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Type of Mutation in the Neurofibromatosis Type 2 Gene (*NF2*) Frequently Determines Severity of Disease

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Summary

The gene predisposing to neurofibromatosis type 2 (*NF2*) on human chromosome 22 has revealed a wide variety of different mutations in NF2 individuals. These patients display a marked variability in clinical presentation, ranging from very severe disease with numerous tumors at a young age to a relatively mild condition much later in life. To investigate whether this phenotypic heterogeneity is determined by the type of mutation in *NF2*, we have collected clinical information on 111 NF2 cases from 73 different families on whom we have performed mutation screening in this gene. Sixty-seven individuals (56.2%) from 41 of these kindreds revealed 36 different putative disease-causing mutations. These include 26 proposed protein-truncating alterations (frameshift deletions/insertions and nonsense mutations), 6 splice-site mutations, 2 missense mutations, 1 base substitution in the 3′ UTR of the *NF2* cDNA, and a single 3-bp in-frame insertion. Seventeen of these mutations are novel, whereas the remaining 19 have been described previously in other NF2 individuals or sporadic tumors. When individuals harboring protein-truncating mutations are compared with cases with single codon alterations, a significant correlation ($P < .001$) with clinical outcome is observed. Twenty-four of 28 patients with mutations that cause premature truncation of the NF2 protein, schwannomin, present with severe phenotypes. In contrast, all 16 cases from three families with mutations that affect only a single amino acid have mild NF2. These data provide conclusive evidence that a phenotype/genotype correlation exists for certain NF2 mutations.

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Introduction

Neurofibromatosis type 2 (NF2), or central neurofibromatosis, is a severe, often fatal condition in which patients usually present with symptoms from tumors affecting the CNS (Eldridge 1981; Martuza and Eldridge 1988). The most common tumors found in NF2 are vestibular schwannomas, schwannomas at other sites, meningiomas, and ependymomas (Evans et al. 1992*b*). These tumors are often benign and slow growing, but their location predominantly within the CNS may have catastrophic effects on sensitive intracranial and intraspinal structures, thus causing a high rate of morbidity and mortality. Affected individuals typically develop symptoms such as hearing loss (often bilateral), imbalance, tinnitus, facial weakness, and headache (Kanter et al. 1980; Martuza and Eldridge 1988). Approximately half of all NF2 patients also develop posterior capsular lens opacities (Pearson-Webb et al. 1986; Kaiser-Kupfer et al. 1989). The overall incidence of NF2 in Western populations is ~1/40,000 live births, and the average age at onset is in the mid 20s (Kanter et al. 1980; Martuza and Eldridge 1988; Evans et al. 1992*b*; Narod et al. 1992).

It has been proposed that NF2 patients may be clinically subdivided into a severe (Wishart) type and a mild (Gardner) subtype (Eldridge et al. 1991; Evans et al. 1992*a*). This classification is based on the age at onset of symptoms, number and type of tumors developing, and duration of disease. Individuals with the severe-Wishart form of the disease usually present before the age of 25 years, develop numerous tumors (more than three), require repeated surgical intervention, and often do not survive past 50 years of age. Patients with the mild-Gardner subtype usually present with symptoms later in life (classically after 25 years of age), develop a smaller number of more slowly growing tumors (often only bilateral vestibular schwannomas [BVS]), and generally survive beyond the 5th decade. It has been reported that in the majority of familial cases of NF2 there is predominantly only one form of the disease, either

Table 1

Clinical Data for 41 NF2 Families with Mutations in the *NF2* Gene

Family	Patient	Present Age (years)	Age at Onset (years)	Lens Opacity[a]	Tumors Present[b]	Family History[c]	Clinical Subtype[d]
799	782402	17	15	Negative	BVS, mM, mSS	Negative	Severe
240	R1923			Unknown		Unknown	
780	R6307	12	11	Negative	BVS, mM	Negative	Severe
736[e]	R6867 (II-1)	74		Unknown		Positive	Asymptomatic
	R6859 (II-2)	71		Unknown		Positive	Asymptomatic
	R6868 (II-3)	70	22	Unknown	BVS	Positive	Mild
	R6873 (II-5)	57	54	Unknown	BVS	Positive	Mild
	R6865 (II-7)	66		Unknown		Positive	Asymptomatic
	R6872 (II-8)	64	40	Negative	BVS	Positive	Mild
	R6869 (II-10)	59	41	Negative	BVS	Positive	Mild
	R6874 (III-4)	36	33	Negative	UVS	Positive	Mild
	R6862 (III-5)	29		Unknown		Positive	Asymptomatic
	R6861 (III-8)	36		Unknown		Positive	Asymptomatic
794	MR111			Unknown		Unknown	
225	G3060	35	12	Negative	BVS, mM	Negative	Severe
261	R5268	37	18	Positive	BVS, mSS, 1 M	Negative	Severe
202	G5789	29	12	Negative	BVS, 1 M	Negative	Severe
214	G6763			Unknown		Unknown	
223	VMR0195			Positive	BVS	Negative	
254	590402	36	16	Negative	BVS, mSS, 1 M, 1 E, 1 N	Negative	Severe
745	G5145	40	19	Unknown	BVS, mN, 1 M	Negative	Severe
201[f]	G4393	61[g]	42	Negative	BVS, mM	Positive	Mild
220	G9823/0391		17	Unknown	VS, mSS, 2 M	Negative	Severe
758	NNFF103	37	6	Positive	BVS, mS, 1 M	Negative	Severe
781	G2815	27	10	Positive	BVS, 1 M, 1 S	Negative	Severe
249	R3188	32	14	Positive	BVS, 2 M, 1 S	Negative	Severe
234[e]	II-1	34[g]	20	Negative	BVS, 1 M	Positive	Severe
	II-5	33[g]	24	Positive	BVS, 1 M	Positive	Severe
	R3681 (III-1)	32		Negative	UVS, mM	Positive	Asymptomatic
	R3807 (III-2)	31	12	Negative	UVS, mM, 1 N	Positive	Severe
	R3572 (III-4)	30	5	Positive	BVS, mM	Positive	Severe
244	R2848	25	18	Positive	BVS, 1 M, 1 N	Negative	Severe
209	G5095	45	42	Positive	BVS, 1 M	Positive	Mild
535	R0004	37	31	Unknown	BVS, 1 S, 1 M	Negative	Moderate
540	R0005	38[g]	21	Unknown	BVS, mSS	Positive	Severe
783	R5866	37	28	Negative	BVS, 2 M	Negative	Moderate
716	552709	39	18	Unknown	BVS, mS	Positive	Severe
	580802	37		Unknown	BVS, 3 M	Positive	Severe
256[e]	I-2	75	40	Unknown	BVS	Positive	Mild
	I-3	65	20	Positive	BVS	Positive	Mild
	II-2	49	20	Unknown	BVS	Positive	Mild
	II-3	36		Unknown		Positive	Asymptomatic
	II-4	37	35	Negative	UVS, 1 S	Positive	Mild
800	800208	15	13	Negative	BVS, mSS	Negative	Severe
222	G9882/0402		18	Unknown	BVS, SS 1 M	Positive	Severe
785	R7122	30	15	Positive		Negative	Severe
205	G4920	37	24	Unknown		Negative	
757	R4519	20	15	Unknown	BVS, mM, 1 S	Negative	Severe
255	3295D	34	24	Positive	BVS, mM	Negative	Severe
213	G9054	48	28	Unknown	BVS, PN	Positive	Mild
744				Unknown		Unknown	
725	R6364	46	28	Unknown	BVS	Positive	Mild
218	G6274			Unknown		Positive	
779	R6816 (II-4)	29	27	Negative	BVS	Positive	Mild
	I-2	57	40	Unknown	BVS	Positive	Mild
	II-2	36		Unknown	BVS	Positive	Mild
	II-3	33		Unknown	BVS	Positive	Mild

