

## Frequency of the Loss of Heterozygosity of the *NF2* Gene in Sporadic Spinal Schwannomas

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**Abstract.** *Background/Aim:* Individuals with type 2 Neurofibromatosis are predisposed for the appearance of schwannomas. In the present study we analyzed the loss of heterozygosity and mutations in the *NF2* gene in patients with sporadic Schwannoma without Neurofibromatosis type 2. *Materials and Methods:* We analyzed 39 patients with sporadic spinal schwannoma. We quantified the number of alleles by FISH and sequenced the *NF2* gene. *Results:* We identified 16/39 patients with point mutations and/or LOHs in the tumor samples analyzed. The LOHs were found in 7/39 patients. Two homozygous mutations were detected in 4/39 tumors, and the presence of the mutation in heterozygosity was revealed in 3/39 patients. In two tumors, we detected the loss of one allele of the *NF2* gene, with no mutation. *Conclusion:* The genetic alterations observed in the *NF2* gene indicated that spinal schwannomas are associated with genetic alterations also found in other schwannomas and type 2 Neurofibromatosis, which reinforces the etiological role of this gene.

Schwannomas are benign, slow-growing tumors, which are typically encapsulated, and originate in the sheath of myelinated nerves (1). The estimated incidence of spinal

schwannomas is 0.3-0.4 cases/100,000 individuals per year, peaking at ages between 30 and 60 years (mean age approximately 47 years), with no predilection for either sex (2-4).

Spinal schwannomas are classified as intramedullar (less than 1%), intradural extramedullar (70-75%), extradural (15%), and intradural-extradural (dumbbell-shaped), with a frequency of 15% (5, 6). Schwannomas are generally found as single lesions, although they may be multiple (schwannomatosis) or associated with type 2 Neurofibromatosis, or *NF2* (7). When associated with type 2 Neurofibromatosis, schwannomas tend to be more aggressive, with distinct and precocious clinical manifestations (6).

A number of studies have shown that sporadic schwannomas may be caused by alterations of the *NF2* gene and/or the loss of heterozygosity (LOH) in this gene (8, 9). The *NF2* gene is located on chromosome 22q12 (10, 11), is a tumor suppressor gene of 110 kb and composed of 16 constitutive exons and an alternative splicing exon (12). The inactivation of this gene is associated with the formation of tumors (13). The product of the *NF2* gene is known as the Merlin or schwannomin protein, a member of the ezrin-radixin-moesin (ERM) protein family, which is involved in the connection of the cytoskeleton to the cellular membrane (14-16).

In the present study, in order to analyze the loss of heterozygosity (LOH) in the *NF2* gene in patients diagnosed with sporadic spinal schwannoma, we sequenced the *NF2* gene and quantified the number of its alleles by fluorescent *in situ* hybridization (FISH), as well as investigating the possible correlation between the molecular defects of the *NF2* gene and the phenotype of the disease in the affected patients.

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**Key Words:** Mutation, LOH, spinal schwannoma, FISH.

## Materials and Methods

**Study population, diagnosis and clinical manifestations.** The present study included 39 patients of both sexes attended by the neurosurgical services of the hospitals of northern Brazil, diagnosed with spinal schwannomas between January 1990 and December 2013. The study was approved by the Ethics Committee of the Ophir Loyola Hospital in Belém (Pará), northern Brazil (approval number 593.717-0) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

The epidemiological and clinical characteristics of the patients were obtained through written forms and the analysis of medical records. Patients diagnosed with type 2 Neurofibromatosis or with symptoms of Schwannomatosis were excluded from the analysis.

All the lesions considered in the present study were diagnosed as spinal schwannomas by two independent neuropathologists, and were located in the vertebral-medullary segment, below the foramen magnum. In addition to the initial histopathological diagnosis, an immunohistochemical diagnosis was also run, in which the tumor cells were strongly positive for the S-100 protein, but negative for SMA, CD34 and CD117.

In the histopathological analysis, the schwannomas were classified as (i) classic, (ii) cellular, (iii) plexiform, and (iv) melanotic. The Antoni A and B patterns were also evaluated and associated with the intraoperative morphology of the lesions.

The morphological characteristics of the tumors were initially evaluated by the nuclear magnetic resonance, with and without contrast, of the affected segment during pre-operative examinations. The patients were classified according to their compartmental morphology, and the affected segment. The lesions were measured and the degree of bone damage was evaluated by tomography with contrast and the reconstruction of the bony window. Following the injection of the contrast medium, the lesions were classified as either solid lesions with homogeneous contrast, solid lesions with heterogeneous or irregular contrast, and heterogeneous multi-cystic lesions.

