Mutation spectrum of NF1 gene in Italian patients with neurofibromatosis type 1 using Ion Torrent PGM™ platform

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ABSTRACT

Neurofibromatosis type 1 (NF1) is caused by mutations of the NF1 gene and is one of the most common human autosomal dominant disorders. The patient shows different signs on the skin and other organs from early childhood. The best known are six or more café au lait spots, axillary or inguinal freckling, increased risk of developing benign nerve sheath tumours and plexiform neurofibromas. Mutation detection is complex, due to the large gene size, the large variety of mutations and the presence of pseudogenes. Using Ion Torrent PGM™ Platform, 73 mutations were identified in 79 NF1 Italian patients, 51% of which turned out to be novel mutations. Pathogenic status of each variant was classified using "American College of Medical Genetics and Genomics" guidelines criteria, thus enabling the classification of 96% of the variants identified as being pathogenic. The use of Next Generation Sequencing has proven to be effective as for costs, and time for analysis, and it allowed us to identify a patient with NF1 mosaicism. Furthermore, we designed a new approach aimed to quantify the mosaicism percentage using electropherogram of capillary electrophoresis performed on Sanger method.

1. Introduction

Neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant inherited disorders, with a prevalence of about 1/2500 individuals (Shen et al., 1996; Williams et al., 2009), caused by heterozygous mutation in the neurofibromin gene (NF1) on chromosome 17q11.2 (Byard, 2007). Diagnosis of NF1 is based on clinical criteria. The main features are prevalently over the skin as follows: café au lait macules, freckling on the arm pits and/or on the groins, sometime subcutaneous or plexiform neurofibromas, iris Lisch nodules. NF1 is one of the largest human genes, composed of 57 exons (transcript reference, NM_000267.3). It encodes for neurofibromin, a large cytoplasmic protein which has a role in tumour suppression. NF1 gene presents with one of the highest mutation rates in the hu-

man genome (Yohay, 2006). More than 2000 mutations have been reported (Ko et al., 2013), most of which are point and splicing mutations; intragenic deletions are rare (Terribas et al., 2013). Half of patients with NF1 have no family history of the disorder.

PCR and Sanger sequencing in large genes is time-consuming and expensive. Next Generation Sequencing, instead, is becoming a major drive providing a powerful and efficient way to study genetic diseases, although this process has still some limitations as for the insertion/deletion detection.

In our study, we used a middle-throughput targeted NGS Ion Torrent PGM, to determine the genetic background in patients with clinical diagnosis of NF1. The mosaicism percentage was then quantified using electropherogram of capillary electrophoresis performed on Sanger method.

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2. Materials and methods

2.1. Patients

NF1 diagnosis was performed by expert clinicians and based on the presence of two or more of the diagnostic criteria proposed by the National Institutes of Health Consensus Development Conference (NIH Consensus Development Conference Statement, 1988). The study was approved by the relevant local Institutional Ethical Committees and informed consent was obtained from each patient. Seventy-nine Italian patients (40 males and 39 females) with suspected or clinically diagnosed NF1 were included in this study. The mean age was 12 for females (range: 1-to-45 years, SD ± 9.79) and 14 for males (range: 2-to-64 years; SD ± 11.50). Fifty-three out of 79 patients included in the study came from the Sicilian island, Italy. Thirty out of 79 were investigated for related affected or unaffected members.

