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

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To Irene, Tatu, Samuli and Aino

Enthusiasm keeps us going

CONTENTS

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	aminoterminus
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 system[61]. 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 manifestations[70]. 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 tumours[207].

The first description of NF2 was probably by Wishart in 1822[3]. 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 1882[213], NF1 and NF2 cases became collectively referred to as von Recklinhausen's disease[182]. The final separation in to two distinct diseases came in 1987, when the *NF1* gene was mapped to chromosome 17[16] and the *NF2* gene to chromosome 22[154]. This led to the establishment of the following diagnostic criteria by the National Institute of Health (NIH) Consensus Group clearly separating NF1 and NF2[1].

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.

<u>The NIH diagnostic criteria for NF2:</u>

1. Bilateral eight nerve masses; or
2. A first-degree relative with NF2 and either a unilateral eight 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 $x10^{-6}$, partly reflecting the low reproductive fitness of severe sporadic cases[56]. 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 individuals[121]. Somatic mosaicism, apparently rather common in NF2[60,100], leads to a lower transmission risk and can cause misleading linkage results[24].

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 33 000-40 562[56]. Antinheimo and associates reported a clearly lower incidence, 1 in 87 410, in southern Finland[11], 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 cases[54], 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, meningioangiomatosis 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 proposed[55], because the original NIH criteria seemed to be too rigid[1].

Current diagnostic criteria for NF2[55]:
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 identical[18].

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 radiation[35]. 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 tumours[96]. 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 uncurable 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 cultures[86], 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 site[216]. 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/100 000[196] and spinal schwannomas 0.3-0.4/100,000[177]. 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 angle[14]. 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 ages[216].

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 schwannomas[165]. 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 schwannomas[186]. NF2 schwannomas demonstrate higher proliferation potential (MIB-1)[2,8] and more foci of high cellularity[186], 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 NF2[166].

Cytogenenetic 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 syndrome[120]. 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 patient[82]; (2) another locus, predisposing to the development of schwannomas; or (3) NF1, although unlikely, since tumours in NF1 are almost exclusively neurofibromas not schwannomas[53].

2.2.3. Meningioma

Meningiomas are usually slowly growing tumours attached to the dura, composed of neoplastic meningothelial (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 1:2[28]. Meningiomas are rare in childhood and adolescence, and their incidence increases with age with a peak in the 7th decade[28]. 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 gene[146]. No cases of familial meningiomatosis were found in a population of 1.6 million in Finland[11].

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 type[115], and demonstrate more aggressive growth (higher MIB-1 indices) than sporadic meningiomas[9].

Cytogenenetic 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 meningiomas[161]. 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 retinoblastoma[101].

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 pancreas[21]. 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 ganglia[68].

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 cells[68]. In the human nervous system, cultured Schwann cells and pure glial cultures express relatively more isoform I than II[68]. 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 8[81] and exon 10[149]. 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 rat[68]. 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 skipped[169]. The biological significance of the isoforms and the mechanisms that regulate their expression is not known.
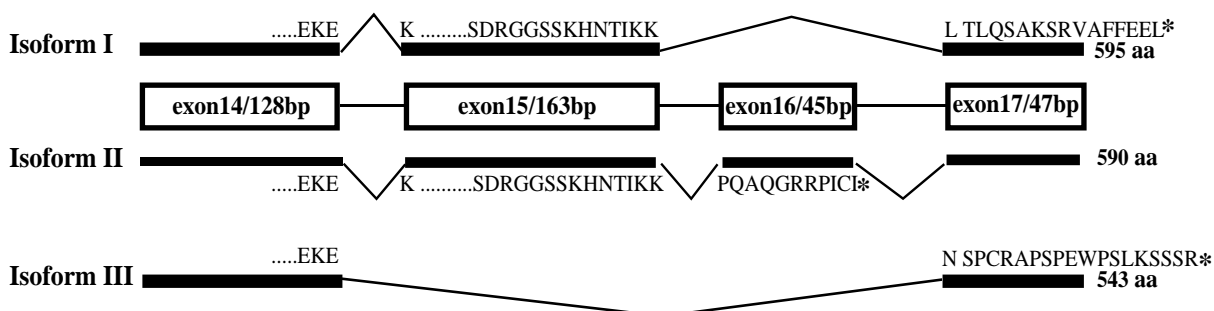

Figure 1. The COOH-terminal *NF2* gene isoforms I, II, and III, respectively, result from alternative splicing of exons 15, 16 and 17. The number of residues contained in each polypeptide is indicated. An asterisk denotes the merlin COOH-terminus.

