European Journal of Human Genetics (2015) 23, 1068–1071

www.nature.com/ejhg

ARTICLE

p.Arg1809Cys substitution in neurofibromin is associated with a distinctive NF1 phenotype without neurofibromas

Valentina Pinna[1,12], Valentina Lanari[1,12], Paola Daniele[1], Federica Consoli[1], Emanuele Agolini[1], Katia Margiotti[1,2], Irene Bottillo[1,3], Isabella Torrente[1,4], Alessandro Bruselles[5], Caterina Fusilli[1], Anna Ficcadenti[6], Sara Bargiacchi[7], Eva Trevisson[8], Monica Forzan[8], Sandra Giustini[9], Chiara Leoni[10], Giuseppe Zampino[10], Maria Cristina Digilio[11], Bruno Dallapiccola[11], Maurizio Clementi[8], Marco Tartaglia[5] and Alessandro De Luca*[,1]

Analysis of 786 *NF1* mutation-positive subjects with clinical diagnosis of neurofibromatosis type 1 (NF1) allowed to identify the heterozygous c.5425C>T missense variant (p.Arg1809Cys) in six (0.7%) unrelated probands (three familial and three sporadic cases), all exhibiting a mild form of disease. Detailed clinical characterization of these subjects and other eight affected relatives showed that all individuals had multiple cafè-au-lait spots, frequently associated with skinfold freckling, but absence of discrete cutaneous or plexiform neurofibromas, Lisch nodules, typical NF1 osseous lesions or symptomatic optic gliomas. Facial features in half of the individuals were suggestive of Noonan syndrome. Our finding and revision of the literature consistently indicate that the c.5425C>T change is associated with a distinctive, mild form of NF1, providing new data with direct impact on genetic counseling and patient management.
European Journal of Human Genetics (2015) **23,** 1068–1071; doi:10.1038/ejhg.2014.243; published online 5 November 2014

INTRODUCTION

Neurofibromatosis type 1 (NF1) (MIM #162200) is an autosomal dominant condition with a birth incidence of 1/3500 live births,[1] manifesting a considerable inter- and intra-familial clinical variability. Major diagnostic characteristics include café-au-lait spots (CaLS), skinfold freckling (SF), Lisch nodules (LN) and neurofibromas (NF), which occur in the majority of patients and represent key features of this disorder. In addition, a number of other features, including macrocephaly, learning problems, short stature, hypertelorism and thorax abnormalities, are found in NF1 patients, but are not pathognomonic for the trait. About a quarter of these patients develop one or more complications leading to significant morbidity and increased mortality. They include various malignant tumors, scoliosis, long-bone dysplasia and cardiovascular manifestations, such as vasculopathies, hypertension or congenital heart disease. Diagnosis is made based on the diagnostic criteria defined at the NIH 1987 NF consensus conference.[2]

NF1 is caused by inactivating intragenic mutations or deletions of the *NF1* gene (MIM #613113), which spans 350 kb and contains 61 translated exons.[3] *NF1* encodes neurofibromin, a multifunctional protein acting as a RAS-specific GTPase activating protein down-regulating RAS function. RasGTPase activating protein activity is mediated by the GTPase activating protein related domain (GRD) (residues 1198–1530). Located adjacent to the C-terminus of the GRD, two additional functionally relevant regions, the Sec-14p homologous (Sec) (residues 1560–1698) and plekstrin homology (PH)-like (residues 1715–1816) domains, form a bipartite lipid-binding module.[4]

To date, only two clinically relevant genotype–phenotype correlations have been reported in NF1. The first applies to patients heterozygous for germline large genomic deletions encompassing the *NF1* gene.[5] These deletions occur in about 4% of all NF1 cases.[6] Differently from subjects with intragenic mutations, these patients display a severe phenotype with facial dysmorphism, cognitive deficits, cardiovascular anomalies and early onset multiple cutaneous neurofibromas (CNFs), together with a higher lifetime risk for developing malignant peripheral nerve sheath tumors. The second correlation relates to the association between a 3-nt deletion in exon 17 of the *NF1* gene (c.2970_2972delAAT, p.Met992del) and absence of CNFs, subcutaneous neurofibromas (SCNFs) and external plexiform neurofibromas (PNFs).[7] The disease in these patients is milder, with an increased rate of pulmonary valve stenosis (PVS). Of note a phenotype resembling NF1, Legius syndrome (LS, MIM #611431), is caused by heterozygous inactivating mutations in *SPRED1* (Sprouty-related, EVH1 domain containing 1, MIM #609291). This disorder is less severe than NF1, and characterized by CaLS and SF, without CNFs and PNFs, LN, osseous lesions and optic pathway gliomas (OPGs).[8]

