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CLINICAL REPORT

Things come in threes: A new complex allele and a novel deletion within the *CFTR* gene complicate an accurate diagnosis of cystic fibrosis

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Abstract

Background: Despite consolidated guidelines, the clinical diagnosis and prognosis of cystic fibrosis (CF) is still challenging mainly because of the extensive phenotypic heterogeneity and the high number of *CFTR* variants, including their combinations as complex alleles.

Results: We report a family with a complicated syndromic phenotype, which led to the suspicion not only of CF, but of a dominantly inherited skeletal dysplasia (SD). Whereas the molecular basis of the SD was not clarified, segregation analysis was central to make a correct molecular diagnosis of CF, as it allowed to identify three *CFTR* variants encompassing two known maternal mutations and a novel paternal microdeletion.

Conclusion: This case well illustrates possible pitfalls in the clinical and molecular diagnosis of CF; presence of complex phenotypes deflecting clinicians from appropriate CF recognition, and/or identification of two mutations assumed to be *in trans* but with an unconfirmed status, which underline the importance of an in-depth molecular *CFTR* analysis.

KEYWORDS

CFTR gene, complex allele, cystic fibrosis, deletion, molecular diagnosis

1 | INTRODUCTION

Cystic fibrosis (CF; MIM#219700) is an autosomal recessive disorder caused by mutations in *CFTR* (CF transmembrane conductance regulator; MIM#602421; GenBank: NM_000492.3), the gene encoding the homonymous chloride-bicarbonate channel (Shteinberg et al., 2021).

Nowadays, CF is one of the most common life-threating diseases with an incidence of 1/3000 in the European population (Scotet et al., 2020; Southern et al., 2007).

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Making a CF diagnosis is a delicate multi-stage process that includes evaluation of typical clinical picture and CFTR dysfunction (typically via sweat chloride test, SCT), as well as identification of biallelic causal *CFTR* variants (Farrell et al., 2017). Even in case of a positive SCT in newborn screening (NBS) (Farrell et al., 2008), all CF subjects should be genotyped, and their variants studied by segregation analysis (Castellani et al., 2009).

Despite well-established directives, the clinical diagnosis and prognosis of CF may still be hampered by the extensive phenotypic heterogeneity, mirroring the high number of *CFTR* variants (466 variants annotated hitherto, https://cftr2.org/mutations_history [last update: 24 September 2021]) and, especially, of their combinations. In these terms, the existence of alleles with two (or more) variants *in cis*, named complex alleles, further challenges the establishment of a clear genotype–phenotype correlation (El-Seedy et al., 2012; Terlizzi et al., 2017). Unless the variants of a complex allele have been found separately, their single functional and phenotypic effect (worsened or ameliorated) or their potential synergistic action could indeed be evaluated only through in vitro studies (El-Seedy et al., 2012; Terlizzi et al., 2017).

Herein, we report a young girl affected by an undefined syndrome, who resulted to have CF. Segregation analysis within her family was central to unveil three *CFTR* variants, including two known CF-causing variants on the same allele and a novel intragenic microdeletion. This case represents a good example to discuss pitfalls in the clinical and molecular diagnosis of CF.

2 | RESULTS

A 6-year-old girl of Albanian descent (II-1, Figure 1) and with an unremarkable family history was referred to the medical geneticists for short stature with some dysmorphic physical features. All the methods and materials used in this study are reported as Supporting Information.

Born at term after a pregnancy complicated by an increased nuchal fold, she presented meconium ileus at the first day of life, but negative CF NBS with only one disease-causing mutation reported (c.1521_1523del; p.Phe508del). Despite the first suspicion of CF, specific clinical evaluations were performed just after two episodes of intestinal sub-occlusions.

The girl had also a history of chronic constipation, cough, and growth delay. The investigations revealed a multifaceted clinical picture, including short-limb dwarfism, delayed bone age, hepatomegaly with mildly elevated transaminases, malabsorption of pancreatic origin, and some lung atelectasis. Morphologic features shared with both the father and the paternal grandfather included midface hypoplasia, macrocephaly, mild frontal bossing, and arched tibia.

Standard karyotyping, as well as molecular karyotyping of the proband and her parents by SNP-array did not identify any potential chromosomal alteration.

The wide plethora of proband's clinical features led us to hypothesize a dominantly inherited skeletal dysplasia (SD) and/or CF. However, it was not possible to determine the SD causative gene neither sequencing the strong candidate *FGFR3* (fibroblast growth factor receptor 3) nor analyzing the other candidates enlisted in Mortier et al. (2019) by whole exome sequencing (WES).

