MUTATION REPORT

Deletions of *NF1* gene and exons detected by multiplex ligation-dependent probe amplification

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To estimate the contribution of single and multi-exon *NF1* gene copy-number changes to the *NF1* mutation spectrum, we analysed a series of 201 Italian patients with neurofibromatosis type 1 (NF1). Of these, 138 had previously been found, using denaturing high-performance liquid chromatography or protein truncation test, to be heterozygous for intragenic *NF1* point mutations/deletions/insertions, and were excluded from this analysis. The remaining 63 patients were analysed using multiplex ligation-dependent probe amplification (MLPA), which allows detection of deletions or duplications encompassing ⩾1 *NF1* exons, as well as entire gene deletions. MLPA results were validated using real-time quantitative PCR (qPCR) or fluorescent in situ hybridisation. MLPA screening followed by real-time qPCR detected a total of 23 deletions. Of these deletions, six were single exon, eight were multi-exon, and nine were of the entire *NF1* gene. In our series, deletions encompassing ⩾1 *NF1* exons accounted for ~7% (14/201) of the *NF1* gene mutation spectrum, suggesting that screening for these should now be systematically included in genetic testing of patients with NF1.

Neurofibromatosis type 1 (NF1; OMIM 162200) is an autosomal dominant disorder with a prevalence of approximately 1 in 3000–4000 individuals worldwide. NF1 is clinically characterised by cutaneous neurofibromas, café-au-lait spots, iris hamartomas (Lisch nodules), and freckling of the axillary and inguinal regions, present in >90% of patients at puberty. Other features occurring in fewer patients include plexiform neurofibromas, optic gliomas, scoliosis, pseudoarthrosis, short stature, macrocephaly, learning disabilities, cardiovascular disease and an increased risk of certain malignancies.[1] Diagnosis is based on National Institutes of Health (NIH) consensus clinical criteria defined in 1987 and revised in 1997.[2 3]

NF1 is caused by mutations in the *NF1* gene, which covers 280 kb of genomic DNA, is divided into 61 exons and encodes a transcript of approximately 12 kb.[4–6] The *NF1* gene product, neurofibromin, is a ubiquitously expressed protein, with structural and functional similarities to the mammalian GTPase-activating protein (GAP)-related protein family, a group of evolutionarily conserved proteins.[7–9] The most highly conserved region of the protein is the NF1 GAP-related domain (GRD), which is encoded by *NF1* exons 20–27a and functions by downregulating Ras.[10] Two additional domains of neurofibromin have been described: the cysteine–serine rich domain (CSRD) and the Sec14p domain.[11 12]

The *NF1* gene is thought to be a tumour suppressor gene, as loss of function mutations are associated with benign and malignant tumours in tissues derived from the neural crest, and by myeloid malignancies.[13 14] Most (~90%) of these mutations are small lesions, such as intraexonic deletions or insertions, splicing mutations, and nonsense or missense mutations.[11 15–17] In these cases, the intrafamilial and interfamilial clinical variability of all symptoms is marked, precluding any prognosis regarding patient outcome even if the disease-causing mutation is known, and thus preventing unambiguous molecular diagnosis.[18] A minority (~4%) of patients carry typical 1.2–1.4 Mb deletions that delete the *NF1* gene and its flanking regions.[19 20] These patients generally exhibit a severe phenotype characterised by more neurofibromas at an earlier age, a lower IQ, non-familial facial dysmorphisms, and possibly a higher incidence of malignant peripheral nerve sheath tumours.[21–24]

Usually, NF1 diagnostic screening strategies employ PCR-based screening methods such as the protein truncation test (PTT),[17 25] single-strand conformational polymorphism (SSCP),[26] or denaturing high-performance liquid chromatography (DHPLC)[15 27] with varying degrees of sensitivity for each method. Direct DNA sequencing is then used to confirm and characterise mutations detected by each of these approaches, and fluorescence in situ hybridisation (FISH) is used to detect large *NF1* deletions.[21 28 29] These techniques detect whole gene deletions and small intraexonic deletions/insertions or point mutations. However, they are rarely able to detect deletions and duplications encompassing ⩾1 *NF1* exons. These lesions have been associated with several conditions, such as Fanconi anaemia group A,[30] hereditary non-polyposis colorectal cancer[31] and hereditary breast–ovarian cancer syndrome.[32] Only recently did Wimmer *et al* use reverse transcriptase PCR in combination with multiplex ligation-dependent probe amplification (MLPA) to screen a large cohort of patients with NF1.[33] Single and multi-exon copy number changes were found in approximately 2% of patients with NF1. To re-evaluate the frequency of single and multi-exon copy-number changes in the NF1 population, and to characterise the *NF1* mutation spectrum, we used MLPA to screen a large series of Italian patients with NF1, who were negative for *NF1* point mutations or small insertions/deletions.[15 16] Taking into account all the cases tested for *NF1* mutations in our laboratory, we estimated the contribution of single and multi-exon rearrangements to the *NF1* mutation spectrum in the Italian NF1 population.

