# Genetic Diagnosis of Neurofibromatosis type 2


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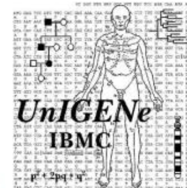



**Mestrado Integrado em Medicina | ICBAS-UP**

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# Genetic Diagnosis of Neurofibromatosis type 2

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**Abstract**

**Introduction** Neurofibromatosis type 2 is an autosomal dominant disease caused by mutations in the *NF2* gene on 22q12.2. Its protein product, merlin, supposedly plays an important role in connecting membrane proteins with the cytoskeleton by coordinating growth-factor signalling. The most common mutations are truncating and splice site mutations, showing a genotype-phenotype correlation. A high rate of somatic mosaicism may account for the difficulty of molecular genetic diagnosis. The main clinical feature is bilateral vestibular schwannomas and multiple neural tumours. Furthermore patients present tinnitus, hearing loss and dysequilibrium, subcapsular cataracts and cutaneous manifestations. The management of the disease includes surgical removal and radiological staging.

**Objectives** It was intended to develop a genetic testing protocol for patients with clinical criteria for this condition comprising sequence analysis and exon dosage study, allowing the observation of genotype-phenotype correlations.

**Methodology** Primers were designed to cover the entire coding region, flanking intronic sequences and 5' and 3' UTR. The obtained sequences were aligned with the reference sequence and checked against SNPs and documented mutations. Multiplex Ligation-dependent Probe Amplification was also performed covering the 17 exons and the gene promoter. Two newly diagnosed cases and one individual with an identified mutation along with his mother were studied.

**Results** It was possible to validate our mutation screening procedure through the detection of a documented mutation in exon 11, although his mother had no identifiable mutations. In a patient with bilateral schwannomas was detected a novel mutation, being a nonsense frameshift insertion in exon 5. In another patient with several neural tumours it was impossible to identify any mutation.

**Conclusions** The main purpose of this study was fulfilled despite the impossibility of drawing conclusions on genotype-phenotype correlation due to sample shortage. The importance of genetic testing in both lymphocyte and tumour DNA with sequencing and Multiplex Ligation-dependent Probe Amplification should not to be neglected.

**Keywords** NF2, VS, merlin, DNA sequencing, MPLA, truncating mutation, mosaicism, genotype-phenotype correlation

## Introduction

Neurofibromatosis type 2 (NF2) is a monogenic disorder whose most frequent manifestation is the vestibular schwannoma (VS), commonly referred to as acoustic neuroma, which is often bilateral.[1] NF2 is an autosomal dominant disease characterized by the development of multiple schwannomas and meningiomas, as opposed to neurofibromatosis type 1 (NF1) whose multiple tumours have a preponderant connective tissue component despite their neural origin, hence its mixed designation.[2] NF2 is mainly constitutional, since it is very unlikely to develop sporadic bilateral VS.

The clinical manifestations are usually related to the compressive nature of the intracranial tumours, including tinnitus, progressive hearing loss and, later, balance dysfunction but generally sparing the facial nerve. The average onset is in the third decade although the diagnosis might take place later due to the low specificity of the early symptoms. Along with the direct involvement of the nervous system many individuals develop posterior subcapsular cataracts leading to a decrease in visual acuity. Even though the cutaneous features in NF2 are much more subtle that in NF1, more than two thirds of the patients present plaque-like pigmented intra-cutaneous lesions as well as nodular subcutaneous tumours.[3] Many cases of a mononeuropathic onset in childhood with facial palsy and involvement of other cranial and peripheral nerves have been recently reported.[4] In order to allow an earlier diagnosis of the disease, improving its sensitivity without compromising its specificity, the diagnostic criteria have been modified according to the table I.[5]

| Initial criteria | Additional criteria |
|---|---|
| • Bilateral VS<br>• First-degree relative with NF2 **and**<br>  ○ Unilateral VS **or**<br>  ○ Any two individual manifestations of: meningioma, schwannoma, glioma, neurofibroma, posterior subcapsular lenticular opacities | • Uniteral VS **and** any two individual manifestations of: meningioma, schwannoma, glioma, neurofibroma, posterior subcapsular lenticular opacities<br>• Multiple meningiomas (two or more) **and**<br>  ○ Unilateral VS **or**<br>  ○ Any two individual manifestations of: schwannoma, glioma, neurofibroma, posterior subcapsular lenticular cataract |

