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ORIGINAL ARTICLE

Expression analysis of *NF1*-mutated alleles in a rare compound heterozygous spinal NF1 patient by digital PCR

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Abstract

Backgroud: Neurofibromatosis type 1 (NF1) is a heterogeneous neurocutaneous disorder. Spinal neurofibromatosis (SNF) is a distinct clinical entity of NF1, characterized by bilateral neurofibromas involving all spinal nerve roots. Although both forms are caused by intragenic heterozygous variants of *NF1*, missense variants have been associated with SNF, according to a dominant inheritance model causing haploinsufficiency. Most patients carry pathogenic variants in one of the *NF1* alleles; nevertheless, patients with both *NF1*-mutated copies have been described. Interestingly, all *NF1* variants carried by the known SNF compound heterozygotes were missense/splicing variants or in-frame insertion-deletions.

Aims: To investigate whether there is a differential expression of *NF1* variant alleles in an *NF1* compound heterozygous SNF patient possibly contributing to clinical phenotype.

Materials & methods: We performed an allele-specific expression study, by chip-based digital PCR, in an SNF family carrying two *NF1* missense variants. We evaluated the expression levels of the two *NF1*-mutated alleles both carried by the compound heterozygous SNF patient and his relatives.

Results: Both alleles were expressed at comparable levels in the patient and hyper-expressed compared to the wild-type alleles of healthy controls.

Discussion: Here we provide new insights into expression studies of *NF1*-mutated transcripts suggesting that a novel pathogenetic mechanism, caused by gain-of-function variants, could be associated with SNF.

Conclusions: Further studies should be performed in larger cohorts, opening new perspectives in the NF1 pathogenesis comprehension.

KEYWORDS

chip-based digital PCR, neurofibromatosis type 1, *NF1* allelic expression, *NF1* double heterozygotes, spinal neurofibromatosis

Paola Bettinaglio and Viviana Tritto contributed equally.

1 | INTRODUCTION

Neurofibromatosis type 1 (NF1) is a monogenic disorder with autosomal dominant inheritance in 50% of cases and de novo in the remaining 50%. It is a clinically heterogeneous neurocutaneous disorder characterized by café-au-lait (CAL) spots, iris Lisch nodules, freckles in the armpits or groin, and multiple neurofibromas (Boyd et al., 2009; Huson et al., 1989). Spinal Neurofibromatosis (SNF) is a distinct clinical entity of NF1, characterized by bilateral neurofibromas involving all spinal nerve roots, with or without other features of classic NF1. SNF shows a greater morbidity than the classic NF1 (Ruggieri et al., 2015).

Overall, 99% of the NF1 cases, genetically analyzed, carry the variant in one of the two copies of the *NF1* gene, consistent with results obtained in animal models, where double *NF1* knock-out variants are lethal (Brannan et al., 1994). Nevertheless, to our knowledge, three patients carrying both the *NF1*-affected copies have been described in the literature. One was a sporadic SNF patient with double *in trans NF1* variants, the missense c.3046T > C (p.Cys1016Arg) in exon 18 and a 3-bp deletion c.8131-8133delGTT (p.2711delVal) in exon 48 (Fauth et al., 2009). The patient showed a severe phenotype, including paraparesis, spinal neurofibromas, and malignant peripheral nerve sheath tumors (MPNSTs) at spinal level, which the authors correlated to the p.Cys1016Arg. The other two patients have been recently described (Paterra et al., 2022). They are two unrelated familial SNF patients: family 1 proband carried the missense c.62T > A (p.Leu21His) and c.528T > A (p.Asp176Glu) variants, family 17 proband carried the splice variant c.3314 + 2T > C, and the missense variant c.7532C > T (p.Ala2511Val). Interestingly, both probands showed a more severe phenotype compared to the other affected relatives, suggesting a contribution of the second *NF1* variant to the clinical phenotype.

Additional reported cases of double variants identified in the *NF1* gene are patients carrying variants *in cis*; therefore, one of the two *NF1* copies remains functional (Hernández-Imaz et al., 2013; Paterra et al., 2022; Terzi et al., 2012).

Given the high mutation rate of the *NF1* gene, it is expected that in a certain percentage of NF1 patients, besides the full *NF1* variant, additional missense *NF1* variant of dubious pathogenic significance may be present in the same affected individual. The widespread application of massive sequencing techniques in diagnosis could reveal compound heterozygotes in *NF1* locus, carrying a full variant and a subclinical variant in the other allele. These genotypes could be correlated to variable expressivity of the NF1 phenotype even in intrafamilial cases.

Another little explored issue is the overall and relative expression level of *NF1* wild-type (WT) and mutated alleles that could be also associated to the phenotype severity.

As RNAs were available from members of family 1, including a compound heterozygote and his relatives described by Paterra et al (2022), we used chip-based digital PCR (cdPCR) to assess the expression profile of the two *NF1*-mutated alleles in the compound heterozygous SNF patient and his relatives, as well as the WT allele in relatives and controls. dPCR is more sensitive than the most widely used real-time PCR (RT-PCR) and, as it is not based on a reference curve, allows allele-specific absolute quantification (D'Alessandra et al., 2022).

The obtained results indicate that both the mutated *NF1* alleles are hyper-expressed in respect to the WT alleles of healthy controls. Our study provides new insight on the expression profile of *NF1*-mutated alleles, little investigated so far, prompting us to speculate on different expression regulations according to the *NF1* variant type, besides opening anew perspectives on NF1 pathogenic models. Further expression studies should be carried out in larger SNF and NF1 cohorts to confirm this hypothesis.

