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SPRED1 Mutations in a Neurofibromatosis Clinic

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Abstract

Legius syndrome, caused by *SPRED1* mutations, has phenotypic overlap with neurofibromatosis type 1 (NF1) without tumorigenic manifestations. Patients fulfilling the NIH diagnostic criteria for NF1 were enrolled from the University of Utah NF Clinic and *SPRED1* mutation analysis performed in order to identify the frequency of Legius syndrome within an NF1 clinic population.

SPRED1 sequencing was performed on 151 individuals with the clinical diagnosis of NF1 and 2 individuals (1.3%) were found to have novel *SPRED1* mutations, p.R18X and p.Q194X. The phenotypes for the two individuals with *SPRED1* mutations included altered pigmentation without tumorigenesis. A specific *SPRED1* haplotype allele was identified in 27 individuals.

The frequency of *SPRED1* mutations in patients meeting diagnostic criteria for NF1 in a hospital-based clinic is 1–2%. The likelihood an individual is harboring a *SPRED1* mutation increases with age if multiple, non-pigmentary NF1 findings are absent. Legius syndrome patients may benefit from altered medical surveillance.

Keywords

neurofibromatosis type 1 (NF1); Legius syndrome; Ras pathway; *SPRED1*

Introduction

Neurofibromatosis type 1 (NF1) is a relatively common autosomal dominant disorder occurring in 1 out of every 3000 births. NF1 shows complete penetrance by adulthood yet there is a high degree of variable clinical expressivity, both inter- and intra-familial variability. Cardinal clinical manifestations are café-au-lait spots and neurofibromas, and other features include Lisch nodules, optic pathway gliomas, malignant peripheral nerve sheath tumors (MPNSTs), learning disorders, and bone abnormalities. While the clinical diagnosis for NF1 is based on NIH clinical diagnostic criteria, other disorders have overlapping phenotypes with NF1. The overlapping phenotype can potentially complicate diagnostic evaluations and lead to medically inappropriate screening protocols. Examples of these disorders include Noonan syndrome, LEOPARD syndrome, cardio-facio-cutaneous (CFC) syndrome, Costello syndrome (Stevenson et al., 2008), and Legius syndrome (Brems

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et al., 2007; Pasmant et al., 2009; Spurlock et al., 2009). The causative genes for all of these disorders encode proteins within the Ras-MAPK signaling pathway.

Legius syndrome, originally termed “neurofibromatosis type 1-like syndrome”, is caused by *SPRED1* mutations on chromosome 15q13.2 (Brems et al., 2007). Individuals with *SPRED1* mutations frequently fulfill the NIH diagnostic criteria for NF1 based on pigmentary manifestations of café-au-lait spots and distinctive freckling patterns (Brems et al., 2007; Pasmant et al., 2009; Spurlock et al. 2009). This overlapping phenotype is likely due to increased Ras signal propagation caused by inactivating *SPRED1* mutations. Normally, SPRED1 protein binds to Ras and blocks phosphorylation of Raf, which diminishes downstream ERK activation (Brems et al., 2007).

