

Genotype-Phenotype Correlation in NF1: Evidence for a More Severe Phenotype Associated with Missense Mutations Affecting *NF1* Codons 844–848

Magdalena Koczkowska,¹ Yunjia Chen,¹ Tom Callens,¹ Alicia Gomes,¹ Angela Sharp,¹ Sherrell Johnson,¹ Meng-Chang Hsiao,¹ Zhenbin Chen,¹ Meena Balasubramanian,² Christopher P. Barnett,³ Troy A. Becker,⁴ Shay Ben-Shachar,⁵ Debora R. Bertola,⁶ Jaishri O. Blakeley,⁷ Emma M.M. Burkitt-Wright,⁸ Alison Callaway,⁹ Melissa Crenshaw,⁴ Karin S. Cunha,¹⁰ Mitch Cunningham,¹¹ Maria D. D'Agostino,¹² Karin Dahan,¹³ Alessandro De Luca,¹⁴ Anne Destrée,¹³ Radhika Dhamija,¹⁵ Marica Eoli,¹⁶ D. Gareth R. Evans,⁸ Patricia Galvin-Parton,¹⁷ Jaya K. George-Abraham,¹⁸ Karen W. Gripp,¹⁹ Jose Guevara-Campos,²⁰ Neil A. Hanchard,²¹ Concepcion Hernández-Chico,²² LaDonna Immken,¹⁸ Sandra Janssens,²³ Kristi J. Jones,²⁴

(Author list continued on next page)

Neurofibromatosis type 1 (NF1), a common genetic disorder with a birth incidence of 1:2,000–3,000, is characterized by a highly variable clinical presentation. To date, only two clinically relevant intragenic genotype-phenotype correlations have been reported for *NF1* missense mutations affecting p.Arg1809 and a single amino acid deletion p.Met922del. Both variants predispose to a distinct mild NF1 phenotype with neither externally visible cutaneous/plexiform neurofibromas nor other tumors. Here, we report 162 individuals (129 unrelated probands and 33 affected relatives) heterozygous for a constitutional missense mutation affecting one of five neighboring *NF1* codons—Leu844, Cys845, Ala846, Leu847, and Gly848—located in the cysteine-serine-rich domain (CSRD). Collectively, these recurrent missense mutations affect ~0.8% of unrelated *NF1* mutation-positive probands in the University of Alabama at Birmingham (UAB) cohort. Major superficial plexiform neurofibromas and symptomatic spinal neurofibromas were more prevalent in these individuals compared with classic NF1-affected cohorts (both $p < 0.0001$). Nearly half of the individuals had symptomatic or asymptomatic optic pathway gliomas and/or skeletal abnormalities. Additionally, variants in this region seem to confer a high predisposition to develop malignancies compared with the general NF1-affected population ($p = 0.0061$). Our results demonstrate that these *NF1* missense mutations, although located outside the GAP-related domain, may be an important risk factor for a severe presentation. A genotype-phenotype correlation at the *NF1* region 844–848 exists and will be valuable in the management and genetic counseling of a significant number of individuals.

Introduction

Neurofibromatosis type 1 (NF1 [MIM: 162200]), one of the most common genetic disorders with a birth incidence of 1 in 2,000–3,000,^{1–3} is characterized by a highly variable

inter- and intrafamilial expressivity (see GeneReviews in [Web Resources](#)). It is caused by loss-of-function genetic variants in *NF1* (MIM: 613113), located on chromosome 17q11.2. *NF1* encodes neurofibromin, a GTPase activating protein (GAP) that downregulates the RAS signal

¹Department of Genetics, University of Alabama at Birmingham, Birmingham, AL 35294, USA; ²Sheffield Clinical Genetics Service, Sheffield Children's NHS Foundation Trust, Sheffield S10 2TH, UK; ³Women's and Children's Hospital/SA Pathology, North Adelaide, SA 5006, Australia; ⁴Medical Genetics, Johns Hopkins All Children's Hospital, St. Petersburg, FL 33701, USA; ⁵The Genetic Institute, Tel-Aviv Sourasky Medical Center and Sackler Faculty of Medicine, Tel-Aviv 6997801, Israel; ⁶Department of Pediatrics, University of São Paulo, São Paulo 05403-000, Brazil; ⁷Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA; ⁸Genomic Medicine, Division of Evolution and Genomic Sciences, Manchester Academic Health Sciences Centre, University of Manchester, Central Manchester University Hospitals NHS Foundation Trust, Manchester M13 9WL, UK; ⁹Wessex Regional Genetics Laboratory, Salisbury NHS Foundation Trust, Salisbury SP2 8BJ, UK; ¹⁰Department of Pathology, School of Medicine, Universidade Federal Fluminense, Niterói 24220-900, Brazil; ¹¹Division of Genetic, Genomic and Metabolic Disorders, Children's Hospital of Michigan, Detroit Medical Center, Detroit, MI 48201, USA; ¹²Department of Medical Genetics, McGill University Health Centre, Montréal, QC H4A 3J1, Canada; ¹³Center for Human Genetics, Institute of Pathology and Genetics (IPG), Gosselies 6041, Belgium; ¹⁴Molecular Genetics Unit, Casa Sollievo della Sofferenza Hospital, IRCCS, San Giovanni Rotondo 71013, Italy; ¹⁵Department of Clinical Genomics and Neurology, Mayo Clinic, Phoenix, AZ 85259, USA; ¹⁶Unit of Molecular Neuro-Oncology, IRCCS Foundation, Carlo Besta Neurological Institute, Milan 20133, Italy; ¹⁷Department of Genetics, Stony Brook Children's, Stony Brook, NY 11794, USA; ¹⁸Dell Children's Medical Center of Central Texas, Austin, TX 78723, USA; ¹⁹Division of Medical Genetics, Al DuPont Hospital for Children, Wilmington, DE 19803, USA; ²⁰Pediatrics Service, Felipe Guevara Rojas Hospital, University of Oriente, El Tigre-Anzoátegui, Venezuela 6034, Spain; ²¹Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA; ²²Department of Genetics, Hospital Universitario Ramón y Cajal, Institute of Health Research (IRYCIS), Madrid 28034, Spain and Center for Biomedical Research-Network of Rare Diseases (CIBERER); ²³Center for Medical Genetics, Ghent University Hospital, Ghent 9000, Belgium; ²⁴Department of Clinical Genetics, the Children's Hospital at Westmead, Westmead, NSW 2145, Australia; ²⁵Division of Human Genetics, Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA; ²⁶Department of Genetics, Valley Children's Healthcare, Madera, CA 93636, USA; ²⁷Division of Clinical Genetics, Center for Genomic Medicine, Miami Cancer Institute, Miami, FL 33176, USA; ²⁸Department of Genetics, Yale University, New Haven, CT 06520, USA; ²⁹St George's University Hospitals NHS Foundation Trust, London SW17 0QT, UK; ³⁰Genomics Medicine Program, Children's Hospital Minnesota, Minneapolis, MN

(Affiliations continued on next page)

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Beth A. Keena,²⁵ Aaina Kochhar,²⁶ Jan Liebelt,³ Arelis Martir-Negron,²⁷ Maurice J. Mahoney,²⁸ Isabelle Maystadt,¹³ Carey McDougall,²⁵ Meriel McEntagart,²⁹ Nancy Mendelsohn,³⁰ David T. Miller,³¹ Geert Mortier,³² Jenny Morton,³³ John Pappas,³⁴ Scott R. Plotkin,³⁵ Dinel Pond,³⁰ Kenneth Rosenbaum,³⁶ Karol Rubin,³⁷ Laura Russell,¹² Lane S. Rutledge,¹ Veronica Saletti,³⁸ Rhonda Schonberg,³⁶ Allison Schreiber,³⁹ Meredith Seidel,³⁵ Elizabeth Siqveland,³⁰ David W. Stockton,¹¹ Eva Trevisson,⁴⁰ Nicole J. Ullrich,⁴¹ Meena Upadhyaya,⁴² Rick van Minkelen,⁴³ Helene Verhelst,⁴⁴ Margaret R. Wallace,⁴⁵ Yoon-Sim Yap,^{46,47} Elaine Zackai,²⁵ Jonathan Zonana,⁴⁸ Vickie Zurcher,³⁹ Kathleen Claes,²³ Yolanda Martin,²² Bruce R. Korf,^{1,50} Eric Legius,⁴⁹ and Ludwine M. Messiaen^{1,*}

transduction pathway through its GAP-related domain (GRD).^{4,5} The most common first signs of NF1 are multiple café-au-lait macules (CALMs) in >95% of infants and skinfold freckling in >80% of children by the age of 7 years.⁶ Other clinical features observed in >90% of adults with NF1 are iris Lisch nodules and cutaneous neurofibromas.⁷ Individuals with a more severe phenotype present with plexiform and/or spinal neurofibromas, symptomatic optic pathway gliomas (OPGs), as well as specific osseous lesions, such as sphenoid wing or tibial dysplasia. Approximately 50% of NF1-affected case subjects have *de novo* mutations, while the remaining individuals inherit the disorder from an affected parent (see GeneReviews in [Web Resources](#)). According to the National Institutes of Health (NIH) diagnostic criteria, at least two of the aforementioned features are required to classify a person as having the clinical diagnosis of NF1.⁸

Due to the variability in clinical presentation, age dependency of most manifestations, the timing and number of second hits in specific cells, and the wide *NF1* allelic heterogeneity, identification of specific genotype-phenotype correlations is extremely challenging. To date, more than 2,800 *different* germline *NF1* pathogenic variants have been identified in the University of Alabama at Birmingham (UAB) cohort, with only 31 unique pathogenic variants present in $\geq 0.5\%$ of all unrelated individuals (L.M.M., unpublished data). Moreover, a mild NF1 phenotype, including only CALMs and skinfold freckles, overlaps with Legius syndrome (MIM: 611431), caused by mutations in *SPRED1* (MIM: 609291).^{9,10}

So far, only three clinically significant genotype-phenotype correlations have been reported. First, individuals with a constitutional *NF1* microdeletion usually show a

more severe phenotype compared to the general NF1-affected population. The *NF1* microdeletion syndrome (MIM: 613675) is typically characterized by a large number of neurofibromas at a young age, dysmorphic facial features (hypertelorism, downslanted palpebral fissures, broad nasal bridge, low-set ears, micrognathia, coarse face, facial asymmetry), and developmental delay and/or intellectual disability. Individuals may present with cardiac defects as well as growth and skeletal abnormalities. *NF1* microdeletions have been associated with an increased lifetime risk for malignant peripheral nerve sheath tumors (MPNSTs). The constitutional co-deletion of *SUZ12* (MIM: 606245) within the common *NF1*-microdeletion region is thought to be a risk factor for the malignant neoplasms.¹¹ Second, individuals with a specific single amino acid *NF1* deletion (c.2970_2972del [p.Met992del]) present with a milder phenotype. These individuals have multiple CALMs with or without freckles, but no externally visible cutaneous or plexiform neurofibromas.¹² A third genotype-phenotype correlation involving *NF1* missense mutations affecting arginine at position 1809 is also associated with a distinct presentation,^{13,14} including developmental delay and/or learning disabilities, pulmonic stenosis, and Noonan-like features, but no external plexiform neurofibromas or symptomatic OPGs. Both of these affected amino acids reside outside the GRD domain.

Another distinct form of NF1 is familial spinal neurofibromatosis (FSNF [MIM: 162210]) originally described by Pulst et al.¹⁵ in six affected members from two unrelated families. It is characterized by bilateral and histologically proven neurofibromas of all spinal dorsal roots with a paucity or absolute lack of cutaneous manifestations.^{16,17} So far, only ~100 individuals (both familial and sporadic)

55404, USA; ³¹Multidisciplinary Neurofibromatosis Program, Boston Children's Hospital, Boston, MA 02115, USA; ³²Department of Medical Genetics, University of Antwerp and Antwerp University Hospital, Antwerp 2650, Belgium; ³³Birmingham Women's and Children's NHS Foundation Trust, Birmingham B15 2TG, UK; ³⁴Department of Pediatrics, Clinical Genetic Services, NYU School of Medicine, New York, NY 10016, USA; ³⁵Department of Neurology and Cancer Center, Massachusetts General Hospital, Boston, MA 02114, USA; ³⁶Division of Genetics and Metabolism, Children's National Health System, Washington, DC 20010, USA; ³⁷University of Minnesota Health, Minneapolis, MN 55404, USA; ³⁸Developmental Neurology Unit, IRCCS Foundation, Carlo Besta Neurological Institute, Milan 20133, Italy; ³⁹Genomic Medicine Institute, Cleveland Clinic, Cleveland, OH 44195, USA; ⁴⁰Clinical Genetics Unit, Department of Women's and Children's Health, University of Padova, Padova, Italy and Italy Istituto di Ricerca Pediatrica, IRP, Città della Speranza, Padova 35128, Italy; ⁴¹Department of Neurology, Boston Children's Hospital, Boston, MA 02115, USA; ⁴²Division of Cancer and Genetics, Cardiff University, Cardiff CF14 4XN, UK; ⁴³Department of Clinical Genetics, Erasmus Medical Center, Rotterdam 3015 GE, the Netherlands; ⁴⁴Department of Paediatrics, Division of Paediatric Neurology, Ghent University Hospital, Ghent 9000, Belgium; ⁴⁵Department of Molecular Genetics & Microbiology, University of Florida College of Medicine, Gainesville, FL 32610, USA; ⁴⁶Division of Medical Oncology, National Cancer Centre Singapore, Singapore 169610, Singapore; ⁴⁷Faculty of Health Sciences, School of Medicine, University of Adelaide, Adelaide, SA 5000, Australia; ⁴⁸Department of Molecular and Medical Genetics, Oregon Health and Science University, Portland, OR 97239, USA; ⁴⁹Department of Human Genetics, KU Leuven - University of Leuven, Leuven 3000, Belgium

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*Correspondence: lmessiaen@uabmc.edu

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have been reported with this form.¹⁷ It has been suggested that individuals with the severe subtype of FSNF more frequently carry an *NF1* missense or splicing mutation.^{18–20} Of particular interest are two families: a two-generation family with three first-degree relatives reported by Pascual-Castroviejo et al.²¹ and a three-generation family with three first-degree relatives reported by Burkitt-Wright et al.¹⁶ Specific *NF1* missense mutations c.2542G>C (p.Gly848Arg) and c.2543G>A (p.Gly848Glu), located in the cysteine-serine-rich domain (CSRD), were present in all individuals affected by multiple spinal dorsal root neurofibromas. Despite the evidence that c.2542G>C (p.Gly848Arg) is a clearly pathogenic mutation, two recent studies using mouse models did not recapitulate the phenotype identified in humans.^{22,23} Genetically engineered mice with c.2542G>C (p.Gly848Arg) mutation developed neither OPGs nor plexiform neurofibromas, demonstrating phenotypic divergence between NF1-affected individuals and mice.^{22,23}

In this study, we report a cohort of 129 unrelated probands and 33 affected relatives heterozygous for a constitutional missense mutation affecting one of five neighboring *NF1* codons—Leu844, Cys845, Ala846, Leu847, and Gly848. These individuals have a high prevalence of a severe phenotype, including plexiform and symptomatic spinal neurofibromas, symptomatic optic pathway gliomas, other malignant neoplasms, and bone abnormalities. The current findings clearly demonstrate that missense mutations outside the GRD are not solely associated with a mild phenotype.

Material and Methods

Individuals and Phenotypic Data

A total of 162 individuals heterozygous for a missense mutation affecting one of five neighboring *NF1* codons (Leu844, Cys845, Ala846, Leu847, and Gly848) were included in the study. Blood samples from 78 individuals (67 probands and 11 relatives) were originally sent to the UAB Medical Genomics Laboratory for molecular *NF1* genetic testing to establish or confirm the diagnosis for NF1. This initial study was expanded to include an additional 84 individuals (62 probands and 22 relatives), molecularly diagnosed in collaborating institutions (as detailed in Table S1).

All individuals included in this study were clinically assessed using the standardized phenotypic checklist form as previously reported (Figure S1).¹⁴ The clinical data were collected at the time of mutation analysis and re-verified for accuracy by referring physicians co-authoring this paper at the time of this study. Additionally, referring physicians updated the phenotypic data at the time of this genotype-phenotype study, when available, i.e., when the individual had been seen and followed at their institution after genetic testing results were reported. The phenotypic data and age provided correspond to the latest clinical evaluation. The phenotypic checklist form consists of two parts: (1) general information including gender, date of birth, ethnicity, height, head circumference (HC), weight, fulfillment of the NIH diagnostic criteria, and mode of inheritance and (2) NF1 signs and symptoms, including CALMs, skinfold freck-

ling, Lisch nodules, cutaneous and subcutaneous, plexiform and spinal neurofibromas, OPGs and other neoplasms, skeletal and cardiac abnormalities, development and education levels, presence/absence of Noonan syndrome features, and segmental phenotype.

Fifteen major clinical features of NF1 were selected for the genotype-phenotype correlation study (Tables 1, 2, and 3). Individuals with missing data for a particular sign and/or symptom were classified as “unknown” or “not specified” and consequently excluded from that part of the genotype-phenotype analysis. Most features were identified by physical examination; ophthalmologic examination for Lisch nodules and imaging to detect asymptomatic OPGs and spinal neurofibromas was not performed in most individuals. Brain and spine/whole-body MRI was done mainly in individuals with signs and/or symptoms indicative of OPGs or internal/spinal neurofibromas; however, depending on institutional policies, some individuals were screened by MRI despite the absence of symptoms. Noonan phenotype was diagnosed if at least two of the following features were observed: short stature, hypertelorism, low-set ears, webbed neck, ptosis, midface hypoplasia, or pulmonic stenosis. To evaluate short stature and macrocephaly, the World Health Organization (WHO) and the Center for Disease Control (CDC) growth charts and the Gerhard Nellhaus’ curve²⁴ were used as previously described.¹⁴ Short stature and macrocephaly were defined as height below or equal to the 3rd percentile ($PC \leq 3$) and as head circumference equal or above the 98th percentile ($PC \geq 98$), respectively. For cognitive impairment/learning disabilities, individuals with attention deficit disorder (ADD) and/or attention deficit hyperactivity disorder (ADHD) but normal development were classified as normal.

To establish a genotype-phenotype association, we used the same approach as previously described.¹⁴ We compared the phenotypes of individuals with missense mutations affecting codons 844–848 with the cohort of 169 individuals with missense mutations affecting p.Arg1809,^{13,14,25–27} 47 individuals heterozygous for c.2970_2972del (p.Met992del) mutations,¹² and previously described large-scale NF1-affected individual cohorts with “classic” NF1.^{7,28–40}

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

Molecular Analysis

In the Medical Genomics Laboratory at UAB, comprehensive *NF1* mutation screening using an RNA-based approach complemented by DNA-dosage analysis was performed as previously described.^{41,42} The status of the specific familial mutation in relatives was ascertained by bidirectional Sanger sequencing (ABI PRISM 3730, Life Technologies).