2.2. NGS sequencing

gDNA was isolated from lymphocytes using the salt chloroform extraction method, checked for degradation on agarose gel, and was quantified by the Qubit 2.0 Fluorometer with the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Foster City, CA). NF1 primers were designed using the AmpliSeq Designer software (Life Technologies, CA, USA), targeting the complete coding sequence of NF1 gene (transcript reference, NM_000267.3) plus flanking intron sequencing whose precise position is indicated in Supplementary Table 1, according to the methods suggested by Balla et al. (2014) and Pasmant et al. (2015). The design target coverage was 99.49%. Exons left out of the design (i.e. Ex 5, see Supplementary Table 1) were sequenced with Sanger method. Amplicon library was prepared using the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific), then pooled together in equimolar concentrations using the Ion Library Equalizer Kit (Thermo Fisher Scientific). Emulsion PCR, and Ion Sphere Particles enrichment were carried out in the Ion One Touch 2 system, then loaded into an Ion 314 sequencing chip. Sequencing runs were performed using the Ion PGM 200 Sequencing kit (Thermo Fisher Scientific). Data of runs were processed using the Ion Torrent Suite 5.0, VariantCaller 5.0, CoverageAnalysis 5.0 (Thermo Fisher Scientific) and the Ion Reporter (Thermo Fisher Scientific) and/or wANNOVAR tools (Chang and Wang, 2012). DNA sequences were displayed by using Integrated Genomics Viewer (Robinson et al., 2011; Thorvaldsdóttir et al., 2013). To confirm mutations identified in patients, Sanger sequencing was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies), with an ABI 3130 instrument (Life Technologies).

Missense variants were assessed using PolyPhen-2, SIFT and Mutation Taster software tools. We removed all the common variants (Minor Allele Frequency, MAF >1%) reported in the following public databases: 1000 Genome Project and Exome Sequencing Project.

2.3. Multiplex ligation-dependent probe amplification (MLPA) analysis

MLPA technique was used to establish whether patients who were negative to NGS analisys bould bear Copy Number Variations (CNVs) mutations in the NF1 gene. To this end, the SALSA P081/P082/P122 kits (MRC Holland, Amsterdam, The Netherlands) was used. This Kit contains reaction mix with probes for all of NF1 gene exons, as well as probes for 15 genes located close to the NF1 gene. Briefly, 100 ng of genomic DNA was added to the MLPA mix and ligation reaction was performed. PCR was performed using (FAM)-labeled primers. The PCR products were then separated on an ABI Prism 3130 Genetic Analyzer (Life Technologies) and the peak areas/heights were measured with GeneScan analysis software (Life Technologies). Finally, data were analyzed with Coffalyser MLPA analysis software (MRC-Holland).

2.4. American College of Medical Genetics and Genomics (ACMG) guidelines to classify genomic variations

Pathogenic status of variants identified was classified using criteria shown in Table 3 of ACMG guidelines (Richards et al., 2015). For example, we have classified the following variations as "Very strong": nonsense, frameshift, canonical ±1 or ±2 splice sites, initiation codon, single or multiexon deletion; as "Strong": De novo (both maternity and paternity confirmed) in a patient with the disease and no family history; and as "Moderate": Absent in controls in Exome Sequencing Project, 1000 Genomes Project.

2.5. Calculation of mosaicism percentage using Sanger-sequencing

In order to quantify the percentage of mosaicism in the c.808C > T (p.Gln270*) mutation, we analyzed the sequencing-electropherogram of capillary electrophoresis performed on ABI 3130 genetic analyzer. Five distinct sequencing-reactions in the patient (#3385) and 20 independent sequencing-reactions in controls were used for the calculation. In order to efficiently process the data, a spreadsheet was generated in Microsoft Excel. Heights of each electropherogram peak of the three bases before (CTA) and one base after (A) the SNP variant (c.808C > T) were analyzed (see Fig. 1). First of all, data relating to each sample (patient's and controls' DNAs) were normalised by dividing each electropherogram height peak value (CTACA) by the average signal strength yielded by the all peaks, in order to generate a Relative Peak Area (RPA) value for each peak. The RPA value for each peak in the patient's DNA was then compared to that of controls, by dividing patient's RPA by average controls' RPA, and this was repeated for each peak. The ratio ~1 stands for 100% of each "CTACA" references variant (controls); the ratio 0.91 stands for 91% of the "C" variant (c.808C > T), and consequently, 9% (100 minus 91) of the base "T" (patient). Standard Deviations (SDs) were calculated for the values as obtained.

2.6. Submission of genomic variations in "Leiden open (source) variation database" (LOVD) public database

All identified variations in NF1 gene were submitted to the LOVD gene variant database at http://www.LOVD.nl/NF1 (Fokkema et al., 2011). The variations shown are described using the NM_000267.3 transcript reference sequence.