The phenotype due to *SPRED1* mutations as described by Brems et al. (2007) includes café-au-lait spots (98%), freckling (30%), and macrocephaly ≥97th centile (42%). Of the 44 reported cases (age ranges 1 to 66 years) in these 5 families, Brems et al. (2007) reported 14 patients with lipomas, 6 with learning problems, 5 with Noonan-like facial features, 3 with depigmented spots, 2 with attention deficit and hyperactivity disorder, and 3 with pectus excavatum. Neurofibromas, Lisch nodules, optic pathway tumors, or malignant tumors were not identified. Two additional studies by Pasmant and Spurlock (Pasmant et al., 2009; Spurlock et al., 2009) reported similar phenotypes in patients with *SPRED1* mutations. Spurlock et al. (2009) tested 85 unrelated cases without detectable *NF1* mutations or neurofibromas, and identified 6 individuals with *SPRED1* mutations. They then screened family members of these 6 cases and identified a total of 12 individuals with *SPRED1* mutations. All had café-au-lait macules, approximately two-thirds had intertriginous freckling, and there was no report of learning problems, Lisch nodules, neurofibromas, or optic gliomas. Pasmant et al. (2009) tested 61 index cases with the clinical diagnosis of NF1 without an identifiable *NF1* mutation and found 5 individuals with *SPRED1* mutations. They also screened additional family members from these 5 cases and identified a total of 18 individuals from 5 families with *SPRED1* mutations. From the report (Pasmant et al, 2009), 9 of the 18 individuals with *SPRED1* mutations fulfilled the NF1 diagnostic criteria assuming that individuals in their age categories of 0–4, 5–9, and 10–14 years are not post-pubertal. This assumption is important given that the NIH diagnostic criteria for café-au-lait macules states “six or more café-au-lait macules over 5mm in diameter in prepubertal individuals and over 15mm in greatest diameter in postpubertal individuals” (NIH, 1988; Gutmann et al., 1997). All 18 individuals had at least 2 café-au-lait macules, 72% had intertriginous freckling, 2 had lipomas, 4 had learning disabilities, 1 had epilepsy, 1 had monoblastic acute leukemia, and none had Lisch nodules, neurofibromas or optic gliomas. A comparison of some of the clinical features of *SPRED1* and *NF1* are summarized in Table 1. It appears from the few reports to date that patients with Legius syndrome do not develop some of the more morbid complications seen with NF1, particularly tumor formation, and an altered health care management plan may be appropriate.

These previous studies (Brems et al., 2007; Pasmant et al., 2009; Spurlock et al. 2009) evaluated cohorts of individuals with clinical features of NF1 who did not have identifiable *NF1* mutations. Approximately 5% of patients who meet the NIH clinical diagnostic criteria for NF1 do not have an identifiable *NF1* mutation by exhaustive mutation analysis (Messiaen et al., 2000). Our aim was to identify the frequency of *SPRED1* mutations in individuals with a clinical diagnosis of NF1 within an unselected Neurofibromatosis Clinic population.

Patients and Methods

NF1 patient phenotyping

Individuals meeting NIH clinical diagnostic criteria for neurofibromatosis type 1 (NF1) evaluated at the University of Utah Neurofibromatosis Clinic were offered enrollment. Prior to *SPRED1* sequencing, a detailed physical examination and review of medical records were performed on all NF1 individuals by one physician investigator between 2002 and 2009 (DS). A standardized NF1 history and exam form created for use for the University of Utah NF1 Registry and modeled after information requested by the Children's Tumor Foundation (formerly National Neurofibromatosis Foundation) International Database (Friedman and Birch, 1997), was modified and used to document the phenotype. For purposes of this study probands were defined as the individual within a family who was first enrolled. Approval by the Institutional Review Board at the University of Utah was obtained.

SPRED1 gene Sequencing

Primer sets were designed for the coding regions and intron/exon boundaries of *SPRED1*. These were designed using LightScanner Primer DesignR software by Idaho Technology Inc. (Salt Lake City, Utah). Selected primer sets were supplied by Integrated DNA Technologies (Coralville, Iowa). Primer sequences are available upon request. Genomic DNAs were isolated from peripheral blood using Puregene DNA extraction kit (Gentra System Inc, Minnesota). Each patient sample was amplified by polymerase chain reaction (PCR). All post PCR products were checked for the appropriate band size using capillary electrophoresis by the Qiagen eGene. PCR primers were then degraded using exoSAP-ITR (USB Corporation, Cleveland, OH), after which bidirectional Sanger sequencing was performed followed by sephadex cleanup. Products were then assessed by an ABI 3730 analyzer. All sequence analysis was performed using Mutation Surveyor® software (SoftGenetics, State College, PA) and compared with the GenBank reference sequence NC_000015. All amplicons were sequenced in both the forward and reverse directions.

Control Population Sample—Normal control population allele studies were performed using DNA from 32 anonymous healthy individuals from the ARUP clinical laboratory (Salt Lake City, UT). These samples were only tested for the specific exons required to identify the *SPRED1* haplotype of interest.

Results

The *SPRED1* gene was sequenced for 151 individuals with the clinical diagnosis of NF1. Demographics are described in Table 2. Within this cohort, 2 prepubertal unrelated individuals with novel *SPRED1* mutations, p.R18X and p.Q194X, were identified.

