

Somatic Instability of the *NF2* Gene in Schwannomatosis

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Context: Schwannomatosis is a newly described form of neurofibromatosis of unclear pathogenesis.

Patient and Methods: We studied the *NF2* locus on chromosome 22 in 7 tumor specimens resected from a 36-year-old man with schwannomatosis of the right ulnar nerve.

Results: Unrelated truncating *NF2* gene mutations were detected in 4 tumor specimens. None of the *NF2* mutations were present in the blood specimen. Loss of heterozygosity at the *NF2* locus was seen in all tumors, and

in every case the same allele was lost. Loss of distal chromosome 22 markers was variable. Fluorescence in situ hybridization results were consistent with monosomy 22 in 4 tumors and mitotic recombination or nondisjunction in 1.

Conclusions: Molecular analysis of tumor specimens distinguishes schwannomatosis from other forms of neurofibromatosis. Further work is needed to understand the natural history and molecular biology of this condition.

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SCHWANNOMAS ARE benign tumors of peripheral myelin-producing cells most commonly seen as an isolated finding in otherwise healthy individuals. The occurrence of multiple schwannomas in the same individual suggests an underlying diagnosis of neurofibromatosis. Most such patients have bilateral vestibular schwannomas and thus neurofibromatosis 2 (NF2). Recently, a small number of patients with multiple pathologically proved schwannomas without vestibular tumors were shown to have a distinct clinical entity termed *schwannomatosis*.^{1,2} Most cases of schwannomatosis are isolated in patients without affected family members,² and one third of cases of schwannomatosis are characterized by localization of tumors to a single arm, leg, or segment of the spine.³ The *NF2* tumor suppressor is the primary tumor suppressor in sporadic and *NF2*-associated schwannomas, with both constitutional and somatic mutations in the latter condition.⁴ Patients with schwannomatosis, like those with NF2, frequently have *NF2* mutations in their tumors,¹ but the germline cause of schwannomatosis remains unknown. Analysis of multiple tumors in a single patient may be especially valuable in understanding the molecular

pathogenesis of this disorder, and we present a unique opportunity to do so.

REPORT OF A CASE

A 36-year-old man had a 12-year history of slowly growing painful tumors on the ulnar aspect of his right forearm and hand. Family history was negative for similar tumors or other manifestations of neurofibromatosis 1 or NF2. Physical examination results revealed several smooth nodular masses extending from the cubital tunnel to the proximal phalanx of the fifth finger. Strength was normal. Sensory deficit was noted in an ulnar distribution. Magnetic resonance imaging of the right arm revealed 8 well-circumscribed lesions surrounding the right ulnar nerve. Contrast material-enhanced cranial magnetic resonance images acquired with 3-mm sections through the internal auditory canals were normal. Eight tumors were surgically resected in 2 procedures. The patient's pain resolved after tumor resection, though minimal sensory deficits remained. Histological examination by one of us (R.A.S.) demonstrated typical features of schwannoma in each tumor. No clinical or pathological differences were seen among the 4 tumors in which

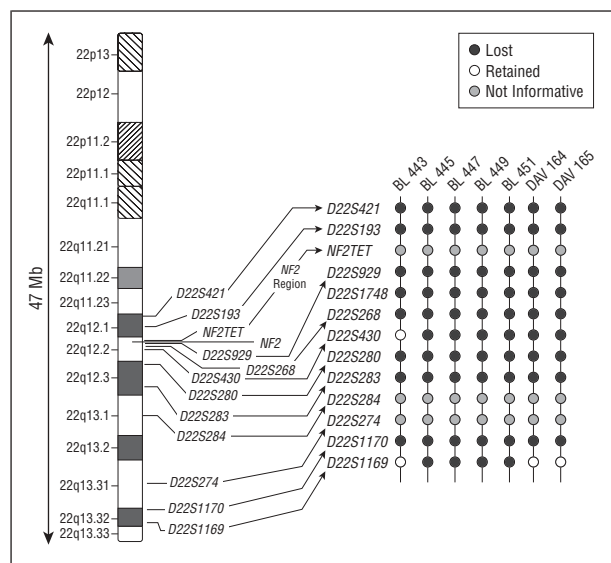
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Molecular Analysis of 7 Tumor Specimens