**Table I**

NF2 is an autosomal dominant disease caused by mutations in the *NF2* gene on 22q12.2. Its consensus sequence spans 95.04 kilobases comprising 16 exons. There is also a further 17$^{th}$ alternatively spliced exon. So far no mutations were detected in this exon and the translated protein domain was not implied in tumourigenesis.[6] The protein product of *NF2* belongs to the 4.1 protein family and was named merlin (moezin-ezrin-radixin-like protein) according to the high homology with the proteins of this family which were described to play a critical role in connecting integral membrane proteins and the actin cytoskeleton. It has been suggested that merlin might coordinate the response to growth-factor receptor signalling, hence having a tumour suppressor activity.[7]

There have been a number of mutations associated with the NF2 phenotype, the vast majority point mutations, in particular the transition C>T in CGA codons leading to a premature stop codon. Furthermore it was observed a correlation between type of mutation and phenotype.[5] The most common mutations are truncating (approximately 90%), either nonsense or frameshift, and generally associated with severe phenotype, regardless of location in the gene. Splice site mutations cause intermediate phenotypes, being less severe as closer to 5' end. Missense mutations and large deletions occur in up to 10% of the cases and are associated with milder disease.[8,9] Chromosomal abnormalities, such as chromosome 22 ring, balanced translocations or cytogenetically detectable deletions, are particularly rare and usually present a more complex phenotype including mental retardation.[10,11] One of the particularities of this disease is the high prevalence of somatic mosaicism also related to a milder course of the disease and a lower probability of transmission to the next generation. The difficulty of the genetic diagnosis in peripheral blood has been attributed to this fact, with the highest success rates never exceeding 75% in confirmed NF2 patients.[12] This has also been pointed as the main reason for the difficulty in establishing values for incidence and prevalence, being the latest estimations respectively 1:25,000 and 1:80,000. This phenomenon may also be related to a supposedly observed anticipation due to the later age of onset in the parent with mosaicism, even though the disease has complete penetrance.[13]

The management of this disease eventually includes the surgical removal of the tumours with radiological guidance. However the outcome is generally less than good, with frequent complete hearing loss and other complications.[14] A medical approach has been proven ineffective to this point. The prognosis is worse in patients with early onset, a high number of meningiomas and molecular diagnosis of a truncating mutation, being the life expectancy particularly low.[15]

It was intended with this study to develop a genetic testing protocol in NF2 patients with already mentioned clinical criteria. The objectives included the optimization of the sequence analysis and exon dosage study, therefore providing the means for observation of correlations between genotype and phenotype.

| Exon | Forward primer | Reverse primer | Length |
|---|---|---|---|
| **1 (5’ UTR)** | 5’CTAGTCGGCCGCTGAGAG3’ | 5’GCCCGTCTCTAGCTACACC3’ | 572 bp |
| **2** | 5’TTTGGAACCTGAGAGTGGAGA3’ | 5’TGCCCCCAGTGATGAGCTAGGC3’ | 303 bp |
| **3** | 5’CAAAGGCTTCTTTGAGGGTAG3’ | 5’ATACAAAGGTGTTTGTGGTCAACT3’ | 292 bp |
| **4** | 5’CATCTGTTGTGATCAGCCTACAC3’ | 5’GTACAGAAAACCCAAAGAAAGAGA3’ | 248 bp |
| **5** | 5’ATGAGATTGGTCCAGCTCTGT3’ | 5’TATTCCTTCAAGTCCTTTGGTTAG3’ | 231 bp |
| **6** | 5’TCTCTCTGTGTGACTATCTCCCTG3’ | 5’ACTGAAGACCAACGTTACTCCC 3’ | 394 bp |
| **7** | 5’GTCAGAATGCTTGATTTGGTG3’ | 5’CTTGGAATGAAGAAGTAACCATTT3’ | 248 bp |
| **8** | 5’ATAAGACAGCTGTGACTTCTGTTG3’ | 5’CTGGGAATTAAAGGACTCTATGAA3’ | 389 bp |
| **9** | 5’GTTGCGCATTTGTGGAATTT3’ | 5’CAAGATGTCACTCTGATATCCTTTA3’ | 247 bp |
| **10** | 5’TGTTTCACTCTATGCATTCATCTT3’ | 5’CCAAAAGGTATAAAACAAGAGCAG3’ | 379 bp |
| **11** | 5’ACTGGAGATGGGGCATCTTT3’ | 5’CTAACTCATGGTTTTCAGGAGAC3’ | 299 bp |
| **12** | 5’TGTCCCATCTCAGTGTTCAAG3’ | 5’AGCTTGAGGACAACTGCTGTA3’ | 399 bp |
| **13** | 5’ACTGTCCTTTTTCACCTCTTTG3’ | 5’ATGTTCTACCCAGTCCCTAACTG3’ | 402 bp |
| **14** | 5’GGTTGTCAACACAGTAGTGTCC3’ | 5’GTTCAGAATGAATGCTGATCTGT3’ | 400 bp |
| **15** | 5’GCCAAGTAGAGACGTGAACC3’ | 5’AGTCTAGTGACTTGGCTACTGAGG3’ | 392 bp |
| **16 (3’ UTR)** | 5’CAACTTCTTGAGCATCTATTTGAA3’ | 5’GAGTCAGGAATCCCACATTAGAC3’ | 470 bp |

