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Heterozygous *RTEL1* mutations are associated with familial pulmonary fibrosis

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ABSTRACT Pulmonary fibrosis is a fatal disease with progressive loss of respiratory function. Defective telomere maintenance leading to telomere shortening is a cause of pulmonary fibrosis, as mutations in the telomerase component genes *TERT* (reverse transcriptase) and *TERC* (RNA component) are found in 15% of familial pulmonary fibrosis (FPF) cases. However, so far, about 85% of FPF remain genetically uncharacterised.

Here, in order to identify new genetic causes of FPF, we performed whole-exome sequencing, with a candidate-gene approach, of 47 affected subjects from 35 families with FPF without *TERT* and *TERC* mutations.

We identified heterozygous mutations in regulator of telomere elongation helicase 1 (*RTEL1*) in four families. *RTEL1* is a DNA helicase with roles in DNA replication, genome stability, DNA repair and telomere maintenance. The heterozygous *RTEL1* mutations segregated as an autosomal dominant trait in FPF, and were predicted by structural analyses to severely affect the function and/or stability of *RTEL1*. In agreement with this, *RTEL1*-mutated patients exhibited short telomeres in comparison with age-matched controls.

Our results provide evidence that heterozygous *RTEL1* mutations are responsible for FPF and, thereby, extend the clinical spectrum of *RTEL1* deficiency. Thus, *RTEL1* enlarges the number of telomere-associated genes implicated in FPF.



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Introduction

Pulmonary fibrosis is a general term used to describe a group of fibrosing interstitial lung diseases (ILDs) characterised by accumulation of extracellular matrix and fibroblasts in the distal lung. Pulmonary fibrosis can result from environmental exposures such as inhalation of fibrogenic dust or aerosolised organic antigens, drug toxicity, or systemic diseases such as connective tissue diseases, or occur as an isolated, sporadic disease without extrapulmonary involvement (idiopathic interstitial pneumonia). Idiopathic pulmonary fibrosis (IPF) is the most frequent (60%) and severe presentation, with a median age at diagnosis of 66 years and median survival of 3–5 years [1, 2].

Most cases of pulmonary fibrosis are sporadic and occur in people with no familial history of the disorder, but the occurrence of pulmonary fibrosis in two or more members of the same family is increasingly recognised and accounts for 5–10% of cases. The most frequent pattern of genetic transmission of pulmonary fibrosis is autosomal dominance. Familial and sporadic pulmonary fibrosis diseases are clinically and histologically indistinguishable, but familial pulmonary fibrosis (FPF) tends to present at an earlier age and might differ slightly in radiological pattern [3].

FPF has been linked to mutations in two main groups of genes [4] involved in surfactant metabolism (*SFTPC* (surfactant protein C), *SFTPA2* (surfactant protein A2) and *ABCA3* (ATP-binding cassette subfamily A, member 3) [5, 6]) or telomere maintenance (*TERT* (reverse transcriptase), *TERC* (RNA component), *DKC1* (dyskerin) and *TINF2* (TRF1-interacting nuclear protein 2 (TIN2)) [7–10]). Mutations of *SFTPC* have rarely been described in FPF [11] and no pathogenic variants of *SFTPC* were found in 73 Newfoundland probands [12]. Heterozygous mutations of *TERT* are the most frequently evidenced mutations, observed in about 15% of FPF, while heterozygous mutations of *TERC* are more rare (a few per cent) [7, 8, 12]. *DKC1* represents the third telomere-related gene recently identified as a genetic cause of FPF in two male probands [13]. Notably, *TERT*, *TERC* and *DKC1* mutations were primarily described being as responsible for dyskeratosis congenita, a bone marrow failure disease associated with short telomeres. Interestingly, more recently, pulmonary disease is found in about 20% of dyskeratosis congenita patients with mutations in *TERT*, *TERC*, *DKC1* and *TINF2* [10, 14], further arguing for a link between telomere deficiency and IPF pathogenesis. As short telomeres are a hallmark of many IPF cases, genes involved in telomere maintenance can be theoretically considered as candidates for IPF. Overall, mutations are identified in about 15% of FPF cases, whereas 85% of cases remain genetically uncharacterised. To uncover new molecular causes of FPF, we performed whole-exome sequencing (WES) analysis of 47 patients from 35 families featuring FPF but without *TERT* and *TERC* mutations. We identified heterozygous mutations in regulator of telomere elongation helicase 1 (*RTEL1*) in four patients (10% of FPF families), identifying *RTEL1* deficiency as a major genetic cause of FPF in our cohort. *RTEL1* is an ATP-dependent DNA helicase [15] that participates in DNA replication, genome stability, DNA repair and telomere maintenance [16]. Very recently, using the same approach, COGAN *et al.* [17] identified heterozygous mutations of *RTEL1* in nine FPF families. Furthermore, a locus containing *RTEL1* has been associated with mean telomere length [18]. Our findings reinforce the link between telomere maintenance and lung fibrosis, and suggest that mutations in genes implicated in telomere maintenance may collectively account for up to 25% of cases of FPF.

