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NF1 Mutational Spectrum

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Abstract

The *NF1* gene is a large and complex gene spread over 280 kb of genomic DNA on chromosome 17q11.2 and comprising 57 constitutive and at least 3 alternatively spliced exons. The *NF1* gene encodes a transcript with an open reading frame of 8,454 nucleotides. Mutation detection is a challenge due to the large size of the gene, the lack of mutational hotspots, the occurrence of a very diverse spectrum of mutation types and the presence of more than 30 unprocessed pseudogenes [1]. Several different techniques have been applied to look for mutations in this complex gene [2–4], which included already early on attempts to apply RNA-based assays, i.e. protein truncation testing (PTT) [5]. We initially started out using this PTT assay [6, 7], but soon realized its limitations which are (i) the necessity for complementary assays capable to detect non-truncating mutations and (ii) the need to overcome the problems inherent to all RNA-based approaches, i.e. illegitimate splicing and nonsense mediated RNA decay. However, we realized the enormous advantages of an RNA-based mutation analysis approach for a large and complex gene like *NF1* that is expressed in the lymphocytes, that has no hotspots for mutations, where mutations of all types exist and where multiple highly homologous unprocessed pseudogene sequences that are spread over the genome hamper specific DNA amplification. Therefore, we further refined and improved the assays so that they now represent the core of a comprehensive mutation analysis cascade that allows highly sensitive and effective NF1 testing. In the following, we will present this approach and the mutational spectrum deduced from its application to a large cohort of patients.

Comprehensive *NF1* Mutation Analysis Using a Cascade of Complementary Techniques – General Overview of the Approach

We applied a multi-step approach to analyze the entire *NF1* coding region, using an RNA-based center assay, complemented with additional methods allowing (i) to fully characterize the cDNA alterations at the gDNA level and (ii) to

identify microdeletions and intragenic copy number alterations. We identified the pathogenic *NF1* mutation in 1,770 unrelated patients submitted for clinical genetic testing to the Medical Genomics Laboratory at UAB. For all patients, an EDTA-blood sample was obtained and split into three aliquots (i) for genomic DNA extraction, (ii) for preparation of cell suspensions for interphase fluorescence in situ hybridization (FISH) and (iii) for short term lymphocyte cultures for RNA extraction. Short-term phytohemagglutinin (PHA)-stimulated lymphocyte cultures were set up to avoid illegitimate splicing, known to lead to multiple aberrant splice variants that impede the detection of mutations in an RNA-based approach [6, 7]. Before RNA extraction, cultures were treated with 200 μg/ml puromycin for 4 h to prevent the nonsense-mediated mRNA decay [6]. Total RNA was extracted and cDNA prepared as described [8]. The entire coding region was amplified in three overlapping fragments of ~4 kb (spanning the *NF1* exons 1–27b, 12a–35 and 22–49). All fragments were analyzed by gel electrophoresis allowing immediate identification of shortened fragments indicative for the presence of splice mutations or deletions [8]. Hereafter, the fragments were used as the starting material for direct cDNA sequencing. To cover the entire coding region 18 sequencing primers were used. Cycle sequencing reactions were performed with commercially available sequencing kits. The sequencing reactions were subsequently run on an automated capillary sequencer and analyzed using the sequence analysis program SeqScape V2.5 (ABI). In addition, exon 1 was analyzed by bidirectional direct sequencing of this exon from genomic DNA.

All alterations/mutations detected at the cDNA level were entirely characterized at the genomic level using exon/intron-specific or breakpoint-specific primers. As prescreen for total gene deletion (with or without deletion of additional flanking genes), microsatellite analysis using four intragenic markers was performed on an ABI3730 capillary sequencer. Patients showing only one allele for all microsatellite markers were further analyzed by multiplex ligation dependent probe amplification (MLPA) using the *NF1* microdeletion MLPA-kit (P122 MRC Holland) [9] and interphase FISH analysis (100 nuclei) using probes flanking the *NF1* gene, i.e. RP5–1002G3 and RP5–926B9, to confirm or rule out the presence of the total *NF1* gene deletion [6]. Finally, MLPA analysis (kits P081 and P082 MRC-Holland) was also performed for all patients in whom no mutation was identified after sequencing the entire coding region at the cDNA level in order to identify intragenic copy number alterations that escaped detection by the RT-PCR fragment analysis and direct sequencing approach [9]. The outline of the cascade of tests used in this comprehensive approach is depicted in figure 1. The 5′ and 3′UTR have not been systematically implemented in this approach, except for analysis of dosage alterations at positions c.1–717 and c.1–680 of the 5′UTR. Furthermore, no cytogenetic