**Table II**

**Materials and Methods**

According to the literature the most effective method of diagnosing NF2 has been sequence analysis with a success rate of 75% in comparison to deletion or duplication analysis or cytogenetic testing, which have not reached more than 15% of positive results.[12] Although some authors consider scanning the first 15 exons sufficient for this purpose due to the lack of evidence of significant mutations outside of this range, it was decided to make a broader sequence analysis in this study, including all the 16 exons and the neighbouring 5’ and 3’ UTRs. This takes into account the previous detection of mutations along the whole gene and also in segments of 5’ and 3’ UTR which were immediately adjacent to codifying regions.[5] Genomic DNA was extracted from lymphocytes using salting-out methods. Large rearrangements (deletions or duplications) were searched by Mutiplex Ligation-dependant Probe Amplification (MLPA) analysis, using Probemix P044, and the instructions provided by the manufacturer (MRC Holland). It was initially intended to gather a sample of patients who had been followed by the neurosurgical service from Hospital de Santo António - Centro Hospitalar do Porto. It was however impossible to obtain blood samples from those patients due to time constraints, thus having been tested four individuals who had been referred to Centro de Genética Preditiva e Preventiva (CGPP) from IBMC-UP. There was a first case of a middle-aged female with three diagnosed meningiomas in addition to a schwannoma, a second case of a young male with bilateral schwannomas and a third case of a family. In this family the son had already a confirmed molecular diagnosis of NF2 being used for the validation of our screening procedure. His mother was also specifically tested for the same mutation given that she possibly presented a schwannoma.

*Primer design*

The primers were designed in order to cover the entire coding region, flanking intronic sequences (approximately 60 bp) to include the splice sites and approximately 350 bp of the 5’ UTR and 250 bp of the 3’ UTR regions. To the primers listed on table II it was added a common M13 tail sequence outwards of the amplicon in order to match the primers used in DNA sequencing. Primers were designed using Primer3Plus software. Optimal melting temperature was 58.0°C (± 3°C). Concentrations of monovalent cations and of annealing oligos were respectively 50.0 mM and 50.0 nM, and the GC content ranged from 30% to 80%, optimally 50%. Primers were tested for specificity, presence of documented SNPs and complimentary regions.

*DNA Amplification*

PCR was performed in a total volume of 12.5 µL using 30 ng of genomic DNA, 1.2 µM of each primer and a final dilution of HotStar Master Mix 1:2. PCR amplification was performed on a Biometra T-Gradient Thermocycler using the following cyclic parameters: 30 cycles of

denaturation (95°C, 45 seconds), annealing (58°C, 1 minute) and extension (72°C, 1 minute); preceded by an initial denaturation 95°C (15 min) and followed by a final extension of 10 minutes (72°C). After this procedure the samples were subjected to a control gel electrophoresis with ethidium bromide in order to verify amplification and to check blanks.