On the contrary, the CF diagnosis was supported by positive SCT (100 mEq/L, 241 mg). Indeed, targeted next generation sequencing (NGS) (detection rate of CF alleles about 97%) revealed two heterozygous variants: the already identified c.1521_1523del (p.Phe508del; MAF = 0.007068) and c.1420G>A (p.[Glu474Lys]; MAF = 0.000004). Representing 66% of *CFTR* alleles worldwide, p.Phe508del has an unquestionable pathogenic effect. The other variant, c.1420G>A, is rare among the CF affected individuals and, in-line with bioinformatic predictions, it is reported as deleterious in databases (see Supporting Information).

However, the segregation analysis revealed that the two mutations were *in cis*, maternally inherited as a complex allele. The data were confirmed by cloning assay (Figure 1) and allelic-specific PCR, ruling out, albeit very rare, a recombination event between the two variants at least in the proband (we could not exclude a recombination occurred in the ancestors of the maternal family branch). Therefore, we hypothesized that the paternal *CFTR* allele could be a variant (e.g., small copy number variation [CNV], deep intronic variant) not explored by the previous analyses. We thus performed a statistical evaluation of the amplicon coverage from the NGS data, which allowed us to predict a deletion of *CFTR* exons 20 and 21 (Figure 2a). GAP PCR (Figure 2b, Table S1) and long-range PCR followed by Sanger sequencing (Figure 2c,d, Table S1) consistently confirmed the paternal inheritance of a novel *CFTR* deletion (c.3158_3468+3219del).

3 | DISCUSSION

The case reported is a good example to discuss pitfalls in the clinical and molecular diagnosis of CF, which traditionally relies on the coexistence of clear clinical picture, positive SCT and identification of biallelic disease-causing *CFTR* variants.

The proband had CF features since the newborn period, including meconium ileus, regarded as the earliest manifestation of CF (20% of infantile cases) (Sathe & Houwen, 2017) and a poor prognostic factor (Gorter