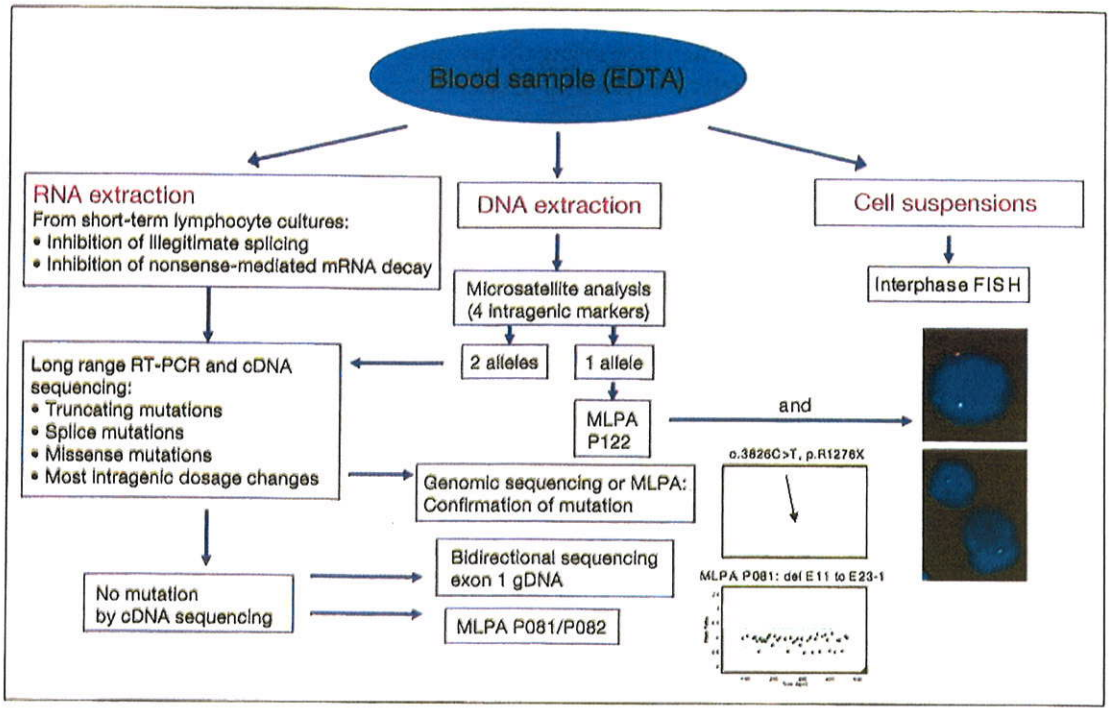

Fig. 1. Comprehensive *NF1* mutation analysis using a cascade of complementary techniques – general overview of the approach.

analysis was performed that would identify a (balanced) translocation or other cytogenetic abnormality resulting in NF1 or a NF1-like phenotype.

Spectrum of Mutations in 1,770 Unrelated Patients as Identified by A Comprehensive Multi-Step Approach

Comprehensive *NF1* mutation analysis using this RNA-based core assay supplemented with methods to identify *NF1* microdeletions as well as smaller copy-number changes identifies a mutation in 95% of non-founder NF1 patients fulfilling NIH criteria [6]. The sensitivity of a comprehensive RNA-based approach was confirmed in an independent cohort of well characterized patients [10]. The efficiency and reliability of this approach is based on the application of a set of complementary techniques that allow the detection of different mutation types, including unusual splicing defects occurring outside the

canonical AG/GT splice site sequences of the *NF1* gene. The higher detection rate compared to other methods is largely explained by the frequent occurrence of unusual splicing defects in the *NF1* gene.

Mutations in the *NF1* gene are spread over the entire coding region and include *NF1* microdeletions, intragenic copy number changes, i.e. deletions/duplications involving one to several exons, frameshift, nonsense, splice, missense mutations and in frame deletions or duplications involving one to several codons.

In total, 1,017 different *NF1* mutations were identified in this cohort of 1,770 unrelated patients in whom a mutation was found. 813 out of these 1,017 mutations were singular mutations. Hence, 46% of the patients (813/1,770) carry a 'private mutation' and 204 different mutations were observed more than once in the remainder of this cohort, i.e. 957 patients. This number includes four different types of *NF1* microdeletions (all encompassing the entire *NF1* gene with or without additional flanking genes) found in 89 unrelated patients as well as 200 minor-lesion mutations and intragenic copy number changes found twice to 30 times in the remaining 868 patients. Twenty-nine *different nonmicrodeletion* mutations residing in 23 different exons each occurred in >0.5% of the patients (ranging from 9/1,770 to 30/1,770) and together account for 413/1,770 of the mutations (23.3%). These mutations will be further discussed under the heading 'recurrent mutations'.