*DNA Sequencing*

Prior to DNA sequencing, sample purification was carried out using the enzymatic mix ExoSAP, according to manufacturer instructions. The sequencing reaction was performed in a final volume of 10 µL with 2.5 µL of purified amplified DNA, 2 µL of diluted (1:1) Big Dye v1.1 and 0.5 µM of forward or reverse primer. The reaction was performed with the following temperature parameters: initial denaturation 95°C (5 min) followed by 30 cycles of denaturation (96°C, 10 seconds), annealing (50°C, 5 seconds) and extension (60°C, 4 minutes) and reaction termination at 4°C. The samples were then purified using DyeEx™ 96 well plates (Qiagen), according to manufacturer instructions. Afterwards samples were loaded on an ABI PRISM 3130xl Genetic analyzer (Applied Biosystems) for capillary electrophoresis. The raw sequence data was analyzed using SeqScape software v2.6 (Applied Biosystems) for comparative sequence analysis.

*Sequence Analysis*

Sequences obtained were aligned against the reference sequence of the NF2 gene (MN_000268.3). All sequence variants detected were analyzed and checked against SNPs, mutation databases and the literature.

*Multiplex Ligation-dependent Probe Amplification (MLPA)*

MLPA® is a multiplex PCR method that allows the detection of copy number variations, deletions or duplications of genomic sequences. For the detection of large gene rearrangements we have used the Salsa MLPA kit P044 (MRC Holland) that contains 33 probes, covering the 17 NF2 exons (additionally two probes are located in the NF2 promoter) and 14 reference probes.

The MLPA analysis was carried out according to manufacturer's instructions. Briefly, 250 ng of genomic DNA were denatured and incubated with the probe mix at 60°C for 16 h. Then, the hybridized products were ligated and amplified by PCR starting with 10 µL of ligation reaction, followed by 35 cycles comprising a denaturation step at 95°C for 30s, annealing at 60°C for 1 min, and then an extension step at 72°C for 1 min and a final extension of 20 min at 72°C. In each test three DNA samples from 3 healthy individuals were used as controls and the method was validated with a known positive control for each gene. Two µL of the amplification product were loaded on an ABI 3130xl Genetic Analyzer (Applied Biosystems), using 250-LIZ (Applied Biosystems) as a size standard and analyzed with GeneMaker software v1.9 (SoftGenetics). The analysis involves the comparison of the peak areas of the samples and the controls; MLPA ratios <0.75 were considered as deletions and ratios >1.30 as duplications.

**Results**

The conception of the diagnosis protocol and its optimizing were successful inasmuch as it was possible to amplify and sequence all exons and their adjacent 5'UTR and 3'UTR as well as clearly read the sequences in the regions in study. Moreover MLPA analysis was successfully implemented for gene dosage ascertainment. Although we were expecting to enlarge our sample of patients clinically diagnosed with NF2 in order to be able to establish genotype-phenotype correlations we were only able to genetically characterize two patients with a clinical diagnosis of NF2.

The first case was a 59-year-old female which presented the first symptoms at the age of 49 showing two meningiomas, which were surgically removed, and has currently a third one. This patient also shows a schwannoma compromising the last pairs of the cranial nerves. No family history was reported for this patient. Sequencing of the entire coding region and gene dosage analysis revealed no disease-causing mutations in this patient (Figure 1).

Two substitutions were found in the 5'UTR, both described as normal polymorphisms (c.-204C>A, rs1800539 and c.-110G>C, rs1800540). Additionally, three substitutions were found in intronic regions, all also described as normal variations (c.364-39A>C, rs2530664; c.1153-51G>T, rs13055076 and c.1575-67G>A, rs140086).

