UNIVERSITÀ
DEGLI STUDI
FIRENZE

FLORE

Repository istituzionale dell'Università degli Studi di Firenze

NF2 mutation screening by denaturing high-performance liquid chromatography and high-resolution melting analysis

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

Original Citation:

NF2 mutation screening by denaturing high-performance liquid chromatography and high-resolution melting analysis / Sestini R; Provenzano A; Bacci C; Orlando C; Genuardi M; Papi L.. - In: GENETIC TESTING. - ISSN 1090-6576. - STAMPA. - 12:(2008), pp. 311-318. [10.1089/gte.2007.0096]

Availability:

This version is available at: 2158/897126 since: 2017-03-16T18:26:36Z

Published version:

DOI: 10.1089/gte.2007.0096

Terms of use:

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

Publisher copyright claim:

(Article begins on next page)

Genetic Testing

Genetic Testing: <http://mc.manuscriptcentral.com/genetic-testing>

NF2 MUTATION SCREENING BY DENATURING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND HIGH RESOLUTION MELTING ANALYSIS

Journal:	<i>Genetic Testing</i>
Manuscript ID:	GTE-2007-0096.R1
Manuscript Type:	Original Articles
Date Submitted by the Author:	n/a
Complete List of Authors:	Sestini, Roberta; University of Florence, Dept. of Clinical Physiopathology, Medical Genetics Unit Provenzano, Aldesia; University of Florence, Dept. of Clinical Physiopathology, Medical Genetics Unit Bacci, Costanza; University of Florence, Dept. of Clinical Physiopathology, Medical Genetics Unit Orlando, Claudio; University of Florence, Dept. of Clinical Physiopathology, Clinical Biochemistry Unit Genuardi, Maurizio; University of Florence, Dept. of Clinical Physiopathology, Medical Genetics Unit Papi, Laura; University of Florence, Dept. of Clinical Physiopathology, Medical Genetics Unit
Keyword:	Neurofibromatosis, Genetic Testing, Mutation Detection

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Sestini

***NF2* MUTATION SCREENING BY DENATURING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND HIGH RESOLUTION MELTING ANALYSIS**

Roberta Sestini¹, Aldesia Provenzano¹, Costanza Bacci¹, Claudio Orlando², Maurizio Genuardi¹, Laura Papi¹

¹ Department of Clinical Physiopathology, Medical Genetics Unit, University of Florence

² Department of Clinical Physiopathology, Clinical Biochemistry Unit, University of Florence

RUNNING TITLE: DHPLC and HRM analysis in *NF2* gene

Corresponding author:

Roberta Sestini

Dipartimento di Fisiopatologia Clinica

Sezione di Genetica Medica

Viale Pieraccini, 6

50139 Firenze

E-Mail: r.sestini@dfc.unifi.it

Phone: +390554271383

Fax: +390557949003

Sestini

ABSTRACT

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder caused by mutations in the *NF2* gene and predisposing to the development of nervous system. Identification of germline mutations is essential to provide appropriate genetic counselling in NF2 patients, but it represents an extremely challenging task, as the vast majority of mutations are unique and spread over the entire coding sequence. Moreover, about 30% of “*de novo*” patients are indeed mosaic and direct sequencing can undetect mutated alleles present in a minority of cells. As most screening techniques do not meet the requirements for efficient NF2 testing, we have developed a semi-automated denaturing high-performance liquid chromatography (DHPLC) method for point mutation detection combined with a MLPA approach to screen for gene rearrangements. In addition, we have evaluated high-resolution melting analysis (HRMA) as a exon scanning procedure to identify point mutations in the *NF2* gene. The results obtained in 92 NF2 patients expand the *NF2* mutational spectrum and indicate DHPLC and HRMA as good systems to screen for point mutations in diseases with a heterogeneous spectrum of alterations.

Sestini

INTRODUCTION

Neurofibromatosis type 2 (NF2; MIM 101000) is an autosomal dominant cancer syndrome that predisposes to the development of nervous system tumours (Evans et al., 1992). Bilateral vestibular schwannomas are pathognomonic of the disorder; furthermore, schwannomas at other locations, meningiomas, ependymomas and juvenile posterior subcapsular lenticular opacities are often associated with the disease (Evans et al., 1992; Parry et al., 1994; Mautner et al., 1996). About half of NF2 patients have no family history of the disease, representing *de novo* mutations.

The disease is caused by mutations in the *NF2* tumour-suppressor gene, located in 22q12 (Trafletti et al., 1993; Rouleau et al., 1993). Since its identification, different germline mutations have been found in up to 91% of NF2 patients with a positive family history using exon-scanning-based methods and Multiplex Ligation-dependant Probe Amplification (MLPA) to detect genomic rearrangements (Kluwe et al., 2005; Evans et al., 2007). Somatic mosaicism has been found in 25-33% of “*de novo*” NF2 patients (Kluwe et al., 2003; Moyhuddin et al., 2003; Evans et al., 2007). Somatic mosaicism may be even more common in sporadic NF2 patients with mild phenotypes, resulting from small proportions of cells harbouring mutant *NF2* alleles: it has been estimated that 60% of sporadic cases with unilateral vestibular schwannoma at presentation might indeed be mosaics (Evans et al., 2007).

