Sensitive Detection of Deletions of One or More Exons in the Neurofibromatosis Type 2 (NF2) Gene by Multiplexed Gene Dosage Polymerase Chain Reaction

Ruth Diebold,* Britta Bartelt-Kirbach,*
D. Gareth Evans,[†] Dieter Kaufmann,[‡] and
C. Oliver Hanemann*

From the Department of Neurology,* Zentrum für klinische
Forschung, and the Department of Human Genetics,[‡] University
of Ulm, Ulm, Germany; and the Department of Medical Genetics
and Regional Genetic Services,[†] St. Mary's Hospital, Hathersage
Road, Manchester, United Kingdom

Mutation detection in the neurofibromatosis type 2 (NF2) gene is challenging because when combining mutation detection methods such as single-strand conformational polymorphism and heteroduplex analysis, denaturing gradient gel electrophoresis, and direct sequencing of aberrant polymerase chain reaction (PCR) fragments only 30 to 60% of the constitutional mutations are detected. Because large deletions and complete chromosome rearrangements are also described methods such as microarray-comparative genomic hybridization and fluorescence *in situ* hybridization are also used. The one type of mutation often missed corresponds to deletions encompassing one or few exons. To detect this type we have developed a swift and reliable method. We perform a gene dosage analysis with two fluorescent multiplex PCR assays that amplify 15 of the 17 NF2 exons. The labeled PCR products are quantified and gene dose is calculated with respect to controls. We tested the reliability of this method with DNA from eight NF2 patients with known heterozygous NF2 deletions, eight controls and four unknown NF2 patients. In all of the patients with known heterozygous deletions we found in several exons a reduction of gene dosage to 50 to 69%. In one NF2 patient with previously unknown mutation and a severe phenotype we found the gene dosage of two exons reduced by 50% indicating a deletion of these two exons on one allele. This finding was validated by reverse transcriptase-PCR on fibroblast and schwannoma cell cultures of this patient and cDNA sequencing. Our gene dosage assay will detect deletions of one or more exons as well as gross deletions of the whole coding region of the gene. It can complement the existing screening methods because it is faster and easier. (J Mol Diagn 2005, 7:97-104)

Neurofibromatosis type 2 (NF2, NM 181835) is a highly penetrant, autosomal dominantly inherited disorder, the classical hallmarks of which are bilateral vestibular schwannomas. Additionally, other tumors of the central and peripheral nervous systems such as schwannomas, meningiomas, ependymomas, as well as ocular abnormalities, can be found.¹ NF2 is caused by mutations in a gene coding for the tumor suppressor merlin or schwannomin²⁻⁶ and tumor formation follows the two-hit model.⁷ The majority of germline mutations detected up to present are of the nonsense, frameshift deletions/insertions, splice-site, and missense^{8,9} types. Mutations resulting in a truncation of the protein represent ~95% of all mutational events¹⁰ and seem to be associated with a severe phenotype, whereas missense mutations are more seldom and are assumed to lead to a milder phenotype.^{8,11} Mutations in both alleles of the NF2 gene also cause sporadic schwannomas and the majority (30 to 70%) of sporadic meningiomas.

At present, even when combining mutation detection methods such as SSCP/HA^{10,12-14} of the coding sequence and intron/exon boundaries, DGGE,¹⁵ and sequencing of aberrant polymerase chain reaction (PCR) fragments,^{10-12,14} only 30 to 60% of the constitutional mutations are detected.^{9,15} Deletions of the NF2 gene or complete chromosome rearrangements that are currently investigated using comparative genomic hybridization and fluorescence *in situ* hybridization are found in 16.8% of schwannomas derived from NF2 patients.¹⁶ Bruder and colleagues¹⁷ have developed a high-resolution array-comparative genomic hybridization covering at least 90% of the region around the NF2 locus. Heterozygous or homozygous deletions down to 40 kb can be detected with this method. So far only one group⁹ has developed a dosage PCR analysis to validate loss of heterozygosity analysis. In this work exons 1, 4, 8, and 15 of the NF2

Supported by the Deutsche Krebshilfe (to D.K. and C.O.H.).

Accepted for publication July 30, 2004.

Address reprint requests to Dieter Kaufmann, Department of Human Genetics, University of Ulm, Albert-Einstein-Allee 11, 89070 Ulm, Germany. E-mail: dieter.kaufmann@medizin.uni-ulm.de. C. Oliver Hanemann, Department of Neurology,* Zentrum für klinische Forschung, University of Ulm, Hemholtzstrasse 8/1, 89081 Ulm, Germany. E-mail: oliver.hanemann@medizin.uni-ulm.de.

