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NF1 Exon 22 Analysis of Individuals with the Clinical Diagnosis of Neurofibromatosis Type 1

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Abstract

Café-au-lait macules are frequently seen in Ras-MAPK pathway disorders and are a cardinal feature of neurofibromatosis type 1 (NF1). Most NF1 individuals develop age-related tumorigenic manifestations (e.g. neurofibromas), although individuals with a specific 3-bp deletion in exon 22 of *NF1* (c.2970_2972delAAT) have an attenuated phenotype with primarily pigmentary manifestations. Previous reports identify this deletion c.2970_2972delAAT in exon 17 of *NF1* using NF Consortium nomenclature. For this report, we elected to use standard NCBI nomenclature, which places this identical deletion within exon 22. *SPRED1* causes Legius syndrome, which clinically overlaps with this attenuated NF1 phenotype. In an unselected cohort of 150 individuals who fulfilled NIH clinical diagnostic criteria from an NF Clinic and did not have *SPRED1* mutations, we sequenced *NF1* exon 22 in order to identify children and adolescents with multiple café-au-lait spots who could be projected to have lower likelihood to develop tumors.

Two individuals with *NF1* exon 22 mutations were identified: an 11-year-old boy with the c.2970_2972delAAT in-frame deletion and a 4-year-old boy with c.2866dupA. The father of the second patient had an attenuated form of NF1 and showed 24% germline mosaicism of the c.2866dupA mutation in whole blood.

These individuals emphasize the need for mutation analysis in some individuals with the clinical diagnosis of NF1 who lack the tumorigenic or classic skeletal abnormalities of NF1. Specifically, with the identification of Legius syndrome, the need to recognize the attenuated phenotype of *NF1* mosaicism and confirmation by mutation analysis is increasingly important for appropriate medical management and family counseling.

Keywords

genotype-phenotype correlation; Legius syndrome; mosaic; neurofibromatosis type 1 (NF1); *NF1* exon 17; *NF1* exon 22; *SPRED1*

INTRODUCTION

Mutations in genes involved in the Ras-MAPK pathway result in a number of syndromes including neurofibromatosis type 1 (NF1), Legius syndrome, LEOPARD syndrome, Costello syndrome, Noonan syndrome, and cardio-facio-cutaneous (CFC) syndrome [Stevenson et al., 2008]. These syndromes are clinically distinct, but there is phenotypic overlap with many of the physical manifestations between the various syndromes. NF1 and Legius syndrome, both autosomal dominant disorders, have significant pigmentary overlap. NF1 is due to mutations in the *NF1* gene leading to activation of downstream signaling of Ras. Mutations in the *SPRED1* gene cause Legius syndrome, the cardinal features of which are multiple café-au-lait macules and freckling. Some individuals with *SPRED1* mutations fulfill NIH clinical diagnostic criteria for NF1 [Brems et al., 2007; Messiaen et al., 2009; Muram-Zborovski et al., 2010; Pasmant et al., 2009; Spurlock et al., 2009] based on pigmentary findings, further confounding accurate differentiation of these syndromes.

Identifying individuals as having NF1 or Legius syndrome is important, since most NF1 individuals develop age-related tumorigenic manifestations, such as neurofibromas and optic pathway tumors. However, a subset of individuals with a specific *NF1* 3-base-pair deletion, generally have an attenuated phenotype with fewer tumor manifestations in adulthood. Previous reports identify this deletion c.2970_2972delAAT in exon 17 of *NF1*. These reports used NF Consortium nomenclature for this classification [Stevenson et al., 2006; Upadhyaya et al., 2007]. For this report, we elected to use standard NCBI nomenclature (National Center for Biotechnology Information nomenclature) which places this identical deletion in *NF1*, c.2970_2972delAAT, within exon 22 (GenBank Reference NM_001042492.1). Milder NF1 phenotypes have been reported with another novel *NF1* mutation [Nystrom et al., 2009a] or mosaicism for a *NF1* mutation [Consoli et al., 2005; Kehrer-Sawatzki et al., 2004; Maertens et al., 2007; Petek et al., 2003]. Due to the age-related occurrence of NF1 manifestations, young patients may also present with only pigmentary findings. These clinical presentations of NF1 without cutaneous neurofibromas can result in a phenotype similar to Legius syndrome and it may be difficult to distinguish them clinically.