Since the vast majority of mutations spread over the entire coding sequence, analysis of the *NF2* gene has been commonly carried out with PCR-SSCP as pre-screening method, followed by DNA sequence analysis of amplicons that show aberrant band shift. Although this strategy is effective; however, turnaround time is relatively long and the limited sensitivity of SSCP does not allow detection of all point mutations. The use of direct sequencing as diagnostic tool is reserved to familial NF2 patients,

Sestini

since it has been shown that this approach does not have a 100% sensitivity in mosaic patients, especially in cases with low proportion of mutated cells (Jones et al., 2001).

To overcome problems deriving from the limited sensitivity of SSCP and other screening techniques, denaturing high performance liquid chromatography (DHPLC) (Underhill et al., 1997) was developed as a suitable mutation scanning method in a large number of single-locus disease genes (Liu et al., 1998; Wu et al., 2001; Marsh et al., 2001; Hegde et al., 2005). DHPLC is a semi-automatic method, that entails limited post-PCR manipulation; it requires heteroduplex formation and need an accurate optimization of running setting prior to chromatography in order to predict conditions for each PCR product. The method allows identification of sequence variants through characteristic peak patterns and shows many advantages including high sensitivity, specificity and throughput. However, DHPLC requires the availability of dedicated and expensive instrumentation.

Recently, high-resolution melting analysis (HRMA) has been described as a new and highly sensitive mutation scanning method to identify point mutations in an amplicon without the need for gel or column separation or labeled oligonucleotides (Wittwer 2003; Liew et al., 2004; Reed et al., 2007). In this technique PCR is performed in the presence of saturating concentrations of double-stranded DNA binding dyes and then amplicons are submitted to high resolution melting analysis monitoring the fluorescence across a defined temperature range (Reed and Wittwer 2004). Data acquisition generates a change in melting curve shape (Graham et al., 2005) that can be used to identify the presence of sequence variation within the amplicon. HRMA has been shown to have near 100% analytical sensitivity and specificity when used on products up to 400 base pairs in length (Chou et al., 2005; Margraf et al., 2006; Krypuy et al., 2006). HRMA was already used to identified germline and somatic point mutations (Kemerson et al., 2007; Margraf et al., 2007; Pal et al., 2007; Dobrowolski et al., 2007; Krypuy et al., 2007; Takano et al., 2007).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Sestini

In the present study, we evaluated DHPLC and HRMA-based approach for point mutation scanning of *NF2* gene.

MATERIALS AND METHODS

Patients and DNA samples

In the present study DHPLC and HRMA were used to evaluate 92 patients from 92 unrelated Italian probands who had been diagnosed with NF2 based on the presence of bilateral vestibular schwannomas. Sixteen NF2 patients in whom a pathogenic mutation had been identified by PCR-SSCP and characterized by cycle sequencing, were used as positive controls for DHPLC and HRMA (Table 1). The second group included 76 cases sporadic patients in which PCR-SSCP and Multiplex Ligation-dependant Probe Amplification (MLPA) (MRC Holland, Amsterdam, The Netherlands), did not reveal single point mutations or genomic rearrangements in *NF2* gene. High molecular weight genomic DNA was extracted by standard phenol/chloroform method from blood samples collected from patients, following provision of informed consent.

Primers and PCR Conditions

Mutation scanning of *NF2* gene requires the analysis of 15 exons, including intron /exon junctions. Genomic DNA was amplified using the intronic primer sets reported in Table 2. For DHPLC analysis, PCR reactions were performed in 30 µl, containing 1X PCR buffer (Applied Biosystems, Milan, Italy), 200 mM of each dNTP, 2mM MgCl₂, specific primers, 0.75 U TaqGold DNA polymerase (Applied Biosystems) and 50 ng of genomic DNA.

Sestini

DHPLC Analysis

DHPLC was performed using the WAVES DNA Fragment Analysis System (Transgenomic, San Jose, CA). The PCR products were denatured at 95°C for 3 min, followed by gradual reannealing for 30 min with a temperature ramp of 11°C/min to optimize the formation of heteroduplexes and homoduplexes. Five µl were applied to a preheated C18 reversed-phase column of nonporous poly(styrene-divinylbenzene) particles (DNA-Sep; Transgenomic). DNA was eluted within 2.5 min at a flow rate of 0.9 ml/min using a linear acetonitrile gradient from 52% to 67% buffer B (0.1M triethylammonium acetate [TEAA]; 25% acetonitrile) at the appropriate denaturing temperature. The temperature for heteroduplex detection was determined using WAVEMAKER software (Transgenomic), based on the melting profiles of the 15 *NF2* amplicons (Table 2). The predicted temperature was optimized by testing specimens of wild-type DNA and different positive controls. Regeneration of the column was achieved by washing with 100% buffer B for 30 sec followed by an equilibration time of 2 min. DNA elution was observed online at 260 nm.