**Tumor samples.** Total genomic DNA was extracted from paraffin-embedded tumor specimens using a JetFlex Genomic DNA Purification kit (Invitrogen, CA, USA) according to the manufacturer's protocol. Samples of the DNA of the tumors were amplified for all 17 exons of the *NF2* gene. The primers used were those described by Merel *et al.* (1995) (17) and Mohyuddin *et al.*, (2002) (18). The amplified products were sequenced in an ABI 3730 automatic sequencer (Applied Biosystems, Foster City, CA, USA) using BigDyeTM Terminator v3.1 Cycle sequencing kits (Applied Biosystems, Foster City, CA, USA). The sequences were analyzed using the SeqScape software (Applied Biosystems, Foster City, CA, USA).

Biopsies of the sural nerve were used as controls for the genetic analysis. These biopsies were conducted in specimens of lower members amputated due to non-tumoral pathologies.

**Molecular cytogenetics.** Interphase fluorescence *in situ* hybridization (I-FISH) was performed using a two-color FISH approach. The DNA derived from the BAC-probe RP11-551L12, which is specific to the *NF2* gene (22q12.2), was labeled with Texas Red, while the BCR Spectrum Green Probe located at 22q11.2 (Abbott Laboratories, Illinois, USA) served as an internal control. The FISH procedure was conducted according to the standard protocols (19, 20). For microscopic evaluation, 200 interphase nuclei were examined in each specimen.

**Statistical analysis.** The data were analyzed using the Chi-square test to evaluate the possible associations among different variables. A  $p < 0.05$  significance level was considered for all analyses.

## Results

The mean age of the patients at diagnosis was 48.82 years, and the mean duration of symptoms was 14.05 months. The 39 patients analyzed in this study included 18 women and 21 men. In all cases, the tumors had been resected completely, and no recurrence of tumoral growth had been observed within a five-year period. During this period, all patients (100%) survived. The clinical details of the patients are provided in Table I.

None of the patients that participated in the present study had any family history of schwannoma or any other type of tumor. Point mutations were identified in the coding sequence of the *NF2* gene in the tumors of a number of the 39 patients analyzed in the present study (Table II).

Point mutations and/or the loss of alleles were identified in 16 (41.03%) of the 39 samples. Cases of LOH resulting from the deletion of one allele and a mutation of the other were recorded in 7/39 (17.94%) of the tumors. Two homozygous mutations (with the presence of the two equal alleles) were detected in 4/39 tumors (10.25%), while heterozygous mutations were found in three (7.69%) cases. The loss of an allele of the *NF2* gene with no mutation was detected in two cases (5.12%). In 23 of the schwannomas (58.97%), no alteration of the *NF2* gene was observed.

Examples of mutations and the results of the I-FISH are shown in Figure 1. We considered the *NF2* allele to have been deleted when 25% or more of the cells in the FISH presented a fluorescent signal. In all the nine cases in which the loss of one allele of the *NF2* gene was detected, the deletion of this gene was the most frequent mechanism detected.

No significant associations ( $p > 0.05$ ) were found in any of the clinical, pathological or genetic variables analyzed in the present study.

## Discussion

Schwannomas are benign tumors that develop in the Schwann cells, which form the myelin sheath that envelopes the peripheral nervous system (21). Alterations of the *NF2* gene have been observed in 60% of the sporadic schwannomas analyzed, indicating an association between the gene and the tumorigenesis of the disease (22, 23). Individuals with Type 2 Neurofibromatosis (NF2) are predisposed to the appearance of schwannomas and other tumors of the nervous system, such as meningiomas (24, 25).

None of the patients analyzed in the present study had any recurrence of tumor growth within a period of five years, probably due to the complete resection of the schwannoma. All the patients also survived this period, given that the prognosis for spinal schwannomas is excellent. The complete

