M, Braverman N, Viskochil D, Ruttledge M, Davis K, Ojemann R, Gusella J, Parry DM. A point mutation associated with a severe phenotype of neurofibromatosis 2. *Ann Neurol* 1996;40:440-445.
120. MacCollin M, Woodfin W, Kronn D, Short MP. Schwannomatosis: a clinical and pathologic study. *Neurology* 1996;46:1072-1079.
121. MacCollin M, Gusella JF. NF2: molecular biology. In: Friedman JM, Gutmann DH, MacCollin M, Riccardi VM, eds. *Neurofibromatosis: phenotype, natural history, and pathogenesis*. 3rd ed. Baltimore: The Johns Hopkins University Press, 1999:351-362.
122. Mangeat P, Roy C, Martin M. ERM proteins in cell adhesion and membrane dynamics. *Trends Cell Biol* 1999;9:187-192.
123. Marquet JF, Forton GE, Offeciers FE, Moeneclaey LL. The solitary schwannoma of the eighth cranial nerve. An immunohistochemical study of the cochlear nerve-tumor interface. *Arch Otolaryngol Head Neck Surg* 1990;116:1023-1025.
124. Martin M, Andreoli C, Sahuquet A, Montcourrier P, Algrain M, Mangeat P. Ezrin NH2-terminal domain inhibits the cell extension activity of the COOH-terminal domain. *J Cell Biol* 1995;128:1081-1093.
125. Martin M, Roy C, Montcourrier P, Sahuquet A, Mangeat P. Three determinants in ezrin are responsible for cell extension activity. *Mol Biol Cell* 1997;8:1543-1557.
126. Matsuda Y, Kawamoto K, Kiya K, Kurisu K, Sugiyama K, Uozumi T. Antitumor effects of antiprogestones on human meningioma cells in vitro and in vivo. *J Neurosurg* 1994;80:527-534.
127. Matsui T, Maeda M, Doi Y, Yonemura S, Amano M, Kaibuchi K, Tsukita S, Tsukita S. Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. *J Cell Biol* 1998;140:647-657.
128. Matsui T, Yonemura S, Tsukita S. Activation of ERM proteins in vivo by Rho involves phosphatidylinositol 4-phosphate 5-kinase and not ROCK kinases. *Curr Biol* 1999;9:1259-1262.
129. Mautner VF, Tatagiba M, Guthoff R, Samii M, Pulst SM. Neurofibromatosis 2 in the pediatric age group. *Neurosurg* 1993;33:92-96.

130. Mautner VF, Tatagiba M, Lindenau M, Funsterer C, Pulst SM, Baser ME, Kluwe L, Zanella FE. Spinal tumors in patients with neurofibromatosis type 2: MR imaging study of frequency, multiplicity, and variety. *Am J Roentgenol* 1995;165:951-955.
131. Mautner VF, Lindenau M, Baser ME, Hazim W, Tatagiba M, Haase W, Samii M, Wais R, Pulst SM. The neuroimaging and clinical spectrum of neurofibromatosis 2. *Neurosurg* 1996;38:880-885.
132. Mautner VF, Lindenau M, Baser ME, Kluwe L, Gottschalk J. Skin abnormalities in neurofibromatosis 2. *Arch Dermatol* 1997;133:1539-1543.
133. Maxwell M, Shih SD, Galanopoulos T, Hedley-Whyte ET, Cosgrove GR. Familial meningioma: analysis of expression of neurofibromatosis 2 protein Merlin. Report of two cases. *J Neurosurg* 1998;88:562-569.
134. McClatchey AI, Saotome I, Mercer K, Crowley D, Gusella JF, Bronson RT, Jacks T. Mice heterozygous for a mutation at the Nf2 tumor suppressor locus develop a range of highly metastatic tumors. *Genes Dev* 1998;12:1121-1133.
135. Merel P, Hoang-Xuan K, Sanson M, Bijlsma E, Rouleau G, Laurent-Puig P, Pulst S, Baser M, Lenoir G, Sterkers J-M, Philippon J, Resche F, Mautner VF, Fischer G, Hulsebos T, Aurias A, Delattre O, Thomas G. Screening for germ-line mutations in the NF2 gene. *Genes Chromosom Cancer* 1995;12:117-127.