High-Resolution Melting Analysis

DNA samples were amplified with the same primers and PCR conditions used for DHPLC analysis. For HRMA, PCR was performed in 20 µL with 50 µM Syto9® (Invitrogen Corp., Carlsbad, CA) as intercalating dye. Amplified products were denatured at 95°C for 1 min and then rapidly cooled to 40°C for 1 min in order to facilitate heteroduplex formation. HRMA was then performed on a Rotor Gene™ 6000 Instrument (Corbett Research, Sydney, Australia). Melt curve data for each PCR product were acquired in a wide temperature range (75°C to 95°C), at a ramping rate of 0.1°C/sec. Results were analyzed as fluorescence versus temperature graphs as previously described (Krypuy M. et al., 2006).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Sestini

DNA sequencing

All cases that exhibited an abnormal melting curve at HRMA and/or aberrant elution profile on DHPLC chromatograms, were subjected to sequence analysis. PCR products were purified using a QIAquick PCR Purification kit (Qiagen Inc., Germany) and then subjected to cycle sequencing in both forward and reverse directions using a BigDye Terminator Cycle Sequencing version 3.1 kit (Applied Biosystems) according to the manufacturer’s instructions. The products from each reaction were analyzed on an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Milan, Italy).

Mutation nomenclature follows the Human Genome Variation Society (URL: <http://www.hgvs.org/mutnomen/>) recommendations. The DNA mutation numbering is based on the *NF2* cDNA sequences (GenBank accession number NM_181832.1) with the A of the ATG translation-initiation codon numbered as + 1. Amino acid numbering starts with the translation initiator methionine as +1.

RESULTS

Analytical sensitivity and specificity of HRMA were firstly validated on a series of 16 DNA samples with previously identified *NF2* mutations, detected by PCR-SSCP and characterized by cycle sequencing. Exons harbouring these mutations were specifically targeted for screening by DHPLC and HRMA. Both methods showed a 100% concordance for the identification of all DNA alterations (Table 1). All samples were distinguishable by significant differences in the melt curves and DHPLC traces of the amplified DNA samples when compared to wild-type DNA (Figure1). To evaluate the diagnostic sensitivity of DHPLC and HRMA analyses and their ability to identify mosaicism in DNA samples, compared to direct sequencing, we performed serial dilutions of a wild-type DNA with genomic DNA deriving from non-mosaic *NF2* patients carrying the following mutations:

Sestini

c.997C>T (exon 10), IVS14+2T>C, IVS15+1G>C. Dilutions were made corresponding to a 1:1, 1:4, 1:10, 1:20, 1:100 and 1:1000 ratio of patient:normal DNA, respectively. In our experimental conditions, DHPLC and HRMA were able to detect the c.997C>T and IVS15+1G>C mutations until a dilution 1:100 in which the mutant allele was present at 0.25% relative to the normal allele (Figure 2) while the IVS14+2T>C mutation was detectable until 1:20 dilution (1.25% of the mutant allele). Direct sequencing detected the c.997C>T and IVS15+1G>C mutations until dilution 1:4 (6.25% of the mutant allele) and the IVS14+2T>C alteration until dilution 1:1 (25% of the mutant allele) (Figure 3). To test “*in vivo*” this capability, DHPLC and HRMA were performed in one previously described mosaic patient with an inactivating mutation in *NF2* gene (Sestini et al., 2000). In this patient, both techniques indicated an abnormal profile related to the presence of a c.1416delC mutation in exon 13 (Figure 4).

Subsequently, we analysed 76 *NF2* samples for which previous mutation screening by PCR-SSCP had not revealed sequence changes. As reported in Figure 5, we identified 7 samples with abnormal DHPLC peaks and showing differences in the melt curves in HRMA. Sequencing analysis of the involved exons demonstrated in all cases the presence of 6 different point mutations. These six different mutation included two nonsense (c.592C>T, p.R198X and c.1090A>T, p.K364X involving exon 6 and exon 11 respectively), one missense (c.447G>C, p.K149N in exon 4), one frameshift (c278delCCTT in exon 3) and two splice site mutations (IVS3+3A>C and IVS7 -1G>T).

DISCUSSION

High sensitivity in detection of DNA sequence variations is essential for mutation analysis in genetic diseases. A wide range of methodologies for exon scanning have been proposed to select samples with

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Sestini

aberrant profiles to be submitted to DNA sequencing, with the aim to reduce laboratory costs and analysis time.