The first individual had a mutation in *SPRED1* with a C>T nucleotide substitution at position c.52, generating a premature stop codon at p.18 (p.R18X). This patient's mutation was confirmed on a clinical basis by the University of Alabama Medical Genomics Laboratory (Birmingham, AL). The observed phenotype for this individual, age 12 years, included 10–20 café-au-lait spots, subtle inguinal and axillary freckling, learning disabilities requiring intervention, and no neurofibromas (Figure 1A,B). His occipital frontal circumference (OFC) was at the 95th centile, weight was at the 75th centile, and height was at the 25th centile. He was reported to have a single Lisch nodule by slit lamp examination, which was clinically reported by an ophthalmologist at an outside institution over multiple evaluations. Given that no other individual with a *SPRED1* mutation has been reported to have Lisch nodules, the patient was evaluated at the University of Utah by one of the authors (DD), a pediatric ophthalmologist who follows a large number of individuals with NF1, for

further investigation of the ophthalmologic findings. The patient did not have classic Lisch nodules; however, he had an unusual pattern of iris pigmentation with multiple iris nevi of varying size (Figure 2A) and abnormally large and wide iris crypts (Figure 2B).

The second individual had a mutation in *SPRED1* with a C>T nucleotide substitution at position c.580, resulting in a premature stop codon at p.194 (p.Q194X). The phenotype for this individual, age 10 years, included more than 10 café-au-lait spots, axillary and groin freckling, learning disabilities requiring intervention, and no neurofibromas (Figure 1C,D). His OFC was >98th centile, height was at the 95th centile, and weight was >98th centile. Ophthalmologic examination at 7 years of age showed no Lisch nodules; however, this individual was reported by a pediatric ophthalmologist at the University of Utah to have “the sort of very convoluted brown iris making detection of Lisch nodules very difficult.”

Demographic and phenotypic information for the clinic cohort was used for comparison between individuals meeting the NIH diagnostic criteria for NF1 with and without a *SPRED1* mutation. Based on the presence or absence of a specific finding or combination of specific findings, the likelihood of having a *SPRED1* mutation is outlined in Figure 3. Within our clinic cohort of individuals fulfilling NIH diagnostic criteria for NF1, we identified 1.32% with *SPRED1* mutations, and 2.60% if selected for only sporadic cases. Selecting for those individuals who lacked Lisch nodules, optic gliomas, neurofibromas, long bone dysplasia and sphenoid wing dysplasia or a family history, this percentage increases to 20%. This percentage increases further with age to 50% for those 10 years or older.

In establishing an assay to identify *SPRED1* mutations, we identified a common haplotype allele matching GenBank sequence NG_008980. This haplotype allele (*SPRED1*^{2}) is defined by 4 single nucleotide polymorphisms (SNPs), differing from the more common *SPRED1*^{1} reference sequence NC_000015. The *SPRED1*^{2} allele was identified in 27 of the 151 individuals sequenced (17.9%). Twenty-five NF1 individuals were heterozygous for *SPRED1*^{2} while two patients were homozygous. The overall *SPRED1*^{2} allele frequency was 9.6%. When only probands are used to calculate the haplotype allele frequency (excluding the affected related individuals), 25 of 127 individuals carry the *SPRED1*^{2} haplotype allele [19.7%, allele frequency 10.6%]. Two of the single nucleotide polymorphisms were found within exons and caused synonymous changes. The first exonic SNP is located in exon 4 (c. 291A>G; p.K97K) and the second exonic SNP is located in exon 8 (c.1044C>T; p.V348V). Both intronic SNPs are located within the intronic region between exons 5 and 6 (c. 424-8A>C and c.424-98C>T). Neither of these two intronic SNPs is predicted to cause splice site alterations (http://www.fruitfly.org/seq_tools/splice.html). None of these 4 SNPs were seen as an isolated event in any of the 151 patients sequenced; indicating that this haplotype block is in high disequilibrium. The allele frequency in our unselected normal population was slightly decreased from the NF1 cohort [12.5% (4/32, p=0.049)]. No *SPRED1*^{2} allele homozygotes were identified in the unselected population. The overall allele frequency was 6.3%, (4/64 alleles). The difference in allele frequency between the NF1 probands only versus the unselected control population is statistically significant (p=0.02). Demographic data was not available for the normal population.

