2015

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RESEARCH ARTICLE

Human Mutation

OFFICIAL JOURNAL
HGVS
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High Incidence of Noonan Syndrome Features Including Short Stature and Pulmonic Stenosis in Patients carrying NF1 Missense Mutations Affecting p.Arg1809: Genotype–Phenotype Correlation

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Additional Supporting Information may be found in the online version of this article.

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Communicated by David N. Cooper

Received 20 March 2015; accepted revised manuscript 19 June 2015.
Published online 14 July 2015 in Wiley Online Library (www.wiley.com/humanmutation). DOI: 10.1002/humu.22832

ABSTRACT: Neurofibromatosis type 1 (NF1) is one of the most frequent genetic disorders, affecting 1:3,000 worldwide. Identification of genotype–phenotype correlations is challenging because of the wide range clinical variability, the progressive nature of the disorder, and extreme diversity of the mutational spectrum. We report 136 individuals with a distinct phenotype carrying one of five different NF1 missense mutations affecting p.Arg1809. Patients presented with multiple café-au-lait macules (CALM) with or without freckling and Lisch nodules, but no externally visible plexiform neurofibromas or clear cutaneous neurofibromas were found. About 25% of the individuals had Noonan-like features. Pulmonic stenosis and short stature were significantly more prevalent compared with classic cohorts ($P < 0.0001$). Developmental delays and/or learning disabilities were reported in over 50% of patients. Melanocytes cultured from a CALM in a segmental NF1-patient showed two different somatic NF1 mutations, p.Arg1809Cys and a multi-exon deletion, providing genetic evidence that p.Arg1809Cys is a loss-of-function mutation in the melanocytes and causes a pigmentary phenotype. Constitutional missense mutations at p.Arg1809 affect 1.23% of unrelated NF1 probands in the UAB cohort, therefore this specific NF1 genotype–phenotype correlation will affect counseling and management of a significant number of patients.

Hum Mutat 36:1052–1063, 2015. Published 2015 Wiley Periodicals, Inc.*

KEY WORDS: neurofibromatosis type 1; NF1; p.Arg1809; phenotype–genotype correlations; Legius syndrome

Introduction

Neurofibromatosis type 1 (NF1; MIM #162200) is one of the most frequent genetic disorders, characterized by café-au-lait macules (CALMs), skinfold freckling and Lisch nodules, neurofibromas, optic pathway gliomas (OPG), and specific osseous lesions such as sphenoid wing and tibial dysplasia. Approximately 50% of patients present as "de novo" cases. NF1, located on chromosome 17q11.2, has 57 constitutive and at least 3 alternatively spliced exons, spanning 282 kb of DNA. NF1 encodes neurofibromin, a GTPase activating protein (GAP) down-regulating the biological activity of normal Ras proteins.

Only a few clinically significant genotype–phenotype correlations are known. The first concerns a constitutional 1.4 Mb microdeletion encompassing the NF1 gene. These patients present with a more severe phenotype, including increased neurofibroma burden with an earlier age of onset, facial dysmorphism including hypertelorism, coarse face, down-slanting palpebral fissures, broad nasal bridge, ptosis, micrognathia, and developmental delay/ intellectual disability or learning problems [Kayes et al., 1994; Upadhyaya et al., 1998; Venturin et al., 2003; Descheemaeker et al., 2004; Mensink et al., 2006; Mautner et al., 2010; Pasmant et al., 2010]. In addition, congenital cardiac defects such as pulmonic stenosis (PS), atrial/ventricular septal defects, valve defects, hypertrophic cardiomyopathy, and patent ductus arteriosus [Venturin et al., 2003; Nguyen et al., 2013]; connective tissue anomalies including joint laxity and hyperextensible skin; and skeletal anomalies [Mensink et al., 2006; Mautner et al., 2010] are more frequently found in these patients. These patients have an increased life-time risk for a malignant peripheral nerve sheath tumor (MPNST) [De Raedt et al., 2003; Mensink et al., 2006]. Childhood overgrowth and bone age acceleration occurs in patients with the common NF1 microdeletion. This contrasts with classical NF1 patients who have short stature and whose prepubertal bone age is delayed by 1–2 years [Carmi et al., 1999].

A second genotype–phenotype correlation involves a specific one amino acid NF1 deletion, p.Met992del, which is associated with a milder NF1 phenotype consisting mainly of pigmentary signs (CALMs and skinfold freckling) and a lack of externally visible cutaneous or plexiform neurofibromas [Upadhyaya et al., 2007]. This mild phenotype of CALMs with or without freckling but no neurofibromas overlaps with and is clinically non-distinguishable from Legius syndrome (LS), caused by mutations in SPRED1 [Brems et al., 2007; Messiaen et al., 2009].

The lack of discovery of more specific genotype–phenotype correlations may be partly due to the methodological approach of lumping mutations in gross categories, such as truncating, versus in frame, splicing, and missense mutations [Sabbagh et al., 2013; van Minkelen et al., 2014]. However, high levels of intra- and interfamilial clinical variability are observed in most NF1 families, even though all affected individuals carry identical NF1 mutations. The confounding problem is that the phenotype is determined, besides by the constitutional mutation, by the patient's age, the timing of second hit mutations in different cells and tissues, potential mosaicism in founder patients and modifying and environmental factors.

We hypothesize that additional genotype–phenotype correlations exist and likely can be identified through analysis of preferentially

postpubertal patients carrying the same non-truncating constitutional mutation. Given the extreme diversity of the NF1 mutational spectrum, with ~46% of patients carrying a private mutation and only 29 different NF1 mutations present in >0.5% of all patients [Messiaen, 2008], identification of unrelated, preferentially non-founder probands, and relatives over 8 years of age with an identical mutation requires large well-characterized patient cohorts.

Multiple (>5) CALMs are usually the first sign indicative of NF1 and present shortly after birth. Up to 90% of the patients thereafter develop skinfold freckling before the age of 8 years, thereby fulfilling the clinical criteria of NF1 [Friedman et al., 1986aa]. Typically, more signs and complications develop thereafter, but few indicators predict the severity of the disorder over time. This uncertainty negatively impacts quality of life [Vranceanu et al., 2013]. Recently 29 patients from 12 families carrying an NF1 missense mutation at Arg1809 were reported with a phenotype of multiple CALMs and freckling, without cutaneous or visible plexiform neurofibromas, Lisch nodules, typical osseous lesions or symptomatic OPGs, and with facial features suggestive of Noonan syndrome in 50% [Pinna et al., 2015; Santoro et al., 2015], in line with our initial findings [Rojnueangnit et al., 2013]. Here, we report a cohort of 136 patients from 98 unrelated families carrying one of five different constitutional missense mutations affecting NF1 codon 1809 in exon 29 [38]. Patients presented with multiple CALM, with or without freckling and Lisch nodules, but externally visible plexiform neurofibromas (0/78 individuals ≥9 years; $P < 0.05$), symptomatic optic pathway glioma (0/98 individuals ≥5 years; $P < 0.02$) (Tables 1 and 2) or clear cutaneous neurofibromas were not found. There was, however, a high incidence of developmental delay/intellectual disability/learning disorder, PS, short stature, and Noonan syndrome features. This mutation was identified in 1.23% of unrelated probands carrying a constitutional NF1 mutation in the UAB cohort (86/7,000). Missense mutations affecting p.Arg1809 are the second most frequent mutation in the UAB cohort, second only to the 1.4 Mb NF1 type 1 microdeletion. This is the third specific NF1 genotype–phenotype correlation, which will affect counseling and management of these patients.