DHPLC has been extensively applied to single nucleotide polymorphism analysis and mutational screening for different disease associated genes (Gross et al., 1999; Nickerson et al., 2000; Dobson-Stone et al., 2000; Jones et al., 2000; Jones et al., 2001). This methodology allows a rapid separation and visualization of homo- and heteroduplex DNA targets by using a reverse-phase liquid chromatography system. Maximal sensitivity in the heteroduplex detection is obtained maintaining the HPLC column at an optimal melting temperature that allows partial strand denaturation in the presence of a single base-pair mismatching.

Recently, HRMA has been proposed as an alternative scanning method to identify changes in DNA sequence using melting-curve analysis of the amplicons. This technique allows characterization of the DNA samples according to their dissociation behaviour in transition from double stranded to single stranded DNA with increasing temperature. As reported for DHPLC, also HRMA identifies heteroduplexes by their lower thermal stability (Reed et al., 2004). However, unlike DHPLC, melting analysis scans through a wide range of temperatures rather than depending on a specific temperature, a feature that normally requires time-consuming optimization for each amplicon to be analyzed.

HRMA relies on the use of fluorescent dsDNA-binding dyes that can be added directly to the PCR mix at saturating concentrations without inhibiting amplification (Wittwer and Reed 2003). This allows the precise monitoring of very low changes in fluorescence, caused by the release of a intercalating dyes consequent to the increase of temperature (Liew et al., 2004). Thus, single base changes can be readily identified as a significant differences in the melting curves of DNA amplicons (Reed et al., 2007). Consequently, HRMA results to be a closed-tube mutation screening method, that can be performed immediately after the amplification and does not require post-PCR handling or separation steps. These

Sestini

features make HRMA particularly suitable for medium to high-throughput applications (Dobrowolski et al., 2007; Margraf 2006; Montgomery et al., 2007) .

In this study we compared for the first time DHPLC and HRMA for mutation scanning of the *NF2* gene in two groups of patients. The first one, included patients with a known pathogenetic mutation. The second group included 76 *NF2* sporadic patients where PCR-SSCP screening was unable to reveal point mutations and MLPA analysis excluded genomic rearrangements. In both groups, HRMA results were compared in blind with those obtained with DHPLC. Our results indicated that both DHPLC and HRMA show a 100% sensitivity in detecting mutations previously characterised by PCR-SSCP, without any false positive samples. In addition, both DHPLC and HRMA detected 7 new samples with abnormal peaks at DHPLC analysis and anomalous melting profile at HRMA. Sequencing analysis revealed mutations in the involved exons including nonsense, missense, frameshift and splice site mutations.

Another very important aspect in the evaluation of methods for *NF2* mutational scanning is their capability to detect mosaicism. The identification of somatic mosaicism in a genetic syndrome is clinically relevant and this information has important implications for genetic counselling. According to our data, DHPLC and HRMA, performed on serial dilution of mutated:normal DNA samples, were able to detect the presence of 1.25% - 0.25% mutated alleles, a theoretical threshold able to identify patients with a very low percentage of mutated cells in blood; this percentage is well under the direct sequencing threshold of detection .

In summary, in our experience that both DHPLC and HRMA are reliable techniques for mutation scanning in *NF2* gene. However, due to its simplicity, rapidity, and lower costs, together with its high sensitivity in detecting germline mutations and mosaicisms and to the absolute concordance with DHPLC results, we propose that HRMA could be used as a diagnostic screening technique for *NF2* patients.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Sestini

REFERENCES

Chou LS, Lyon E, Wittwer CT. (2005) A comparison of high-resolution melting analysis with denaturing high-performance liquid chromatography for mutation scanning: cystic fibrosis transmembrane conductance regulator gene as a model. *Am J Clin Pathol.* 124:330-338

Dobrowolski SF, Ellingson C, Caldovic L, Tuchman M. (2007) Streamlined assessment of gene variants by high resolution melt profiling utilizing the ornithine transcarbamylase gene as a model system. *Hum Mutat.* 12; [Epub ahead of print]

Dobson-Stone C, Cox RD, Lonie L, Southam L, Fraser M, Wise C, Bernier F, Hodgson S, Porter DE, Simpson AH, Monaco AP. (2000) Comparison of fluorescent single-strand conformation polymorphism analysis and denaturing high-performance liquid chromatography for detection of EXT1 and EXT2 mutations in hereditary multiple exostoses. *Eur J Hum Genet.*8:24-32.

Evans DG, Huson SM, Donnai D, Neary W, Blair V, Teare D, Newton V, Strachan T, Ramsden R, Harris R. (1992). 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

Evans DG, Ramsden R, Shenton A, Gokhale C, Bowers NL, Huson SM, Pichert G, Wallace A.(2007) Mosaicism in NF2 an update of risk based on uni/bilaterality of vestibular schwannoma at presentation and sensitive mutation analysis including MLPA. *J Med Genet.* 44:424-428.