2 | MATERIALS AND METHODS

2.1 | Subjects and segregation analysis of *NF1* variants in compound heterozygotes

Based on a previously reported analysis by Paterra et al. (2022) out of 106 classic NF1 and 75 SNF patients, three familial SNF patients, carrying two variants in the *NF1* gene, were identified (Paterra et al., Table 3) by NGS (Paterra et al. supplementary materials, file S1). The segregation analysis revealed that in family 1, the two *NF1* variants were inherited *in trans* (Figure 1 and Paterra et al., Table 3).

In family 1, the proband (1136) is a 40-year-old man, affected by a severe form of SNF. At age 26, he performed his first spinal magnetic resonance imaging (MRI) due to the occurrence of weakness of the lower limbs showing symmetrical bilateral intraforaminal neurofibromas along all the nerve roots. Seven years later, he developed tetra paresis and MRI showed the occurrence of myelopathy at cervical level. The patient had spinal surgery; however, after 2 years, his conditions start to deteriorate again. Now he is unable to walk with severe weakness of the upper limbs and sphincter paralysis.

The brother (1140), aged 39, is also affected by an SNF with also a plexiform neurofibroma. However, he performed his first spinal MRI at 32 when he was asymptomatic. At present, despite the presence of neurofibromas along all nerve roots, both intradural and paraspinal (i.e.,

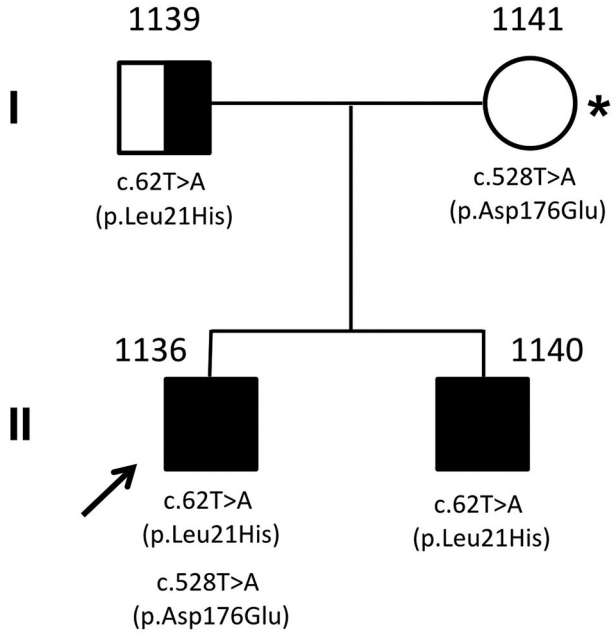

FIGURE 1 Pedigree of family 1 showing neurofibromatosis type 1 (*NF1*) variants. The segregation analysis showed that patient 1136 is a compound heterozygote in the *NF1* locus. Black filled symbols, spinal neurofibromatosis (SNF) patients; black and white filled symbol, subject with multiple neurofibromas few spinal root (MNFSR); *, suspected diagnosis of NF1; arrow, proband.

tumor epicentered lateral to the neuroforamina, having a large soft-tissue component outside the spinal canal), he can walk, complains mild upper and lower limbs weakness and lumbar pain. A subcutaneous neurofibromas in the left leg was removed.

The father (1139), aged 65, is affected by neurofibromas of multiple but not all spinal roots (MNFSR, multiple neurofibromas few spinal roots). The mother (1141) is a 57-year-old woman, not affected by NF1 according to the revised diagnostic criteria for NF1 but showing two CAL spots and at MRI scan with gadolinium several small (diameter of less than 1 cm) nodular-enhancing lesions in the laterocervical soft tissues, suggestive of neurofibromas, but no further sign of NF1.

The proband (1136) carries the NM_001042492.3: c.62T > A (p.Leu21His) missense variant in exon 2 of *NF1*, inherited from his MNFSR father (1139) and shared with his SNF brother (1140). Furthermore, patient 1136 has a second missense variant NM_001042492.3: c.528T > A (p.Asp176Glu) in *NF1* exon 5 inherited from the mother (1141) assessing that the 2 missense variants are inherited *in trans* by the proband.

The patient 1493, aged 42, is a symptomatic sporadic case of SNF with cutaneous (1–10) and subcutaneous (1–10) neurofibromas, CAL spots (11–100), axillar frecklings, Lisch nodules, and macrocephaly.

All medical records were surveyed, and data were collected at the time of genetic screening and reverified at IRCSS C. Besta Neurological Institute. The RNA samples of family 1 were provided by the IRCSS C. Besta Neurological Institute biobank.

All the patients gave informed consent to the approved study by the Fondazione IRCCS Istituto Neurologico Carlo Besta Ethical Committee and Scientific Board (No 50-19/3/2018).

2.2 | Chip-based digital PCR on *NF1* double variants

The cdPCR analysis has been carried out on all members of family 1 and from six healthy male controls with an average age of 29 years (Figure S1).

The RNA was isolated from the peripheral blood mononuclear cells, by using the "TempusTM Spin RNA Isolation" Kit (Applied Biosystems). Subsequently, 500 ng of total RNA from each patient was reverse transcribed with the Maxima H Minus cDNA Synthesis Master mix.

Each cDNA sample was quantified by RT-PCR method using 18S rRNA as the target (Figure S2).

