

## Mutational analysis of the NF2 gene in sporadic meningiomas by denaturing high-performance liquid chromatography

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**Abstract.** The NF2 tumor suppressor gene, located in chromosome 22q12, is involved in the development of sporadic meningiomas of the nervous system. In order to evaluate the role of the NF2 gene in sporadic meningiomas, we analyzed the entire coding regions of the NF2 gene in a group of 42 sporadic meningiomas: 17 meningothelial, 11 transitional, 11 fibrous, one secretory, one atypical, and one malignant subtype, using denaturing high-performance liquid chromatography (DHPLC) and sequence analysis. Twenty-one mutations were identified in 20 patients with an overall mutation detection rate of 47.6%. The mutations included nine deletions (exons 1, 2, 5, 10, and 12), resulting in a frameshift, four non-sense mutations (exons 1, 2, and 7), four splice errors (exons 4, 5, 7, and 12), two missense mutations (exon 5) and two silent mutations (exon 11). Among these, 14 novel mutations were also identified in the present study. All mutations were noted in the first 12 exons, the region of homology with the ezrin-moesin-radixin protein. Furthermore, an association between NF2 mutations and histologic subtypes were observed; NF2 mutations were more frequent in fibrous meningiomas (8/11, 73%) and transitional meningiomas (6/11, 55%), than in meningothelial variant (5/17, 29%). These results provide evidence that mutations in the NF2 gene play an important role in the development of sporadic meningiomas as well as indicating a different tumorigenesis of these meningioma variants.

### Introduction

Meningiomas are common tumors of the human nervous system and are classified by the World Health Organization as grade I, II, and III according to their recurrence and aggressive behavior (1). Meningiomas are also frequently observed in patients with neurofibromatosis 2 (NF2) as a part of the disease spectrum (2).

The NF2 gene consists of 16 constitutive exons and 1 alternatively spliced exon and encodes a 595 amino-acid-protein, merlin or schwannomin, similar to the EMR (ezrin-moesin-radixin) subgroup of the protein 4.1 family (3,4). EMR family proteins are structural and functional components of the actin cytoskeletal network in the cell membrane, involved in cell morphogenesis and contact formation, that have a fundamental role in the regulation of cell growth, motility and differentiation (5). The function of merlin protein suggests that meningiomas may be formed when cells lose the ability to accurately regulate cell movement, shape, or communication, leading to a loss of growth control.

The NF2 tumor suppressor gene, located in chromosome 22q12, is known to be involved in the development of neurofibromatosis 2-associated neoplasms and also of their sporadic counterparts and other unrelated tumors (3,6). However, NF2 mutations in sporadic meningiomas are not associated with neurofibromatosis 2. Previous studies show that the loss of heterozygosity of chromosome 22q is a frequent event in approximately 60% of meningiomas (7) and screening of the NF2 gene by single-strand conformational polymorphism (SSCP) technique, identified mutations in 15.9% for eight of the 16 NF2 exons or 61.4% of sporadic meningiomas (8,9).

Denaturing high-performance liquid chromatography (DHPLC), based on the detection of heteroduplexes in PCR products by ion pair reverse-phase HPLC under partially denaturing conditions, is a highly sensitive and specific technique for mutation detection (10). Though DHPLC was used as a prescreening technique for heteroduplex analysis so as to detect mutations, the accurate prevalence of NF2 mutations in sporadic meningiomas using DHPLC has been rarely reported (11).

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Table I. Primer sequences used in this study.