Table 1. Primers for the Multiplex PCR

Gene	Exon	Forward primer	Reverse primer	(bp)
NF2	1	5'AGCTCTCTCAAGAGGAAGCAAC3'	5'GAGAACCTCTCGAGCTTCCAC3'	146
	2	5'TCCTTCCCCATTGGTTTGTATTG3'	5'CAGGCCACTGTGTCTTGATTGT3'	134
	3	5'TTGAGGGTAGCACAGGAGGAAG3'	5'GGATAAAATTTGGCCAAGAAGTGAA3'	129
	5	5'TGGAGCTGGGAGGGAATGAGAT3'	5'CTTGTGAACACTGGGGTCTGTAG3'	125
	7	5'GTCTTCCGTCTCCCCACAGG3'	5'TAGTTCACACCGTACATCTCCAG3'	83
	8	5'CCACAGAATAAAAAAGGGCACAGAG3'	5'AGGGGGGCAGACAGGGAAAGA3'	198
	9	5'GTTCTGCTTCATTCTCCAGTTTAC3'	5'TGCGCCAAGTGAGATACCATT3'	217
	10	5'TGTATCGGGAACCATGATCTATTTAT3'	5'TGTATCGGGAACCATGATCTATTTAT3'	158
	11	5'GCCCTGTGATTCAATGACTGTTT3'	5'TTGGCCATTGTTGCTTCTTCTTC3'	153
	12	5'TCTGGGCGGGAGAACAGCACA3'	5'ACCTCGGCTTCCAGCACCTTC3'	269
	13	5'GGAGCGAAGAGCCAAGCAGAAG3'	5'CGGGAGGAAAGAGAACATCACC3'	116
	14	5'CACCGTTGCCTCCTGACATACC3'	5'GGGGCTACATACTTTTCTTCTCT3'	121
	15	5'CTGTCTGCCCAAGCCCTGATG3'	5'CTGCCACCCCTGTCTGGAGTTC3'	181
	16	5'GATTTTCAGGCCTATCCAAGCATTTT3'	5'GAGGGCAGAAGCACCATCACCACAT3'	191
	17	5'GGGAGCTGGCTGGGGGTTTC3'	5'AGGGGCTGGGCTCTTCACTCA3'	68
FANCC	5	5'CTGATGTAATCCTGTTTGCAGCGTG3'	5'TCCTCTCATAACCAAACCTGATACA3'	186

Primers used for the detection of deletions in the neurofibromatosis 2 (NF2) gene and the internal control Fanconi anemia complementation group C (FANCC) gene.

gene are amplified together with control amplicons in a multiplex reaction.^{9,14} With this method smaller deletions in the NF2 gene can also be found, but still the loss of any one exon other than the four investigated will be missed. There is currently no data as to how frequently such small deletions occur, either as constitutive or somatic mutation. Methods such as DGGE using cDNA can detect small as well as larger deletions but their sensitivity is often not sufficient. These deficiencies call for fast and reliable methods that can complement the existing screening techniques. With this in mind we have designed a multiplex dosage-PCR assay able to screen the DNA sequences of 15 of the 17 exons (exon 4 and 6 are missing) as well as their flanking introns. We validated our assay using DNA from NF2 patients with known deletions and found it to be extremely reliable. Analysis of patient DNA with previously uncharacterized germline mutations showed this method to be very sensitive and revealed in one case a heterozygous deletion of three exons. We verified the latter result using reverse transcriptase (RT)-PCR and sequence analysis using primary schwannoma and fibroblast cell cultures derived from this patient.

Materials and Methods

DNA Samples

Gene dosage analysis was performed on eight healthy individuals carrying two copies of the NF2 gene, eight NF2 patients with known heterozygous partial deletions of the NF2 gene, four NF2 patients with unknown mutations, and on microdissected cells from a 5- μ m paraffin-embedded formalin-fixed cross-section of a healthy peripheral nerve. All NF2 patients matched the criteria of the National Institutes of Health Consensus Statement on neurofibromatosis in 1987 and additional criteria described by Evans and colleagues.¹ Additionally, two schwannoma cell cultures derived from two different schwannomas of one of the four NF2 patients (NF2/6 tumors 1 and 3) with unknown mutation and a fibroblast

culture of the same patient were used. As a control we used Schwann cell cultures derived from peripheral nerves obtained from surgical patients not carrying any disease predisposing to a peripheral neuropathy. Microdissection was performed on a PixCell2 laser dissection microscope (Arcturus, Moerfelden-Walldorf, Germany). DNA was isolated and preamplified as previously described.¹⁸ For the extraction of genomic DNA from peripheral blood leukocytes of controls and NF2 patients a salting-out procedure was used.¹⁹ DNA was isolated from the schwannoma cell culture with the QiaQuick kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Multiplex PCR

