

Clinical and Mutational Spectrum of Neurofibromatosis Type 1–like Syndrome

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See also p 2150 and Patient Page.

Context Autosomal dominant inactivating sprouty-related EVH1 domain-containing protein 1 (*SPRED1*) mutations have recently been described in individuals presenting mainly with café au lait macules (CALMs), axillary freckling, and macrocephaly. The extent of the clinical spectrum of this new disorder needs further delineation.

Objective To determine the frequency, mutational spectrum, and phenotype of neurofibromatosis type 1–like syndrome (NFLS) in a large cohort of patients.

Design, Setting, and Participants In a cross-sectional study, 22 unrelated probands carrying a *SPRED1* loss-of-function (LOF) mutation identified through clinical testing participated with their families in a genotype-phenotype study (2007-2008). In a second cross-sectional study, 1318 unrelated anonymous samples collected in 2003-2007 from patients with a broad range of signs typically found in neurofibromatosis type 1 (NF1) but no detectable *NF1* germline mutation underwent *SPRED1* mutation analysis.

Main Outcome Measures Comparison of aggregated clinical features in patients with or without a *SPRED1* or *NF1* mutation. Functional assays were used to evaluate the pathogenicity of missense mutations.

Results Among 40 *SPRED1* LOF-positive individuals from the clinical cohort, 20 (50%; 95% confidence interval [CI], 34%-66%) fulfilled National Institutes of Health (NIH) NF1 diagnostic criteria based on the presence of more than 5 CALMs with or without freckling or an NF1-compatible family history. None of the 40 *SPRED1* LOF-positive individuals (0%; 95% CI, 0%-7%) had discrete cutaneous or plexiform neurofibromas, typical NF1 osseous lesions, or symptomatic optic pathway gliomas. In the anonymous cohort of 1318 individuals, 34 different *SPRED1* mutations in 43 probands were identified: 26 pathogenic mutations in 33 probands and 8 probable nonpathogenic missense or silent mutations in 10 probands. Of 94 probands with familial CALMs with or without freckling and no other NF1 features, 69 (73%; 95% CI, 63%-80%) had an *NF1* mutation and 18 (19%; 95% CI, 12%-29%) had a pathogenic *SPRED1* mutation. In the anonymous cohort, 1.9% (95% CI, 1.2%-2.9%) of individuals with the clinical diagnosis of NF1 according to the NIH criteria had NFLS.

Conclusions A high *SPRED1* mutation detection rate was found in *NF1* mutation-negative families with an autosomal dominant phenotype of CALMs with or without freckling and no other NF1 features. Among individuals in this study, NFLS was not associated with the peripheral and central nervous system tumors seen in NF1.

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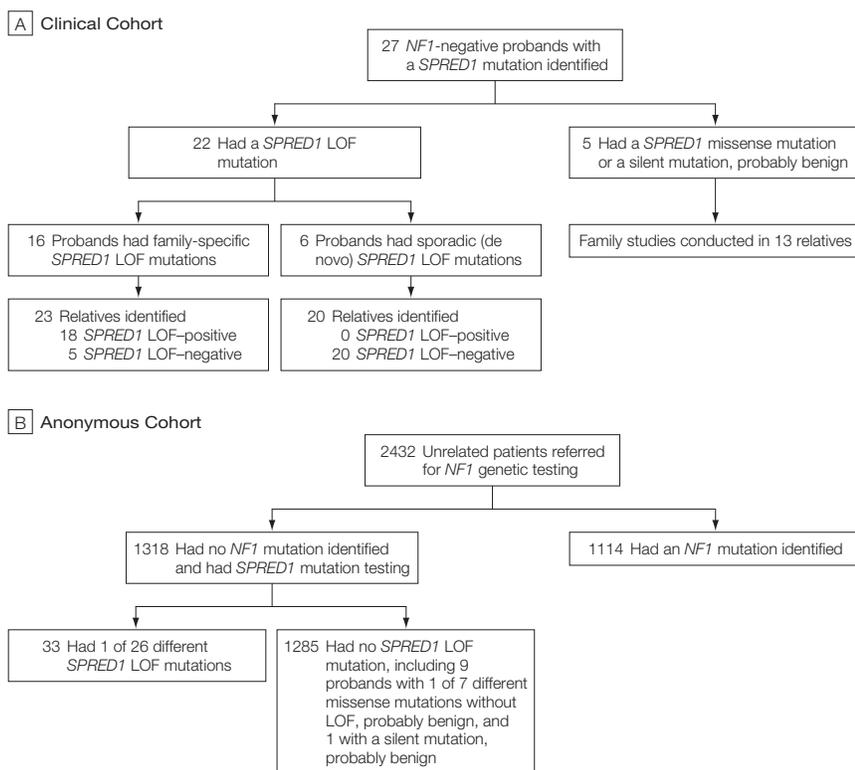
NEUROFIBROMATOSIS TYPE 1 (NF1) (Online Mendelian Inheritance in Man [OMIM] 162200), an autosomal dominant disorder affecting approximately 1 in 3000 individuals worldwide,^{1,2} results from inactivating mutations in the *NF1* tumor suppressor encoding neurofibromin. Neurofibromatosis type 1 is characterized by multiple café au lait macules (CALMs), skin-fold freckling, iris Lisch nodules, and tumors of the nervous system such

as optic pathway gliomas, astrocytomas, neurofibromas, and malignant peripheral nerve sheath tumors. Other frequently observed features are specific bone abnormalities, short stature, macrocephaly, and learning problems.