Discussion

The overlap of disease phenotype within the disorders of the Ras-MAPK pathway is well established (Brems et al., 2007; Pasmant et al., 2009; Spurlock et al. 2009; Stevenson et al., 2008; Stevenson et al., 2006; Nyström et al., 2003; Johnson et al., 1998; Lopez-Rangel, 2007; Diglio et al., 2002; Tassabehji et al., 1993; Allanson et al., 1991). One of the most common Ras-MAPK pathway disorders is NF1. In individuals fulfilling the NIH diagnostic

criteria for NF1, 95% have identifiable mutations of the *NF1* gene (Messiaen et al. Hum Mut: 2000). Thus *NF1* mutations are not identified in ~5% of patients who meet NIH clinical diagnostic criteria for NF1. If diagnostic criteria are met solely by pigmentary findings (with or without a first-degree relative), a proportion of these individuals could have Legius syndrome with mutations in *SPRED1*. It is also possible that the remaining individuals have an *NF1* mutation that is undetectable by current methods, a different overlapping Ras pathway disorder, or mutations in other unknown genes. There are several reports of families with isolated multiple café-au-lait macules inherited in an autosomal dominant pattern (Charrow et al., 1993; Arnsmeier et al., 1994; Nyström et al., 2009; Abeliovich et al., 1995; Brunner et al., 1993). Several of these families showed no linkage to the *NF1* locus (Charrow et al., 1993; Nyström et al., 2009; Brunner et al., 1993), and one family's phenotype did not segregate with *SPRED1* (Nyström et al., 2009). This suggests that other unknown genes can result in a similar pigmentary phenotype similar to Legius syndrome and NF1.

Our data show that 1.3% of individuals who meet the clinical diagnostic criteria for NF1 and seeking medical care at an NF Clinic have *SPRED1* mutations. This percentage increases if the cohort is stratified to eliminate those with an affected parent, an optic pathway tumor, *bona fide* Lisch nodules, neurofibromata, long bone dysplasia or sphenoid wing dysplasia. In this circumstance, the percentage of patients with *SPRED1* mutations is 20%. Because of the age-related penetrance of non-pigmentary manifestations of NF1, as an individual's age increases the absence of certain characteristic features of NF1 becomes more predictive of Legius syndrome. For individuals 10-years of age or older who do not have other NF1 features, yet fulfill diagnostic criteria for NF1 by pigmentary features, 50% harbor a *SPRED1* mutation (Figure 3). While, the chance of having both an *NF1* mutation and a *SPRED1* mutation is possible, co-occurrences of 2 rare disorders are extremely infrequent (Wilken et al., 2009).

The phenotypes of the 2 individuals with *SPRED1* mutations were consistent with the previous reports to date (Table 1) (Brems et al., 2007; Pasmat et al., 2009; Spurlock et al., 2009), including café-au-lait spots, intertriginous freckling, learning disabilities, and no neurofibromas. Both children were older than 10 years of age and had not yet developed neurofibromas. One patient with a *SPRED1* mutation was initially reported to have a single identified Lisch nodule, although a subsequent evaluation did not confirm the findings. To date, there are no reported cases of individuals with *SPRED1* mutations who have Lisch nodules, including those adults (>18-years-old) identified by previous authors (Brems et al., 2007; Pasmat et al., 2009; Spurlock et al., 2009).

As shown in Figure 2, it is possible that individuals with Legius syndrome have increased pigment deposits in the iris which may lead some physicians to confuse increased pigment with Lisch nodules, especially if one does not frequently evaluate patients with NF1. It is important to accurately diagnose Lisch nodules in individuals with isolated café-au-lait macules and/or intertriginous freckling, as the presence of Lisch nodules would impact the delineation of Legius syndrome versus NF1, and the diagnosis could subsequently change medical management and counseling. Since fewer than 90 Legius syndrome patients have been reported, it may be that a small percentage of patients can develop increased pigment deposits or other iris findings. Further investigation is necessary to determine the degree of association and types of ocular abnormalities for individuals with *SPRED1* mutations. The absence of a Lisch nodule in the above reported patient is consistent with Legius syndrome.