The primers (Thermo Hybaid, Ulm, Germany) for 15 of the NF2 exons and exon 5 of the FANCC gene²⁰ (NM 000136) were chosen within the exon or in the flanking intronic region to generate products that could be exactly identified by their size. The maximum product size was chosen to be under 250 bp so that the assay can also be used on partly degraded DNA, such as that extracted from formalin-fixed paraffin sections. The forward primers were 5'-labeled with the fluorescent dye carboxy-tetramethylrhodamine (TAMRA). The sequences and predicted PCR product sizes are shown in Table 1. According to the different annealing temperatures and the product lengths the primers were combined in two mixes of seven and eight pairs of NF2 primers, respectively, and additionally the primer pair for the internal control FANCC. Mix 1 comprises primers for NF2 exons 2, 11, 12, 13, 14, 15, and 16, and mix 2 for NF2 exons 1, 3, 5, 7, 8, 9, 10, and 17. The PCR reaction was performed in a total volume of 50 μ l using 100 ng of genomic DNA, 0.2 μ mol/L of each primer, 1 $\times$ Qiagen Multiplex PCR Master Mix and for primer mix 2, 0.5 $\times$ Q-Solution. PCR amplification was performed on a MJ Research PTC 100 thermal cycler (Biozym Diagnostik GmbH, Hess. Oldendorf, Germany) using the following

parameters: initial denaturation 95°C (15 minutes), 25 cycles of denaturation (94°C, 30 seconds), annealing (60°C for primer mix 1 and 58°C for primer mix 2, 1 minute 30 seconds) and extension (72°C, 1 minute 30 seconds) followed by a final extension of 1 hour (72°C). For determination of the exponential phase of the PCR amplification for each primer pair, the peak area was plotted against the cycle number. The amount of the multiplex reaction analyzed on the sequencer was optimized for best peak heights. Each peak could be discriminated from additional products and also only negligible amounts of unwanted byproducts occurred.

Quantification of PCR Products and Calculation of Gene Dose

For measuring the gene dose, the labeled PCR products were diluted 1:10 with sterile water. Four μ l of this solution was mixed with 11 μ l of HiDi formamide (Applied Biosystems, Foster City, CA) and analyzed on the ABI Prism 3100 genetic analyzer (Applied Biosystems). The areas of the peaks of interest were measured with the ABI Prism 3100 Data Collection Software, version 1.1. The average of the peak areas of the internal control was defined as a gene dose of 100%. For the patients' DNAs the percentage of the reduction compared to the external controls was determined. All experiments were repeated at least three times. Because of limited amounts of DNA, the gene dosage of one of the schwannoma cell cultures (NF2/6 tumor 1) was only repeated twice. In all cases the gene dosage of the individually amplified fragment was within the same range.

Cell Culture and RNA Isolation

Primary human schwannoma cells derived from a NF2 patient (NF2/6) were cultured as previously described.^{21,22} To determine the degree of fibroblast contamination in these cultures immunostaining with an anti-cow S100 antibody (DAKO, Hamburg, Germany) was performed as described previously.²¹ The fibroblasts of patient NF2/6 were isolated from schwannoma cell culture by seeding the culture in fibroblast medium (Dulbecco's modified Eagle's medium with 5% fetal calf serum). Total RNA of the cell cultures was isolated by a modified guanidinium isothiocyanate method²³ and with the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The cDNA first strand synthesis was performed by using the First Strand cDNA synthesis kit for RT-PCR (Roche, Mannheim, Germany) according to the manufacturer's instructions. The cDNA was incubated with 2 U of RNase H (Invitrogen, Karlsruhe, Germany) for 20 minutes at 37°C. On average 4×10^5 cells were used for one RT-PCR reaction. To amplify part of the *NF2* cDNA we used the oligomers E2_H.3 5'TGGACTGCAGTACA-CAATCAAG3' lying in exon 2 and E11_L.3 5'CAGCCTC-CTCTCCAACATCATCC3' lying in exon 11 yielding a product of 874 bp in normal control cDNA. The products were separated on an 1.5% agarose gel, single fragments eluted from the gel and used for a second or third PCR

with the same primers under the following conditions: 35 cycles of 1 minute at 94°C, 1 minute at 55°C and 1 minute 30 seconds at 72°C.

Quantification of DNA

For quantification of each DNA sample the optical density at 260 nm was measured using a UV spectrophotometer (Ultrospec 3000; Amersham, Amersham Biosciences Europe GmbH, Freiburg, Germany).

Sequencing

Sequence analysis of the PCR products was performed by cycle sequencing with the Big Dye Terminator cycle sequence kit, version 3.1 (Applied Biosystems). Sequences were detected and processed by an ABI Prism 3100 genetic analyzer.