MATERIALS AND METHODS

Patients

Between 2000 and 2005, our laboratory tested 201 NF1 unrelated patients by DHPLC and/or PTT, and found 138

Abbreviations: CSRD, cysteine-serine rich domain; DHPLC, denaturing high-performance liquid chromatography; FISH, fluorescence in situ hybridisation; GAP, GTPase-activating protein; MLPA, multiplex ligation-dependent probe amplification; NIH, National Institutes of Health; NF1, neurofibromatosis type 1; OMIM, Online Mendelian Inheritance in Man; PTT, protein truncation test; qPCR, quantitative PCR; SSCP, single-strand conformational polymorphism

Table 1 Primer sequences used for quantitative real-time PCR reactions

Exon	Forward primer	Reverse primer
3	TTTCACTTTTCAGATGTGTGTTG	CTTTGTGAATTTGATCTTGAG
4a	GTTTGAAAATTTTCATAATAGAAA	CTCACAGCAGCTTTGACCTCC
10a	CTACAGTGATAAACAGAGCAT	ATTCCTGCTGCTTTGGTT
11	GAAAGAGCTCAATTTCTTAGC	ACCATAAAACCTTTGGAAGTG
15	ACTTGGCTGTAGCTGATTGA	TCAAGAGTCGCTCAGTAAAGT
22	TGCTACTCTTTAGCTTCCTAC	GGCTGATTGTCTTCTTTTAAGG
23.1	TTTGTATCATTCATTTTGTGTGTA	CTTTTCACATAGAACCGCTGTTTTTT
26	GCTTTGTCTAATGTCAAGTCA	GATAGTGAACACTCTCCGTTTAA
27a	ATGGTCCTGAGGTCTTTTTG	GCCACCAGGCCACTTGTTAG
OMG	GGGTAGAACATGGAGTCCC	AGTTCCAACCAACATGCCC
30	GAAAAAATTTTGGAACTATAAGG	TAACAATTATTCTAAGAGAATTCAAAG
34	TTCTAAATTCAAAATGAAACATGG	AAAAACACTTGCATGGACTG
35	GCATGGACTGTGTTATTGGTA	TCTGTGGATCTTTTAATTGCA
36	GCTGGACCAGTGGACAGAAC	GACGTTTAAATTTGAGGTCAATGA

subjects who were positive for *NF1* mutations.[15 16] The current study group includes the remaining 63 people in whom mutation analysis did not find any pathogenic *NF1* mutation. All patients were diagnosed with NF1 according to NIH diagnostic criteria,[2 3] except for three sporadic patients (patients 131, 182 and 18), who presented only café-au-lait spots at the ages of 1, 2 and 5 years, respectively. All participants were informed about the study and their consent was obtained.

MLPA analysis

Genomic DNA was purified from peripheral blood leucocytes as previously described.[15] Screening for *NF1* single and multi-exon deletions was perfomed using the SALSA P081/082 NF1 V.04 MLPA assay (MRC-Holland, Amsterdam, The Netherlands), as instructed by the manufacturer. This assay consists of two reaction mixes containing probes for all constitutive *NF1* exons, with the exception of exons 5, 7, 17, 19a, 45, and 47. An aliquot of ~100 ng of denatured genomic DNA was used in the overnight annealing of the exon-specific probes and subsequent ligation reaction. PCR was carried out with FAM-labelled primers using 10 µl of ligation reaction. Separation and relative quantification of the amplification products were carried out using an ABI Prism 3100 Genetic Analyzer (Applied Biosystem, Foster City, California, USA). The peak area for each fragment was measured with GeneScan Analysis software V.3.7 (Applied Biosystems), and normalised by dividing it by the combined area of all peaks in that lane. This normalised peak area was then divided by the average normalised peak area from five normal controls. With this method, the results given are allele copy numbers compared with normal controls, and a ratio of ~1 should be obtained if both alleles are present. A reduction or increase in the peak area values to <0.7 or >1.3 was considered an indication of a deletion or a duplication, respectively. DNA samples showing such a reduction or increase in the MLPA peak area values were reanalysed by MLPA, and only the samples showing consistent results between the two experiments were considered positive for a deletion or duplication. Another MLPA (SALSA P122 NF1 area) assay specifically designed to detect whole *NF1* gene deletions was also used, and the same procedure was followed. In particular, the SALSA P122 NF1 area assay contained four probes centromeric to *NF1* (in genes *CRLF3*, *FLJ12735*, *CENTA2*, and *RNF135*), five intragenic probes (*NF1* exons 1, 12b, 23–2, 40, 48), and three probes telomeric to the *NF1* gene (in *HCA66*, *JJAZ1* and the *KIAA0563-related gene*).

Real-time PCR

DNA copy-number changes identified by MLPA were confirmed using an ABI 7000 Sequence Detection System (Applied Biosystems) and the DNA-binding dye SYBR Green (Invitrogen Corporation, Carlsbad, California, USA). To account for possible variation related to DNA input amounts or the presence of PCR inhibitors, the reference gene *ZNF80* was simultaneously quantified in a separate tube for each patient sample. SYBR Green amplification mixtures (25 µl) contained SYBR Green master mix, 150 nmol/l of each forward and reverse primer, and 60 ng of template DNA. The PCR cycling conditions were as follows: 2 minutes at 50℃, 2 minutes at 95℃, 40 cycles of 95℃ for 15 seconds and 60℃ for 30 seconds, and a final step at 72℃ for 30 seconds; primer sequences are shown in table 1. After PCR amplification, a melting curve was generated for every PCR product to check the specificity of the PCR reaction (absence of primer dimers or other nonspecific amplification products). Each assay included a no-template control, 60 ng of a normal control DNA used as a calibrator, and approximately 10 ng of test DNA (in triplicate). Each sample was combined with two non-deleted negative controls (in triplicate) and three deleted positive controls (in triplicate). The deleted control had previously been proven to carry a whole *NF1* gene deletion by FISH using probes specific for the NF1 locus (data not shown). The threshold cycle (Ct) values of SDS software V.2.3 (Applied Biosystems) were exported to Excel (Microsoft Corp., Seattle, Washington, USA) for further analysis. The ΔΔCt calculation for the relative quantification of target was used as follows