(*continued*)

Table 1 (continued)

Family	Patient	Present Age (years)	Age at Onset (years)	Lens Opacity[a]	Tumors Present[b]	Family History[c]	Clinical Subtype[d]
204	G5434		25	Unknown	BVS, mM, 2 S	Positive	Severe
793	MR112			Unknown		Unknown	
260	R5528 (II-1)	21	20	Positive	BVS, mS, 1 G	Positive	Severe
	II-2	19	19	Unknown	BVS	Positive	Severe
	II-3	16	16	Unknown	BVS	Positive	Severe
	I-2	45	29	Unknown	BVS, 1 M	Positive	Severe
203	G4924	46	41	Unknown	BVS, 2 SS	Positive	Mild
	G5144	50	30	Unknown	BVS	Positive	Mild
	G4913	35	30	Unknown	mSS	Positive	Mild
226	R0622	32	12	Positive	BVS, mS	Negative	Severe

NOTE.—Clinical data are provided only for the families in which sequence variants were identified in NF2.

[a] Eye examination to detect posterior subcapsular lens opacities was performed whenever possible.

[b] Tumors with which each patient has been diagnosed (either by direct histopathological examination of tumor tissue or by radioimaging techniques): M = meningioma (mM = multiple meningiomas); SS = spinal schwannoma (mSS = multiple spinal schwannomas); UVS = unilateral vestibular schwannoma; E = spinal ependymoma; N = neurofibroma (mN = multiple neurofibromas); VS = vestibular schwannoma; S = schwannoma (mS = multiple schwannomas); PN = peripheral neurofibroma; and G = glioma.

[c] Patients are regarded as having a family history if at least one first-degree relative has been diagnosed with NF2.

[d] Patients have been classified according to whether they display a phenotype consistent with the severe or mild form of NF2. This classification is based on age at onset of symptoms, number and type of tumors present, and survival time from diagnosis. Two affected individuals have been classified as having a moderate phenotype, since they do not clearly fall into either of the above two categories.

[e] Pedigrees for three of the largest previously undescribed families (736, 234, and 256) are shown in figure 1.

[f] Clinical data for 22 other affected members of this family may be found in the work of Wertelecki et al. (1988).

[g] Age at death.

the mild-Gardner or severe-Wishart type (Eldridge et al. 1991; Evans et al. 1992*a*). However, this is not exclusively the case, since families with both extremes and with intermediate cases have been observed (Kanter et al. 1980).

We wished to determine whether there is a correlation between the molecular defects in the NF2 gene (*NF2*) and the disease phenotype in affected individuals. That is, does the site or type of mutation play a major role in the severity of NF2, or are there additional factors that play a significant part in the development of this disease? Phenotype/genotype associations have been identified in other disorders, such as colon cancer (for review, see Foulkes, in press), and in some cases this has resulted in a better understanding of the disease and in improvement of the clinical care of patients. Moreover, this type of analysis often results in identification of specific parts of the protein that are critical for normal functioning. To this end, we have screened 73 NF2 families (comprising 111 NF2 individuals) for mutations in the entire coding region of *NF2*, using single-strand conformational analysis (SSCA). For each of the individuals (n = 67) in whom a mutation was identified we have evaluated, wherever possible, the clinical presentation of the disease. For 59 of these cases we have determined age at onset of symptoms, present age, number and type of tumors present, and whether the patient developed posterior capsular lens opacities. Our results demonstrate a statistically significant phenotype/genotype correlation in NF2, for the majority of mutations identified.

Subjects, Material, and Methods

Patient Material

A total of 73 NF2 families containing 111 individuals were screened for mutations in *NF2* (clinical data for the 41 families in which a mutation was identified are given in table 1). These patients were collected as part of our ongoing efforts to elucidate the role of *NF2* and its protein product, schwannomin (SCH), in the disease process. Most cases were referred to us by various clinics in Canada and the United States, but a number were received from Japan, Germany, and Poland. Patients developing at least three tumors (schwannomas, meningiomas, or ependymomas) at an early age (usually before age 25 years) are classified as having severe-Wishart NF2 (n = 49). Individuals presenting later in life (after 30 years of age) and with three tumors or fewer are considered as mild-Gardner cases (n = 38). Four individuals (R0004, R5866, D1956, and D1204) were classified as having moderate NF2, since they did not clearly fall into either of the above two categories (two of these cases are shown in table 1). A further 12 individuals who are presently asymptomatic are known to have NF2 either because a mutation is present or because there is radioimaging evidence of intracranial or intraspinal tumors.