[1]IRCCS-Casa Sollievo della Sofferenza Hospital, Mendel Institute, Rome, Italy; [2]PRABB-Centro Integrato di Ricerca, Campus Bio-Medico University, Rome, Italy; [3]Division of Medical Genetics, Department of Molecular Medicine, San Camillo-Forlanini Hospital, Sapienza University, Rome, Italy; [4]San Camillo-Forlanini Hospital, Rome, Italy; [5]Dipartimento di Ematologia, Oncologia e Medicina Molecolare, Istituto Superiore di Sanità, Rome, Italy; [6]Pediatric Division, Department of Clinical Sciences, Rare Diseases Regional Centre, Polytechnic University of Marche Ospedali Riuniti, Ancona, Italy; [7]Genetics and Molecular Medicine Unit, Anna Meyer Children's University Hospital, Florence, Italy; [8]Clinical Genetics Unit, Department of Woman and Child Health, University of Padova, Padova, Italy; [9]Department of Dermatology, Sapienza University of Rome, Policlinico Umberto I, Rome, Italy; [10]Istituto di Clinica Pediatrica, Università Cattolica del Sacro Cuore, Rome, Italy; [11]Bambino Gesù Children Hospital, IRCCS, Rome, Italy
[12]These authors contributed equally to this work.
*Correspondence: Dr A De Luca, Istituto CSS-Mendel, Viale Regina Margherita 261, 00198 Rome, Italy. Tel: +39 06 44160510; Fax: +39 06 44160548; E-mail: a.deluca@css-mendel.it
Received 17 June 2014; revised 26 September 2014; accepted 1 October 2014; published online 5 November 2014

Here, we report on a recurrent missense *NF1* variant, predicting the p.Arg1809Cys amino-acid change, identified in six unrelated families sharing a distinctive disorder within the NF1 phenotypic spectrum, but characterized by high prevalence of CaLS and SF, without NFs and other NF1-associated malignancies.

MATERIALS AND METHODS

Patients were referred for diagnostic testing to the Molecular Genetics Laboratory of CSS-Mendel Institute (Rome, Italy) and the Medical Genetics Unit of Padua University Hospital (Padua, Italy), because of a clinical suspect of NF1. Participating practitioners filled out a clinical questionnaire about each patient. Clinical and genetic analyses were conducted with the approval of the institutional review boards of the participating institutions. Informed consent was obtained from all patients or their legal guardians. Permission for publication of photographs was obtained. *NF1*, *PTPN11* and *SPRED1* mutation analysis was performed by direct sequencing on all their coding exons and flanking intronic portions (*NF1*, NM_000267.3; *PTPN11*, NM_002834.3; *SPRED1*, NM_152594.2). *NF1* exon numbering followed that used in the NCBI Reference Sequence (NG_009018.1), which places c.5425C>T variant within exon 38. This exon corresponds to exon 29 according to the 'historical NF Consortium *NF1* exon numbering'.[9] Bidirectional Sanger sequencing was performed using the ABI BigDye Terminator Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA) and ABI 3130, and ABI3100 Genetic Analyzers (Applied Biosystems). Primer sequences and PCR conditions are available upon request. The multiplex ligation-dependent probe amplification kits no. P081/P082 and no. P295-B1 (MRC-Holland, Amsterdam,the Netherlands) were used to screen for the presence of intragenic deletions or duplications in the *NF1* and *SPRED1* genes, respectively, according to the manufacturer's instructions. High-resolution melting (HRM) scanning was used to search for the occurrence of the c.5425C>T change in population-matched unaffected controls. HRM optimization strategies for genotyping are available on request. In familial cases, paternity was confirmed by STR analysis. The six probands plus three additional family members carrying c.5425C>T have been entered in the LOVD NF1 database, including a description of their phenotype (www.LOVD.nl/NF1, patient IDs 00019904, 00019941–00019948).