Results

Development and Optimization of the Gene Dosage PCR Assay

In two multiplex PCR reactions we amplified eight and nine products, respectively, products thus yielding a specific pattern of fluorescent peaks, each peak corresponding to an exon. To minimize unwanted byproducts, we optimized the amount of genomic DNA, annealing temperature, cycle number, and addition of property stabilizers such as Q-solution (Qiagen). For optimization we tested 20, 40, 60, 80, 100, and 300 ng of genomic DNA. The amplified products could only be reliably detected and definitely distinguished from unwanted byproducts with 100 or 300 ng of DNA. Under 100 ng, unwanted byproducts reached the same peak height as specific products and the peaks began to broaden, limiting the quality of quantification. For further analysis 100 ng were therefore used. The exponential phase for each primer pair was determined in both multiplex reaction mixes, using 100 ng of DNA and 23 to 34 cycles. The exponential phase was found to start at cycle 24 (data not shown) and at cycle 25 all primer pairs had comparable efficiencies and were in the exponential phase. After 25 cycles the peaks began to split into two, reducing the resolution of the gel matrix and the accuracy of the quantification. We consequently limited the number of cycles to 25 for both mixes. The PCR product of exon 11 was detected as two peaks in all samples, both peak areas were here summated. In Figure 1 electropherograms of one control (Figure 1, a and b) are shown as a representative example.

We compared the peak area of each *NF2* exon fragment with the *FANCC* amplicon. Multiplex PCRs of eight control samples generated similar patterns. Table 2 shows the variation among three controls. The peak areas were first normalized on the *FANCC* amplicon. Gene dosage of control 1 was then defined as 100% and the

Figure 1. Electropherograms of multiplex PCRs. Multiplex PCR of 15 exons of the *NF2* gene performed on DNA of a healthy control, a NF2 patient, and on DNA from microdissected cells from a paraffin section of a normal peripheral nerve. The PCR products were separated on an ABI Prism 3100 genetic analyzer and analyzed with Data Collection software, version 1.1. The y axis displays fluorescence intensity in arbitrary units. Product sizes are written below the respective peaks. **a:** Multiplex PCR of eight *NF2* exons and the internal control *FANCC* (mix 1) on DNA of a healthy control. **b:** Multiplex PCR of the remaining nine *NF2* exons plus *FANCC* (mix 2) on DNA of a healthy control. **c** and **d:** Mix 1 and 2, respectively, multiplex PCR on DNA of NF2 patient 2/6. **e:** Mix 1 on DNA from microdissected cells after preamplification. The percentage of the reduction in relation to the controls for the presented run is written above the respective peaks.

Table 2. Variation amongst Three Controls

	Control 1	Control 2	Control 3
Exon 1	100%	+0.2%	-7%
Exon 2	100%	-9%	-12%
Exon 3	100%	-5%	-7%
Exon 5	100%	+1%	-6%
Exon 7	100%	+0.9%	-12%
Exon 8	100%	-5%	-9%
Exon 9	100%	-2%	-14%
Exon 10	100%	+2%	-11%
Exon 11	100%	-15%	-16%
Exon 12	100%	-11%	-10%
Exon 13	100%	-7%	-14%
Exon 14	100%	-14%	-12%
Exon 15	100%	-16%	-12%
Exon 16	100%	-4%	-6%
Exon 17	100%	+1%	-12%

The peak areas were first normalized on the *FANCC* amplicon, gene dosage of control 1 was then defined as 100% and the variation of the other two from control 1 is shown.

variation of each exon of the other two from control 1 is shown and is less than 17% between all exons.

To validate the method we used eight DNA samples of NF2 patients in which mutations had been defined by a different semiquantitative assay investigating heterozygous genomic deletions in exons 1, 4, 8, and 15 (D.G.E.). The results of this assay are summarized in Table 3. In each sample we detected the expected deletion by a peak reduction to 50 to 67% (average, 58%) of the control peak, indicating a heterozygous deletion (Table 4). As our optimized assay examines more than the four exons, in which the deletions had been described, we were also able to identify additional deleted exons (Tables 3 and 4).

Investigation of NF2 Patients with Unknown Mutations

DNA from four NF2 patients with unknown germline mutations was then investigated. All had previously been screened by direct sequencing of all 17 exons plus part of the flanking intronic sequence. In two cases mutations had been detected (data not shown); one was a point mutation in the splice site acceptor (intron 13), the second a small deletion in exon 15, resulting in a frameshift. Using our gene dosage PCR assay for the other two patients we found in one (patient NF2/6) a deletion of exons 5 and 7 (Figure 1, c and d; Table 5) with a reduction to 52% and 51%, respectively. The other exons tested were not deleted, exon 6 is not included in our assay. The deletion of these two exons corresponds to a novel mutation of the *NF2* gene. In the remaining patients no reduction in gene dosage was detected. We additionally tested genomic DNA from two Schwann cell cultures derived from two different schwannomas of patient NF2/6. In the culture of tumor one (NF2/6 tumor 1) we detected a reduction of exons 5 and 7 to 11% and 13%, respectively, and in culture 2 (NF2/6 tumor 3) no peaks for either of these exon amplicons could be detected. The other exons showed a reduction of ~50% (Table 5). In summary we were able to verify the reduction of the two