The NF1 diagnostic criteria were established at the National Institutes of

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Figure 1. Patients Included in the Study

LOF indicates loss of function; *NF1*, neurofibromatosis type 1; *SPRED1*, sprouty-related EVH1 domain-containing protein 1.

Health (NIH) NF consensus conference in 1988,³ were updated in 1997,⁴ and are widely used to make the diagnosis using information obtained from physical examination, family history, and radiologic studies.

Neurofibromin is a negative regulator of RAS and pathogenic *NF1* (NM_000267.2) mutations result in increased active RAS (guanosine triphosphate-bound RAS) and increased signaling through the downstream effectors such as the mitogen-activated protein kinase (MAPK) pathway.⁵ Recently, a genetically distinct but phenotypically similar disorder caused by heterozygous *SPRED1* (NM_152594.2) mutations was identified (OMIM 611431).^{6–8} Sprouty-related EVH1 domain-containing protein 1 (*SPRED1*), a member of the *SPROUTY/SPRED* family, negatively regulates MAPK signaling by inhibition of RAS-RAF interaction.^{9–11} Inacti-

vating *SPRED1* mutations, resulting in increased MAPK signaling, were found in individuals from 5 families with multiple CALMs, freckling, and macrocephaly, with many fulfilling the NIH criteria for *NF1* based on the presence of CALMs, freckling, or presence of a positive family history.⁶

In the original study,⁶ families suitably sized for linkage analysis had specifically been collected based on individuals with multiple CALMs with or without freckling and absence of a detectable *NF1* mutation, which may imply that the observed phenotype and possible complications associated with the disorder might have been biased or underestimated. Similarly, the study by Spurlock et al⁸ and Pasmant et al⁷ primarily focused on *SPRED1* analysis in patients with the mild pigmentary phenotype. Given the absence of neurofibromas in any patient described so far,^{6–8} it has been proposed to refer to this new

syndrome as Legius syndrome (as named in OMIM 611431)^{7,8} and discontinue referring to it as neurofibromatosis type 1–like syndrome (NFLS).

In this study, we determined the phenotype in 22 unrelated probands and 18 relatives carrying a *SPRED1* loss-of-function (LOF) mutation identified through clinical genetic testing. In addition, we carried out a cross-sectional study in an anonymous cohort of 1318 unrelated patients referred for *NF1* genetic testing, in whom no *NF1* mutation was found, allowing delineation of the *SPRED1* mutational spectrum and estimation of the frequency of NFLS.

METHODS

The flow of participants included in the studies is shown in FIGURE 1.

Clinical Cohort

In a cross-sectional study, a *SPRED1* LOF mutation was identified in 26 *NF1*-negative probands (with clinical *NF1* testing before July 2007) for whom a blood sample was received (August 2007–September 2008) for clinical *SPRED1* mutation testing (this test became available by August 2007 and is described at <http://www.genetics.uab.edu/medgenomics>). Probands were referred from 19 different centers in the United States and Canada. Written informed consent to collect phenotypic and genotypic data was obtained from study participants and parents or guardians of children. Clinical notes and phenotypic information as available through the referring physician were reviewed.

Height and head circumference at a given age were converted to percentiles using the Centers for Disease Control and Prevention US growth charts (<http://www.cdc.gov/nchs/data/ad/ad314.pdf>). An individual was recorded as macrocephalic when head circumference was above the 97th percentile. An individual was recorded to have relative macrocephaly when head circumference was above the 97th percentile at the age when height would have been at the 50th percen-

Table 1. Aggregated Clinical Features in 40 Individuals From 22 Families Carrying a Pathogenic *SPRED1* LOF Mutation, by Age Group (Clinical Cohort)

Age, y	Total	F/S/U	No. of Patients With Size and No. of CALMs Fulfilling First NIH Criterion	No. of Patients				No. of Patients With Clinical NF1 Diagnosis (Fulfilling ≥ 2 NIH Criteria)	Absolute or Relative Macrocephaly
				Freckling					
				Axillary and Inguinal	Axillary	Inguinal	None		
0-7 ^a	21	15/5/1	20/21 ^b	3/21	4/21	2/21	13/21	9/21	4/21
8-19 ^c	5	4/1/0	5/5	1/5 Plus 1 with thoracic freckling	1/5	1/5	1/5	4/5	0/4 ^d
20-68 ^e	14	14/0/0	10/14 ^f	2/14 Plus 1 with freckling in neck	3/14	0/14	9/14	7/14	3/6

Abbreviations: CALMs, café au lait macules; F, familial; LOF, loss of function; NF1, neurofibromatosis type 1; NIH, National Institutes of Health; S, sporadic, *SPRED1*, sprouty-related EVH1 domain-containing protein 1; U, unknown.