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 spots[121]. 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 frequent[164]. The nonsense, frameshifting and splice site mutations predicted to cause premature translation termination and protein truncation account for a vast majority of all NF2 mutations[121] (Table 1).

Table 1. Germ-line NF2 mutations[a]

Exons:	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Mutations (no):	13	26	15	6	10	19	11	22	1
Exon size (bp)[b]:	114	126	123	84	69	83	76	135	75

Exons:	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Mutations (no)	11	26	18	10	10	17	0	0
Exon size (bp):	114	123	218	106	128	163	45	47

[a]Reported *NF2* mutations; a mutation map is accessible at http://neuro-trials1.mgh.harvard.edu/nf2/

[b]Exon 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 mutations[99]. 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* allele[18]. Altogether, in individual cases, cautiousness is suggested in the prediction of outcome based on the mutation type[59].

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 schwannomas[88]. 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 substitutions[121]. The mutations in schwannomas span throughout the *NF2* gene[88], 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 *ß*-adaptin (BAM22)[144] and the *MN1* gene[112]. 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 cases[38]. 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 malignancies[111].

2.4. NF2 gene product, merlin

2.4.1. Homology with ERM proteins

The *NF2* gene product, merlin[197] or schwannomin[155] 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 talin[122]. 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 strech 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 VIIA[40].
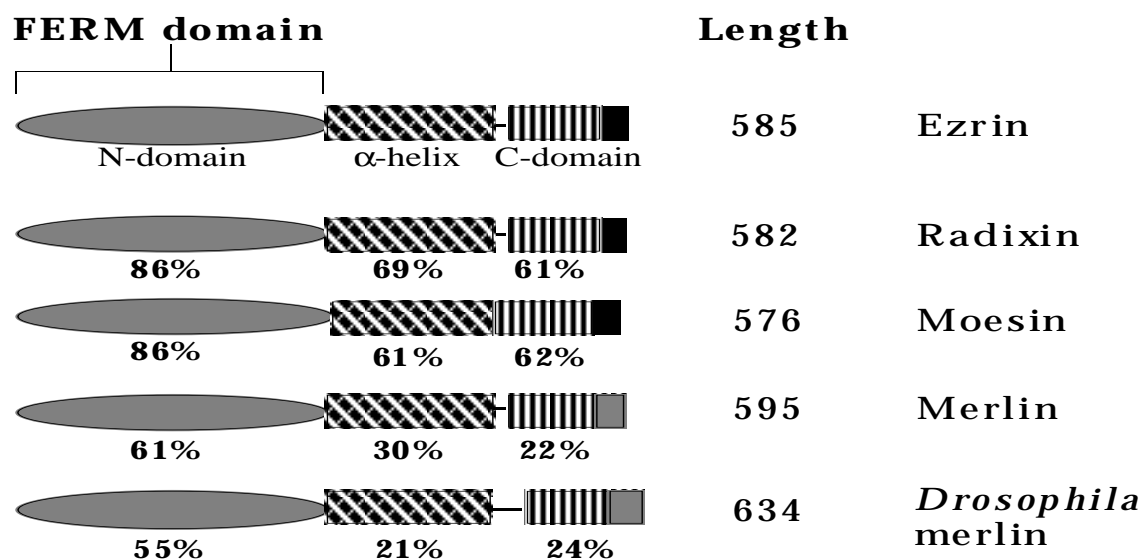
FERM domain   **Length**

N-domain	α-helix C-domain	585 Ezrin
86%	69% 61%	582 Radixin
86%	61% 62%	576 Moesin
61%	30% 22%	595 Merlin
55%	21% 24%	634 *Drosophila* merlin

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 separarated 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 ruffles[33]. 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 specific[20]. 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 uropods[178].

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 oligonucleotides[194]. 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 proteins[153]. 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 (PIP_2), 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 unmasks 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, PIP$_2$, known to conformationally regulate several cytoskeletal components, binds the N-terminal part of ezrin and enhances the interaction between ezrin and transmembrane proteins[79].