We applied *SPRED1* and *NF1* exon 22 sequence screening to an unselected NF1 population consisting of a spectrum of disease severity, followed in a primarily pediatric NF Clinic, in order to identify those who may be projected to have an attenuated clinical course. We identified 2 individuals with *SPRED1* mutations [Muram-Zborovski et al., 2010], and here we report on the clinical and molecular findings of three individuals [an 11-year-old boy (Patient #1), and a 4-year-old boy (Patient #2) and his father (Patient #3)] with *NF1* exon 22 mutations. The attenuated phenotype and family history of these three individuals suggested the possibility of Legius syndrome.

MATERIALS AND METHODS

NF1 Patient Phenotyping

Phenotypic information for an unselected cohort of individuals fulfilling the NIH clinical diagnostic criteria for NF1 recruited through the University of Utah Neurofibromatosis Clinic was reviewed. The phenotype was previously documented through a medical records review and a standardized NF1 history and exam form by one physician (DS). This NF1 history and exam form was created for use by the University of Utah NF1 Registry and modeled after the Children's Tumor Foundation International Database [Friedman and Birch, 1997]. Peripheral blood was obtained on all individuals for DNA extraction. *SPRED1* sequencing was previously performed to exclude individuals with *SPRED1* mutations, and

the remaining individuals with wild-type *SPRED1* were Sanger sequenced specifically for *NF1* exon 22 mutations.

***NF1* gene Sequencing**

DNA was isolated from peripheral blood samples using the Gentra Puregene DNA extraction kit (Qiagen, Valencia, CA). Each DNA sample was amplified by PCR for exon 22 of *NF1*, including intron/exon boundaries, using the forward primer 5'-TGCACCTTACTCTGTGTGTTAG-3' and the reverse primer 5'-TACCAGTATCAGTGTGTAAGAGG-3' designed using LightScanner Primer Design® software by Idaho Technology Inc. (Salt Lake City, Utah) and supplied by Integrated DNA Technologies (Coralville, Iowa). Bidirectional Sanger sequencing was performed on the ABI 3730 analyzer (Applied Biosystems, Foster City, CA) and data was analyzed using Mutation Surveyor® software (SoftGenetics, State College, PA). All sequences were compared with GenBank reference sequence NM_001042492.1 for *NF1*.

Genotype Confirmation Testing for Patients #2 and #3

Single Nucleotide Extension—Amplification of DNA for patients #2 and #3 was performed using the primers for *NF1* exon 22 described above. Products were analyzed using the ABI Prism SNaPshot Multiplex Kit (Foster City, CA) with extension primer 5'-CTTTCTTTAGGTTTTATTGACTGATA-3'. This primer covers the nucleotide at position c.2866 to allow interrogation of the next base in the forward direction. All steps were performed according the manufacturer's recommendations. Products were analyzed on the ABI Prism 3100 Genetic Analyzer. Result analysis was performed using GeneMarker software version 1.6 (SoftGenetics, State College, PA).

Pyrosequencing—DNA for patients #2 and #3 was further analyzed by pyrosequencing to quantify the percentage of alleles harboring the suspected duplication. First, DNA was amplified as described above using the *NF1* exon 22 primers, with a biotinylated form of the reverse primer. PCR products were immobilized to streptavidin-coated beads and denatured to produce single stranded products. Pyrosequencing was performed on the PyroMark Q24 (Qiagen, Valencia, CA) using the sequencing primer 5'-TTTCTTTAGGTTTTATTGACC-3'. The sequence to analyze was set to TGATA[A]CCAATAC, corresponding to positions c.2862_2873 of *NF1*. Analysis of the percentage of alleles harboring a duplicated "A" nucleotide at the bracketed ([]) position was performed the PyroMark Q24 version 2.0.6 software in the AQ (allele quantification) analysis mode.