136. Murthy A, Gonzalez-Agosti C, Cordero E, Pinney D, Candia C, Solomon F, Gusella J, Ramesh V. NHE-RF, a regulatory cofactor for Na(+)-H+ exchange, is a common interactor for merlin and ERM (MERM) proteins. *J Biol Chem* 1998;273:1273-1276.
137. Nakamura F, Huang LQ, Pestonjamas K, Luna EJ, Furthmayr H. Regulation of F-actin binding to platelet moesin in vitro by both phosphorylation of threonine 558 and polyphosphatidylinositides. *Mol Biol Cell* 1999;10:2669-2685.
138. Narod SA, Parry DM, Parboosingh J, Lenoir GM, Ruttledge M, Fischer G, Eldridge R, Martuza RL, Frontali M, Haines J, Gusella JF, Rouleau GA. Neurofibromatosis type 2 appears to be a genetically homogeneous disease. *Am J Hum Genet* 1992;51:486-496.
139. Ogose A, Hotta T, Morita T, Otsuka H, Hirata Y. Multiple schwannomas in the peripheral nerves. *J Bone Joint Surg Br* 1998;80:657-661.
140. Pakkanen R. Immunofluorescent and immunochemical evidence for the expression of cytovillin in the microvilli of a wide range of cultured human cells. *J Cell Biochem* 1988;38:65-75.
141. Parry DM, Eldridge R, Kaiser-Kupfer MI, Bouzas EA, Pikus A, Patronas N. Neurofibromatosis 2 (NF2): clinical characteristics of 63 affected individuals and clinical evidence for heterogeneity. *Am J Med Genet* 1994;52:450-461.
142. Parry DM, MacCollin MM, Kaiser-Kupfer MI, Pulaski K, Nicholson HS, Bolesta M, Eldridge R, Gusella JF. Germ-line mutations in the neurofibromatosis 2 gene: correlations with disease severity and retinal abnormalities. *Am J Hum Genet* 1996;59:529-539.
143. Pestonjamas K, Amieva MR, Strassel CP, Nauseef WM, Furthmayr H, Luna EJ. Moesin, ezrin, and p205 are actin-binding proteins associated with neutrophil plasma membranes. *Mol Biol Cell* 1995;6:247-259.
144. Peyrard M, Fransson I, Xie YG, Han FY, Ruttledge MH, Swahn S, Collins JE, Dunham I, Collins VP, Dumanski JP. Characterization of a new member of the human beta-adaptin gene family from chromosome 22q12, a candidate meningioma gene. *Hum Mol Genet* 1994;3:1393-1399.
145. Pietromonaco SF, Simons PC, Altman A, Elias L. Protein kinase C-theta phosphorylation of moesin in the actin-binding sequence. *J Biol Chem* 1998;273:7594-7603.
146. Pulst SM, Rouleau GA, Marineau C, Fain P, Sieb JP. Familial meningioma is not allelic to neurofibromatosis 2. *Neurology* 1993;43:2096-2098.
147. Pulst SM, Riccardi V, Mautner V. Spinal schwannomatosis. *Neurology* 1997;48:787-788.
148. Purcell SM, Dixon SL. Schwannomatosis. An unusual variant of neurofibromatosis or a distinct clinical entity? *Arch Dermatol* 1989;125:390-393.

149. Pykett MJ, Murphy M, Harnish PR, George DL. The neurofibromatosis 2 (NF2) tumor suppressor gene encodes multiple alternatively spliced transcripts. *Hum Mol Genet* 1994;3:559-564.
150. Ragge NK, Baser ME, Riccardi VM, Falk RE. The ocular presentation of neurofibromatosis 2. *Eye* 1997;11:12-18.
151. Rausing A, Ybo W, Stenflo J. Intracranial meningioma-a population study of ten years. *Acta Neurol Scand* 1970;46:102-110.
152. Reczek D, Berryman M, Bretscher A. Identification of EBP50: A PDZ-containing phosphoprotein that associates with members of the ezrin-radixin-moesin family. *J Cell Biol* 1997;139:169-179.
153. Reczek D, Bretscher A. The carboxyl-terminal region of EBP50 binds to a site in the amino-terminal domain of ezrin that is masked in the dormant molecule. *J Biol Chem* 1998;273:18452-18458.