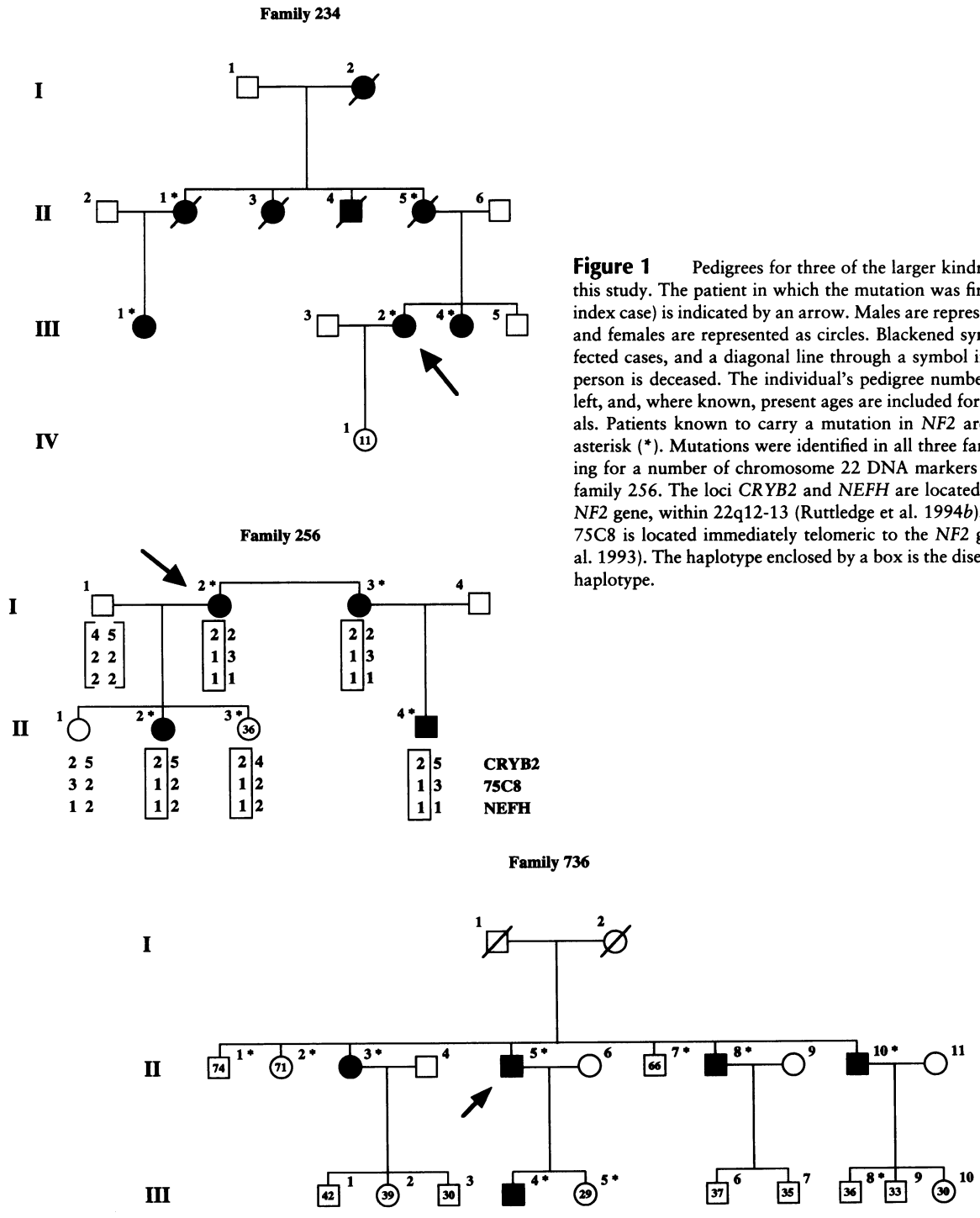

Figure 1 Pedigrees for three of the larger kindreds examined in this study. The patient in which the mutation was first identified (the index case) is indicated by an arrow. Males are represented as squares, and females are represented as circles. Blackened symbols denote affected cases, and a diagonal line through a symbol indicates that the person is deceased. The individual's pedigree number is given to the left, and, where known, present ages are included for at-risk individuals. Patients known to carry a mutation in *NF2* are denoted by an asterisk (*). Mutations were identified in all three families. Allelotyping for a number of chromosome 22 DNA markers are included for family 256. The loci *CRYB2* and *NEFH* are located proximal to the *NF2* gene, within 22q12-13 (Ruttledge et al. 1994*b*), and the marker 75C8 is located immediately telomeric to the *NF2* gene (Rouleau et al. 1993). The haplotype enclosed by a box is the disease-predisposing haplotype.

Of the 73 index cases investigated, 28 (38.4%) had a previous family history of the disease, 37 (50.7%) had no other family members presenting evidence of CNS tumors or peripheral nervous system tumors, and in the 8 (11%) remaining cases no other family information was available. Family 201 is the large NF2 pedigree (BANF 1/family 1) that was originally used to genetically map the *NF2* gene to chromosome 22 (Rouleau et al. 1987, 1990; Wertelecki et al. 1988). We have previously performed presymptomatic diagnosis on many members of this pedigree, using DNA markers flanking the *NF2* gene (Ruttledge et al. 1993). Subsequently, the disease-predisposing mutation has been identified in this family (MacCollin et al. 1993; present report). Families 203 and 218 are two NF2 cohorts (BANF 2 and BANF 9, respectively) that were used for presymptomatic diagno-

Table 2

Oligonucleotide Primerrs for SSCA and Direct Sequencing Analysis of the *NF2* Gene