Table 3. Deleted and Present Exons in the Known *NF2* Patients

NF2 patient	94/0380	02/3886	02/2890	94/1899	03/2771	03/2772	03/2773	03/2774
Exon 1	—	—	—	—	—	—	—	—
Exon 4	+	+	—	+	—	—	—	—
Exon 8	+	+	—	+	—	—	—	—
Exon 15	+	+	—	+	—	—	—	—

Known heterozygous partial deletions of the *NF2* gene of eight *NF2* patients determined by gene dosage PCR (Mohyuddin et al.⁹). All samples are DNA from peripheral white blood cells.
+, Present; —, deleted.

exons as had already been seen in leukocyte DNA, thus confirming the germline mutation (deletion of exons 5 and 7). Also, the further reduction of these exons in both tumor cultures indicates a homozygous deletion and the 50% reduction of all other exons permits to define the second hit mutation in the tumors as a deletion of the complete *NF2* coding region. The still detectable PCR amplicons in culture 1 could be because of a fibroblast contamination of the cell culture of ~14% as shown by immunostaining with an anti-cow S100 antibody (data not shown). In culture 2 no fibroblast contamination could be detected. So we confirmed the germline mutation, the deletion of exon 5 and 7, and additionally we detected the second hit mutation in the tumor: the deletion of the whole *NF2* coding region.

Validation of the Mutation Identified by Our Assay

So as to substantiate the mutation mentioned above, we performed RT-PCR on RNA derived from one of the schwannoma cell cultures of this *NF2* patient (*NF2/6* tumor 1) and compared the result to a RT-PCR from control Schwann cells. Additionally, we used fibroblasts for RT-PCR. The primers lie in exons 2 and 11 (Figure 2a). In both schwannoma cells and fibroblast culture of patient *NF2/6* we amplified a shortened fragment of ~704 bp versus the expected 874-bp fragment shown in a control (Figure 2, b and c). In the schwannoma cell culture we additionally detected two weak bands, one having the

same length as the undeleted fragment. This is in agreement with the contamination with fibroblasts and the reduction to 11% and 13% in the gene dosage assay instead of 0%. The other band is either a nonspecific product or represents heteroduplexes.

To further analyze the origin of the undeleted wild-type fragments in our schwannoma cell cultures we enriched fibroblasts from this tumor. In the RNA from the fibroblasts of *NF2/6* we detected both the wild-type and the shortened fragment at about the same intensity (Figure 2d), thus again supporting the notion that the weak wild-type band in the schwannoma cell culture was because of fibroblast contamination.

RT-PCR products were sequenced in both forward and reverse orientation using the same primer-pair as for RT-PCR. We confirmed the identical germline deletion of exons 5 and 7 in both schwannoma cells and fibroblasts. Additionally, we found that in both cultures exon 6, which is not included in our multiplexed gene dosage PCR, was deleted. Interestingly, parts of introns 5 and 7 remained in the cDNA, suggesting an activation of cryptic splice sites (Figure 2b). This all results from an in-frame deletion of 76 amino acids and an in-frame insertion of 18 amino acids coming from intronic sequences.

We finally tested our assay on DNA isolated from microdissected cells from a formalin-fixed paraffin section of a normal peripheral nerve. Because this DNA was degraded, we preamplified it by whole genome amplification.¹⁸ Using primer mix 1 we detected all fragments

Table 4. Percentage of the Reduction of Exon Peaks in the Known *NF2* Patients

NF2 patient	94/0380	02/3886	02/2890	94/1899	03/2771	03/2772	03/2773	03/2774
Exon 1	51%—	53%—	55%—	53%—	56%—	55%—	56%—	59%—
Exon 2	91%+	98%+	65%—	94%+	63%—	62%—	62%—	56%—
Exon 3	99%+	103%+	59%—	102%+	55%—	55%—	58%—	57%—
Exon 5	99%+	104%+	57%—	103%+	54%—	55%—	59%—	61%—
Exon 7	96%+	98%+	58%—	97%+	54%—	54%—	57%—	60%—
Exon 8	97%+	99%+	58%—	98%+	60%—	60%—	61%—	52%—
Exon 9	96%+	100%+	54%—	100%+	55%—	54%—	56%—	50%—
Exon 10	93%+	100%+	59%—	100%+	58%—	54%—	59%—	61%—
Exon 11	91%+	95%+	67%—	95%+	63%—	65%—	66%—	61%—
Exon 12	92%+	97%+	63%—	98%+	59%—	58%—	56%—	60%—
Exon 13	93%+	96%+	63%—	97%+	57%—	58%—	58%—	58%—
Exon 14	92%+	94%+	65%—	95%+	62%—	62%—	64%—	58%—
Exon 15	93%+	96%+	60%—	96%+	59%—	60%—	60%—	59%—
Exon 16	95%+	98%+	55%—	98%+	55%—	57%—	55%—	60%—
Exon 17	95%+	96%+	67%—	97%+	58%—	58%—	59%—	53%—

The percentage of the reduction of exon peaks of the eight *NF2* patients with known heterozygous partial deletions of the *NF2* gene (Table 2) as compared to controls. Results are the mean values of at least three independent experiments. The previously examined exons (Table 2) are indicated in gray coloring. In all patients the deletions could be detected by a reduction to 50 to 68% of control value. As we investigated more than four exons in our assay additional deleted exons were found.