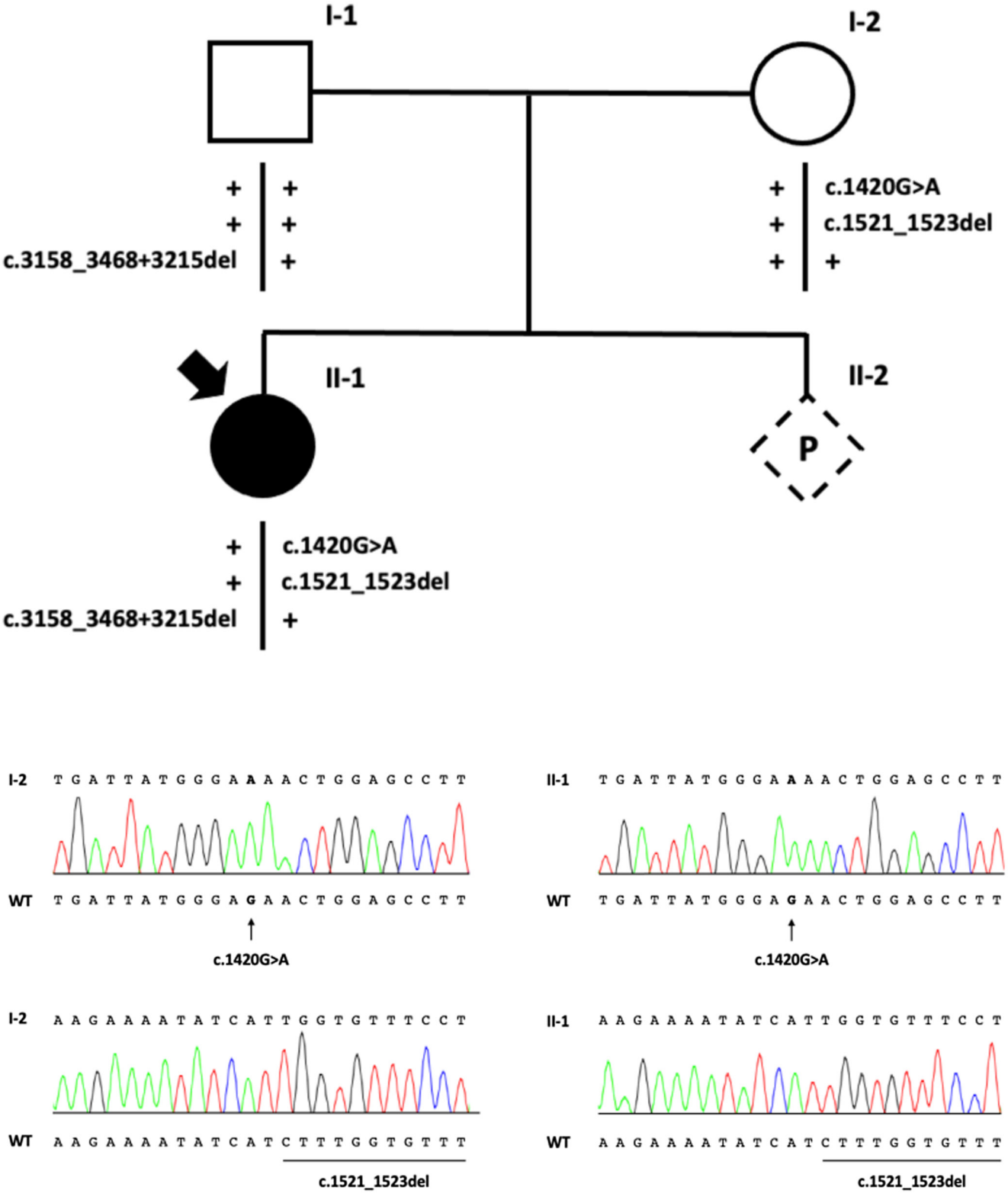

FIGURE 1 Clinical and genetic analyses of the family. Familial pedigree; the black arrow indicates the proband, the plus symbols (+) represent the wild-type alleles at different loci. The electropherograms show the c.[1420G>a;1521_1523del] variants found in the proband and her mother

et al., 2010), and reduced weight gain due to pancreatic insufficiency. Nevertheless, she tested negative for NBS (protocol: single immunoreactive trypsinogen [IRT] assay on blood spots+ DNA analysis), thus leading clinicians to focus on the SD signs and preventing CF diagnosis. Our patient fell within the several cases of false-negative CF

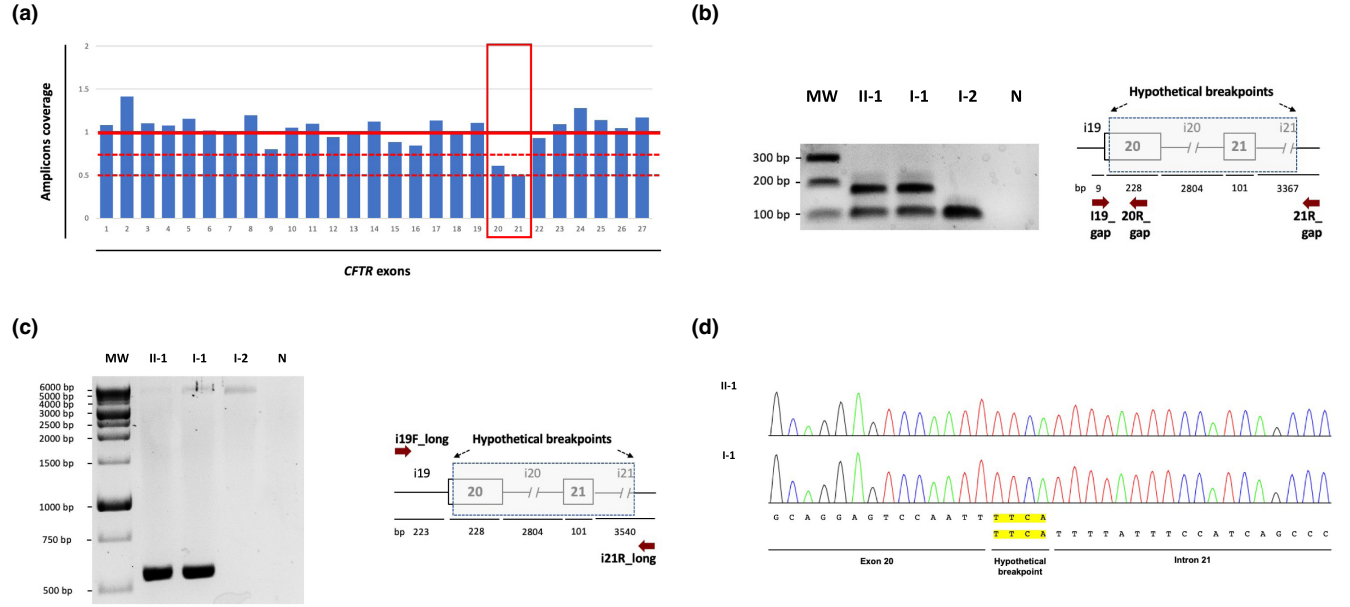

FIGURE 2 Characterization of paternal deleted allele. (a) Detection of potential deletion of exon 20 and 21 of CFTR gene (GenBank: NM_000492.3) by NGS coverage data analysis. The parameters of the *x*-axis and *y*-axis indicate CFTR exons (1–27) and the average of the corresponding amplicons (mapped probes), respectively. The average (red continuous bar) of the amplicons of exons 20–21 (red box) appeared to be lower than 1 (disomic condition). (b) GAP PCR performed in the proband (II-1), the father (I-1), and the mother (I-2) with primers (black arrows) in intron 19 (black bar), exon 20 (box), intron 21 (black bar); MW and N represent the DNA size markers (100 bp DNA ladder) and the blank, respectively. WT product length: 101 bp; deleted product (tinted boxes) length: 175 bp. (c,d) Long-range PCR performed with primers in introns 19 and 21 of all three family members (MW: 1 kb DNA ladder) (c) and Sanger sequencing analysis confirming the c.3158_3468+3219 del deletion. WT product length: 6896 bp; deleted product (tinted boxes) length: 561 bp