3. Results and discussion

More than two thousand different NF1 mutations have been reported in the Human Gene Mutation Database (Stenson et al., 2014). NF1 gene shows one of the highest mutation rates, and different populations present with different exon sets of recurrent mutations (Ko et al., 2013). De novo mutations include almost half of all NF1 patients (Valero et al., 1997). Our study counts 73 mutations, detected in 79 NF1 patients, 37 (51%) of which are novel. The list of all NF1 gene variations detected (pathogenic and not) is reported in Table 1. We observed the following types of variations: missense variations (30%), a value that is higher than previously reported (i.e., ~10% see van Minkelen et al., 2014), twenty five nonsense mutations (34%),
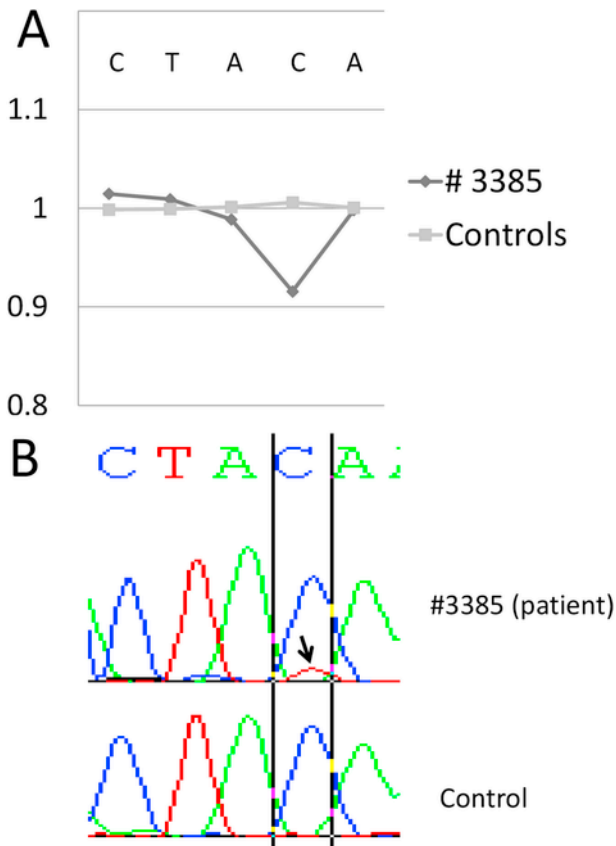
Fig. 1. . **Sequencing analysis of the c.808C > T (p.Gln270*) mutation in NF1 gene performed with Sanger in patient (#3385) and controls**. (A) Patient's and controls' Relative Peak Areas (RPA) ratios. Reference values: ~1 refers to 100% of "C" variant in controls; ratio 0.91 refers to 91% of "C" variant, and consequently, 9% (100 minus 91) of the base "T" in the patient (see methods for more details). (B) The electropherograms show results from Patient and Controls. The peack "T" (under the C peak) in the patient (arrow) is absent in controls (n. 20, see text).

one insertion mutation, two large deletions (whole gene), 6 one-base deletions, 4 two-base deletions, 3 three-base deletions, 3 four-base deletions, 1 six-base deletion and 1 twenty-two-base deletion. In one patient, clinical evidence of segmental NF1 due to mosaicism was obtained. Splicing mutations represented only 7%, unlike percentages previously reported (21%; Bianchessi et al., 2015; Griffiths et al., 2007; van Minkelen et al., 2014). Forty out of 57 exons carry at least one causative mutation, and there are no exons particularly rich in mutations (up to 4 in exon 23).

NGS method presents some limitations as for the large insertion/deletion detection. DNA sequencing, indeed, would fail due to the masking effect by the not-deleted allele (Cali et al., 2010). MLPA, instead, efficiently detects gene or partial exon deletions/duplications in heterozygotes. Consequently, we genotyped NF1 patients by MLPA and found two patients (#3304 and #3860, see Table 1) with a known deletion of the whole gene (1.4 Mb) and one patient (#3638) with an altered amplification pattern, compatible with a heterozygous deletion of exon 4; simultaneously, this latter after being tested by DNA sequencing, was found to have c.6786_6807del22 (p.Asp2262fs) causative mutation. This mutation has thus prevented efficient ligation of the MLPA probes, producing the false-positive result of an apparent exon 4 deletion (Bunyan et al., 2003).