Methods

Subjects

200 probands with FPF and their relatives were referred for molecular exploration of *TERT* and *TERC* genes by rare pulmonary disease expert centres in France from 2008 to 2013. The genetics department of Bichat hospital was the only French laboratory that performed this genetic test during this period. All patients gave signed consent for genetic analysis including for research. An institutional review board of the French learned society for respiratory medicine, Société de Pneumologie de Langue française, approved the study protocol. By bidirectional Sanger sequencing, we identified 22 heterozygous *TERT* mutations in 24 patient and four heterozygous variations of *TERC* in six patients [19]. *TERT* and *TERC* mutations were ruled out in 170 probands from 170 independent families. As an exploratory step, we selected 35 “unexplained” families without mutations identified within the telomerase complex for WES analysis. The clinical charts for patients were reviewed and data were collected on a standardised, anonymised collection form. Clinical data, chest computed tomography (CT) scan and lung histology findings were systematically reviewed, analysed by a multidisciplinary team, and classified according to the 2011 American Thoracic Society (ATS)/European Respiratory Society (ERS)/Japanese Respiratory Society/Latin American Thoracic Society statement on IPF and the 2013 ATS/ERS classification of idiopathic interstitial pneumonias [2, 20]. Families with the largest number of affected cases were included and/or families with a DNA sample available for several affected individuals in order to retain the variants that cosegregate between both individuals. 35 unexplained families of European origin were selected for exome sequencing analysis according to several criteria: confirmation of FPF (at least two cases of pulmonary fibrosis), and availability of clinical data, chest CT scan and lung histology findings. For 12 families among the 35

selected, a DNA sample was also available for another affected subject. The 35 unexplained families included 14 families with two known cases of pulmonary fibrosis, 17 families with three cases, three families with four cases, and one family with five cases. DNA of the 47 affected subjects (35 male and 10 female) were submitted for WES.

WES and bioinformatic analysis

Whole human exome sequencing was performed by Otogenetics (Norcross, GA, USA), which used Agilent V5 (Agilent Technologies, Santa Clara, CA, USA) and PE Illumina HiSeq2000 (Illumina, San Diego, CA, USA) sequencing with an estimated 50-fold average coverage. Sequences were aligned to the reference human genome hg19 by using the Burrows–Wheeler Aligner. Downstream processing involved the Genome Analysis Toolkit (GATK) (<https://www.broadinstitute.org/gatk/>), SAMtools (www.htslib.org) and Picard (<http://broadinstitute.github.io/picard/>), following documented best practises [21]. Creation of variant calls involved the GATK Unified Genotyper. All variants were annotated with use of a software system developed by the Paris Descartes University Bioinformatics platform (Paris, France). The annotation process was based on the ENSEMBL v72 database (www.ensembl.org). Querying and filtering involved use of POLYWEB, an in-house bioinformatics tool (Imagine Institute, Université Paris Descartes). We combined WES with a candidate-gene approach to identify novel FPF genes.

RTEL1 variants were retained as disease-causing mutations according to several criteria: the absence or rare frequency of the mutations (<1%) in a database such as the Exome Variant Server (<http://evs.gs.washington.edu/EVS/>) or 1000 Genome database (<http://browser.1000genomes.org/index.html>); the presence of rare variants in all affected individuals of several independent families; and prediction of the variants as being pathogenic based on structure prediction after molecular modelling (e.g. frameshift mutation; nonsynonymous mutation affecting a conserved amino acid, and predicted to affect the three-dimensional (3D) structure and the function of the protein).

Analysis of WES data from the 35 families confirmed the absence of mutations in *TERT* and *TERC*, and did not reveal any mutations in the telomerase-associated genes *DKC1* and *TINF2*, nor in the *SFTPC*, *SFTPA2* and *ABCA3* genes.