RESULTS

SPRED1 and *NF1* exon 22 sequencing was performed on 152 enrolled individuals with the clinical diagnosis of NF1. We identified two individuals with *SPRED1* mutations [Muram-Zborovski et al., 2010] who were subsequently excluded. Mutations were identified in *NF1* exon 22 in 2 out of the 150 remaining individuals. A family member of one of these individuals was subsequently enrolled and found to also harbor an *NF1* exon 22 mutation. Prior to mutation analysis, these 3 individuals were thought to potentially have Legius syndrome based on the clinical phenotype and family history.

Patient #1

An *NF1* 3-base-pair deletion (c.2970_2972delAAT) was identified by Sanger sequencing for *NF1* exon 22 in an 11-year-old boy (Fig 1). His phenotype includes >6 café-au-lait spots of >1.5cm in diameter, bilateral groin and axillary freckling, attention deficit and hyperactivity disorder (ADHD) with learning difficulties and speech delay, and pectus excavatum. This

individual had not developed any neurofibromas, Lisch nodules, or optic pathway tumors at the time of this report.

Patients #2 and #3

An *NF1* single base duplication, c.2866dupA, was identified in a 4-year-old male child (Fig 2A). This heterozygous mutation results in a frameshift, leading to a premature termination codon, 19 amino acids downstream and likely, nonsense-mediated decay. The child's phenotype includes >6 café-au-lait spots of >0.5cm in diameter, left groin and mild bilateral axillary freckling, no neurofibromas, no Lisch nodules, no optic pathway tumors, speech delay, and a very mild pectus carinatum. This child has two siblings without reported manifestations of NF1, but his father was reported to have a history of a "few birthmarks" without any other manifestations of NF1. The 4-year-old boy's 36-year-old father was subsequently evaluated and enrolled. His phenotype includes normal cognition without learning difficulties, macrosomia (height >97%; weight >97%; head circumference >97%), bilateral axillary freckling, and 9 café au lait macules >1.5cm in greatest diameter involving multiple body regions without a segmental distribution. He had no Lisch nodules and he denied any neurofibromas, but physical examination showed a small fleshy mass ~0.5cm in diameter on the right arm. He reports that a dermatologist informed him that this was a sebaceous cyst, but the appearance of the mass upon examination by one author (DS) was consistent with a single cutaneous neurofibroma. Based upon these physical findings and family history, the father satisfied NIH clinical criteria for the diagnosis of NF1. From whole blood, a single *NF1* exon 22 base duplication, c.2866dupA, identical to his son, was suspected in a small percentage of alleles by Sanger sequencing (Figure 2A).

Confirmation of the genotype was performed by single nucleotide extension with results shown in Figure 2B. In this case, the 3' end of the extension primer is located over the adenosine at c.2866. In a wildtype sequence the next incorporated base would be cytosine. In the son's sample, both cytosine and adenosine residues are incorporated, indicating both wildtype alleles and alleles harboring the duplicated base. The father's DNA also shows evidence of adenosine incorporation, corresponding to the duplicated base, at this position (Figure 2B). Although single nucleotide results are not quantitative, it appears that the adenosine in the father's sample occurs in a lower percentage of the alleles than in the son's sample.

Results of pyrosequencing analysis also indicate the incorporation of an additional adenosine in a subset of alleles for both the son and the father at position c.2866 (Figure 2C). This is indicated by the increased peak height at dispensation 6, as compared to the peak height at dispensation 4 where a single adenosine base is incorporated. Allele quantification analysis showed that the son harbored the duplication in 48% of alleles, indicating a heterozygous state in all cells. Analysis of the father's DNA showed the duplication in 12% of the alleles. Since this is a heterozygous condition, an allele percentage of 12% reflects an approximate level of mosaicism of 24% in the father's peripheral blood cells.