The *NF1* microdeletions found in 89 patients (5%) were of variable size. Seventy-six were 1.4 Mb in size (referred to as type I deletion [11, 12]) or larger; 9 were 1.2 Mb in size (referred to as type II deletions [13]), and 4 deletions were smaller than 1.2 Mb but still encompassed the entire *NF1* gene. 367 patients carried a nonsense mutation (20.7%). 478 patients had splicing mutations (27%). Of these, 186 affected the canonical AG/GT splice dinucleotides (10.5%), 46 affected intronic sequences at position +3 to +30 from the adjacent exon border (2.6%) and 38 affected intronic sequences at the position –3 to –30 from the adjacent exon border (2.1%), 172 patients carried a mutation within one of the *NF1* exons affecting splicing (9.7%), although at the gDNA level they presented as a nonsense, missense or silent alteration. Thirty-six patients carried a deep intronic alteration (2%) resulting in the creation of an intronic 5′ or 3′ splice site that is used in conjunction with a cryptic 3′ or 5′ splice site present in the wild type intronic sequence. 497 patients carried small (one to several nucleotides) deletions or duplications/insertions (28%). Forty-four of them were non-frameshifting mutations, i.e. a deletion (40/44) or duplication (4/44) leading to loss/gain of 1 (24 patients), 2 (16 patients), 3 (2 patients), 5 (1 patient) or 8 (1 patient) codons. The remaining 453 deletions/insertions caused a frameshift with 21 patients carrying a frameshift mutation due to insertion of 1 to 10 nucleotides (1.2%), 136 due to duplication of 1 to 13 nucleotides (7.7%) and 296 patients carried a frameshift mutation due to deletion of 1 to 20 nucleotides (16.7%). In

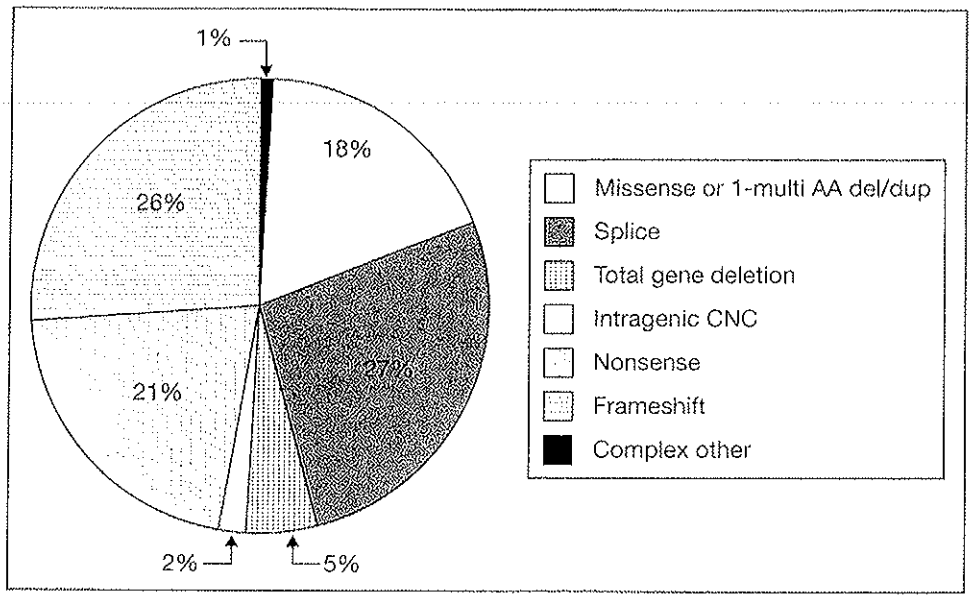

Fig. 2. Spectrum of mutations in 1,770 unrelated patients as identified by a comprehensive multi-step approach.

13 patients (0.7%), a unique complex mutation was identified, such as indels, multiple substitutions in nearby but non-adjacent nucleotides or a substitution plus deletion in nearby but non-adjacent nucleotides. 282 patients carried a missense mutation (15.9%). In 39 patients (2.2%), an intragenic copy number change was found to delete (35/39) or duplicate (4/39) one (12/39) to several (27/39) exons. Finally, in 4 patients, the methionine start codon was mutated (M1V, M1R, M1I, c.1–16_c.1del17), which may result in use of an alternative start codon and in one patient, a balanced translocation was found, t(14;17)(q32;q11.2) [6]. Although rare, the contribution of gross chromosomal aberrations might be slightly underestimated in the current study. An overview of the spectrum of mutations is depicted in figure 2.