The nomenclature of the mutations is based on *NF1* mRNA sequence GenBank: NM_000267.3 according to the recommendations of the Human Genome Variation Society (HGVS). For exon numbering we used the NCBI numbering, followed by the historical numbering in square brackets originally developed by the NF1 community.⁴²

In Silico Prediction of Effect of Missense Mutations

Eight software programs were used to predict the effects of missense variants: two online *in silico* prediction tools (CADD v.1.3 and PolyPhen-2) and six complementary tools (Grantham Difference, SIFT v.4.0.3, SpliceSiteFinder-like, MaxEntScan, NNSplice v.0.9, and Human Splicing Finder v.2.4.1) embedded

Table 1. Demographic and Clinical Characterization of Individuals with a Missense Mutation Affecting Codons 844–848

Mutation [Proband:Relative]	Codon 844			Codon 845			Codon 846			Codon 847			Codon 848			All Codons 844–848	Total		
	c.2530C>T (p.Leu844Phe) [10:1]; c.2531T>A (p.Leu844His) [2:0]; c.2531T>C (p.Leu844Pro) [7:0]; c.2531T>G (p.Leu844Arg) [6:0]			c.2533T>C (p.Cys845Arg) [3:1]; c.2534G>A (p.Cys845Tyr) [8:0]			c.2536G>C (p.Ala846Pro) [1:2]; c.2537C>A (p.Ala846Asp) [5:2]			c.2540T>C (p.Leu847Pro) [58:12]; c.2540T>G (p.Leu847Arg) [8:0]			c.2542G>A (p.Gly848Arg) [6:0]; c.2542G>C (p.Gly848Arg) [8:11]; c.2543G>A (p.Gly848Glu) [7:4]						
Mutation-positive individuals [Proband:Relative]	26 [25:1]			12 [11:1]			10 [6:4]			78 [66:12]			36 [21:15]			162 [129:33]			
Age group, years	≤8	9–18	≥19	≤8	9–18	≥19	≤8	9–18	≥19	≤8	9–18	≥19	≤8	9–18	≥19	≤8	9–18	≥19	all ages
Total	12	5	9	4	2	6	3	1	6	28	14	36	13	5	18	60	27	75	162
Proband:Relative	12:0	5:0	8:1	4:0	2:0	5:1	2:1	1:0	3:3	27:1	12:2	27:9	6:7	4:1	11:7	51:9	24:3	54:21	129:33
Age range, years	1–8	9–16	24–55	1–2	15–16	19–48	4–5	18	33–69	1–8	9–18	19–72	1–7	10–17	19–74	1–8	9–18	19–74	1–74
Male: Female	6:6	4:1	1:8	1:3	1:1	1:5	2:1	0:1	1:5	10:18	5:9	19:17	9:4	2:3	5:13	28:32	12:15	27:48	67:95
Fulfilling the NIH criteria if the family history is taken into account	10/11	4/5	9/9	2/4	1/2	4/5	3/3	1/1	6/6	17/28	14/14	35/36	4/11	4/5	17/18	36/57	24/27	71/74	131/158
Fulfilling the NIH criteria if solely taking the physical signs into account	10/11	4/5	9/9	2/4	1/2	4/5	2/3	1/1	6/6	17/28	14/14	33/36	4/11	4/5	13/18	35/57	24/27	65/74	124/158
>5 CALMs	12/12	5/5	8/8	4/4	1/2	4/5	3/3	1/1	4/6	27/28	14/14	32/35	5/11	3/5	7/18	51/58	24/27	55/72	130/157
Freckling	10/10	4/5	6/7	0/4	1/2	4/5	2/2	1/1	5/5	12/23	13/13	31/34	4/10	3/5	8/18	28/49	22/26	54/69	104/144
Lisch nodules	2/9	1/4	4/4	0/1	0/0	1/2	0/1	0/1	2/2	4/19	3/9	17/19	2/8	0/5	6/14	8/38	4/19	30/41	42/98
Skeletal abnormalities ^a	2/11	2/5	5/9	2/4	1/2	2/4	0/2	0/1	0/5	3/25	3/14	17/28	3/11	3/5	5/18	10/53	9/27	29/64	48/144
Plexiform neurofibromas	0/11	2/5	3/9	0/3	2/2	2/5	0/2	1/1	1/2	6/24	3/13	19/33	0/11	1/5	7/17	6/51	9/26	32/66	47/143
Cutaneous neurofibromas ^b	1/11	1/5	7/9	0/4	0/2	3/4	0/2	1/1	4/5	1/26	4/14	28/33	1/11	1/5	5/18	3/54	7/27	47/69	57/150
Subcutaneous neurofibromas ^b	1/9	0/5	6/8	1/4	0/2	1/4	0/2	0/0	3/5	1/26	4/13	17/30	1/11	0/5	6/18	4/52	4/25	33/65	41/142
Cutaneous and subcutaneous ^b	0/9	0/5	5/8	0/4	0/2	1/3	0/2	0/0	3/5	0/25	1/13	17/30	0/11	0/5	4/18	0/51	1/25	30/64	31/140
Symptomatic spinal neurofibromas	0/10	0/3	0/8	0/2	1/2	0/4	0/2	0/0	0/2	1/23	1/13	2/27	0/11	1/4	7/16	1/48	3/22	9/57	13/127
Spinal neurofibromas by MRI ^c	0/1	0/0	0/5	0/0	1/2	1/1	0/1	0/0	0/1	1/5	2/6	3/16	0/1	2/3	10/11	1/8	5/11	14/34	20/53

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Table 1. Continued

Mutation [Proband:Relative]	Codon 844			Codon 845			Codon 846			Codon 847			Codon 848			All Codons 844–848	Total		
	c.2530C>T (p.Leu844Phe) [10:1]; c.2531T>A (p.Leu844His) [2:0]; c.2531T>C (p.Leu844Pro) [7:0]; c.2531T>G (p.Leu844Arg) [6:0]			c.2533T>C (p.Cys845Arg) [3:1]; c.2534G>A (p.Cys845Tyr) [8:0]			c.2536G>C (p.Ala846Pro) [1:2]; c.2537C>A (p.Ala846Asp) [5:2]			c.2540T>C (p.Leu847Pro) [58:12]; c.2540T>G (p.Leu847Arg) [8:0]			c.2542G>A (p.Gly848Arg) [6:0]; c.2542G>C (p.Gly848Arg) [8:11]; c.2543G>A (p.Gly848Glu) [7:4]						
Symptomatic OPGs ^d	1/11	1/5	0/9	0/3	0/2	0/5	1/3	1/1	0/3	2/25	1/13	2/27	1/11	1/5	1/13	5/53	4/26	3/57	12/136
Asymptomatic OPGs ^e	2/6	1/2	2/4	0/1	0/2	0/2	0/1	0/0	0/3	1/8	6/9	4/13	1/4	0/2	1/6	4/20	7/15	7/28	18/63
Other neoplasms ^f	1/11	0/4	1/8	0/2	0/1	0/4	0/2	0/1	0/3	1/24	3/14	11/34	2/11	1/5	1/15	4/50	4/25	13/64	21/139
Cognitive impairment and/or learning disabilities	3/11	3/4	0/6	1/4	0/2	3/4	3/3	0/1	1/5	10/26	7/13	12/26	5/11	5/5	3/17	22/55	15/25	19/58	56/138
Noonan syndrome features	0/9	1/5	1/8	0/2	1/1	0/4	0/2	0/1	0/4	3/27	0/13	3/26	1/10	0/5	0/17	4/50	2/25	4/59	10/134
Short stature ^g	1/7	0/2	0/4	0/3	1/1	0/1	0/2	0/0	1/2	0/11	3/10	4/21	3/10	0/3	2/14	4/33	4/16	7/42	15/91
Macrocephaly	2/11	1/4	1/2	1/3	0/1	0/0	2/2	0/0	0/2	8/21	2/11	10/17	3/11	1/4	5/9	16/48	4/20	16/30	36/98
Pulmonic stenosis	0/8	1/5	0/6	0/2	0/2	1/1	0/3	0/0	0/5	0/23	0/13	0/20	0/8	0/3	0/14	0/44	1/23	1/46	2/113

^aAll bone abnormalities included, i.e., scoliosis (n = 27), pectus excavatum (n = 4), pectus carinatum (n = 6), long bone dysplasia (n = 4), pseudarthrosis (n = 2), bone cysts (n = 2), sphenoid wing dysplasia (n = 2), ulnar aplasia, dural ectasia, 4th lumbar vertebrae fragmentation, bowed long bones, tibial dysplasia, clinodactyly, postaxial polydactyly, and cherubism.

^bAt least two cutaneous/subcutaneous neurofibromas were required to be considered as “positive for the criterion of neurofibromas.”

^cThe frequency of both symptomatic and asymptomatic spinal neurofibromas in individuals who had done MRI examination.

^dThe presence or absence of symptomatic OPGs was determined by ophthalmological examination and confirmed by MRI.

^eIncluding only individuals without signs of symptomatic OPGs who underwent MRI examination.

^fIncluding benign and malignant neoplasms, except for OPGs and neurofibromas.

^gAs no specific growth curves are available for the Hispanic and Asian populations, Hispanic and Asian individuals were excluded as having short or normal stature.

Table 2. Frequency of Clinical Features in Cohorts of Individuals with a Missense Mutation Affecting Leu844, Cys845, Ala846, Leu847, and Gly848

NF1 Feature	Number of Individuals (%) [95% Confidence Interval]				
	Leu844	Cys845	Ala846	Leu847	Gly848
>5 CALMs	25/25 (100) [86.7–100]	9/11 (81.8) [52.3–94.9]	8/10 (80) [49–94.3]	73/77 (94.8) [87.4–98]	15/34 (44.1) [28.9–60.6]
Skinfold freckling ^a	10/12 (83.3) [55.2–95.3]	5/7 (71.4) [35.9–91.8]	6/6 (100) [61–100]	44/47 (93.6) [82.8–97.8]	11/23 (47.8) [29.2–67]
Lisch nodules	7/17 (41.2) [21.6–64]	1/3 (33.3) [6.2–79.2]	2/4 (50) [15–85]	24/47 (51.1) [37.2–64.7]	8/27 (29.6) [15.9–48.5]
Plexiform neurofibromas ^a	5/14 (35.7) [16.3–61.2]	4/7 (57.1) [25–84.2]	2/3 (66.7) [20.8–93.9]	22/46 (47.8) [34.1–61.9]	8/22 (36.4) [19.7–57]
Cutaneous neurofibromas ^b	7/9 (77.8) [45.3–93.7]	3/4 (75) [30.1–95.4]	4/5 (80) [37.6–96.4]	28/33 (84.9) [69.1–93.4]	5/18 (27.8) [12.5–50.9]
Subcutaneous neurofibromas ^b	6/8 (75) [40.9–92.9]	1/4 (25) [4.6–69.9]	3/5 (60) [23.1–88.2]	17/30 (56.7) [39.2–72.6]	6/18 (33.3) [16.3–56.3]
Symptomatic spinal neurofibromas ^a	0/11 (0) [0–25.9]	1/6 (16.7) [3–56.4]	0/2 (0) [0–65.8]	3/40 (7.5) [2.6–19.9]	8/20 (40) [21.9–61.3]
Spinal neurofibromas by MRI ^{a,c}	0/5 (0) [0–43.5]	2/3 (66.7) [20.8–93.9]	0/1 (0) [0–79.4]	5/22 (22.7) [10.1–43.4]	12/14 (85.7) [60.1–96]
Symptomatic OPGs, age ≥ 5 years ^d	1/21 (4.8) [0.9–22.7]	0/7 (0) [0–35.4]	2/5 (40) [11.8–76.9]	5/47 (10.6) [4.6–22.6]	3/24 (12.5) [4.3–31]
Asymptomatic OPGs, age ≥ 5 years ^e	4/10 (40) [16.8–68.7]	0/4 (0) [0–49]	0/3 (0) [0–56.2]	11/25 (44) [26.7–62.9]	1/10 (10) [1.8–40.4]
Other neoplasms ^f	2/23 (8.7) [2.4–26.8]	0/7 (0) [0–35.4]	0/6 (0) [0–39]	15/72 (20.8) [13.1–31.6]	4/31 (12.9) [5.1–28.9]
Skeletal abnormalities	9/25 (36) [20.3–55.5]	5/10 (50) [23.7–76.3]	0/8 (0) [0–32.4]	23/67 (34.3) [24.1–46.3]	11/34 (32.4) [19.1–49.2]
Noonan syndrome features	2/22 (9.1) [2.5–27.8]	1/7 (14.3) [2.6–51.3]	0/7 (0) [0–35.4]	6/66 (9.1) [4.2–18.5]	1/32 (3.1) [0.6–15.8]
Pulmonic stenosis	1/19 (5.3) [0.9–24.6]	1/5 (20) [3.6–62.5]	0/8 (0) [0–32.4]	0/56 (0) [0–6.4]	0/25 (0) [0–13.3]
Short stature ^g	1/13 (7.7) [13.7–33.3]	1/5 (20) [3.6–62.5]	1/4 (25) [4.6–69.9]	7/42 (16.7) [8.3–30.6]	5/27 (18.5) [8.2–36.7]
Macrocephaly	4/17 (23.5) [9.6–47.3]	1/4 (25) [4.6–69.9]	2/4 (50) [15–85]	20/49 (40.8) [28.2–54.8]	9/24 (37.5) [21.2–57.3]
Cognitive impairment and/or learning disabilities	6/21 (28.6) [13.8–50]	4/10 (40) [16.8–68.7]	4/9 (44.4) [18.9–73.3]	29/65 (44.6) [33.2–56.7]	13/33 (39.4) [24.7–56.3]
Severe phenotype, age ≥ 19 years ^h	7/9 (77.8) [45.3–93.7]	4/6 (66.7) [30–90.3]	1/6 (16.7) [3–56.4] ⁱ	32/36 (88.9) [74.7–95.6]	12/18 (66.7) [43.8–83.7]

^aIn individuals ≥ 9 years.^bIn individuals ≥ 19 years.^cThe frequency of both symptomatic and asymptomatic spinal neurofibromas in individuals who had undergone MRI examination.^dThe presence or absence of symptomatic OPGs was determined by ophthalmological examination and confirmed by MRI.^eIncluding only individuals without signs of symptomatic OPGs who underwent MRI examination.^fIncluding benign and malignant neoplasms, except for OPG and neurofibromas.^gAs no specific growth curves are available for the Hispanic and Asian populations, Hispanic and Asian individuals were excluded as having short or normal stature.^hIndividual was classified as having a severe phenotype if at least one of the following features was observed: plexiform and/or symptomatic spinal neurofibroma, symptomatic OPG, malignant neoplasm, or osseous lesions.ⁱAmong individuals with a missense mutation affecting codon 846, the status of plexiform and spinal neurofibromas was known only for 2/6 individuals (UG-R0781-S and UG-R665-F), thus a severe phenotype cannot be excluded in the remaining four individuals with missing data.

Table 3. Comparison of Clinical Features of the Studied Group with the *NF1* Arg1809 Cohort, the *NF1* Met992del Cohort, and Large-Scale Previously Reported Cohorts of Individuals with “Classic” *NF1*

NF1 Feature	Number of Individuals (%)				p Value (2-Tailed Fisher’s Exact Test)		
	aa 844–848	Arg1809 ^a	Met992del ^b	Previously Reported NF1 Cohorts	aa 844–848 versus Arg1809	aa 844–848 versus Met992del	aa 844–848 versus “Classic” NF1
>5 CALMs	130/157 (82.8)	157/169 (92.9)	46/47 (97.9)	1,537/1,728 (89) ^c	0.0060* ↘	0.0067* ↘	0.0263 ↘
Skinfold freckling	104/144 (72.2)	95/161 (59)	32/47 (68.1)	1,403/1,667 (84.2) ^c	0.0164 ↗		0.0007** ↘
Lisch nodules	42/98 (42.9)	12/120 (10)	3/38 (7.9)	729/1,237 (58.9) ^c	<0.0001** ↗	<0.0001** ↗	0.0028* ↘
Major external plexiform neurofibromas ^d	36/92 (39.1)	0/105 (0)	0/41 (0)	120/648 (18.5) ^{e,f}	<0.0001** ↗	<0.0001** ↗	<0.0001** ↗
Cutaneous neurofibromas ^g	47/69 (68.1)	0/57 (0)	0/18 (0)	656/723 (90.7) ^{f,h,i,j}	<0.0001** ↗	<0.0001** ↗	<0.0001** ↘
Subcutaneous neurofibromas ^g	33/65 (50.8)	0-5/57 (0-8.8) ^k	ND	297/515 (57.7) ^{f,i,j}	<0.0001** ↗		
Symptomatic spinal neurofibromas ^{d,i}	12/79 (15.2) 13/127 (10.2)	0/40 (0) 0/76 (0)	1/41 (2.4) 1/47 (2.1)	2/119 (1.7) ^e 36/2,058 (1.8) ^{e,f,m}	0.0080* ↗ 0.0022* ↗	0.0341 ↗	0.0004** ↗ <0.0001** ↗
Symptomatic OPGs, age ≥ 5 years ^{l,n}	11/104 (10.6) 12/136 (8.8)	0/114 (0) 0/139 (0)	0/46 (0) 0/47 (0)	7/180 (3.9) ^{e,o} 64/1,650 (3.9) ^c	0.0002** ↗ 0.0002** ↗	0.0186 ↗ 0.0384 ↗	0.0404 ↗ 0.0125* ↗
Asymptomatic OPGs, age ≥ 5 years ^{l,p}	16/52 (30.8) 18/63 (28.6)	0/35 (0) 0/38 (0)	ND	2/45 (4.4) ^o 70/519 (13.5) ^{q,r,s}	0.0001** ↗ <0.0001** ↗		0.0012** ↗ 0.0043* ↗
Other malignant neoplasms ^t	13/139 (9.4)	2/155 (1.3) ^u	0/47 (0)	18/523 (3.4) ^f	0.0023* ↗	0.0409 ↗	0.0061* ↗
Skeletal abnormalities ^{d,i}	38/91 (41.8) 48/144 (33.3)	14/72 (19.4) 21/126 (16.7)	8/41 (19.5) 9/47 (19.2)	14/96 (14.6) ^e 144/948 (15.2) ^{e,f,i,v}	0.0025* ↗ 0.0020* ↗	0.0174 ↗	<0.0001** ↗ <0.0001** ↗
Scoliosis ^g	20/64 (31.3)	6/48 (12.5)	2/18 (11.1)	51/236 (21.6) ^{b,j}	0.0241 ↗		
Noonan syndrome features	10/134 (7.5)	46/148 (31.1)	4 (all from 1 family)	57/1,683 (3.4) ^c	<0.0001** ↘		0.0276 ↗
Pulmonic stenosis	2/113 (1.8)	14/132 (10.6)	4/47 (8.5)	25/2,322 (1.1) ^w	0.0076* ↘		
Short stature	15/91 (16.5)	32/111 (28.8)	5/47 (10.6)	109/684 (15.9) ^{e,i}	0.0451 ↘		
Macrocephaly	36/98 (36.7)	31/107 (29)	4/45 (8.9)	239/704 (33.9) ^{e,i}		0.0005** ↗	
Cognitive impairment and/or learning disabilities	56/138 (40.6)	80/159 (50.3)	8/47 (17)	190/424 (44.8) ^{e,f}		0.0042* ↗	

Statistically significant p values with false discovery rates of 0.05 (indicated by *) and 0.01 (indicated by **) after correction for multiple testing using Benjamini-Hochberg procedure (see details in Table S10). After applying the Benjamini-Hochberg correction, $p \leq 0.0125$ remained statistically significant at FDR of 0.05, while p values ≤ 0.0012 were still be considered as significantly different at FDR of 0.01. The black arrows indicate the statistically significant differences of the NF1 clinical features prevalence between the studied group and the cohort(s) used for the comparison, with the up and down arrows representing an increase and a decrease of the prevalence in the studied group, respectively. Abbreviation: ND, no data

^aBased on data from Pinna et al.,¹³ Rojnuangnit et al.,¹⁴ Nyström et al.,²⁵ Ekvall et al.,²⁶ and Santoro et al.²⁷

^bBased on data from Upadhyaya et al.¹²

^cPrevious NF1 cohort used for comparison: Friedman and Birch.³²

^dIn individuals ≥ 9 years in this study and Arg1809, ≥ 10 years in Met992del and other studies.

^ePrevious NF1 cohort used for comparison: Huson et al.⁷

^fPrevious NF1 cohort used for comparison: McLaughran et al.³⁴

^gIn individuals ≥ 19 years in this study and Arg1809, ≥ 20 years in Met992del and other studies.

^hPrevious NF1 cohort used for comparison: Huson et al.^{28,29}

ⁱPrevious NF1 cohort used for comparison: Khosrotehrani et al.³⁸

^jPrevious NF1 cohort used for comparison: Plotkin et al.³⁹

^kFive individuals with few (1–6) small, subcutaneous “possible” neurofibromas, none were biopsied and therefore none have been histologically confirmed.¹⁴

^lSecond value is the frequency of a particular feature regardless of the individuals’ age.

^mPrevious NF1 cohort used for comparison: Thakkar et al.³⁵

ⁿThe presence or absence of symptomatic OPGs was determined by ophthalmological examination and confirmed by MRI.

^oPrevious NF1 cohort used for comparison: Van Es et al.³¹

^pIncluding only individuals without signs of symptomatic OPGs who underwent MRI examination.

^qPrevious NF1 cohort used for comparison: Listerneck et al.³⁰

^rPrevious NF1 cohort used for comparison: Blazo et al.³⁷

^sPrevious NF1 cohort used for comparison: Blanchard et al.⁴⁰

^tOnly malignant neoplasms, hence excluding neurofibromas and OPGs, have been taken into account.

^uBreast cancer (n = 1) and Ewing sarcoma (n = 1) were found in the *NF1* Arg1809 cohort, no follow-up information on these individuals was available.¹⁴

^vPrevious NF1 cohort used for comparison: Cnossen et al.³³

^wPrevious NF1 cohort used for comparison: Lin et al.³⁶

in Alamut visual software v.2.9.0 (Interactive Biosoftware). The presence or absence of the variants was checked in population databases, including the Genome Aggregation Database (gnomAD), 1000 Genomes, and the Exome Variant Server (EVS) as well as in disease databases: the Leiden Open Variation Database (LOVD), ClinVar, and the Human Gene Mutation Database (HGMD) (last accessed May 2017). Evolutionary conservation for human neurofibromin GenBank: NP_000258.1 residues 804–950 was evaluated using Clustal software v.2.0.12. The palindromic sequences and quadruplex forming G-Rich sequences (QGRS) were identified by Palindrome search and QGRS Mapper, respectively.

Interpretation of variant pathogenicity was performed based on the American College of Medical Genetics and Genomics (ACMG) recommendations.⁴³

Statistical Analysis

For univariate analysis, two-tailed Fisher's exact test was used to compare categorical variables with a *p* value < 0.05 considered as statistically significant. The resulting *p* values were adjusted for multiple comparisons using Benjamini-Hochberg (B-H) procedure with false discovery rates (FDRs) of 0.05 and 0.01. The 95% confidence interval (CI) was also calculated when appropriate. All statistical analyses were performed with GraphPad and VassarStats softwares.

Results

Description of Missense Mutations Affecting Codons 844–848

Exon 21 [16] is the largest *NF1* exon (441 nucleotides), and in it we identified, besides the missense variants affecting the codons 844–848, a total of 19 different missense variants in 35 unrelated individuals from the UAB cohort. Fourteen of these alterations were classified as variants of uncertain significance (8/19) or likely benign (6/19) and reported 1–3 times in the UAB cohort (Figure S2). Only five variants were classified as pathogenic (4/19) or likely pathogenic (1/19) according to the current recommendations.⁴³ Region 844–848 in exon 21 [16] stood out due to its high frequency of variants compared with the neighboring codons, indicating functional importance (Figures S2 and S3). A similar distribution and spectrum of missense alterations in the *NF1* exon 21 [16] was observed in the publicly available databases (ClinVar, LOVD, and HGMD). Besides a clear cluster of recurrent variants in codons 844–848, other alterations spread over the entire exon 21 [16] were mostly classified as variants of uncertain significance and reported 1–2 times in these databases (Figure S2). The frequency of this cluster of variants in aa 844–848 is ~0.8% (67/8,400) in unrelated *NF1* mutation-positive individuals from the UAB cohort, second only to the p.Arg1809 (~1.2%), and therefore represents a significant hotspot for missense mutations within *NF1*.