Exon	Oligonucleotide Sequences[a]	PCR Product Size (bp)	Nucleotides Screened[b]
1	Forward 5′-AGG CCT GTG CAG CAA CTC-3′ Reverse 5′-GAG AAC CTC TCG AGC TTC CAC-3′	261	−60→114 (174)
3	Forward 5′-GCT TCT TTG AAG GTA GCA CA-3′ Reverse 5′-GGT CAA CTC TGA GGC CAA CT-3′	275	241→363 (123)
4	Forward 5′-CCT CAC TTC CCC TCA CAG AG-3′ Reverse 5′-CCC ATG ACC CAA ATT AAC GC-3′	188	364→447 (84)
6	Forward 5′-CAT GTG TAG GTT TTT TAT TTT GC-3′ Reverse 5′-GCC CAT AAA GGA ATG TAA ACC-3′	161	517→599 (83)
7	Forward 5′-CAG TGT CTT CCG TTC TCC-3′ Reverse 5′-AGC TCA GAG AGG TTT CAA-3′	123	600→675 (76)
9	Forward 5′-GTT CTG CTT CAT TCT TCC-3′ Reverse 5′-GTA ATG AAA ACC AGG ATC-3′	138	811→885 (75)
10	Forward 5′-CCT TTT AGT CTG CTT CTG-3′ Reverse 5′-TCA GTT AAA ACA AGG TTG-3′	166	886→999 (114)
13	Forward 5′-GGT GTC TTT TCC TGC TAC CT-3′ Reverse 5′-GGG AGG AAA GAG AAC ATC AC-3′	227	1341→1446 (106)
14	Forward 5′-TGT GCC ATT GCC TCT GTG-3′ Reverse 5′-AGG GCA CAG GGG GCT ACA-3′	253	1447→1574 (128)
15	Forward 5′-TCT CAC TGT CTG CCC AAG-3′ Reverse 5′-GAT CAG CAA AAT ACA AGA AA-3′	245	1575→1737 (163)
16	Forward 5′-CTC TCA GCT TCT TCT CTG CT-3′ Reverse 5′-CCA GCC AGC TCC TAT GGA TG-3′	178	1738→1876 (149)
17	Forward 5′-GGC ATT GTT GAT ATC ACA GGG-3′ Reverse 5′-GGC AGC ACC ATC ACC ACA TA-3′	148	

[a] Oligonucleotide sequences for exons 2, 5, 8, and 12 have been given by Ruttledge et al. (1994*x*). Sequences for exons 3, 4, 6, 13, 14, 16, and 17 were provided by Dr. Lee Jacoby. The antisense oligonucleotide from each primer set was biotinylated at the 5′ end for sequencing.

[b] From Rouleau et al. (1993). The total number of nucleotides examined is given in parentheses.

sis and genetic homogeneity testing (Narod et al. 1992; Ruttledge et al. 1993). Family 752 has been described by Pastores et al. (1991). Clinical details for family 213 (BANF 14) will be described elsewhere. Pedigrees for families 736, 234, and 256 (which have not been described previously) are given in figure 1. Substantial clinical information was unavailable for other affected family members in the remaining kindreds.

Mutation Screening by SSCA

Isolation of constitutional DNA directly from peripheral blood leukocytes or from transformed cell lines was performed as described elsewhere (Ruttledge et al. 1993). All 17 exons of the *NF2* gene were examined for point mutations by using SSCA with the primers listed in table 2. Approximately 50–100 ng of genomic DNA was used for PCR (with S^{35}-dATP) in a total reaction volume of 12.5 μl. The conditions for PCR were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of alternate steps of annealing, polymerization, and denaturation as outlined elsewhere (Ruttledge et al. 1994*a*). To this reaction 8 μl of stop buffer (95% formamide, 10 mM EDTA pH 8.0, 0.025% each of xylene cyanol FF and bromophenol blue) was added, and 5 μl of this mix was heated to 85–90°C for 2 min and then loaded onto a 6% nondenaturing polyacrylamide gel (with and without 5%–7% glycerol). A nondenatured control was also included, so that the single-stranded fragments could be identified easily. The samples were electrophoresed at a constant current of 20 mA at 4°C for 4–8 h, the time being dependent on the size and pattern of migration of the fragment being

Table 3

Families in Which Constitutional Mutations in the *NF2* Gene Have Been Identified

Family	Patient	Exon[a]	Type of Mutation	Nucleotide(s) Affected	Codon(s) Affected	Predicted Consequence on SCH[b]	Family History	Clinical Subtype[c]
799	780224	1	Nonsense	CAA(Gln)→TAA(Stop) at 52	18	X at 18	Negative	Severe
240	R1923	1	Deletion	TC at 41-42	14	F→X at 47	Unknown	
780	R6307	1	Insertion	A at 27-28	9–10	F→X at 48	Negative	Severe
736	R6873	2	Insertion	GAT TTG→GAT TTG TTG[d]	49	I at 49	Positive	Mild
794	MR111[e]						Unknown	
225	G3060	3	Deletion	A at 270	90	F→X at 122	Unknown	Severe
261	R5268	3	Deletion	T at 287	96	F→X at 122	Unknown	Severe
202	G5789	3	Complex	GAGA[A]TGCTGAA→GAGATTAAATTGCTGAA[f]	103–104	F→X at 122	Negative	Severe
214	G6763	4	Insertion	CTCCTGG at 417	139	F→X at 154	Unknown	
223	VMR0195	6	Nonsense	TAT(Tyr)→TAA(Stop) at 531	177	X at 177	Negative	
254	590402	6	Nonsense	CGA(Arg)→TGA(Stop) at 586	196	X at 196	Unknown	Severe
745	G5145	6	Nonsense	CGA(Arg)→TGA(Stop) at 592	198	X at 198	Negative	Severe
201	G4393	7	Missense	AAC(Asn)→TAC(Tyr) at 658	220	M at 220	Positive	Mild
220	G9823/0391	8	Nonsense	TGG(Trp)→TGA(Stop) at 774	258	X at 258	Negative	Severe
758	NNFF103	8	Nonsense	CGA(Arg)→TGA(Stop) at 784	262	X at 262		Severe
781	G2815	8	Nonsense	CGA(Arg)→TGA(Stop) at 784	262	X at 262	Negative	Severe
249	R3188	8	Deletion	C at 768	256	F→X at 295	Negative	Severe
234	R3807	8	Splice	GAG:gta→GAA:gta		SE	Positive	Severe
244	R2848	10	Deletion	T at 945	315	F→X at 321	Negative	Severe
209	G5095	11	Nonsense	CGA(Arg)→TGA(Stop) at 1021	341	X at 341	Positive	Mild
535	R0004	11	Nonsense	CGA(Arg)→TGA(Stop) at 1021	341	X at 341	Negative	Moderate
540	R0005	11	Nonsense	CGA(Arg)→TGA(Stop) at 1021	341	X at 341	Positive	Severe