$\Delta\Delta Ct$ = (Ct, target *NF1* exon – Ct, *ZNF80*)χ – (Ct, target *NF1* exon – Ct, *ZNF80*)y,

where χ = unknown NF1 sample and y = calibrator. Results for each sample were expressed in N-fold changes in χ *NF1* gene copies, and normalised to *ZNF80* relative to the copy number of the *NF1* gene in the calibrator according to the following equation: amount of target = $2^{-\Delta\Delta Ct}$.[34]

Cases showing N-fold ⩽ the maximum N-fold copy number observed among the deleted positive controls were considered deleted. Cases showing N-fold > the maximum N-fold copy number observed among the deleted positive controls and < the minimum N-fold copy number observed among the non-deleted negative controls were considered equivocal. Cases showing N-fold ⩾ the minimum N-fold copy number observed among the non-deleted negative controls were considered non-deleted.

Fluorescence in situ hybridisation

FISH analysis was undertaken using four probes (RP11-353O18, RP11-17I16, CTD-2283L18 and CTD-3060L5) selected from a public database (http//genome.ucsc.edu). The RP11-353O18 clone spans from *NF1* intron 1 to *NF1* intron 27b. The RP11-17I16 probe covers the residual area of *NF1* and part of the flanking *RAB11-FIP4* gene. The CTD-2283L18 and

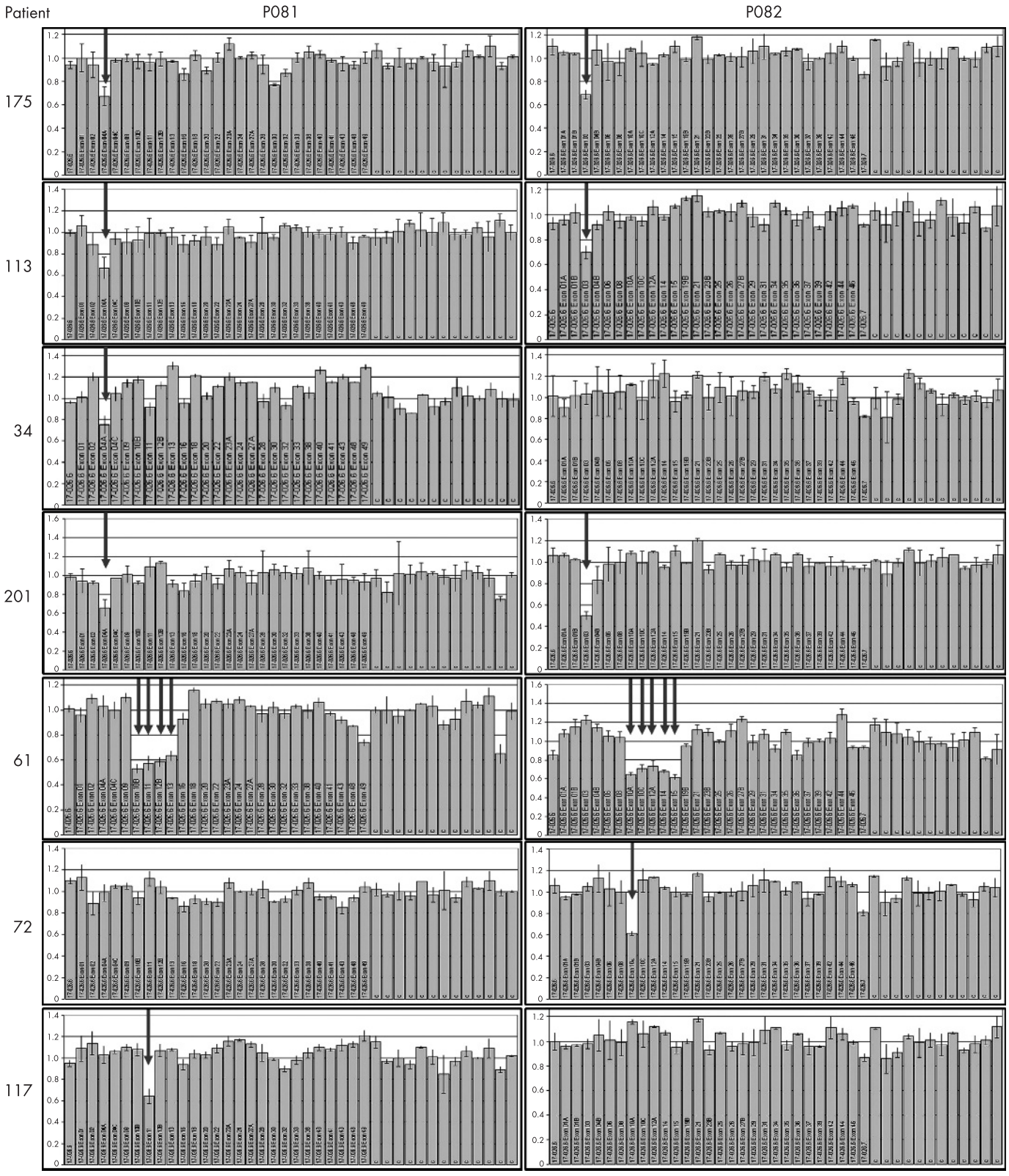

Figure 1 Single deletions detected by multiplex ligation-dependent probe amplification and confirmed by quantitative real-time PCR. Normalised relative peak areas of all *NF1* gene-specific and control probes are shown. Sequences present in two copies of the genome have a relative peak area value of approximately 1.0. A reduction in the peak area values to <0.7 indicates a deletion (black arrows).