In the 129 unrelated individuals reported here, we identified 12 different *NF1* missense alterations affecting one of five neighboring codons in exon 21 [16] (Table 1 and Figure 1). Within the group of individuals with p.Gly848Arg, two different substitutions were observed:

c.2542G>A (6/14) and c.2542G>C (8/14). Detailed characteristics of the identified missense mutations are shown in Tables S2–S4 and Figure 1. All variants identified in this study with confirmed origin of the variant were submitted to the LOVD and ClinVar databases. Based on the data accumulated in this report (Tables S1 and S2), these variants can all be classified as pathogenic (Table S4) according to current recommendations.⁴³

Among the aforementioned variants, 8/12 were present in the LOVD database with 5/8 classified as pathogenic (c.2533T>C [p.Cys845Arg], c.2536G>C [p.Ala846Pro], c.2537C>A [p.Ala846Asp], c.2540T>C [p.Leu847Pro], and c.2543G>A [p.Gly848Glu]) and 3/8 as variants of uncertain significance (c.2534G>A [p.Cys845Tyr], c.2540T>G [p.Leu847Arg], and c.2542G>C [p.Gly848Arg]). Eight of the 12 were present in ClinVar, including 3/8 classified as pathogenic (c.2531T>G [p.Leu844Arg], c.2540T>C [p.Leu847Pro], and c.2542G>C [p.Gly848Arg]), 1/8 as likely pathogenic (c.2534G>A [p.Cys845Tyr]), 1/8 as a variant of uncertain significance (c.2533T>C [p.Cys845Arg]), and 3/5 with no significance provided (c.2530C>T [p.Leu844Phe], c.2531T>C [p.Leu844Pro], and c.2543G>A [p.Gly848Glu]) (Tables S2 and S3). One individual (UAB-R4444) with c.2531T>A (p.Leu844His) carried another novel alteration (c.2524G>A); assuming both variants reside in *cis*, this alteration should be described as c.2524_2531delinsAGCTTCCA (p.Gly842_Leu844delinsSerPheHis). None of these variants, except for c.2531T>G (p.Leu844Arg), has been reported in 129,639 unrelated controls of the gnomAD and EVS databases or in the 1000 Genomes Project; c.2531T>G (p.Leu844Arg) was reported once in Latino (the variant's frequency in all populations is 0.00041%). Based on *in silico* analysis, all alterations are predicted to be deleterious (SIFT) and probably or possibly damaging (PolyPhen-2). Additionally, CADD classified all variants as more likely to have deleterious effects (range: 22.6 to 31). In contrast to results of *in silico* analysis, suggesting a possible effect of two identified alterations (c.2542G>A and c.2543G>A) on splicing through creation of a novel exonic splice acceptor sequence, transcript analysis and sequencing showed a minor effect on splicing only for c.2542G>A in three individuals (UAB-R9493, UAB-R1474, and UAB-R0008), i.e., low levels of r.2410_2543del. The other individuals with c.2542G>A screened with an RNA-based approach (UAB-R3513 and UAB-R4476) in whom no missplicing was observed also carried the nearby benign variant c.2544G>A (p.Gly848=) (rs17883704) with both variants proven to reside in *cis* through next-generation sequencing. As missplicing was observed only in individuals carrying c.2542G>A in the absence of rs17883704 (Figure S4), rs17883704 is hypothesized to have a modifying effect. All missense mutations, except for c.2536G>C (p.Ala846Pro), were proven to be *de novo* in at least one proband; a total of 26 probands with unaffected parents were proven to have a *de novo* mutation, but formal confirmation of paternity/maternity by identity testing was pursued only for individuals tested in the Netherlands

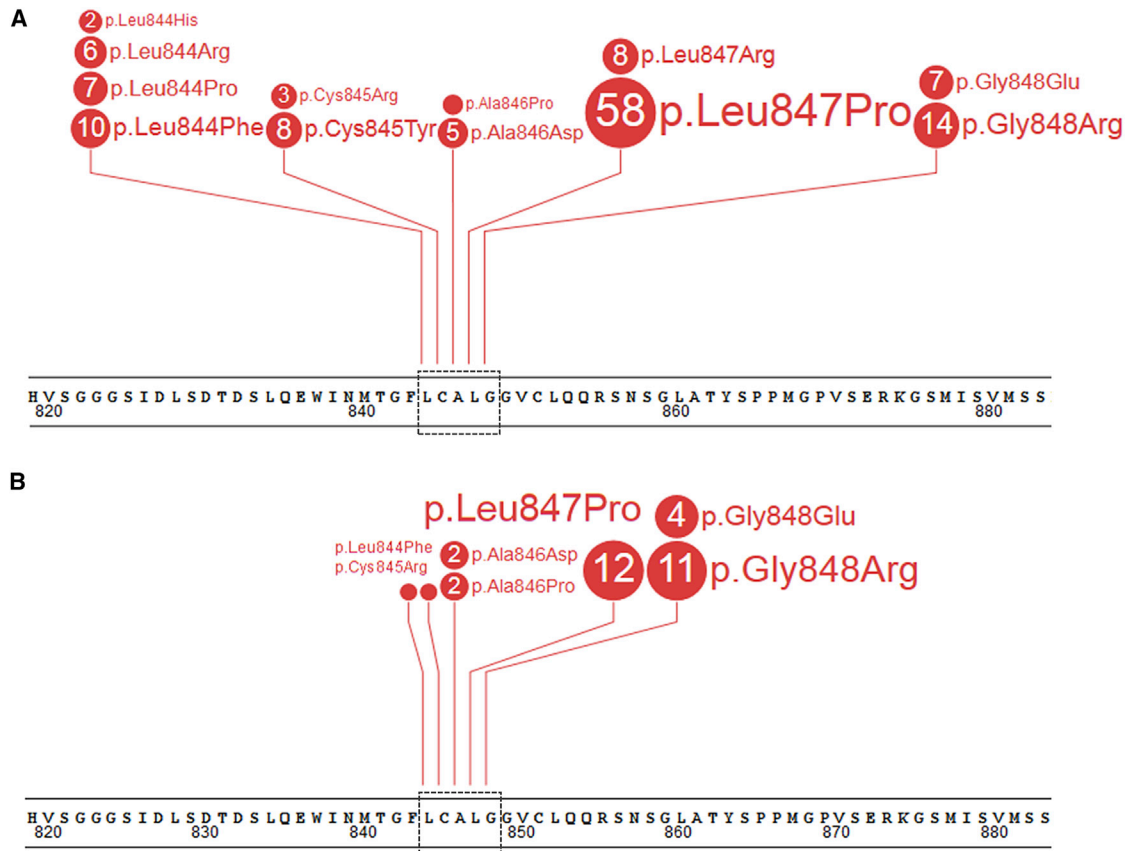


Figure 1. Spectrum of Missense Mutations Affecting *NF1* Codons 844–848 in the Cohort of 129 Proband and 33 Relatives
 Shown are 129 probands (A) and 33 relatives (B). Each number in circle corresponds with the total number of individuals heterozygous for a specific mutation. The black dotted lines on the panels present the region 844–848. The figure was prepared using the ProteinPaint application.⁴⁴

(ROT-R02233, ROT-R22853, and ROT-R17435). Additionally, 7/12 missense mutations (c.2530C>T [p.Leu844Phe], c.2533T>C [p.Cys845Arg], c.2536G>C [p.Ala846Pro], c.2537C>A [p.Ala846Asp], c.2540T>C [p.Leu847Pro], c.2542G>C [p.Gly848Arg], and c.2543G>A [p.Gly848Glu]) segregated with the phenotype (at least one individual per family) in 23 affected first-degree relatives from 15 families (Tables S1 and S2 and Figure S5). Finally, all missense mutations affecting amino acids 844–848 are located in a highly conserved region of the CSRD (amino acids 543–909; Figure S6). Besides cysteine at position 845 that is conserved up to zebrafish, all remaining amino acids are evolutionarily conserved up to *Drosophila melanogaster* (Ala846 and Gly848) and even to yeast IRA1 and/or IRA2 (Leu844 and Leu847). In chimpanzee, rat, and mouse all amino acids from 775 to 856 are fully evolutionarily conserved. None of these variants has been functionally characterized.

Demographic and Clinical Characterization of the Studied Cohort

A total of 162 individuals from 129 unrelated families were enrolled in the study, including 37/129 (28.7%) familial and 89/129 (69%) sporadic case subjects; 3/129 (2.3%) individuals had an unknown family history

(ROT-R13734, ROT-R89874, and CAR-R8012M6). Detailed demographic and clinical descriptions of the individuals included in the study are shown in Tables 1 and S1 and Figure S5.

The complete phenotypic checklist forms were collected from 151/162 individuals (93.2%). Of these, 125/151 (82.8%) fulfilled the NIH diagnostic criteria and 118/151 (78.2%) fulfilled the NIH diagnostic criteria if family history was excluded as a criterion. Among 26/151 individuals who did not fulfill the NIH diagnostic criteria (with 20/26 being ≤ 8 years), multiple CALMs-only (>5) were present in 16/26, <6 CALMs-only were present in 8/26, and 2/26 did not have any pigmentary manifestations but had externally visible plexiform neurofibromas (UAB-R9135 and UG-R5831) (Table S5). CALMs-only (<6) were observed mostly in individuals with a missense mutation at codon 848 (5/8 with c.2542G>C [p.Gly848Arg], 1/8 with c.2542G>A [p.Gly848Arg], 1/8 with c.2543G>A [p.Gly848Glu], and 1/8 with c.2534G>A [p.Cys845Tyr]).

Among 102 individuals ≥ 9 years, more than 5 CALMs and skinfold freckling were present in 79.8% (79/99) and 80% (76/95), respectively (Table 1). Both clinical features were found in 71.6% (68/95) of case subjects. Out of 20 individuals ≥ 9 years with only few or absolute lack of

CALMs (Table S1), 11 case subjects fulfilled the NIH diagnostic criteria based on presence of other clinical signs, such as skinfold freckles, Lisch nodules, neurofibromas, and/or osseous lesions (UG-R0781, UAB-R3618-M, MIL-R192/982-F, UAB-R4476, MIL-R999/399, MIL-R999/399-M, ROT-R95424, UG-R923-S, UAB-R3237, MAN-R95417G, and MAN-R95417G-C). Among these individuals, 8/11 (72.7%) carried a missense mutation at codon 848. Lisch nodules were reported less frequently (42/98 all ages, but in 34/60 ≥ 9 years).

Cutaneous and subcutaneous neurofibromas were found in 68.1% (47/69 ≥ 19 years) and 50.8% (33/65 ≥ 19 years) of the case subjects, respectively. Thirty adults had both types of tumors (30/64 ≥ 19 years, 46.9%). Ten individuals ≥ 17 years had >100 cutaneous and/or subcutaneous nodules, including a 47-year-old man previously reported⁴⁵ with $>1,400$ neurofibromas (individual counts of externally visible neurofibromas; BRA-R38) and a 17-year-old woman (ROT-R1CMUL) with >500 cutaneous neurofibromas, >100 subcutaneous neurofibromas, and >100 intradermal neurofibromas. Nine out of ten individuals with a very high number of neurofibromas carried a missense mutation at codon 847: c.2540T>G (p.Leu847Arg) (2/9) or c.2540T>C (p.Leu847Pro) (7/9, including two individuals with metastasized MPNSTs). In 16 case subjects with “several” neurofibromas, a more precise estimated number was not reported. Eight individuals (UAB-R5776, UAB-R3618, UAB-R4624, UAB-R7447, UAB-R1002, UAB-R1037-M, UAB-R3237, PAD-R500-C1) were reported to have a single cutaneous or subcutaneous nodule (none histopathologically confirmed); these individuals were considered as “negative for the criterion of neurofibromas” as ≥ 2 cutaneous/subcutaneous neurofibromas are required according to the NIH clinical criteria.

45% of the individuals ≥ 9 years had known plexiform neurofibromas (41/92 ≥ 9 years; 47/143 all ages), including externally visible ($n = 36$) and internal ($n = 5$) tumors. For six case subjects, the information was not provided whether plexiform neurofibromas were identified clinically or by MRI. Among all individuals with plexiform neurofibromas, 31/47 presented with one plexiform tumor and 16/47 with ≥ 2 plexiform neurofibromas. Plexiform tumors were found in the head, face, and neck area (35.7%, 25/70), limbs (34.3%, 24/70), trunk (17.1%, 12/70), back ($n = 3$), abdomen ($n = 3$), pelvis ($n = 2$), and chest ($n = 1$).

Symptomatic spinal neurofibromas visible by MRI were found in 15.2% of individuals (12/79 ≥ 9 years; 13/127 all ages). Forty asymptomatic individuals received MRI screening, leading to the identification of another seven case subjects with spinal tumors (Table S6). Approximately one-third of the individuals with spinal tumors (6/20) had fewer than 6 CALMs and no skinfold freckling, whereas in 60% (12/20) plexiform neurofibromas were observed (with 11/12 being externally visible).

Symptomatic OPGs, confirmed by MRI imaging, were found in 11/104 of individuals older than 5 years (10.6%), whereas asymptomatic OPGs were present in

16/52 additional individuals who underwent MRI examination (30.8% ≥ 5 years). In 19 of 27 symptomatic and asymptomatic OPGs, the detailed information about the tumor's location was collected, involving optic nerves (2 symptomatic OPGs and 7 asymptomatic OPGs), chiasm (1 symptomatic OPG and 1 asymptomatic OPG), or both locations (6 symptomatic OPGs and 2 asymptomatic OPGs). Three children were diagnosed with a symptomatic OPG (PAD-R300) or asymptomatic OPGs (UAB-R3714 and UAB-R3513) before age 4 years (Table S7).

Skeletal abnormalities were frequently reported (48/144 all ages) and included scoliosis (27/144 all ages, but 20/64 ≥ 19 years) and pectus anomalies (10/144 all ages: pectus carinatum 6/10 and excavatum 4/10). In addition, long bone dysplasia ($n = 4$), pseudarthrosis ($n = 2$), tibial dysplasia ($n = 1$), bone cysts ($n = 2$), sphenoid wing dysplasia ($n = 2$), ulnar aplasia, likely representing the severe end of ulnar pseudarthrosis with bone resorption and absence of ulnar bone ($n = 1$), dural ectasia ($n = 1$), 4th lumbar vertebrae fragmentation ($n = 1$), bowed long bones ($n = 1$), clinodactyly ($n = 1$), postaxial polydactyly ($n = 1$), and cherubism ($n = 1$) were observed in the studied group.

Noonan syndrome features were observed in 10/134 (7.5%) individuals. One previously reported individual (UAB-R624) with a family history of *PTPN11*-positive (MIM: 176876) Noonan syndrome (MIM: 163950) had a severe phenotype of pulmonic stenosis and aortic coarctation, dysmorphic features (high forehead, hypertelorism, downslanting palpebral fissures, short neck with a low posterior hair line), short stature, pectus carinatum, >5 CALMs, axillary and inguinal freckling, plexiform and cutaneous neurofibromas, and symptomatic OPG with signs of hydrocephalus.⁴⁶ Besides the familial *PTPN11* c.1529A>G (p.Gln510Arg) inherited from the individual's father, the *NF1* missense mutation c.2531T>G (p.Leu844Arg) was found *de novo* in the proband (Figure S5). In other individuals with Noonan syndrome features (UAB-R2696, UAB-R5001, UAB-R3725, and UAB-R4676) no pathogenic or likely pathogenic variants in Noonan-related disorders genes (*PTPN11* [MIM:176876], *SPRED1* [MIM:609291], *BRAF* [MIM: 164757], *CBL* [MIM: 165360], *HRAS* [MIM: 190020], *KRAS* [MIM: 190070], *MAP2K1* [MIM: 176872], *MAP2K2* [MIM: 601263], *NRAS* [MIM: 164790], *RAF1* [MIM: 164760], *SHOC2* [MIM: 602775], *SOS1* [MIM: 182530], *RIT1* [MIM: 609591], *RASA2* [MIM: 601589], and *SOS2* [MIM: 601247]) were identified. Cardiovascular abnormalities observed in the studied group included hypertension ($n = 7$, one related to renal artery stenosis), pulmonic stenosis ($n = 2$), mitral valve stenosis, atrial septal defect, ventricular septal defect, Moyamoya disease, pericarditis carcinomatosa, mitral valve insufficiency, mild pulmonic insufficiency, and hypertrophic cardiomyopathy (each observed in a single individual). Short stature (PC ≤ 3) and macrocephaly (PC ≥ 98) were found in 15/91 (16.5%) and 36/98 (36.7%), respectively. Of the 138 case subjects with

provided developmental data, 56 individuals had abnormal development presenting with at least one of the following forms of cognitive impairment and/or learning difficulties: learning disabilities (n = 43), developmental delay (n = 30), speech delay (n = 8), ADD (n = 8), ADHD (n = 10), motor delay (n = 5), autism spectrum (n = 2), or Asperger syndrome (n = 1). Seven individuals had significant global developmental delay with/without speech delay, learning difficulties, and/or AD(H)D, including one with a full scale intelligence quotient (FSIQ) score 59. Additionally, three individuals were reported to have frequent migraine headaches and two had epilepsy and/or psychiatric problems.

For 139/162 individuals, data on the presence or absence of tumors other than neurofibromas and OPGs was available. 13 of 139 (9.4%) individuals were diagnosed with malignant neoplasms (Table S8), including embryonal rhabdomyosarcoma (3/13), MPNST (7/13, including one woman with MPNST and *BRCA1/2*-negative breast cancer), colon cancer (1/13), medullary thyroid carcinoma (1/13), and juvenile myelomonocytic leukemia (JMML) (1/13). Individuals ≥ 14 years old with c.2540T>C (p.Leu847Pro) had a higher number of malignant neoplasms compared to individuals carrying other missense mutations in the studied region (p = 0.0448; Table S9). Moreover, this mutation was present in most case subjects with MPNST (5/7), except for one each carrying c.2543G>A (p.Gly848Glu) or c.2530C>T (p.Leu844Phe). Four of seven individuals with MPNST died before age 30 years (Table S8). Hypothalamic glioma (n = 1), lipoma (n = 1), cerebral tumors (n = 3), non-ossifying fibroma (n = 2), and odontogenic fibroma (n = 1) were also reported.

The frequency of clinical features in individuals heterozygous for missense mutations affecting one of five neighboring codons 844–848 is presented in Table 2. A lower number of CALMs, freckling, and cutaneous neurofibromas was observed in case subjects with missense mutations at codon 848 (all p < 0.0001; Table S9); however, these individuals had a higher prevalence of symptomatic spinal neurofibromas (p = 0.0012; Table S9).

Taken together, a severe phenotype, including at least one of the following features (plexiform and/or symptomatic spinal neurofibromas, symptomatic OPGs, malignant neoplasm, or osseous lesions) was observed in 75% of adult NF1-affected individuals (56/75 ≥ 19 years; Table 2).

Comparison of Clinical Features Observed in the Studied Cohort with Individuals Heterozygous for p.Arg1809 and p.Met992del Mutations and Cohort of Individuals with “Classic” NF1 Phenotype

Comparison of clinical features of the studied group with the *NF1* p.Arg1809 and p.Met992del cohorts as well as previously described large-scale cohorts of individuals with “classic” NF1 is shown in Table 3. The complete list of adjusted p values with FDRs at 0.05 and 0.01 after B-H correction for multiple testing is presented in Table S10. All p values ≤ 0.0125 and p values ≤ 0.0012 remained

statistically significant after applying the B-H correction at FDRs of 0.05 and 0.01, respectively.

In the current study, we observed a significantly higher number of major external plexiform neurofibromas compared with the *NF1* p.Arg1809 and the *NF1* p.Met992del cohorts, as well as classic NF1-affected population (all p < 0.0001; statistically significant after B-H correction at FDR of 0.01). Importantly, while none of the individuals carrying the p.Arg1809 and p.Met992del had external plexiform, cutaneous, and/or subcutaneous neurofibromas, $\sim 71\%$ of the individuals ≥ 19 years with a missense mutation affecting codons 844–848 had cutaneous and/or subcutaneous neurofibromas (p < 0.0001; statistically significant after B-H correction at FDR of 0.01) and $\sim 39\%$ of the individuals ≥ 9 years had externally visible plexiform neurofibromas (p < 0.0001; statistically significant after B-H correction at FDR of 0.01). Compared with p.Arg1809, p.Met992del, and classic NF1-affected cohorts, at least 5-fold greater prevalence of symptomatic spinal neurofibromas was reported in the studied group (0%–2.1% versus 10.2%) which was statistically significant at FDR of 0.01 for the general NF1-affected population (p < 0.0001) and at FDR of 0.05 for the p.Arg1809 cohort (p = 0.0022).

Symptomatic and asymptomatic OPGs were more frequent compared to individuals with p.Arg1809, p.Met992del, and classic NF1, with symptomatic and asymptomatic OPGs statistically increased after B-H correction at FDR of 0.05 in the 844–848 cohort compared to the classic NF1-affected cohorts (p = 0.0125 and p = 0.0043, respectively) and at FDR of 0.01 compared with the p.Arg1809 cohort (p = 0.0002 and p < 0.0001, respectively). The overall prevalence of malignant neoplasms, other than neurofibromas and OPGs, was also higher in the studied group compared to a large cohort of classic NF1-affected individuals (9.4% versus 3.4%; p = 0.0061, statistically significant at FDR of 0.05 after B-H correction).