5 ng of cDNA was amplified (in triplicate) in 10 μL containing 5 μL of "TaqMan Fast Advanced Master Mix, Thermofisher," 0.25 μL of "TaqMan SNP Gene expression assay 40×, Thermofisher" FAM and VIC labeled. The Taqman probes were designed at the level of the *NF1* (NF1:NM_001042492.3) c.62T > A, c.528T > A, and c.2446C > T variants, which are included in a region of homology of type I and type II *NF1* transcripts. The specific probes for the WT c.62T, c.528T, and c.2446C alleles were VIC-dye labeled and that specific for the mutated c.62A, c.528A, and c.2446T alleles were FAM-dye labeled (Table S1).

RT-PCR was carried out on the QuantStudio 12K (Thermofisher), using a pre-PCR step of 20 s at 95°C, followed by 40 cycles of 1 s at 95°C and 20 s at 60°C. One no template control (water) sample was run.

cdPCR was performed on 2.5 ng of cDNA amplified in 15 μL containing the following reagents: 7.5 μL of "QuantStudio 3D Digital PCR Master Mix v2, Thermofisher," 0.375 μL of "TaqMan SNP Gene expression assay 40x, Thermofisher" FAM and VIC labeled. The mix was loaded on the chip using the QuantStudio 3D Digital PCR Chip Loader.

The chips were then loaded on the Proflex PCR System (Thermofisher), and the PCR was carried out using a pre-PCR step of 10 min at 96°C, followed by 39 cycles of 2 min at 60°C and 30 s at 98°C, followed by 2 min at 60°C. Data were analyzed with "QuantStudio 3D Analysis Suite Cloud Software."

2.3 | LOH analysis in patients' tumors

The loss of heterozygosity (LOH) study has been carried out on the DNA obtained from a resected cervical root neurofibroma from the proband (1136) and on the DNA from dermal neurofibromas resected from his brother (1140), of family 1. The LOH detection has been assessed by Sanger sequencing by verifying the presence or loss of heterozygosity at the NM_001042492.3: c.62T *NF1* locus, where both patients shared the NM_001042492.3: c.62T > A *NF1* variant and the WT allele at constitutional level.

2.4 | *In-silico* prediction of protein stability of *NF1* variants

We submitted the pdb files of *NF1* to the DynaMut tool (http://biosig.unimelb.edu.au/dynamut/) for the prediction of protein stability changes and conformational changes upon NF1: c.62T > A (p.Leu21His) and c.528T > A (p.Asp176Glu) variants of family 1.

A difference in Gibbs free energy ($\Delta\Delta G$) < −0.05 indicates a significant destabilizing effect of the variant, whereas a $\Delta\Delta G > +0.05$ indicates a significant stabilizing effect.

3 | RESULTS

3.1 | Expression analysis of the *NF1*-mutated alleles by cdPCR

To establish whether the mutated alleles were both expressed, and which was their level in the analyzed members of family 1, we performed cdPCR with specific labeled probes (Figure S1a,b). The cdPCR analyses have been carried out by using probes specific for c.62T and c.62A *NF1* variants, detecting the expression of the WT and the NM_001042492.3: c.62T > A (p.Leu21His) mutated allele, and for the c.528T and c.528A *NF1* variants, detecting the expression of the WT and the NM_001042492.3: c.528T > A (p.Asp176Glu) mutated allele, to establish the amount of each transcript in the compound heterozygous patient 1136, in the relatives carrying one of the two mutated *NF1* copies and in a control. The overall amount of the *NF1* transcripts is about 4 times higher in the proband compared to the control, 1.7 times higher compared to his father and brother (Figure 2a), and 1.4 times compared to his mother (Figure 2b). The overall amount of *NF1* mRNA, including WT and mutated transcripts, expressed by the affected relatives, is more than twice higher compared to the *NF1* expression in the healthy control (Figure 2a,b). Moreover, the mutated alleles are expressed at comparable level in the compound heterozygous, as well as the mutated and WT alleles in the relatives. Interestingly, not only the mutated transcripts but also the WT transcripts are hyper-expressed in the relatives compared to the control.

To evaluate possible different effects on the *NF1* expression, associated to missense or to a stop gain variant normally present in NF1 patients, we carried out a cdPCR assay in the 1493 patient, a sporadic SNF case, carrying an *NF1* stop gain variant, NM_001042492.3: c.2446C > T (p.Arg816*), in heterozygous condition. We used as healthy control the same RNA sample analyzed for the cdPCR study carried out on family 1 and an RNA pool from five healthy controls to minimize the effect of the *NF1* expression variability.

The overall amount of WT and mutated transcript in the patient is 1.3 times higher compared to the *NF1* expression in the WT and 1.5 in the pooled WT RNAs. The cdPCR analysis showed that the total *NF1* transcript consisted of 22% of mutated allele c.2446C and 78% of WT allele, as expected for stop gain variants (Figure 3).

3.2 | LOH study in patients' tumors

To verify whether there is preferentially loss of one of the two mutated alleles, the LOH study has been carried out, by Sanger sequencing, on resected cervical root neurofibroma of patient 1136 and on dermal neurofibromas of his brother (1140), of family 1.

No LOH within the *NF1* locus was observed in the tumoral tissues of the composite heterozygous proband (1136) and in his SNF brother (1140), both showing the germinal WT and the mutated c.62A allele. A similar picture was obtained in the proband after sequencing of NM_001042492.3: c.528T > A locus (Figure 4a–c). Nevertheless, patient 1140 showed in tumoral tissue the stop gain variant NM_001042492.3: c.5839C > T (p.Arg1947*) that should inactivate the function of one *NF1* copy (Figure 4d).