154. Rouleau GA, Wertelecki W, Haines JL, Hobbs WJ, Trofatter JA, Seizinger BR, Martuza RL, Superneau DW, Conneally PM, Gusella JF. Genetic linkage of bilateral acoustic neurofibromatosis to a DNA marker on chromosome 22. *Nature* 1987;329:246-248.
155. Rouleau GA, Merel P, Lutchman M, Sanson M, Zucman J, Marineau C, Hoang-Xuan K, Demczuk S, Desmaze C, Plougastel B, Pulst SM, Lenoir G, Bijlsma E, Fashold R, Dumanski J, de Jong P, Parry D, Eldridge R, Aurias A, Delattre O, Thomas G. Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. *Nature* 1993;363:515-521.
156. Roy C, Martin M, Mangeat P. A dual involvement of the amino-terminal domain of ezrin in F- and G-actin binding. *J Biol Chem* 1997;272:20088-20095.
157. Rubinstein LJ. The malformative central nervous system lesions in the central and peripheral forms of neurofibromatosis. A neuropathological study of 22 cases. *Ann New York Acad Sci* 1986;486:14-29.
158. Rudzki Z, Jothy S. CD44 and the adhesion of neoplastic cells. *Mol Pathol* 1997;50:57-71.
159. Rutledge MH, Narod SA, Dumanski JP, Parry DM, Eldridge R, Wertelecki W, Parboosingh J, Faucher MC, Lenoir GM, Collins VP, Nordenskjöld M, Rouleau GA. Presymptomatic diagnosis for neurofibromatosis 2 with chromosome 22 markers. *Neurology* 1993;43:1753-1760.
160. Rutledge MH, Sarrazin J, Rangaratnam S, Phelan CM, Twist E, Merel P, Delattre O, Thomas G, Nordenskjöld M, Collins VP, Dumanski JP, Rouleau GA. Evidence for the complete inactivation of the NF2 gene in the majority of sporadic meningiomas. *Nature Genet* 1994;6:180-184.
161. Rutledge MH, Xie YG, Han FY, Peyrard M, Collins VP, Nordenskjöld M, Dumanski JP. Deletions on chromosome 22 in sporadic meningioma. *Genes Chromosom Cancer* 1994;10:122-130.
162. Rutledge MH, Andermann AA, Phelan CM, Claudio JO, Han FY, Chretien N, Rangaratnam S, MacCollin M, Short P, Parry D, Michels V, Riccardi VM, Weksberg R, Kitamura K, Bradburn JM, Hall BD, Propping P, Rouleau GA. Type of mutation in the neurofibromatosis type 2 gene (NF2) frequently determines severity of disease. *Am J Hum Genet* 1996;59:331-342.
163. Sainz J, Huynh DP, Figueroa K, Ragge NK, Baser ME, Pulst SM. Mutations of the neurofibromatosis type 2 gene and lack of the gene product in vestibular schwannomas. *Hum Mol Genet* 1994;3:885-891.
164. Sainz J, Figueroa K, Baser ME, Mautner VF, Pulst SM. High frequency of nonsense mutations in the NF2 gene caused by C to T transitions in five CGA codons. *Hum Mol Genet* 1995;4:137-139.
165. Samii M, Matthies C. Management of 1000 vestibular schwannomas (acoustic neuromas): the facial nerve--preservation and restitution of function. *Neurosurg* 1997;40:684-694.
166. Samii M, Matthies C, Tatagiba M. Management of vestibular schwannomas (acoustic neuromas): auditory and facial nerve function after resection of 120 vestibular schwannomas in patients with neurofibromatosis 2. *Neurosurg* 1997;40:696-705.

167. Sanson M, Marineau C, Desmaze C, Lutchman M, Rutledge M, Baron C, Narod S, Delattre O, Lenoir G, Thomas G, Aurias A, Rouleau GA. Germline deletion in a neurofibromatosis type 2 kindred inactivates the NF2 gene and a candidate meningioma locus. *Hum Mol Genet* 1993;2:1215-1220.
168. Scherer SS, Gutmann DH. Expression of the neurofibromatosis 2 tumor suppressor gene product, merlin, in Schwann cells. *J Neurosci Res* 1996;46:595-605.
169. Schmucker B, Tang Y, Kressel M. Novel alternatively spliced isoforms of the neurofibromatosis type 2 tumor suppressor are targeted to the nucleus and cytoplasmic granules. *Hum Mol Genet* 1999;8:1561-1570.