RESULTS

Mutation screening allowed to identify 786 *NF1* mutation-positive subjects. Among them, six unrelated patients were found to be heterozygous for c.5425C>T transition in exon 38 (Supplementary Figure S1). The predicted amino-acid change is non-conservative, resulting in the replacement of a positively charged residue with a cysteine residue (p.Arg1809Cys). Different evidences supported the pathogenic role of this missense change. First, Arg^{1809} is invariantly conserved among orthologs (Supplementary Figure S2). Consistently, this amino-acid change was considered as deleterious by different *in silico* prediction programs (SIFT, score =0.00; PolyPhen-2, score= 1.00; Mutation Taster, $P=0.99$). Second, p.Arg1809Cys was the only variant within the entire coding region (CDS) and exon–intron junctions of *NF1* gene identified in all affected individuals. Copy number changes involving *NF1* and *SPRED1*, and functionally relevant variants in the entire CDS of *SPRED1* and *PTPN11* were excluded in all subjects. To exclude that c.5425C>T was a common variant in the Italian population, 460 healthy unrelated subjects were screened by means of HRM scanning (Supplementary Figure S3), and none of them was positive for this missense change. In agreement with our finding, this change had not been reported in public databases (dbSNP138 and 1000 Genomes). Finally, genotyping of parental DNAs demonstrated the *de novo* origin of this missense variant in the three families with sporadic occurrence of disease, as well as cosegregation in the three families transmitting the trait (Figure 1a).

Clinical features of the subjects heterozygous for the transition are shown in Figure 1b, summarized in Table 1, and compared with those reported in available large NF1 cohorts in Supplementary Table S1. All examined individuals with the c.5425C>T change displayed a distinctive and mild form of NF1. Specifically, they exhibited six or more CaLS, which were frequently associated with SF. Remarkably, none of the patients had discrete CNFs, SCNFs, evident PNFs, OPG or other malignancies. Other major NF1 features, including LN and typical osseous lesions were not found in any of these subjects. Macrocephaly, thoracic anomalies, reduced growth and learning problems were observed to occur in these subjects with similar

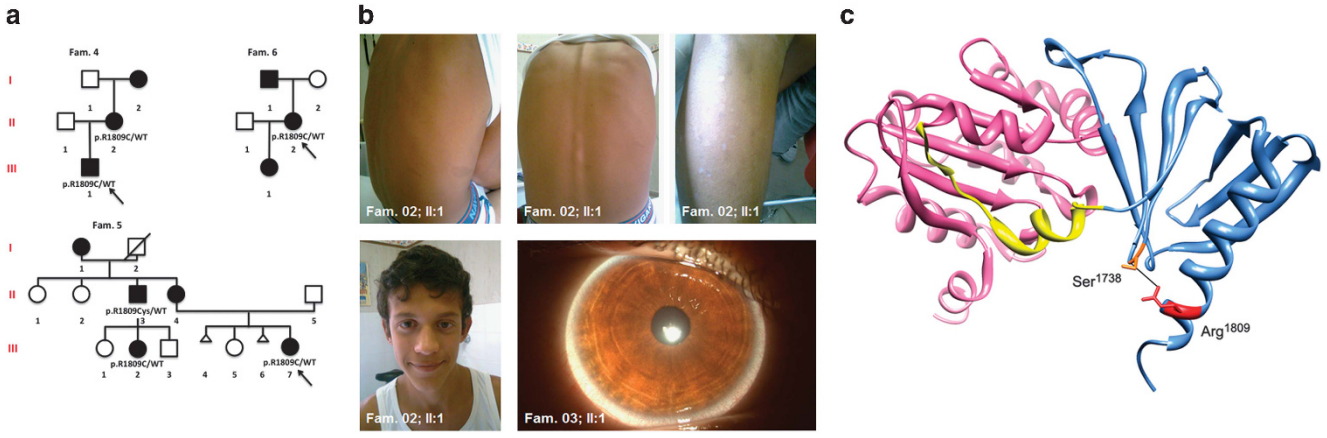

Figure 1 The c.5425C>T change in *NF1* is associated with a distinctive NF1 form. (**a**) Pedigrees of the three families cosegregting NF1 and the *NF1* mutation. Squares and circles indicate males and females, respectively; open symbols indicate unaffected individuals, filled symbols indicate affected individuals; arrows indicate index cases, and symbols with a slash indicate deceased family members. WT, wild-type allele. (**b**) Representative clinical features of subjects heterozygous for the c.5425C>T missense change in *NF1*. This mutation is associated with a mild phenotype consisting mainly of pigmentary signs (CaLS and SF) and lack of NFs. Other typical findings, including LN, OPGs and osseous dysplasia are uncommon or absent, whereas a high incidence of facial features resembling NS is observed. (**c**) Location of Arg^{1809} in the tridimensional structure of the neurofibromin Sec/PH-like bipartite module. The cartoon shows the ribbon representation of the module together with location of the residue (lateral chain reported as stick representation) mutated in the studied NF1 subjects. The Sec domain (residues 1560–1698) (pink), which is implicated in lipid binding, is connected to a regulatory PH-like domain (residues 1715–1816) (light blue) by a partly helical linker (residues 1699–1714) (yellow). Arg^{1809} (red) is in close contact with the backbone of Ser^{1738} (dark blue). The H-bond between these residues is also shown (black line).