783	R5866	11	Nonsense	CGA(Arg)→TGA(Stop) at 1021	341	X at 341	Negative	Moderate
716	552709	11	Nonsense	CGA(Arg)→TGA(Stop) at 1021	341	X at 341	Positive	Severe
256	I-2	11	Missense	CTG(Leu)→CCG(Pro) at 1079	360	M at 360	Positive	Mild
800	800802	11	Insertion	AG at 1032-1033	344–345	F→X at 364	Negative	Severe
222	G9882/0402	11	Splice	CTG:gtg→CTG:ttg		SE	Positive	Severe
785	R7122	12	Nonsense	CAG(Gln)→TAG(Stop) at 1165	389	X at 389	Negative	Severe
205	G4920	12	Deletion	G at 1177	393	F→X at 425	Negative	
757	R4519	12	Deletion	AGAGAGGAG:gtg→AGGAG:gtg	445–446	F→X at 453	Negative	Severe
255	3295D	12	Deletion	AG at 1336-1337	446	F→X at 493	Negative	Severe
213	G9054	13	Splice	cag:GGC→caa:GGC		SE	Positive	Mild
744		14	Deletion	atccgaaatttctcattaacag:CC→atag:CC		SE	Unknown	
725	R6364	14	Insertion	T at 1518–1520	506–507	F→X at 513	Positive	Mild
218	G6274	14	Deletion	T at 1499	500	F→X at 514	Positive	
779	R6816	15	Splice	cag:AGT→cac:AGT		SE	Positive	Mild
204	G5434	15	Nonsense	GAA(Glu)→TAA(Stop) at 1580	527	X at 527	Positive	Severe
793	MR112						Unknown	
260	R5528	14	Deletion	GAGA at 1564–1567	522–523	F→X at 549	Positive	Severe
203	G4924	15	Insertion	200 bp at AG:gtacccaggg[g]		SE	Positive	Mild
226	R0622	16	Substitution	G→A at 1833	3′ UTR	Unknown	Negative	Severe

NOTE.—A total of 73 NF2 families were screened for mutations in all 17 exons of the *NF2* gene by SSCA. The mutations identified in 41 of these families are described here.

[a] Exons of the *NF2* gene that were screened by SSCA; this includes intronic splice-site sequences on both sides of the exon in all cases except for exons 1 and 16, where one of each pair of the PCR primers was situated in the 5′ and 3′ UTR of the *NF2* cDNA, respectively.

[b] According to the amino acid sequence reported by Rouleau et al. (1993): X = stop at the codon indicated; F→X = frameshift to a stop at the codon indicated; I = insertion of a single amino acid at the codon indicated; M = missense at the codon indicated; and SE = putative splicing error.

[c] Patients have been classified as in table 1.

[d] The 3-bp in-frame insertion in exon 2 is predicted to introduce an additional leucine residue into SCH at position 49.

[e] The mutation has been described by M. Sainio (personal communication).

[f] Complex rearrangement involves deletion of an A nucleotide (boxed) at position 311 of the *NF2* cDNA (Rouleau et al. 1993) and concomitant insertion of a novel 6-bp sequence (underlined), the net result being insertion of 5 bp into the open reading frame and a frameshift resulting in the introduction of a premature stop codon at position 122 of the NF2 protein, SCH.

[g] An ~200-bp insertion in the intron between exons 15 and 17, located 10 bp 3′ of the end of exon 15 and presumably causing aberrant splicing of the *NF2* transcript.

tested. The gel was then dried and exposed to X-ray film for 1–3 d. When an abnormal migration pattern was detected, the PCR was repeated and run together with negative controls on a new nondenaturing polyacrylamide gel.

Direct Sequencing of Aberrant PCR Fragments

The exons showing an abnormal SSCA pattern were amplified by PCR from ~300–500 ng of genomic DNA in a total reaction volume of 100 μl by using the same conditions as described above. The amplified fragments were excised from a 1% agarose gel and were purified as described elsewhere (Ruttledge et al. 1994*a*). After precipitation, the DNA was resuspended in 50 μl of TE buffer (10 mM Tris-Cl pH 7.6, 1 mM EDTA pH 8), and the complementary strands were separated by using streptavidin-coated magnetic beads (Dynal) as described elsewhere (Ruttledge et al. 1994*a*). The nucleotide sequence of both single strands was independently determined by using the Sequenase version 2 DNA sequencing kit (United States Biochemical). Mutations were verified by repeating the sequencing from a second PCR, and, in cases where other family members were available, these were also included. The primers that were employed for PCR reactions were also used for sequencing, except that nonbiotinylated oligonucleotides were used in the antisense direction.

Results

Clinical details for the 41 NF2 families (67 individuals) in which mutations were identified in *NF2* are given in table 1. Pedigrees for three of the larger families that have not been described elsewhere are shown in figure 1. A total of 36 different putative disease-causing mutations were identified in these 41 kindreds after SSCA and direct sequencing analysis of constitutional DNA (table 3). Of these 36 variants, 15 are insertions or deletions presumably causing frameshifts, 11 are nonsense mutations, 6 are believed to result in defective splicing, 2 are missense changes, 1 is an in-frame insertion of 3 bp in exon 2, and in a single case a base substitution in the 3′ UTR of *NF2* was identified. The age at onset of symptoms in 83 of the 95 NF2 individuals for whom data were available ranged from 1 to 54 years (mean 24.8 years). In the remaining 12 cases either a mutation in *NF2* has been identified or there is diagnostic imaging evidence of intracranial tumors, but the patient remains asymptomatic at ages ranging from 10 to 74 years (mean 37.3 years). On the basis of these ages at onset, the number of years since diagnosis, and the number and type of tumors presenting, we have classified these patients as either mild-Gardner NF2 or severe-Wishart NF2.