Using the NGS and MLPA methods and the criteria set by ACMG (see Table 3 in Richards et al., 2015), we could classify 96% of the all identified variations as highly or potentially pathogenic. Seventy

percent of these latter were classified as Very Strong, 23% as Supporting and 4% as Moderate (see Table 1 and Methods), except for two mutations (c.1722-11T > A and c.2851-6_2851-3del4, see Note[b], in Table 1), for which results are still uncertain, although the in silico prediction analysis performed with "Mutation Taster" shows them as being Pathogenic too. Further RNA studies should confirm any splicing effects of these mutations on NF1 transcripts. Only one mutation (c.493A > G, p.Thr165Ala) shown in Table 1, relating to patient #4070, can be considered as a polymorphism, since it was inherited from the mother, who does not show the clinical phenotype. In silico analyses performed with SIFT and Polyphen softwares support this data.

We have estimated a mutation detection rate of at least 80%. This value takes into account: (i) the possible occurrence of mutations in the NF1 gene other than exons and canonical splicing sites and (ii) the possible presence in our sample of undiagnosed Legius's syndrome patients who display overlapping phenotypes with NF1 patients before the age of 3 (19% of our patients are below this age). As a matter of fact, we have recently developed a NGS panel for the simultaneous detection of mutations in both NF1 and SPRED1 (Legius syndrome) genes (Pasmant et al., 2015) and have identified in a 2-year-old patient a known nonsense mutation (c.217G > T, p.Glu73*) in the SPRED1 gene causing Legius syndrome (Spurlock et al., 2009).

Mosaicism might reasonably be expected to be frequent among sporadic cases in NF1 and the prevalence of segmental NF1, in the general population, has been estimated to be 0.002% (Ruggieri and Huson, 2001; Ogose et al., 2000), that is 10–20 times lower than the frequency of generalized NF1 (0.029%; Friedman, 1999). In our study, mosaicism was found in a female patient only (#3385), age 9 years, bearing a heterozygous nonsense mutation c.808C > T (p.Gln270*) in 9% of her blood cells (44 of 472 reads). Somatic or gonosomal mosaicism was not investigated. Results were then confirmed by Sanger method, comparing the electropherogram peak height of the normal "C" base in this patient with that of 20 subjects (see Fig. 1). The height of the electropherogram peack for the base "C" in the patient turned out to be lower (0.915, SD ± 0.009) than that of controls (1.005, SD ± 0.011), matching to 9% of mosaicism cells (see Methods). The patient showed, as expected, NF1 disease features throughout the whole body (neck, back, abdomen, medial portions of thighs), with a few areas of normal pigmentation (suggestive of mosaicism). In addition this patient presented with mild attention deficits, cognitive level below the normal range as also reported in other NF1 patients (Tanito et al., 2014).

Our sample of Italian patients displays a striking clinically variable phenotype (Table 2) which makes difficult to assess a reliable genotype-phenotype relationship. Such a difficulty is documented by the lack of the genotype-phenotype correlation in siblings (Ko et al., 2013) and even in identical twins (Vogt et al., 2011). A possible explanation for these inconsistencies could be ascribed to the action of other factors (i.e. modifying loci, environmental factors) (Sabbagh et al., 2009). Some exceptions have been described in patients with a larger common deletion (deletion of exons 1–57, 1.4 Mb) involving NF1 and additional 14 protein-coding genes. Patients bearing this large deletion are associated with a more severe clinical phenotype including intellectual disability, morphological anomalies, cardiovascular malformations, overgrowth, higher benign tumor burden (Shofty et al., 2015). Two of our patients (#3304 and #3860, Table 1) present with the same clinical phenotype, as described above, but one shows neither cardiovascular malformations, nor overgrowth, or higher benign tumor burden. Moreover, genotype-phenotype correlation was successfully performed by Shothy et al. (2015) in a group of

Table 1
NF1 mutations in 79 unrelated patients with neurofibromatosis type 1.