The nomenclature of the mutations is based on *NF1* mRNA sequence NM_000267.1, with 1 being the first nucleotide from the methionine startcodon. The *NF1* exons are named according to the most widely used and best known nomenclature, which does not use strictly consecutive numbers (see [4]).

Advantages of Using an RNA-Based Core Assay

RNA-based mutation detection techniques are a powerful, reliable and efficient means to identify mutations, especially in large and complex genes expressed in easily accessible cells such as lymphocytes and are more sensitive,

especially in genes with a mutational spectrum showing a high prevalence of splicing mutations not residing at the canonically conserved GT/AG splice sites [2, 6, 10]. Moreover, the approach is very cost-effective: indeed for the *NF1* gene the entire coding region can be analyzed in only 3–5 large overlapping fragments using ~18–20 sequencing primers, instead of the 57 amplicons needed if all exons are analyzed at the gDNA level. Full benefit of the advantages of the RNA-based assays is however only possible if the necessary precautions are taken to prevent illegitimate splicing as well as nonsense-mediated mRNA decay.

Correct Classification of Mutations that Affect Splicing but Mimic Nonsense, Missense or Silent Mutations

In this study, we identified a total of 63 *different* exonic mutations in 192 patients, affecting correct splicing. Seventeen mimic nonsense mutations at the gDNA, 34 missense, 10 silent alterations and 1 a frameshift mutation, c.6792delC, leading to in-frame skipping of exon 37. Whereas the mutations mimicking a nonsense mutation would still overall be classified as 'pathogenic' in a gDNA direct sequencing approach, their classification and assessment of effect would be incorrect, which eventually may impact downstream studies such as genotype-phenotype correlations. The decisive evaluation of the 'missense' and 'silent' alterations would not be possible without a functional evaluation of the effect of these alterations on the splicing of the exons harboring the alterations.

One class of exonic alterations create de novo splice sites, which are used despite the presence of the intact wild-type splice sites and result in the loss of a part of the exon. c.1466A>G ('p.Y489C') in exon 10b exemplifies this class. It is the most recurrent minor lesion mutation in our cohort (30/1770) and creates a novel 5′ splice site (ss) that is used instead of the authentic wild-type and leads to skipping of the last 62 nucleotides of exon 10b [14]. Other examples include c.2709G>A ('p.V903V'), c.3278T>A ('p.V1093E') and c.3831C>T ('p.G1277G') [10].

Another class of exonic alterations leads to exon skipping most likely by disruption/creation of splicing regulatory elements such as exonic splicing enhancers (ESEs) or exonic splicing silencers (ESSs). Data strongly support the notion that p.Y2264X (c.6792C>A or C>G), one of the more frequently recurring mutations resulting in exon 37 skipping [15], acts through the disruption of an ESE [16]. Other examples include p.R304X, p.W336X and p.E1907X. Although ESE/ESS prediction programs have proven to be useful for understanding the mechanisms of mutation-induced exon skipping, experimental data from patient tissues remains pivotal to assess the effect on splicing of silent or conservative amino acid changes.

Avoidance of the Detection of Rare Benign Intronic Variants with No Significance

Intronic sequences without functional impact diverge faster and accumulate more variation in the population [17]. These rare variants, besides the other more commonly observed SNPs, will come to attention by gDNA-based exon-by-exon sequencing, which usually assesses the intronic regions flanking each exon from –30 to +30, however their significance will be unknown. An effect on splicing can only be safely assumed for alterations affecting the conserved canonical GT/AG dinucleotides. As all new intronic variants found in this region may or may not impact correct splicing, they will need further formal evaluation by RNA-based analysis of the region of interest starting from a new blood draw.