Additionally, the aa 844–848 cohort had a significantly increased frequency of skeletal abnormalities compared to individuals with p.Arg1809 and classic NF1 phenotypes (both statistically significant after B-H correction at FDR of 0.05), regardless of the age. Scoliosis was reported more frequently compared with p.Arg1809 individuals (31.3% versus 12.5% in ≥ 19 years), but this difference was not statistically significant after B-H correction.

The prevalence of CALMs was lower than in p.Arg1809 and p.Met992del cohorts (both significant at FDR of 0.05 after B-H correction), while skinfold freckles occurred more commonly in classic NF1-affected cohorts than in the studied group (significant at FDR of 0.01 after B-H correction). Noonan syndrome features were significantly less frequent in the studied group compared to individuals with p.Arg1809 (significant at FDR of 0.01 after B-H correction). In line with this finding, pulmonic stenosis was very rarely observed in the cohort (1.8% versus 10.6% in the p.Arg1809 cohort; significant at FDR of 0.05 after B-H correction). All cohorts, except for the p.Met992del, shared

a similar frequency of cognitive impairment and/or learning difficulties (~45%).

Discussion

We present 162 individuals heterozygous for a constitutional *NF1* missense mutation in one of five neighboring codons 844–848 who have a high prevalence of a severe NF1 phenotype, including plexiform and/or symptomatic spinal neurofibromas, symptomatic OPGs, and other malignant neoplasms, as well as bone abnormalities. The frequency of the cluster of these mutations is ~0.8% (67/8,400) in unrelated *NF1* mutation-positive individuals from the UAB cohort, second only to the p.Arg1809 (~1.2%) among the missense variants.

One of the most severe complications in NF1-affected individuals are clinically apparent plexiform neurofibromas affecting 15%–30% of the NF1-affected general population.^{7,34,47–50} In this study, externally visible plexiform neurofibromas were found in ~39% of individuals ≥9 years, therefore significantly higher compared with p.Arg1809 and p.Met992del and classic NF1-affected cohorts (significant at FDR of 0.01 after B-H correction; Tables 3 and S10). Individuals in this study did not undergo whole-body MRI; therefore, the frequency provided here is a likely underestimate, as internal asymptomatic plexiform neurofibromas were not accounted for.

As plexiform neurofibromas have been suggested to be associated with a higher lifetime risk for the development of MPNSTs,^{50–53} the finding of MPNSTs in 5% (7/139) of the affected in our cohort, which is twice as high as reported by Huson et al. in the South-East Wales cohort,^{28,29} is in line with expectations.

Approximately 24%–40% of NF1-affected individuals develop spinal neurofibromas,^{35,39,52} but they are most often asymptomatic and not detectable by physical examination. The estimated prevalence of *symptomatic* spinal neurofibromas in the general NF1-affected population is less than 2%.^{7,34,35} In the current study, a high number of individuals with *symptomatic* spinal neurofibromas was reported, compared to the classic NF1-affected cohorts (statistically significant at FDR of 0.01 after B-H correction): 13/127 (10.2%) for all ages and 12/79 (15.2%) for ≥9 years. Kluwe et al. suggested that spinal neurofibromas cause symptoms mainly in older case subjects (mean age 32.8 years),¹⁸ but 4 of 13 symptomatic individuals in our cohort were below age 18 (range: 7–17 years). In 40 individuals who underwent MRI examination, an additional seven case subjects with asymptomatic spinal neurofibromas were found. Among all affected individuals, five belonged to two previously reported multi-generation families (UG-R923 and MAN-R95417G) where the spinal tumors segregated within the family.^{16,21} For two relatives of these probands, the spinal neurofibromas were recognized only after MRI, although the tumor burden was extensive. None of the individuals had >5 CALMs,

including 2/5 who had <6 CALMs and 3/5 had none. This rare form of NF1 is called familial spinal neurofibromatosis (FSNF).

Plexiform and spinal tumors as well as subcutaneous neurofibromas are associated with a severe NF1 phenotype and may result in significant morbidity in children and adults.^{54,55} OPGs, the most common brain tumors in children, are another complication in the general NF1-affected population.⁵⁶ The *overall* prevalence of OPGs in the NF1-affected population is ~11%–20%,^{39,50,57} but only ~30% of these individuals have clinically *symptomatic* OPGs and present with impaired visual acuity, visual field loss, abnormal color vision, squint, proptosis, and/or hypothalamic dysfunction.⁴⁹ Most symptomatic OPGs are diagnosed before age 7 years⁵⁷ with the mean age of 5 years.⁵⁸ In the studied group, symptomatic OPGs were found in 11/104 (10.6%) of individuals ≥5 years, which is more frequent compared with p.Arg1809 and p.Met992del cohorts (none of the individuals had OPGs) and with classic NF1-affected population (3.9%); however, after applying the B-H correction, only the result of comparison with p.Arg1809 cohort and the general NF1-affected population remained statistically significant at FDR of 0.05 (Tables 3 and S10). Furthermore, there was a higher prevalence of asymptomatic OPGs in 16/52 (30.8%) individuals ≥5 years who underwent MRI examination (statistically significant at FDR of 0.01).

Individuals with NF1 are at higher risk to develop specific malignancies compared with the general population, significantly increasing mortality.^{59,60} Besides the high-grade gliomas, the most common malignancies in NF1-affected children are rhabdomyosarcomas, JMML, and neuroblastomas, but accurate estimates on prevalence are not available due to the rarity of these tumors.^{61,62} Based on the data provided by Sung et al. and Crucis et al.,^{63,64} the prevalence of rhabdomyosarcomas in children with NF1 is estimated at 0.4%–0.5%, while Chang and Shannon reported that the individual risk of JMML in NF1 is ~0.04%.⁶⁵ In the studied group, three NF1-affected children younger than 5 years developed embryonal rhabdomyosarcomas, including one individual, now >26 years, who survived both a rhabdomyosarcoma and astrocytoma grade II, diagnosed at the age 2 and 15 years, respectively. Furthermore, one 5-year-old girl (out of 50 children ≤8 years) presented with <6 CALMs and JMML. This girl was heterozygous for two pathogenic *NF1* mutations in the blood, c.2542G>A (p.Gly848Arg) as well as c.1246C>T (p.Arg416*), with p.Gly848Arg being the first hit given the absence of p.Arg416* in buccal swabs, indicating somatic mosaicism for p.Arg416*. A UK population-based hospital admission and death certificate study found that individuals with NF1 have, after excluding the well-established risks of nervous systems tumors, a 2.7-fold increased risk of developing cancers of the esophagus, stomach, colon, liver, lung, bone, thyroid, malignant melanoma, non-Hodgkin lymphoma, chronic myeloid leukemia, breast, and ovary.⁶⁶ In the current study, we

noted recurrent malignant tumors, such as MPNSTs (7/139; 5%) (Tables S1 and S8). Among these individuals, one 44-year-old woman previously described with the missense mutation c.2540T>C (p.Leu847Pro) had MPNST, *BRCA1/2*-negative (MIM: 113705 and 600185) breast cancer as well as a high number of cutaneous neurofibromas (>100).⁶⁷ In addition, one individual developed a medullary thyroid carcinoma and three first-degree relatives of a Belgian proband with c.2540T>C (p.Leu847Pro) died from malignancies (a metastasized colon adenocarcinoma and two MPNSTs, both deceased before age 26). Taken together, the overall prevalence of malignant neoplasms in the studied group was substantially higher than in the published datasets of the general NF1-affected population (significant at FDR of 0.05 after B-H correction; Tables 3 and S10). Furthermore, specifically mutation p.Leu847Pro seems to confer a high predisposition to develop malignant tumors compared to other missense variants reported in this study ($p < 0.0448$; Table S9), although the CADD score of this variant is not the highest among the studied region (only 26.1; Table S2). Given the predominance of the p.Leu847Pro mutations in the studied cohort (70/162 individuals), larger datasets are required to further refine the increased tumor risk associated with the other mutations within the studied region.

Skeletal abnormalities, including long bone dysplasia with or without pseudarthrosis, scoliosis, sphenoid wing dysplasia, bone cysts, including cherubism, non-ossifying fibromas and osseous giant cell lesions, hand anomalies, anterior chest wall anomalies, and short stature, can lead to serious clinical consequences and significant morbidity.⁶⁸ We observed a clear overall increase in the number of skeletal anomalies compared with p.Arg1809 (FDR of 0.05 after B-H correction) and the general NF1-affected population (FDR of 0.01 after B-H correction). As many as 33.3% of the NF1-affected individuals (48/144) presented with one or more osseous lesion, scoliosis ($n = 27$), and pectus anomalies ($n = 10$) being most frequent (18.8% and 6.9%, respectively). The overall frequency would be higher if individuals with short stature (40.3%; 58/144) are included. Rarely reported complications possibly associated with *NF1* status included cherubism, chronic arthritis of multiple joints with elbow contractures, clinodactyly of the 3th–5th toes, postaxial polydactyly, and ulnar aplasia, likely representing the severe end of ulnar pseudarthrosis with bone resorption and absence of the ulnar bone. Interestingly, the latter has been reported only in two NF1-affected case subjects.⁶⁹ Mild to moderate scoliosis was reported in only 18% of *NF1*-positive individuals with bilateral neurofibromas of all spinal roots;¹⁷ however, in our study we observed co-occurrence of scoliosis and spinal tumors in 45% (9/20) of individuals with confirmed symptomatic or asymptomatic spinal neurofibromas (not necessarily affecting all dorsal roots) (Table S6). An additional 11 individuals had scoliosis without evidence of spinal neurofibromas by MRI (Table S1).

Cohorts of individuals with *NF1* missense mutations affecting codons 844–848 and classic NF1-affected population shared a similar frequency for short stature and macrocephaly. Noonan syndrome features were rarely observed in the studied group compared with the p.Arg1809 cohort (significant at FDR of 0.01 after B-H correction). In line with previous studies,^{7,34,39,70} intellectual disability, developmental delay, and/or learning difficulties were frequently observed in the current study (40.6%).

Among the 129 unrelated probands with a missense mutation affecting codons 844–848, p.Leu847Pro and p.Gly848Arg are the most recurrent variants, found in 58 and 14 unrelated individuals, respectively (Table S2 and Figure 1). Both alterations are associated with a severe NF1 phenotype, including a high prevalence of plexiform neurofibromas and skeletal abnormalities, compared to the general NF1-affected population. However, missense mutations at p.Gly848 predispose with a greater frequency to symptomatic or asymptomatic spinal tumors, which were found in ~70% of probands carrying the p.Gly848Arg or p.Gly848Glu mutations (9/13 ≥ 9 years, but in 9/10 ≥ 9 years who received MRI screening), which is slightly higher than in individuals presenting with a severe phenotype caused by a total *NF1* deletion (8/13 ≥ 9 years).⁷¹ Several of the severely affected individuals with a missense mutation at p.Gly848 had only few or no pigmentary skin findings. So far, ~100 case subjects have been reported with the true “spinal NF” phenotype¹⁷ and these individuals more frequently carry a splice site or missense mutation spread over the entire *NF1* coding region.^{18–20} So far, no single mutation has been correlated with this severe clinical presentation. We provide the specific genotype-phenotype association between a particular *NF1* mutation and the spinal phenotype. Individuals with missense mutations at p.Gly848 appear to constitute a distinct group of NF1-affected individuals with a high prevalence of symptomatic spinal neurofibromas and a clear decrease of pigmentary manifestations (CALMs and skinfold freckles) as well as cutaneous neurofibromas (Tables 2 and S9). Because of the limited number of individuals ≥ 9 years old with the missense mutations at codons 844–846, it is still difficult to establish a genotype-phenotype correlation among these cohorts; however, so far these variants also seem to be associated with a severe phenotype, including a high prevalence of plexiform neurofibromas in the p.Cys845 and p.Ala846 cohorts (57.1% and 66.7%, respectively) and OPGs in p.Leu844 cohort (~24% for both symptomatic and asymptomatic OPGs in ≥ 5 years). At this moment, it cannot be excluded that two specific genotype-phenotype correlations exist within this small region of *NF1* with the *NF1* codon 847 associated with an increased risk for malignant neoplasia and the *NF1* codon 848 associated with a high prevalence of symptomatic spinal neurofibromas. The current study, however, intended to show that the whole region of 844–848 codons stood out due to its high frequency of

variants compared with the neighboring codons, indicating functional importance. In addition, the cluster of missense mutations here described, although located outside the GRD important for RAS regulation, is clearly associated with a severe phenotype, not reported so far in literature. As the current study necessarily still underestimates the internal tumor burden, as systematic whole-body imaging was not performed, close clinical management seems warranted for individuals presenting with a missense variant affecting aa 844–848.

As NF1 is known for its variable expressivity and age dependency, it is challenging to establish genotype-phenotype correlations. Although we performed a comparative analysis on a large well-described cohort using a standardized phenotypic data collection form, one limitation of the study is that clinical information was collected by physicians from different referral centers, although all were NF1 specialists. Data in this and the previously reported p.Arg1809 cohort were “double-checked” through verification of the originally submitted phenotypic checklist forms and subsequent update of the clinical notes, so data should be highly accurate.

Clinical variability, both inter- and intrafamilial, has been widely reported in the past two decades.^{72–74} Although significant progress has been made over the last 20 years, the mechanisms underlying this phenotypic heterogeneity only gradually start to be unraveled. The factors contributing to the phenotypic variability include (1) age dependency of some of the NF1 features,^{29,75,76} (2) timing, cell of origin, and number of second hits in specific cells, resulting in presence and number of CALMs, freckling, tibial dysplasia, neurofibromas, and other tumors,⁷⁷ (3) post-zygotic mosaicism for the first NF1 hit in mosaic individuals,⁷⁷ (4) the enormous NF1 allelic heterogeneity,⁷⁸ (5) occasional presence of two different NF1 pathogenic variants segregating within a family (see MAD-R9.232; Table S1 and Figure S5) or the occurrence of two independent mutations, one in NF1 and the other in a different gene, within an individual (see UAB-R624 with the NF1/PTPN11 mutations and UF-R1 with the NF1/KIT mutations; Table S1), (6) modifying genes,⁷⁹ and (7) environmental factors (e.g., number of pregnancies).⁸⁰ To date, two studies have identified potential modifying genes, unlinked to the NF1 locus, associated with the severity of NF1 presentation.^{81,82} Pasmant et al. demonstrated that a high number of plexiform neurofibromas has been significantly associated with allele T of SNP rs2151280 of ANRIL (MIM: 613149).⁸¹ Pemov et al. reported a correlation of two common SNPs (rs4660761 and rs7161), located between DPH2 (MIM: 603456) and ATP6VOB (MIM: 603717), as well as of SNP rs1800934 in MSH6 (MIM: 600678) with the number of CALMs.⁸² Further studies are needed to confirm these findings.

Missense mutations affecting NF1 codons 844–848 described in this study are clearly pathogenic and individuals with these missense mutations have a statistically

higher risk of developing spinal neurofibromas, plexiform neurofibromas, and OPGs. Functional studies in mutant mice harboring the missense mutation c.2542G>C (p.Gly848Arg) did not recapitulate this human phenotype, as neither optic pathway gliomas nor plexiform neurofibromas developed.^{22,23} Western blot analysis showed that c.2542G>C (p.Gly848Arg) resulted in 38%–50% reduction of neurofibromin levels.^{22,23} These mutations reside outside the GRD (amino acids 1,217–1,511), known to have tumor-suppressor activity through downregulation of members of the Ras family of small GTP-binding proteins. Although NF1 was cloned in 1990, the cellular functions performed by this huge 2,818-amino acid multi-domain protein are still incompletely understood. The cluster of recurrent missense mutations involving aa 844–848 described in the current study are located within the CSRD (amino acids 543–909), located N-terminal to the GRD. The CSRD domain, originally described by Fahsold et al.,⁸³ is likely functionally important, which is further implied by the presence of multiple missense variants in this segment of the gene in NF1-affected individuals. The 3D structure of this region has not been resolved and its precise functions and interactors have not been described. Ras GAP activity is enhanced through phosphorylation by Protein Kinase C α of serine and threonine residues within this domain.⁸⁴ Based on the 2D modeling of the CSRD using PredictProtein server,⁸⁵ the region 831–847 might form the C-part of a helix and be buried in the protein. Missense mutations affecting codons 844–848, especially those substituting smaller hydrophobic amino acids to large ones, may result in breaking of the helix and exposure of the buried protein domain, consequently affecting the function of the protein. No functional studies confirming the aforementioned bioinformatics analysis have been performed, however. In any case, missense mutations in this region seem to act through a loss-of-function mechanism and not gain-of-function or dominant-negative, at least in melanocytes and JMML. Indeed, the c.2540T>C (p.Leu847Pro) was observed as a “second hit” in one CALM, biopsied from a 13.5-year-old girl with >5 CALMs and skinfold freckling carrying the NF1 constitutional mutation c.5547–1G>A (Table S11), confirming that two hits are required to cause a phenotypic effect. Additionally, we reported a 5-year-old girl with JMML (UAB-R9493; Table S1) who carried two pathogenic NF1 mutations in the blood: c.2542G>A (p.Gly848Arg) as a “first hit” mutation and c.1246C>T (p.Arg416*) as a “second hit.” There is a need to improve our understanding of the physiological functions of neurofibromin and to determine how each domain regulates the function of this protein.

Six amino acids in the region aa 804–950 are evolutionarily conserved down to yeast (IRA1 and IRA2), Leu844, Gly849, Leu852, Glu924, Leu933, and Phe934 (Figure S6) and would therefore be expected to be of particular functional importance.⁸⁶ Only Leu844 and Leu933 have, however, been observed in NF1-affected

individuals to predispose to recurrent missense mutations (HGMD, LOVD, ClinVar, and our cohort). The tumorigenic potential of aa 844 is further highlighted by identification of somatic mutations in the COSMIC database: one glioma with c.2531T>C (p.Leu844Pro), one glioma and four malignant melanomas with c.2530C>T (p.Leu844Phe).

Palindromic structures belong to the non-B DNA structures and are often the site of replication errors resulting in substitutions.⁸⁷ The *NF1* missense mutation hotspot (aa 844–848) is located in the highly conserved amino acid region, suggesting that it is functionally important. The genomic sequence encoding the human *NF1* aa 845–853 is a part of two palindromic structures (Figure S7); therefore the high rate of recurrent missense mutations affecting Leu847 and Gly848 may partially be due these being both located in the loop of the palindrome. In *NF1* exon 21 [16], other palindromic nucleotide sequences, specifying the amino acid residues aa 828–832, aa 865–868, aa 908–911, and aa 933–937 are observed, resulting in four additional stem-loop structures. However, these structures do not predispose to recurrent missense mutations as none were found either in the UAB, HGMD, or LOVD cohort, except for c.2798T>C (p.Leu933Pro), whose location does not include the loop of the palindrome. The complex interplay between functional significance and genomic architecture needs to be considered when analyzing the recurrence of mutations.

Although only a few clear genotype-phenotype correlations have been so far reported,^{11–14} the data presented here show that additional clinically relevant *NF1* genotype-phenotype correlations exist. A renewed interest in such studies is needed to come to a timely unfolding of additional correlations, as so far only the surface has been scratched. This will require close collaboration between NF1 clinicians and molecular geneticists. The lack of discovery of more specific genotype-phenotype correlations may be partly due to the methodological approach, including lumping mutations in large categories (truncating versus microdeletion, splice, missense mutations).^{88,89} Identification of *mutation-specific* genotype-phenotype correlations depends on the dataset size with a large number of individuals, preferentially postpubertal, carrying the *same* non-truncating constitutional mutation, with the associated phenotype recorded in a standardized way. As there are only a limited number of truly recurrent non-truncating mutations, prioritization on individuals carrying such recurrent mutations is indicated. Although each of the recurrent mutation affects only a small percentage of *NF1*-affected individuals (3%–8% with the microdeletion type I, ~0.8% with p.Met992del, ~1.2% with the p.Arg1809 missense mutation, and ~0.8% for the cluster of missense mutations affecting codons 844–848), together they may affect counseling and surveillance in a significant fraction of the *NF1*-affected population.

In conclusion, the present findings indicate that missense mutations affecting one of five neighboring codons 844–848 located outside the GAP-related domain are an important risk factor for a severe phenotype in *NF1*-affected individuals. We report that these individuals have a high prevalence of plexiform and/or spinal neurofibromas, symptomatic and asymptomatic OPGs, malignant neoplasms, and skeletal abnormalities. A severe phenotype was observed in 75% of adult *NF1*-affected individuals with these mutations, clearly demonstrating that missense mutations outside the GRD can be associated with a severe clinical presentation. The current study identified a genotype-phenotype correlation in this region that may be valuable in the management and genetic counseling of a significant number of *NF1*-affected individuals. These data suggest that there is a potential need for increased disease surveillance in individuals with these mutations enabling genotype-driven personalized medicine.

Supplemental Data

Supplemental Data include 7 figures and 11 tables and can be found with this article online at <https://doi.org/10.1016/j.ajhg.2017.12.001>.