Family number	Patient ID	gender	age	Nucleotide Change	Affected proteins	Variant/ Type	Variant/Predicted effect	Exon	Novelty	SIFT (score)	Polyphen (score)	FamilyHistory (FH)/de novo	pathogenic variants (ACMG criteria)
1	3269	F	29	c.61-2A > C	p.?	substitution	splicing	1	Novel			FH	very strong
2	4008	F	1	c.311T > G	p.Leu104*	substitution	nonsense	4	Mattocks et al., 2004			de novo	very strong
3	3706	F	2	c.422delT	p.Leu142fs	deletion	frameshift	4	Novel				very strong
4	4070	M	9	c.493A > G	p.Thr165Ala	substitution	missense	5	Novel	T (0,38)	B (0.07)	Note [a]	Likely non-pathogenic
5	3986	F	2	c.499_502del4	p.Cys167fs	deletion	frameshift	5	Toliat et al., 2000			FH	very strong
6	4026	M	13	c.574C > T	p.Arg192*	substitution	nonsense	5	De Luca et al., 2004				very strong
7	3533	F	3	c.667T > C	p.Trp223Arg	substitution	missense	7	Griffiths et al., 2007	D (0.00)	D (1.00)	de novo	very strong
8	3851	F	2	c.721G > A	p.Asp241Asn	substitution	missense	7	Novel	D (0.03)	D (0.99)		supporting
9	3385	F	9	c.808C > T	p.Gln270*	substitution	nonsense	8	Novel				very strong
10	3126	M	5	c.998dupA	p.Tyr333*	duplication	nonsense	9	Novel				very strong
11	3885	F	26	c.910C > T	p.Arg304*	substitution	nonsense	9	Upadhyaya et al., 2008	D (0.00)	D (1.00)		very strong
12	3950	M	3	c.1117dupG	p.Val373fs	duplication	frameshift	10	Novel			FH	very strong
13	4192	F	10	c.1414delG	p.Val472*	deletion	nonsense	13	Fokkema et al., 2011				very strong
14	3151	F	18	c.1541_1542delAG	p.Gln514fs	deletion	frameshift	14	Sabbagh et al., 2013				very strong
15	3300	F	7	c.1632_1633insTAA	p.Ala545*	insertion	nonsense	14	Novel				very strong
16	3352	F	25	c.1632_1633insTAA	p.Ala545*	insertion	nonsense	14	Novel			de novo	very strong
17	4194	M	18	c.1658A > G	p.His553Arg	substitution	missense	15	Li et al., 2007	D (0.00)	D (0.99)		supporting
18	3485	M	25	c.1682G > A	p.Trp561*	substitution	nonsense	15	Novel				very strong
19	3487	M	19	c.1721+2T > C	p.?	substitution	splicing	15	Pros et al., 2008			de novo	very strong
20	3692	M	14	c.1722-11T > A	p.?	substitution	splicing	16	Novel			Note[b]	
21	3452	M	64	c.1851_1854del4	p.Asp618fs	deletion	frameshift	17	Sabbagh et al., 2013				very strong
22	3730	F	6	c.1885G > A	p.Gly629Arg	substitution	missense	17	De Luca et al., 2004	T (0.03)	D (1.00)		supporting
23	3486	F	18	c.2056A > T	p.Lys686*	substitution	nonsense	18	Novel			FH	very strong
24	3100	F	14	c.2342A > C	p.His781Pro	substitution	missense	20	Fahsold et al., 2000	D (0.01)	B (0.36)	FH	supporting
25	3441	M	7	c.2446C > T	p.Arg816*	substitution	nonsense	20	Maynard et al., 1997			de novo	very strong
26	3109	M	12	c.2326-1G > T	p.?	substitution	splicing	20	Novel				very strong
27	3731	M	15	c.2540T > C	p.Leu847Pro	substitution	missense	21	De Luca et al., 2004	D (0.00)	D (1.00)		Moderate
28	3545	M	10	c.2536G > C	p.Ala846Pro	substitution	missense	21	Novel	D (0.00)	D (1.00)	de novo	very strong
29	3949	F	9	c.2851-6_2851-3del4	p.?	deletion	splicing	22	Novel			Note[b]	
30	3639	M	10	c.2970_2972delAAT	p.Met992del	deletion	in-frame deletion	22	Fokkema et al., 2011			de novo	very strong
31	3855	M	10	c.2998C > T	p.Arg1000Cys	substitution	missense	23	Fokkema et al., 2011	D (0.00)	D (1.00)		supporting
32	3887	F	8	c.3017delT	p.Val1006fs	deletion	frameshift	23	Novel				very strong
33	3090	M	19	c.3053T > A	p.Leu1018*	substitution	nonsense	23	Fokkema et al., 2011				very strong
34	3556	F	2	c.3088_3089delTC	p.Ser1030fs	deletion	frameshift	23	Novel			de novo	very strong
35	3857	M	3	c.3318C > G	p.Tyr1106*	substitution	nonsense	26	Novel				very strong
36	3799	F	12	c.3568G > A	p.Gly1190Ser	substitution	missense	27	Novel	D (0.03)	D (1.00)	FH	supporting
37	3994	F	12	c.3586C > T	p.Leu1196Phe	substitution	missense	27	Fokkema et al., 2011	T (0.21)	D (1.00)		supporting
38	3761	F	45	c.3827G > A	p.Arg1276Gln	substitution	missense	28	Fahsold et al., 2000	D (0.00)	D (1.00)		supporting
39	4190	F	11	c.3957delT	p.Phe1319fs	deletion	frameshift	29	Novel				very strong