Detection of Deep Intronic Splice Mutations

In this study we identified 23 different deep intronic mutations in 36 unrelated patients. Eighteen of them were 'private' mutations only observed in one unrelated patient/family. These mutations alter a single nucleotide often within very large introns, creating de novo 5′ or 3′ intronic splice sites that are used in conjunction with an already available intronic 'partner' cryptic splice site leading to inclusion of a cryptic exon. One deep intronic mutation, c.5749 + 332A>G in intron 30, reported previously in three independent studies [10, 18, 19], was identified in 7 unrelated patients in this study. This mutation creates a strong de novo 5′ss and leads to the insertion of a 177-bp cryptic exon between exons 30 and 31. Interestingly, the 3′ss at c.5749 + 154, used by c.5749 + 332A>G to create the 177-bp cryptic exon is predicted to be very weak by the currently available in silico prediction tools [10]. This finding has two immediate implications: it suggests that other intronic sequences help to define this cryptic exon [20] and demonstrates that the available in silico prediction tools (so far) lack the power to predict the outcome of an array of variants that would be encountered by direct sequencing of entire introns and to decide based on these predictions which of these variants should be pursued as likely pathogenic.

Another novel deep intronic splice mutation in the 17.7 kb long intron 6, i.e. c.888 + 651t>a, is illustrated in figure 3. According to the splice site prediction by neural network (SSPNN) program (http://www.fruitfly.org/seq_tools/splice.html) this 'private' mutation, found as de novo mutation in a sporadic patient with spinal NF, creates a strong 3′ss that is used in conjunction with a cryptic strong 5′ss leading to inclusion of a cryptic 132-bp exon containing a premature stop codon. Interestingly, the cDNA data indicate that the cryptic exon only gets included in part of the mutant alleles, indicative of poor cryptic exon recognition at least in the investigated tissue.

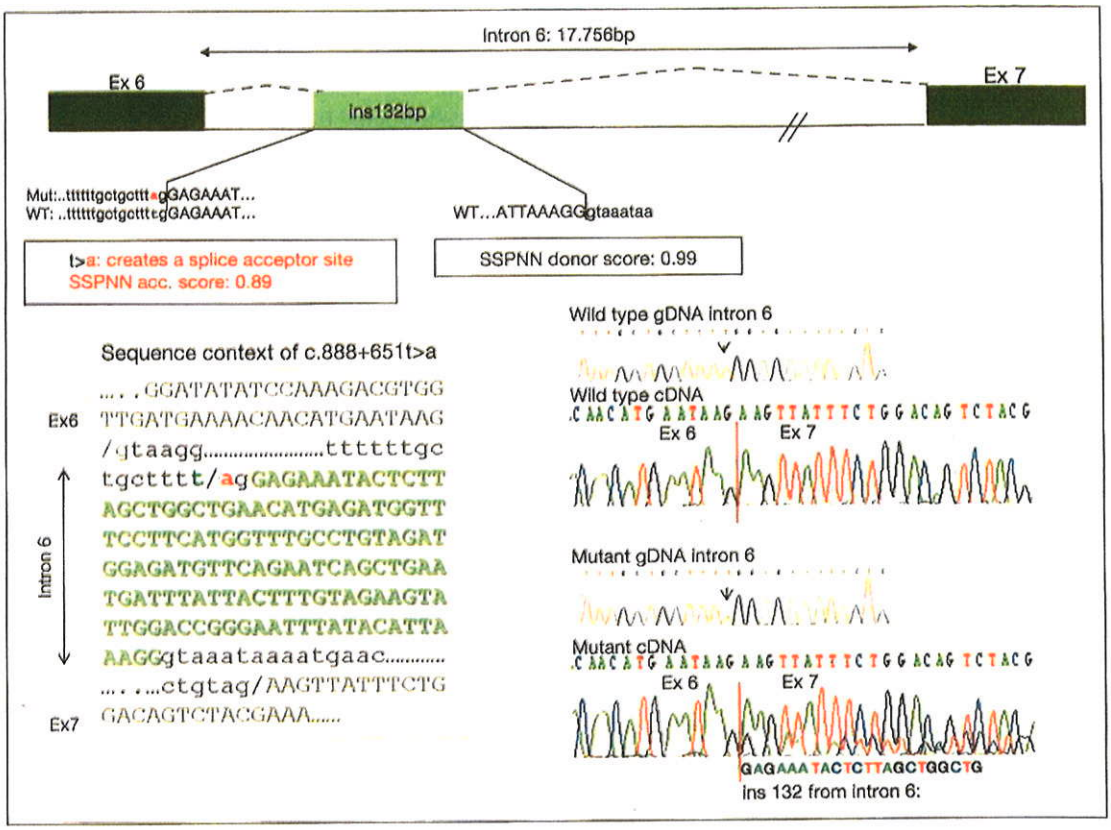

Fig. 3. Deep intronic de novo splice mutation in the 17.7 kb long intron 6, i.e. c.888 + 651t>a, leading to exonisation of 132 bp from intron 6 due to the use of a newly created splice acceptor site that gets used in conjunction with a cryptic splice donor site.