Table 1 Clinical features of 14 subjects from six families with c.5425C>T change in *NF1*

	Cases														
Features	*Family 1*	*Family 2*	*Family 3*	*Family 4*			*Family 5*					*Family 6*			
				I:2	*II:2*	*III:1*	*I:1*	*II:3*	*II:4*	*III:2*	*III:7*	*I:1*	*II:2*	*III:1*	*Total*
Age (years) at observation	17	16	10	54	35	6	87	65	62	29	21	60	39	3.3	
Sex	M	M	F	F	F	M	F	M	F	F	F	M	F	F	5M/9F
CaLS	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	14/14 (100%)
Freckling	Yes	Yes	Yes		Yes	No		Yes	Yes	Yes	Yes		Yes		9/10 (90%)
Lish nodules	No	No	No			No			No	No	No	No	No	No	0/10 (0%)
Cutaneous neurofibromas	No	No	No		No	No	No	No	No	No	No	No	No	No	0/13 (0%)
Subcutaneous neurofibromas	No	No	No		No	No	No	No	No	No	No	No	No	No	0/13 (0%)
External plexiform neurofibromas	No	No	No		No	No	No	No	No	No	No	No	No	No	0/13 (0%)
Spinal neurofibromas			No												0/1 (0%)
Optic pathway glioma	No[a]	No	No				No	No	No[a]	No	No[a]	No	No[a]	No	0/11 (0%)
Short stature	No	No	No		Yes	No			No		No	No	No	No	1/10 (10%)
Macrocephaly	Yes	No	Yes		No	No			No		No	No	No	No	2/10 (20%)
Other neoplasias	No	No	No		No	No	No		No		No	No	No	No	0/11 (0%)
Thoracic anomalies	PE	PE, SW	PE		No	No	No		No		No	No	No	No	3/11 (27%)
Learning problems	No	Yes	No		No	No	No	No	No	No	No	No	No	No	1/13 (8%)
Scoliosis	No	Yes	No		No	No			No		No	No	Yes	No	2/10 (20%)
Pulmonary valve stenosis	No	No	No		No	No			No		No	No	No	No	0/10 (0%)
NS features		P, TF, CP LSPRE	H, LSPRE, SN		LSE, P	LSE, H			No		No	No	No	No	4/9 (44%)
Others	No	MJL, SH, EEoE, FF, CD, NoPVF	No		No	No			No		No		No	No	

Abbreviations: CD, celiac disease; CP, close palate; EEoE, easy extensibility of the ears; F, female; FF, flat feet; H, Hypertelorism; LSE, low set ears; LSPRE, low set posteriorly rotated ears; M, male; MJL, mild joint laxity; NoPVF, narrowing of the peripheral nasal visual field; NS, Noonan syndrome; P, ptosis; PE, pectus excavatum; SH, skin hyperelasticity; SN, short neck; SW, scapular winging; TF, triangular face.
[a]Absent on MRI scanning of the brain.

prevalence compared with the NF1 general population. None of them had PVS or other congenital heart disease. Notably, half of the examined cases displayed facial features suggestive of Noonan syndrome (NS, MIM #163950), including ptosis, low-set posteriorly rotated ears, hypertelorism, triangular face, as well as short neck.[10]

DISCUSSION

The c.5425C>T missense change had previously been reported as either a rare variant[11] or a NF1-causing mutation.[12,13] In the present study, we provided evidence for its *de novo* occurrence in three sporadic individuals, and cosegregation with the trait in other three pedigrees, including families with three generations. This *per se* provides unequivocal proof that c.5425C>T has functional relevance and underlies a trait within the NF1 phenotypic spectrum. Based on the examined NF1 cohort, we estimate that this variant might account for approximately 1% of *NF1* mutation-positive cases. This frequency, however, may represent an underestimate of its prevalence in the general population as the mild phenotype, which is expected to result in an overlooked diagnosis.