Table 1 (*Continued*)

Family number	Patient ID	gender	age	Nucleotide Change	Affected proteins	Variant/Type	Variant/Predicted effect	Exon	Novelty	SIFT (score)	Polyphen (score)	FamilyHistory (FH)/de novo	pathogenic variants (ACMG criteria)
40	3794	M	19	c.3974G > A	p.Arg1325Lys	substitution	missense	29	Novel	T (0.27)	PD (0.62)	FH	supporting
41	4191	M	17	c.3972_3974delCAG	p.Arg1325del	deletion	in-frame deletion	29	Novel			FH	very strong
42	4133	M	16	c.4120C > T	p.Gln1374*	substitution	nonsense	31	Novel	T (0.08)	D (0.93)		supporting
43	3811	F	10	c.4154delG	p.Gly1385fs	deletion	frameshift	31	Fokkema et al., 2011				very strong
44	4087	M	27	c.4256A > T	p.Lys1419Met	substitution	missense	32	Novel	D (0.00)	D (0.99)		supporting
45	3384	M	3	c.4256A > T	p.Lys1419Met	substitution	missense	32	Novel	D (0.00)	D (1.00)		supporting
46	3224	M	17	c.4333_4352dup20	p.Phe1451fs	duplication	frameshift	32	Novel				very strong
47	3455	M	4	c.4414_4423dup10	p.Leu1475fs	duplication	frameshift	33	Novel			FH	very strong
48	4162	M	19	c.4402A > G	p.Ser1468Gly	substitution	missense	33	Fokkema et al., 2011	T (0.39)	D (0.95)		Moderate
49	3735	M	2	c.4535G > C	p.Arg1512Thr	substitution	missense	34	Novel	D (0.00)	D (1.00)		supporting
50	3557	F	37	c.4537C > T	p.Arg1513*	substitution	nonsense	34	De Luca et al., 2004				very strong
51	3614	M	2	c.4537C > T	p.Arg1513*	substitution	nonsense	34	De Luca et al., 2004			de novo	very strong
52	3886	M	18	c.5007dupC	p.Lys1670fs	duplication	frameshift	36	Fokkema et al., 2011				very strong
53	4096	M	31	c.4786C > T	p.Gln1596*	substitution	nonsense	36	Novel			FH	very strong
54	3758	F	14	c.5425C > T	p.Arg1809Cys	substitution	missense	37	Fokkema et al., 2011	D (0.00)	D (1.00)		supporting
55	3947	F	10	c.5425C > T	p.Arg1809Cys	substitution	missense	37	Fokkema et al., 2011	D (0.00)	D (1.00)		Moderate
56	4166	M	14	c.5242C > T	p.Arg1748*	substitution	nonsense	37	Fokkema et al., 2011				very strong
57	3580	F	3	c.5380C > T	p.Gln1794*	substitution	nonsense	38	Heim et al., 1995			de novo	very strong
58	4092	F	7	c.5589T > G	p.Phe1863Leu	substitution	missense	38	Novel	D (0.02)	D (0.99)		supporting
59	3351	F	7	c.5602G > T	p.Glu1868*	substitution	nonsense	39	Novel			de novo	very strong
60	3251	F	3	c.5870T > C	p.Leu1957Pro	substitution	missense	39	Novel	D (0.00)	D (1.00)	de novo	very strong