CTD-3060L5 probes encompass the *JJAZ1* and *LRRC37B* genes, respectively. Clones were obtained from the Sanger Institute (http://www.sanger.ac.uk). Probe labelling and hybridisation were carried out as previously reported.[35]

RESULTS

Molecular analysis

In total, 63 subjects who tested negative for *NF1* point mutations and intragenic insertions/deletions were analysed

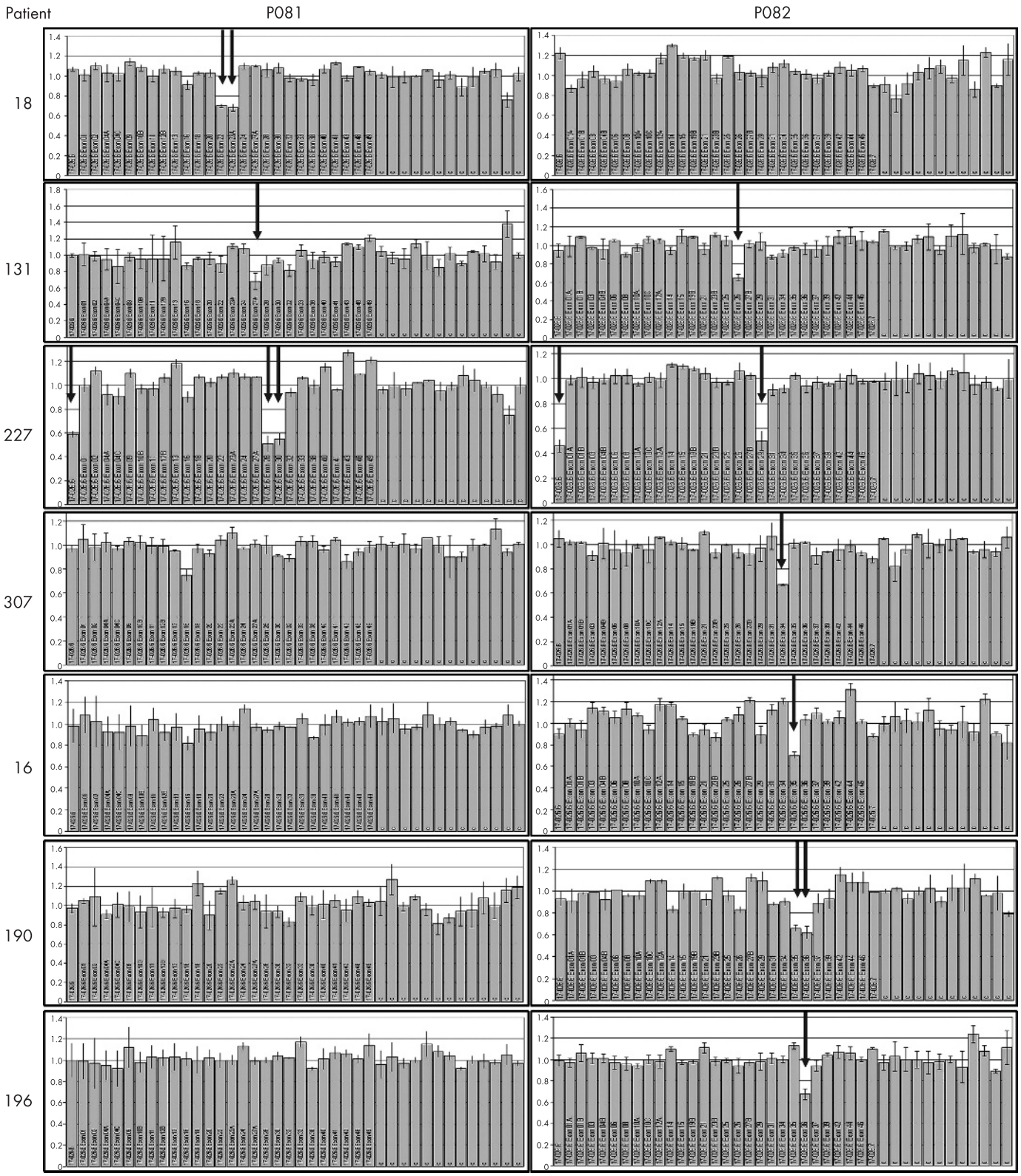

Figure 1 cont'd Multi-exon deletions detected by multiplex ligation-dependent probe amplification and confirmed by quantitative real-time PCR. Normalised relative peak areas of all *NF1* gene-specific and control probes are shown. Sequences present in two copies of the genome have a relative peak area value of approximately 1.0. A reduction in the peak area values to <0.7 indicates a deletion (black arrows).