Arg^{1809} is located within the PH-like domain of the GTPase. This domain is adjacent to the C-terminus of the GRD, and forms a bipartite module with the Sec domain.[4] Specifically, Arg^{1809} is located at the end of the C-terminal α-helix, close to the end of PH-like domain. A number of crystal structures of PH domains have been determined. Although residue conservation is not high, their overall structure is well preserved, and characterized by two orthogonal β-sheets made of seven β-strands, followed by an α-helix. The loops of the β-sheets are very different in length, and their structures are difficult to be determined due to their high flexibility,[14] which likely underlies the functional specificity of individual domains. Based on the available Sec/PH-like bipartite module of neurofibromin (RCSB-PDB accession number 2D4Q) Arg^{1809} is solvent exposed, facing away from the protein core (Figure 1c), with its lateral chain hydrogen bonded with the backbone of residue Ser^{1738}, contributing to the intradomain interaction between the C-terminal α-helix and the loop connecting the β2- and β3-strands. These considerations suggest that substitution of Arg^{1806} to cysteine might affect such intradomain interaction, and possibly lead to a rearrangement of the entire secondary structure of the domain with possible impact on lipid-binding properties of the adjacent Sec domain.[4]

The clinical phenotype associated with the c.5425C>T substitution was observed to be mild in all cases, with no patient developing CNFs, SCNFs or evident PNFs. This is remarkable considering that CNFs are an hallmark of NF1, and virtually all NF1 adult patients develop CNFs. These benign tumors are a major cause of morbidity in NF1 and present observation has immediate clinical impact. The absence of subcutaneous and plexiform lesions in present cohort should be taken with more caution. Whereas CNFs are mostly visible and palpable, SCNFs and internal PNFs are more difficult to recognize and quantify. MRI scanning studies should be performed to investigate the presence of internal plexiform tumors among subjects carrying the c.5425C>T change. We also observed a significant lower incidence of LN among these subjects, compared with the general NF1 population (Supplementary Table S1). LN represent one of the most common features of the disease. Based on published data, they were found in two of three members of a family with c.5425C>T substitution and mild features of NF1 and NS without congenital heart disease and CNFs.[13] In another family, this change was associated with CaLS and a severe NS phenotype with several additional defects, such as Arnold–Chiari I malformation, hypoplastic corpus callosum, syringomyelia, hydronephrosis, malrotation of the bowel and premature menopause, coexisting, however, with a *de novo PTPN11* mutation (p.Phe285Leu) in the index patient.[12] It is likely that the athypical symptoms observed in this patient resulted from the additive effect of two mutations

affecting different genes, as reported in some subjects with neurofibromatosis-Noonan syndrome (MIM #601321).[15,16] These observations are in line with the present findings pointing to an association between c.5425C>T change and facial features of NS, including ptosis, low-set posteriorly rotated ears, hypertelorism, short neck and triangular face. Although none of the patients exhibited PVS, accurate cardiac screening on larger cohorts might be considered to assess whether the c.5425C>T substitution results in a higher risk of developing PVS or other congenital heart diseases.

Multigenerational families with multiple CaLS were initially recognized by Riccardi,[17] who suggested the possibility of a disorder distinct from NF1. Consistent with this hypothesis, prior *NF1* mutation testing, linkage studies argued for genetic heterogeneity of this phenotype.[18,19] Successively, studies allowed to identify a 3-bp deletion in exon 22 of the gene (c.2970_2972delAAT, p.Met992del) as responsible for a mild form of NF1 with multiple CaLS and SF as cardinal signs, in the absence of CNFs.[7] Genetic heterogeneity for familial CaLS in association with SF without LN, NFs or other features of NF1, was confirmed by the identification of inactivating mutations in *SPRED1*[8] as the molecular event underlying the NF1 clinically related LS. The present data add a novel genotype–phenotype correlation to the clinical spectrum of NF1. Similarly to the previously reported c.2970_2972delAAT, c.5425C>T causes a mild phenotype with major features consisting in pigmentary signs (CaLS and SF), without neurofibromas or other NF1-related malignancies. The present and published data recommend to consider *NF1* mutation screening in adults with multiple CaLS, with or without SF and no other NF1 diagnostic features, starting from exons 22 and 38 (exons 17 and 29, following the previously used *NF1* exon numbering), before to eventually extend the analysis to the remaining coding exons.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We would like to express our gratitude to the patients and their family, who made this study possible. We thank Angela Alberico and Stefania Cavone for skilfull technical assistance. This work was supported, in part, by funding from AIRC (IG 13360) and Telethon-Italy (GGP13107) to MT, and Italian Ministry of Health (RF-2010-2310935 and RC-2014) to ADL.

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Supplementary Information accompanies this paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)