61	4193	F	6	c.5817C > A	p.Cys1939*	substitution	nonsense	39	Novel				very strong
62	3537	M	8	c.5791T > A	p.Trp1931Arg	substitution	missense	40	Hudson et al., 1997	D (0.00)	D (1.00)		supporting
63	3125	F	13	c.6334_6335delCT	p.Leu2112fs	deletion	frameshift	41	Fokkema et al., 2011				very strong
64	4132	M	16	c.6743G > A	p.Arg2248His	substitution	missense	44	Novel	T (0.07)	D (0.99)		supporting
65	3638	M	4	c.6786_6807del22	p.Asp2262fs	deletion	frameshift	45	Novel			FH	very strong
66	3534	M	6	c.6791dupA	p.Tyr2264*	duplication	nonsense	46	Fokkema et al., 2011			FH	very strong
67	3252	F	3	c.6792C > A	p.Tyr2264*	substitution	nonsense	46	Robinson et al., 1995			de novo	very strong
68	3620	F	20	c.6792C > A	p.Tyr2264*	substitution	nonsense	46	Robinson et al., 1995				very strong
69	3862	M	8	c.6949_6951delCTT	p.Leu2317del	deletion	in-frame deletion	46	Novel				very strong
70	3801	M	4	c.7096_7101del6	p.Asn2366_Phe2367del	deletion	in-frame deletion	47	Novel			FH	very strong
71	3993	F	5	c.7106T > G	p.Leu2369Trp	substitution	missense	48	Novel	D (0.00)	D (0.99)		supporting
72	3948	F	6	c.7202_7203delAA	p.Lys2401fs	deletion	frameshift	48	Fokkema et al., 2011				very strong
73	3807	M	6	c.7203delA	p.Lys2401fs	deletion	frameshift	48	Fokkema et al., 2011				very strong
74	3389	M	3	c.7253T > G	p.Leu2418*	substitution	nonsense	49	Novel			FH	very strong
75	3536	M	19	c.7552G > T	p.Gly2518*	substitution	nonsense	50	Heim et al., 1995				very strong
76	3390	F	12	c.7486C > T	p.Arg2496*	substitution	nonsense	51	Fokkema et al., 2011				very strong

Table 1 (*Continued*)

Family number	Patient ID	gender	age	Nucleotide Change	Affected proteins	Variant/ Type	Variant/Predicted effect	Exon	Novelty	SIFT (score)	Polyphen (score)	FamilyHistory (FH)/de novo	pathogenic variants (ACMG criteria)
77	3349	F	8	c.7846C > T	p.Arg2616*	substitution	nonsense	53	De Luca et al., 2004			de novo	very strong
78	3304	M	35	deletion of exons1–58	p.?	deletion	deletion	1–58	Fokkema et al., 2011				very strong
79	3860	F	18	deletion of exons1–58	p.?	deletion	deletion	1–58	Fokkema et al., 2011				very strong

SIFT: D = deleterious T = tolerated.
  Polyphen: B = benign, D = damaging, PD = possibly damaging.

[a] Note: inherited by mother who does not show the clinical phenotype.

[b] Mutation Taster: Pathogenetic.

Table 2
Clinical features (according to clinical criteria) of 70/79 NF1 patients.