The number of individuals from each clinical group with a specific type of mutation is summarized in table 4. When protein-truncating mutations are compared with single codon changes (missense mutations in families 201 and 256 and a 3-bp in-frame insertion in family 736), a significant correlation with phenotype is found (by χ^2 test; $P < .001$). Two of 28 patients with protein-truncating mutations have mild NF2, whereas all 16 cases with single codon changes are classified as having this phenotype. When all individuals in table 4 are taken into account, a total of nine different alterations (table 3) are detected in 27 individuals (table 1) who have been classified as mild-Gardner NF2 ($n = 20$) or who are asymptomatic ($n = 7$). Twenty-five (92.6%) of these 27 cases are found to have either a splice-site alteration ($n = 9$) or a single amino acid change—that is, two missense mutations ($n = 6$) or a single 3-bp insertion ($n = 10$)—in *NF2*. In contrast, only 5 (15.6%) of the 32 severe-Wishart ($n = 30$) or moderate ($n = 2$) NF2 cases (constituting 18 different mutations) have one of the latter types of alteration. The vast majority (26/32) of severe-Wishart or moderate NF2 cases have nonsense or frameshift-causing deletion/insertion mutations, all of which are predicted to result in the production of a substantially truncated SCH protein.

With the possible exceptions of the two missense changes (families 201 and 256; table 3) and the single base substitution in the 3′ UTR of *NF2*, we did not identify any rare polymorphisms in these 111 NF2 cases. Furthermore, in direct sequencing of all 17 exons in 28 unrelated individuals we did not encounter any sequence changes that were not believed to be associated with the disease process.

The 3-bp in-frame insertion (table 3) that has been identified in kindred 736 (fig. 1) is present in 10 family members, only 5 of whom are affected with NF2 (table 1 and fig. 1). A further seven, unaffected individuals (family 736, cases III-1, III-2, III-3, III-6, III-7, III-9, and III-10 in fig. 1) do not harbor this alteration. The clinical phenotype associated with this mutation is very mild-Gardner NF2. This is represented by the fact that five mutation carriers (average age 55.2 years) still show little or no signs of disease. One of these individuals (III-5) has experienced some balance problems, but magnetic-resonance imaging (MRI) at age 28 years failed to detect intracranial tumors. Furthermore, the five affected cases appear to develop only vestibular schwannomas with no evidence of other intracranial or intraspinal tumors, even though MRI or computed-tomography scans have been performed in all cases.

The two missense mutations that have been identified are present in a total of 23 individuals from two NF2 kindreds, families 201 and 256 (table 3 and fig. 1). The first of these variants was found in family 256 (fig. 1), and it results in the substitution of a proline for a leucine residue at amino acid position 360 of the protein sequence reported by Rouleau et al. (1993). This mutation

Table 4

Number of Individuals in Each Clinical Group, for Different Classes of Mutation in *NF2*

	Protein-Truncating Mutations (n = 26)[a]	Splice-Site Mutations (n = 6)	Single Codon Changes (n = 3)[b]	3′ UTR (n = 1)	Total
Mild	2	9	16	0	27
Moderate	2	0	0	0	2
Severe	24	5	0	1	30
Unknown	7	1	0	0	8
Total	35	15	16	1	67

NOTE.—Sixty-seven individuals from 41 families with 36 different mutations are presented; sufficient clinical data were available for 59 cases to be classified as mild, moderate, or severe NF2.

[a] Includes nonsense changes and frameshift insertions/deletions leading to a premature translation stop.

[b] Two missense mutations in families 201 and 256 and the 3-bp in-frame insertion in family 736 (table 3).

has previously been identified in a presumably unrelated individual displaying a mild-Gardner phenotype (Rouleau et al. 1993; Merel et al. 1995). In family 256, this mutation is present in four individuals (I-2, I-3, II-2, and II-4) with the mild-Gardner subtype of NF2 (fig. 1 and table 1). An additional family member (II-3) also has the missense mutation but was asymptomatic on the basis of normal MRI results of the entire neuraxis at age 36 years. Individual II-3 previously had been predicted to carry the mutated *NF2* gene, on the basis of haplotype analysis using DNA markers flanking the *NF2* gene on chromosome 22 (fig. 1). Two affected members of family 256 (I-3 and II-4) have been examined by an ophthalmologist for lens abnormalities. No evidence of lens opacities was found in case II-4. Individual I-3 presented with a subcapsular cataract of the right lens at age 65 years, the relationship to NF2 being unclear.

The second missense mutation identified in this study (patient G4393 in family 201; table 1) has been reported independently elsewhere (MacCollin et al. 1993). It results in the conversion of an asparagine to a tyrosine residue at amino acid position 220 of SCH. This change is found in 12 other affected members and in 5 presently asymptomatic members of family 256 whom we have tested. We had previously predicted that all 18 of these patients—that is, individuals 37, 16, 20, 18, 49, 53, 52, 60, 57, 46, 67, 69, 79, 81, 82, 85, 75, and 76 in figure 2 of the report by Ruttledge et al. (1993)—would carry the mutated *NF2* gene. Another 20 unaffected members of this pedigree were found not to harbor this missense mutation, results consistent with our previous haplotype analysis (Ruttledge et al. 1993).

An insertion of ~200 bp was identified in the intron between exons 15 and 16 in constitutional DNA from patient G4924 (table 1; family 203), a member of a large Italian/American family that previously has been used in genetic linkage studies to narrow down the *NF2* locus on chromosome 22 (Narod et al. 1992; Ruttledge et al. 1993). In addition to the normal 245-bp PCR product for exon 15 (table 3), a second novel band, of ~450 bp, was seen in this patient by agarose-gel electrophoresis (data not shown). After direct sequencing of both the normal and novel fragments, an ~200-bp insertion was found in the larger fragment. The 200-bp insertion is located 10 bp 3′ of the splice-donor junction of exon 15 (table 1). No significant sequence homology was identified between this 200-bp fragment and sequences in the database. The smaller, 245-bp band contained the normal published sequence for exon 15 and its surrounding introns. The variant identified in patient G4924 is also present in seven other affected family members and in four presently unaffected at-risk individuals. The clinical course of NF2 in three of these affected cases whom we have followed is one of relatively late onset (in the 4th and 5th decades) with predominantly BVS and a limited number of tumors at other sites (table 1). The clinical picture in this kindred is thus most consistent with the mild-Gardner form of NF2.

Discussion

Previous studies have provided evidence of an association between the severe-Wishart form of NF2 and protein-truncation mutations (Bourn et al. 1994*a*, 1994*b*; Merel et al. 1995). We confirm this correlation, since 80% of our 30 severe NF2 cases have nonsense changes or frameshift causing deletion/insertion mutations. Our data, however, also reveal a highly significant ($P < .001$) association between mild-Gardner NF2 and single codon alterations. Furthermore, there is also a predominance of mild NF2 in splice-site-mutation carriers. In total, six mutations are associated with a milder phenotype, and these include two missense changes, three splice-site alterations, and a single 3-bp in-frame insertion (tables 1 and 3). In all but 2 of the 27 mild-phenotype or asymptomatic NF2 patients in whom mutation data were available, one of these three types of mutation was observed.