170. Schrell UM, Rittig MG, Anders M, Koch UH, Marschalek R, Kiesewetter F, Fahlbusch R. Hydroxyurea for treatment of unresectable and recurrent meningiomas. II. Decrease in the size of meningiomas in patients treated with hydroxyurea. *J Neurosurg* 1997;86:840-844.
171. Scoles DR, Baser ME, Pulst SM. A missense mutation in the neurofibromatosis 2 gene occurs in patients with mild and severe phenotypes. *Neurology* 1996;47:544-546.
172. Scoles DR, Huynh DP, Morcos PA, Coulsell ER, Robinson NG, Tamanoi F, Pulst SM. Neurofibromatosis 2 tumour suppressor schwannomin interacts with betaII-spectrin. *Nature Genet* 1998;18:354-359.
173. Seizinger BR, de la Monte S, Atkins L, Gusella JF, Martuza RL. Molecular genetic approach to human meningioma: loss of genes on chromosome 22. *Proc Natl Acad Sci USA* 1987;84:5419-5423.
174. Seizinger BR, Martuza RL, Gusella JF. Loss of genes on chromosome 22 in tumorigenesis of human acoustic neuroma. *Nature* 1986;322:644-647.
175. Seizinger BR, Rouleau G, Ozelius LJ, Lane AH, St George-Hyslop P, Huson S, Gusella JF, Martuza RL. Common pathogenetic mechanism for three tumor types in bilateral acoustic neurofibromatosis. *Science* 1987;236:317-319.
176. Sekido Y, Pass HI, Bader S, Mew DJ, Christman MF, Gazdar AF, Minna JD. Neurofibromatosis type 2 (NF2) gene is somatically mutated in mesothelioma but not in lung cancer. *Cancer Res* 1995;55:1227-1231.
177. Seppälä MT, Haltia MJ, Sankila RJ, Jääskeläinen JE, Heiskanen O. Long-term outcome after removal of spinal schwannoma: a clinicopathological study of 187 cases. *J Neurosurg* 1995;83:621-626.
178. Serrador JM, Alonso-Lebrero JL, del Pozo MA, Furthmayr H, Schwartz-Albiez R, Calvo J, Lozano F, Sanchez-Madrid F. Moesin interacts with the cytoplasmic region of intercellular adhesion molecule-3 and is redistributed to the uropod of T lymphocytes during cell polarization. *J Cell Biol* 1997;138:1409-1423.
179. Shaw RJ, McClatchey AI, Jacks T. Regulation of the neurofibromatosis type 2 tumor suppressor protein, merlin, by adhesion and growth arrest stimuli. *J Biol Chem* 1998;273:7757-7764.
180. Sherman L, Xu HM, Geist RT, Saporito-Irwin S, Howells N, Ponta H, Herrlich P, Gutmann DH. Interdomain binding mediates tumor growth suppression by the NF2 gene product. *Oncogene* 1997;15:2505-2509.
181. Shishiba T, Niimura M, Ohtsuka F, Tsuru N. Multiple cutaneous neurilemmomas as a skin manifestation of neurilemmomatosis. *J Am Acad Dermatol* 1984;10:744-754.
182. Short PM, Martuza RL, Huson SM. Neurofibromatosis 2: clinical features, genetic counselling and management issues. In: Huson SM, Hughes RAC, eds. *The Neurofibromatoses: A Pathogenetic and Clinical overview*. London: Chapman and Hall Medical, 1994:414-444.
183. Sieb JP, Pulst SM, Buch A. Familial CNS tumors. *J Neurol* 1992;239:343-344.
184. Simons PC, Pietromonaco SF, Reczek D, Bretscher A, Elias L. C-terminal threonine phosphorylation activates ERM proteins to link the cell's cortical lipid bilayer to the cytoskeleton. *Biochem Biophys Res Commun* 1998;253:561-565.
185. Slattery Wr, Brackmann DE, Hitselberger W. Hearing preservation in neurofibromatosis type 2. *Am J Otol* 1998;19:638-643.

186. Sobel RA. Vestibular (acoustic) schwannomas: histologic features in neurofibromatosis 2 and in unilateral cases. *J Neuropathol Exp Neurol* 1993;52:106-113.
187. Stangl AP, Wellenreuther R, Lenartz D, Kraus JA, Menon AG, Schramm J, Wiestler OD, von Deimling A. Clonality of multiple meningiomas. *J Neurosurg* 1997;86:853-858.