DISCUSSION

Evaluation of an individual presenting with only multiple café-au-lait spots with or without freckling is complicated, and the differential diagnosis includes but is not limited to generalized NF1 at a young age without emergence of other manifestations, NF1 with a 3-base-pair deletion in *NF1* exon 22 (c.2970_2972delAAT), *NF1* mosaicism, and Legius syndrome. Other disorders within the Ras pathway as well as additional genetically unknown causes of autosomal dominant café-au-lait spots must also be considered [Nystrom et al., 2009b].

These three reported patients were initially thought to potentially have Legius syndrome because of their mild phenotypes and family history. The phenotype of the 11-year old boy (Patient #1) with a 3-base-pair deletion (c.2970_2972delAAT) is consistent with other reports associating a mild phenotype with this genotype [Stevenson et al., 2006; Upadhyaya et al., 2007]. This deletion results in the loss of one of two adjacent methionine residues [Stevenson et al., 2006; Upadhyaya et al., 2007]. The genotype-phenotype correlation with this deletion may be due to the maintenance of a partially functional *NF1* protein product, neurofibromin [Upadhyaya et al., 2007]. This individual emphasizes the usefulness of mutation analysis in providing anticipatory guidance for this child and his family.

Initially, the 4-year-old child (Patient #2) was thought to also potentially have Legius syndrome because of his café-au-lait spots and lack of non-pigmentary findings. This suspicion was strongly supported by his father's reported phenotype of multiple café-au-lait spots with axillary freckling without tumorigenic manifestations. However, the child did not have either a *SPRED1* mutation or the *NF1* 3-bp deletion. Instead, this young child had a frameshift mutation (*NF1* exon22 c.2866dupA) with presumed premature protein truncation and nonsense-mediated decay of the mRNA. In this situation, the overlapping phenotypes of Legius syndrome and NF1 in young individuals, demonstrates the usefulness of genetic testing in specific circumstances. Presumably, the mild NF1 phenotype seen in this child is secondary to his young age and development of further NF1 manifestations are likely to arise with age. Conversely, the attenuated phenotype of his father is likely due to his low level of germline mosaicism and the higher dosage of functional neurofibromin. The level of mosaicism in other tissue, including gonadal mosaicism, is not available. However, since both the father and son have been shown to have identical *NF1* mutations, we suggest that the father has some degree of gonadal mosaicism.

Exhaustive mutation analysis of *NF1* identifies approximately 95% of individuals fulfilling the diagnostic criteria for NF1 [Messiaen et al., 2000]. However, *NF1* sequencing of genomic DNA is not a sensitive method for detection of low-level mosaicism since the limit of detection is approximately 20%, based on our internal validation. The affected father (Patient #3) is estimated to have approximately 12% mutant alleles, corresponding to 24% germline mosaicism in the peripheral blood. This degree of mosaicism however, may not be consistent between tissue types and may be dependent on the sample type obtained. While not essential, knowing the familial mutation was helpful in identifying the father's low level of mosaicism. Given the overlapping phenotype of Legius syndrome and other causes of autosomal dominant café-au-lait spots with NF1, the father/son pair we report highlights the importance of identifying individuals with *NF1* mosaicism in order to provide appropriate genetic counseling. Clinically it would be advantageous to know the level of gonadal mosaicism through sperm analysis in order to counsel this family in regards to recurrence risks.