Graham R, Liew M, Meadows C, Lyon E, Wittwer CT. (2005) Distinguishing different DNA heterozygotes by high-resolution melting. *Clin Chem.* 51:1295-1298.

Gross E, Arnold N, Goette J, Schwarz-Boeger U, Kiechle M. (1999) A comparison of BRCA1 mutation analysis by direct sequencing, SSCP and DHPLC. *Hum Genet.* 105:72-78.

Sestini

- Hegde M, Blazo M, Chong B, Prior T, Richards C. (2005) Assay validation for identification of hereditary nonpolyposis colon cancer-causing mutations in mismatch repair genes MLH1, MSH2, and MSH6. *J Mol Diagn.* 7:525-34.
- Jones AC, Sampson JR, Hoogendoorn B, Cohen D, Cheadle JP. (2000) Application and evaluation of denaturing HPLC for molecular genetic analysis in tuberous sclerosis. *Hum Genet.* 106:663-668.
- Jones AC, Sampson JR, Cheadle JP Low level mosaicism detectable by DHPLC but not by direct sequencing. (2001) *Hum Mutat.* 17:233-234.
- Kennerson ML, Warburton T, Nelis E, Brewer M, Polly P, De Jonghe P, Timmerman V, Nicholson GA (2007) Mutation scanning the GJB1 gene with high-resolution melting analysis: implications for mutation scanning of genes for Charcot-Marie-Tooth disease. *Clin Chem.* 53:349-352.
- Kluwe L, Mautner V, Heinrich B, Dezube R, Jacoby LB, Friedrich RE, MacCollin M. (2003). Molecular study of frequency of mosaicism in neurofibromatosis 2 patients with bilateral vestibular schwannomas. *J Med Genet* 40: 109-114.
- Kluwe L, Nygren AO, Errami A, Heinrich B, Matthies C, Tatagiba M, Mautner V. (2005) Screening for large mutations of the NF2 gene. *Genes Chromosomes Cancer.* 42:384-91
- Krypuy M, Newnham GM, Thomas DM, Conron M, Dobrovic A. (2006) High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer. *BMC* 21:295
- Krypuy M, Ahmed AA, Etemadmoghadam D, Hyland SJ, Group AO, Brenton JD, Fox SB, Defazio A, Bowtell DD, Dobrovic A. (2007) High resolution melting for mutation scanning of TP53 exons 5-8. *BMC Cancer.* 31:168
- Liew M, Pryor R, Palais R, Meadows C, Erali M, Lyon E, Wittwer C (2004) Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clin Chem.* 50:1156-1164

Sestini

Liu W, Smith DI, Rechtzigel KJ, Thibodeau SN, James CD. (1998) Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. *Nucleic Acids Res.* 26:1396-400.

Margraf RL, Mao R, Highsmith WE, Holtegaard LM, Wittwer CT. (2006) Mutation scanning of the RET protooncogene using high-resolution melting analysis. *Clin Chem.* 52:138-141.

Margraf RL, Mao R, Highsmith WE, Holtegaard LM, Wittwer CT. (2007) RET proto-oncogene genotyping using unlabeled probes, the masking technique, and amplicon high-resolution melting analysis. *J Mol Diagn.* 9:184-196.

Marsh DJ, Theodosopoulos G, Howell V, Richardson AL, Benn DE, Proos AL, Eng C, Robinson BG. (2001) Rapid mutation scanning of genes associated with familial cancer syndromes using denaturing high-performance liquid chromatography. *Neoplasia.* 3:236-244.

Mautner VF, Lindenau M, Baser ME, Hazim W, Tatagiba M, Haase W, Samii M, Wais R, Pulst SM. (1996) The neuroimaging and clinical spectrum of neurofibromatosis 2. *Neurosurgery* 38: 880-885

Moyhuddin A, Baser ME, Watson C, Purcell S, Ramsden RT, Heiberg A, Wallace AJ, Evans DG. (2003). Somatic mosaicism in neurofibromatosis 2: prevalence and risk of disease transmission to offspring. *J Med Genet* 40: 459-463.

Montgomery J, Wittwer CT, Kent JO, Zhou L (2007) Scanning the Cystic Fibrosis Transmembrane Conductance Regulator Gene Using High-Resolution DNA Melting Analysis. *Clin Chem.* 21; [Epub ahead of print]

Nickerson ML, Weirich G, Zbar B, Schmidt LS. (2000) Signature-based analysis of MET proto-oncogene mutations using DHPLC. *Hum Mutat.* 16:68-76.

Parry DM, Eldridge R, Kaiser-Kupfer MI, Bouzas EA, Pikus A, Patronas N. (1994) Neurofibromatosis 2 (NF2): clinical characteristics of 63 affected individuals and clinical evidence for heterogeneity. *Am J Med Genet* 52: 450-461.