+, Present; —, deleted.

Table 5. Percentage of Reduction of Exon Peaks in NF2 Patient 2/6 Compared to Controls

	NF 2/6 peripheral leukocytes	NF 2/6 tumor 1	NF 2/6 tumor 3
Exon 1	96%+/+	59%+/-	55%+/-
Exon 2	91%+/+	63%+/-	53%+/-
Exon 3	102%+/+	66%+/-	54%+/-
Exon 5	52%+/-	11%-/-	0%-/-
Exon 7	51%+/-	13%-/-	0%-/-
Exon 8	91%+/+	69%+/-	66%+/-
Exon 9	96%+/+	63%+/-	59%+/-
Exon 10	97%+/+	60%+/-	63%+/-
Exon 11	93%+/+	57%+/-	64%+/-
Exon 12	93%+/+	59%+/-	51%+/-
Exon 13	96%+/+	55%+/-	56%+/-
Exon 14	93%+/+	58%+/-	63%+/-
Exon 15	97%+/+	57%+/-	54%+/-
Exon 16	100%+/+	57%+/-	51%+/-
Exon 17	90%+/+	57%+/-	51%+/-

Gene dosage analysis was performed on DNA isolated from peripheral blood cells (constitutive mutation) and on genomic DNA of two Schwann cell cultures derived from two different tumors. Results are mean values of three independent experiments. In the peripheral white blood cells, exons 5 and 7 are heterozygously deleted (constitutive mutation) with a reduction to 52% and 51%, respectively. In the two tumors these two exons are homozygously deleted. Additionally, the remaining wild-type allele in the tumor seems to be missing completely, since all exons show a heterozygous reduction to 50 to 69%.

+/+, Present; +/-, heterozygously deleted; -/-, homozygously deleted.

(Figure 1e). To show that the gene dosage is also maintained after the preamplification additional studies need to be done in future work.

Discussion

We present a semiquantitative gene dosage assay for the detection of deletions of either one or several exons or of large regions in the *NF2* gene to complement the existing mutational detection methods. In two multiplex reactions 7 and 8 different exon fragments, respectively, of the 17 exons were amplified plus the internal control, part of exon 5 of the *FANCC* gene. Each primer-pair amplifies parts of the exons, except for the primer pairs for exons 8 and 9, which include the whole exon sequence. The positions were optimized to allow a combination of the primers in only two separate mixes and to permit the discrimination of the amplicons. Additionally the fragment length of our assay was designed to be on the one hand short enough to reduce differences in amplification efficiency and allows its use on partially degraded DNA and on the other hand long enough not to miss smaller deletions in the exons. We optimized the gene dosage assay for an amount of 100 ng DNA. Lower amounts of template DNA resulted in unwanted products reaching the same intensity as the products of interest. We found that it is absolutely necessary to use equal amounts of DNA to assure correct detection of gene dosage. This was achieved by quantification of the samples by measuring the optical density at 260 nm. Further, the preparation procedure of the DNA seems to be important, because it

was difficult to compare directly DNAs isolated with different methods. The DNA of the control samples and the NF2 patients with unknown mutations were isolated by the same method (salting-out procedure¹⁹). The DNA of the schwannoma cell culture was isolated by use of Qia-Quick kit (Qiagen) and that of the patients with previously determined heterozygous deletions by using phenol-chloroform extraction and a different salting-out method. Two exons, 4 and 6, were not included in our assay because we could not find primer pairs compatible with the two primer mixes when considering temperature and product length. However, this will not have any influence on the detection of gross deletions and the majority of small deletions will also be identified by our assay. In addition, this assay was developed to function also on partially degraded DNA extracted from formalin-fixed paraffin sections, thus limiting the fragment size to 250 bp.¹⁸ For primer mix 1 we have already been able to detect all of the products using preamplified DNA from microdissected cells. In one of four NF2 patients with unknown mutations we were able to identify a deletion of three exons which was then validated by RT-PCR and subsequent sequencing of cDNA. This mutation had not been detected by direct sequencing beforehand.

We also detected all previously reported deletions of our eight control patients by a peak reductions ranging from 50 to 69% of the control peak, indicating heterozygous deletions. Because of our optimized assay we were also able to identify additional deleted exons. We suggest that the wide reduction range of less than the theoretical 50% up to 69% is because of small variations in the DNA amount for multiplex reaction. In three of the samples (94/0380, 02/3886, 94/1899) only exon 1 showed a reduction indicating a heterozygous deletion of at least this exon, whereas all of the other exons showed no reduction. In the other five samples all tested exons showed peak areas near to 50%. Therefore it can be assumed that the whole *NF2* locus is deleted on one allele. The deletion of only one exon would be missed by commonly used methods such as microarray comparative genomic hybridization¹⁷ or loss of heterozygosity,²⁴ which are only able to detect large deletions of at least 40 kb or the whole allele, respectively.