Probably the most striking example of a phenotype/genotype correlation is in family 736, where a 3-bp in-frame insertion is observed between codons 48 and 49 of *NF2* (tables 1 and 3). The clinical picture observed in this kindred is remarkable because thus far, at a relatively late age (29 to 74 years), five mutation carriers have not developed any detectable tumors. In addition, the five affected mutation carriers have presented only with vestibular schwannomas. Two of these patients (individuals II-8 and II-3 in fig. 1) are in their 7th and 8th decades, respectively. The presence of this 3-bp insertion in the five known affected individuals in family 736 suggests that either this is the pathogenic mutation in this kindred or it is segregating with the disease allele. Support for a causative role of this variant in NF2 is provided by the observations that (1) it has not been identified in >500 unrelated individuals whom we have screened by SSCA, (2) it is segregating with the disease in five affected individuals, and (3) it is found in a highly conserved region of *NF2* (Rouleau et al. 1993; Trofatter et al. 1993). In addition, given that the predicted result of this alteration is the introduction of a single leucine residue, without any other known consequence on SCH, it is conceivable that such a mild phenotype may result. On the basis of both the unusual nature of this insertion and the clinical phenotype, it is possible that some residual protein activity still exists in tumors, and this may account for their generally slow-growing nature. This hypothesis of a partially acting protein differs from the all-or-none concept that generally is proposed for tumorigenesis in NF2 (Seizinger et al. 1987; Rouleau et al. 1993; Trofatter et al. 1993).

The two missense mutations that are found in 23 affected individuals in families 201 and 256 (mutations affecting codons 220 and 360, respectively) are presumably located in regions of SCH that are of functional importance, since both amino acids are highly conserved between SCH and the human band 4.1 proteins (Rouleau et al. 1993; Trofatter et al. 1993). Whatever the function of these codons, it appears that their substitution by other amino acids results in a predominantly mild form of NF2. These missense mutations do allow for the specific formation of vestibular schwannomas and other tumors associated with NF2, but they do so at a lower frequency than is seen in cases that have protein-truncation mutations. Family 201 has been examined in detail elsewhere (Rouleau et al. 1987; Wertelecki et al. 1988; MacCollin et al. 1993; Ruttledge et al. 1993), with affected individuals displaying a generally mild form of NF2 in which no presenile lens opacities (believed to be associated with NF2) have been identified. In the original report of this family (Wertelecki et al. 1988), only 3 (13.0%) of the 23 affected individuals presented with symptoms of NF2 before 20 years of age, and 13 (56.5%) of 23 remained asymptomatic until at least the 4th decade. Therefore, although a small proportion of affected members of this family have developed numerous tumors (some at a relatively young age), the overall clinical picture is one of mild-Gardner NF2, with the majority of carriers displaying one or two neoplasms.

The milder symptoms observed in most of the individuals with missense mutations may be attributable to the fact that these changes only partially disrupt SCH or alter its half-life. Thus, residual SCH activity may lead to slower tumor growth in these cases. In contrast, by deleting a substantial portion of SCH (e.g., in cases with protein-truncation mutations), total loss of SCH function occurs after loss of the wild-type allele in tumor tissue. In this situation the rate at which tumors grow usually increases, resulting in both earlier onset of symptoms and a larger number of tumors presenting (on average) in each individual.

An association between splice-site mutations and mild NF2 is also evident in our data. Six mutations listed in table 3 are likely to affect splicing, and three of these (in families 213, 779, and 203) are associated with a mild phenotype in eight different affected individuals. Such mutations might result in partial splicing, where the mutant site is used less frequently than its normal counterpart, thus allowing a certain amount of normal mRNA and protein to be produced and resulting in a less severe form of the disease. Further support for a relationship between splice-site mutations and a mild-Gardner NF2 phenotype is provided in a recent report by Merel et al. (1995). That report describes a total of four mild NF2 cases, three of which harbor splice-donor mutations; however, a further six splice-site mutations are found in patients with a severe clinical course. Moreover, two of the remaining three splice-site mutations in the present study are associated with a severe phenotype. When the results from both data sets are combined, there is no clear indication as to which type of splice-site alteration (e.g., splice-donor or -acceptor mutations) gives rise to a mild or severe phenotype.

The role of the base substitution in the 3′ UTR of the NF2 cDNA in family 226 is unclear. Conceivably, it may affect the half-life of the *NF2* transcript in this individual, or it may be involved in deregulating the splicing machinery of the cell. On the other hand, it is possible that it is a benign polymorphism that is not the causative mutation of *NF2* in this case. It should be noted that, after the entire coding region of the *NF2* gene was screened by SSCA, only ~60% of affected families were shown to have mutations. This figure may represent genetic heterogeneity in NF2 (Narod et al. 1992). A more likely explanation for the lack of mutation detection in the remaining 40% of affected cases, however, is that either there is a shortfall in sensitivity of SSCA or mutations lie outside the coding exons of the gene (e.g., in the introns or regulatory regions). Thus, it is conceivable that the pathogenic mutation in family

226 has not yet been identified. To aid in the determination of the role of this base substitution in NF2, it would be helpful to examine both parents for the presence or absence of this variant. If this mutation occurred de novo in individual 226, it may be of greater significance, since there is no known family history of the disease. Unfortunately, neither parent was available for study, and thus the role of this base substitution in the development of NF2 remains to be determined

We were interested to determine whether a particular type of mutation in NF2 is associated with the development of presenile lens opacities. Of the 111 individuals included in this study, 57 were examined for lens opacities, with 26 being found positive for them. Fourteen of these 26 cases were also found to have a mutation in *NF2*, with 12 cases harboring a splice-site or protein-truncation mutation. The role of the remaining two mutations (in families 256 and 226, discussed above) in the disease process is uncertain. Thus, our data neither support nor refute the hypothesis of a truncated-protein/presenile-lens-opacity correlation.