Materials and Methods

Patients and Phenotypic Characterization

Phenotypic data of all individuals carrying an NF1 constitutional missense mutation affecting codon 1809, as provided by the referring physicians using a standardized phenotypic checklist (Supp. Fig. S1), were reviewed. Initial results on 67 probands and 17 relatives from UAB were reported at the 2013 American College of Medical Genetics meeting [Rojnueangnit et al., 2013]. This initial study was expanded to include 31 additional probands and 21 relatives (from UAB and collaborating centers) and refined by requesting referring physicians to (i) verify whether the information in the originally submitted checklist was accurate and (ii) provide an update on their patient(s) if additional information had become available. The phenotypic data documented in the standardized phenotypic checklist include: age; sex; ethnicity; height; head circumference; NIH criteria including CALMs, freckling, cutaneous, subcutaneous, plexiform and spinal neurofibromas, optic gliomas, Lisch nodules, osseous lesion, and inheritance (familial, sporadic); Noonan syndrome features including short stature, apparently low set ears, hypertelorism, midface hypoplasia, webbed neck, and PS; cardiovascular defect (hypertension, pulmonic, aortic or renal artery stenosis, Moyamoya, ASD, or VSD); development (normal, abnormal, ADD, ADHD, learning disability, autism, speech delay, pervasive developmental delay, IQ) and education; presence/absence of other neoplasms. The UAB cohort comprises 111 individuals (86 probands and 25 relatives). In addition, 25 individuals (12 probands and 13 relatives) from four other centers were included: three Belgian families (six affected individuals) referred to the Medical Genetics Laboratory at the University Ghent, Belgium (UG), one Spanish family (three affected individuals) referred to the Catalan Institute of Oncology, Barcelona, Spain (BARC), four Spanish families (11 affected individuals) referred to the genetic department of the "Hospital Universitario Ramon y Cajal", Madrid, Spain (MADR) and four UK probands (three familial with genetic and full clinical examination available on four individuals and one proven sporadic individual) evaluated at the Institute of Medical Genetics, University Hospital of Wales, UK (CARD) were included.

The data for any given sign/symptom reflect the number of patients for whom we received information, that is, after exclusion of those with entry "unknown" or "Not Specified." Screening for Lisch nodules was not routinely performed in most patients. None of the patients presented with ophthalmological problems indicative of a symptomatic OPG, but an MRI to exclude presence of an asymptomatic OPG was not routinely performed, except when stated. Similarly, imaging or whole body MRI was not available in patients without signs or symptoms indicative of internal or spinal tumors.

A patient was classified as having short stature when height was below or equal to the 3rd percentile (PC), using the World Health Organization (WHO) growth curve for age less than 2 years, and the Center for Disease Control (CDC) growth curve for age above 2 years in Caucasian and African-American ethnicity. For the Spanish individuals, Spaniards' growth charts were used as provided at http://www.webpediatrica.com/endocrinoped/antropometria.php. As no specific growth curves are available for the Hispanic and Asian populations, but average height is lower than in the Caucasian or African-American population [Hur et al., 2008; Chen and Chang, 2010; de Wilde et al., 2014], we conservatively excluded Hispanic or Asian patients as having short or normal stature. We still calculated the PC values using the WHO or CDC growth curves and these values are provided between square brackets in the Supp. Table S1. Macrocephaly was defined as the head circumference above the 97th percentile using the WHO curve for age up to 2 years old, and the Gerhard Nellhaus' curve [Nellhaus, 1968] for age over 2 years old, for all ethnic backgrounds.

A patient was classified as having "NF-Noonan" when at least two of the following features were present: short stature, low set ears, hypertelorism, midface hypoplasia, webbed neck, or PS.

Molecular Analyses

All unrelated UAB, UG, BARC, and MADR probands had undergone comprehensive NF1-mutational analysis using an RNA-based approach complemented by DNA-based dosage analysis, as previously described [Ars et al., 2000; Messiaen et al., 2000; Messiaen and Wimmer, 2005; Messiaen, 2008; Valero et al., 2011]. Melanocytes from a CALM of patient UAB-RSeNF23 were cultured as previously described [Maertens et al., 2007]. RNA and DNA were extracted from cultured cells and subjected to comprehensive NF1-mutational analysis. Mutations in CARD individuals were identified using DNA-based Sanger sequencing of all coding exons. NF1 mutations are described following recommendations of the Human Genome Variation Society using NM_000267.3 as the reference sequence. Exon numbering uses the historical numbering used by the NF1 consortium, followed by the NCBI numbering in square brackets.

Low level mosaicism for the mutation affecting codon 1809 in the blood of parents with 1–4 CALMs (i.e., parent of UAB-R3933,

Table 1. Demographics and Clinical Features as Present According to Age Group (0-≤8; 9-≤18; ≥19 years) in Cohorts of Individuals Carrying a Germline Missense Mutation p.Arg1809Cys, p.Arg1809Leu, p.Arg1809Pro, p.Arg1809Ser, or p.Arg1809Gly