There are a few published reports of *NF1* mosaicism [Consoli et al., 2005; Kehrer-Sawatzki et al., 2004; Maertens et al., 2007; Petek et al., 2003]. One report specifically addresses the transmission of a familial *NF1* mutation due to gonadal mosaicism in a phenotypically normal father [Lazaro et al., 1994]. Lazaro et al. present this report of mitotic germ-line mutation as a mechanism for NF1 transmission, since male germ cells have greater mitotic activity and the risk of mutation increases with the number of cell cycles [Lazaro et al., 1994]. In general, approximately half of those individuals who are evaluated in the clinical setting in North America and Europe are sporadic, which indicates that 50% of *NF1* mutations are *de novo* [Ars et al., 2003; Lazaro et al., 1994]. However, the risk of having a second child affected with NF1 may be higher than the normal population, due to the chance of gonadal mosaicism [Lazaro et al., 1994; van der Meulen et al., 1995]. This is a concern when counseling a family on future reproduction as some individuals may wish to perform

NF1 mutation analysis after a single affected child is diagnosed. For the family herein reported, it is reasonable to assume that the father, with a 24% peripheral blood germline mosaicism and one affected child, has gonadal mosaicism as well. However, the degree of gonadal mosaicism cannot be determined from our results without sperm analysis, making recurrence risk calculations difficult. Sperm analysis was offered and was declined at the time of this report.

The phenotype of Legius syndrome decreases the specificity of the clinical NIH diagnostic criteria for NF1, as these patients may meet diagnostic criteria based on pigmentary findings [Brems et al., 2007; Messiaen et al., 2009; Muram-Zborovski et al., 2010; Pasmant et al., 2009; Spurlock et al., 2009]. The criteria used in this manner cannot distinguish NF1 from Legius syndrome, although clinical assessment of parents can be quite helpful. Accurate diagnosis is important since anticipatory guidance differs. These individuals emphasize the need for mutation analysis in some individuals with café-au-lait macules and freckling who lack the tumorigenic or classic skeletal abnormalities of NF1. Specifically, with the identification of Legius syndrome, the need to recognize the attenuated phenotype of *NF1* mosaicism and confirmation by mutation analysis is increasingly important for appropriate medical management and family counseling. While accurate diagnosis is possible in most cases using strict clinical criteria, genetic testing may be necessary in specific circumstances, although the costs of the genetic testing may be prohibitive for some families. Accurate diagnosis is crucial as it directly impacts future expectations of disease severity, genetic counseling for families and future reproduction, as well as ongoing research where individuals with mild phenotypes may be clinically diagnosed erroneously as either NF1 or Legius syndrome and enrolled in clinical trials.

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Figure 1.

Patient #1 sequencing results showing a heterozygous inframe 3-base pair deletion, c. 2970-2972delAAT, (red squares) resulting in the loss of one of two adjacent methionine residues (green circle). The allele with the 3-base pair deletion is displayed along with the normal allele sequence. This deletion results in an allele, which shifts a guanine in the forward direction (black arrows) and a cytosine in the reverse direction (blue arrows), 3-base pairs.

Figure 2.

A) Sanger sequencing results showing a heterozygous insertion of a single adenosine base resulting in a frameshift sequence seen in both the forward and reverse directions in patient 2 and at a very lower level in patient 3 (blue arrows). Black circles indicate sequence position analyzed by single nucleotide extension (results shown in frame B). Orange boxes indicate sequence position analyzed by pyrosequencing (results shown in frame C). B) Single nucleotide extension results showing incorporation of both a cytosine and adenosine residue (green arrow) in NF1 exon 22 c.2867, confirming the frameshift at the first mixed base position in patient 2 and at a lower concentration in patient 3. C) Pyrosequencing results showing incorporation of bases corresponding to positions c.2862_2866 of *NF1* in patients 2 and 3. Dispensed bases are shown along the X-axis, with the adenosine at dispensation 6 corresponding to c.2866. In both samples, the increased height of this peak as compared to the corresponding adenosine peak height at dispensation 4, shows that multiple adenosine bases were incorporated in the sequence. Allele quantification analysis indicates that 48% of the son's alleles and 12% of the father's alleles harbor the duplicated adenosine. Since this mutation in a heterozygous state, an allele percentage of 12% reflects an approximate level of mosaicism of 24% in the father's peripheral blood cells.