3.3 | *In-silico* prediction of protein stability of *NF1* variants

In family 1, variations are present within *NF1* exons 2 and 5, out of the region coding the Cysteine Serine Rich Domain. No specific proteins interacting in this N-terminal part of neurofibromin have been reported or predicted by *in-silico* analysis. However, we cannot exclude that the conformational changes, due to the presence of the variants, could

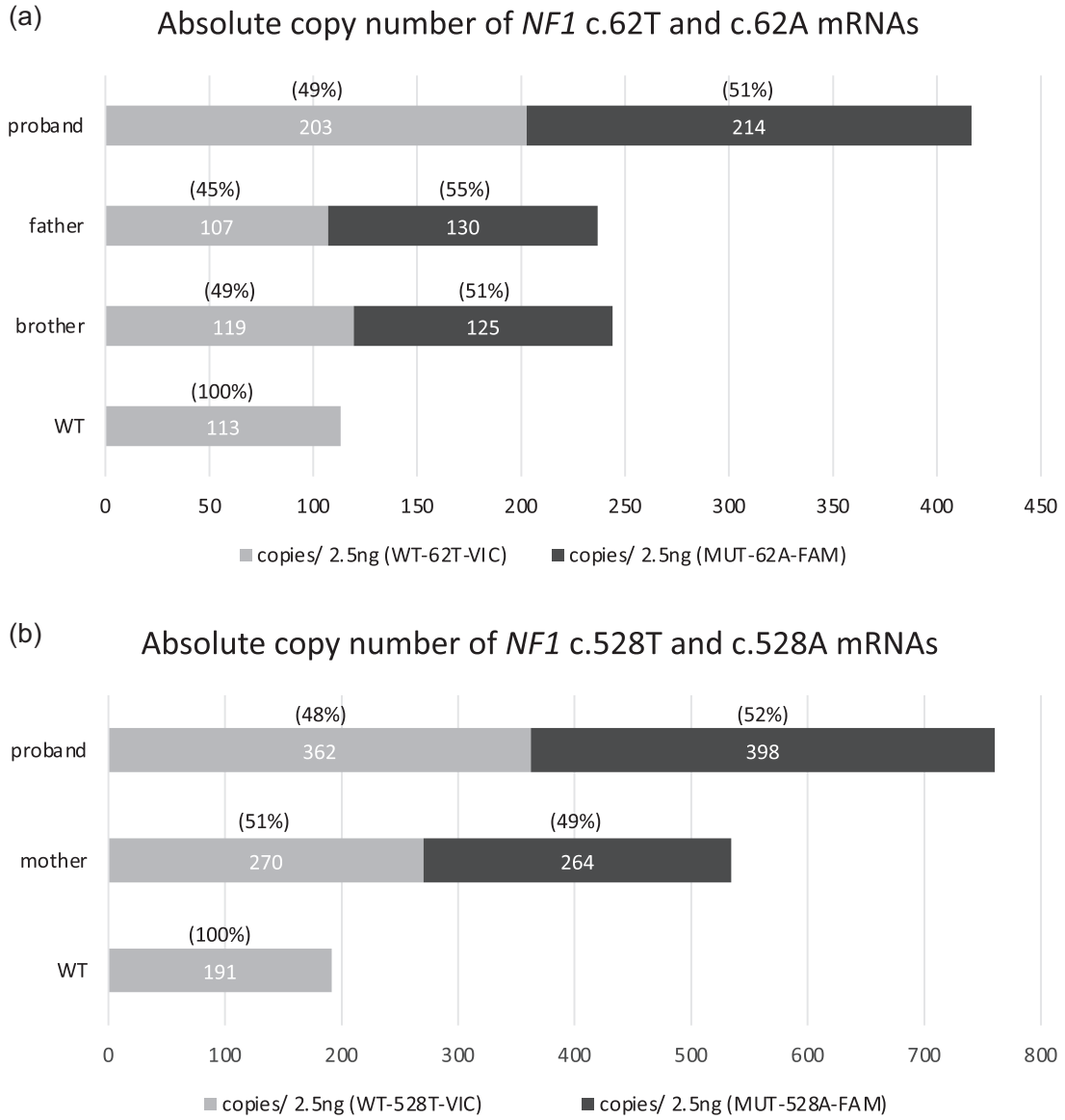

FIGURE 2 Absolute quantification of neurofibromatosis type 1 (*NF1*) c.62T > A (a) and c.528T > A (b) allelic-specific mRNAs in proband (patient 1136) and relatives of family 1 by chip-based digital PCR (cdPCR). The number of mRNA copies from each allele present in 2.5 ng of total mRNA is indicated in the histogram as well as the relative percentage.

affect protein–protein interactions. To address this issue, we calculated the energy variation expressed in Gibbs free energy between the wild-type and mutated proteins by a bioinformatic tool (DynaMut). The prediction tool calculated a $\Delta\Delta G$ of +1.913 kcal/mol, suggesting a stabilizing effect of the c.62T > A (p.Leu21His) missense variant, whereas it was calculated a $\Delta\Delta G$ of +0.131 kcal/mol for the c.528T > A (p.Asp176Glu) missense variant, suggesting a possible, but not significant, stabilizing effect. Interatomic interactions are altered in the mutant compared to the wild-type proteins upon c.62T > A (p.Leu21His) (Figure 5, panels a and b) and c.528T > A (p.Asp176Glu) (Figure 5, panels c and d) missense variants.