Mutation	p.Arg1809Cys (c.5425C>T)			p.Arg1809Leu (c.5426G>T)			p.Arg1809Pro (c.5426G>C)		p.Arg1809Ser (c.5425C>A)		p.Arg1809Gly (c.5425C>G)		All Arg1809			Total
Mutation positive individuals (proband:relatives)	107 (75:32)			18 (15:3)			4 (4:0)		3 (3:0)		4 (1:3)		136 (98:38)			136 (98:38)
Proband:Relative	33:6	31:5	11:21	10:1	3:0	2:2	2:0	2:0	2:0	1:0	1:1	0:2	47:7	37:6	14:25	98:38
Age group	0–≤8 years	9–≤18 years	≥19 years	0–≤8 years	9–≤18 years	≥19 years	0–≤8 years	9–≤18 years	0–≤8 years	≥19 years	9–≤18 years	≥19 years	0–≤8 years	9–≤18 years	≥19 years	All ages
Age range/group	8 month–8.5 years	9–18 years	19–59 years	6 month–8 years	9–18 years	19–42 years	1–4.5 years	9–11 years	7 years	19 years	10 years	46–50 years	6 month–8 years	9–18 years	19–59 years	6 months–59 years
Total (Male:female)	39 (20:19)	36 (21:15)	32 (13:19)	11 (8:3)	3 (1:2)	4 (2:2)	2 (2:0)	2 (2:0)	2 (1:1)	1 (0:1)	2 (1:1)	2 (1:1)	54 (31:23)	43 (25:18)	39 (16:23)	136 (72:64)
Short stature[a]	5/21 (6/21)	11/25 (14/25)	4/15 (4/15)	5/9 (5/9)	0/1 (0/1)	0/3 (2/3)	0/1 (0/1)	1/1 (1/1)	0/1 (1/1)	1/1 (1/1)	1/2 (1/2)	1/2 (1/2)	10/32 (12/32)	13/29 (16/29)	6/21 (8/21)	29/82 (36/82)
Macrocephaly[b]	4/27 (7/27)	10/27 (10/27)	4/10 (5/10)	2/8 (3/8)	0/1 (0/1)	0/2 (0/2)	0/1 (0/1)	0/1 (0/1)	0/1 (0/1)	NS	0/2 (0/2)	NS	6/37 (10/37)	10/31 (10/31)	4/12 (5/12)	20/80 (25/80)
Fulfilling the NIH criteria if the family history is taken into account	22/39	27/36	23/32	6/11	3/3	4/4	0/2	2/2	2/2	1/1	2/2	2/2	30/54	34/43	30/39	94/136
Fulfilling the NIH criteria if solely taking the physical signs into account	15/39	26/36	18/32	5/11	3/3	3/4	0/2	2/2	2/2	1/1	1/2	1/2	22/54	32/43	23/39	77/136
>5 CALMs	37/39	36/36	26/32	11/11	3/3	3/4	1/2	2/2	2/2	1/1	1/2	1/2	51/54	42/43	31/39	124/136
Freckling	15/38	25/34	19/32	5/11	3/3	2/4	0/2	1/2	2/2	1/1	1/2	2/2	22/53	30/41	24/39	76/133
Lisch nodules	3/27	3/25	0/12	1/10	0/3	2/4	0/1	0/2	0/2	0/1	1/2	0/2	4/40	4/32	2/19	10/91
Cutaneous or subdermal neurofibromas*	0/39 (0%)	2/36*	5/32*	0/11	0/3	0/4	0/1	0/2	0/2	0/1	0/2	0/2	0/53	2/43*	5/39*	7/135 (7/82)*
Plexiform neurofibromas	0/39 (0%)	0/34	0/31	0/11	0/3	0/4	0/2	0/1	0/2	0/1	0/2	0/2	0/54	0/40	0/38	0/132
Optic pathway glioma by MRI	0/13	0/16	0/1	0/3	0/1	0/1	ND	ND	ND	ND	0/2	0/2	0/16	0/19	0/4	0/39
Pectus abnormalities	2/39	6/33	0/25	0/11	0/3	0/4	0/2	0/1	1/2	0/1	0/2	0/2	3/54	6/39	0/32	9/125
Bone abnormalities[c]	5/39	8/33	4/25	0/11	0/3	0/4	1/2	1/1	1/2	0/1	0/2	0/2	7/54	9/39	4/32	20/125
Noonan syndrome features[d]	11/38 (14/38)	11/34 (14/34)	3/25 (8/25)	2/9 (5/9)	0/2 (0/2)	1/4 (1/4)	0/2 (0/2)	0/2 (1/2)	1/1 (1/1)	0/1 (1/1)	1/2 (2/2)	2/2 (2/2)	14/50 (20/50)	12/40 (17/40)	6/32 (12/32)	32/122 (49/122)
Pulmonic Stenosis/other cardiac defect	5/35 (6/35)	3/28 (4/28)	3/22 (6/22)	1/7	0/3	0/2	0/1	0/1	1/1	0/1	0/2 (1/2)	0/2	7/44 (8/44)	3/34 (5/34)	3/27 (6/27)	13/105 (19/105)
Cognitive impairment/ learning disabilities	21/37	27/36	12/27	5/10	2/3	0/4	0/2	1/2	2/2	1/1	0/2	0/1	28/51	30/43	13/33	71/127

*Presence of cutaneous or subdermal neurofibromas (NFs) questionable in all cases (7/135 patients of all ages or 7/82 patients of ≥9 years old), none biopsied for pathology: three patients with 1 small subdermal lesion (NF vs. lipoma), two patients with 2 "possible" small subdermal lesions (NF vs. lipoma), one patient with few (2–5) possible peripheral neurofibromas, one patient with nodules (8–10 mm) internal part lower lip, tongue tip and basis by MRI, no histology.

[a]First value PC≤3; second value PC≤5.

[b]First value PC≥98; second value PC≥90.

[c]All bone abnormalities included, that is, scoliosis (8×), pectus excavatum (7×), pectus carinatum (1×), long bone dysplasia (3×), 2nd and 5th bilateral short toes, 4–5 toe syndactyly R foot, long 2nd and 3rd toes, club foot, bone cysts, congenital talus deformity and hip dysplasia, flat rib cage. None of the patients had a pseudarthrosis or sphenoid wing dysplasia.

[d]First value: patients with ≥2 Noonan signs (short stature, low set ears, hypertelorism, downslanting palpebral fissures, midface hypoplasia, webbed neck, pectus abnormality, or pulmonic stenosis); second value: patients with only 1 Noonan sign. Patients with ≥2 Noonan signs had PTPN11 testing, all negative.

Table 2. Comparison of Clinical Features of the Arg1809 Cohort with the NF1 p.Met992del Cohort (Upadhyaya et al., 2007) and Previously Reported Large-Scale NF1 Patient Cohorts

	Number of patients (%)				*P* value (2-tailed Fisher's exact test)		
NF1 feature	p.Arg1809[a]	p.Met992del[b]	Legius[c]	Previous NF cohorts[d]	p.Arg1809 versus p.Met992del	p.Arg1809 versus Legius syndrome	p.Arg1809 versus previous NF cohorts
>5 CALMs (and size appropriate for age)	124/136 (91.2)	46/47 (97.9)	110/142 (77.5)	1210/1297 (93.3)[B,C,E]		0.0018 ↗	
Skinfold freckling (SF)[e]	54/80 (67.5)	30/47 (63.8)	42/89 (47.2)	661/960 (68.9) [B,C,E]		0.0086 ↗	
>5 CALMs and SF, age ≥9 year	52/80 (65)	22/34 (64.7)	35/92 (38)	estimated at 95% (95)		0.0005 ↗	<0.0001 ↘
Lisch nodules	10/91 (11.0); 10/119 (8.4)	3/30 (10)	0 (n unknown)	82/88 (93.2) [A]			<0.0001 (<0.0001) ↘
Cutaneous neurofibromas[f]	5[g]/39 (12.8); 5[g]/59 (8.5)	0/18 (0)	0/68 (0)	99/99 (100) [A]			<0.0001 (<0.0001) ↘
Major external plexiform neurofibromas[e]	0/78 (0); 0/107 (0)	0/41 (0)	0/95 (0)	7/115 (6.1) [A]			0.0429 (0.0146) ↘
Symptomatic optic pathway glioma, age ≥5 yr	0/98 (0)[h]; 0/119 (0)[i]	0/46 (0)	0/118 (0)	66/1383 (4.8)[A,E, G,H,I]			0.0193 (0.0081) ↘
Noonan syndrome features[j]	32/122 (26.2)	4 (all from 1 family)	19 reported	12/94 (12.8)			
Pulmonic stenosis	13/105 (12.4)	4/47 (8.5)	3 reported	25/2322 (1.1) [F]			<0.0001 ↗
Short stature[k]	29/82 (35.4)	5/47 (10.6)	10/85 (11.8)	109/684 (15.9) [A,B]	0.0019 ↗	0.0004 ↗	<0.0001 ↗
Macrocephaly	20/80 (25)	4/45 (8.9)	25/111 (22.5)	239/704 (33.9) [A,B]	0.0335 ↗		
Scoliosis[l]	6/71 (8.5)	2/20 (10)	5 reported	11/96 (11.5) [A]			0.0467 ↘
Pectus abnormalities	9/125 (7.2)	7/45 (15.6)	15 reported	36/127 (28.3) [D]			<0.0001 ↘
Cognitive impairment/ learning disorders	71/127[m]	8/47 (17)	15/53 (learning disabilities); 12/56 (psychomotor developmental delays); 11/52 (A(D)HD, autistic behaviour)[n]	190/424 (44.8) [A,E]			