Tumor Identification No.	NF2 Region				FISH	
	Exon	Sequence*	Result	LOH	NF2	BCR
BL443	12	1340 + 1 G to A	Splice	Yes	Deleted	Deleted
BL445	2	240 + 2 T to C	Splice	Yes	Deleted	Deleted
BL447	None	Yes	Deleted	Deleted
BL449	3	331 C to T	Nonsense	Yes	Deleted	Deleted
BL451	None	Yes	Not deleted	Not deleted
DAV164	7	1-bp deletion (G) 616	Frameshift	Yes	NA	NA
DAV165	None	Yes	NA	NA

Abbreviations: bp, base pair; ellipses, not applicable; FISH, fluorescence in situ hybridization; LOH, loss of heterozygosity; NA, not available.

*Nucleotide positions are numbered according to the complementary DNA sequence with the first nucleotide of the ATG initiation codon as 1, using nomenclature previously described.¹



Loss of heterozygosity patterns in 7 tumors studied. Position of markers on chromosome 22 was determined by using the resources of the human genome browser (University of California, Santa Cruz Genome Bioinformatics, <http://genome.ucsc.edu/>). Mb indicates megabases.

we subsequently detected NF2 mutation, as compared with findings in the other 4 tumors.

METHODS

SPECIMEN COLLECTION AND DNA EXTRACTION

A lymphoblast line was established from a peripheral blood sample as previously described.⁴ Paraffin-embedded blocks of 5 of 6 tumors resected during the first procedure were retrieved from department archives. After pathological examination was completed from the second procedure, excess tissue from 2 anatomically separate tumors was collected and frozen on dry ice. High-molecular-weight DNA was extracted from peripheral blood leukocytes, frozen pulverized tumor tissue, and archived paraffin-embedded specimens as previously described.¹ The institutional review board of the Massachusetts General Hospital approved this study, and the study subject provided written informed consent.

MOLECULAR ANALYSIS OF THE NF2 LOCUS

Single-stranded conformational polymorphism (SSCP) analysis of the NF2 gene was performed as previously described^{1,4} in 7 tumor specimens. Single-stranded conformational polymorphism analysis and sequencing of genomic DNA from peripheral blood leukocytes was confined to exons 2, 3, 7, and 12 of the NF2 gene.

Loss of heterozygosity (LOH) in the NF2 region was determined using the proximal marker D22S193, 2 intragenic markers (NF2tet and D22S929), and 3 distal markers (D22S1748, D22S268, and D22S430). The distance from the most centromeric marker (D22S193) to the most telomeric (D22S430) was 2.4 megabases. Loss of heterozygosity was then determined at the pericentromeric marker D22S421 and at 6 markers spaced relatively evenly on 22q distal to the NF2 locus itself: D22S280, D22S283, D22S284, D22S274, D22S1170, and D22S1169. Alleles were amplified using primers and conditions available at the Genome Database (<http://gdbwww.gdb.org/>).

FLUORESCENCE IN SITU HYBRIDIZATION

Dual-color fluorescence in situ hybridization (FISH) was performed on formalin-fixed paraffin-embedded tumors obtained from the first procedure as previously described.⁵ Two probes were used—1 for the BCR region proximal to NF2 at 22q11.23 (Vysis Inc, Downers Grove, Ill) and 1 for the NF2 region at 22q12 (n3022 and n24f20; UK Human Genome Mapping Project Resource Centre, <http://www.hgmp.mrc.ac.uk/>). All specimens were counted twice by a single observer (A.P.) in a blinded fashion.

RESULTS

MOLECULAR ANALYSIS

Genomic DNA of adequate quality for mutational analysis was obtained from 5 of 6 paraffin-embedded tumors and both frozen specimens. Typical truncating mutations were found in 4 of the 7 tumors studied, but no mutations were seen at the level of SSCP or direct sequence analysis in the blood specimen (**Table**). Microsatellite analysis revealed LOH of the same allele in the NF2 region and the proximal marker D22S421 in all 7 tumors studied (**Figure**). Four of 7 tumors also lost heterozygosity at all markers telomeric to the NF2 region, while 3 retained a single marker at the tip of chromosome 22.

FISH ANALYSIS

Five of 6 tumors from the first procedure produced reproducible FISH analysis results. Four of the 5 tumors were deleted with both the *NF2* and *BCR* probes, which is consistent with monosomy of this region of chromosome 22. A single tumor revealed 2 hybridization signals at *NF2* and *BCR*. Since this tumor also showed LOH at all markers tested, this result is consistent with mitotic recombination or nondisjunction.