Sestini

Pal T, Napierala D, Becker TA, Loscalzo M, Baldrige D, Lee B, Sutphen R (2007) The presence of germ line mosaicism in cleidocranial dysplasia. *Clin Genet.* 71:589-591.

Reed GH, Wittwer CT (2004) Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clin Chem.* 50:1748-1754.

Reed GH, Kent JO, Wittwer CT. (2007) High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics* 8:597-608

Rouleau GA, Merel P, Lutchman M, Sanson M, Zucman J, Marineau C, Hoang-Xuan K, Demczuk S, Desmaze C, Plougastel B, Pulst SM, Lenoir G, Bijlsma E, Fahsold R, Dumanski J, de Jong P, Parry D, Eldridge R, Aurias A, Delattre O, Thomas G. (1993). Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. *Nature* 363: 515-521.

Sestini R, Vivarelli R, Balestri P, Ammannati F, Montali E, Papi L (2000) Neurofibromatosis type 2 attributable to gonosomal mosaicism in a clinically normal mother, and identification of seven novel mutations in the NF2 gene. *Hum Genet.* 107:366-371.

Takano T, Ohe Y, Tsuta K, Fukui T, Sakamoto H, Yoshida T, Tateishi U, Nokihara H, Yamamoto N, Sekine I, Kunitoh H, Matsuno Y, Furuta K, Tamura T. (2007) Epidermal growth factor receptor mutation detection using high-resolution melting analysis predicts outcomes in patients with advanced non small cell lung cancer treated with gefitinib. *Clin Cancer Res.* 15:5385-5390

Trofatter JA, MacCollin MM, Rutter JL, Murrell JR, Duyao MP, Parry DM, Eldridge R, Kley N, Menon AG, Pulaski K, Haase VH, Ambrose CM, Munroe D, Bove C, Haines JL, Martuza RL, MacDonald ME, Seizinger BR, Short MP, Buckler AJ, Gusella JF. (1993). A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. *Cell* 72: 791-800.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Sestini

Underhill PA, Jin L, Lin AA, Mehdi SQ, Jenkins T, Vollrath D, Davis RW, Cavalli-Sforza LL, Oefner PJ. (1997) Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. *Genome Res.* 7:996-1005.

Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ (2003) High-resolution genotyping by amplicon melting analysis using LCGreen.. *Clin Chem* 49:853-860.

Wu G, Wu W, Hegde M, Fawcner M, Chong B, Love D, Su LK, Lynch P, Snow K, Richards CS. (2001) Detection of sequence variations in the adenomatous polyposis coli (APC) gene using denaturing high-performance liquid chromatography. *Genet Test.* 5:281-290.

Sestini

Table 1: Known mutations in the *NF2* gene detected with DHPLC and HRMA.

MUTATION	
EXON 1	c.71-72insT
EXON 2	c.169C>T p.R57X c.169C>T p.R57X c.169C>T p.R57X c.169C>T p.R57X IVS2+2T>C
EXON 5	c.463delCCinsT
EXON 7	c.656T>A p.V219E c.663C>G p.Y221X
EXON 8	c.773G>A p.W257X
EXON 10	c.997C>T p.Q333X
EXON 12	c.1279G>T p.E427X
EXON 13	c.1416delC c.1396C>T p.R466X
EXON 14	IVS14+2T>C
EXON 15	IVS15+1G>C

Sestini

Table 2: Primers for PCR amplification of NF2 gene and temperatures for heteroduplex detection in DHPLC analysis