[a] First value are data from this study; second values are the data combined from this and previous studies (Ekvall et al., 2014; Nyström et al., 2009; Pinna et al., 2015; Santoro et al., 2015); between round brackets: percentages.
[b] Upadhyaya et al. (2007).
[c] Data compiled from Brems et al. (2007) (excluding UAB31,43,48,88, as these are included in Messiaen et al. (2009)) (Denayer et al., 2011; Messiaen et al., 2009; Pasmant et al., 2009; Spurlock et al., 2009). For some features, total number of cases assessed for a given feature is unknown; only the number of observations is known and is stated here as summarized by Brems et al. (2012).
[d] Previous NF1 cohorts used for comparison: A: Huson et al. (1988); B: Khosrotehrani et al. (2005); C: Huson et al. (1989aa, Huson et al., 1989b); D: Cnossen et al. (1998); E: McGaughran et al. (1999); F: Lin et al. (2000); G: Listernick et al. (1994); H: Singhal et al. (2002); I: Blazo et al. (2004).
[e] In patients ≥9 years in this study and ≥10 years in other studies.
[f] In patients ≥19 years in this study and ≥20 years in other studies.
[g] Five individuals with few (1–6) small, subdermal "possible neurofibromas," none were biopsied and therefore none have been histologically confirmed, therefore this is a very conservative estimate.
[h] Including 31 aged ≥5 years who had an MRI and no asymptomatic OG present.
[i] Including 34 aged ≥5 years who had an MRI and no asymptomatic OG present.
[j] A patient was classified as having "Noonan-syndrome features" when at least 2 of the following features were present: short stature, low set ears, hypertelorism, midface hypoplasia, webbed neck, or pulmonic stenosis.
[k] Short stature defined as ≤PC3 in the Arg1809 and Met992del cohort, in Huson et al. (1988), but as <PC5 in the Legius cohort.
[l] In patients ≥9 years in this and previous studies.
[m] Three individuals with said normal development and ADD (2) and ADHD (1) were counted as normal but included toward the total number of cases in the denominator.
[n] Data as summarized in Brems et al. (2007).

UAB-R1593, UAB-R9044, UAB-R6935, and UAB-R3885) was investigated using High Resolution Melting Curve analysis, as described [Vossen et al., 2009]. Genomic DNA was diluted to a concentration of 15 ng/μl. A DNA sample from a non-founder individual carrying the constitutional mutation c.5425C>T (present in all cells, 50% of all alleles) was serially diluted with wild-type control DNA to generate a calibration panel with DNA containing the mutation in 50%, 25%, 12.5%, 6.25%, 3.13%, and 1.56% of the alleles. All samples were amplified in duplicate using Lightscanner® Master Mix (BioFire Defense) and the PCR products were subsequently analyzed by the LightScanner® System (BioFire Defense).

PTPN11 mutation analysis was performed in probands and relatives with "NF-Noonan" phenotype by Sanger sequencing of all coding exons and 20 nucleotides of the flanking introns (primer sequences available upon request).

In Silico Prediction on the Mutations and their Effects on 3D Structure

The following in silico prediction software programs were used to assist with interpretation of pathogenicity: SIFT (sift.jcvi.org), PolyPhen (genetics.bwh.harvard.edu/pph2), MutationTaster (http://www.mutationtaster.org), MutPred Splice

prediction (mutdb.org/mutpredsplice), and Grantham Difference as embedded in Alamut visual v2.7.1.(http://www.interactive-biosoftware.com) and CADD (cadd.gs.washington.edu). Presence or absence of the variants against large control populations was checked: 1000Genomes (http://www.1000genomes.org), the Exome Variant Server (http://evs.gs.washington.edu/EVS), and the Exome Aggregation Consortium (http://exac.broadinstitute.org).

Evolutionary conservation for human neurofibromin NP_000258.1 residues 1730–1815 was evaluated using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). The structures of human NF1 p.Arg1809 mutations (Arg1809Cys, Arg1809Leu, Arg1809Pro, Arg1809Gly, Arg1809Ser, and Arg1809His) were predicted through the Analysis function of UCSF Chimera (version 1.10.1), based on the crystal structure of human NF1 (RefSeq NP_000258.1: PDB-accession code 2D4Q, residues 1560–1816 and 2E2X, residues 1567–1816).

This study was approved by the Institutional Review Boards of all participating institutions offering clinical genetic testing.

Results

The study includes 98 probands and 38 relatives carrying a constitutional missense mutation affecting codon Arg1809, and one patient with segmental presentation in whom we identified the missense p.Arg1809Cys in melanocytes cultured from a CALM, but not in the blood.

Germline missense mutations affecting codon Arg1809 were identified in 86/7,000 unrelated NF1-positive probands through clinical testing in the UAB Medical Genomics Laboratory (July 2003 to July 2014). Therefore, the frequency of missense mutations affecting this codon is ~1.23% in individuals submitted for clinical testing with a positive genetic testing result. This is the codon most frequently mutated in NF1 in our cohort.

Characteristics and Location of the Mutations

Amongst 98 unrelated probands carrying mutations affecting codon 1809, five different missense mutations were observed: p.Arg1809Cys (75/98), p.Arg1809Leu (15/98), p.Arg1809Pro (4/98), p.Arg1809Ser (3/98), and p.Arg1809Gly (1/98) and their characteristics are summarized in Supp. Table S2. None of these variants have been found in the 1000Genomes, Exome Variant Server or the ExAC control populations (61,486 unrelated individuals). We never observed these missenses in a patient carrying a different clear-cut pathogenic NF1 mutation (out of 7,000 NF1-mutation positive probands), although a mother and daughter (family UAB-R5153) carried a complex allele p.[Arg1809Cys;Leu1789Val], with p.Leu1789Val never observed or reported before and classified as a variant of unknown significance. Arginine at position 1809 is evolutionarily conserved up to Drosophila melanogaster and yeast IRA2, whereas the very similar basic amino acid, Lysine, is found at that position in IRA1 (Supp. Fig. S2). All in silico prediction programs classify these variants as deleterious (SIFT), disease-causing (MutationTaster), or probably damaging (PolyPhen). Combined Annotation Dependent Depletion (CADD) v1.0 classified all missenses as being more likely pathogenic (PHRED scores ranging from 21.9 to 34). In addition, MutPred prediction software predicted p.Arg1809Leu to be a splice affecting variant (score 0.69), whereas the other mutations were predicted to be Splice Neutral Variants. RNA-based sequencing showed that p.Arg1809Leu and p.Arg1809Pro (but not p.Arg1809Cys, p.Arg1809Ser, or p.Arg1809Gly) lead to out-of-frame skipping of exon 29 [38] and exon 29 + 30 [38 + 39] in a minor fraction of the transcripts. The missense mutations were proven to segregate with the phenotype in 29 familial cases and to be de novo in 21 sporadic probands. Taken together, these variants can all confidently be classified as pathogenic according to current recommendations [Richards et al., 2015].