^aOther features included bilateral polydactyly hands and feet (n=1); pectus excavatum (n=3); hyperactivity (n=3); attention deficit (n=1); speech/language delay (n=4); pervasive developmental delay; seizures; hypotonia (n=1); simple ears (n=1); and bright blue eyes, down-slanting palpebral fissures, short neck, and Noonan syndrome–like (n=1).

^bIn addition, 1 individual with 4 CALMs larger than 5 mm and 1 individual (age 2 y) with no spots.

^cOther features included meatal stenosis, hypertelorism, hearing loss, and speech delay (n=1); attention deficit (n=1); and large venous anomaly and progressive dystonia (n=1).

^dOne individual with no head circumference information.

^eOther features included mild hearing loss (n=2); congenital pulmonic stenosis, mitral valve prolapse (n=1); angioliopoma, tenosynovial giant cell tumor (n=1); dermoid tumor ovary (n=1); history of seizures (n=1); vascular anomaly leg (n=1); and 2 lipomas (n=1).

^fIn addition, 2 individuals with 1 to 2 CALMs and 3 individuals with 4 to 5 CALMs.

tile. Information on race/ethnicity was recorded by the referring health care clinician as African American, Asian, Hispanic, Native American, Caucasian, other (eg, Pacific Islander) or “not provided.” Additional detail on ethnic background was requested if essential for interpretation of the genetic results. The study was approved by the University of Alabama at Birmingham (UAB) Institutional Review Board.

Anonymous Cohort of NF1-Positive and NF1-Negative Samples

Samples received at the UAB Medical Genomics Laboratory for *NF1* genetic testing (August 2003–July 2007) were included in an anonymous study if a phenotypic checklist (<http://www.genetics.uab.edu/medgenomics>) summarizing the *NF1*-related clinical signs at the time of blood sampling had been completed by the referring physician. This is a cross-sectional, referral-based study with samples received from more than 250 different referral centers in the United States (85%), Canada (8%), Latin America (2%), Australia and New Zealand (2%), Asia (1.5%), and Europe (1.5%). This inevitably implies heterogeneity in the quality of the

data set, which is based on these phenotypic data forms. In total, 2432 unrelated samples with phenotypic data were available by July 2007, which equals approximately 85% of samples received.

Search for genotype-phenotype correlations using a Microsoft Access (Microsoft, Redmond, Washington) database containing phenotypic and mutation data was approved by the UAB Institutional Review Board. The following data were entered through the password-protected Access database: year of birth, age of the individual at time of completion of the phenotypic checklist, country of origin, sex, race/ethnicity, sporadic or familial, standardized information pertaining to the *NF1*-related signs, and a unique identifier not linked to date of birth, name, or other database connected to such information. The same unique identifier was labeled on an aliquot of the left-over sample from the patients referred for *NF1* clinical testing. Entries were exported to an Microsoft Excel table format to facilitate the subsequent statistical analyses.

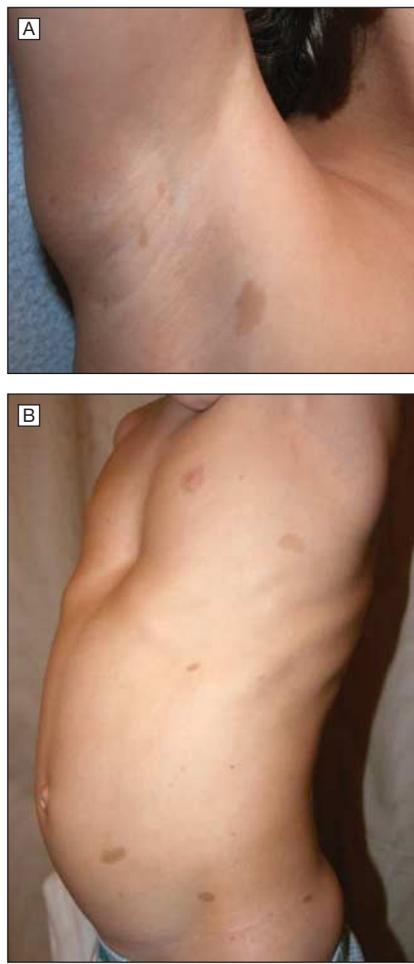
In 1114 of 2432 individuals, an *NF1* mutation had been identified. Anonymous DNA samples from 1318 *NF1*-

negative probands underwent further research-based *SPRED1* mutation analysis, resulting in 3 groups of phenotypic data: *NF1* mutation–positive, *SPRED1* LOF mutation–positive, and *NF1* and *SPRED1* LOF mutation–negative cohorts. This *NF1*-negative cohort is not biased toward the initially reported phenotype in patients with a *SPRED1* mutation.⁶ Genetic research on the anonymous samples was approved by the UAB Institutional Review Board. The 26 probands in the clinical cohort are most likely also part of the anonymous cohort because these patients previously had an *NF1*-negative test result during the study period.