using the SALSA P081/082 NF1 MLPA assay for *NF1* copy-number changes. Gene electropherograms showed reductions of specific MLPA fluorescence signals in 23 cases compared with controls. After MLPA testing, all cases showing abnormal signals were reanalysed by real-time PCR using SYBR Green as the detection system. Single exon deletions were corroborated by a single real-time PCR assay corresponding to the deleted exon, and multi-exon deletions were confirmed by reanalysing, using two separate real-time PCR assays, the most distal and the most proximal exons encompassed by the

deletions predicted by MLPA. All putative deletions identified by MLPA were confirmed by real-time PCR. In total, MLPA followed by real-time quantitative PCR (qPCR) detected 23 *NF1* deletions including 6 single exon deletions, 8 multi-exon deletions, and 9 large deletions encompassing the entire *NF1* gene. In patient 111, MLPA gave ambiguous results with all exons showing area values higher than those of deleted exons, but lower than undeleted exons, suggesting the presence of a mosaic whole-gene deletion. In patient 307, carrying a multi-exon deletion, the real-time confirmation for the most proximal MLPA probe (*OMG* gene) gave ambiguous results ($2^{-\Delta\Delta Ct} = 0.57$). However, results were borderline compared with deleted positive controls ($0.49 < 2^{-\Delta\Delta Ct} < 0.56$) and clearly below non-deleted negative controls ($0.92 < 2^{-\Delta\Delta Ct} < 0.99$). Furthermore, the most distal MLPA probe (exon 30) of this deletion was consistently deleted by real-time PCR, thus confirming this deletion. In total, single and multi-exon *NF1* deletions were found in 14/201 (~7%) patients with NF1 (fig 1), whereas whole *NF1* gene deletions were detected in 9/201 (~4.5) NF1 individuals. Real-time qPCR results are reported in table 2; a list of all single and multi-exon deletions detected by MLPA and confirmed by quantitative real-time PCR is reported in table 3. To verify the absence of a point mutation residing within the corresponding MLPA probe, the DNA from all patients carrying single exon deletions or the recurrent deletion of exons 3 and 4a were sequenced. Sequence analysis did not reveal any point mutation in the DNA fragment recognised by MLPA probe in these exons (supplementary fig 1; available at http://jmg.bmj.com/supplemental).

The SALSA P081/082 NF1 MLPA assay detected nine cases carrying a whole *NF1* gene deletion. These results were corroborated using the SALSA P122 NF1 area assay, which consists of 12 probes covering the entire *NF1* gene and its flanking genes. The SALSA P122 NF1 area assay was previously proven to distinguish between the 1.4 Mb deletions (type I) encompassing 14 genes, with breakpoints in the *NF1* low-copy repeats, and the 1.2 Mb deletions (type II), which cover 13 genes and are mediated by recombination between the *JJAZ1* gene and its pseudogene.[33 36] The SALSA P122 AREA MLPA assay confirmed each of the whole gene deletions identified by SALSA P081/082 NF1 MLPA assay. In particular, SALSA P122 NF1 area revealed six cases carrying a type I deletion and three cases with a type II deletion (table 4).

All deletions covering the entire *NF1* gene detected by MLPA were confirmed by FISH using a set of probes previously proven to distinguish between type I and II deletions. FISH was performed on peripheral blood cells by analysing a total of 30 metaphases for each case (table 4). In patient 111, FISH analysis detected a type I mosaic deletion in which 66% of 50 metaphases showed a single chromosome 17 signal, whereas the remaining 34% had two signals.

In all, single and multi-exon deletions were found in 14/201 (~7%) cases, whereas entire gene deletions were detected in 9/201 (~4.5%) patients.

Clinical results

After genetic testing, the clinical charts of patients with single and multi-exon deletions and of patients carrying whole gene deletions were reviewed. The group of patients with single and multi-exon deletions comprised 14 unrelated patients (12 female and 2 male); 9 cases were sporadic and 5 had a family history of NF1. Mean age at the time of the examination was 23.9 (range 1–48) years. A mean of 2.7 diagnostic criteria was present in each patient. All patients with single and multi-exon deletions fulfilled the NIH Consensus Criteria for NF1, except for two sporadic patients (131 and 18), who presented only café-au-lait spots at the age of 1 and 5 years, respectively. In the subjects with whole gene deletions (four males and five female patients, median age of 27.2 (range 8–47) years), a mean of 3.1 diagnostic criteria was observed. In this group, only one subject had a family history of NF1. The clinical manifestations identified in patients with either single and multi-exon *NF1* deletions or whole *NF1* gene deletions are summarised in table 5.

Table 2 Quantitative real-time PCR results*

		Sample†	Non-deleted controls‡			Deleted controls§		
Patient no	Exon	Mean	Min	Max	Mean	Min	Max	Mean
175	3	0.70	0.96	1.03	1.00	0.69	0.72	0.70
	4a	0.46	0.86	1.14	0.99	0.44	0.53	0.50
113	3	0.55	0.96	1.03	1.00	0.69	0.72	0.70
	4a	0.26	0.86	1.14	0.99	0.44	0.53	0.50
34	4a	0.44	0.86	1.14	0.99	0.44	0.53	0.50
201	3	0.58	0.96	1.03	1.00	0.69	0.72	0.70
	4a	0.37	0.86	1.14	0.99	0.44	0.53	0.50
61	10a	0.68	0.81	0.95	0.89	0.64	0.68	0.67
	15	0.32	0.85	1.00	0.95	0.44	0.56	0.49
72	10a	0.65	0.89	1.05	0.98	0.64	0.68	0.67
117	11	0.69	0.89	0.97	0.93	0.67	0.70	0.69
18	22	0.48	0.84	0.92	0.87	0.49	0.55	0.51
	23.1	0.49	0.87	1.09	0.96	0.47	0.53	0.49
131	26	0.43	0.87	1.20	1.01	0.44	0.53	0.47
	27a	0.57	0.83	0.94	0.89	0.47	0.57	0.51
227	34	0.65	0.95	1.02	0.97	0.64	0.69	0.66
307	*OMG*	0.57	0.92	0.99	0.95	0.49	0.56	0.53
	30	0.56	0.94	0.96	0.95	0.52	0.58	0.56
16	35	0.60	0.88	0.96	0.92	0.59	0.65	0.61
190	35	0.33	0.88	0.96	0.92	0.59	0.65	0.61
	36	0.55	0.74	1.20	0.90	0.54	0.59	0.56
196	36	0.55	0.74	1.20	0.90	0.54	0.59	0.56