Table I. *The general characteristics of all 39 cases of spinal schwannoma.*

Case	Gender (age)	Neurological manifestations	Location of the schwannomas in the spinal column	Location of the lesion	Associated morbid conditions	Antoni pattern
1	Female (20)	Topical pain/radicular pain (extremity)	Sacral	Intradural-Extramedullary	Thyroid dysfunction	A and B
2	Female (22)	Urinary incontinence/loss of feeling/neurogenic claudication/radicular pain (extremity)	Lumbar	Extradural	Coronary arterial disease	A and B
3	Female (33)	Motor deficit/myelopathy/radicular pain (extremity)/topical pain	Lumbar	Intradural-Extramedullary	Thyroid dysfunction	A and B
4	Female (37)	Urinary incontinence/neurogenic claudication/topical pain	Cervical	Extradural	Coronary arterial disease	A and B
5	Female (39)	Motor deficit/myelopathy/radicular pain (extremity)	Lumbar	Intradural-Extramedullary	Previous surgery	A and B
6	Female (41)	Chest pain/loss of feeling/myelopathy/topical pain	Cervical	Extradural	Smoker	B
7	Female (50)	Motor deficit/myelopathy/radicular pain (extremity)/topical pain	Sacral	Extradural	Diabetes Mellitus	A and B
8	Female (52)	Unstable walk/myelopathy/radicular pain (extremity)	Thoracic	Intradural-Extramedullary	Smoker	A and B
9	Female (56)	Myoclonus/loss of feeling/myelopathy/radicular pain (extremity)	Lumbar	Extradural	Hypertension	A
10	Female (59)	Headache/loss of feeling/radicular pain (extremity)/topical pain	Lumbar	Extradural	Diabetes Mellitus, Stroke	B
11	Female (66)	Urinary incontinence/neurogenic claudication/radicular pain (extremity)	Thoracic	Intradural-Extramedullary	Smoker, Hypertension	A and B
12	Female (72)	Motor deficit/myelopathy/radicular pain (extremity)	Lumbar	Extradural	Hypertension	A
13	Female (75)	Unstable walk/myelopathy/radicular pain (extremity)	Lumbar	Intradural-Extramedullary	Hypertension, Diabetes Mellitus	A and B
14	Female (66)	Motor deficit/loss of feeling/myelopathy/topical pain	Cervical	Intradural-Extramedullary	Smoker, Hypertension	A
15	Female (32)	Neurogenic claudication/myelopathy/radicular pain (extremity)	Lumbar	Extradural	Previous surgery	A and B
16	Female (68)	Neurogenic claudication/Motor deficit/radicular pain (extremity)	Thoracic	Extradural	Hypertension	A and B
17	Female (50)	Motor deficit/loss of feeling/myelopathy/topical pain	Thoracic	Extradural	Previous surgery	A and B
18	Female (51)	Motor deficit/loss of feeling/myelopathy/topical pain	Lumbar	Intradural-Extramedullary	Multiple sclerosis	A
19	Male (26)	Motor deficit/loss of feeling/myelopathy/radicular pain (extremity)	Cervical	Extradural	Previous surgery	A and B
20	Male (28)	Loss of feeling/radicular pain (extremity)/topical pain	Lumbar	Intradural-Extramedullary	Previous surgery	B
21	Male (29)	Loss of feeling/radicular pain (extremity)/topical pain	Cervical	Intradural-Extramedullary	Previous surgery	A and B
22	Male (34)	Neurogenic claudication/myelopathy/topical pain	Lumbar	Intradural-Extramedullary	Smoker	A
23	Male (35)	Motor deficit/myelopathy/topical pain	Thoracic	Extraintradural	Smoker, thyroid dysfunction	A
24	Male (37)	Motor deficit/myelopathy/radicular pain (extremity)/topical pain	Thoracic	Extradural	Smoker	A and B
25	Male (38)	Loss of feeling/myelopathy/topical pain	Lumbar	Extradural	Thyroid dysfunction	A and B
26	Male (58)	Swelling of the neck/Motor deficit/myelopathy/topical pain	Lumbar	Intradural-Extramedullary	Previous surgery	A and B
27	Male (4)	Motor deficit/myelopathy/topical pain	Thoracic	Extraintradural	Smoker	A
28	Male (5)	Headache/ unstable walk	Cervical	Extradural	Previous surgery	B
29	Male (11)	Motor deficit/myelopathy/topical pain	Cervical	Intradural-Extramedullary	Smoker	A and B
30	Male (20)	Chest pain/radicular pain (extremity)	Lumbar	Intradural-Extramedullary	Coronary arterial disease	A
31	Male (7)	Motor deficit/myelopathy/topical pain	Lumbar	Intradural-Extramedullary	Smoker	A and B
32	Male (16)	Urinary incontinence/neurogenic claudication/topical pain	Thoracic	Extradural	Smoker	A
33	Male (21)	Myoclonus/radicular pain (extremity)/topical pain	Cervical	Intradural-Extramedullary	Hypertension	A
34	Male (7)	Headache/unstable walk/myelopathy/topical pain	Lumbar	Extradural	Diabetes Mellitus	A and B
35	Male (39)	Loss of feeling/radicular pain (extremity)/topical pain	Thoracic	Intradural-Extramedullary	Smoker, Hypertension	B
36	Male (34)	Loss of feeling/radicular pain (extremity)/topical pain	Sacral	Intradural-Extramedullary	Hypertension, Diabetes Mellitus	A
37	Male (14)	Urinary incontinence/neurogenic claudication/topical pain	Cervical	Intradural-Extramedullary	Smoker, Hypertension	A and B
38	Male (2)	Myelopathy/radicular pain (extremity)/topical pain	Lumbar	Intradural-Extramedullary	Smoker	A
39	Male (9)	Neurogenic claudication/loss of feeling	Thoracic	Intradural-Extramedullary	Smoker	A and B