Mutation Analysis, Functional Analysis of Missense Mutations, and Homology Modeling

Comprehensive *NF1* and *SPRED1* mutational testing was performed as described in the eAppendix and eTable 1. The pathogenicity of identified missense mutations was analyzed using 2 different functional assays: (1) neurite outgrowth of pheochromocytoma 12 (PC12) cells in vitro after stimulation with nerve growth factor and (2) Elk-1 activation and phosphorylation.

Figure 2. Café au Lait Macules and Freckling in Right Axilla (A) and Pectus Excavatum (B) of Individual S12



Both functional assays are described in detail in the eAppendix. Secondary structure prediction of mutant p.Thr102Arg and p.Cys74Phe *SPRED1* was carried out as described in the eAppendix.

Statistical Analysis

Ninety-five percent confidence intervals (CIs) of proportions were calculated with the exact binomial method using Statcalc version 1.1 (<http://www.ucs.louisiana.edu/~kxk4695/StatCalc.htm>). We also calculated sensitivity, specificity, and positive and negative predictive values for detection of a *SPRED1* mutation in the *NF1*-negative cohort, also using Statcalc.

RESULTS

Clinical Cohort

In the clinical cohort, a *SPRED1* mutation was identified in 27 *NF1*-negative probands (eTable 2); 22 had a *SPRED1* LOF mutation (including 2 LOF missense mutations: p.Thr102Arg and p.Pro415Ala); 4 of 6 missense mutations and 1 silent mutation were classified as rare, probably benign variants: p.Cys74Arg, p.Ser149Asn, p.Asp398Asn, p.Cys433Tyr, and c.42T>C (eTable 3). Forty-three relatives of the 22 probands with a *SPRED1* LOF mutation participated in the study and 18 of 43 carried the family-specific *SPRED1* mutation. Six of 22 were de novo cases (27%; 95% CI, 11%-50%). Phenotypic details of 40 individuals with a *SPRED1* LOF mutation are provided in TABLE 1 (aggregated data) and eTable 2 (individual data); 14 of 40 had more than 5 CALMs and axillary/inguinal freckling. An additional 6 individuals fulfill *NF1* criteria if family history is taken into account. The majority of individuals (35/40) had more than 5 CALMs even at a young age. Axillary/inguinal freckling was mild or faint in 7 of 14 patients with freckling (FIGURE 2A).

Noonan syndrome (OMIM 163950; an autosomal dominant disorder characterized by a variable combination of facial dysmorphism, short stature, pectus deformity, and congenital heart defects¹²) was suspected in 1 child (individual S10-III1; individual/family numbering is shown in eTable 2), a mild pulmonic valve stenosis was present in individual S11-I2, and mild to severe pectus excavatum was seen in 3 individuals (Figure 2B and eTable 2). Five children had abnormal language and speech development and 5 were reported to have attention deficit, hyperactivity, or both. None of the individuals carrying a *SPRED1* mutation showed neurofibromas, typical osseous lesions, or a symptomatic optic pathway glioma.

We observed 1 occurrence each of tenosynovial giant cell tumor, angioliopoma, breast cancer, and dermoid tumor of the ovary and 2 lipomas in 1 individual (eTable 2). No tumor ma-

terial was available to study the causal involvement of *SPRED1*.

In family S23, the *NF1* mutation c.2755delG had previously been identified in a classically affected patient, S23-III1 (eTable 2), but no *NF1* mutation was found in several of his relatives with *NF1* signs (individuals S23-II2, S23-II3, S23-I2, and S23-III3). A truncating *SPRED1* mutation was found in individual S23-II3 (the younger brother of individual S23-II2), in individual S23-I2 (the mother of individual S23-II2 and S23-II3), and in individual S23-III3 (the child of individual S23-II3) but was absent in individuals S23-II2 and S23-III1.

Anonymous Cohort

Spectrum of *SPRED1* Mutations. We identified 34 different *SPRED1* mutations in 43 probands in the anonymous cohort of 1318 unrelated *NF1*-negative patients (eFigure 1). The majority of mutations (23/34) are predicted to result in a premature stop codon. Furthermore, 1 in-frame deletion (c.242_256del15bp; p.Ile81_Val85del), 9 different missense mutations, and 1 silent mutation were found. Mutations were spread over the entire gene; 23 are novel. Eight different mutations (p.Arg16X; p.Arg18X, p.Arg24X, p.Arg64X, p.Arg117X, p.Thr313Met, p.Arg325X, and p.Asp398Asn), accounting for 12 of the 43 mutations, can be attributed to deamination at methylated CpG dinucleotides. No *SPRED1* copy number changes were detected.

***SPRED1* Missense Mutation.** The phenotype of individuals carrying a (probably) benign missense mutation is described in eTable 3.

Functional and *In Silico* Analysis of Missense Mutations. *SPRED1* missense mutations p.Thr102Arg and p.Pro415Ala are pathogenic and mutants p.Cys74Phe, p.Ser149Asn, p.Met188Ile, p.Thr196Ile, p.Thr313Met, p.Asp398Asn, and p.Cys433Tyr are probably benign variants. Data supporting classification of missense mutations, including the functional effect on the RAS-MAPK pathway using the PC12 differentiation assay and Elk-1 reporter assay

and secondary structure prediction by *in silico* homology modeling, are summarized in the eAppendix, eTable 4, and eFigure 2, eFigure 3, and eFigure 4.