Avoidance of Amplification of Non-Processed Pseudogenes

In addition to the functional *NF1* gene, over 30 non-processed pseudogenes are present in the human genome [1]. The pseudogenes on chromosomes 2, 14 and 22, containing the middle part of the *NF1* gene, without the GAP-encoding segment, have a sequence homology of more than 95%. Hence, the design of primers to *specifically* and *uniquely* amplify those *NF1* exons that also have been duplicated during evolution and are part of these pseudogenes, has been a real challenge and greatly impeded accurate mutation analysis solely based on DNA-dependent methods in the past. This challenge has recently become more manageable due to the public availability of the sequences of the entire human genome as well as through more powerful primer design and

homology search software [21]. Nevertheless, it is advisable when designing primers for gDNA sequencing to allow the amplification of at least 30 nucleotides of the adjacent intronic sequences. Primers too close to the exon-intron border (see e.g. primer pair for exon 6 [21]) will miss splicing mutations outside the canonical GT/AG splice site dinucleotides. Furthermore, primer pairs with only one mismatch at the terminal bases between one or more of the *NF1* pseudogenes and the real *NF1* gene may still coamplify some pseudogene sequences, since the extension of the primers from the pseudogene template may continue if terminal mismatching has weak-destabilizing effect.

Pathogenic Versus Rare Benign Variant

The distinction between a pathogenic and rare benign variant is of utmost importance when mutation analysis is performed as clinical testing and this distinction is, apart from the silent and deep intronic sequence changes, particularly challenging for missense alterations.

In the absence of functional assays, rigorous criteria must be applied before a novel missense alteration in the *NF1* gene can be unequivocally classified as the disease causing mutation in order to avoid diagnostic errors. Sometimes, a final decision will necessarily need to be suspended until additional data from larger cohorts of patients become available. Importantly, we have found missense mutations spread over the entire gene from exon 1 to exon 46. The missense mutations were not restricted uniquely to any of the so far more characterized regions with known or putative functions (GRD, CSRD, Sec14). No missense alterations were detected (so far?) in the exons 5, 9br, 23a, 34, 35, 37, 39, 42–45, 47–49.

We propose the following criteria for the evaluation of missense alterations: (i) absence of any other possible deleterious mutation after comprehensive analysis of the whole coding region. This is a necessary but insufficient criterion. This analysis *must* include screening for a total gene deletion, smaller dosage alterations (one to multiple exons deletions/duplications) and splice mutations including deep intronic mutations affecting splicing. 'Missense' mutations shown to have an effect on splicing if RNA-based mutation analysis is performed, can be considered to be deleterious; (ii) absence of the sequence alteration in a large number of unrelated control samples. This is a necessary but insufficient criterion. We *still* find novel benign variants on the wild-type *NF1* allele, inherited from the unaffected parent, even after sequencing the entire coding region in >3000 patients. Furthermore, no sufficient data are available from all ethnic backgrounds; (iii) alterations at amino acids that have been conserved over a long evolutionary distance in the *NF1* orthologs of *Pan*

troglodytes, Mus musculus, Rattus norvegicus, Gallus gallus, Danio rerio, Canis familiaris, Anopheles gambiae, Takifugu rubripes and *Drosophila melanogaster* have a high likelihood of being deleterious. In addition, algorithms that differentiate one variant from another at a given codon based on chemical differences (such as PolyPhen, SIFT or Grantham score A-GVGD), can add further support; (iv) however, finally and most importantly, clinical and molecular genetic assessment of the relevant family members is needed before a final conclusion should be made for all novel missense alterations. Although NF1 is a progressive and phenotypically highly variable disorder, the disease is fully penetrant. In a sporadic patient, the missense mutation needs to be *proven* to be a de novo event and clinical evaluation of both parents needs to confirm absence of signs of NF1 in them. De novo occurrence of a missense alteration after comprehensive mutation analysis, along with other supportive data such as evolutionary conservation and Grantham score, provides the strongest argument in favor of pathogenicity at this time. In a familial patient, the missense mutation needs to be proven to segregate with the disorder in the family by analysis of at least one affected relative. The latter argues that the missense alteration segregates with the symptoms in the family, but does not provide in itself proof of its pathogenicity.

Recurrent Mutations with a Frequency >0.5% in the NF1 Patient Population

1,017 different *NF1* mutations were identified in this cohort of 1,770 unrelated patients in whom a mutation was found. 813 out of these 1,017 mutations were singular mutations, indicating that ~46% of the patients (813/1,770) carry a 'private mutation'. We expect that the number of novel mutations will further steadily increase as more patients get analyzed and that we will continue to witness that almost half of the newly analyzed patients carry a private mutation.