Theoretically, substitution of a single nucleotide of the Arg1809 codon CGC can result in six different missense variants: c.5425C>T, p.Arg1809Cys (codon TGC); c.5426G>C, p.Arg1809Pro (codon CCC); c.5426G>T, p.Arg1809Leu (codon CTC); c.5425C>G, p.Arg1809Gly (codon GGC); c.5425C>A, p.Arg1809Ser (codon AGC) and c.5426G>A, p.Arg1809His (codon CAC). As C>T and G>A transitions are the most frequently observed mutations at CpG dinucleotides, due to spontaneous deamination after cytosine-methylation, we expected finding p.Arg1809Cys and p.Arg1809His with equal frequency. However, p.Arg1809His was not found as a constitutional mutation in mutation-positive clinical samples submitted at UAB for NF1 testing (0/7,000 vs. 67/7,000 for p.Arg1809Cys; $P < 0.0001$), whereas both are absent in the ExAC control cohort (61,486 individuals). Arg1809 resides within the C-terminal α-helix of the Pleckstrin Homology (PH) domain of neurofibromin, encompassing the amino acids 1,713 to 1,816 (Supp. Fig. S2). The function of this α-helix within the domain is unknown, but might be important for protein-protein interactions given its location at the surface of the domain. 3D-modeling indicates that all Arg1809 missense mutations, including p.Arg1809His, are predicted to lose a hydrogen bond with Ser1738 (Supp. Fig. S3). To further investigate the discrepancy in prevalence of p.Arg1809Cys versus p.Arg1809His, we verified whether partners were present within suitable distance from p.Arg1809Cys to form disulfide bridges, but none were found (Supp. Fig. S4).

Phenotypic Description of the Patient Cohort

The NF1 clinical features of 136 individuals with a constitutional missense mutation affecting Arg1809 and the pedigrees of familial cases are summarized in Table 1, Table 2, Supp. Table S1, Supp. Table S3, Supp. Figure S5, and Supp. Figure S6. Forty of 98 probands were familial cases, 21/98 were proven to have a de novo mutation (with no formal paternity/maternity identity testing performed), 16/98 were reported as sporadic cases but one or both parents were unavailable for testing, and for 21/98 cases family history was unknown, including two adopted probands.

A 22-year old woman with segmental NF1, UAB-RSeNF23, (>6 CALMs and axillary freckling all restricted to the left side of the body, Lisch nodules in left eye, no learning disabilities, no OPG, no PNF or cutaneous neurofibromas, no scoliosis or skeletal dysplasia) had no mutations identified in the blood, but two different somatic NF1 mutations in the melanocytes cultured from a CALM: c.5425C>T (p.Arg1809Cys) and an in-frame deletion of 228 residues, c.(63_205)_(888_958)del (p.Arg69_Lys296del).

Age of the individuals ranged from 6 months to 59 years, with 54/136 individuals younger than 9 years, 43/136 between 9 and 18 years, and 39/136 over 19 (10/39 between 19 and 26 years, 8/39 between 27 and 35 years, 16/39 between 36 and 50 years and 5/39 older than 50 years). Seventy-two were male, 64 were female.

Ninety-four out of 136 individuals fulfilled NIH clinical criteria, but only 77/136 fulfilled NIH criteria if family history was excluded as a criterion. Fifty-two of 80 (65%) individuals ≥9 years had >5CALMs and skinfold freckling (Tables 1 and 2). Three adults (familial cases: UAB-R008-I1, UAB-R5863-I1, UAB-R6673-I1) had only 1–2 CALMs (with irregular margins) without other external signs. Lisch nodules were rarely present (6/51 individuals ≥9 years; 10/91 all ages). A symptomatic OPG was absent in all 98 individuals

≥5 years. Moreover, an asymptomatic optic glioma was excluded by MRI imaging in 39 individuals, including 32/39 ≥5 years. Externally visible plexiform neurofibroma or symptomatic spinal neurofibromas were not found in the 136 individuals in this cohort. Furthermore, no spinal neurofibromas were found by MRI in six asymptomatic individuals.

Cutaneous neurofibromas often first appear around the time of puberty and increase in size and number with age. None of the individuals in this cohort had overt cutaneous neurofibromas, including 39 individuals ≥19 years old (and 21/39 >35 years). In five patients ≥19 years (UAB-R744, 33y; UAB-R7641-I2, 47y; UAB-R0143-I1, 55y; UAB-R4174-I1, 40y; UAB-R4835, 28y; Supp. Tables S1 and S3), 1–2 small lesions called possibly subdermal/peripheral neurofibromas by the referring physicians were reported; none had been histopathologically confirmed. In one patient (UAB-R3412, 15y), multiple intramucosal oral nodules, suspected to be neurofibromas, were identified by MRI, but no histology was available.

Scoliosis was found in 6/71 patients older than 9 years. In 3/125 individuals, a long bone dysplasia was present, but pseudarthrosis or sphenoid wing dysplasia was not seen. Pectus anomalies (pectus carinatum or excavatum, flat rib cage) were reported in 9/125; macrocephaly (PC ≥ 98) was present in 20/80.

Noonan syndrome features were present in 32/122 individuals. No PTPN11 mutation was found in those available for testing (0/22). PS was present in 13/105 and short stature (PC ≤ 3) in 29/82. Seven individuals had a phenotype previously referred to as "Watson syndrome," given the combination of multiple CALMs, PS and learning disabilities and/or intelligence at the lower end (UAB-R9216, UG-575, UAB-R8141, UAB-R9851, UAB-R7626, UAB-R2635, and UAB-R7961). Out of 136 individuals with constitutional missense mutations at p.Arg1809, no developmental data were provided for 9; 53 were stated as having normal development; three as having normal development but ADD was reported in two and ADHD in the one. The remaining 71 individuals were reported to have abnormal development presenting with one or a combination of the following forms of cognitive impairment or learning difficulties: learning disabilities ($n = 62$), developmental delays ($n = 3$), ADHD ($n = 13$), Asperger syndrome ($n = 1$), pronunciation problems ($n = 1$), speech delay ($n = 12$), language delay ($n = 1$), nasal speech and speech apraxia ($n = 1$), dyspraxia ($n = 1$), dyslexia ($n = 1$), motor delay ($n = 3$) (Supp. Table S1 and Table 1). Seven patients had significant learning difficulties and intellectual disability, including four with a Full Scale Intelligence Quotient (FSIQ) between 58 and 75.

A venous malformation (multiple subcutaneous hemangiomas/lymphangiomas, six nevi flammei, mildly bluish discoloration) and a Becker nevus were found in a child and mother respectively (family UAB-R9813). A likely regressed hemangioma (UAB-R0844), a nevus anemicus (MADR-690), multiple cherry angiomas (UAB-R9266-I1), hemangiomas on left upper chest (UAB-R3154-I1), and lumbar hemangioma plus involuted cutaneous hemangioma (UAB-R2135) were each found in a single patient.

One or multiple lipomas were reported in three patients. A 24-year-old woman had a diagnosis of breast cancer, a 50-year-old woman had a breast fibroadenoma, and an 8-year-old boy had a Ewing Sarcoma. No follow-up information on these patients was available.