The phenotype of Legius syndrome is currently based on a cross-sectional phenotype of a small number of patients (Brems et al., 2007; Pasmat et al., 2009; Spurlock et al., 2009). The clinical manifestations of Legius syndrome will continue to become better defined with the

identification of more individuals. Long-term studies will be required to confirm the current suggested phenotype and establish the lifetime risk of tumorigenesis and whether it is increased compared to the general population. Within our cohort, the phenotype primarily consists of altered pigmentary patterns and learning disorders as demonstrated in previously reported cases (Brems et al., 2007; Pasmant et al., 2009; and Spurlock et al., 2009). This is also consistent with the relative lack of manifestations of tumorigenesis in Legius syndrome. Pasmant et al. identified one *SPRED1* mutation positive patient with acute myelogenous leukemia. While no second hit in *SPRED1* could be identified in this patient, the authors could not definitively discern causality in relation to Legius syndrome. Our data support the lack of tumorigenesis in Legius syndrome.

An altered management plan may be appropriate for young individuals fulfilling the pigmentary diagnostic criteria for NF1 who have *SPRED1* mutations rather than *NF1* mutations. Since the absence of characteristic NF1 findings appears consistent across multiple studies, this absence may help identify individuals with *SPRED1* mutations. For those patients meeting NIH diagnostic criteria for NF1 based on pigmentary findings alone, the likelihood of an individual having a *SPRED1* mutation increases as multiple additional characteristic NF1 findings are absent (Figure 3), with increasing age. However, the possibility of an individual mildly affected with NF1 or NF1 mosaicism must always be considered.

A common *SPRED1* haplotype allele was identified in 17.9% of our NF1 cohort (allele frequency of 9.6%), which is significantly more frequent than the 12.5% of an unselected population (allele frequency of 6.3%) (p=0.049). However, because demographic data for the unselected population are not available, ethnicity may be a confounding factor. In addition, it is possible that this is a founder effect within the Utah population. *SPRED1* sequence variants could potentially modify the NF1 phenotype and future studies will be important in examining the role of the *SPRED1* haplotypes on the clinical manifestations in individuals with NF1.

Previous reports have shown that many individuals with *SPRED1* mutations do not meet the NIH diagnostic criteria for NF1 (Brems et al., 2007; Pasmat et al., 2009; Spurlock et al., 2009). Our cohort only included individuals who fulfilled the NIH diagnostic criteria. Hence, the incidence of *SPRED1* mutations in individuals without the clinical diagnosis of NF1 cannot be determined from our results. There is also a potential bias in this study as the NF1 Clinic is staffed primarily by pediatricians at a tertiary children's hospital, thus the cohort is primarily a pediatric population.

Conclusion

In summary, 1–2% of patients meeting NIH clinical diagnostic criteria for NF1 in a hospital-based clinic may harbor *SPRED1* mutations. Patients who fulfill only the pigmentary component of the NF1 diagnostic criteria are more likely to have a *SPRED1* mutation, especially at an older age. Patients with *SPRED1* mutations may require altered medical surveillance, based on the current Legius syndrome phenotype showing altered pigmentation with a lack of tumorigenesis.