In our cohort, 89 unrelated patients carried microdeletions that fall in at least 4 different types/size classes (all encompassing the entire *NF1* gene with or without additional flanking genes). 868 patients carried 200 different recurrent minor lesion mutations and intragenic copy number changes found twice to 30 times in our cohort.

Twenty-nine of these *different nonmicrodeletion* mutations residing in 23 different exons each occurred in >0.5% of the patients (ranging from 9/1,770 to 30/1,770) and together account for 413/1,770 of the mutations (23.3%) (table 1 and fig. 4). Another 10 different mutations residing in 7 different exons/introns occurred in >0.3% and <0.5% of the patients and account for 68/1,770 of the mutations (4%) (table 1 and fig. 4). Fifty-one different mutations found in 167

Table 1. Summary of all recurrent mutations presenting in >0.3% of the patients in this cohort

Mutation	Exon/ intron	Type/ effect	No. of patients
Mutations found in >0.5% of the patients			
c.499_502delTGTT	E4b	frameshift	22
c.574C>T; p.R192X	E4b	nonsense	10
c.910C>T; p.R304X	E7	nonsense/splice	12
c.1246C>T; R416X	E9	nonsense	13
c.1318C>T; p.R440X	E10a	nonsense	12
c.1381C>T; p.R461X	E10a	nonsense	10
c.1466A>G; 'Y489C'	E10b	splice	30
c.1541_1542delAG	E10c	frameshift	13
c.1756_1759delACTA	E12a	frameshift	22
c.1885G>A; 'p.G629R'	E12b	splice	10
c.2033dupC	E13	frameshift	20
c.2041C>T; p.R681X	E13	nonsense	10
c.2446C>T; p.R816X	E15	nonsense	15
c.2970_2972delAAT; p.991delM	E17	1 AA deletion	14
c.3457_3460delCTCA	E20	frameshift	14
c.3721C>T; p.R1241X	E22	nonsense	10
c.3826C>T; p.R1276X	E22	nonsense	13
c.4084C>T; p.R1362X	E23-2	nonsense	16
c.4267A>G; p.K1423E	E24	missense	17
c.4537C>T; p.R1513X	E27a	nonsense	18
c.5242C>T; p.R1748X	E29	nonsense	13
c.5425C>T; p.R1809C	E29	missense	13
c.5546G>A; 'p.R1849Q'	E29	splice	17
c.5839C>T; p.R1947X	E31	nonsense	10
c.6709C>T; p.R2237X	E36	nonsense	9
c.6792C>A; 'p.Y2264X'	E37	splice	16
c.7096_7101delAACTTT; p.2366_2367delNF	E39	2 AA deletion	9
c.7486C>T; p.R2496X	E42	nonsense	9
c.7846C>T; p.R2616X	E45	nonsense	16
Mutations found in >0.3% but <0.5% of the patients			
c.1019_1020delCT	E7	frameshift	6
c.2325+1G>A	I14	splice	7
c.3827G>A; p.R1276Q	E22	missense	6
c.3916C>T; p.R1306X	E23-1	nonsense	7
c.5749+332A>G	I30	deep intronic splice	7
c.6789_6792delTTAC	E37	frameshift	6
c.6791dupA	E37	frameshift	8

Table 1. (continued)

Mutation	Exon/ intron	Type/ effect	No. of patients
c.6792C>G; 'p.Y2264X'	E37	splice	7
c.7267dupA	E41	frameshift	6
c.7285C>T; p.R2429X	E41	nonsense	8

Mutations that can be explained by spontaneous methyl-cytosine deamination-mediated C>T/G>A transitions at 19 of the 120 CpG dinucleotides of the *NF1* coding region are shown in bold.