Min, minimum; Max, maximum.
*Results for unknown NF1 samples, non-deleted negative controls, and deleted positive controls are expressed in N-fold changes in *NF1* gene copies, normalised to *ZNF80* relative to the copy number of the *NF1* gene in the calibrator, according to the following equation: amount of target $= 2^{-\Delta\Delta Ct}$.[34]
The ΔΔCt calculation used for the relative quantification of target was as follows: ΔΔCt = (Ct, target *NF1* exon − Ct, *ZNF80*)χ−(Ct, target *NF1* exon−Ct, *ZNF80*)y, where χ = unknown NF1 sample (†), nondeleted negative control (‡) or deleted positive control (§), and y = calibrator.

Table 3 Single and multi-exon deletions detected by multiplex ligation-dependent probe amplification and confirmed by real-time PCR

Patient no	Deleted exons	Type of deletion
175	3 and 4a*	Multi-exon
113	3 and 4a*	Multi-exon
34	4a*	Single exon
201	3 and 4a*	Multi-exon
61	10a to 15	Multi-exon
72	10a*	Single exon
117	11*	Single exon
18	22 and 23.1	Multi-exon
131	26 and 27a	Multi-exon
307	IVS27b(*OMG*) to 30	Multi-exon
227	34*	Single exon
16	35*	Single exon
190	35 and 36	Multi-exon
196	36*	Single exon

*These deletions were sequenced and none of the corresponding exons were found to carry a point mutation within the corresponding multiplex ligation-dependent probe amplification (MLPA) probe (supplementary fig 1; available at http://jmg.bmj.com/supplemental).

DISCUSSION

Hundreds of mutations have been reported in the *NF1* gene, although no clear genotype–phenotype correlation has been identified to date.[37] The only exceptions are deletions of the entire *NF1* gene, which are present in approximately 4% of patients with NF1, generally associated with a severe form of the disease,[21 22 24] and a 3-bp deletion in *NF1* exon 17, which has been recently associated with the absence of neurofibromas.[38] Although entire gene deletions have been intensely studied, smaller rearrangements encompassing ⩾1 *NF1* exons have been investigated to a lesser extent as they are difficult to detect using standard molecular genetics techniques. To better investigate smaller *NF1* rearrangements, we performed MLPA screening of a large series of patients affected by NF1 for whom the presence of point mutations, small deletions and insertions had been previously excluded by DHPLC and/or PTT.[15 16]

Single and multi-exon *NF1* copy-number changes, exclusively represented by intragenic deletions in our series, were found in approximately 7% of the patients with NF1. This frequency is lower than in a previous smaller study, in which single or multi-exon deletions were found in 3/30 (10%) of patients with NF1 with high/low grade malignant peripheral nerve sheath tumours,[39] but is higher than in a second very large study in which single and multi-exon copy-number changes were detected in only 25/1100 (~2%) of the cases.[33] In the latter study, most of the samples were screened using an RNA-based approach, whereas all our samples were screened by MLPA using genomic DNA as a starting material. Very recently, the same authors reported the identification of 5/97 (~5%) intragenic deletions in a cohort of Austrian patients with NF1 meeting the NIH criteria,[40] a result more commensurate with our findings.

The spectrum of genomic rearrangements disclosed by MLPA was characterised by a wide range of single and multi-exon deletions, distributed along almost the entire sequence of the *NF1* gene. Most of the deletions were unique, although the deletion of exons 3 and 4a was found in several patients. The presence of this lesion was confirmed by two independent real-time qPCR assays using primers for exon 3 and exon 4. No point mutations that might alter the binding of the MLPA probe to genomic DNA were detected in exons 3 and 4a in any of the patients carrying this lesion. Alone, deletion of exons 3 and 4a represents 13% of all lesions detected by MLPA. However, considering all the participants of this study, this lesion is responsible for only 1.5% of patients with NF1, and therefore priority screening for this lesion is unnecessary, in our opinion. With the exception of the multi-exon deletion of exon 22 and 23.1, which has been previously reported,[33] all other lesions detected in this study were new. Most of the deletion breakpoints were unique, although some introns harboured more breakpoints than did others. For example, four breakpoints were mapped to intron 4a, three to introns 2 and 34, and two to intron 36. The fine characterisation of these breakpoints is ongoing in our laboratory with the intention of determining the molecular mechanisms underlying these deletions and of designing long-range PCR assays for their rapid confirmation.