Exon	Product size (bp)	Annealing temp. (°C)	Forward	Reverse
1	244	63	AACTCCAGGGGGGCTAAAGG	ACCTCTCGAGCTTCCACC
2	200	63	TTGCTCACAGTGTCTTCCC	TCAGCCCCACCAGTTTCATC
3	271	63	GCTTCTTTGAGGGTAGCACA	GGTCAACTCTGAGGCCAACT
4	187	58	CCTCACTTCCCCTCACAGAG	CCCATGACCCAAATTAACGC
5	173	58	ATCTTTAGAATCTCAATCGC	AGCTTTCTTTTAGACCACAT
6	159	58	ATGTGTAGGTTTTTTATTTTGCTC	GCCCATAAAGGAATGTAACC
7	190	63	CCGCTCTCCACCCATCTCAC	TGGCCCTCACTCAGTCTCTG
8	179	58	CCACAGAATAAAAAGGGCAC	GATCTGCTGGACCCATCTGC
9	122	58	GTTCTGCTTCATTCTTCC	GTAATGAAAACCAGGATC
10	198	63	GGCAGTGAAGTAAATTTGTGGAT	AGGCCAGGACTGACCACACA
11	212	63	TCGAGCCCTGTGATTCAATG	AAGTCCCCAAGTAGCCTCCT
12	284	63	CCCACTTCAGCTAAGAGCA	CTCCTCGCCAGTCTGGTG
13	202	63	TACCTGCCCTCTTCTGTGAA	AGAACATCACCAGGACTAAG
14	212	63	ATGACCCAAGCTCCTAATCC	AGTCTAGTTCACAGCTGCCC
15	245	58	TCTCACTGTCTGCCCAAG	GATCAGCAAAATACAAGAAA
16	207	63	GCAGATGGCACTTATGGCAT	AGGACAGAGACAGGCAAGCT

Therefore, we performed a DHPLC analysis of the entire coding regions of the NF2 gene and direct sequencing to investigate the accurate prevalence of NF2 mutations and to detect novel mutations in a group of 42 sporadic meningiomas.

## Materials and methods

**Samples and DNA extraction.** Forty-two tumor specimens and peripheral blood samples were obtained from patients during surgery at Keimyung University Dongsan Medical Center from 2000 to 2004. The patients consisted of 12 males and 30 females, and ranged in age from 30 years to 75 years. H&E-stained histologic sections were reviewed in each case. Of the 42 sporadic meningiomas, there were 17 meningothelial, 11 transitional, 11 fibrous, one secretory, one atypical, and one malignant meningioma. None of the patients had a clinical manifestation of neurofibromatosis 2. The use of DNA material derived from human subjects has been approved by the institutional review board of Keimyung University Dongsan Medical Center. High molecular weight DNAs derived from tumor tissue snap frozen by immersion in liquid nitrogen and corresponding peripheral blood leukocytes were isolated and purified as described by James *et al* (12).

**Amplicon synthesis.** Polymerase chain reaction (PCR) amplification of 16 exons of the NF2 gene, with the flanking intronic sequences, was performed using a set of primer pairs displayed in Table I. Each exon of the NF2 gene was

amplified from 50 ng of genomic DNA using 0.5 U AmpliTaq Gold DNA polymerase (Perkin Elmer, Branchburg, NJ, USA), 0.5 mM of each dNTP, 1.6  $\mu$ M of exon-specific primer pair and 1.5 mM MgCl<sub>2</sub>. PCR amplification was performed as follows: 12 min at 94°C for hot-start and initial denaturing followed by 35 cycles of 94°C for 40 sec, 58/63°C for 40 sec, and 72°C for 1 min, and followed by final extension of 10 min at 72°C. The PCR products were verified as a single band on 4% metaphor agarose gel.

**DHPLC analysis.** DHPLC was carried out using the Wave nucleic acid fragment system (Transgenomic, San Jose, CA, USA). PCR products of tumor DNA were combined with PCR products of wild-type DNA in a ratio of 4:1 in order to allow the formation of heteroduplex between mutated and wild-type DNAs. Each PCR product (4-7  $\mu$ l) was denatured for 5 min at 95°C and then gradually reannealed with 43 cycles of 1.6°C decrement temperature for 1 min. Mixed PCR (18-15  $\mu$ l) products were injected into a preheated column and separated at a flow rate of 0.9 ml/min through a 2% linear acetonitrile gradient.