4 | DISCUSSION

Here we report on the expression analysis of *NF1*-mutated alleles transcribed by heterozygous and compound heterozygous *NF1* mutated genotypes of previously described family 1's members (Paterra et al., 2022). Mutation analysis of the *NF1* gene in family 1 with both MNFSR and SNF patients revealed a composite heterozygous condition for the *NF1* locus in the SNF proband 1136. Interestingly, the SNF patient carrying the *in trans* double *NF1* missense variants, as well as another unrelated SNF patient carrying *in trans NF1* variants, showed a more severe and complex phenotype than their relatives, suggesting a contribution

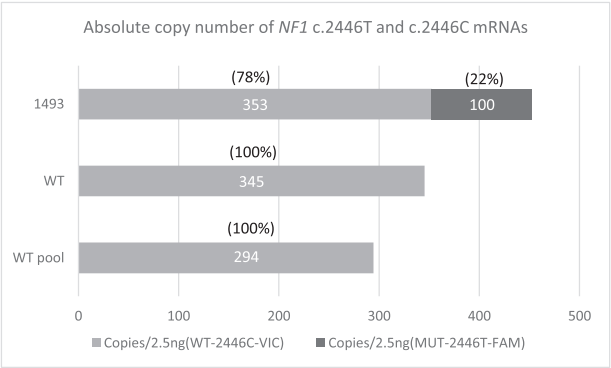

FIGURE 3 Absolute quantification of neurofibromatosis type 1 (*NF1*) c.2446C > T allelic-specific mRNAs in patient 1493 with the *NF1* stop gain variant, in a healthy control (wild-type [WT]) and in a pool of five healthy controls (WT pool) by chip-based digital PCR (cdPCR). The number of mRNA copies from each allele, present in 2.5 ng of total mRNA, is indicated in the histogram as well as the relative percentage.

of the second variant to the phenotype. A similar pattern has been described for family 17 by Paterra et al. (2022).

According to Mauda-Havakuk et al. (2017) in NF1 patients with spinal involvement, two features correlate with clinical presentation and outcome: tumor burden and neurofibromas subtype, in particular, the presence of kissing neurofibromas at cervical level is a risk factor for a greater morbidity. Furthermore, an association between clinical outcome and the presence of neurofibromas at cervical region and intradural involvement has also been reported (Patronas et al., 2001; Taleb et al., 2011).

In family 1, both brothers displayed a severe burden of spinal phenotype presenting with multilevel disease. However, patient 1136 had symmetrical bilateral kissing neurofibromas and showed an early and quick tumor growth according to REINS criteria (Plotkin et al., 2013) and a greater morbidity due to cervical myelopathy.

It is worth noting that the two pairs of *NF1* variants inherited in families 1 and 17, described by Paterra et al. (2022), have different pathogenicity scores and different $\Delta\Delta G$s calculated by an *in-silico* conformational analysis. In family 1, the NM_001042492.3: c.62T > A (p.Leu21His) missense variant was shared by the father, the proband, and his brother and seems to be associated to an SNF condition. In fact, the pathogenicity predictive results (ANNOVAR; Wang et al., 2010) showed 17/20 deleterious predictors. Furthermore, this variant, never described in ClinVar (Landrum et al., 2014) is not present in the populations of the 1000 genomes (Auton et al., 2015) and Exac databases (Lek et al., 2016). The $\Delta\Delta G$ between the wild-type and mutated protein upon the c.62T > A (p.Leu21His) variant is 1.913 kcal/mol, suggesting a significant stabilizing effect of the c.62T > A (p.Leu21His) missense variant. The mother (1141) carries the missense variant NM_001042492.3: c.528T > A (p.Asp176Glu) and displays three CAL spots, suggesting a subclinical significance of the *NF1* variant which should maintain a residual function of neurofibromin. The association of this variant with a subclinical phenotype is consistent with the pathogenicity predictive results showing 10/20 deleterious predictors (ANNOVAR). Furthermore, according to the InterVar analysis, the variant is classified as benign, whereas ClinVar predicts the conflicting interpretation of variant pathogenicity. The *in-silico* conformational analysis on c.528T > A (p.Asp176Glu) showed a not significant difference in stability between WT and mutated protein upon this variant.

Similarly, in the family 17 the splice variant NM_001042492.3: c.3314 + 2T > C is present in the SNF or MNFSR patients, indicating an association with the SNF form, whereas the second missense variant NM_001042492.3: c.7532C > T (p.Ala2511Val) is present alone only in the NF1 classic subject, displaying plexiform neurofibromas.

The splicing variant c.3314 + 2T > C is located at 1 bp far from the canonical splicing site, and it has been described as pathogenic in ClinVar in another patient with NF1. We conducted an *in-silico* analysis by interrogating 10 predictors: 8 predictors (dbscSNV, Eigen, Eigen PC, FATHMM, Polyphen2 HDIV, Polyphen2 HVAR, BayesDel addAF, and BayesDel noAF) out of 10 classified the variant as "pathogenic," whereas 2 predictors (Dann and Mutation Taster) out of 10 as "uncertain," according to the ACMG criteria. The variant c.7532C > T (p.Ala2511Val) is predicted to be damaging by 10/20 predictors (ANNOVAR).

Interestingly, the proband of family 17, carrying both *NF1* variants, shows CAL spots, axillar freckling, UBOs, Lisch nodules, and cutaneous and subcutaneous neurofibromas, besides the SNF form, usually without the cutaneous traits. The copresence of the SNF-associated allele with a second *NF1* mutated allele in both probands, correlated to a more severe phenotype, indicates that the neurofibromin encoded by one of the two alleles maintains a partial function.