Table 4 MLPA and FISH results showing type I and type II deletions of the entire *NF1* gene detected in patients with NF1

Patients	55	248	208	45	71	111	27	318	305
MLPA									
Centromeric probes									
CRLF3	−	−	−	−	−	−	−	−	−
FLJ12735	−	−	−	−	−	−	−	−	−
CENTA2	−	−	−	−	−	−	−	−	−
RNF135	−	−	−	−	−	−	−	−	−
Intragenic probes									
Exon 1	−	−	−	−	−	−	−	−	−
Exon 12B	−	−	−	−	−	−	−	−	−
Exon 23–2	−	−	−	−	−	−	−	−	−
Exon 40	−	−	−	−	−	−	−	−	−
Exon 48	−	−	−	−	−	−	−	−	−
Telomeric probes									
HCA66	−	−	−	−	−	−	−	−	−
JJAZ1	+	−	−	+	−	−	−	+	−
KIAA0563–related gene	+	−	−	+	−	−	−	+	−
FISH									
RP11–353O18	Del	Del	Del	Del	Del	Del	Del	Del	Del
RP11–17I16	Del	Del	Del	Del	Del	Del	Del	Del	Del
CTD–2283L18	Non-Del	Del	Del	Non-Del	Del	Del	Del	Non-Del	Del
CTD–3060L5	Non-Del	Del	Del	Non-Del	Del	Del	Del	Non-Del	Del
Deletion type	II	I	I	II	I	I	I	II	I

+, MLPA probes showing peak area values between 0.7 and 1.3; −, MLPA probes showing peak area values <0.7. Del, deleted; Non-del, non-deleted.

Table 5 Clinical features in 23 patients with neurofibromatosis type 1 carrying either single and multi-exon *NF1* deletions or whole *NF1* gene deletions

Patients	Age at observation (years)	Sex	Family history	CLS	CNf	SNf	PNf	AF	Sc	OG	MR	PA	TD	Other tumours	Other features
Patients carrying single or multi-exon deletions															
201	28	F	S	+	−	−	+	NE	−	−	−	−	−	Astrocytoma	−
131	1	F	S	+	−	−	−	−	−	−	−	−	−	−	−
113	26	F	S	+	−	+	−	+	−	−	−	−	+	−	Hypothyroidism, UBOs, seizures
175	48	F	FH	+	−	+	−	−	+	−	−	−	−	Neurinoma, meningioma, adrenal adenoma	−
227	30	F	FH	+	+	−	−	+	−	−	−	−	−	−	−
61	37	M	S	+	+	+	+	−	−	−	−	−	−	−	H
117	30	F	S	+	+	−	−	−	−	−	−	−	−	Acoustic neurinoma	PE
72	26	F	FH	+	+	−	+	+	+	−	−	−	−	Schwannoma	BN, thyroid nodules
196	25	F	S	+	−	−	−	+	−	−	−	−	−	−	−
34	9	F	FH	+	−	−	−	−	−	−	−	−	−	−	−
190	15	M	S	+	−	−	−	+	−	−	−	−	−	−	−
16	20	F	S	+	+	−	−	−	−	+	−	−	−	−	−
18	5	F	S	+	−	−	−	−	+	−	−	−	−	−	−
307	34	F	FH	+	−	+	−	+	−	−	−	−	−	−	−
Patients carrying whole gene deletions															
55	35	M	S	+	+	+	+	+	+	−	−	−	−	−	−
248	35	F	S	+	+	+	+	+	+	−	+	−	−	−	Facial dysmorphism
208	11	F	S	+	+	−	−	+	+	−	+	−	−	−	Chest anomalies, PVS
45	26	M	S	+	+	+	−	+	−	−	−	−	−	−	P
71	36	M	S	+	+	−	+	+	+	−	−	−	−	−	−
111	47	F	S	+	+	−	−	+	+	−	−	−	−	−	H, PE
27	33	M	FH	+	−	+	+	+	+	−	−	+	+	−	E
318	8	F	S	+	−	−	−	+	−	−	−	−	−	−	Facial dysmorphism
305	14	F	S	+	+	−	−	+	−	−	+	−	−	−	−

+, Present; −, absent; AF, axillary freckling; BN, Becker naevus; CLS, café-au-lait spots; CNf, cutaneous neurofibromas; E, epilepsy; F, female; FH, positive family history; H, hypertension; M, male; MR, mental retardation; NE, not evaluated; OG, optic glioma; P, ptosis; PE, pectus excavatum; PNf, plexiform neurofibromas; PA, pseudoarthrosis; PVS, pulmonar valvular stenosis; Sc, scoliosis; S, sporadic; SNf, subcutaneous neurofibromas; TD, tibial dysplasia; UBOs, unidentified bright objects.