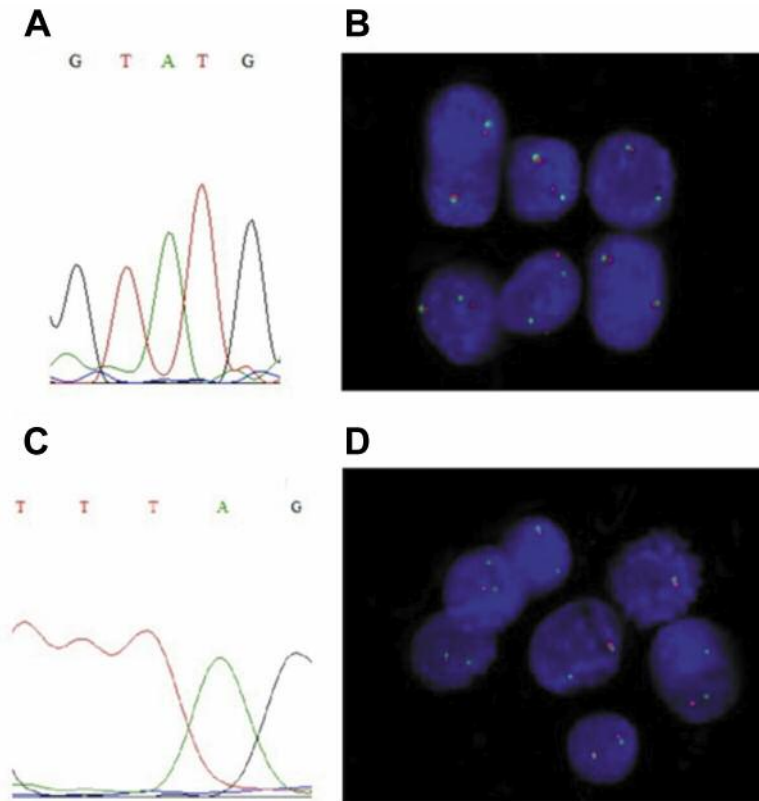


Figure 1. Mutations and LOHs in two of the patients with spinal schwannomas analyzed in the present study. A and B: The missense mutation p.V219M in a homozygous condition, with the presence of two alleles of the NF2 gene (patient 12 in Table I). C and D: The nonsense mutation 955 C-T and the deletion of an allele of the NF2 gene (patient 31 in Table I).

Table II. Point mutations of the NF2 gene identified in the 39 study patients with spinal schwannoma.

Patient	Sequence alteration <sup>a</sup>	Codon Change <sup>b</sup>	Consequence	Type of mutation	Number of alleles
1	c.784 C-T (exon 8)	p.R262X	Nonsense	Homozygous	2
6	c.1021 C-T (exon 11)	p.R341X	Nonsense	Homozygous	2
8	c.136 T-G (exon 2)	p.L46R	Missense	Homozygous	2
10	c.655G-A (exon 7)	p.V219M	Missense	Homozygous	2
12	c.655G-A (exon 7)	p.V219M	Missense	LOH	1
14	c.148T-C (exon 2)	p.C50R	Missense	LOH	1
18	c.1079 T-C (exon 11)	p.L360P	Missense	LOH	1
23	c.397T-C (exon 4)	p.C133R	Missense	LOH	1
25	c.122 G-A (exon 2)	p.W41X	Nonsense	LOH	1
28	c.169 C-T (exon 2)	p.R57X	Nonsense	LOH	1
29	c.133 C-G (exon 12)	p.S444X	Nonsense	LOH	1
31	c.955 C-T (exon 10)	p.Q319X	Nonsense	Heterozygous	2
32	c.934 A-T (exon 10)	p.Lys312X	Nonsense	Heterozygous	2
34	c.934 A-T (exon 10)	p.Lys312X	Nonsense	Heterozygous	2
36	No alteration	-	-	No mutation	1
39	No alteration	-	-	No mutation	1