To our knowledge, besides this study and the data provided by Paterra et al., only one describes an *NF1* compound heterozygous patient (Fauth et al., 2009). Three studies reported on two *in cis* double mutated NF1 patients. As the other *NF1* copy was WT for both patients, thus these variants mimic the genetic condition of a typical NF1 patient (Hernández-Imaz et al., 2013; Paterra et al., 2022; Terzi et al., 2012).

Interestingly, the compound heterozygous case for the *NF1* locus described by Fauth et al. (2009) was an SNF patient, carrying a missense and 3-bases deletion allele. The authors assumed that p.2711delVal is a most likely

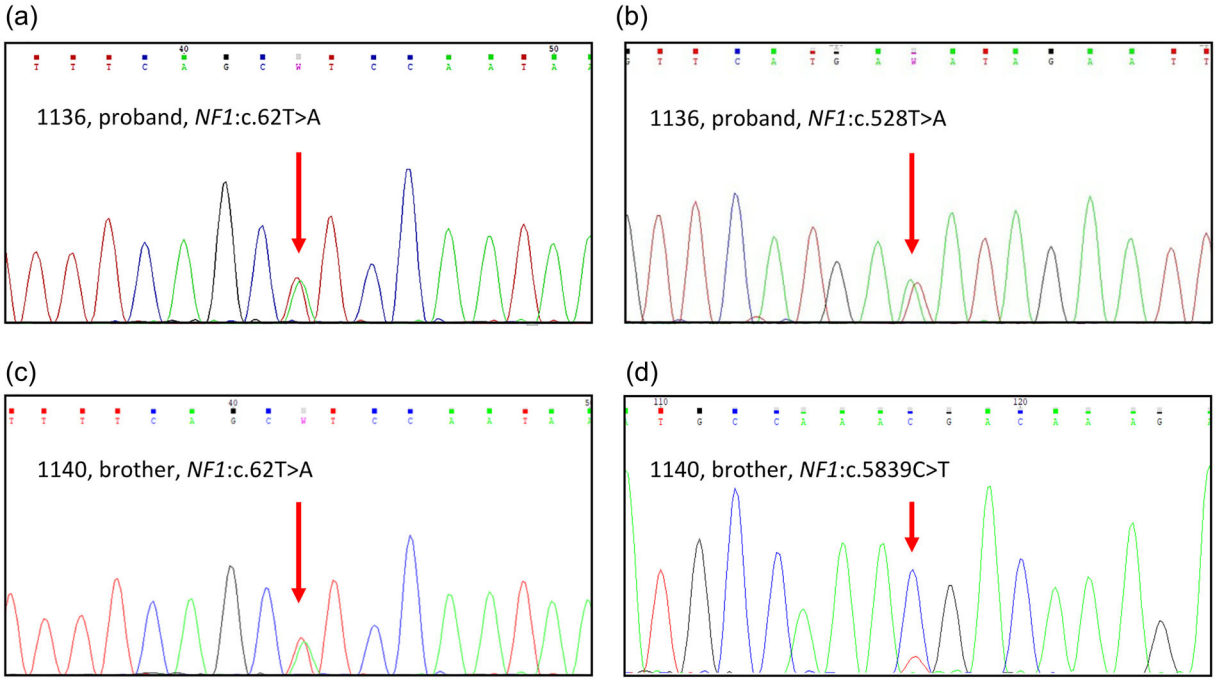

FIGURE 4 Partial electropherogram of neurofibromatosis type 1 (*NF1*) exon 2 (a and c), exon 5 (b), and exon 31 (d) of DNA sequences obtained from neurofibromas resected from patients 1136 and 1140: (a) DNA from patient 1136 heterozygous for the *NF1* c.62T > A variant in exon 2. (b) DNA from subject 1136 heterozygous for *NF1* c.528T > A variant in exon 5. (c) DNA from patient 1140 (brother) heterozygous for the *NF1* c.62T > A variant in exon 2, and (d) DNA from patient 1140 (brother) heterozygous for the *NF1* c.5839C > T variant in exon 31.

benign unclassified variant, and p.Cys1016Arg represents the pathogenic variant responsible for a severe phenotype of patient, characterized by a mild dermal feature, paraparesis, spinal neurofibromas, and MPNSTs at spinal level. All the described compound heterozygous cases reported in this study and in literature show, besides the *NF1* causative variant, a subclinical *NF1* variant on the homologous chromosome 17. The compound heterozygous condition is consistent with a viable genotype. In fact, the loss-of-function *NF1* variants have been never detected in homozygous status, having been demonstrated to be lethal in the null $nf1^{-/-}$ mouse (Brannan et al., 1994).

Interestingly, the compound heterozygote here described and those reported in literature (Fauth et al., 2009) are affected by SNF. One explanation could be that they carry *NF1* missense variants, recently associated to SNF form and probably causing a gain of function by the mutant neurofibromin that seems to be correlated to the development of the SNF form (Paterra et al., 2022; Ruggieri et al., 2015). Nevertheless, additional cases should be studied to verify a specific association between the heterozygous compound genotype and SNF.

The condition of *NF1* compound heterozygotes is not usually verified in NF1 patients due to the complex setting up of analytical methods of *NF1* variant detection, before NGS diagnostic application. The *NF1* variants with uncertain pathogenic significance are probably underestimated, as well as the *NF1* compound heterozygotes that could account for the variable expressivity of the disease even in familial cases (Easton et al., 1993; Sabbagh et al., 2009; Szudek et al., 2002). The widespread use of NGS analysis in diagnosis could provide a correct estimation of this condition.