188. Stemmer-Rachamimov AO, Gonzalez-Agosti C, Xu L, Burwick JA, Beauchamp R, Pinney D, Louis DN, Ramesh V. Expression of NF2-encoded merlin and related ERM family proteins in the human central nervous system. *J Neuropathol Exp Neurol* 1997;56:735-742.
189. Stemmer-Rachamimov AO, Xu L, Gonzalez-Agosti C, Burwick JA, Pinney D, Beauchamp R, Jacoby LB, Gusella JF, Ramesh V, Louis DN. Universal absence of merlin, but not other ERM family members, in schwannomas. *Am J Pathol* 1997;151:1649-1654.
190. Subach BR, Kondziolka D, Lunsford LD, Bissonette DJ, Flickinger JC, Maitz AH. Stereotactic radiosurgery in the management of acoustic neuromas associated with neurofibromatosis type 2. *J Neurosurg* 1999;90:815-822.
191. Takahashi K, Sasaki T, Mammoto A, Takaishi K, Kameyama T, Tsukita S, Takai Y. Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein. *J Biol Chem* 1997;272:23371-23375.
192. Takahashi K, Sasaki T, Mammoto A, Hotta I, Takaishi K, Imamura H, Nakano K, Kodama A, Takai Y. Interaction of radixin with Rho small G protein GDP/GTP exchange protein Dbl. *Oncogene* 1998;16:3279-3284.
193. Takamiya Y, Friedlander RM, Brem H, Malick A, Martuza RL. Inhibition of angiogenesis and growth of human nerve-sheath tumors by AGM-1470. *J Neurosurg* 1993;78:470-476.
194. Takeuchi K, Sato N, Kasahara H, Funayama N, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S. Perturbation of cell adhesion and microvilli formation by antisense oligonucleotides to ERM family members. *J Cell Biol* 1994;125:1371-1384.
195. Tikoo A, Varga M, Ramesh V, Gusella J, Maruta H. An anti-Ras function of neurofibromatosis type 2 gene product (NF2/Merlin). *J Biol Chem* 1994;269:23387-23390.
196. Tos M, Charabi S, Thomsen J. Incidence of vestibular schwannomas. *Laryngoscope* 1999;109:736-740.
197. Trofatter JA, MacCollin MM, Rutter JL, Murrell JR, Duyao MP, Parry DM, Eldridge R, Kley N, Menon AG, Pulaski K, Haase VH, Ambrose CM, Munroe D, Bove C, Haines JL, Martuza RL, MacDonald ME, Seizinger BR, Short MP, Buckler AJ, Gusella JF. A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. *Cell* 1993;72:791-800.
198. Tsukita S, Oishi K, Sato N, Sagara J, Kawai A, Tsukita S. ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. *J Cell Biol* 1994;126:391-401.
199. Tsukita S, Yonemura S. ERM (ezrin/radixin/moesin) family: from cytoskeleton to signal transduction. *Curr Opin Cell Biol* 1997;9:70-75.
200. Tsukita S, Yonemura S, Tsukita S. ERM proteins: head-to-tail regulation of actin-plasma membrane interaction. *Trends Biochem Sci* 1997;22:53-58.
201. Turunen O, Wahlström T, Vaheri A. Ezrin has a COOH-terminal actin-binding site that is conserved in the ezrin protein family. *J Cell Biol* 1994;126:1445-1453.
202. Turunen O, Sainio M, Jääskeläinen J, Carpén O, Vaheri A. Structure-function relationships in the ezrin family and the effect of tumor-associated point mutations in neurofibromatosis 2 protein. *Biochim Biophys Acta* 1998;1387:1-16.
203. Twist EC, Ruttledge MH, Rousseau M, Sanson M, Papi L, Merel P, Delattre O, Thomas G, Rouleau GA. The neurofibromatosis type 2 gene is inactivated in schwannomas. *Hum Mol Genet* 1994;3:147-151.
204. Vaheri A, Carpén O, Heiska L, Helander TS, Jääskeläinen J, Majander-Nordenswan P, Sainio M, Timonen T, Turunen O. The ezrin protein family:

- membrane-cytoskeleton interactions and disease associations. *Curr Opin Cell Biol* 1997;9:659-666.