Phenotypic Characteristics. We identified 33 LOF *SPRED1* mutations (eFigure 1) in an anonymous *NF1*-negative cohort of 1318 unrelated probands presenting with a broad range of *NF1*-related signs, not biased toward the phenotype initially described in patients with a *SPRED1* mutation.⁶ Demographic and phenotypic features of a cohort with and without *SPRED1* mutations were compared with a cohort of 1114 patients with a definitive *NF1* mutation (TABLE 2 and TABLE 3). Mean age was 14.9 years in the *NF1*-positive cohort, 9.5 years in the *SPRED1*-positive cohort, and 12.4 years in the *NF1/SPRED1*-negative cohort. The average number of NIH criteria fulfilled was 2.39 in the *NF1*-positive cohort, 1.84 in the *SPRED1*-positive cohort, and 0.89 in the *NF1/SPRED1*-negative cohort.

The high proportion of sporadic patients likely reflects the greater uptake of genetic testing among patients with diagnostic uncertainties in the absence of a family history. Thirty-one of 33 *SPRED1* LOF-positive individuals had more than 5 CALMs with or without freckling and no other *NF1* criteria, 13 (39%; 95% CI, 23%-58%) of which were sporadic cases.

The ratio of *SPRED1/NF1* mutations detected in the total group of 2432 patients was 33/1114 (3.0%; 95% CI, 2.0%-4.1%): 20/251 (8.0%; 95% CI, 4.9%-12.0%) in the familial group vs 13/863 (1.5%; 95% CI, 0.8%-2.6%) in the sporadic group.

Of 1086 patients fulfilling NIH criteria for clinical diagnosis of *NF1*, an *NF1* mutation was found in 823 (76%; 95% CI, 73%-78%), a *SPRED1* mutation in 21 (1.9%; 95% CI, 1.2%-2.9%), and no *NF1/SPRED1* mutation in 243 (22%; 95% CI, 20%-25%), with 211 of 243 being sporadic cases.

No *SPRED1* mutations were found in any *NF1*-negative patients with neurofibromas, optic pathway glioma, Lisch nodules, or typical osseous lesions. The

sensitivity, specificity, and positive and negative predictive values to detect a *SPRED1* mutation in the analyzed *NF1*-negative cohort are reported in eTable 5. The highest positive predictive value was observed in familial patients with more than 5 CALMs with or without freckling and no other criteria (0.720; 95% CI, 0.506-0.879), with a sensitivity of 0.545 (95% CI, 0.364-0.719) and a specificity of 0.995 (95% CI, 0.988-0.998).

COMMENT

We investigated the clinical spectrum of a neurofibromatosis type 1–like syndrome, recently named Legius syndrome (OMIM 611431), and estimated its frequency relative to *NF1* in an anonymous cohort of patients with a broad range of signs typically found

in *NF1*. Individuals with *SPRED1* mutations presented with multiple CALMs with or without freckling. The dermatologic phenotype in young children with a *SPRED1* mutation could not be differentiated from *NF1* and half of individuals (20/40) with a *SPRED1* mutation fulfilled the *NF1* diagnostic criteria based on presence of more than 5 CALMs with or without skinfold freckling and with or without familial history.

Most patients presented with a mild phenotype compared with *NF1*, although in family S24, severe progressive dystonia, a large temporal venous anomaly, and a vascular anomaly were present. In the report by Spurlock et al,⁸ 1 patient had an inguinal heman-gioma, which is of interest given the recently identified association of *RASA1*

Table 2. Demographic Characteristics of Anonymous Cohorts of *NF1*-Positive Patients, *NF1*- and *SPRED1* LOF-Negative Patients, and *SPRED1* LOF-Positive Patients^a

Demographic Subgroup (N = 2432)	No. of Patients		
	<i>NF1</i> -Positive Cohort (n = 1114)	<i>NF1</i> -/ <i>SPRED1</i> LOF-Negative Cohort (n = 1285) ^b	<i>SPRED1</i> LOF-Positive Cohort (n = 33)
Age, y			
0-7 (n = 1211)	525	662	24
8-19 (n = 640)	236	399	5
20->60 (n = 581)	353	224	4
Sex			
Female (n = 1174)	573	585	16
Male (n = 1258)	541	700	17
Race/ethnicity			
African American (n = 164)	70	91	3
Asian (n = 49)	33	15	1
Hispanic (n = 108)	55	51	2
Native American (n = 7)	3	4	0
White (n = 1304)	590	696	18
Other (n = 91)	45	45	1
Not reported (n = 709)	318	383	8

Abbreviations: LOF, loss of function; *NF1*, neurofibromatosis type 1; *SPRED1*, sprouty-related EVH1 domain-containing protein 1.