The elution buffer consisted of buffer A, 0.1 M triethylammonium acetate (TEAA), and buffer B, 0.1 M TEAA with 25% acetonitrile, and the gradient slope was an increase of 2% of buffer B per min. The oven temperatures for optimal heteroduplex separation were determined using Wave-maker software (Transgenomic), which gives a computer-assisted determination of melting profile and analytical conditions for each fragment. The temperature giving 70 to 80% of double

Table II. Summary of NF2 mutations detected in 42 sporadic meningiomas.

Exon	Tumor No.	Histologic subtype	Nucleotide change	Codon change	Consequence
1	29	Transitional	112 G→T	Glu 38 Stop (GAG→TAG)	Non-sense
	80	Fibrous	70 del 1bp (G)*	Val 24→29 Stop	Frame shift
	137	Transitional	94 G→T*	Glu 32 Stop (GAG→TAG)	Non-sense
2	90	Fibrous	234 del 1bp (C)*	Asp 78→83 Stop	Frame shift
	99	Fibrous	193 C→T	Gln 65 Stop (CAG→TAG)	Non-sense
	153	Transitional	138~139 del 2bp (CT)*	Leu 46→47 Stop	Frame shift
4	117	Meningothelial	364-1 g→c	-	Splice acceptor (intron 3/exon 4)
5	118	Fibrous	448-3~455 del 11bp*	-	Splice acceptor (intron 4/exon 5)
	133	Meningothelial	468 T→A*	Ser 156 Arg (AGT→AGA)	Missense
	134	Transitional	488 del 1bp (T)*	Leu 163→173 Stop	Frame shift
	145	Fibrous	501 A→T*	Glu 167 Asp (GAA→GAT)	Missense
7	140	Atypical	634 C→T*	Gln 212 Stop (CAG→TAG)	Non-sense
	150	Fibrous	675+2 del 1bp (T)	-	Splice acceptor (exon 7/intron 8)
10	119	Fibrous	892 del 1bp (C)*	Gln 298→308 Stop	Frame shift
11	20	Meningothelial	1113 C→T	Asn 371 Asn (AAC→AAT)	Silent
	111	Fibrous	1113 C→T	Asn 371 Asn (AAC→AAT)	Silent
12	24	Transitional	1191 del 1bp (T)*	Leu 397→425 Stop	Frame shift
	111	Fibrous	1264 del 1bp (G)*	Glu 422→425 Stop	Frame shift
	127	Transitional	1254 del 1bp (C)*	Arg 418→425 Stop	Frame shift
	146	Meningothelial	1340+1 G→A	-	Splice acceptor (exon 12/intron 13)
	149	Meningothelial	1334~1337 del 4bp	Glu 445→454 Stop	Frame shift

\*Mutation is novel compared with Neurofibromatosis Research at Massachusetts General Hospital (<http://Neurosurgery.MGH.Harvard.Edu/NFCLinic/NFResearch.htm>).

helical fraction of wild-type DNA was defined and confirmed experimentally by performing separation at this calculated temperature and at others above and below it. Heteroduplex can be observed as separate peaks or as a shoulder on the leading edge of homoduplex peaks or as broader than the wild-type DNA peak.

**Sequence analysis.** Samples with DHPLC elution profiles indicating the presence of heteroduplexes were analyzed in order to identify the sequence alteration. The fragments were reamplified from tumor DNA and purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany). Direct sequencing was performed by an automatic sequencer ABI/HITACHI 3100 genetic analyzer (PE Biosystem, Foster City, CA, USA) using the forward primer.

**Statistical analysis.** Significant differences in the numbers detected by DHPLC according to histologic subtypes of

meningiomas were tested by Chi-square test, and  $p < 0.05$  was used as the criterion of significance.