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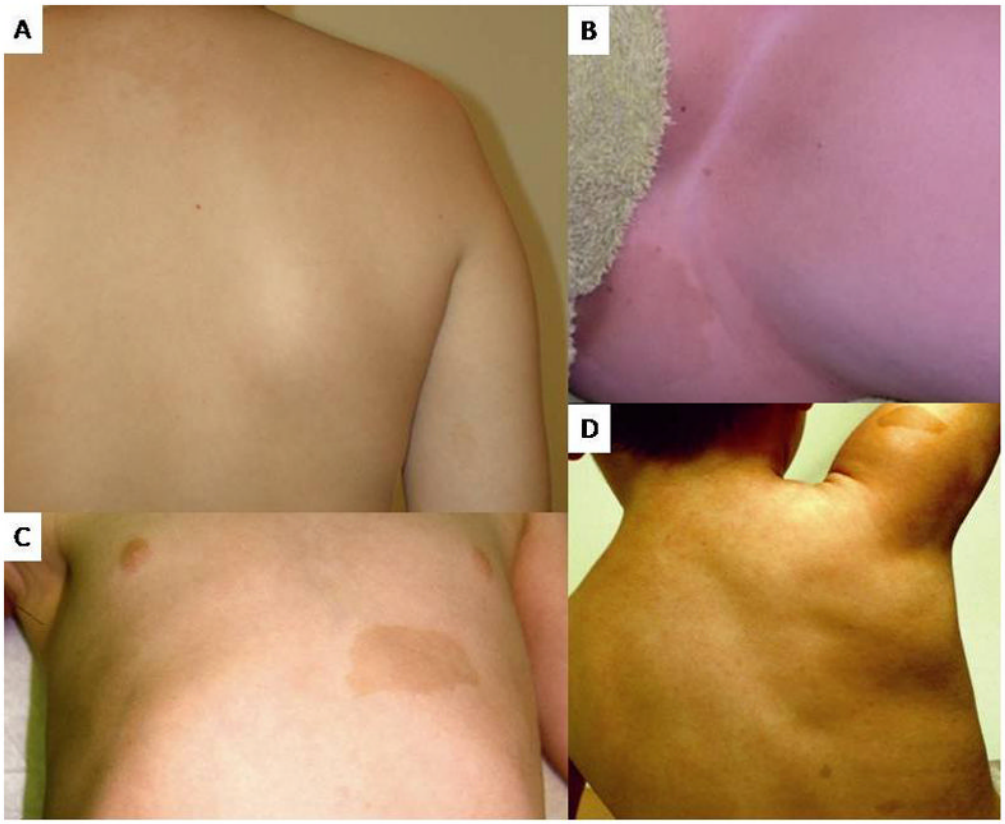

Fig. 1.

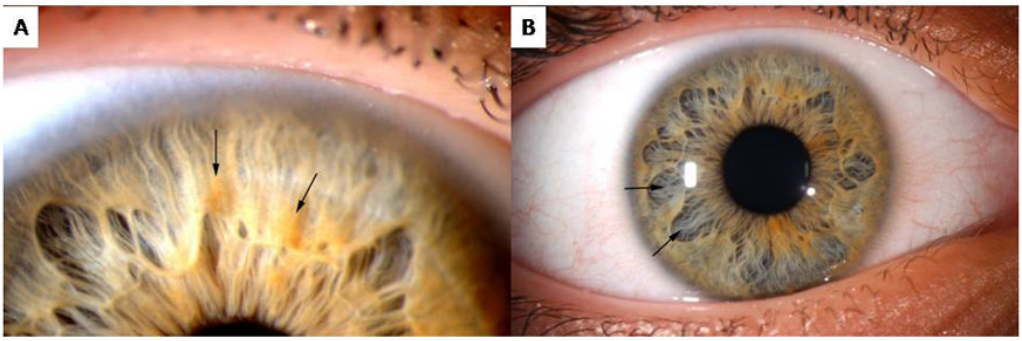

Fig. 2.