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Web Resources

1000 Genomes, <http://www.internationalgenome.org/>
CADD, <http://cadd.gs.washington.edu/>
ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>
Clustal, <http://www.clustal.org/clustal2/>
COSMIC, <http://cancer.sanger.ac.uk/cosmic>
dbSNP, <https://www.ncbi.nlm.nih.gov/projects/SNP/>
GenBank, <https://www.ncbi.nlm.nih.gov/genbank/>
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Supplemental Data

Genotype-Phenotype Correlation in NF1: Evidence for a More Severe Phenotype Associated with Missense Mutations Affecting *NF1* Codons 844–848

Magdalena Koczkowska, Yunjia Chen, Tom Callens, Alicia Gomes, Angela Sharp, Sherrell Johnson, Meng-Chang Hsiao, Zhenbin Chen, Meena Balasubramanian, Christopher P. Barnett, Troy A. Becker, Shay Ben-Shachar, Debora R. Bertola, Jaishri O. Blakeley, Emma M.M. Burkitt-Wright, Alison Callaway, Melissa Crenshaw, Karin S. Cunha, Mitch Cunningham, Maria D. D'Agostino, Karin Dahan, Alessandro De Luca, Anne Destrée, Radhika Dhamija, Marica Eoli, D. Gareth R. Evans, Patricia Galvin-Parton, Jaya K. George-Abraham, Karen W. Gripp, Jose Guevara-Campos, Neil A. Hanchard, Concepcion Hernández-Chico, LaDonna Immken, Sandra Janssens, Kristi J. Jones, Beth A. Keena, Aaina Kochhar, Jan Liebelt, Arelis Martir-Negron, Maurice J. Mahoney, Isabelle Maystadt, Carey McDougall, Meriel McEntagart, Nancy Mendelsohn, David T. Miller, Geert Mortier, Jenny Morton, John Pappas, Scott R. Plotkin, Dinél Pond, Kenneth Rosenbaum, Karol Rubin, Laura Russell, Lane S. Rutledge, Veronica Saletti, Rhonda Schonberg, Allison Schreiber, Meredith Seidel, Elizabeth Siqveland, David W. Stockton, Eva Trevisson, Nicole J. Ullrich, Meena Upadhyaya, Rick van Minkelen, Helene Verhelst, Margaret R. Wallace, Yoon-Sim Yap, Elaine Zackai, Jonathan Zonana, Vickie Zurcher, Kathleen Claes, Yolanda Martin, Bruce R. Korf, Eric Legius, and Ludwine M. Messiaen

MEDICAL GENOMICS LABORATORY: *NF1/SPRED1* PHENOTYPIC CHECKLIST FORM



Patient ID: _____ Referring Physician: _____ Date of Exam: __/__/__

DEMOGRAPHIC INFORMATION

Gender: Male Female Date of Birth: __/__/__

Ethnicity: Mother: White Black Native American Hispanic Asian Other: White Black Native American Hispanic Asian Other:

DIAGNOSIS

NIH criteria: >6 CAL spots >5mm, postpubertal >15mm Otic glioma
 >2 neurofibromas or 1 plexiform NF >2 Lisch nodules
 Axillary or inguinal freckling A distinct osseous lesion
 First degree relative diagnosed with NF1 by above criteria
 Does patient fulfill NIH diagnostic criteria for NF1? Yes No

Clinical diagnosis: NF1 Multiple CAL spots Familial multiple CAL spots
 Spinal NF Isolated neurofibromas Segmental NF1
 NF Noonan Single NF1 feature Watson Syndrome
 Legius syndrome Unknown

Family history: Sporadic (proband is a "founder") Familial (proband is a "non-founder") Unknown
 Consanguinity: Yes No Unknown

GENERAL INFORMATION

Height: ____ cm Head circumference: ____ cm Weight: ____ kg

NF SIGNS AND SYMPTOMS

1) **CAL spots:** 0 1-5 >6 to 100 >100
 General impression on the borders of the CAL-spots:
 typical well-defined smooth borders diameter: _____
 irregular margins, ragged borders
 for detail on size and location of the CAL-spots and other hyperpigmentation areas: page 3.

2) **Skin fold freckling:** None Left Right Unknown
 Comments (e.g. very faint,....): _____
 Groin Axilla Submammary

3) **Lisch nodules:** None Left Right Unknown

4) **Cutaneous neurofibromas (soft nodules that project above the skin):** histopathologically confirmed: Y / N

0 2-6 6-99 100-500 >500

5) **Intradermal neurofibromas (soft depression within the skin w/ pinkish overlying discoloration):** histopathologically confirmed: Y / N

0 2-6 6-99 100-500 >500

6) **Subdermal neurofibromas (firm nodules palpable underneath the skin):** histopathologically confirmed: Y / N

0 2-6 6-99 100-500 >500

MEDICAL GENOMICS LABORATORY: *NF1/SPRED1* PHENOTYPIC CHECKLIST FORM



7) **Plexiform neurofibromas:** histopathologically confirmed: Y / N

None visible from outside with hyperpigmentation
 internal without hyperpigmentation

Head Neck Trunk L Arm L Hand L Leg L Foot
 Abdomen Pelvis Genital area R Arm R Hand R Leg R Foot

8) **Spinal neurofibromas (neurofibromas arising from the spinal nerve root):** histopathologically confirmed: Y / N

Unknown Absent by MRI Present, asymptomatic
 Present, symptomatic

If present: please provide detail on figure page 4
 unilateral or bilateral;
 C: ____ T: ____ L: ____ S: ____ regions.

9) **Optic glioma:**

Unknown Absent by MRI Familial multiple CAL spots
 Present by MRI, asymptomatic Present by MRI, symptomatic
 Nerve (L and/or R) Chiasm
 Present by MRI, asymptomatic Nerve (L and/or R) Chiasm

10) **Other neoplasms:**

None Hypothalamic glioma Brainstem glioma Other glioma
 MPNST JNML Rhabdomyosarcoma
 Pheochromocytoma Colonic polyps Lipoma
 Juvenile xanthogranuloma Breast cancer
 schwannoma Other, specify: _____

11) **Skeletal Abnormalities:**

None Long bone dysplasia Pseudoarthrosis Sphenoid wing dysplasia
 Bone cysts scoliosis Dysplastic vertebrae
 pectus excavatum pectus carinatum Other: _____

12) **Cardiovascular disease:**

Absent Unknown Present:
 Hypertension Pulmonic stenosis Aortic stenosis
 Renal artery stenosis Moya moya ASD
 VSD Other: _____

13) **Development:**

Normal Exam not done
 Abnormal ADD Hyperactivity Learning disability
 speech delay autism pervasive developmental delay
 IQ: Full scale: _____ Verbal: _____ Performance: _____

14) **Education:**

Too young for school At or above age level Below age level Unknown
 HS completion College graduate Higher degree

MEDICAL GENOMICS LABORATORY: *NF1/SPRED1* PHENOTYPIC CHECKLIST FORM

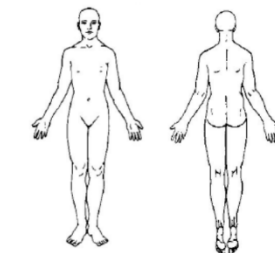


15) **Noonan phenotype:**

Absent Possible Unknown
 Present: Short stature Low set ears Pulmonic Stenosis
 Hypertelorism Webbed neck peak jet velocity ____ m/s
 Midface hypoplasia peak gradient ____ mmHg across pulmonic valve
 PTPN11 testing done? Result: _____

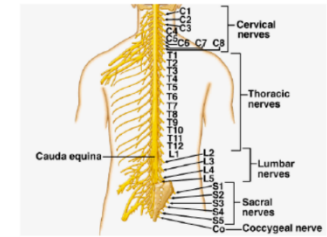
16) **Segmental NF phenotype:** Absent Possible

location/size of pigmentary lesions and/or neurofibromas



Indicate size and location of
 Neurofibromas
 CAL-spots
 Freckling
 Hyperpigmented region

17) **location of spinal tumors (if present)**



18) **any other comments/remarks:**

Figure S1. Standardized phenotypic checklist used to document the phenotype of the individuals included in this study.





Figure S2. The spectrum of missense mutations in the exon 21 [16] of *NF1* (codons 804-950): a comparison of variants deposited in the publicly available disease databases (LOVD, ClinVar and HGMD, as of 05/09/2017) and variants identified in ~8400 unrelated individuals from UAB cohort.




All individuals identified in UAB cohort had been analyzed using comprehensive *NF1* mutation analysis using a core RNA-based approach and MLPA as previously described.^{1,2} The variants reported in UAB cohort were classified according to the ACMG recommendations.³ The classifications of the variants deposited in the publicly available databases (LOVD, ClinVar and HGMD) are shown here as reported by the original author(s). Additionally, the evidence provided in the original publications for those variants deposited in the HGMD database were reviewed and re-classified, if needed, based on currently accepted criteria for pathogenicity.³

HGMD re-verification:

c.2514C>G (p.Ile838Met) - reported in 1/91 unrelated *NF1* individuals fulfilling the NIH diagnostic criteria, also carrying the second *NF1* variant c.2531T>C (p.Leu844Pro); no phenotypic details available; no evidence for pathogenicity provided (Mattocks et al.⁴); **c.2509T>C (p.Trp837Arg)**, **c.2585C>G (p.Thr862Ser)**, **c.2606C>T (p.Pro869Leu)**, **c.2798T>A (p.Leu933Gln)** - absence in normal controls, segregation studies, proven *de novo* with paternity confirmation, *in silico* analysis (van Minkelen et al.⁵); **c.2509T>G (p.Trp837Gly)**, **c.2617C>G (p.Arg873Gly)**, **c.2638G>A (p.Val880Met)**, **c.2684T>G (p.Met895Arg)** - no evidence for pathogenicity provided by the original author(s) (Sabbagh et al.⁶ and Pasmant et al.⁷); **c.2693T>C (p.Leu898Pro)** - reported once in sporadic case of 465 unrelated *NF1* individuals; no phenotypic details available; no evidence for pathogenicity provided (Maynard et al.⁸); **c.2759T>C (p.Leu920Pro)** - reported once in sporadic case of 17 unrelated *NF1* individuals; 2 CALMs and spinal neurofibromas; no evidence for pathogenicity provided (Kluwe et al.⁹); **c.2617C>T (p.Arg873Cys)**, **c.2663A>G (p.Asp888Gly)** - both variants reported in the same Sicilian individual; the same person also carrying two *NF1* frameshift alterations: c.2480delA, c.2632delC and other missense and synonymous variants (Bongiorno et al.¹⁰); **c.2764G>A (p.Gly922Ser)** - RNA-based approach analysis demonstrated that variant p.Gly922Ser results in cryptic 5' splice site/ 2761del90 (Ars et al.¹¹); **c.2786T>C (p.Leu929Pro)** - reported once, absent in >1000 normal chromosomes, no further evidence for pathogenicity provided (Ribeiro et al.¹²).

Each number in the circle corresponds with the total number of probands with a specific variant. The dotted black line indicates the region studied in this report (codons 844-848). For graph presenting the variants deposited in the HGMD database, right panel shows the variants' classifications originally reported by the author(s), while left panel indicates the classifications based on our re-verification and following the ACMG recommendations.³ Different colors correspond with the pathogenicity score according to the legend presented below ("exonic splice variants" are not *true* missense mutations; these variants cause missplicing as observed by RNA-based analysis in blood). The phenotypes of individuals from UAB cohort carrying a particular alteration are presented below in the table. The figure was prepared using the ProteinPaint application.¹³

-  Pathogenic
-  Likely pathogenic
-  Variant of uncertain significance
-  Likely benign
-  Benign

-  Clinical significance not reported
-  Lack of evidence for pathogenicity
-  Exonic splice variants

Variant	Phenotype	Classification
c.2410G>A (p.Ala804Thr)	30-yo Japanese individual with >5 CALMs, freckling, 6-99 cutaneous and 6-99 intradermal nf	VUS
c.2441A>G (p.Lys814Arg)	6-yo Asian individual with >5 CALMs and freckling, also carrying <i>NF1</i> c.1338delA (p.Leu447Phefs*26); p.Lys814Arg ALSO present in the unaffected mother	Likely benign
c.2486C>T (p.Ser829Phe)	16-yo Native American proband with right axillary freckling, abdominal internal nf, also carrying <i>NF1</i> c.2894T>G (p.Ile965Arg); p.Ser829Phe ALSO present in the unaffected mother, whereas p.Ile965Arg was <i>de novo</i>	Likely benign
c.2509T>C (p.Trp837Arg)	24-yo individual with >5 CALMs and freckling	VUS
c.2509T>G (p.Trp837Gly)	>5 CALMs and freckling (all individuals, from 3-yo to 20-yo); other complications: abnormal development and asymptomatic OPG (3-yo) and plexiform nf (20-yo)	Pathogenic
c.2521A>C (p.Thr841Pro)	>5 CALMs and freckling (3-yo); <i>de novo</i> but no identity test done	VUS
c.2570A>G (p.Asn857Ser)	2.5-yo individual with >5 CALMs; p.Asn857Ser ALSO present in the unaffected mother	Likely benign
c.2573C>G (p.Ser858Cys)	>5 CALMs and freckling, 2-6 cutaneous and 2-6 intradermal nf, long bone dysplasia (31-yo); presence of second nonsense variant c.4108C>T (p.Gln1370*)	Likely benign
c.2585C>G (p.Thr862Ser)	5-yo proband with 1-5 CALMs and pre B-cell acute lymphoblastic leukemia; p.Thr862Ser ALSO present in affected 33-yo mother with 4-5 CALMs, bilateral Lisch nodules and plexiform nf on the leg	Pathogenic
c.2643G>A (p.Met881Ile)	2-yo proband with 1-5 CALMs and freckling; variant ALSO found in 32-yo father with 3 CALMs	VUS
c.2681T>C (p.Phe894Ser)	10-yo proband with >5 CALMs and freckling; variant ALSO found in 12-yo sibling with >5 CALMs and freckling	VUS
c.2693T>C (p.Leu898Pro)	>5 CALMs and freckling (all individuals, from 1-yo to 38-yo); 2 individuals >26-yo have numerous cutaneous and subcutaneous nf and plexiform nf; proven <i>de novo</i> but no identity test done; p.Leu898Pro ALSO found in father and son of the one individual (son with OPG)	Pathogenic
c.2728G>C (p.Gly910Arg)	only 3-4 CALMs and abnormal development; the presence of second missense variant c.3632T>G (p.Leu1211Arg); familial history, but ONLY variant p.Leu1211Arg was identified in the individual's father and paternal grandfather with the NF1 features; p.Gly910Arg ALSO present in the unaffected mother	Likely benign
c.2740C>T (p.Arg914Trp)	both individuals with >5 CALMs; individual with abnormal development and pulmonic stenosis has the presence of the second variant (<i>NF1</i> deletion exons 2-3)	Likely benign
c.2747A>G (p.Asn916Ser)	10-mo individual with 1-5 CALMs, brainstem glioma and pectus excavatum (sporadic); 12-yo individual only with >5 CALMs and variant ALSO present in the individual's father with <6 CALMs and in 5-yo brother with >5 CALMs and freckling	Likely pathogenic
c.2750T>A (p.Val917Asp)	only >5 CALMs; the individual's mother has the NF1 features, but she had not been tested	VUS
c.2786T>C (p.Leu929Pro)	7-mo individual with >6 CALMs and freckles, proven <i>de novo</i> ; for other individuals no data provided	VUS
c.2794A>G (p.Met932Val)	4-mo Asian individual with only >5 CALMs	VUS
c.2798T>C (p.Leu933Pro)	>5 CALMs and freckles; Lisch nodules (in 2/6 individuals), cutaneous (2/6) and intradermal (2/6) nf, plexiform (1/6) and spinal (1/6) nf, abnormal development (3/6); others: juvenile pilocytic astrocytoma, brainstem hamartoma and pseudarthrosis	Pathogenic

yo - years old; mo - months old; nf - neurofibromas; OPG - optic pathway glioma; VUS - variant of uncertain significance

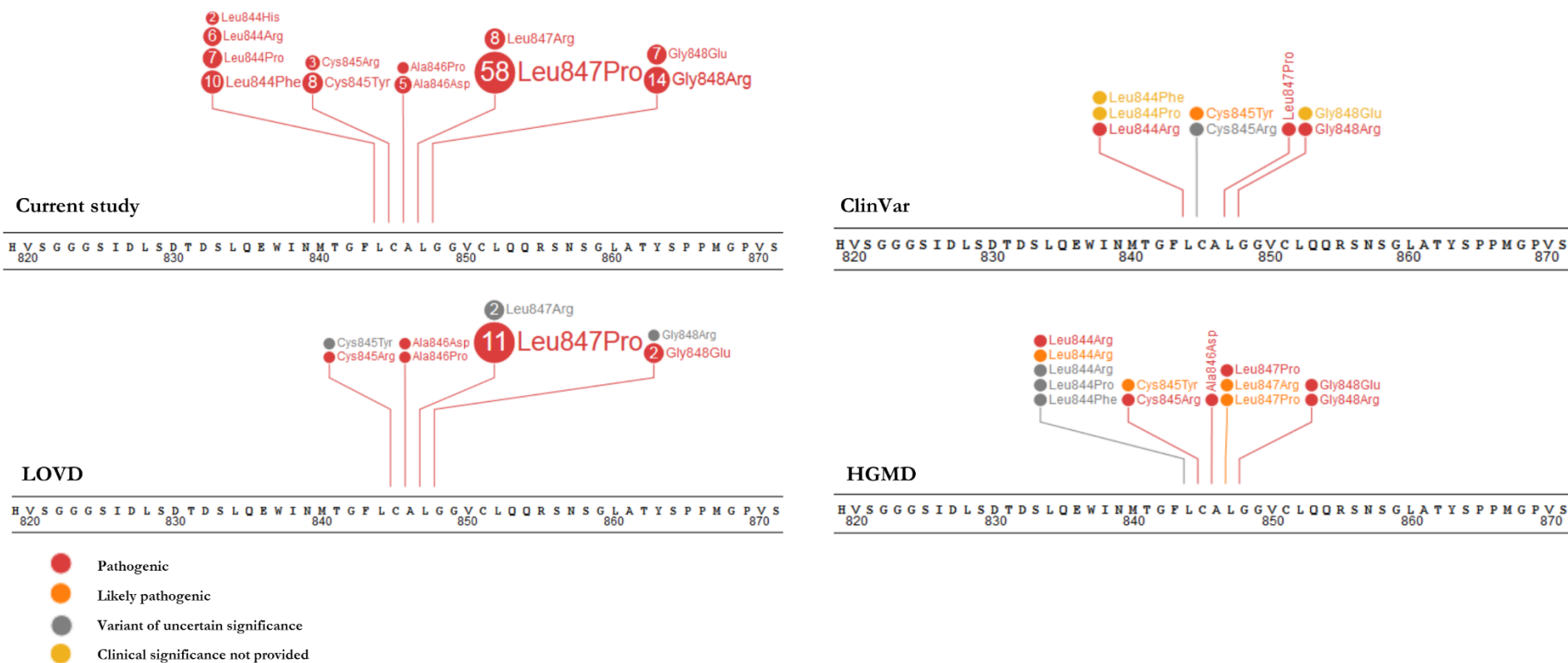


Figure S3. The classification of *NFI* missense mutations affecting codons 844-848: a comparison of the classification in the publicly available disease databases (LOVD, ClinVar and HGMD, as of 05/09/2017) with the current study, classifying variants according to the ACMG recommendations (details in Tables S2-S4).

The classifications of the variants deposited in the publicly available LOVD and ClinVar databases are shown here as reported by the original author(s). For variants deposited in the HGMD database, the originally reported variants' classifications were reviewed and if necessary re-classified according to the ACMG guidelines³ based on the evidence for pathogenicity provided in the published papers by the authors (details in Table S3). Each number in the circle corresponds with the total number of probands with a specific variant. Different colors correspond with the pathogenicity score. The figure was prepared using the ProteinPaint application.¹³

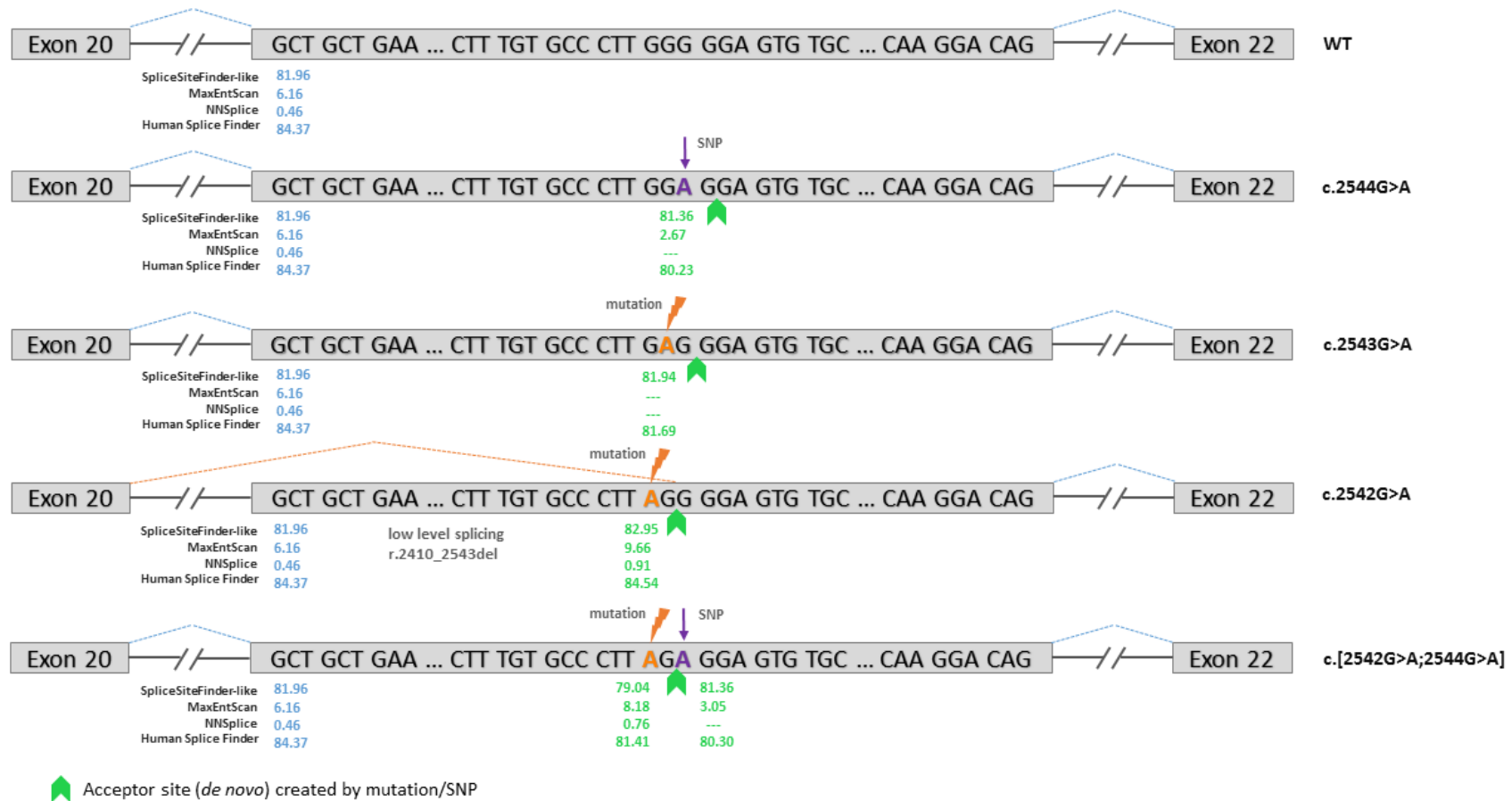


Figure S4. *In silico* prediction and *in vivo* analysis of the effect of the *NF1* c.2542G>A (p.Gly848Arg) and c.2543G>A (p.Gly848Glu) missense mutations as well as the single nucleotide polymorphism c.2544G>A (p.Gly848=) on splicing in short-term cultured blood lymphocytes.