To understand the pathogenic potential of the *NF1* variants, it should be important to evaluate the expression level of the overall amount of the *NF1* transcripts and the differential allelic expression of the *NF1* alleles. This aspect is poorly investigated, and little is known about the differential allelic expression in NF1 patients. In the 1990s, differential expression studies of *NF1* were carried out that showed unequal expression of the two *NF1* alleles in a portion of NF1 patients ranging from 28% to 75% (Cowley et al., 1998; Hoffmeyer et al., 1994; Skuse & Cappione, 1997). In addition, in three patients, it has been shown that the allele with reduced expression was the one mutated (Hoffmeyer et al., 1994). Jentarra et al. (2012) stated that the association between *NF1* variants and reduced *NF1* expression levels might suggest that the pathogenetic mechanism underlying the NF1 is the *NF1* haploinsufficiency, rather than toxic gain-of-function. Furthermore, they have provided evidence that approximately 30% of healthy control individuals showed significant variation in the expression level of the two *NF1* alleles. Therefore, differential allelic expression of *NF1* in subjects carrying pathogenic variants could

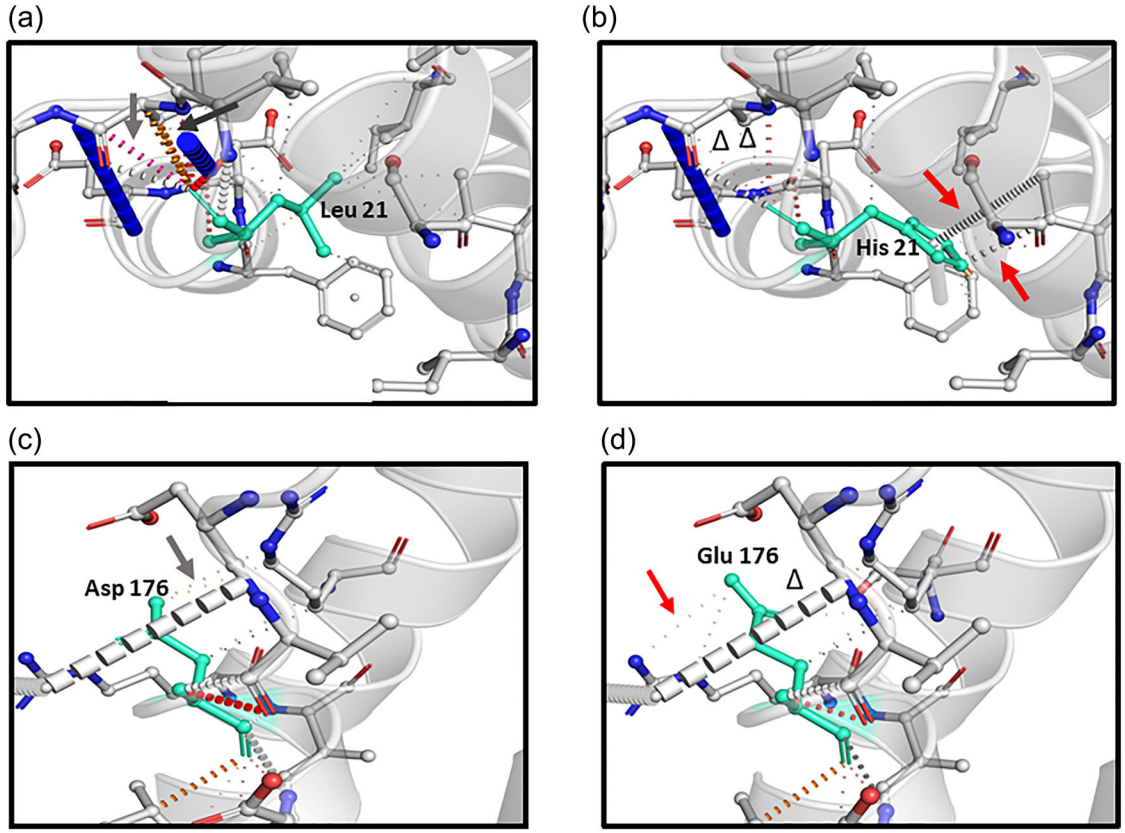

FIGURE 5 Models of wild-type and mutated neurofibromatosis type 1 (NF1) proteins. (a) Prediction of interatomic interactions due to the wild-type Leu residue at 21 position. The black arrow indicates a weak hydrogen bond that is disrupted in the mutant protein (Δ symbol, panel b), and the gray arrow indicates carbonyl contacts that are disrupted in the mutant protein (Δ symbol, panel b). (b) Prediction of interatomic interactions due to substitution of Leu residue 21 by Cys. The mutant protein acquires two new ionic bonds (red arrows, panel b). The variant is stabilizing: $\Delta\Delta G$: 1.913 kcal/mol. (c) Prediction of interatomic interactions due to the wild-type Asp residue at 176 position. The grey arrow indicates two ionic bonds that are disrupted (Δ symbol, panel d), in the mutant protein. (d) Prediction of interatomic interactions due to substitution of Asp residue 176 by Glu. The mutant protein acquires two ionic bonds (red arrow, panel d). The variant is stabilizing: ΔΔG: 0.131. Color legend: the amino acidic residues at 21 and 176 positions in light green, weak hydrogen bonds in orange, carbonyl contacts in pink, water mediated hydrogen bonds in light red dotted lines, ionic bonds in gray dotted lines, and halogen bonds in dark blue dotted lines.

be a possible mechanism for the phenotypic variability shown by patients (Jentarra et al., 2012).