COMMENT

We present the case of a 36-year-old man with schwannomatosis confined to the right ulnar nerve and slowly enlarging painful masses that responded well to surgical resection. Molecular genetic analysis of his tumor specimens showed typical truncating mutations in the *NF2* gene in 4 tumors, which were not shared by other tumors or present in his blood. This pattern of somatic instability of the *NF2* gene contrasts sharply with that found in patients with *NF2* or sporadic tumors. Microsatellite analysis of the 7 tumors investigated demonstrated LOH of most chromosome 22 markers, which is consistent with loss of all or most of the long arm of chromosome 22. In addition, the lost allele was not random but consistent in each tumor, which implies that the somatic mutations accumulate on the same retained allele. Varying patterns of LOH in distal markers (loss in 4 tumors and retention in 3) excluded the possibility of segmental mosaicism for monosomy 22 or large interstitial deletion. The FISH analysis with 2 chromosome 22 probes also revealed heterogeneity, which is consistent with monosomy in 4 tumors and mitotic recombination or nondisjunction in 1.

Genetic inactivation of the *NF2* tumor suppressor is seen in nearly all schwannomas. For example, in our work we detected grossly truncating mutations in 62 (78%) of 80 sporadic schwannomas, with 2 mutations detected in 14 of the tumors.^{4,6} Frameshifting deletions were especially common, with more than half of these mutations involving the removal of 1 to 34 base pairs. Forty (51%) of 79 tumors showed LOH of markers around the *NF2* locus. At least 1 inactivating event (mutation or LOH) was seen in 72 (90%) of 80 tumors. Others have published similar results implicating *NF2* in most if not all schwannomas,⁷⁻¹⁰ and our immunohistochemical results implicate loss of the *NF2* protein product merlin, even in tumors lacking evidence of *NF2* gene inactivation.¹¹ Comparison of schwannomas in culture and with primary normal human Schwann cells reveals striking cytoskeletal differences between the 2.¹² Our work with sporadic, *NF2*-related, and schwannomatosis-related schwannomas has shown that these alterations in cellular architecture are reversed by transduction of merlin,¹³ which strongly supports a specific pathogenetic role of the mutations seen at the *NF2* locus in schwannoma development.

Several cases of schwannomatosis have been presented in the literature, and debate has been raised regarding its distinction from the more common condition of *NF2*.¹⁴⁻¹⁶ Recent epidemiological results,² along

with our own observations,^{1,3} suggest that when vestibular schwannomas are carefully excluded, these diseases can be definitively distinguished from one another. To date, the causative genetic change of schwannomatosis has not been identified, but results of this study and others have shown a unique pattern of acquired mutation, with LOH as a common "second hit." This pattern of tumor-suppressor gene inactivation is fundamentally different from any other reported in human disease. In approximately one third of cases of schwannomatosis, tumors are confined to a single anatomical location, as in this patient, which might suggest mosaicism or noncontiguous spread of a single tumor. We were able to exclude mosaicism for *NF2* locus change and noncontiguous spread of a single clonal tumor as possible pathogenetic mechanisms in this patient. Because previous work strongly supports merlin as the tumor suppressor for schwannoma,¹³ and because this patient's phenotype is limited to a single nonmalignant tumor type, we think it unlikely that he has a widespread tendency to accumulate mutation in multiple loci.

Loss of heterozygosity was universal in the tumors in this patient, and rates of LOH were also high in our previous study of schwannomatosis.¹ Although some researchers have published study results on *patterns* of LOH, less is known about the *mechanisms* leading to LOH in any specific tumor type. In neurofibromatosis 1, deletion or monosomy appears to account for most LOH in plexiform and malignant tumors,¹⁷ but mitotic recombination was found in a small number of benign neurofibromas.¹⁸ There is limited evidence that monosomy for chromosome 22 leads to most LOH in sporadic and *NF2*-related schwannomas,^{7,19} but to our knowledge, this issue has not previously been addressed in schwannomatosis-derived tumors. Our results indicate that monosomy may be a common but not universal mechanism of LOH in sporadic schwannomatosis, but additional comparative studies are needed to address this issue. Our current work is focused on addressing this and other molecular aspects of this disorder.

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