All the mutations had a somatic origin. <sup>a</sup>The numbering of the bases indicating the alteration is given relative to the cDNA sequence, with the initiator ATG beginning at base 1; <sup>b</sup>The original amino acid and position of the residues in the protein (with the initiator Met numbered as 1) are followed by an "X" in the case of the nonsense mutations.

resection rate is 85-90% in modern cases, and recurrence is extremely uncommon after complete resection (26, 6). The survival of patients with spinal schwannomas is similar to that of the general population (26).

To our knowledge, this is the first South American study to analyze the *NF2* gene in patients with spinal schwannomas without type 2 Neurofibromatosis. All the mutations identified in the present study, except for Gln319X, which has been found in a patient with spinal schwannoma (27), are reported here for the first time in relation to spinal schwannoma. These mutations have nevertheless been described previously in patients with type 2 Neurofibromatosis, Schwannomatosis, and vestibular schwannomas.

Twelve types of mutations were identified in the present study, of which, seven (p.Arg262X; p.Arg341X; p.Arg57X; p.Gln319X; Trp41X; p.Val219Met; p.Leu360Prol) have been recorded in the database of the *NF2* gene (<https://databases.lovd.nl/shared/variants/NF2/unique>; <http://www.hgmd.cf.ac.uk/nf2/>). The other five mutations have only been described in patients with Schwannomatosis, and sporadic vestibular schwannomas (p.L312X; p.L46R, p.W41X, p.S44X and p.Q319X (28, 29).

The functional inactivation of a tumor suppressor gene requires two genetic hits in the same gene and cell (30). In the present study, seven patients (17.94%) presented point mutations and LOHs, which represent a second genetic hit (29). In this case, when two mutations or one mutation together with the loss of an allele, are found in the tumor, one of them may be constitutional, and their descendants should be tested for possible abnormalities (31).

In all the cases in which the *NF2* gene was lost, the most frequent mechanism was deletion. Few cells presented monosomy of chromosome 22. This is different from meningiomas, which may present deletions or monosomy in isolation in different samples of the tumor (20, 32). We know of no other studies in which a two-color FISH was used to analyze sporadic schwannomas.

The Val219Met and Lys312X mutations were the most common in the present study, each being recorded in two patients. Even so, Lys312X was found in heterozygosity, together with the mutations Q319X and as the *NF2* gene is a tumor suppressor, based on the hypothesis of Knudson (30), these mutations would not have an effect on these patients.

The Val219Met mutation was homozygous in one patient, and in the other, it was recorded together with the loss of the second allele, which was identified as a LOH. This type of alteration has been observed in schwannomas in patients with Type 2 Neurofibromatosis (18, 33). The valine at position 219 affects the normal configuration of the helicoidal alpha domain of the Merlin protein (34, 18).

The nonsense mutations Arg341X, Arg262X and Arg57X, which were each found in a single patient in the present study, have been described in vestibular schwannomas in

individuals with Type 2 Neurofibromatosis (33, 35). The Arg262X mutation has also been recorded in a patient with schwannomatosis (27). These modifications, together with the Gln319X mutation, provoke the formation of a premature stop codon, resulting in the truncation of the Merlin protein, which may be defective (36).

Other mechanisms, such as mutations in other genes (SMARCB1; LZTR1) (37) and/or epigenetic factors may be involved in the parthenogenesis of schwannomas (38). It is also possible that other sequences of the *NF2* gene not analyzed in the present study are involved in the etiology and development of the disease. Previous studies have shown that 20-40% of sporadic schwannomas are activated by the methylation of the *NF2* gene (39, 40), which may account for the fact that some of the tumors analyzed did not have either identifiable point mutations or LOHs (31).

The present study revealed that the etiology of development of schwannomas involves genetic alterations of the *NF2* gene and/or other mechanisms that are still not fully understood. The genetic alterations observed in the *NF2* gene indicated that spinal schwannomas are associated with genetic alterations also found in other schwannomas and type 2 Neurofibromatosis, which reinforces the etiological role of this gene.

## Conflicts of Interest

The Authors declare that they have no conflicts of interest.

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