^aThe first column indicates how many patients with a specific trait/phenotype (age, sex, CALMs only, etc) are present in the entire cohort of 2432 patients. The second column indicates how many of the patients with a specific trait/phenotype as specified in column 1 carry an *NF1* mutation. The third column indicates how many of the patients with a specific trait/phenotype as specified in column 1 do not carry an *NF1* mutation or *SPRED1* LOF mutation. The fourth column indicates how many of the patients with a specific trait/phenotype as specified in column 1 carry a *SPRED1* mutation.

^bThis cohort includes 9 patients with a *SPRED1* missense mutation classified as benign or likely benign. This cohort also includes 16 patients (none of whom had a *SPRED1* LOF or missense mutation classified as benign or likely benign) with more than 5 CALMs and acute lymphoblastic leukemia (aged 3 y), astrocytoma (2 patients aged 11 and 54 y), breast cancer and freckling (2 patients aged 47 and 39 y), gastrointestinal stromal tumors (aged 37 y), glioblastoma multiforme and freckling (aged 12.5 y), juvenile xanthogranuloma (aged 3.4 y), neuroblastoma (2 patients aged 6 and 5.5 y), ovarian cysts (aged 15 y), malignant thoracic spinal cord tumor (aged 5 y), parathyroid tumor (aged 28 y), pituitary adenoma and freckling (2 patients aged 25 and 59 y), Wilms tumor (aged 7 y); and including 12 patients with 1 to 5 CALMs and neuroblastoma (aged 5 mo), rhabdomyosarcoma (aged 3.5 y), oligodendroglioma (aged 17 y), brainstem glioma (2 patients aged 13 and 1.5 y), ependymoma (aged 10 y), ganglioneuroma (aged 24 y), lipoma (aged 14 y), multiple meningiomas in craniocervical junction and thoracic spine (aged 10 y), thalamic glioma (aged 12.5 y), tubular adenoma (aged 39 y), and colon polyps and lipomas (aged 46 y).

with abnormal angiogenesis. RASA1 (p120-RASGAP; OMIM 139150), another guanosine triphosphate–ase-activating protein, down-regulates the RAS-MAPK pathway similarly to neu-

rofibrin.¹³⁻¹⁵ The potential association of NF1s with vascular malformations warrants further investigation. The RAS-MAPK pathway syndromes show a large variability in tumor predispo-

sition, congenital malformations, and intellectual disabilities.¹⁵

Because SPRED1 is involved in regulation of the MAPK pathway, patients with SPRED1 mutations may be at in-

Table 3. Phenotypic Characteristics of Anonymous Cohorts of *NF1*-Positive Patients, *NF1*- and *SPRED1* LOF–Negative Patients, and *SPRED1* LOF–Positive Patients^a

Demographic Subgroup (N = 2432)	No. of Patients (%) [95% Confidence Interval]		
	<i>NF1</i> -Positive Cohort (n = 1114)	<i>NF1</i> -/ <i>SPRED1</i> LOF–Negative Cohort (n = 1285) ^b	<i>SPRED1</i> LOF–Positive Cohort (n = 33)
Sporadic (n = 2109)	863 (40.9) [38.8-43.0]	1233 (58.5) [56.3-60.6]	13 (0.6) [0.3-1.1]
>5 CALMs (n = 1607)	970 (60.4) [57.9-62.8]	606 (37.7) [35.3-40.1]	31 (1.9) [1.3-2.7]
Sporadic and >5 CALMs (n = 1354)	751 (55.5) [52.8-58.1]	590 (43.6) [40.9-46.3]	13 (0.9) [0.5-1.6]
Sporadic, >5 CALMs with or without freckling, and no other NIH criterion (n = 957)	414 (43.3) [40.1-46.5]	530 (55.4) [52.1-58.6]	13 (1.3) [0.7-2.3]
Sporadic, >5 CALMs with or without freckling, and ≥1 additional NIH criterion (n = 389)	337 (86.6) [82.8-89.9]	52 (13.4) [10.1-17.2]	0 [0-0.8]
Familial and >5 CALMs (n = 253)	219 (86.6) [81.7-90.5]	16 (6.3) [3.7-10.1]	18 (7.1) [4.3-11.0]
Familial, >5 CALMs with or without freckling, and no additional NIH criterion (n = 94)	69 (73.4) [63.3-82.0]	7 (7.5) [3.0-14.7]	18 (19.1) [11.7-28.6]
Familial, >5 CALMs with or without freckling, and ≥1 additional NIH criterion (n = 159)	150 (94.3) [89.5-97.4]	9 (5.7) [2.6-10.5]	0 [0-1.9]
With neurofibromas (n = 659)	475 (72.1) [68.5-75.5] ^c	184 (27.9) [24.5-31.5] ^d	0 [0-0.4]
Sporadic with neurofibromas only (n = 145)	27 (18.6) [12.6-25.9] ^e	118 (81.4) [74.0-87.4] ^f	0 [0-2.0]
With OPG (n = 166)	79 (47.6) [39.8-55.5] ^g	87 (52.4) [44.5-60.2] ^h	0 [0-1.8]
Sporadic with OPG only (n = 74)	3 (4.1) [0.8-11.4]	71 (95.9) [88.6-99.2]	0 [0-4.0]
Sporadic OPG and >5 CALMs with or without freckling only (n = 27)	23 (85.2) [66.3-95.8]	4 (14.8) [4.2-33.7]	0 [0-10.5]
Sporadic OPG, 0-5 CALMs, and ≥1 additional NIH criterion (n = 65)	53 (81.5) [70.0-90.1]	12 (18.5) [9.9-30.0] ⁱ	0 [0-4.5]
With Lisch nodules	176 ^j	39 ^k	0 [0-8.4]
With osseous lesion (n = 81)	46 (56.8) [45.3-67.7]	35 (43.2) [32.2-54.7]	0 [0-3.6]
Fulfills 0-1 NIH criterion (n = 1345)	291 (21.6) [19.5-23.9]	1042/1345 (77.5) [75.1-79.7]	12 (0.9) [0.5-1.6]
Fulfills 2 NIH criteria (n = 568)	349 (61.5) [57.3-65.5]	203 (35.7) [31.8-39.8]	16 (2.8) [1.6-4.5]
Fulfills 3 NIH criteria (n = 295)	255 (86.4) [82.0-90.1]	35 (11.9) [8.4-16.1]	5 (1.7) [0.6-3.9]
Fulfills ≥4 NIH criteria (n = 224)	219 (97.8) [94.9-99.3]	5 (2.2) [0.7-5.1]	0 [0-1.3]