EXON	PRODUCT SIZE (bp)	PRIMER SEQUENCE		Tm PCR*	DHPLC ANALYSIS**
		FORWARD	REVERSE		
1	235	5'-GGG CTA AAG GGC TCA GAG TG-3'	5'-AAC CTC TCG AGC TTC CAC CT-3'	59°C	64.5°C
2	265	5'-CCT GAG AGT GGA GAG TGC AG-3'	5'-GGG AAA AAC TGG AAA GCT CA-3'	62°C	58.5°C - 60.9°C
3	241	5'-TGA GGG TAG CAC AGG AGG AA-3'	5'-CAA CTC TGC AAC CAC TCC TG-3'	57°C	58.7°C
4	237	5'-CAC AGA GTA TCA TGT CTC CCT TG-3'	5'-CAC TTT CTG GGG CAA GTC TC-3'	58°C	57.5°C - 58.3°C - 61.2°C
5	188	5'-AGC TGG GAG GGA ATG AGA TT-3'	5'-TCC TTC AAG TCC TTT GGT TAG C-3'	59°C	56.5°C - 59.5°C
6	192	5'-AAA AGT GGC AAA CAA TAC CAA A-3'	5'-AAG CCC ATA AAG GAA TGT AAA CC-3'	61°C	54.7°C - 59.5°C
7	177	5'-GCTCTCCACCCATCTCACTT-3'	5'-TTT AGC AGT CTG GCC CTC AC-3'	59°C	59.7°C - 60.6°C
8	258	5'-TGC CAG ATT CTT TGG AAG GT-3'	5'-GCA GAC AGG GAA AGA TCT GC-3'	57°C	58.8°C - 60.5°C
9	248	5'-GGT AAC ATT CCA GGC TGT CG-3'	5'-GCG CCA AGT GAG ATA CCA TT-3'	59°C	55.9°C - 56.5°C
10	234	5'-TAG TGG GCC AGT AGG CAG TG-3'	5'-GCC AGG ACT GAC CAC ACA G-3'	60°C	57.2°C - 58.8°C - 60.0°C
11	212	5'- CTC GAG CCC TGT GAT TCA A -3'	5'-AGT CCC CAA GTA GCC TCC TG-3'	62°C	60.5°C - 61.8°C - -63.0°C
12	284	5'- CCC ACT TCA GCT AAG AGC AC -3'	5'-CTC CTC GCC AGT CTG GTG-3'	64°C	62.1°C - 63.8°C
13	227	5'- GGT GTC TTT TCC TGC TAC CT -3'	5'-ACC ACT ACA AGA GAA AGG AGG G-3'	60°C	60.7°C - 61.9°C - 64.5°C
14	246	5'- AGG ATC GGT TGT CAA CAC AG -3'	5'-GGC CCC AAT CAC TCA GTC TA-3'	61°C	58.9°C - 60.0°C
15	255	5'- TGT CTC ACT GTC TGC CCA AG--3'	5'-TGG TCC TGA TCA GCA AAA A-3'	60°C	56.6°C - 60.5°C

* Annealing temperature for PCR amplification

**Optimum temperatures for DHPLC analysis were empirically determined using the predicted fragment melting profile generated by WAVEMAKER software.

Sestini

Figure legends

Figure 1

DHPLC chromatograms and HRMA melting profiles for known *NF2* gene mutations in exons 2, 7, 12, 14, 15. The arrows indicates variant elution profiles and change in melting curve shape.

MUT – mutant

Figure 2

Evaluation of the sensitivity in detecting mosaicism of DHPLC and HRMA. Serial dilutions of a wild-type DNA with genomic DNA carrying a c.997C>T mutation in exon 10, deriving from a non-mosaic *NF2* patient were prepared. Dilutions corresponding to a 1:1, 1:4, 1:10, 1:20, 1:100 and 1:1000 ratio of patient : normal DNA were amplified in PCR and analysed by DHPLC and HRMA.

A) DHPLC chromatograms showing the double peak corresponding to the mutation in dilutions 1:1, 1:4, 1:10, 1:20, 1:100.

B) HRMA difference plots of patient : normal DNA dilutions. All dilutions were compared to the median wild-type control sample to produce the plot. The mutated allele was easily detectable in all the dilution tested except 1:1000.

Figure 3

Evaluation of the sensitivity in detecting mosaicism of HRMA and direct sequencing. HRMA difference plots (on the left) and direct sequencing (on the right) of patient : normal DNA dilutions.

A) Mutation IVS15+1G>C. The mutated allele was detectable, until dilution 1:100 and 1:20 respectively by HRMA and direct sequencing.

B) Mutation IVS14+2T>C. The mutated allele was detectable, until dilution 1:20 and 1:1 respectively by HRMA and direct sequencing.

1 Sestini

2
3 **Figure 4**

4
5 DHPLC chromatograms and HRMA melting curves representative of the mutation detected in a mosaic
6 patient carrying a mutation in *NF2* exon 13, namely c.1416delC.
7
8
9

10
11
12 **Figure 5**

13 DHPLC (left) and HRMA (right) profiles of the mutations lost at SSCP analysis.
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1 Sestini

2
3 Roberta Sestini
4 Dipartimento di Fisiopatologia Clinica
5 Sezione di Genetica Medica
6 Viale Pieraccini, 6
7 50139 Firenze
8 E-Mail: r.sestini@dfc.unifi.it
9 Phone: +390554271383, Fax: +390557949003
10
11

12 Aldesia Provenzano
13 Dipartimento di Fisiopatologia Clinica
14 Sezione di Genetica Medica
15 Viale Pieraccini, 6
16 50139 Firenze
17 E-Mail: aldesia@libero.it
18 Phone: +390554271383, Fax: +390557949003
19
20
21

22 Costanza Bacci
23 Dipartimento di Fisiopatologia Clinica
24 Sezione di Genetica Medica
25 Viale Pieraccini, 6
26 50139 Firenze
27 E-Mail: costanzab@virgilio.it
28 Phone: +390554271383, Fax: +390557949003
29
30
31

32 Claudio Orlando
33 Dipartimento di Fisiopatologia Clinica
34 Sezione di Biochimica Clinica
35 Viale Pieraccini, 6
36 50139 Firenze
37 E-Mail: c.orlando@dfc.unifi.it
38 Phone: +390554271440, Fax: +390554271413
39
40