## Results

In order to detect mutations of the NF2 gene in 42 sporadic meningiomas, DHPLC was performed using sixteen primer pairs for constitutive exons 1-16. PCR was performed using multiple primer pairs. The integrity of the DNA was confirmed in each case by a successful amplification of the pseudogene for methylthioadenosine phosphorylase. DHPLC elution profile variants were observed in 21 DNA-exonic fragments, 19 of which showed two or three distinct peaks (exons 1, 2, 4, 5, 10, 11, and 12) and two displayed a shoulder on a homoduplex peak (exon 7). In contrast, their corresponding blood DNAs presented a wild-type elution profile for all exons, indicating that all the mutations in the present study were somatic. Both normal and variant DNA fragments were

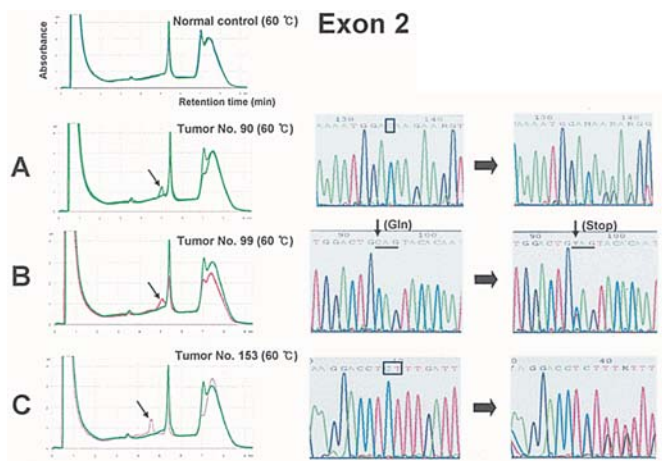


Figure 1. NF2 exon 2 mutation. A, NF2 exon 2 deletion mutation. DHPLC elution profile at the denaturing temperature (60°C, left) revealed two distinct peaks. Direct sequencing of the amplicon confirmed one bp deletion, resulting in a frameshift (right). B, NF2 exon 2 point mutation. The double-peak elution profile detected on DHPLC analysis at denaturing temperature (60°C, left) corresponded with the non-sense mutation found on direct DNA sequencing (right). C, NF2 exon 2 deletion mutation. DHPLC elution profile at the denaturing temperature (60°C, left) revealed two distinct peaks. Direct sequencing of the amplicon confirmed two-bp deletion, resulting in a frameshift (right).

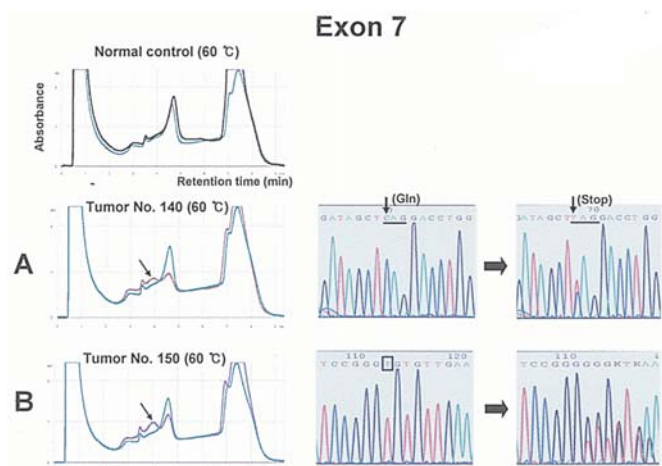


Figure 2. NF2 exon 7 mutation. A, NF2 exon 7 point mutation. The shoulder on a homoduplex peak elution profile detected on DHPLC analysis at denaturing temperature (60°C, left) corresponded with the non-sense mutation found on direct DNA sequencing (right). B, NF2 exon 7 deletion mutation. The shoulder on a homoduplex peak elution profile detected on DHPLC analysis at denaturing temperature (60°C, left) corresponded with the splice error found on direct DNA sequencing (right).

compared by direct sequence analysis in order to identify the precise base change involved. The results showed that all the amplicons with a DHPLC detected shift were due to DNA sequence variation.