Abbreviations: CALMs, café au lait macules; LOF, loss of function; *NF1*, neurofibromatosis type 1; NIH, National Institutes of Health; OPG, optic pathway glioma; *SPRED1*, sprouty-related EVH1 domain–containing protein 1.

^aThe first column indicates how many patients with a specific trait/phenotype (age, sex, CALMs only, etc) are present in the entire cohort of 2432 patients. The second column indicates how many of the patients with a specific trait/phenotype as specified in column 1 carry an *NF1* mutation. The third column indicates how many of the patients with a specific trait/phenotype as specified in column 1 do not carry an *NF1* mutation or *SPRED1* LOF mutation. The fourth column indicates how many of the patients with a specific trait/phenotype as specified in column 1 carry a *SPRED1* mutation.

^bThis cohort includes 9 patients with a *SPRED1* missense mutation classified as benign or likely benign. This cohort also includes 16 patients (none of whom had a *SPRED1* LOF or missense mutation classified as benign or likely benign) with more than 5 CALMs and acute lymphoblastic leukemia (aged 3 y), astrocytoma (2 patients aged 11 and 54 y), breast cancer and freckling (2 patients aged 47 and 39 y), gastrointestinal stromal tumors (aged 37 y), glioblastoma multiforme and freckling (aged 12.5 y), juvenile xanthogranuloma (aged 3.4 y), neuroblastoma (2 patients aged 6 and 5.5 y), ovarian cysts (aged 15 y), malignant thoracic spinal cord tumor (aged 5 y), parathyroid tumor (aged 28 y), pituitary adenoma and freckling (2 patients aged 25 and 59 y), Wilms tumor (aged 7 y); and including 12 patients with 1 to 5 CALMs and neuroblastoma (aged 5 mo), rhabdomyosarcoma (aged 3.5 y), oligodendroglioma (aged 17 y), brainstem glioma (2 patients aged 13 and 1.5 y), ependymoma (aged 10 y), ganglioneuroma (aged 24 y), lipoma (aged 14 y), multiple meningiomas in craniocervical junction and thoracic spine (aged 10 y), thalamic glioma (aged 12.5 y), tubular adenoma (aged 39 y), and colon polyps and lipomas (aged 46 y).

^cOne hundred eighty-nine of 475 had plexiform neurofibroma.

^dEighty-seven of 184 had plexiform neurofibroma and 13 had positive family history; remainder sporadic.

^eEight of 27 had plexiform neurofibroma.

^fSixty-seven of 118 had plexiform neurofibroma.

^gTwenty-three familial.

^hAll sporadic.

ⁱSeven patients had freckling, 1 had plexiform neurofibroma, 2 had Lisch nodules, and 2 had osseous lesions.

^jOne hundred seventy-six patients had ophthalmologically confirmed Lisch nodules, but status for Lisch nodules was unknown for majority of patients when sample was submitted for *NF1* testing.

^kThirty-nine of 1284 *NF1*- and *SPRED1*-negative patients had ophthalmologically proven Lisch nodules, but status for Lisch nodules was unknown for majority of patients when sample was submitted for *NF1* testing.

creased risk of developing specific tumors or learning problems. In our study, 5 children had abnormal language and speech development and 5 were reported to have attention deficit/hyperactivity. The importance of *SPRED1* for hippocampal-dependent learning was recently documented in mice.¹⁶ Future studies need to systematically investigate potential problems with speech, learning, and behavior in children with *SPRED1* mutations.