Clinical features	Total number of patients (%) M = males F = females	Family number # males	Family number # females
Café au lait spots	M=36/36(100%) F=34/34(100%)	4, 6, 12, 17–21, 25–28, 30, 33, 35, 40–42, 44, 45, 47, 48, 49, 52, 53, 56, 62, 64–66, 69, 70, 73–75, 78	1, 3, 5, 7, 9, 11, 13, 15, 16, 22, 23, 24, 29, 32, 34, 36–39, 43, 50, 54, 55, 57–59, 60, 61, 67, 68, 71, 72, 76, 77
Cutaneous neurofibromas or plexiform neurofibroma	M=17/36(47%) F=9/34(26%)	17-19, 21, 25, 27, 40, 41, 44, 48, 52, 53, 64, 66, 69, 75, 78	1, 11, 16, 22, 23, 43, 50, 59, 71
Axillary or inguinal freckling	M=27/36(75%) F=24/34(70%)	6, 12, 17–21, 25, 27, 30, 33, 40, 41, 42, 44, 45, 47, 48, 52, 53, 56, 62, 64, 73–75, 78	1, 3, 7, 9, 11, 13, 15, 16, 22, 23, 29, 32, 36, 39, 43, 50, 57, 59, 61, 68, 71, 72, 76, 77
Lisch nodules	M=13/36(36%) F=8/34(24%)	6, 19, 27, 33, 40, 45, 48, 53, 56, 62, 69, 75, 78	1, 11, 16, 22, 43, 50, 68, 71
Optic nerve glioma	M=1/36 (3%) F=2/34(6%)	25	1, 76
A first-degree relative with NF1	M=10/36(28%) F=5/34(15%)	12, 40, 41, 45, 47, 53, 65, 66, 70, 74	1, 5, 23, 24, 36
A distinctive osseous lesion (sphenoid wing dysplasia, thinning of the long bone cortex with or without pseudoarthrosis)	M=3/36 (8%) F=1/34(3%)	33, 40, 78	1
unavailable information	M=4/40 (10%) F=5/39(13%)	10, 31, 46, 51,	2, 8, 14, 63, 79

different missense mutations affecting codon Arg1809. Patients bearing these missense mutations present with multiple café-au-lait spots and skinfold freckling, but lack of plexiform neurofibromas, Lisch nodules, typical NF1 osseous lesions and symptomatic optic gliomas. Finally, patients with these mutations have been described by Shothy et al. (2015) as having the following characteristics: increased Noonan-like features, pulmonary valve stenosis and learning disabilities (50% of patients). This latter mutation (p.Arg1809Cys) was also found in two of our patients (#3758 and #3947). The clinical features of the first patient (#3758) only included multiple café-au-lait spots and learning disabilities. She lacked of skinfold freckling, plexiform neurofibromas, Lisch nodules, typical NF1 osseous lesions and symptomatic optic gliomas. There were no Noonan-like features nor pulmonary valve stenosis. Instead, the second patient (#3947) had multiple café-au-lait spots and a pulmonary valve stenosis; psychomotor development was within the normal range, and Noonan-like syndrome traits have never been detected. We have also reported a patient (#3639, see Table 1) with 3-bp in-frame deletion c.2970_2972delAAT (p.Met992del) of the NF1 gene, which was as-

sociated with a known peculiar clinical phenotype, characterized by the absence of cutaneous neurofibromas, as reported in Upadhyaya et al., 2007 as well. Finally, no significant effect of sex (the F/M Sex ratio was close to 1) was observed for each of the clinical features (Table 2).

NGS, compared to the traditional Sanger method, is significantly cheaper, quicker, and makes it possible to analyze multiple genes or one gene of very large size. This study has demonstrated the efficacy and effectiveness of the NGS approach to identify mutations in the NF1 gene; it can also be used as a first-choice method to effectively identify mosaic variations.

Web resources

PolyPhen-2: http://genetics.bwh.harvard.edu/pph2/
SIFT: http://sift.jcvi.org/
Mutation Taster: http://www.mutationtaster.org/
1000 Genome Project: http://www.1000genomes.org/
Exome Sequencing Project: ESP6500, http://evs.gs.washington.edu/EVS/
LOVD Database: http://www.LOVD.nl/NF1.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmg.2016.11.001.

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