An Arnold Chiari I malformation was present in three patients (UAB-R0143-I1, UAB-R5913-II1, and MADR-694-II2). One patient (UAB-R4517) had abnormal morphologic appearance with decreased transverse dimensions of the superior frontal lobes bilaterally and an unusual sulcation pattern in the superior right frontal lobe, possibly indicating a multifocal cortical dysplasia. Four patients had a brain lesion: a stable brain mass of unknown type (UAB-R9216-I2), glial nodules by MRI (UAB-R3133), a stable dorsal medullar lesion suspicious for glioma (UAB-R5625) and a right thalamic glioma, histologically confirmed as being an astrocytoma at age 17 years (UAB-R3773) and treated with stereotactic radiosurgery and chemotherapy due to changes perceived on imaging. The tumor has been stable post treatment. No material was available to investigate by genetic analyses whether the astrocytoma was NF1-associated or coincidental.

There is no indication that the severity of the phenotype differs amongst the five observed missense mutations, although numbers are small for p.Arg1809Gly, p.Arg1809Pro, and p.Arg1809Ser.

Comparison of Clinical Features in Patients Carrying Missense Mutations Affecting Codon 1809 with Patients Carrying The NF1 P.Met992del Mutation, Patients With LS and Patients with "Classic NF1"

Comparison of clinical features of the p.Arg1809 cohort with individuals carrying the NF1 mutation p.Met992del [Upadhyaya et al., 2007], LS [Brems et al., 2012, and references therein and patients with "classic NF1" as previously reported in large scale NF1 patient cohorts [Huson et al., 1988; Huson et al., 1989a; Tonsgard et al., 1998; McGaughran et al., 1999; Lin et al., 2000; Khosrotehrani et al., 2005] is summarized in Table 2, with statistical significance calculated using two-tailed Fisher's exact test (http://graphpad.com/quickcalcs/contingency1/).

Compared to large cohorts of classic NF1 patients [Huson et al., 1988; Huson et al., 1989aa; Listernick et al., 1994; Cnossen et al., 1998; McGaughran et al., 1999; Lin et al., 2000; Singhal et al., 2002; Blazo et al., 2004; Khosrotehrani et al., 2005], individuals with a missense mutation at p.Arg1809 have statistically significantly less cutaneous and externally visible plexiform neurofibromas, symptomatic optic gliomas and pectus abnormalities. Fewer patients have Lisch nodules. Importantly, no individual described in this and previous cohorts [Nyström et al., 2009; Ekvall et al., 2014; Pinna et al., 2015] showed visible plexiform neurofibromas (0/107 individuals ≥9 years) or symptomatic optic gliomas (0/119 individuals ≥5 years).

Compared with classic NF1 cohorts, individuals with a p.Arg1809 missense mutation had significantly increased frequency of PS ($P < 0.0001$) and short stature ($P < 0.0001$). Short stature was also significantly more frequent compared with individuals with NF1 p.Met992del or LS (all P values <0.002).

Phenotype in individuals carrying a p.Arg1809 missense mutation is similar to that of individuals with the NF1 p.Met992del mutation [Upadhyaya et al., 2007], except for short stature being more prevalent in the p.Arg1809-positive individuals. In addition, learning disabilities were more frequently reported in the p.Arg1809-positive cohort.

95% of individuals with classic NF1 fulfill ≥2 of the NIH physical criteria (i.e., not taking family history into account) by ≥9 years of age according to De Bella, National Neurofibromatosis Foundation International Database [Friedman et al., 1986a]. In contrast, individuals with mutation at p.Arg1809, p.Met992del or with LS less likely fulfill these criteria at ≥9 years of age (all $P < 0.0001$). Furthermore, individuals with a mutation at p.Arg1809 or p.Met992del differ significantly from individuals with LS, with individuals with LS being the least likely to fulfill NIH criteria by age 9 years. Lisch nodules were found in patients with a p.Arg1809 mutation, although rarely (10/91), but have never been reported in LS.

Unusual Families within this Cohort

In 5/21 probands, with proven de novo mutation, one (UAB-R9044, UAB-R3885, and UAB-R6935) or both parents (UAB-R3933 and UAB-R1583) had 1–4 CALMs. Probands of these families invariably had >5 CALMs. p.Arg1809 was not detected in the parental blood samples by Sanger sequencing or by High Resolution Melting Curve Analysis (HRMCA), which would detect the mutation if present at ≥3% minor allele frequency (data not shown). This does, however, not completely exclude parental mosaicism for the mutation.

Tumor-related phenotypes were strikingly discordant in affected individuals within two families (Supp. Fig. S6). Comprehensive NF1 genetic testing showed two different mutations segregating within these two families. In Family UG-575, the 7-year-old proband carrying the missense mutation p.Arg1809Ser had multiple CALMs and skinfold freckling, Noonan phenotype with PS and severe learning disabilities with FSIQ of 58. Her father with >6 CALMs, >100 cutaneous neurofibromas, a large previously resected plexiform neurofibroma in the left leg and enlarged lumbar and sacral nerve roots by MRI did not carry the mutation p.Arg1809Ser (de novo in UG-575, with biological relationship of both parents proven by identity testing). Further comprehensive NF1 mutation analysis in him showed a splice mutation, c. 6365-2A>G, resulting in out-of-frame skipping of exon 34[43], and absent in his daughter.

In family UAB-R7916, the 41-year-old p.Arg1809Cys-positive proband had >8 CALMs, skinfold freckling, and PS. Four first-degree p.Arg1809Cys-negative relatives had 1–3 pigmentary lesions (CALMs or hypopigmentation or freckle). A 2-year-old niece of the proband with >6 CALM, skinfold freckling and an internal plexiform neurofibroma did not carry the mutation p.Arg1809Cys, but a de novo nonsense mutation c.539T>G, p.Leu180*. Therefore, if a substantial deviation from the here described phenotype is found in an inferred (i.e., a relative of an individual with a proven p.Arg1809 missense mutation) as well as in a proven p.Arg1809 missense mutation carrier, further genetic investigations should be pursued.

Discussion

We describe a distinct phenotype associated with five different NF1 missense mutations (p.Arg1809Cys, p.Arg1809Leu, p.Arg1809Pro, p.Arg1809Ser, and p.Arg1809Gly) in a large cohort of 136 individuals. Missense mutations affecting NF1 p.Arg1809 were found in 1.23% of unrelated NF1 mutation-positive individuals in the UAB cohort, therefore the current findings will impact management and counseling of a large number of families.