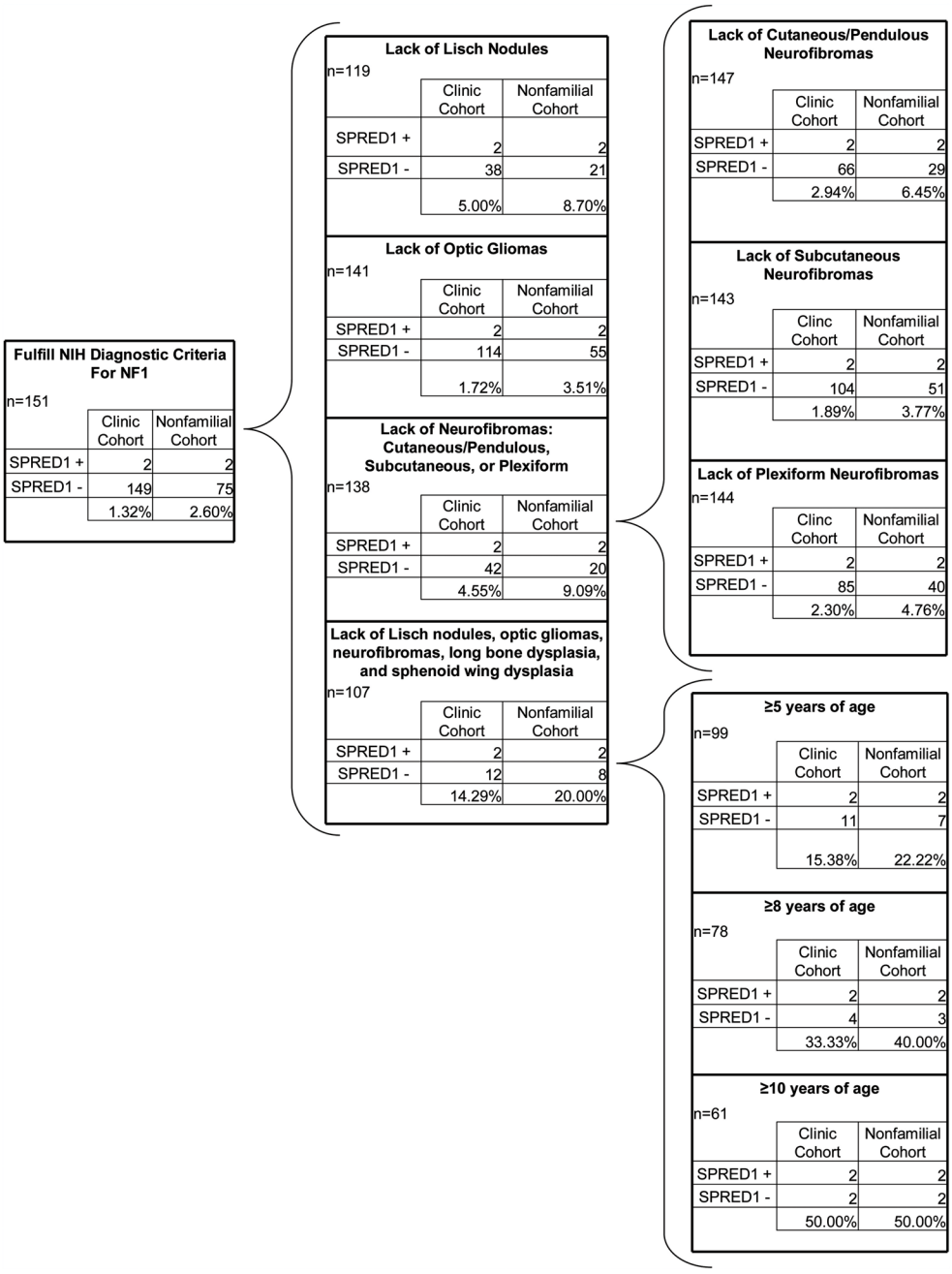

Fig. 3.

Table 1

Clinical Features of *NF1* vs *SPRED1*

	NF1	SPRED1
Café-au-lait spots	+	+
Intertriginous freckling	+	+
Lisch nodules	+	
Neurofibromas	+	
Tibial bowing	+	
Pseudarthrosis	+	
Macrocephaly	+	+
Learning disabilities	+	+
MPNST	+	
Optic glioma	+	

❖ Comparison of some features reported to be associated with the diagnosis of neurofibromatosis type 1 (NF1) and Legius syndrome. MPNST: malignant peripheral nerve sheath tumor

Table 2

Clinic Cohort Demographics

Individuals Enrolled	151
males	79
females	72
Age Range	1 to 34 years
Average Age	10.9 years
Ethnicity	
white	122
black	1
Hispanic	6
Asian	1
>1 ethnicity indicated	18
unknown	3
Inheritance Pattern	
Sporadic cases	72
Familial cases	73
Unknown pattern	6

❖ All individuals included meet NIH clinical criteria for the diagnosis of NF1.