As the number of mutations identified in the *NF2* gene by methods optimized for detection of point mutations, deletions of several bp or large deletions is still low, it could be hypothesized that such smaller deletions encompassing only one or a small number of exons may occur quite frequently. Our assay can help to shed light on this. Assays similar to our optimized gene dosage assay are currently in use for other genes such as *MLH1*, *MSH6*,^{25,26} the dystrophin gene,²⁷ and the *SMN* gene²⁸ and its principle has been proven to be very reliable, especially in the detection of heterozygous states.

Acknowledgment

We thank Dr. T. Utermark for the technical assistance in introducing us to the Schwann cell culture.

Figure 2. Validation of the deletion of *NF2* exons 5 and 7 in *NF2* patient 2/6 by RT-PCR on cultured Schwann cells and fibroblasts, respectively, derived from tumor 1. **a:** Schematic representation of the position of the primers used for RT-PCR. The forward primer E2.H.3 corresponds to exon 2, the reverse primer E11.R.3 to exon 11. The product length is indicated for the undeleted fragment. **b:** Schematic representation of the situation found in patient *NF2*/6. Exons 5 to 7 are deleted and parts of introns 5 and 7 are retained in the cDNA. **c:** RT-PCR products amplified from cultured schwannoma cells derived from a healthy control and a tumor of *NF2*/6, respectively. **Arrow 1** shows the 874-bp fragment in a healthy control and also a weak one in the tumor-derived cells. This is probably because of the contamination of the Schwann cell culture with fibroblasts. **Arrow 2** indicates the deleted product of ~704 bp in the *NF2*/6 tumor-derived cells. An additional weak product of ~820 bp in the tumor-derived cells may represent a nonspecific product or heteroduplexes. **d:** RT-PCR on fibroblasts cultured from a tumor of patient *NF2*/6. Two products of ~874 bp and ~704 bp, respectively, with nearly the same intensity are visible. They correspond to the deletion of the three *NF2* exons detected by gene dosage PCR (del) and the wild-type fragment (wt).

References

- Evans DG, Huson SM, Donnai D, Neary W, Blair V, Teare D, Newton V, Strachan T, Ramsden R, Harris R: 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* 1992, 29:841-846
- 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, Fashold R, Dumanski J, de Jong P, Parry D, Eldridge R, Aurias A, Delattre O, Thomas G: Alteration in a new gene encoding a putative membrane organizing protein causes neurofibromatosis type 2. *Nature* 1993, 363:515-521
- Trofatter JA, MacCollin MM, Rutter JL, Murrell JR, Duyao MP, Parry DM, Eldridge R, Kley N, Menon AG, Pulaski K: A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. *Cell* 1993, 75:791-800
- Seizinger BR, Martuza RL, Gusella JF: Loss of genes on chromosome 22 in tumorigenesis of human acoustic neuroma. *Nature* 1986, 322: 644-647
- Wolff RK, Frazer KA, Jackler RK, Lanser MJ, Pitts LH, Cox DR: Analysis of chromosome 22 deletions in neurofibromatosis type 2-related tumors. *Am J Hum Genet* 1992, 51:478-485
- Rouleau GA, Merel P, Lutchman M, Sanson M, Zucman J, Marineau C, Hoang-Xuan K, Demczuk S, Desmaze C, Plougastel B: Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. *Nature* 1993, 363:515-521
- Knudson Jr AG: Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci USA* 1971, 68:820-823
- Rutledge MH, Andermann AA, Phelan CM, Claudio JO, Han FY,