Mutational screening of the NF2 gene of 42 sporadic meningiomas identified 21 different mutations in 20 individuals, including nine deletions (exons 1, 2, 5, 10, and 12), which resulted in a frameshift, four non-sense mutations (exons 1, 2, and 7), four splice errors (exons 4, 5, 7, and 12), two missense mutations (exon 5) and two silent mutations (exon 11) (Table II).

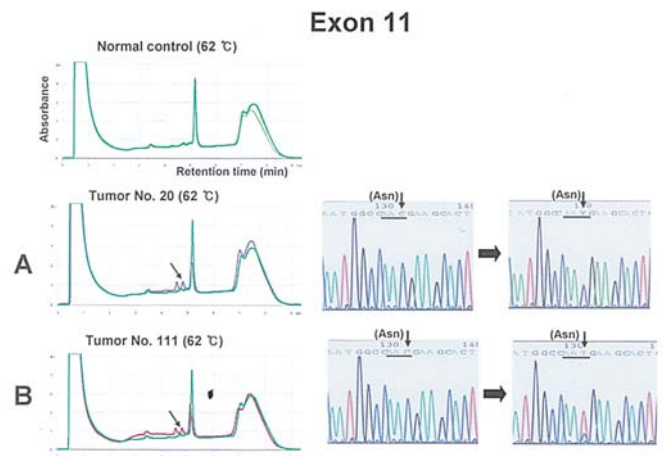


Figure 3. NF2 exon 11 mutation. A, NF2 exon 11 point mutation. The triple-peak elution profile detected on DHPLC analysis at denaturing temperature (62°C, left) corresponded with the silent mutation found on direct DNA sequencing (right). B, NF2 exon 11 point mutation. DHPLC elution profile at the denaturing temperature (62°C, left) revealed three distinct peaks. Direct sequencing of the amplicon confirmed one base substitution, resulting in a silent mutation (right).

The most frequently detected lesions were deletions (11 out of 21). Deletions of 1, 2, 4, and 11 bp were found in exons 1, 2, 5, 7, 10, and 12 of DNA from tumors no. 80, 90 (Fig. 1A), 153 (Fig. 1C), 118, 134, 150 (Fig. 2B), 119, 24, 111, 127, and 149. These mutations caused splice errors and frameshifts, leading to stop codon in the same exon or in the next one (Table II).

The next frequent lesions detected were base pair substitutions (10 out of 21), leading to stop codons at residues 38 of exon 1 (tumor no. 29), 32 of exon 1 (tumor no. 137), 65 of exon 2 (tumor no. 99; Fig. 1B) and 212 of exon 7 (tumor no. 140; Fig. 2A), or affecting the splice acceptor site consensus sequence in intron 3 and exon 12 (tumor no. 117 and tumor no. 146). Two tumors showed a base pair substitution not resulting in an amino-acid change (silent mutation) (tumor no. 20; Fig. 3A and tumor no. 111; Fig. 3B). The two remaining tumors with missense mutations showed T to A transition in exon 5, leading to Ser→Arg substitution at codon 156 (tumor no. 133) and A to T transition in exon 5, leading to Glu→Asp substitution at codon 167 (tumor no. 145), respectively. This study also detected two distinct mutations in tumor no. 111 (fibrous meningioma), a base pair substitution in exon 11 (silent mutation) and one bp deletion in exon 12 (frameshift mutation). All mutations were localized in the first 12 exons of the NF2 gene, the region of homology with the EMR protein, showing a more frequent occurrence of the frameshift mutations. The mutations are predicted in order to cause a premature translation termination and protein truncation, resulting in presumably inactive protein. Fourteen mutations were novel compared with Neurofibromatosis Research at Massachusetts General Hospital (<http://Neurosurgery.MGH.Harvard.Edu/NF2Clinic/NF2Research.htm>) (Table II).

As far as the relationship between the histological subtypes and NF2 gene mutations is concerned, NF2 mutations were more frequent in fibrous meningiomas (8/11, 73%) and transitional meningiomas (6/11, 55%) than in meningothelial variant (5/17, 29%), but not significant statistically ( $p=0.073$ ).