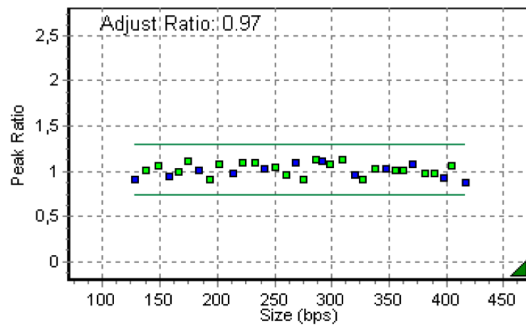


**Figure 1** - MLPA plot from the first patient studied showing a normal dosage for all the NF2 exons.

The second case was a 14 years old male, showing bilateral schwannomas of the VIII pair and unilateral (left) schwannomas of the V and XII pairs. This patient's mother presented with neurofibromas. In this patient we were able to detect, by direct sequencing analysis, a heterozygous insertion (c.490_491insT), in exon 5, resulting in a frameshift and a premature stop codon (p.Ala164ValfsX39) (Figure 2). Additionally, the two substitutions in the 5'UTR, observed in the previous patient, were also found (c.-204C>A, rs1800539 and c.-110G>C, rs1800540) as well as two of the intronic substitutions (c.1153-51G>T, rs13055076 and c.1575-67G>A, rs140086). Gene dosage analysis was also performed in this patient allowing the confirmation of two copies of all exons in this patient.

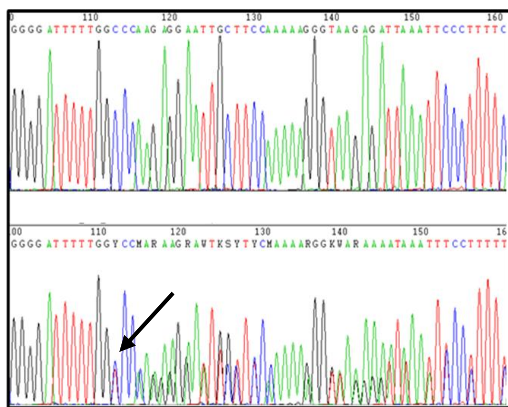

**Figure 2** - Electropherograms of exon 5 in a control (upper panel) and patient (lower panel) showing the c.490_491insT mutation

Furthermore we have confirmed the presence of a c.119del15ins13 at the end of exon 11, in a third patient (molecular diagnosis performed in an external laboratory) (Figure 3). This mutation affects the donor splice site of intron 11 probably resulting in exon skipping. The analysis of this patient allowed the validation of our mutation screening procedure, as well as the study of the patient's mother, which additionally showed a possible schwannoma. Sequencing of exon 11 in DNA samples from peripheral blood and from the tumour of the patient's mother failed to detect this mutation in both samples.

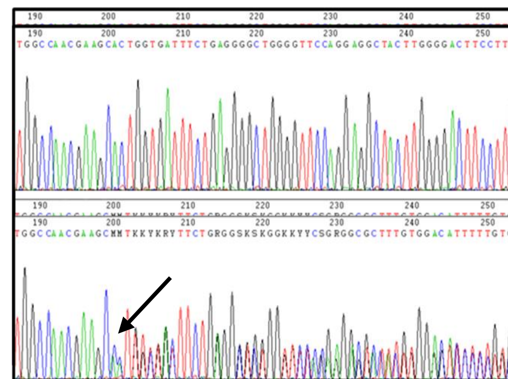

**Figure 3** - Electropherograms of exon 11 in a control (upper panel) and patient (lower panel) showing the c.119del15ins13 mutation

## Discussion

The main purpose of this study was to implement the mutation screening of the *NF2* gene by direct sequencing analysis of the entire coding region and intronic flanking regions, as well as, the detection of large gene rearrangements by MLPA, which was completely fulfilled. Our methodology was validated by the detection of a previously detected mutation in a NF2 patient. In the first case described it would be advisable to revise the clinical diagnosis as both no mutations were found in the sequencing analysis and the whole gene deletion or complete exon deletion was already excluded. The high prevalence of somatic mosaicism can account for the inability

to identify a disease-causing mutation in the first case tested and particularly in the mother of the third patient, in which the mutation identified in her son was absent in DNA samples of both peripheral blood and of the tumour. In this third case, the mutation identified in the son is a *de novo* mutation which probably occurred only at the gamete level.[16]

By mutation analysis we were able to identify a novel mutation in *NF2* in a patient with clinical features compatible with the diagnosis of Neurofibromatosis type 2. This mutation results in a truncated protein lacking more than half of the amino acids and thus several of merlin's functional domains. Similar to what has been largely described, this mutation results in a truncated protein which accounts for approximately 90% of the NF2 patients.[17,18]