Patients carrying whole *NF1* gene deletions are usually affected by a more severe form of NF1, characterised by a high number of neurofibromas and plexiform neurofibromas, facial dysmorphism, mental retardation and a higher risk of malignancies.[21 22 24] These patients represent approximately 4% of the entire NF1 population, according to a large FISH study.[20] Using MLPA, we were able to detect a whole gene deletion in 9/201 (4.5%) patients with neurofibromatosis type 1, indicating that MLPA sensitivity for whole gene deletions is quite comparable with FISH. Most of the whole *NF1* gene deletions are of two types: (1) type I is a 1.4 Mb germline deletion, with breakpoints mapping in low-copy repeats termed NF1-LCR,[41] and (2) type II spans 1.2 Mb and is caused by aberrant recombination of the *JJAZ1* gene and its pseudogene.[36] Most patients with sporadic NF1 who have type II deletions are mosaic with normal cells and usually show a less severe phenotype than patients with type I deletions.[36] Consequently, the development of sensitive, reliable and easy to use methods to differentiate between type I and type II deletions has important clinical implications for the management of patients with NF1. In our study, the SALSA P122 NF1 area assay, which is specifically designed to detect and characterise whole gene deletions, was able to distinguish between type I and II deletions. The MLPA results were corroborated by FISH using a probe set previously proven to distinguish between type I and II deletions. Similarly, another study has previously shown that the SALSA P122 NF1 area assay can be used to distinguish between type I and II deletions.[33] Previous and current results confirm that the SALSA P122 NF1 area assay could be used as a reliable alternative to identify whole gene deletions in NF1 laboratories where FISH is not available. Both MLPA and FISH were not able to confirm the mosaic status of type II deletions in our cases, but MLPA did detect a mosaic for a type I deletion, which was further confirmed by FISH. However, this type I deletion mosaic affected 66% of peripheral blood cells, whereas mosaics for type II deletions usually involve >90% of peripheral blood cells,[36] and thus would have been detected with difficulty by our FISH, which was performed by analysing 30 peripheral blood metaphases.

In general, subjects with whole gene deletions presented with a more severe phenotype than those carrying single and multi-exon deletions. For example, plexiform neurofibromas were found in 44% of patients with a whole gene deletion, but occurred in only 25% of the cases with single and multi-exon deletions. Similarly, scoliosis was found at higher frequency in patients with whole gene deletions compared with those with partial gene deletions. Facial dysmorphism and mental retardation, which are hallmarks of whole gene deletions, were observed in two and three patients with a whole gene deletion, respectively, but were not observed in patients with single or multi-exon deletions. The severe NF1 phenotype associated to whole gene deletions is usually explained by the large size of the lesion, which spans approximately 1.2–1.4 Mb of genomic DNA, resulting in the haploinsufficiency of 14 different genes.[36 41] In comparison, single and multi-exon deletions of *NF1* are relatively small lesions, usually involving no genes other than *NF1*. Interestingly, all patients with mental retardation (patients 248, 208 and 305) carried a type I germline deletion, but none had a type II deletion. This observation is in accordance with the notion that patients with NF1 with type I deletions generally show a more severe phenotype than patients with type II deletions.[36] In one case

(patient 307), we identified a deletion involving three *NF1* exons and the small *OMG* gene in *NF1* intron 27b. This woman presented with café-au-lait spots, axillary freckling and subcutaneous neurofibromas at 34 years of age, suggesting that *OMG* is unlikely to be the gene responsible for the NF1 complications (ie mental retardation and facial dysmorphism) observed in whole gene deletion carriers. Accordingly, a previous screening of *OMG* gene for point mutations in patients with non-syndromic mental retardation failed to demonstrate nucleotide variants of clear significance.[42] Patient 208, carrying a whole *NF1* gene deletion, had Watson syndrome, presenting with café-au-lait spots, neurofibromas, mental retardation, thoracic abnormalities and pulmonary stenosis. Watson syndrome has been reported previously in a patient carrying an *NF1* tandem duplication, as well as in other patients carrying small deletions and point mutations,[37] thus showing that this syndrome is associated with a wide range of *NF1* gene abnormalities.

In our hands, the MLPA technique gave several false positive results, including reduced MLPA signals for exons 13a and 18, and for both probes in intron 27b recognising the *OMG* gene. For this reason, it is our recommendation to (1) reassess every MLPA-positive sample by a second MLPA experiment, and (2) confirm every putative lesion identified by MLPA with an alternative technique. In this regard, quantitative real-time PCR with SYBR Green has several advantages, including the requirement for small amounts of DNA and low costs owing to use of the same primers used for DHPLC and sequence analysis. Furthermore, samples carrying whole gene deletions can be used as reliable positive controls for real-time PCR. We also suggest running each sample with a non-deleted negative control as normal DNA reference for real-time PCR. It has also been reported that false positive MLPA signals could be due to the presence of subtle point mutations under the MLPA probe, which may impair probe hybridisation and mimic the presence of a deletion.[33] To exclude this possibility, we suggest sequencing the MLPA probe corresponding region in all cases carrying recurrent deletions or single exon deletions.

In our series, single and multi-exon *NF1* deletions were responsible for ~7% of the mutations in our patient group. The spectrum of these lesions was heterogeneous, with a similar proportion of single and multi-exon deletions. In previous studies, whole gene deletions were found in 4% of the patients,[20] and using DHPLC followed by direct sequencing, we identified a point mutation or a small deletion/insertion in 138/201 (68.7%) patients with NF1. By combining the mutations previously identified by DHPLC with the genomic rearrangements detected in this study by MLPA, we have been able to find the disease-causing lesion in 161/201 (80.1%) of our patients with NF1. We hypothesise that mutations affecting regulatory portions of the gene might also have a pathogenic role in NF1. Although mutations affecting the *NF1* promoter have not been reported to date, lesions in other non-coding portions are to be expected.[43] Furthermore, we cannot exclude the possibility that some mutations have not been detected by our protocol.

In conclusion, MLPA analysis followed by real-time PCR revealed 23 genomic rearrangements in a series of 201 patients with NF1. These data suggest the possibility of adding MLPA to DHPLC in routine diagnostic procedures for patients with NF1. Furthermore, to reduce the time of analysis, MLPA should be used as a priority in patients presenting with severe NF1, possibly reflecting deletion of the entire *NF1* gene.

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