p.Arg1809Cys is the most recurrent missense mutation in the NF1 gene, found in 68/7,000 (0.97%) unrelated mutation-positive individuals in the UAB cohort. This variant was initially misclassified as a rare polymorphic variant [Ars et al., 2003] due to finding this variant in a sporadic individual and presence of the variant in several "unaffected" individuals (mother and sibling). [Ars et al., 2003] acknowledged that for some families the clinical information in their cohort was limited. Nyström et al. (2009) was hesitant how to classify p.Arg1809Cys in a family consisting of a proband with severe Noonan syndrome with a de novo PTPN11 as well as inherited NF1 p.Arg1809Cys missense mutation and four relatives with multiple CALMs: 2 carrying (sibling and father) and two not carrying (paternal uncle and cousin) NF1 p.Arg1809Cys. As detailed examination of the CALMs in the paternal uncle and cousin was not possible, a different genetic disorder in this branch of the family cannot be excluded. As other loci for familial CALMs remain to be discovered [Messiaen et al., 2009], whole exome sequencing may shed light on the genetic make-up in this and other families. In addition, several other families have been reported where mutations in more than one gene, involved in disorders with an overlapping phenotype, segregated within a family [Bertola et al., 2005; Messiaen et al., 2009; this study]. Understanding of the phenotypic spectrum associated with NF1 mutations has expanded significantly with the increased use of NF1 genetic testing and the identification of patients with a specific mutation such as p.992delMet, who—amongst other features—do not develop the typical neurofibromas with age and not all fulfill NIH criteria even in late adulthood [Upadhyaya et al., 2007]. Furthermore, guidelines and recommendations have been established and refined to help with the standardized classification of genetic variants [Richards et al., 2008; Richards et al., 2015]. Novel NF1 missense variants rarely can be confidently classified based on observations in a single proband/family and often remain a "variant of unknown significance" until more evidence becomes available. Based on the evaluation of >100 unrelated families and one segmental NF individual (this study, [Nyström et al., 2009; Ekvall et al., 2014; Pinna et al., 2015; Santoro et al., 2015]) sufficient evidence is available to classify all five missense mutations presented here as pathogenic. The family originally included in the study by [Ars et al., 2003] was clinically re-evaluated in this study (BARC-123) and the clinical phenotype was found to be concordant with the distinct phenotype described here. Nevertheless, further studies in large Arg1809-missense mutation positive families will refine our understanding of the phenotypic variability within families and allow determining whether non-penetrance could exist at this locus, as suggested by the presence of three individuals with only 1–2 CALMs in our cohort (UAB-R008-I1, UAB-R5863-I1, and UAB-R6673-I1; it remains unknown if these individuals were founders, and therefore might have been mosaic).

p.Arg1809 is located at the end of the C-terminal α-helix, close to the end of the PH-like domain which forms a bipartite module with the Sec-14 domain. 3D-modeling predicts a loss of a hydrogen bond between p.Ser1738 in both the solvent and lipid bound model for all six missense variants that can arise after a substitution of the Arg1809 CGC codon, suggesting this may cause a change in the secondary structure of the PH-like domain with possible effect on domain interaction and lipid-binding properties of the adjacent Sec-14 domain. However, other domains involving, for example, the amino acids 1817–2818, whose crystal structures remain unresolved, may affect the stability and structure of this domain, therefore the predicted loss of the hydrogen bond with p.Ser1738 may or may not be crucial. p.Arg1809His, expected to be equally frequent as p.Arg1809Cys, given the equal likelihood for both variants to originate as a consequence of deamination after methylation of the CpG dinucleotide of the CGC codon, has never been observed as a constitutional mutation in patients or in 61486 ExAC control samples. On the other hand, p.Arg1809His is present as a somatic mutation in the COSMIC database in large intestine carcinomas (1/131 vs. 2/131 for p.Arg1809Cys), which does however not imply pathogenicity as p.Arg1809His could just be a passenger subsequent spontaneous deamination at this site. Interestingly, Arg is evolutionary conserved down to yeast IRA2 whereas Lys is present at this site in IRA1. As Arg, His, and Lys all share a similar basic side chain, we postulate this charge at position 1809 is important for normal function which may explain why p.Arg1809His has not been observed in patients so far. We therefore hypothesize that p.Arg1809His is either not pathogenic or results in a different phenotype.

The present data indicate that p.Arg1809 missense mutations are associated with a distinct NF1 phenotype of multiple CALM, with or without skinfold freckling and Lisch nodules, but a significantly

reduced frequency of NF1-associated benign and malignant tumors. Multiple (>5) CALMs are usually the first sign indicative of NF1 and present shortly after birth. Up to 90% of the patients thereafter develop skinfold freckling before the age of 8 years, thereby fulfilling the clinical criteria of NF1 [Friedman et al., 1986a]. Typically, more signs and complications develop thereafter, but few indicators predict the severity of the disorder over time. This uncertainty negatively impacts quality of life [Vranceanu et al., 2013].

The most striking finding is the paucity of cutaneous, subdermal and externally visible plexiform neurofibromas. Cutaneous and subcutaneous neurofibromas are benign tumors that start growing during puberty. Typically, adult patients have multiple to thousands of them. As they cause esthetic and social disability with a significant effect on the quality of life in post-pubertal individuals, the current findings are important for a large group of patients.

In addition, summarizing all available evidence so far, no externally visible plexiform neurofibromas were found in 107 individuals older than 9 years [Nyström et al., 2009; this study; Ekvall et al., 2014; Pinna et al., 2015; Santoro et al., 2015]. Plexiform neurofibromas are prone to undergo malignant transformation, with an NF1 patient's lifetime risk of developing a MPNST estimated at 5.9%–13% [Evans et al., 2002; McCaughan et al., 2007]. Importantly, no MPNSTs were observed in 59 individuals between 19 and 87 years of age [Nyström et al., 2009; Ekvall et al., 2014; Pinna et al., 2015; Santoro et al., 2015]. Caution remains needed however, as minor lesions might be missed on a routine clinical examination. Moreover, full body imaging would be needed to exclude the presence of internal peripheral nerve sheath tumors.

NF1-related optic gliomas occur in 15%–20% of children and are most often asymptomatic [Listernick et al., 2007]. Although routine MRI is not indicated in an asymptomatic child, visual assessment is recommended as standard of care in NF1 patients, even in younger children, to detect decreased visual acuity and proptosis, the most common symptoms caused by optic glioma requiring MRI. The mean age of diagnosis of a symptomatic optic glioma is 5 years [Nicolin et al., 2009]. No symptomatic optic pathway glioma (0/153, with 114/153 ≥5 years), including no optic pathway glioma by MRI in all 42 individuals ≥5 years was found (this study and [Pinna et al., 2015]). A brain lesion was present in four individuals in our study, including one patient where the physician decided to treat (right thalamic, histologically confirmed astrocytoma treated with stereotactic radiosurgery and chemotherapy at the age of 17 years, due to changes in the tumor perceived on imaging prior to NF1 genetic testing). Collection of greater datasets is needed to further refine the decreased tumor risk associated with Arg1809 missense mutations.

No systematic occurrence of any other tumor, except lipomas (10/136 individuals, this study and [Nyström et al., 2009] has been associated with missense mutations at p.Arg1809. Absence of a clear tumor phenotype may indicate that Arg1809 acts differently compared to truncating NF1 mutations and may be a hypomorphic, gain-of-function or dominant-negative acting mutation. However, p.Arg1809Cys behaves as a loss-of-function mutation in melanocytes from CALMs as evidenced by the identification of 2 mutations in the patient with segmental NF1 presentation, UAB-RSeNF23, in line with our previous finding of NF1 acting as a tumor suppressor in these cells [Maertens et al., 2007; De Schepper et al., 2008].This finding questions but does not exclude for other tissues the often raised suspicion that NF1 missense mutations associated with a NF-Noonan phenotype may exert a gain of function or dominant-negative effect. Additional functional studies would be needed to shed further light on this.

A co-occurrence of p.Arg1809 and vascular abnormalities was observed, of interest given the known association between RASA1 (p.120-RASGAP; MIM #139150), another guanosine triphosphatase activating protein with similar RAS-MAPK-downregulating function as neurofibromin, and abnormal angiogenesis [Revencu et al., 2008].