(Table II). These results provide evidence that mutations in the NF2 gene might play an important role in the development of sporadic meningiomas as well as implicating a different tumorigenesis of these meningioma variants.

## Discussion

The identification of mutations in the NF2 gene causing neurofibromatosis 2 has presented a considerable challenge because of the large size of the gene, the lack of significant mutational clustering, and the diversity of the underlying pathological lesion. DHPLC, a high throughput, non-hazardous and largely automated heteroduplex-based technique, is in many ways ideally suited to mutation detection in this condition (13-17). The present study is the second to apply DHPLC methodology to screen the NF2 gene mutations in the sporadic meningiomas. We detected NF2 mutations in 47.6% (20/42 cases) of these tumors. Previous to this study, Szijan *et al* (11) was the first to apply DHPLC methodology to the screening of the entire NF2 gene for mutations of sporadic meningiomas, reporting a detection rate of 38.5% (5 out of 13 meningiomas). The entire coding regions of the NF2 gene have been previously examined for mutations in 70 sporadic meningiomas, and 43 mutations (61.4%) was detected, using a single strand conformational polymorphism and DNA sequencing (9).

In our study, 21 NF2 gene mutations in 20 meningiomas were detected. Most frequently, we found deletions in the coding regions that, in nine of 42 tumors, resulted in an altered reading frame. Four tumors had non-sense mutations leading to stop codon and, in four tumors, there were splice site alterations. Two tumors had a missense mutation. All 21 mutations were seen in the first 12 exons of the NF2 gene. This region of merlin shows homology with the ERM protein and contains an  $\alpha$ -helix domain (4). Thirteen mutations were noted at the N-terminus of the protein 4.1 subfamily of ERM proteins, while eight mutations occurred in the  $\alpha$ -helix domain. Mutations in these regions would cause a premature translation termination and protein truncation, which could impair the function of merlin (18).

Fourteen novel mutations were identified in the present study. Of the nine frameshift mutations detected, eight were novel. The six remaining novel mutations consisted of three non-sense, two missense, and one splice error. These data showed that the prevalence of NF2 mutations of sporadic meningiomas in Korean patients is different from that in previous data (<http://Neurosurgery.MGH.Harvard.Edu/NFclinic/NFResearch.htm>). The high proportion of novel mutations in the present study may be due to a high level of mutational heterogeneity in the NF2 gene and racial difference.

Clear discrepancies in the incidence of NF2 gene mutations observed in meningiomas of different histopathological subtypes have been reported (9,19-22). Some authors report no difference in the mutation rate of the NF2 gene in meningo-thelial compared with non-meningothelial meningiomas (19-21), whereas others report rates that vary considerably between these tumor types (9,22). Recently, a wide variation in mutation frequency of the histologic subtypes of meningiomas has been found (23). It has been found that mutations of the NF2 gene occurred only in one (5%) of 20 meningo-thelial meningiomas and three (43%) of seven non-meningothelial

meningiomas. Our results indicate that mutations inactivating the NF2 gene were more frequent in fibrous meningiomas (8/11, 73%) and transitional meningiomas (6/11, 55%) than in meningo-thelial variant (5/17, 29%). When all the data reported here and elsewhere are taken into consideration, it is clear that different mechanisms of tumorigenesis, independent of the loss of function of the NF2 gene, are responsible for the development of meningo-thelial meningiomas, compared with other types of meningiomas. Further efforts are needed to investigate other molecular mechanisms of tumorigenesis in meningo-thelial meningioma.

In conclusion, our study shows that the NF2 gene is mutated in the majority of sporadic meningiomas, indicating that the NF2 gene plays a critical role in the pathogenesis of these tumors. The significant difference in the incidence of NF2 mutations between distinct histological variants of meningiomas strongly suggests that meningiomas arise through different molecular genetic pathways.

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