As mRNAs were available from four family 1's members, including the compound heterozygote, we applied cdPCR technique to assess the expression level of the two mutated alleles, both comparing them in the *NF1* compound heterozygotes, and considering the expression of each mutated allele in respect to the WT allele in both the *NF1* heterozygous patients and in normal controls. Unlike RT-PCR, cdPCR is more sensitive and does not need any reference curve, also providing allele-specific absolute quantification (Basu et al., 2017; D'Alessandra et al., 2022). Our study provides original data on the expression of mutated and WT *NF1* alleles in blood samples from SNF and NF1 patients of the same family. We observed the hyper-expression of both mutated alleles in the proband and of mutated and WT alleles in his relatives. As the analyzed *NF1* mutated transcripts carry missense variants, we performed a differential allelic expression study in a patient carrying an *NF1* stop-gain variant. Our findings indicate that the expression of stop-gain mutated allele is strongly reduced compared to the WT allele, as expected according to the nonsense-mediated-decay mechanism, commonly activated when stop-gain variants occur (Doma & Parker, 2007). Moreover, we included a healthy control derived by a pool of five healthy individuals allowing us to verify the variability of the WT expression level. If the hyperexpression of a missense mutated *NF1* allele could be associated to a specific variation type, the observed hyperexpressed WT allele in heterozygous patients suggests the involvement of an *in trans* epigenetic mechanism affecting the WT allele regulation. Furthermore, the detection of increasing level of missense mutated *NF1* transcripts indicates that not only the *NF1* haploinsufficiency is the causative mechanism of NF1, but also the hyperexpression of missense variants causing gain-of-function in a protein

as complex as neurofibromin could play a role in NF1 pathogenesis.

The hyperexpression of the overall amount of *NF1* in the family 1's patients as compared to healthy controls has been also confirmed by qPCR (data not shown), confirming the results obtained by cdPCR.

This is a pilot study that suggests investigating whether there is a correlation between the type of variant and the expression level of *NF1*. For this purpose, it would be necessary to compare the levels of both total and differential expression of the two *NF1* alleles in a larger cohort of patients, carrying missense and stop-gain variants.

We also checked the *NF1* LOH in tumor samples derived from cervical roots and peripheral nerve, available from the SNF proband and his brother of family 1, respectively. The cervical root tumor did not show any LOH of the *NF1* region, whereas the peripheral nerve tumor showed, besides the constitutional variant, a further stop gain variant. According to the findings provided by studies on tumoral DNA, generally in tumors, occurs a second hit inactivating the WT. In patient 1140, it is not possible to establish whether the tumoral stop gain variant is *in cis* or *in trans* with respect to the constitutional *NF1* variant, even if it is expected to inactivate the WT allele.

Most of the studies addressing the LOH of *NF1* in the literature are performed on cutaneous neurofibromas, reporting a percentage of LOH in the tumoral tissues ranging from 2.26% to 32% (Colman et al., 1995; Dä et al., 1997; Serra, et al., 1997). The only study performed on spinal neurofibromas in SNF patients, by Upadhyaya et al. (2009), identified *NF1* LOH in 8/22 of the studied spinal tumor tissues. The absence of LOH in our SNF patient is consistent with the previously reported findings and may be consistent with a gain-of-function significance of both variants, thus contributing to the tumoral phenotype of the patients.

5 | CONCLUSIONS

The missense variants could have a functional significance in the pathogenesis of the different forms of *NF1*. Among the data provided by a wide literature in the field, the frequency of compound heterozygotes is missing. The copresence of a subclinical variant with the full *NF1* variant could explain the different degrees of the phenotype severity, as it could contribute to the clinical phenotype by an additive effect on molecular pathway/s activation. Moreover, the relative variable expression of *NF1* WT and mutated alleles could concur to the variable expressivity of the disease. The hyperexpression of the alleles with missense *NF1* variants and, in general, the overall expression of *NF1* missense mutated transcripts is a new insight suggesting the involvement of additional unexplored NF1 pathogenic models. These results, if confirmed in a wider cohort of NF1 and SNF patients, raise an obvious question: could be a new pathogenic mechanism, different from *NF1* haploinsufficiency, caused by loss-of-function variants, possibly associated to SNF form? Further expression studies should be carried out in larger cohorts to confirm this hypothesis, allowing to elucidate the role of specific *NF1* gene alterations in the different forms of NF1, thus improving a more effective diagnosis, counseling, patient management, and the development of a tailored personalized medicine.

AUTHOR CONTRIBUTIONS

Conceptualization: Paola Bettinaglio, Paola Riva. *Methodology*: Paola Bettinaglio, Viviana Tritto, Rosina Paterra, Marica Eoli. *Investigation*: Paola Bettinaglio, Viviana Tritto, Rosina Paterra, Marica Eoli. *Resources*: Paola Riva, Marica Eoli. *Data curation*: Paola Bettinaglio. *Writing—original draft preparation*: Paola Bettinaglio, Paola Riva. *Writing—review and editing*: Paola Bettinaglio, Paola Riva, Viviana Tritto, Marica Eoli. *Supervision*: Paola Riva. *Project administration*: Paola Riva. *Funding acquisition*: Paola Riva. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw reads of NGS data are available in NCBI Short-read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra/) under the accession number PRJNA8509016.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Fondazione IRCCS Istituto Neurologico Carlo Besta Ethical Committee and Scientific Board (No 50- 19/3/2018).

INFORMED CONSENT STATEMENT

Written informed consent was obtained from all subjects involved in the study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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