We have considered all of the affected individuals in family 234 as having severe-Wishart NF2—except patient III-1, who, at age 32 years, still shows no clinical signs of the disease, even though she has radioimaging evidence of several intracranial tumors (table 1). This severity is evident when the pedigree for this family is examined (fig. 1); all four sibs in the second generation of this kindred died at an early age (34 and 33 years for individuals II-1 and II-5, respectively), because of the serious complications associated with NF2. In contrast, all four affected cases in family 256 (fig. 1) are alive and are coping well. This comparison highlights the disastrous affects of some mutations in *NF2*—and the relatively benign nature of others—on the individuals who harbor them. In conclusion, we believe that most protein-truncation mutations are associated with severe-Wishart NF2, whereas other specific mutations (two missense mutations, some splice-site alterations, and the 3-bp insertion in family 736) are associated with a more mild-Gardner form of the disease. However, other factors certainly play a role in determining the severity of this condition in certain individuals.

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References

Bourn D, Carter SA, Evans DGR, Goodship J, Coakham H, Strachan T (1994*a*) 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 55:69–73

Bourn D, Carter SA, Mason S, Evans DGR, Strachan T (1994*b*) Germline mutations in the neurofibromatosis type 2 tumor suppressor gene. Hum Mol Genet 3:813–816

Eldridge R (1981) Central neurofibromatosis with bilateral acoustic neuroma. Adv Neurol 29:57–65

Eldridge R, Parry DM, Kaiser-Kupfer MI (1991) Neurofibromatosis 2: clinical heterogeneity and natural history in 39 individuals in 9 families and 16 sporadic cases. Am J Hum Genet Suppl 49:A133

Evans DGR, Huson SM, Donnai D, Neary W, Blair V, Newton V, Strachen T, et al (1992*a*) A genetic study of type 2 neurofibromatosis in the United Kingdom. II. Guidelines for genetic counseling. J Med Genet 29:847–852

Evans DGR, Huson SM, Donnai D, Neary W, Blair V, Teare D, Newton V, et al (1992*b*) 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 29:841–846

Foulkes WD. A tale of four syndromes: familial adenomatous polyposis, Gardner syndrome, attenuated APC and Turcot syndrome. Q J Med (in press)

Kaiser-Kupfer MI, Friedlin V, Datiles MB, Edwards PA, Sherman JL, Parry D, McCain LM, et al (1989) The association of posterior capsular lens opacities with bilateral acoustic neuromas in patients with neurofibromatosis type 2. Arch Ophthalmol 107:541–544

Kanter WR, Eldridge R, Fabricant R, Allen JC, Koerber T (1980) Central neurofibromatosis with bilateral acoustic neuroma: genetic, clinical, and biochemical distinctions from peripheral neurofibromatosis. Neurology 30:851–859

MacCollin M, Mohney T, Trofatter J, Wertelecki W, Ramesh V, Gusella J (1993) DNA diagnosis of neurofibromatosis 2. JAMA 270:2316–2320

Martuza RL, Eldridge R (1988) Neurofibromatosis 2 (bilateral acoustic neurofibromatosis). N Engl J Med 318:684–688

Merel P, Hoang-Xuan K, Sanson M, Bijlsma E, Rouleau GA, Laurent-Puig P, Pulst S, et al (1995) Screening for germ-line mutations in the NF2 gene. Genes Chromosom Cancer 12: 117–127

Narod SA, Parry DM, Parboosingh J, Lenoir GM, Ruttledge M, Fischer G, Eldridge R, et al (1992) Neurofibromatosis type 2 appears to be a genetically homogeneous disease. Am J Hum Genet 51:486–496

Pastores GM, Michels VV, Jack CR (1991) Early childhood diagnosis of acoustic neuromas in presymptomatic individuals at risk for neurofibromatosis 2. Am J Med genet 41: 325–329

Pearson-Webb MA, Kaiser-Kupfer MI, Eldridge R (1986) Eye findings in bilateral acoustic (central) neurofibromatosis: association with presenile lens opacities and cataracts but absence of Lisch nodules. N Engl J Med 315: 1553–1554

Rouleau GA, Merel P, Lutchman M, Sanson M, Zucman J, Marineau C, Hoang-Zuan K, et al (1993) Alteration in a new gene encoding a putative membrane-organizing protein causes neurofibromatosis type 2. Nature 363:515–521

Rouleau GA, Seizinger BR, Wertelecki W, Haines JL, Superneau DW, Martuza RL, Gusella JF (1990) Flanking markers bracket the neurofibromatosis type 2 (NF2) gene on chromosome 22. Am J Hum Genet 46:323–328

Rouleau GA, Wertelecki W, Haines JL, Hobbs WJ, Trofatter JA, Seizinger BR, Martuza RL, et al (1987) Genetic linkage of bilateral acoustic neurofibromatosis to a DNA marker on chromosome 22. Nature 329:246–248

Ruttledge MH, Narod SA, Dumanski JP, Parry D, Eldridge R, Wertelecki W, Parboosingh J, et al (1993) Pre-symptomatic diagnosis for neurofibromatosis 2 with chromosome 22 markers. Neurology 43:1753–1760

Ruttledge MH, Sarrazin J, Rangaratnam S, Phelan CM, Twist E, Merel P, Delattre O, et al (1994*a*) Evidence for the complete inactivation of the NF2 gene in the majority of sporadic meningiomas. Nat Genet 6:180–184

Ruttledge MH, Xie Y-G, Han F-Y, Giovannini M, Janson M, Fransson I, Werelius B, et al (1994*b*) Physical mapping of the NF2/meningioma region on human chromosome 22q12. Genomics 19:52–59

Seizinger BR, Rouleau G, Ozelius LJ, Lane AH, St George-Hyslop P, Huson S, Gusella JF, et al (1987) Common pathogenic mechanism for three tumor types in bilateral acoustic neurofibromatosis. Science 236:317–319

Trofatter JA, MacCollin MM, Rutter JL, Murrell JR, Duyao MP, Parry DM, Eldridge R, et al (1993) A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. Cell 72:791–800

Wertelecki W, Rouleau GA, Superneau DW, Forehand LW, Williams JP, Haines JL, Gusella JF (1988) Neurofibromatosis 2: clinical and DNA linkage studies of a large kindred. N Engl J Med 319:278–283