NBS documented, mainly attributable to the strategy adopted (e.g., NBS algorithm, cutoff, and sensitivity) and various conditions at birth (Steven et al., 2006; Taccetti et al., 2020). In this situation, a SCT could have been decisive to resolve the CF suspicion even in concomitance with other diseases; but it was performed only at patient's age of six, indeed providing a positive result.

Consistently, proband's mutational screening revealed two known *CFTR* mutations (p.[Glu474Lys] and p.Phe508del), whose parental origin was ascertained by segregation analysis within the family (Castellani et al., 2009). The study was critical to unveil the maternal inheritance of both the anomalies as a *CFTR* complex allele (c.[1420G>A;1521_1523del]). This suggested the presence of another disease-causing variant on the paternal chromosome, which was confirmed as a deletion affecting exons 20 and 21 and characterized as c.3158_3468+3219del. Taken together these results stress the importance to perform segregation analysis, and to consider CNVs among the disease-causing mutations for accurate *CFTR* genotyping.

The identification of a complex allele lead us to question on the pathogenetic role of the single variants, which might have different effects when combined *in cis* (Lucarelli et al., 2010). In the case of (c.[1420G>A;1521_1523del]), the deleterious effect of p.Phe508del is unquestionable. For p.(Glu474Lys), we queried CFTR2 database (https://cftr2.org/) and found that it was reported in five subjects. In addition to p.(Glu474Lys), two of the five affected individuals are heterozygous for known missense or frameshift variants, and three—like our proband—for the p.Phe508del mutation. Segregation analysis is not available for any case, as the CFTR2 database is built on genetic information of CF registries assuming that the two variants identified are in *in trans* phase. We questioned if any or all the three subjects with p.Phe508del and p.(Glu474Lys) carried the variants *in cis* as a complex allele, together with an unidentified third causative one on the other chromosome.

The detection of c.1420G>A (p.(Glu474Lys)) *in cis* with a known CF-causing mutation raises question about its deleterious effect. However, p.(Glu474Lys) is likely to be pathogenic for the following aspects: (i) damaging effect predicted by bioinformatic tools; (ii) functional assay based on chloride conductance (1.2% compared to wild-type as reported in CFTR2 database; (iii) found in individuals with mutations other than c.1521_1523del (as reported in CFTR2); (iv) rarity in the general population (not reported in gnomAD).

Although the disease-causing role of p.(Glu474Lys) seems not to be in doubt, additional CF subjects with the same c.[1420G>A;1521_1523del] complex allele would be

needed to establish any genotype–phenotype relationship, as well as more in-depth study to investigate the concerted effect of the two mutations.

The characterization and determination of the exact prevalence of complex alleles (including those with the well-described p.Phe508del variant) (Baatallah et al., 2018) still remain a thorny issue. Mutational search protocols are generally designed on panels including only the most common *CFTR* anomalies and/or end following the detection of two variants on different alleles, often excluding additional *in cis* mutations (Lucarelli et al., 2010). This represents a severe limitation for an accurate genetic testing, which could be instead solved by scanning the entire *CFTR* gene (Lucarelli et al., 2010). Providing a complete *CFTR* genotypization appears to be even more important today in view of the recent CF etiological therapies; these approaches are mainly directed to c.1521_1523del (p.Phe508del), given its major frequency, but their effectiveness on this variant within a complex allele is rarely proven (Baatallah et al., 2018; Chevalier & Hinzpeter, 2020). The detection of a further *in cis* variant could indeed improve patients' recruitment, expected response to current treatments, and combinatorial targeting of distinct defects.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

All experimental protocols were approved by the ethical committee of IRCCS "Burlo Garofolo" hospital. All subjects provided written informed consent for the study, which was conducted in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

IP and AF collect and analyze data, review the literature, and drafted the manuscript. RB and AS organized this study, reviewed clinical and laboratory data, and finalized this manuscript. FS, FF, and MMa performed genetic counselling, patient management and reviewed clinical data. KSR revised the draft critically for important intellectual content. MF and GF performed Sanger sequencing and analysis of deletion. MLB, SS, MMo and APD performed NGS and SNP array. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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