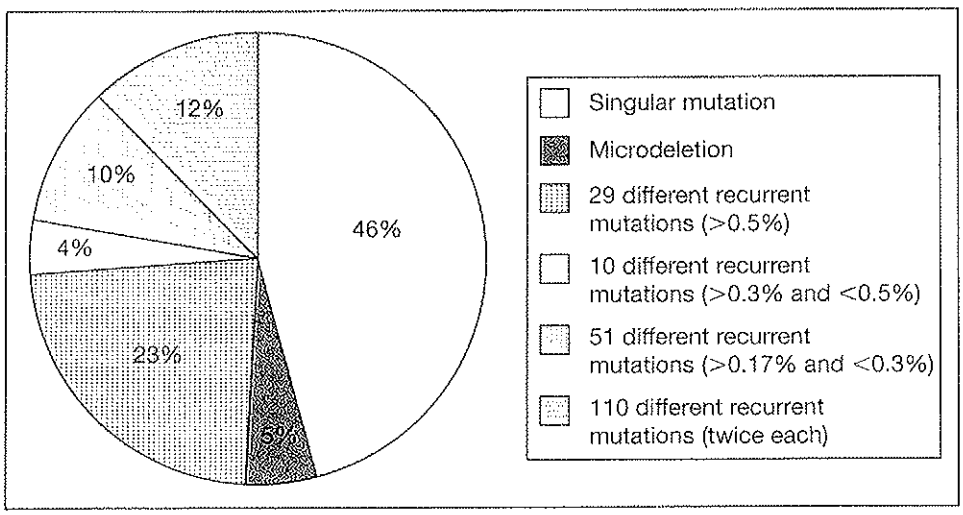

Fig. 4. Overview of the fraction of recurrent versus singular mutations in the cohort of 1,770 unrelated patients.

unrelated patients, occurred 3 to 5 times in this cohort and 110 different mutations were identified twice in this cohort.

Of the 29 recurring mutations with a prevalence of >0.5%, 19 can be explained by spontaneous methyl-cytosine deamination-mediated C>T/G>A transitions at 19 of the 120 CpG dinucleotides of the *NF1* coding region, see mutations presented as bold in table 1. Three deletions are tandem-repeat mediated deletions (c.499_502delTGTT, c.1756delACTA and c.7096_7101delAAC TTT). Mutation c.2033dupC can be caused by DNA-polymerase slippage within a C_7-homonucleotide tract. p.991delM is suggested to be mediated by palindrome correction [22]. Mutation c.1541_1542delAG may be explained by

excision of two mispaired nucleotides in an inverted repeat [23]. c.1466A>G and c.4267A>G are probably induced by spontaneous adenine deamination at the sense strand [24]. p.Y2264X in exon 37 is located within a hypermutable region in exon 37 containing a 'quasi-symmetric' element [25]. The reason for the recurrence of c.3457_3460delCTCA remains so far unexplained.

Final Remarks

We have described and defined the overall unbiased spectrum of constitutional *NF1* mutations as found in a large cohort of 1,770 *unrelated* patients analyzed in the Medical Genomics Laboratory at UAB using a comprehensive mutation analysis consisting of an RNA-based core assay supplemented with methods to identify *NF1* microdeletions as well as smaller copy number changes. We do not expect to see further significant fluctuations in the *percentages* contributed by the different types of mutations by analyzing larger cohorts of patients. However, the spectrum of mutations within each of the subtypes (e.g. missense, deep intronic splice mutations, etc.) will need to be defined/described in more detail to further our understanding of the genomics of the *NF1* gene. This spectrum will serve as a baseline to compare against specific *subgroups* of patients presenting with variant forms/subtypes of NF1. It will also allow exploring in further depth whether the spectrum of second hit mutations as identified in different cells/tumors differs from the constitutional spectrum.

There is a need for a *reliable, specific* and *sensitive NF1* mutational detection approach, to help resolve diagnostic dilemmas using clinical genetic testing in patients not fulfilling the NIH diagnostic criteria, especially young children but also atypical patients, such as patients with spinal neurofibromatosis or segmental NF. Only about half of patients with sporadic *NF1* mutations (i.e. founder patients) fulfill the NIH diagnostic criteria by one year of age and *still* 5% will not fulfill these criteria by the age of 8 years [26]. CAL-spots are often the first signs of NF1, increasing in number during the first years of life. Waiting for more symptoms to appear with time in order to ascertain the diagnosis on a clinical basis can be very stressful for families. Furthermore, distinction between NF1 and the recently reported autosomal dominant *NF1-like disorder*, caused by mutations in the *SPRED1* gene and characterized by multiple CAL-spots, freckling, macrocephaly but absence of cutaneous neurofibromas, will become essential once the symptoms and complications associated with this new disorder are documented in more detail [27].

Earlier diagnosis allows to offer genetic counseling to parents and relatives earlier as well as to initiate interventions for learning or developmental problems sooner. Earlier diagnosis will become even more important once more

therapeutic options become available. A direct genetic test may help to establish the diagnosis earlier, especially in sporadic patients, but *only* when the testing has a high sensitivity, i.e. finds the mutation in (almost) all patients that eventually will fulfill the NIH criteria (low false negative results) *and*, equally important, does not confuse a benign variant with a pathogenic mutation (no false positive results).

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