- Chretien N, Rangaratnam S, MacCollin M, Short P, Parry D, Michels V, Riccardi VM, Weksberg R, Kitamura K, Bradburn JM, Hall BD, Proping P, Rouleau GA: Type of mutation in the neurofibromatosis type 2 gene (NF2) frequently determines severity of disease. *Am J Hum Genet* 1996, 59:331–342
9. Mohyuddin A, Baser ME, Watson C, Purcell S, Ramsden RT, Heiberg A, Wallace AJ, Evans DG: Somatic mosaicism in neurofibromatosis 2: prevalence and risk of disease transmission to offspring. *J Med Genet* 2003, 40:459–463
10. MacCollin M, Ramesh V, Jacoby LB, Louis DN, Rubio MP, Pulaski K, Trofatter JA, Short MP, Bove C, Eldridge R: Mutational analysis of patients with neurofibromatosis 2. *Am J Hum Genet* 1994, 55:314–320
11. Evans DG, Trueman L, Wallace A, Collins S, Strachan T: Genotype/phenotype correlations in type 2 neurofibromatosis (NF2): evidence for more severe disease associated with truncating mutations. *J Med Genet* 1998, 35:450–455
12. Jacoby LB, MacCollin M, Louis DN, Mohney T, Rubio MP, Pulaski K, Trofatter JA, Kley N, Seizinger B, Ramesh V: Exon scanning for mutation of the NF2 gene in schwannomas. *Hum Mol Genet* 1994, 3:413–419
13. Evans DG, Wallace AJ, Wu CL, Trueman L, Ramsden RT, Strachan T: Somatic mosaicism: a common cause of classic disease in tumor-prone syndromes? Lessons from type 2 neurofibromatosis. *Am J Hum Genet* 1998, 63:727–736
14. Mohyuddin A, Neary WJ, Wallace A, Wu CL, Purcell S, Reid H, Ramsden RT, Read A, Black G, Evans DG: Molecular genetic analysis of the NF2 gene in young patients with unilateral vestibular schwannomas. *J Med Genet* 2002, 39:315–322
15. Merel P, Khe HX, Sanson M, Bijlsma E, Rouleau G, Laurent-Puig P, Pulst S, Baser M, Lenoir G, Sterkers JM: Screening for germ-line mutations in the NF2 gene. *Genes* 1995, 2:117–127
16. Merel P, Haong-Xuan K, Sanson M, Moreau-Aubry A, Bijlsma EK, Lazaro C, Moisan JP, Resche F, Nishisho I, Estivill X: Predominant occurrence of somatic mutations of the NF2 gene in meningiomas and schwannomas. *Genes* 1995, 3:211–216
17. Bruder CE, Hirvela C, Tapia-Paez I, Fransson I, Segraves R, Hamilton G, Zhang XX, Evans DG, Wallace AJ, Baser ME, Zucman-Rossi J, Hergersberg M, Boltshauser E, Papi L, Rouleau GA, Poptodorov G, Jordanova A, Rask-Andersen H, Kluwe L, Mautner V, Sainio M, Hung G, Mathiesen T, Moller C, Pulst SM, Harder H, Heiberg A, Honda M, Niimura M, Sahlen S, Blennow E, Albertson DG, Pinkel D, Dumanski JP: High resolution deletion analysis of constitutional DNA from neurofibromatosis type 2 (NF2) patients using microarray-CGH. *Hum Mol Genet* 2001, 10:271–282
18. Heinmoller E, Liu Q, Sun Y, Schlake G, Hill KA, Weiss LM, Sommer SS: Toward efficient analysis of mutations in single cells from ethanol-fixed, paraffin-embedded, and immunohistochemically stained tissues. *Lab Invest* 2002, 82:443–453
19. Miller SA, Dykes DD, Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988, 16:1215
20. Morgan NV, Tipping AJ, Joenje H, Mathew CG: High frequency of large intragenic deletions in the Fanconi anemia group A gene. *Am J Hum Genet* 1999, 65:1330–1341
21. Rosenbaum C, Kluwe L, Mautner VF, Friedrich RE, Mueller HW, Hanemann CO: Isolation and characterization of Schwann cells from neurofibromatosis type 2 patients. *Neurobiol Dis* 1998, 5:55–64
22. Utermark T, Kaempchen K, Hanemann CO: Pathological adhesion of primary human schwannoma cells is dependent on altered expression of integrins. *Brain Pathol* 2003, 13:352–363
23. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987, 162:156–159
24. Legoix P, Legrand MF, Ollagnon E, Lenoir G, Thomas G, Zucman-Rossi J: Characterisation of 16 polymorphic markers in the NF2 gene: application to hemizyosity detection. *Hum Mutat* 1999, 13:290–293
25. Charbonnier F, Raux G, Wang Q, Drouot N, Cordier F, Limacher JM, Saurin JC, Puisieux A, Olschwang S, Frebourg T: Detection of exon deletions and duplications of the mismatch repair genes in hereditary nonpolyposis colorectal cancer families using multiplex polymerase chain reaction of short fluorescent fragments. *Cancer Res* 2000, 60:2760–2763
26. Charbonnier F, Olschwang S, Wang Q, Boisson C, Martin C, Buisine MP, Puisieux A, Frebourg T: MSH2 in contrast to MLH1 and MSH6 is frequently inactivated by exonic and promoter rearrangements in hereditary nonpolyposis colorectal cancer. *Cancer Res* 2002, 62:848–853
27. Yau SC, Bobrow M, Mathew CG, Abbs SJ: Accurate diagnosis of carriers of deletions and duplications in Duchenne/Becker muscular dystrophy by fluorescent dosage analysis. *J Med Genet* 1996, 33:550–558
28. Saugier-Verber P, Drouot N, Lefebvre S, Charbonnier F, Vial E, Munnich A, Frebourg T: Detection of heterozygous SMN1 deletions in SMA families using a simple fluorescent multiplex PCR method. *J Med Genet* 2001, 38:240–243