Three individuals with the p.Arg1809Cys mutation in this cohort and 1 patient with both the p.Arg1809Cys and PTPN11 p.Phe285Leu mutations [Nyström et al., 2009] presented with an Arnold-Chiari type 1 malformation. While Arnold Chiari type 1 malformation has been more frequently reported in PTPN11, its association with NF1 is still under discussion [Plümpe N et al., 2010]. The true incidence of this malformation is only recently being understood with the increased use of MRI, and the prevalence in the general population may be as high as 1/1280 according to recent estimates [Meadows et al., 2000; Boyles et al., 2006]. Therefore, the data presented here may suggest a possible association between Arnold-Chiari 1 malformation and the NF1 p.Arg1809Cys mutation.

Pectus abnormalities (pectus excavatum, pectus carinatum) were significantly less frequent in the Arg1809-missense mutation-positive individuals compared to classic NF1 ($P < 0.0001$).

Learning disabilities, cognitive impairment and abnormal development were frequently reported in this cohort, especially speech and language delays, AD(H)D, gross and fine motor delays. Significant learning disabilities or intellectual disability were reported in 8 individuals. As the learning disabilities remain a significant concern in this group of patients, we propose to refer to the associated phenotype as being distinct, rather than mild.

As patients referred for genetic testing were coming from many different clinics, it was not possible to obtain standardized data on the extent of the learning disabilities. Therefore, further studies are needed to systematically investigate problems with speech, language, learning, and behavior in Arg1809-missense mutation-positive individuals.

Features/complications belonging to the Noonan-spectrum were present in significant excess (26.2%). Perception of facial and dysmorphic features is however somewhat subjective, whereas short stature and PS are objective findings. Although more than 80% of NF1 patients have an average height below expected for age, only 5%–7% have true short stature (height more than 3 standard deviations below average) [Friedman et al., 1986b]. The cohorts described by Huson et al. (1988) and Khosrotehrani et al. (2005) report short stature in a higher percentage of individuals (15.9%) and therefore, we used the latter frequency, conservatively, for our comparisons. Quantitative data on height for age were evaluated rigorously in this study, taking ethnic background into consideration and only considering short stature when height was ≤PC3. Short stature (≤PC3) was present in 35.4%, highly significantly more prevalent than in classic NF1 ($P < 0.0001$).

PS was also present in excess in p.Arg1809-missense mutation-positive individuals compared with the classic NF1 cohorts. The current finding supports that PS in NF1 might be more frequently found in patients carrying a non-truncating mutation [Ben-Shachar et al., 2013], although none of the previous studies had identified this recurrent p.Arg1809 missense mutation in their cohort. The association of Arg1809 with short stature and PS remained undetected in the study by Pinna et al., 2015 (short stature in 1/10 individuals and PS in 0/10) and Santoro et al., 2015 (short stature in 0/15 and PS in 0/13). However, the power to detect differences in the frequency of specific complications in this cohort versus classic NF1, with a significance at the 5% level, is lower in a cohort of 23 and 25 individuals: power of 69% to detect the increased frequency of PS (12.5% in this cohort vs. 1.1% in classic NF1) and power of 60% to

detect the increased frequency of short stature (35% in this cohort vs. 15.9% in classic NF1).

Cohorts of individuals with the NF1 mutations at p.Arg1809, p.Met992del, and LS share the low frequency of benign and malignant tumors. Based on our epidemiological data, 86/7,000 mutation-positive unrelated individuals carry a germline mutation at p.Arg1809 and 55/7,000 at p.Met992del (Messiaen, unpublished). In addition, as ~2% of individuals fulfilling NIH criteria have LS (Messiaen et al., 2009) and, as only ~38% of reported cases with LS fulfill NIH criteria by the age of 9 years, a larger group of individuals with CALMs with or without skinfold freckling exist for whom identification of the aforementioned genetic changes may relieve the psychosocial burden and anxiety associated with uncertainty and waiting for tumor symptoms to develop.

Considering complications that can be found in the general population with a frequency of ~1/1,000 (e.g., phaeochromocytoma, Arnold-Chiari type 1 malformation, etc.), combining data from this ($n = 39$) and previous studies ($n = 21$) on 60 Arg1809-missense mutation-positive adults (≥19 year) results in a power estimate of more than 85% to detect complications as being associated with this mutation and occurring in at least 5% of Arg1809-positive individuals. Combining data from this ($n = 111$) and previous studies ($n = 33$) on 143 Arg1809-positive individuals aged ≥5 years, we estimated in post-hoc power calculation that this sample size would allow detection of complications with a prevalence of at least 3% with a power of 92%, and of at least 2% with a power of 75%. Complications that are even rarer would require larger cohorts to show up as being associated with this mutation. This calls for careful collection of additional genotype and phenotype data as 250 well-characterized, preferably adult patients are needed to identify complications present with a frequency of only 1%. We therefore call for an international effort to collect accurate phenotypic data on these patients.

A limitation of the current study is the heterogeneity of the clinical data, with many clinical NF experts involved, although the same standardized questionnaire was used to collect the data. Data were interrogated extensively, first through the initial submission of the questionnaire as part of the clinical testing, followed by a request to confirm and update the data and to clarify unclear data. This approach may result in over-reporting of certain signs and symptoms compared to classic cohorts of aggregated data that were only interrogated once.

Therefore, for those symptoms that are statistically significantly less frequent in the Arg1809-missense positive cohort, the findings are even more significant as information was collected and verified twice: therefore we do not anticipate underreporting for these specific features in our cohort.

On the other hand, we have features that are clearly more frequent in the Arg1809-missense positive cohort compared with classic NF1 cohorts, especially short stature (35.4%; $P < 0.0001$) and PS (12.5%; $P < 0.0001$). As length for a given age is expressed as a discrete quantitative measurement there cannot be bias in the resulting data. Also, the excess of PS in individuals carrying an Arg1809-missense mutation cannot be attributed to ascertainment bias. It is standard of care for children to be evaluated with echocardiograms if a heart murmur is present or suspected. Moreover, the difference in PS frequency between the Arg1809 cohort and the classic NF1 cohorts (12.5% vs. 1.1%) is very large.

Missense mutations affecting p.Arg1809 are the second most frequent mutation in the UAB cohort, second only to the 1.4 Mb NF1 type 1 microdeletion. Therefore, the current findings affect a large number of individuals. Finding this genotype in an individual or family will impact counseling and management, thus providing an incentive to perform constitutional NF1 mutation analysis. Moreover, if a substantial deviation from the here described phenotype is found in an inferred (i.e., a relative of an individual with a proven p.Arg1809 missense mutation) as well as in a proven p.Arg1809 missense mutation carrier, further genetic investigations should be pursued. Communicating this information to the patients including the necessary nuances and detail is best done by genetic counselors and clinicians specialized in the complexity of NF1. It is important that clinicians involved in the care of patients with NF1 are aware of these emerging genotype–phenotype correlations that may alter surveillance and management.

The current data demonstrate that genotype–phenotype correlations in NF1 exist and may be relevant to management and surveillance of these patients. Therefore a renewed interest in these studies and close collaboration between NF1 clinicians and molecular geneticists are needed to come to a timely unfolding of additional such correlations.

Acknowledgments

We thank the patients, their family and their referral physicians for the information. We thank the genetics counselor students who helped to recontact all referral physicians for confirming information. This work was supported though the Children's Tumor Foundation by the Isaac and Sadie Fuchs Genotype-Phenotype Study (to L.M.) and by internal funds from the Medical Genomics Laboratory at UAB.

Disclosure statement: The authors declare no conflict of interest.

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