None of the individuals in our study had neurofibromas, typical osseous lesions, or symptomatic optic pathway gliomas. In this and previous studies, no systematic occurrence of any tumor type was observed, except possibly subcutaneous lipomas in adults, also frequently observed in the general population. Tenosynovial giant cell tumor, dermoid tumor of the ovary, Wilms tumor, tubular colon adenoma, acute leukemia, and non-small cell lung cancer were each observed in only 1 individual once.

Combining data on 53 adults with NFLS from this study and in previous reports⁶⁻⁸ results in a post hoc power estimate of 80% to detect complications occurring in at least 5% of affected adults. Using the binomial distribution, the chance of detecting 2 cases with a specific complication with a prevalence of 5% in a group of 53 individuals is 76%. Combining data from this and previous reports⁶⁻⁸ on individuals aged 5 years or older with a *SPRED1* mutation (n=79), we estimated, in post hoc power calculation, that this sample size would allow detecting complications with a prevalence of at least 3% with a power estimate of 80%. None of these 79 individuals had evidence of plexiform neurofibroma, discrete neurofibroma, or symptomatic optic pathway glioma, suggesting that the frequency of these complications is lower than in NF1 (plexiform neurofibromas, 24%; discrete neurofibromas, 53%; symptomatic optic pathway gliomas, 4.3%¹⁷).

However, even these pooled data are underpowered to detect rare complications associated with a prevalence of

only 1%; a study of 250 well-characterized, preferably adult patients would be needed. Other limitations of the present study are the referral bias (mainly diagnostic uncertainty) and heterogeneity of clinical data (many clinical NF experts involved).

As of July 2007, no *NF1* mutation had been identified through clinical testing in 1318 of 2432 unrelated patients who fulfilled inclusion criteria for further anonymous studies at the UAB Medical Genomics Laboratory. Many samples were submitted because of a diagnostic uncertainty: 55% fulfilled fewer than 2 NIH criteria and 87% were sporadic. *SPRED1* analysis of this anonymous *NF1*-negative cohort revealed a *SPRED1* LOF mutation in 33 of 1318. No *SPRED1* copy number changes were found in this or previous studies⁶⁻⁸ but we recently identified 3 different multi-exon deletions, indicating the need for dosage analysis in the clinical setting (L.M., unpublished data).

Clinical features of the cohort with a *SPRED1* LOF mutation (n=33) were compared with the cohort without mutations in *NF1* and *SPRED1* (n=1285) and with the cohort with an *NF1* mutation (n=1114). Twenty-one of 1087 individuals (approximately 2%) fulfilling NIH diagnostic criteria in the entire anonymous cohort carried a *SPRED1* mutation. All individuals carrying a pathogenic *SPRED1* mutation were found in the subgroup of patients with multiple CALMs with or without freckling but fulfilling no other NIH criterion. No *SPRED1* mutations were found if patients had neurofibromas, optic pathway glioma, ophthalmologically proven Lisch nodules, or a typical NF1-associated osseous lesion. The data from this anonymous cohort further underscore that association of these features with NFLS must be rare.

In this study of 957 sporadic patients with CALMs with or without freckling and no other criterion, 414 (43%) had a *NF1* mutation in the blood and 13 (1.3%) had a *SPRED1* mutation. An *NF1* mutation was found in 150 of 159 familial patients (94%) with

more than 5 CALMs with or without freckling and an additional criterion, usually the presence of neurofibromas, but in 69 of 94 familial patients (73%) with CALMs with or without freckling only. In this last group, 18 of 94 families (19%) showed a *SPRED1* mutation, and in 7 of 94 (7%), no mutation could be identified in *NF1* or *SPRED1*.

The NIH NF1 criteria, originally designed to help identify families suitable for linkage studies that led to the positional cloning of the *NF1* gene, are widely used and allow establishment of a clinical diagnosis of NF1 in most cases. In individuals with CALMs with or without freckling and no other specific distinguishing features, presenting sporadically or with a family history of CALMs with or without freckling only, the NIH criteria cannot reliably distinguish NF1 from NFLS. In such patients, a correct diagnosis has important implications for prognosis, counseling, and potential prenatal genetic diagnosis. Although an NF1 diagnosis may become apparent with the passage of time, the diagnosis will remain uncertain for individuals who do not develop other signs of NF1.

Molecular genetic testing can resolve the diagnosis in most such cases. In case of diagnostic uncertainty, we recommend that *NF1* should be analyzed first and, if negative, *SPRED1* testing should be considered in patients with CALMs with or without freckling and no other NF1 diagnostic features. Identification of a *SPRED1* mutation may relieve a psychological burden from families who otherwise would be in a waiting mode for potential serious NF1-associated manifestations.

We currently are conservative regarding the clinical surveillance of *SPRED1*-positive patients and recommend the same medical follow-up as that for patients with NF1. Less stringent surveillance may possibly be recommended for these patients if clinical data from several hundreds of patients confirm the low frequency of benign and malignant tumors.

For all of these reasons, it is important that clinicians, including general practitioners, clinical geneticists, pediatricians, ophthalmologists, dermatologists, neurologists, and oncologists, who are involved in the care, diagnosis, and treatment of individuals with NF1, should be aware that Legius syndrome can resemble NF1.

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