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 Dbl[191,192] and a regulatory subunit of PKA[49]. 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 cells[80]. 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 proliferation[94] 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* alleles[134], and in *Drosophila*, loss of the respective NF2 homologue (Dmerlin) results in cellular overproliferation[107]. *In vitro*, merlin suppresses the growth rate of NIH-3T3 cells[116] and reverses the malignant phenotype, *i.e.* anchorage-independent growth, of Ha-ras-transformed cells[195]. Also, an increase in the expression of merlin due to increased confluency suggests a role in the contact inhibition of cell growth[179]. This is in line with the cell detachment and increased proliferation seen in response to antisense oligonucleotide inhibition of merlin expression[83]. 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 with 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 Otology, 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 -80º 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 gene[56]. 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). Fibrobast 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 $[^{32}P]$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 t_{1737+3} and subsequently ATP to detect the wild-type a_{1737+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) 1398NF2[46] against residues 508-533 and (2) mAb HB7[46] against residues 192-209 (both kindly provided by Drs. den Bakker and Zwarthoff), (3) anti-SCH[116] 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 3C12[37] and CD44 with mAb Hermes-3[90] (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 chemiluminescense. 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 pSVß-Galactosidase-transfected CHO cells, stained with FITC-WGA and anti-SCH or anti-ß-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 ß-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 CD44[4], 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 periferal 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 ß-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 ß-galactosidase transfection. The transfected CHO cells were much more elongated ($p<0.0001$) when expressing merlin than ß-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 years[59]. 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 disease[18] 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 mutations[99]. 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 function[69]. 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 schwannomas[186]. 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 schwannomas[177] (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 analyses[58].

Recently, the molecular analysis of tumours from schwannomatosis patients suggested four alternative molecular backgrounds in schwannomatosis[89]. (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 schwannomas[88]. This is supported by the detection of schwannomas with no alterations in the *NF2* gene but deletions elsewere on 22q[36]. (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 lineage[89]. Indeed, somatic mosaicism has been recently proposed to be a fairly common (15% of cases) phenomenon in NF2[60]. (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 allele[89].

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 mutations[142]. 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 cases[97-99]. Somatic mosaicism, shown to occur occasionally in sporadic schwannomatosis[89], 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 NF2[142] 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 cells[46] and at the ruffling edges in primary meningioma cells[66]. 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 demonstrated[67]. 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 filopodia[78]. 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]. Intrestingly, 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 adhesion[179].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 funtional actin-binding site is suggested when C-domain expressed as GST-fusion proteins or by *in vitro* transcription/translation does not bind polymerized actin[217](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 178-367[217]. 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, βII-spectrin[172].

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 metastasis[158]. 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 adhesion[205]. 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 carcinogenesis[19]. 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 adhesion[77]. 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 β4-integrin in differentiating keratinocytes[104]. Thus, neurofibromin may also have a role in connecting cytoskeletal components and cell adhesion molecules.
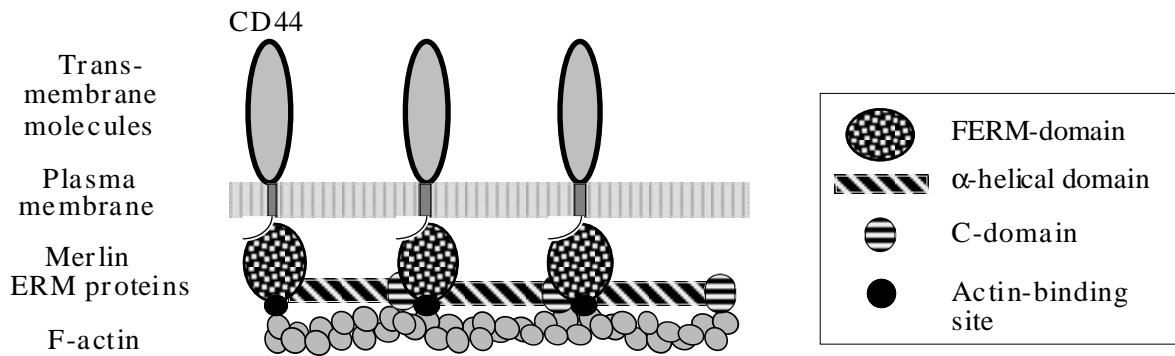
Figure 3. Membrane-cytoskeleton linker model for merlin. In the cortical cytoskeleton, merlin and ERM proteins form head-to-tail oligomers. They interact with cytoplasmic tails of transmembrane adhesion molecules (CD44) with the N-terminal FERM domains. An F-actin binding site in FERM-domain anchors 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 extensions[77]. 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 transfectants[116]. 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.

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