205. von Andrian UH, Hasslen SR, Nelson RD, Erlandsen SL, Butcher EC. A central role for microvillous receptor presentation in leukocyte adhesion under flow. *Cell* 1995;82:989-999.
 206. von Deimling A, Kraus JA, Stangl AP, Wellenreuther R, Lenartz D, Schramm J, Louis DN, Ramesh V, Gusella JF, Wiestler OD. Evidence for subarachnoid spread in the development of multiple meningiomas. *Brain Pathol* 1995;5:11-14.
 207. von Deimling A, Krone W. Neurofibromatosis 1. In: Kleihues P, Cavenee WK, eds. *Pathology and Genetics of Tumours of the Nervous System*. Lyon: International Agency for Research on Cancer, 1997:172-174.
 208. Watson CJ, Gaunt L, Evans G, Patel K, Harris R, Strachan T. A disease-associated germline deletion maps the type 2 neurofibromatosis (NF2) gene between the Ewing sarcoma region and the leukaemia inhibitory factor locus. *Hum Mol Genet* 1993;2:701-704.
 209. Weber RG, Bostrom J, Wolter M, Baudis M, Collins VP, Reifenberger G, Lichter P. Analysis of genomic alterations in benign, atypical, and anaplastic meningiomas: toward a genetic model of meningioma progression. *Proc Natl Acad Sci USA* 1997;94:14719-14724.
 210. Wellenreuther R, Waha A, Vogel Y, Lenartz D, Schramm J, Wiestler OD, von Deimling A. Quantitative analysis of neurofibromatosis type 2 gene transcripts in meningiomas supports the concept of distinct molecular variants. *Lab Invest* 1997;77:601-606.
 211. Wertelecki W, Rouleau GA, Superneau DW, Forehand LW, Williams JP, Haines JL, Gusella JF. Neurofibromatosis 2: clinical and DNA linkage studies of a large kindred. *N Engl J Med* 1988;319:278-283.
 212. Wiestler OD, von Siebenthal K, Schmitt HP, Feiden W, Kleihues P. Distribution and immunoreactivity of cerebral micro-hamartomas in bilateral acoustic neurofibromatosis (neurofibromatosis 2). *Acta Neuropathol* 1989;79:137-143.
 213. Wilkins RH, Brody IA. Von Recklinghausen's neurofibromatosis. *Arch Neurol* 1971;24:374-377.
 214. Winckler B, Gonzalez Agosti C, Magendantz M, Solomon F. Analysis of a cortical cytoskeletal structure: a role for ezrin-radixin-moesin (ERM proteins) in the marginal band of chicken erythrocytes. *J Cell Sci* 1994;107:2523-2534.
 215. Wolkenstein P, Benchikhi H, Zeller J, Wechsler J, Revuz J. Schwannomatosis: a clinical entity distinct from neurofibromatosis type 2. *Dermatology* 1997;195:228-231.
 216. Woodruff JM, Kourea HP, Louis DN. Schwannoma. In: Kleihues P, Cavenee WK, eds. *Pathology and Genetics of Tumours of the Nervous System*. Lyon: International Agency for Research on Cancer, 1997:126-128.
 217. Xu HM, Gutmann DH. Merlin differentially associates with the microtubule and actin cytoskeleton. *J Neurosci Res* 1998;51:403-415.
 218. Yonemura S, Nagafuchi A, Sato N, Tsukita S. Concentration of an integral membrane protein, CD43 (leukosialin, sialophorin), in the cleavage furrow through the interaction of its cytoplasmic domain with actin-based cytoskeletons. *J Cell Biol* 1993;120:437-449.
 219. Yonemura S, Hirao M, Doi Y, Takahashi N, Kondo T, Tsukita S. Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of CD44, CD43, and ICAM-2. *J Cell Biol* 1998;140:885-895.
 220. Zhu J, Maruyama T, Jacoby LB, Herman JG, Gusella JF, Black PM, Wu JK. Clonal analysis of a case of multiple meningiomas using multiple molecular genetic approaches: Pathology case report. *Neurosurg* 1999;45:409-416.
 221. Zucman-Rossi J, Legoix P, Der Sarkissian H, Cheret G, Sor F, Bernardi A, Cazes L, Giraud S, Ollagnon E, Lenoir G, Thomas G. NF2 gene in neurofibromatosis type 2 patients. *Hum Mol Genet* 1998;7:2095-2101.