Based on the results of *in silico* analysis (SpliceSiteFinder-like, MaxEntScan, NNSplice and Human Splice Finder embedded in Alamut visual software v.2.9.0), a novel splice acceptor site is created by the presence of *NF1* mutations (c.2542G>A and c.2543G>A) as well as by the single nucleotide polymorphism (SNP) at c.2544G>A (rs17883704). *In vivo* transcript analysis and sequencing revealed a minor effect on splicing ONLY for c.2542G>A, resulting in low level of r.2410_2543del transcript. No missplicing effect was observed in individuals carrying the *NF1* mutation c.2542G>A and SNP; both variants proven to reside in cis through next-generation sequencing.

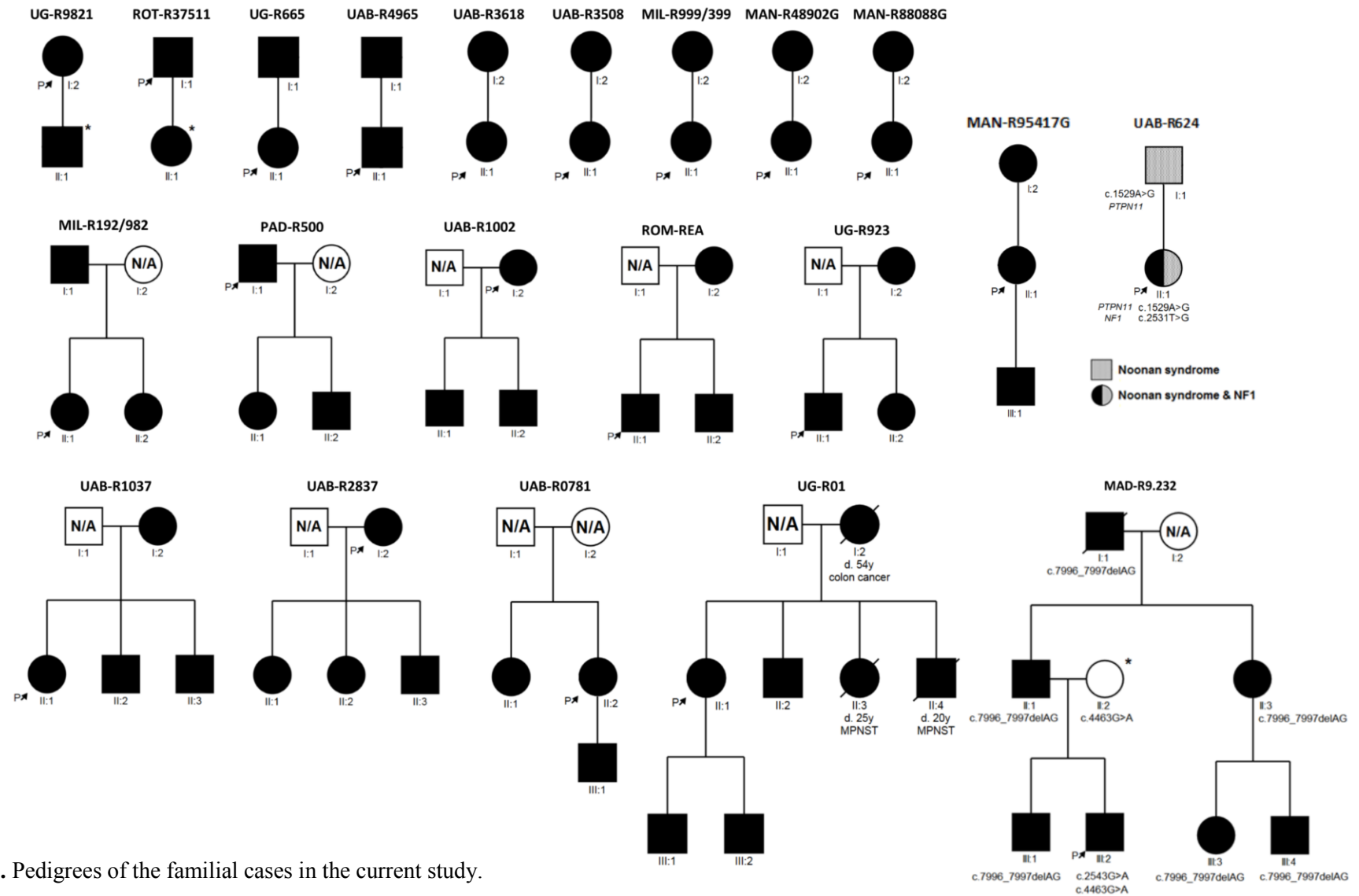


Figure S5. Pedigrees of the familial cases in the current study.

The filled black symbols represent the individuals who are positive for an *NF1* missense mutation affecting codons 844-848, while the open symbols represent relatives who are negative for the family specific mutations. The arrow indicates the proband of the family. For the first generation, father is always I:1 and mother is I:2.

Abbreviations: N/A - the relatives not available for genetic analysis; * - no clinical information available for these individuals.

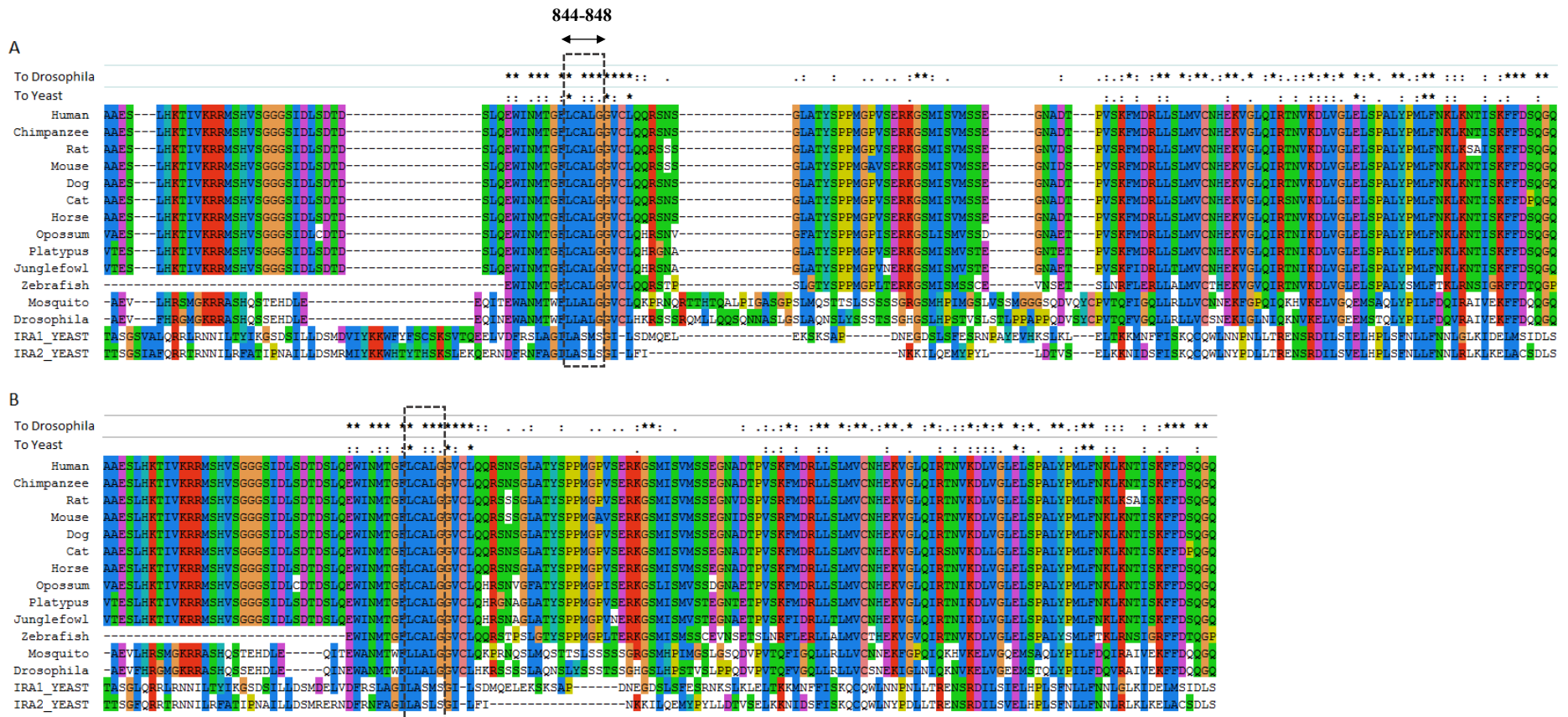


Figure S6. Multiple sequence alignment of the exon 21 [16] of *NFI* (A) and multiple sequence alignment of the same region adjusted to the human *NFI* without gaps (B).

Human (RefSeq: NP_000258.1 residues 804-950), Chimpanzee (TrEMBL: K7DMZ3 residues 804-950), Rat (SWISSPROT: P97526 residues 806-952), Mouse (SWISSPROT: Q04690-2 residues 806-952), Dog (RefSeq: XP_013971967.1 residues 799-945), Cat (RefSeq: XP_011287367.1 residues 807-953), Horse (TrEMBL: F6XRM7 residues 739-885), Opossum (TrEMBL: F6ZHU9 residues 564-710), Platypus (RefSeq: XP_007664859.1 residues 840-986), Junglefowl (RefSeq: XP_003642464.1 residues 799-945), Zebrafish (TrEMBL: E7FBD0 residues 751-865), Mosquito (TrEMBL: B0WYP5 residues 771-936), Drosophila (TrEMBL: O01397 residues 804-969), yeast IRA1 (SWISSPROT: IRA1_YEAST residues 1253-1419) and IRA2 (SWISSPROT: IRA2_YEAST residues 1263-1411) were included in the alignment. The multiple sequence alignment was performed using *Clustal 2.0.12* software with manually adjustment. The black dotted lines on the panels present the region 844-848. An asterisk (*), a colon (:), and a period (.) indicate the following positions: (*) a single fully conserved residue, (:) conservation between groups of strongly similar properties and (.) conservation between groups of weakly similar properties.

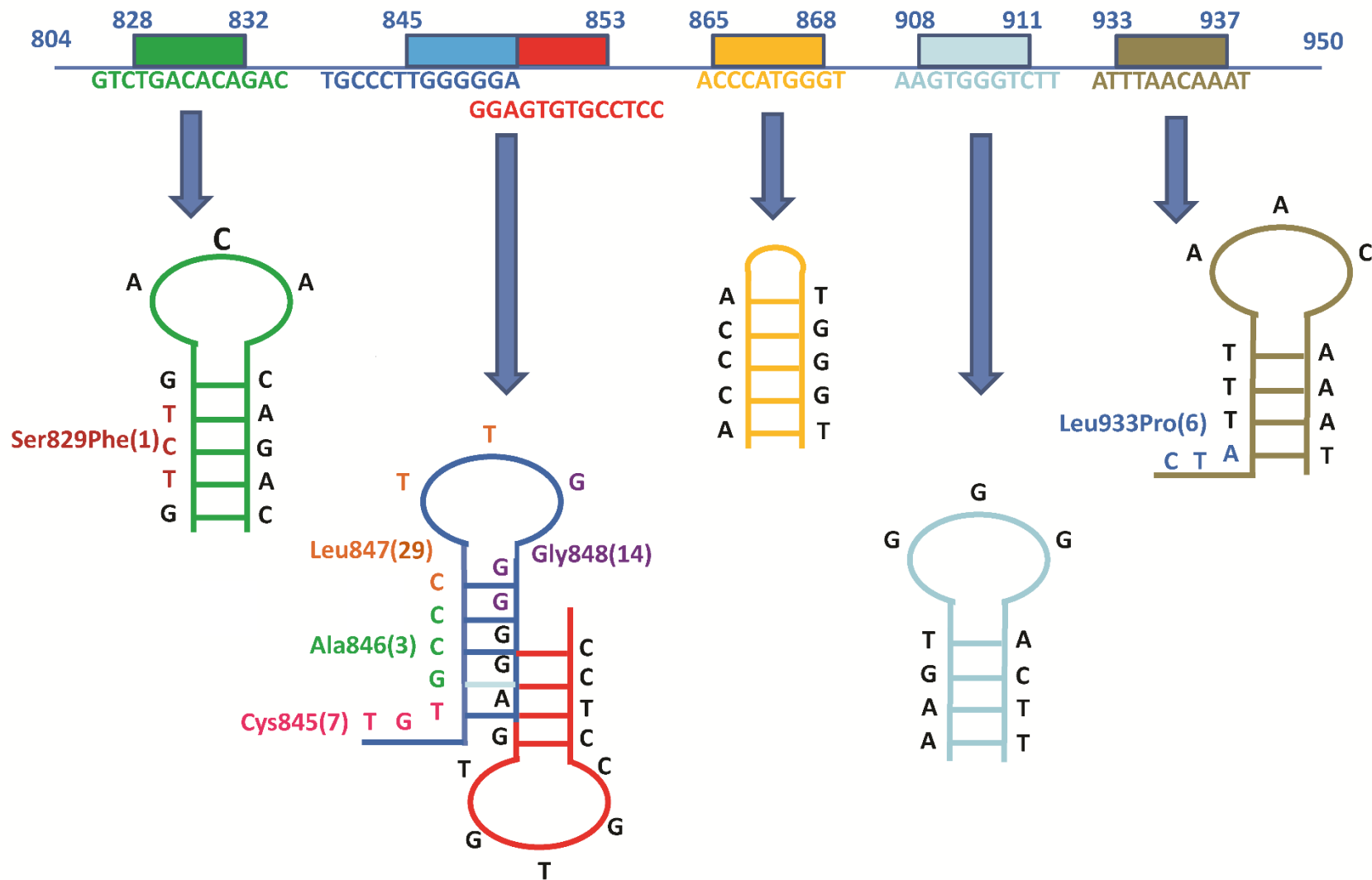


Figure S7. Schematic of five palindromic structures in the *NF1* codons 804-950.

Missense mutations with a total number of samples identified in the UAB cohort were annotated next to the palindromic structures with each color corresponding to the variant motif. Particularly, two palindromic sequences TGCCCTTGGGGGA (AA845-849) and GGAGTGTGCCTCC (AA849-853) may form a unique non-B DNA structure contributing recurrent missense mutations.

Table S1. Clinical details for 162 individuals with an *NF1* missense mutation affecting one of five codons 844-848 from 129 different families.

Table S1 is included as a separate Excel file.

^A For individuals with one asterisk (*) the standardized phenotypic checklist forms were not updated by the referring physicians, the data are based on the originally submitted forms; individuals with two asterisks (**) had incomplete phenotypic checklist forms; individuals with three asterisks (***) were the newborn infants without the NF1 features at birth or without the clinical data provided.

^B **UAB:** University of **A**labama at **B**irmingham (67 probands and 11 relatives); **BRA:** Fluminense Federal University, Niterói, **B**razil (one proband); **CAR:** i/ Division of Cancer and Genetics, Cardiff University, **C**ardiff, UK; ii/ Birmingham Women's and Children's NHS Foundation Trust, Birmingham, UK; iii/ Wessex Regional Genetics Laboratory, Salisbury NHS Foundation Trust, Salisbury, UK; iv/ St George's University Hospitals NHS Foundation Trust, London, UK; v/ Division of Medical Oncology, National Cancer Centre, Singapore (5 probands); **MAD:** Hospital Universitario Ramón y Cajal, Institute of Health Research (IRYCIS) and Center for Biomedical Research-Network for Rare Diseases (CIBERER), **M**adrid, Spain (6 probands); **MAN:** University of **M**anchester, UK (4 probands and 4 relatives); **MIL:** Carlo Besta Neurological Institute, **M**ilan, Italy (6 probands and 3 relatives); **PAD:** University of **P**adova, Italy (5 probands and 2 relatives); **SGR:** Molecular Genetics Unit, Casa Sollievo della Sofferenza Hospital, IRCCS, **S**an **G**iovanni **R**otondo, Italy (4 probands and 2 relatives); **ROT:** Erasmus Medical Center, **R**otterdam, the Netherlands (13 probands); **SHE:** Sheffield Children's NHS Foundation Trust, **S**heffield, UK (one proband); **UL:** University of **L**euven, Leuven, Belgium (one proband); **UF:** University of **F**lorida College of Medicine, Gainesville, Florida, USA (one proband); **UG:** University Hospital, **G**hent, Belgium (15 probands and 11 relatives).

^C **F:** familial; **PrS:** proven sporadic; **RS:** reportedly sporadic; **NS:** not specified

^D Exact age was used to calculate Height and Head Circumferences, but provided as age-groups in Table S1: 0-24 months; 2-4 years; 5-8 years; 9-13 years; 14-18 years; 19-26 years; >26 years.

^E **F:** female; **M:** male

^F **W:** White; **His:** Hispanic; **AA:** African American; **As:** Asian; **NaA:** Native American; **Pa:** Pakistani; **Fil:** Filipino; **A:** African; **Jew:** Jewish

^G An individual was classified as having „NF-Noonan” if at least two of the following features were present: short stature (SS), low set ears (LSE), hypertelorism (HTL), midface hypoplasia (MH), webbed neck (WN) or pulmonic stenosis (PS).

^H Height percentiles for Hispanic and Asian individuals were provided in square brackets to indicate that they were excluded from the data analysis on frequency of short or normal stature due to the lack of ethnic-specific growth charts.

^I Individuals with ADD/ADHD, but normal development were still classified as normal.

Abbreviations:

NS: not specified; **bil:** bilateral; **gr:** groin; **ax:** axillary; **OPG:** optic pathway glioma; **N, UN:** no symptomatic OPG or symptomatic spinal neurofibroma, unknown if any asymptomatic OPG or asymptomatic spinal neurofibromas are present; **N, MRI:** no symptomatic and asymptomatic OPG or spinal neurofibroma detected by **MRI**; **Y, MRI:** yes, presence of OPG or spinal neurofibroma confirmed by **MRI**; **ASD:** atrial septal defect; **LD:** learning disability; **HTL:** hypertelorism; **MH:** midface hypoplasia; **SS:** short stature; **PT:** ptosis; **PS:** pulmonic stenosis; **DPF:** downslanting palpebral fissures; **LSE:** low set ears; **ADD:** attention deficit disorder; **ADHD:** attention deficit hyperactivity disorder; **SD:** speech delay; **F:** father; **M:** mother; **S:** sibling; **C:** child.












































Table S2. *In silico* prediction of pathogenicity and *in vivo* splicing analysis of *NF1* missense mutations affecting codons 844-848 in the studied group.