41 Maurizio Genuardi
42 Dipartimento di Fisiopatologia Clinica
43 Sezione di Genetica Medica
44 Viale Pieraccini, 6
45 50139 Firenze
46 E-Mail: m.genuardi@dfc.unifi.it
47 Phone: +390554271420, Fax: +390554271413
48
49

50 Laura Papi
51 Dipartimento di Fisiopatologia Clinica
52 Sezione di Genetica Medica
53 Viale Pieraccini, 6
54 50139 Firenze
55 E-Mail: l.papi@dfc.unifi.it
56 Phone: +390554271382, Fax: +39055794900
57
58
59
60

Table 1: Known mutations in the *NF2* gene detected with DHPLC and HRMA.

MUTATION	
EXON 1	c.71-72insT
EXON 2	c.169C>T p.R57X c.169C>T p.R57X c.169C>T p.R57X c.169C>T p.R57X IVS2+2T>C
EXON 5	c.463delCCinsT
EXON 7	c.656T>A p.V219E c.663C>G p.Y221X
EXON 8	c.773G>A p.W257X
EXON 10	c.997C>T p.Q333X
EXON 12	c.1279G>T p.E427X
EXON 13	c.1416delC c.1396C>T p.R466X
EXON 14	IVS14+2T>C
EXON 15	IVS15+1G>C

Table 2: Primers for PCR amplification of NF2 gene and temperatures for heteroduplex detection in DHPLC analysis

EXON	PRODUCT SIZE (bp)	PRIMER SEQUENCE		T _m PCR*	DHPLC ANALYSIS**
		FORWARD	REVERSE		
1	235	5'-GGG CTA AAG GGC TCA GAG TG-3'	5'-AAC CTC TCG AGC TTC CAC CT-3'	59°C	64.5°C
2	265	5'-CCT GAG AGT GGA GAG TGC AG-3'	5'-GGG AAA AAC TGG AAA GCT CA-3'	62°C	58.5°C - 60.9°C
3	241	5'-TGA GGG TAG CAC AGG AGG AA-3'	5'-CAA CTC TGC AAC CAC TCC TG-3'	57°C	58.7°C
4	237	5'-CAC AGA GTA TCA TGT CTC CCT TG-3'	5'-CAC TTT CTG GGG CAA GTC TC-3'	58°C	57.5°C - 58.3°C - 61.2°C
5	188	5'-AGC TGG GAG GGA ATG AGA TT-3'	5'-TCC TTC AAG TCC TTT GGT TAG C-3'	59°C	56.5°C - 59.5°C
6	192	5'-AAA AGT GGC AAA CAA TAC CAA A-3'	5'-AAG CCC ATA AAG GAA TGT AAA CC-3'	61°C	54.7°C - 59.5°C
7	177	5'-GCTCTCCACCCATCTCACTT-3'	5'-TTT AGC AGT CTG GCC CTC AC-3'	59°C	59.7°C - 60.6°C
8	258	5'-TGC CAG ATT CTT TGG AAG GT-3'	5'-GCA GAC AGG GAA AGA TCT GC-3'	57°C	58.8°C - 60.5°C
9	248	5'-GGT AAC ATT CCA GGC TGT CG-3'	5'-GCG CCA AGT GAG ATA CCA TT-3'	59°C	55.9°C - 56.5°C
10	234	5'-TAG TGG GCC AGT AGG CAG TG-3'	5'-GCC AGG ACT GAC CAC ACA G-3'	60°C	57.2°C - 58.8°C - 60.0°C
11	212	5'- CTC GAG CCC TGT GAT TCA A -3'	5'-AGT CCC CAA GTA GCC TCC TG-3'	62°C	60.5°C - 61.8°C - -63.0°C
12	284	5'- CCC ACT TCA GCT AAG AGC AC -3'	5'-CTC CTC GCC AGT CTG GTG-3'	64°C	62.1°C - 63.8°C
13	227	5'- GGT GTC TTT TCC TGC TAC CT -3'	5'-ACC ACT ACA AGA GAA AGG AGG G-3'	60°C	60.7°C - 61.9°C - 64.5°C
14	246	5'- AGG ATC GGT TGT CAA CAC AG -3'	5'-GGC CCC AAT CAC TCA GTC TA-3'	61°C	58.9°C - 60.0°C
15	255	5'- TGT CTC ACT GTC TGC CCA AG--3'	5'-TGG TCC TGA TCA GCA AAA A-3'	60°C	56.6°C - 60.5°C

* Annealing temperature for PCR amplification

**Optimum temperatures for DHPLC analysis were empirically determined using the predicted fragment melting profile generated by WAVEMAKER software.

190x254mm (96 x 96 DPI)

190x254mm (96 x 96 DPI)

A

B

190x254mm (96 x 96 DPI)

190x254mm (72 x 72 DPI)