Genotype-phenotype correlation was impaired by the small sample and by the confirmation of the diagnosis in only two patients. A frameshift mutation such as the one detected is usually associated to a premature stop codon in addition to a complete disruption in the structure of the translated protein, which is frequently hydrolysed. As a truncating mutation could explain the severe phenotype of the proband who, at a young age, already had developed bilateral VS. In the other confirmed case the mutation affects the splice site donor region and probably results in exon 11 skipping. The consequence of this type of mutation at the protein level is more difficult to predict although usually results in a truncated protein. Unfortunately it was not possible to gather a wider sample of patients due to time constraints, and to the difficulty of obtaining access to patient information yet as a student because of confidentiality issues. This would enable a study of the correlation between phenotype and genotype that has often been mentioned as well as clearer perspective on somatic mosaicism and its diagnostic difficulties.[19]

Even though one can argue that the diagnosis success rate is low, it was shown that 93% of mutations are detectable only in the second generation. Although the DNA sequencing has been shown as the best diagnostic technique for this condition, most experts recommend the simultaneous testing with MLPA which enables the detection of large deletions since there is a high frequency of complete or first exon *NF2* deletions. Hence it would be recommendable to include this procedure to the normal diagnostic protocol.[20]

Although the diagnosis of NF2 is essentially clinical due to its specific manifestations, the importance of genetic testing should not by any means be ignored. In sporadic cases it is suggested to evaluate patients thoroughly, clinical and by the use of neuroimaging. The first case suggests that testing the tumoural DNA concomitantly with lymphocyte DNA could enable a more accurate molecular diagnosis in patients with somatic mosaicism, in order to identify the constitutive mutation which can be carried over to the next generations. Its identification provides each family with a specific test for the mutation and allows an accurate screening for the disease as well as appropriate Genetic Counselling. It offers advantages to both relatives at risk and for the cases where it is possible to exclude a mutation. Moreover the exclusion of the mutation in a family member avoids frequent screening with expensive neuroimaging techniques and on the other hand an early diagnosis might favour the prognosis of carriers as it allows the surgical removal of tumours in initial stages avoiding significant hear loss.[21] Furthermore and if well established genotype-phenotype correlations are described, the molecular diagnosis could predict the course of disease and put therapy measures into context, allowing a more effective management of this condition, which currently has a fairly poor prognosis. It could also enable, after adequate genetic counselling, prenatal diagnosis for pregnancies of affected/at-risk individuals along with preimplantation genetic diagnosis.[15]

With this study we were able to implement the molecular diagnosis for NF2 assessing both sequence variants and large gene

rearrangements. We have detected a novel mutation in *NF2* resulting in a truncated protein confirming the clinical diagnosis in one patient. In order to establish genotype-phenotype correlations we need to enlarge our sample of clinically diagnosed NF2 patients.

**Acknowledgements**

First of all I would like to thank Professor Jorge Sequeiros for having introduced me to this opportunity to explore my theoretical interest in genetics and putting it into practice in a real research context. Furthermore I must thank my thesis supervisors, Jorge Pinto Basto, MD, and Isabel Alonso, PhD, for their relentless support, enormous help and patience with the constant doubts of the newbie, as well as the whole Labteam in UnIGENe from CGPP, IBMC-UP. I would also like to express my deepest gratitude to Professor Ernesto Carvalho and Doctor Isabel Ribeiro from the Neurosurgical Service of Hospital de Santo António, Centro Hospitalar do Porto, for their interest and effort in helping me through the difficult task of fighting against hospital bureaucracy. And finally a particular *herzlichen Dank* for my *Stellvertreter* in Portugal Pedro and my *Masterarbeitgenossen* Carolina and Fábio in Berlin. Wir haben es geschafft!

**Abbreviations**

MPLA: mutiplex ligation-dependant probe amplification; NF1: neurofibromatosis type 1, von Recklinghausen disease; NF2: neurofibromatosis type 2; PCR: polymerase chain reaction; SNP: single nucleotide polymorphism; UTR: untranslated region; VS: vestibular schwannoma, acoustic neuroma

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