Variant	Protein	844-848 positive probands	844-848 positive UAB probands	gnomAD (123,136)	EVS (6,503)	1000 G	HGMD ^A	LOVD ^A (3,384)	ClinVar ^A	Grantham distance	SIFT	PolyPhen	CADD	SSF/ MaxEntScan/ NNSplice/HSF	RNA observed	Other evidence ^B
c.2530C>T	p.Leu844Phe	10/129	5/67	0	0	0	Present (DM)	0	Present (NP)	22	Deleterious (0)	Probably damaging (0.997)	27.2	No change	Normal	Proven <i>de novo</i> (UAB-R2425; UAB-R8475; UAB-R0903); segregation in family (MAN-R48902G)
c.2531T>A	p.Leu844His	2/129	1/67	0	0	0	0	0	0	99	Deleterious (0)	Probably damaging (0.999)	26.6	No change	Normal	Proven <i>de novo</i> (UAB-R4444)
c.2531T>C	p.Leu844Pro	7/129	5/67	0	0	0	Present (DM)	0	Present (NP)	98	Deleterious (0)	Probably damaging (0.997)	25.9	No change	Normal	Proven <i>de novo</i> (UAB-R3714; UAB-R2087; UAB-R8047; UAB-R0075)
c.2531T>G	p.Leu844Arg	6/129	3/67	0.00041%	0	0	Present (DM)	0	Present (P)	102	Deleterious (0)	Probably damaging (0.997)	26.8	No change	Normal	Proven <i>de novo</i> (UAB-R624)
c.2533T>C	p.Cys845Arg	3/129	1/67	0	0	0	Present (DM)	1 (P)	Present (VUS)	180	Deleterious (0.010)	Possibly damaging (0.571)	22.6	No change	Normal	Proven <i>de novo</i> (UAB-R3112); segregation in family (MAN-R88088G)
c.2534G>A	p.Cys845Tyr	8/129	6/67	0	0	0	Present (DM?)	1 (VUS)	Present (LP)	194	Deleterious (0.01)	Possibly damaging (0.891)	23.2	No change	Normal	Proven <i>de novo</i> (UAB-R9135; UAB-R7836; UAB-R2696)
c.2536G>C	p.Ala846Pro	1/129	0/67	0	0	0	0	1 (P)	0	27	Deleterious (0.03)	Probably damaging (0.999)	27	No change	Normal	Segregation in family (UG-R0781)
c.2537C>A	p.Ala846Asp	5/129	3/67	0	0	0	Present (DM)	1 (P)	0	126	Deleterious (0.01)	Probably damaging (0.999)	31	No change	Normal	Proven <i>de novo</i> (ROT-R02233); segregation in family (UAB-R3618; UG-R665)
c.2540T>C	p.Leu847Pro	58/129	27/67	0	0	0	Present (DM)	11 (P)	Present (P)	98	Deleterious (0.01)	Probably damaging (0.997)	26.1	No change	Normal	Proven <i>de novo</i> (UAB-R9676; UAB-R2266; ROT-R22853 ; ROT-R17435 ; UG-R5831); segregation in multiple families (UAB-R4965; UAB-R3508; UG-R01; PAD-R500; MIL-R192/982)
c.2540T>G	p.Leu847Arg	8/129	2/67	0	0	0	Present (DM?)	2 (VUS)	0	102	Deleterious (0)	Probably damaging (0.997)	27	No change	Normal	Proven <i>de novo</i> (UAB-R6913; UG-R399)
c.2542G>C	p.Gly848Arg	8/129	5/67	0	0	0	Present (DM)	1 (VUS)	Present (P)	125	Deleterious (0.02)	Probably damaging (1)	27.2	No change	Normal	Proven <i>de novo</i> (UAB-R45401FN.201); segregation in multiple families (UAB-R1002; UAB-R1037; UAB-R2837; UG-R923; MIL-R999/399)
c.2542G>A	p.Gly848Arg	6/129	5/67	0	0	0	0	0	0	125	Deleterious (0.02)	Probably damaging (1)	28.2	Creates a novel splice acceptor sequence in the exon that is used in a small fraction of transcripts: from WT cc/cc/cc to MUT - 83.0 / 9.7 / 0.9 / 84.5 (Fig S4)	Low level of r.2410_2543del	Proven <i>de novo</i> (UAB-R4476; UAB-R3513)
c.2543G>A	p.Gly848Glu	7/129	4/67	0	0	0	Present (DM)	2 (P)	Present (NP)	98	Deleterious (0.03)	Probably damaging (1)	27.2	Creates a novel splice acceptor sequence in the exon: from WT cc/cc/cc/ to MUT - 81.9 / - / - / 81.7 (Fig S4)	No missplicing observed	Proven <i>de novo</i> (UAB-R1333; MAD-R9.232); segregation in family (SGR-REA; MAN-R95417G)


Abbreviations - **gnomAD**: the **G**enome **A**ggregation **D**atabase; **EVS**: **E**xome **V**ariant **S**erver; **1000 G**: **1000 G**enomes Project; **HGMD**: **H**uman **G**ene **M**utation **D**atabase; **LOVD**: **L**eiden **O**pen **V**ariation **D**atabase; **CADD**: **C**ombined **A**nnotation **D**ependent **D**epletion; **SSF**: **S**plice**S**ite**F**inder-like; **HSF**: **H**uman **S**plicing **F**inder; **VUS**: **v**ariant of **u**ncertain **s**ignificance; **P**: **p**athogenic; **LP**: **l**ikely **p**athogenic; **DM**: **d**isease causing; **DM?**: likely disease causing; **NP**: clinical significance **n**ot **p**rovided.


^A The variants' classifications presented in round brackets have been exactly the same as reported by the original author(s). ^B Individuals proven *de novo* with the confirmed biological relationships are shown in bold.


Table S3. The classification of *NF1* missense mutations affecting codons 844-848 reported in the studied group: a comparison of the publicly available disease databases (LOVD, ClinVar and HGMD, as of 05/09/2017) with the current study.


Variant	Protein	LOVD ^A	ClinVar ^A	HGMD ^B	This study ^C
c.2530C>T	p.Leu844Phe			 ^{a,b}	
c.2531T>A	p.Leu844His				
c.2531T>C	p.Leu844Pro			 ^b	
c.2531T>G	p.Leu844Arg			   ^{c-e}	
c.2533T>C	p.Cys845Arg			 ^f	
c.2534G>A	p.Cys845Tyr			 ^f	
c.2536G>C	p.Ala846Pro				
c.2537C>A	p.Ala846Asp			 ^f	
c.2540T>C	p.Leu847Pro			   ^{g,h}	
c.2540T>G	p.Leu847Arg			 ^f	
c.2542G>A	p.Gly848Arg				
c.2542G>C	p.Gly848Arg			 ⁱ	
c.2543G>A	p.Gly848Glu			 ^h	


Benign 

Likely benign 

Variant of uncertain significance 

Likely pathogenic 

Pathogenic 

Clinical significance not provided 

^A The variants' classification is provided as reported by the original author(s); ^B the originally reported variants' classifications have been reviewed and if necessary re-classified based on evidence for pathogenicity provided by the authors in the published papers and according to the ACMG recommendations; ^C the variants' classification according to the ACMG recommendations (details in Table S4).

HGMD: ^a reported in 1/93 unrelated NF1 individuals fulfilling the NIH diagnostic criteria; no phenotypic details available; no evidence for pathogenicity provided (Girodon-Boulant et al.¹⁹); ^b reported in 2/91 unrelated NF1 individuals fulfilling the NIH diagnostic criteria; no phenotypic details available; no evidence for pathogenicity provided (Mattocks et al.⁴); ^c reported once in sporadic case of 465 unrelated NF1 individuals; no phenotypic details available; no evidence for pathogenicity provided (Maynard et al.⁸); ^d proven *de novo*, no other evidence for pathogenicity provided (Wang et al.²⁰); ^e proven *de novo*, absence in normal controls, phenotype highly specific for a disease (Bertola et al.¹⁴); ^f absence in normal controls, segregation studies, proven *de novo* with paternity confirmation, *in silico* analysis (van Minkelen et al.⁵); ^g reported in 1/521 unrelated NF1 individuals, *in silico* analysis, no other evidence for pathogenicity provided (Fahsold et al.²¹); ^h absence in normal controls, disease phenotype segregated with the mutation in familial cases, *in silico* analysis (De Luca et al.²²; De Luca et al.²³); ⁱ disease phenotype segregated with the mutation in familial cases, phenotype highly specific for a disease (Pascual-Castroviejo et al.¹⁷)

Table S4. The classification of *NF1* missense mutations affecting codons 844-848 reported in the studied group according to the ACMG recommendations.³

Variant	Protein	PS1	PS2 ^A	PS3	PS4	PM1 ^B	PM2	PM3	PM4	PM5 ^C	PM6 ^D	PP1 ^E	PP2	PP3	PP4 ^F	PP5 ^G	Variant classification ^H
c.2530C>T	p.Leu844Phe	N/A	N/A	?	YES	?	YES	N/A	N/A	YES	YES	N/A	?	YES	YES	NO	VUS → Pathogenic
c.2531T>G	p.Leu844Arg	N/A	N/A	?	YES	?	NO	N/A	N/A	N/A	YES	YES	?	YES	YES	YES ^{2,3}	Pathogenic → Pathogenic
c.2531T>C	p.Leu844Pro	N/A	N/A	?	YES	?	YES	N/A	N/A	YES	YES	N/A	?	YES	YES	NO	VUS → Pathogenic
c.2531T>A	p.Leu844His	N/A	N/A	?	YES	?	YES	N/A	N/A	YES	YES	N/A	?	YES	YES	N/A	? → Pathogenic
c.2533T>C	p.Cys845Arg	N/A	N/A	?	YES	?	YES	N/A	N/A	N/A	YES	N/A	?	YES	YES	YES ^{1,3}	Pathogenic → Pathogenic
c.2534G>A	p.Cys845Tyr	N/A	N/A	?	YES	?	YES	N/A	N/A	YES	YES	N/A	?	YES	YES	NO	VUS → Pathogenic
c.2536G>C	p.Ala846Pro	N/A	N/A	?	YES	?	YES	N/A	N/A	N/A	N/A	YES	?	YES	YES	YES ¹	Pathogenic → Pathogenic
c.2537C>A	p.Ala846Asp	N/A	YES	?	YES	?	YES	N/A	N/A	N/A	N/A	N/A	?	YES	YES	YES ^{1,3}	Pathogenic → Pathogenic
c.2540T>C	p.Leu847Pro	N/A	YES	?	YES	?	YES	N/A	N/A	N/A	N/A	YES	?	YES	YES	YES ^{1,3}	Pathogenic → Pathogenic
c.2540T>G	p.Leu847Arg	N/A	N/A	?	YES	?	YES	N/A	N/A	YES	YES	N/A	?	YES	YES	NO	VUS → Pathogenic
c.2542G>A	p.Gly848Arg	YES	N/A	?	YES	?	YES	N/A	N/A	YES	YES	N/A	?	YES	YES	N/A	? → Pathogenic
c.2542G>C	p.Gly848Arg	N/A	N/A	NO ¹	YES	?	YES	N/A	N/A	N/A	YES	YES	?	YES	YES	YES ^{2,3}	Pathogenic → Pathogenic
c.2543G>A	p.Gly848Glu	N/A	N/A	?	YES	?	YES	N/A	N/A	N/A	YES	YES	?	YES	YES	YES ^{1,3}	Pathogenic → Pathogenic

PS1: same amino acid change as a previously established pathogenic variant regardless of nucleotide change; **PS2:** proven *de novo* (both maternity and paternity confirmed); **PS3:** well-established functional studies; **PS4:** the prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls; **PM1:** located in a mutational hot spot and/or in critical functional domain; **PM2:** absent from controls; **PM3:** for recessive disorders, detected in *trans* with a pathogenic variant; **PM4:** protein length changes due to in-frame or stop-loss variants; **PM5:** novel missense change at amino acid residue where a different pathogenic missense change has been seen before; **PM6:** assumed *de novo*, but without confirmation of paternity and maternity; **PP1:** co-segregation with disease in multiple affected family members; **PP2:** missense variant in a gene that has a low rate of benign missense variation; **PP3:** multiple lines of computational evidence support a deleterious effect on the gene or gene product; **PP4:** individual's phenotype or family history is highly specific for a disease; **PP5:** reputable source reports variant as pathogenic; **N/A:** not applicable; **YES:** the criterion has been fulfilled; **NO:** the criterion has not been fulfilled; **?:** due to the lack of evidence, it is difficult to establish whether the criterion has been fulfilled or not.

^A based on the data from LOVD database (only individuals confirmed as *de novo* and identified in the Netherlands where biological relationships were confirmed as part of the *de novo* assessment have been taken into account); ^B CSRD domain has not been proved to be critical to protein function (as function is largely unknown still), thus this evidence could not be considered as moderate evidence of pathogenicity; ^C variants classified as *pathogenic* in the publicly available disease databases (LOVD, ClinVar, HGMD) have been taken into account; ^D only individuals confirmed as *de novo* at UAB have been taken into account, except for variants fulfilling the PS2 criterion (p.Ala846Asp and p.Leu847Pro); ^E segregation with at least three affected family members needed to fulfill this criterion; ^F at least one individual with "classic" NF1 phenotype, including neurofibroma(s) and/or Lisch nodules was considered required to fulfill this criterion; ^G based on the variant's classification as pathogenic reported in LOVD (1), ClinVar (2) and/or HGMD (3) databases (details in Table S3); ^H prior variants classification as reported in the publicly available disease databases (LOVD, ClinVar, HGMD) and *a posteriori* classification based on our study and following the ACMG recommendations; ¹ based on functional studies by Li et al.²⁴ and Toonen et al.²⁵

To classify variant as pathogenic the following criteria need to be fulfilled: ≥2 strong (PS1-PS4) OR 1 strong (PS1-PS4) and ≥3 moderate (PM1-PM6) OR 1 strong (PS1-PS4) and 2 moderate (PM1-PM6) and ≥2 supporting (PP1-PP5) OR 1 strong (PS1-PS4) and 1 moderate (PM1-PM6) and ≥4 supporting (PP1-PP5).

Table S5. The age of the individuals who did present with CALMs-only (either >5 or < 6), but carried a missense mutation affecting one of five *NF1* codons 844-848 in this study.

Family ID	cDNA level	Protein level	Age group	Family ID	cDNA level	Protein level	Age group
>5 CALMs				<6 CALMs			
UAB-R8475	c.2530C>T	p.Leu844Phe	14-18 years	UAB-R2696 ^A	c.2534G>A	p.Cys845Tyr	14-18 years
UAB-R8047	c.2531T>C	p.Leu844Pro	0-24 months	* UAB-R9135 ^B	c.2534G>A	p.Cys845Tyr	19-26 years
ROT-R13734	c.2534G>A	p.Cys845Tyr	0-24 months	* UG-R5831 ^C	c.2540T>C	p.Leu847Pro	5-8 years †
UG-R391	c.2534G>A	p.Cys845Tyr	2-4 years	UAB-R9493	c.2542G>A	p.Gly848Arg	5-8 years
ROT-R36375	c.2540T>G	p.Leu847Arg	0-24 months	UAB-R1037-S2	c.2542G>C	p.Gly848Arg	0-24 months
UAB-R6913	c.2540T>G	p.Leu847Arg	5-8 years	UAB-R2837-C1	c.2542G>C	p.Gly848Arg	2-4 years
UAB-R09001FN.101	c.2540T>C	p.Leu847Pro	0-24 months	UAB-R2837-C3	c.2542G>C	p.Gly848Arg	2-4 years
UAB-R6106	c.2540T>C	p.Leu847Pro	0-24 months	UAB-R2837-C2	c.2542G>C	p.Gly848Arg	5-8 years
UAB-R3537	c.2540T>C	p.Leu847Pro	0-24 months	UAB-R1037-M	c.2542G>C	p.Gly848Arg	>26 years
ROT-R2CMUL	c.2540T>C	p.Leu847Pro	0-24 months	MAD-R9.232	c.[2543G>A;4463G>A]	p.[Gly848Glu;Arg1488His]	5-8 years
CAR-R655763	c.2540T>C	p.Leu847Pro	0-24 months				
UG-R746	c.2540T>C	p.Leu847Pro	0-24 months				
UAB-R9676	c.2540T>C	p.Leu847Pro	2-4 years				
ROT-R49013	c.2540T>C	p.Leu847Pro	>26 years				
UAB-R45401FN.201	c.2542G>C	p.Gly848Arg	9-13 years				
UAB-R1333	c.2543G>A	p.Gly848Glu	0-24 months				

^A Individual had also two superficial plexiform neurofibromas on trunk and back and symptomatic spinal neurofibroma; ^B Individual had one superficial plexiform neurofibroma on trunk and no CALMs; ^C Individual had very severe phenotype, presenting shortly after birth; three plexiform neurofibromas, including right orbital tumor, one located in right middle ear and one on neck, visible from outside; protrusion of the right eye with hemangiomas on the right cheek confirmed by MRI; severe epilepsy with attacks occurring mostly in sleep or during feeding, causing vomiting; individual was not able to eat (tube feeding); deceased at age of ~8 years.

* None CALMs had been observed in these individuals.

Table S6. Spinal neurofibromas in individuals carrying a missense mutation affecting one of five codons 844-848 in this study.

Family ID	cDNA level	Protein level	Family history	Age	CALMs	Freckles	Neurofibromas	Others	Comments
<u>SYMPTOMATIC SPINAL NEUROFIBROMAS</u>									
UAB-R2696	c.2534G>A	p.Cys845Tyr	PrS	14-18 years	<6	-	plexiform neurofibromas on trunk and back (visible from outside)	mild scoliosis, hypertension, Noonan phenotype features	MRI: innumerable spinal, intercostal, brachial and lumbosacral plexiform neurofibromas; large right-sided paraspinous neurofibroma in the lumbosacral region (12 cm in length)
UG-R399	c.2540T>G	p.Leu847Arg	PrS	5-8 years	>5	NS	plexiform neurofibroma on trunk (visible from outside and internal, without hyperpigmentation)	scoliosis, Noonan-phenotype features, abnormal development, gross motor delays, LD, ADHD, SD	extensive spinal neurofibromas on the costal nerves T8-11 right side; visible externally but much larger on MRI
UAB-R3508	c.2540T>C	p.Leu847Pro	F	14-18 years	>5	+	plexiform neurofibromas on neck, trunk and left arm (visible from outside, with hyperpigmentation)	symptomatic OPG, abnormal development, LD, ADD, ADHD	none
UAB-R01701FN.103	c.2540T>C	p.Leu847Pro	F	19-26 years	>5	+	plexiform neurofibromas on neck, left leg, left foot and abdomen (visible from outside); multiple cutaneous and subcutaneous neurofibromas on all parts of the body (#unknown)	Lisch nodules, LD, ADD	spinal neurofibromas on T5-T6 and L4-L5 regions
UF-R1	c.2540T>C	p.Leu847Pro	F	19-26 years	>5	-	large nodular neurofibroma on right thigh, surgically removed during the teenage years; some cutaneous (<10) and subcutaneous neurofibromas (#unknown)	scoliosis, chronic arthritis multiple joints, possible OPG (small lesion on optic nerve by MRI scan), Noonan phenotype features (PT, LSE, DPF), macrocephaly, non-ossifying fibroma on femur/knee, bilateral ankle clonus, knee hyperreflexia and esotropia	individual has suffered from proximal weakness in lower extremities that can be associated with the nerve root enlargements, significant difficulty walking, weakness of iliopsoas and quadriceps muscles; in addition, the individual has depigmented spots on back and left chest (vitiligo) and piebaldism (presence of white forelock); genetic analysis of <i>KIT</i> and <i>SNAI2</i> revealed presence of the <i>KIT</i> missense alteration c.1195G>A (p.Val399Ile), likely contributing to the piebaldism phenotype in this case
UAB-R0008	c.2542G>A	p.Gly848Arg	RS	14-18 years	>5	+	-	scoliosis, LD (dyslexia associated with working memory and phonological awareness issues), history of recurrent migraine headaches	p.Gly848Arg causing low level missplicing r.2410_2543del; father was diagnosed with a bilateral distal sensory neuropathy, his spinal MRI was normal
ROT-R95424	c.2542G>C	p.Gly848Arg	F	>26 years	<6	+	2-6 cutaneous and 2-6 subcutaneous neurofibromas	normal development, concentration problems, macrocephaly	individual's mother has many small CALMs spots and some neurofibromas
UAB-R1002	c.2542G>C	p.Gly848Arg	F	19-26 years	>5	+	mediastinal mass on chest (internal) and large plexiform neurofibroma bilateral on both sides of neck; 1 cutaneous neurofibroma in the right ear filling the entire canal	scoliosis, hypertension, renal artery stenosis, conductive hearing loss in the right ear, likely secondary to right ear being filled with a neurofibroma	individual's brother, father and paternal uncle have NF1
UG-R923	c.2542G>C	p.Gly848Arg	F	19-26 years	0	-	plexiform neurofibromas on both sides of the neck	moderate scoliosis, LD, ADHD	clumsiness with impaired walking, moderate atrophy of the distal leg muscles; generalized nerve sheath tumors, presence of symmetric neurofibromas in all spinal nerves of the body following the peripheral course of the nerves; case reported by Pascual-Castroviejo et al. ¹⁷
UAB-R2492	c.2543G>A	p.Gly848Glu	F	>26 years	>5	-	plexiform neurofibroma on right leg (visible from outside); 100-500 cutaneous/subcutaneous neurofibromas	macrocephaly	none

SYMPTOMATIC SPINAL NEUROFIBROMAS

UAB-R3237	c.2543G>A	p.Gly848Glu	RS	>26 years	<6	+	plexiform neurofibromas on head, neck, left and right leg (visible from outside, without hyperpigmentation); 1 cutaneous neurofibroma	asymptomatic OPG, Lisch nodules	none
MAN-R95417G	c.2543G>A	p.Gly848Glu	F	>26 years	0	+	multiple cutaneous and subcutaneous neurofibromas developed from her teenage years	SS, macrocephaly, normal development, possible Noonan phenotype (specific facial features, but no details available)	MRI: tumors present on nerve roots throughout the neuroaxis, particularly around the cauda equina, and a large retroperitoneal mass; repeated few years later MRI showed a very extensive burden of nerve sheath tumors, involving the large majority of spinal nerve roots; case reported by Burkitt-Wright et al. ¹⁸
MAN-R95417G-C	c.2543G>A	p.Gly848Glu	F	>26 years	0	-	-	Lisch nodules, pectus carinatum, normal development, possible Noonan phenotype (specific facial features, but no details available)	MRI: a very extensive burden of nerve sheath tumors, including a particularly large lobulated lesion in the occipital/posterior neck region; case reported by Burkitt-Wright et al. ¹⁸

ASYMPTOMATIC SPINAL NEUROFIBROMAS

UAB-R0147	c.2534G>A	p.Cys845Tyr	RS	19-26 years	>5	+	innumerable solitary and plexiform neurofibromas on left arm, neck, trunk, left leg, right leg, abdomen (visible from outside, with hyperpigmentation); left arm extensive neurofibroma confirmed histopathologically as a focally cellular neurofibroma with degenerative atypia; 6-99 cutaneous and 6-99 subcutaneous neurofibromas	abnormal development, LD	numerous subcentimeter subcutaneous scalp solitary T2 hyperintense nodules; likely small peripheral nerve sheath tumors?
ROT-R21382	c.2540T>C	p.Leu847Pro	F	>26 years †	>5	+	left nervus ischiadicus, surgically removed; >500 cutaneous and 6-99 subcutaneous neurofibromas	pericarditis carcinomatosa, metastasized MPNST, superior vena cava syndrome (SVCS), dyspnea on effort	individual died of MPNST at age of 30 years; family history: affected mother and sister
UG-R01-C1	c.2540T>C	p.Leu847Pro	F	14-18 years	>5	+	plexiform neurofibroma on trunk (visible from outside); 2-6 cutaneous neurofibromas	scoliosis, short stature, odontogenic fibroma lower jaw (removed at age of 11)	individual attends vocational training; muscular build; paravertebral plexiform neurofibroma at the site of scoliosis surgery
MIL-R999/399	c.2542G>C	p.Gly848Arg	F	19-26 years	<6	-	2-6 cutaneous and 2-6 subcutaneous neurofibromas	scoliosis, mitral insufficiency, Lisch nodules	none
UG-R923-S	c.2542G>C	p.Gly848Arg	F	14-18 years	<6	-	several cutaneous neurofibromas, #unknown	symptomatic OPG, scoliosis, short stature, cerebellar cystic tumors, borderline mental level, LD	<u>brain MRI</u> : glial cystic tumor of low degree of malignancy in the left cerebellar hemisphere; symmetrical spinal neurofibromas that affected all the spinal nerves; case reported by Pascual-Castroviejo et al. ¹⁷
UG-R923-M	c.2542G>C	p.Gly848Arg	F	>26 years	<6	-	several cutaneous-subcutaneous neurofibromas	-	individual showed asymmetrical spinal neurofibromas in all the spinal nerves; her father died at 40 years of age of multiple hepatic neurofibromas, but reportedly had no cutaneous manifestations of NF1; case reported by Pascual-Castroviejo et al. ¹⁷
SGR-REA	c.2543G>A	p.Gly848Glu	F	>26 years	>5	-	-	mild pulmonic insufficiency, Lisch nodules	none

PrS: proven sporadic; **F:** familial; **RS:** reportedly sporadic; **NS:** not specified; **LD:** learning difficulties; **SD:** speech delay; **ADD:** attention deficit disorder; **ADHD:** attention deficit hyperactivity disorder; **OPG:** optic pathway glioma; **SVCS:** superior vena cava syndrome; **MPNST:** malignant peripheral nerve sheath tumor; **PT:** ptosis; **LSE:** low set ears; **DPF:** downslanting palpebral fissures

Table S7. Optic pathway gliomas in individuals carrying a missense mutation affecting one of five codons 844-848 in this study.

Family ID	cDNA level	Protein level	Family history	Age	CALMs	Freckles	Neurofibromas	Others	Comments
SYMPTOMATIC OPTIC PATHWAY GLIOMAS									
UAB-R624	c.2531T>G	p.Leu844Arg	PrS	14-18 years	>5	+	plexiform neurofibroma on right cheek; cutaneous neurofibromas (#unknown)	bowled long bones, pectus carinatum, pulmonic stenosis, aortic coarctation, Noonan-phenotype features (and presence of pathogenic <i>PTPN11</i> mutation), short stature, pilocytic astrocytoma, Lisch nodules	MRI: bulky optic nerves, especially on the right, and a solid expansive lesion in the suprasellar region involving the optic chiasm and deforming the third ventricle; at 7 years of age, the individual complained of visual loss and cranial MRI showed progression with signs of hydrocephalus; the tumor, a pilocytic astrocytoma, was partially resected at age of 8 years; no further therapy was administered and the individual experienced progressive visual loss; case reported by Bertola et al. ¹⁴
PAD-R300	c.2531T>C	p.Leu844Pro	RS	2-4 years	>5	+	-	hypothalamic glioma, abnormal development, SD	OPG involved optic nerves and chiasm
ROT-R02233	c.2537C>A	p.Ala846Asp	PrS	14-18 years	>5	+	plexiform neurofibroma on head (visible from outside, without hyperpigmentation); 2-6 cutaneous neurofibromas	normal development, executive function problems	none
UG-R665	c.2537C>A	p.Ala846Asp	F	5-8 years	>5	+	-	LD, ADHD, hemangioma left (2-3 cm)	OPG involved optic nerves, especially on the left, and chiasm
UL-R83988968	c.2540T>G	p.Leu847Arg	RS	5-8 years	>5	-	-	hypertension, Noonan-like features, macrocephaly, short stature, juvenile xanthogranulomas, LD, Lisch nodules	OPG involved optic nerves; individual needed treatment for pubertas precox ; hyper nasal speech after adenoidectomy and tonsillectomy
UAB-R3508	c.2540T>C	p.Leu847Pro	F	14-18 years	>5	+	plexiform neurofibromas on neck, trunk and left arm (visible from outside, with hyperpigmentation); symptomatic spinal neurofibromas (unilateral C1-C3)	short stature, abnormal development, LD, ADD, ADHD	OPG involved optic nerves; individual treated with bevacizumab and chemotherapy for 9-10 months; OPG is no longer symptomatic
CAR-R8012M6	c.2540T>C	p.Leu847Pro	NS	5-8 years	>5	NS	NS	individual attends special school (LD?)	none
MAD-R3.793	c.2540T>C	p.Leu847Pro	RS	19-26 years	>5	+	plexiform neurofibroma on left side of the face (visible from outside) and orbital neurofibroma resected	scoliosis, sphenoid wing dysplasia, short stature, macrocephaly, LD, Lisch nodules	OPG involved left optic nerves and chiasm; hemimegalencephaly with parenchymal atrophy and complex partial seizures
UG-R01-S1	c.2540T>C	p.Leu847Pro	F	>26 years	>5	+	-	short stature, focal epilepsy, psychiatric problems	-
UAB-R1474	c.2542G>A	p.Gly848Arg	RS	5-8 years	>5	+	2-6 intradermal neurofibromas	pectus carinatum, macrocephaly, embryonal rhabdomyosarcoma, abnormal development, gross motor delays, LD, ADHD, Lisch nodules	OPG involved optic nerves and chiasm; p.Gly848Arg causing low level missplicing r.2410_2543del
UG-R923-S	c.2542G>C	p.Gly848Arg	F	14-18 years	<6	-	several cutaneous neurofibromas (#unknown); asymptomatic spinal neurofibromas affected all the spinal nerves	scoliosis, short stature, cerebellar cystic tumors, LD	OPG involved left optic nerve and chiasm; MRI: glial cystic tumor with low degree of malignancy in the left cerebellar hemisphere
SGR-REA-S	c.2543G>A	p.Gly848Glu	F	19-26 years †	>5	-	plexiform neurofibroma on neck (visible from outside, without hyperpigmentation)	short stature, MPNST	OPG involved optic chiasm

PrS: proven sporadic; **F:** familial; **RS:** reportedly sporadic; **LD:** learning difficulties; **SD:** speech delay; **ADD:** attention deficit disorder; **ADHD:** attention deficit hyperactivity disorder; **MPNST:** malignant peripheral nerve sheath tumor; **OPG:** optic pathway glioma; **NS:** not specified

Family ID	cDNA level	Protein level	Family history	Age	CALMs	Freckles	Neurofibromas	Others	Comments
<u>ASYMPTOMATIC OPTIC PATHWAY GLIOMAS</u>									
UAB-R0903	c.2530C>T	p.Leu844Phe	PrS	9-13 years	>5	+	absent	LD, special education	none
UAB-R0713	c.2531T>G	p.Leu844Arg	RS	5-8 years	>5	+	2-6 cutaneous neurofibromas	macrocephaly, abnormal development	OPG involved optic nerves
MIL-R168	c.2531T>G	p.Leu844Arg	RS	>26 years	>5	-	2-6 cutaneous and 2-6 subcutaneous neurofibromas	scoliosis, possible brainstem glioma, Lisch nodules	OPG involved optic nerves
SGR-RSO	c.2531T>G	p.Leu844Arg	RS	19-26 years	>5	+	absent	scoliosis	none
UAB-R3714	c.2531T>C	p.Leu844Pro	PrS	0-24 months	>5	+	absent	Lisch nodules	none
UAB-R0252	c.2540T>C	p.Leu847Pro	RS	19-26 years	>5	+	plexiform neurofibroma on trunk (with hyperpigmentation); 2-6 cutaneous, 2-6 subcutaneous and 2-6 intradermal neurofibromas	-	none
UAB-R2934	c.2540T>C	p.Leu847Pro	F	19-26 years	>5	+	2-6 cutaneous, 2-6 subcutaneous and 2-6 intradermal neurofibromas	Lisch nodules	bilateral OPG, no OPG treatment
UAB-R4965	c.2540T>C	p.Leu847Pro	F	14-18 years	>5	+	a few subcutaneous neurofibromas on lower back	Lisch nodules, bone cysts, LD, ADD, Asperger syndrome, anger problems, mild hearing loss, migraines, non-ossifying fibroma of the right knee and anger/impulse control issues	OPG involved optic nerves and chiasm; individual had a tethered cord repair and had complaints of chronic pain in various areas
UAB-R2546	c.2540T>C	p.Leu847Pro	RS	14-18 years	>5	+	a few subcutaneous neurofibromas	ADD, migraine headaches (very strong family history of migraines); unusual bone infarctions at the site of some probable non-ossifying fibromas	OPG involved left chiasm and has been present for many years; it has remained asymptomatic and unchanged in size
UAB-R1928	c.2540T>C	p.Leu847Pro	RS	19-26 years	>5	+	6-99 cutaneous, 2-6 subcutaneous and 2-6 intradermal neurofibromas	dural ectasia, 4 th lumbar vertebrae fragmentation, mild pervasive developmental delay, LD	OPG involved optic nerves
MAD-R3.7001	c.2540T>C	p.Leu847Pro	RS	5-8 years	>5	+	plexiform neurofibroma on head (visible from outside); 2-6 cutaneous neurofibromas	Lisch nodules, macrocephaly, LD	OPG involved optic nerves
MAD-R3.409	c.2540T>C	p.Leu847Pro	RS	9-13 years	>5	+	absent	LD	MRI: anomalous posterior fossa venous drainage; OPG involved left optic nerve
MIL-R361/018	c.2540T>C	p.Leu847Pro	F	14-18 years	>5	+	absent	abnormal development	OPG involved optic nerves, especially on the left; MRI: unidentified bright objects (UBOs)
ROT-R89874	c.2540T>C	p.Leu847Pro	F	9-13 years	>5	+	absent	abnormal development	none
UG-R004	c.2540T>C	p.Leu847Pro	F	9-13 years	>5	+	2-6 subcutaneous neurofibromas	ADD, ADHD, special education (LD?)	OPG involved left optic nerve and chiasm; brother was diagnosed with ADHD, but no NF1
UG-R01	c.2540T>C	p.Leu847Pro	F	>26 years	>5	+	2-6 cutaneous and 2-6 intradermal neurofibromas (symptomatic internal neurofibroma in left knee)	Lisch nodules, bone cysts in femur	OPG has remained asymptomatic for 16 years
UAB-R3513	c.2542G>A	p.Gly848Arg	F	2-4 years	>5	+	2-6 cutaneous neurofibromas	-	individual's brother has developmental delay, but he does not have any NF pigmentary signs
UAB-R3237	c.2543G>A	p.Gly848Glu	RS	>26 years	<6	+	plexiform neurofibromas on head, neck, left and right leg (visible from outside, without hyperpigmentation); 1 cutaneous neurofibroma, symptomatic spinal neurofibroma	-	OPG involved optic nerves

PrS: proven sporadic; F: familial; RS: reportedly sporadic; LD: learning difficulties; ADD: attention deficit disorder; ADHD: attention deficit hyperactivity disorder; OPG: optic pathway glioma

Table S8. Malignant neoplasms in individuals carrying a missense mutation affecting one of five codons 844-848 in this study.

Family ID	cDNA level	Protein level	Family history	Age	CALMs	Freckles	Neurofibromas	Others	Comments
RHABDOMYOSARCOMA									
UAB-R1227	c.2540T>C	p.Leu847Pro	RS	2-4 years	>5	+	plexiform neurofibroma on back (visible from outside, with hyperpigmentation)	normal development	MRI: mass in left lobe of liver with possible involvement of the right atrium (diagnosis: embryonal rhabdomyosarcoma)
ROT-R49013	c.2540T>C	p.Leu847Pro	RS	>26 years	>5	NS	-	scoliosis, macrocephaly	embryonal rhabdomyosarcoma of left testis at 2 years old and astrocytoma grade II at 15 years old
UAB-R1474	c.2542G>A	p.Gly848Arg	RS	5-8 years	>5	+	2-6 intradermal neurofibromas	Lisch nodules, symptomatic OPG, pectus carinatum, macrocephaly, abnormal development, gross motor delays, LD, ADHD	diagnosis: embryonal rhabdomyosarcoma; p.Gly848Arg causing low level missplicing r.2410_2543del
MPNST									
MAN-R48902G	c.2530C>T	p.Leu844Phe	F	>26 years	>5	+	6-99 cutaneous, 2-6 subcutaneous and 2-6 intradermal neurofibromas	hypertension	low grade MPNST in right distal femur
CAR-R18010M61	c.2540T>C	p.Leu847Pro	F	>26 years	>5	+	6-99 cutaneous neurofibromas	-	individual has two sisters with multiple neurofibromas and brother who had colorectal cancer in his 40s
CAR-RNIS	c.2540T>C	p.Leu847Pro	F	>26 years	<6	-	plexiform neurofibroma in pelvis; 100-500 cutaneous neurofibromas	-	MPNST and breast cancer ; genetic <i>BRC1/2</i> testing was negative by whole exome sequencing (WES); WES analysis revealed the presence of p.Leu847Pro in all the three tumors and independent somatic "second hits" in <i>NF1</i> as well as multiple copy number changes in the breast cancer and MPNST, but not in the plexiform neurofibroma; case reported by McPherson et al. ¹⁶
ROT-R21382	c.2540T>C	p.Leu847Pro	F	>26 years †	>5	+	left nervus ischiadicus, surgically removed; >500 cutaneous and 6-99 subcutaneous neurofibromas, asymptomatic spinal neurofibromas (from T11 and up)	pericarditis carcinomatosa, superior vena cava syndrome (SVCS) and dyspnea on effort	metastasized MPNST; individual died of MPNST at age of 30 years
UG-R01-S2	c.2540T>C	p.Leu847Pro	F	19-26 years †	>5	+	cutaneous and subcutaneous neurofibromas (#unknown); possible internal tumors	-	malignant MPNST of mediastinum and abdomen; individual died of MPNST at age of 25 years
UG-R01-S3	c.2540T>C	p.Leu847Pro	F	19-26 years †	NS	NS	NS	NS	individual died of MPNST at age of 20 years ; individual was diagnosed with NF1, no further information available
SGR-REA-S	c.2543G>A	p.Gly848Glu	F	19-26 years †	>5	-	plexiform neurofibroma on neck (visible from outside, without hyperpigmentation)	symptomatic OPG, short stature	individual died of MPNST at age of 26 years
OTHERS									
UG-R01-M	c.2540T>C	p.Leu847Pro	F	>26 years †	>5	+	cutaneous neurofibromas (#unknown); possible internal neurofibroma	Lisch nodules	individual died of metastasized colon cancer (adenocarcinoma) at age of 54 years
MIL-R192/982-F	c.2540T>C	p.Leu847Pro	F	>26 years	<6	+	plexiform neurofibroma on palate (without hyperpigmentation); 6-99 cutaneous and 2-6 subcutaneous neurofibromas	Lisch nodules, scoliosis, severe mood depression	medullary thyroid carcinoma
UAB-R9493	c.2542G>A	p.Gly848Arg	F	5-8 years	<6	-	-	-	NF1-associated JMML (second hit identified: c.1246C>T, p.Arg416*)

F: familial; RS: reportedly sporadic; NS: not specified; LD: learning difficulties; ADHD: attention deficit hyperactivity disorder; OPG: optic pathway glioma; JMML: juvenile myelomonocytic leukemia

Table S9. Comparison of clinical features (malignant neoplasms, >5 CALMs, skinfold freckles, cutaneous and symptomatic spinal neurofibromas) in cohorts of individuals carrying different missense mutations affecting codons 844-848 - additional statistical calculations.

NF1 clinical features	Number of individuals (%)		p value (2-tailed Fisher's exact test)
	p.Leu847Pro	Other mutations in AA 844-848	
Malignant neoplasms \geq 14 years	8/38 (21.1)	2/40 (5)	0.0448
	p.Gly848	Other mutations in AA 844-848	
Symptomatic spinal neurofibromas \geq 9 years	8/20 (40)	4/59 (6.8)	0.0012
>5 CALMs	15/34 (44.1)	115/123 (93.5)	<0.0001
Skinfold freckles \geq 9 years	11/23 (47.8)	65/72 (90.3)	<0.0001
Cutaneous neurofibromas \geq 19 years	5/18 (27.8)	42/51 (82.4)	<0.0001

Table S10. List of all adjusted p-values after applying the Benjamini-Hochberg correction for multiple testing with false discovery rates at 0.05 and 0.01.

p-value	index	Statistical significance at p<0.05		Statistical significance at p<0.01	
		B-H critical ^A	value ^B	B-H critical ^A	value ^B
0.8794	1	0.0250		0.0050	
0.5830	2	0.0246		0.0049	
0.5727	3	0.0242		0.0048	
0.4489	4	0.0237		0.0047	
0.4296	5	0.0233		0.0047	
0.3590	6	0.0229		0.0046	
0.2968	7	0.0225		0.0045	
0.2916	8	0.0220		0.0044	
0.1349	9	0.0216		0.0043	
0.1323	10	0.0212		0.0042	
0.1160	11	0.0208		0.0042	
0.1029	12	0.0203		0.0041	
0.0693	13	0.0199		0.0040	
0.0619	14	0.0195		0.0039	
0.0451	15	0.0191		0.0038	
0.0409	16	0.0186		0.0037	
0.0404	17	0.0182		0.0036	
0.0384	18	0.0178		0.0036	
0.0341	19	0.0174		0.0035	
0.0276	20	0.0169		0.0034	
0.0263	21	0.0165		0.0033	
0.0241	22	0.0161		0.0032	
0.0186	23	0.0157		0.0031	
0.0174	24	0.0153		0.0031	
0.0164	25	0.0148		0.0030	
0.0125	26	0.0144	*	0.0029	
0.0080	27	0.0140	*	0.0028	
0.0076	28	0.0136	*	0.0027	
0.0067	29	0.0131	*	0.0026	
0.0061	30	0.0127	*	0.0025	
0.0060	31	0.0123	*	0.0025	
0.0043	32	0.0119	*	0.0024	
0.0042	33	0.0114	*	0.0023	
0.0028	34	0.0110	*	0.0022	
0.0025	35	0.0106	*	0.0021	
0.0023	36	0.0102	*	0.0020	
0.0022	37	0.0097	*	0.0019	
0.0020	38	0.0093	*	0.0019	
0.0012	39	0.0089	*	0.0018	*
0.0007	40	0.0085	*	0.0017	*
0.0005	41	0.0081	*	0.0016	*
0.0004	42	0.0076	*	0.0015	*
0.0002	43	0.0072	*	0.0014	*
0.0002	44	0.0068	*	0.0014	*
0.0001	45	0.0064	*	0.0013	*
0.0001	46	0.0059	*	0.0012	*
0.0001	47	0.0055	*	0.0011	*
0.0001	48	0.0051	*	0.0010	*
0.0001	49	0.0047	*	0.0009	*
0.0001	50	0.0042	*	0.0008	*
0.0001	51	0.0038	*	0.0008	*
0.0001	52	0.0034	*	0.0007	*
0.0001	53	0.0030	*	0.0006	*
0.0001	54	0.0025	*	0.0005	*
0.0001	55	0.0021	*	0.0004	*
0.0001	56	0.0017	*	0.0003	*
0.0001	57	0.0013	*	0.0003	*
0.0001	58	0.0008	*	0.0002	*
0.0001	59	0.0004	*	0.0001	*

^A according to Thissen et al.²⁶; ^B an asterisk (*) indicates the statistically significant p-value after B-H correction

Table S11. A mosaic neurofibromatosis type-1 case (13.5-year-old girl presenting with >5 CALMs and skinfold freckling) with the *NFI* c.2540T>C (p.Leu847Pro) mutation found as the “second hit” in the cultured melanocytes from one CALM.

Family ID	Biopsy	Mutation	
		“First hit” ^A	“Second hit”
UAB-R921FNES	1 st CALM biopsy		c.2540T>C (p.Leu847Pro)
* analysis performed on successfully cultured melanocytes from three biopsies of CALMs	2 nd CALM biopsy	c.5547-1G>A	absent
	3 rd CALM biopsy		c.1432A>G (p.Lys478Glu)

^A mutation leads to out-of-frame skipping of exon 38 [29] during *NFI* mRNA splicing and out-of-frame skipping exons 38 and 39 [29 and 30] in a proportion of *NFI* mRNA transcripts; the c.5547-1G>A mutation was not found in the blood lymphocytes by next-generation sequencing (0/600 reads, 3 different amplicons)

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