RTEL1 sequencing

To confirm the presence of mutations identified by WES of proband DNA and to study cosegregation of the mutation with the phenotype in families, we sequenced exons carrying *RTEL1* mutations by bidirectional fluorescent sequencing (primer sequences are available on request) and compared them to reference sequences for the two main *RTEL1* transcripts expressed in human cells [22] (1219 amino acid (aa) isoform: NM_016434.3/NP_057518.1; 1300-aa isoform: NM_032957.4/NP_116575.3), which differ in exons 34 and 35. For the cDNA sequence NM_032957.4, some sequence discrepancies exist between the reference databases.

Telomere length measurement

Telomere length was measured by telomeric restriction fragment (TRF) assay. Genomic DNA (800 ng) extracted from whole-blood cells was digested with *HinfI* and *RsaI* enzymes, resolved on a 0.7% agarose gel, transferred onto a nylon membrane, and hybridised using EasyHyb solution (Roche, Basel, Switzerland) with a γ -³²P-labelled (TTAGGG)₄ probe. After washes, telomere-probed Southern blots were exposed over a phosphorimager, and analysed with ImageJ (<http://imagej.nih.gov/ij/>). Digitised signal data were then transferred to Excel (Microsoft, Redmond, WA, USA) for calculating mean TRF length with the formula:

$$\text{Length} = \frac{\sum \text{OD}_i}{\sum \frac{\text{OD}_i}{L_i}}$$

where OD_{*i*} is the integrated signal intensity at position *i* and *L_i* is the length of the DNA fragment in position *i*. Healthy, age-matched controls (n=13) were recruited in France and gave consent. Measurement of telomere length of the controls was performed using anonymised DNA samples.

Molecular modelling and 3D structure visualisation

We used the sequence of the N-terminal Rad3-related DNA helicase core of *RTEL1* to search for similarities with known 3D structures using HH-PRED [23], Phyre2 [24] and I-TASSER [25]. Based on their results, we created a model of the 3D structure for this core, using Modeller v9.14 [26] and the *Thermoplasma acidophilum* XPD (taXPD) as a template (Protein Data Bank entries 2vsf and 4a15 [27, 28]; HH-PRED E-value 1.1×10^{−74}, Phyre 2 confidence 100 and I-TASSER C-score −0.73). Although the two sequences share only 21% sequence identity, the DNA helicase core is well conserved (see the online supplementary material

for the details of the alignment). The RTEL1 sequence displays some large insertions within loops not present in the archaeal template. Moreover, the alignment of archaeal XPD and eukaryotic RTEL1 ARCH domains displays higher sequence divergence, and could be refined only by using sensitive methods of alignment (our unpublished results). The quality of the 3D model was assessed by examining various structural features and stereochemical properties (data not shown). However, it should be noted that the variability between *Homo sapiens* RTEL1 and taXPD sequences did not affect the reliability of our analysis because mutations are in regions that are highly conserved between the two proteins (see the online supplementary material). The 3D structure model for the first harmonin-N-like domain was from FAURE *et al.* [29].

Results

Heterozygous RTEL1 mutations identified in FPF patients

To identify new genetic causes of FPF, we performed WES analysis of 47 patients with FPF from 35 families. Before performing the WES, Sanger sequencing had been used to exclude mutations of the main known FPF-associated genes, namely *TERT* and *TERC*. The filters and query used were rare variants predicted to be pathogenic in genes related to telomere biology. Thus, we identified heterozygous *RTEL1* mutations in four probands from four independent families (families A, B, C and D) (fig. 1a and table 1). Three of the four *RTEL1* mutations were missense mutations, corresponding in patient AII.2 to the c.146C>T, p.(Thr49Met) substitution in exon 3; in patient BII.1, to the c.637C>T, p.(Arg213Trp) substitution in exon 8; and in patient CII.2, to the c.2890T>C, p.(Phe964Leu) substitution in exon 30. The fourth *RTEL1* mutation, identified in patient DII.1, was the duplication of 1 bp in exon 33, leading to a frameshift and a premature stop codon (c.3493dupC, p.(Gln1165Profs*22)) (fig. 1b). The heterozygous amino acid change p.Phe964Leu found in patient CII.2 was previously reported in a homozygous state in a patient with Hoyerall-Hreidarsson (HH) syndrome [30] with an alternative nomenclature based on a different *RTEL1* splice variant (c.2964T>G, p.(Phe988Leu)) and corresponding to a different nucleotide change (nomenclature in the 1219- and 1300-aa isoforms: 2890T>C in our patient and c.2892T>G in the

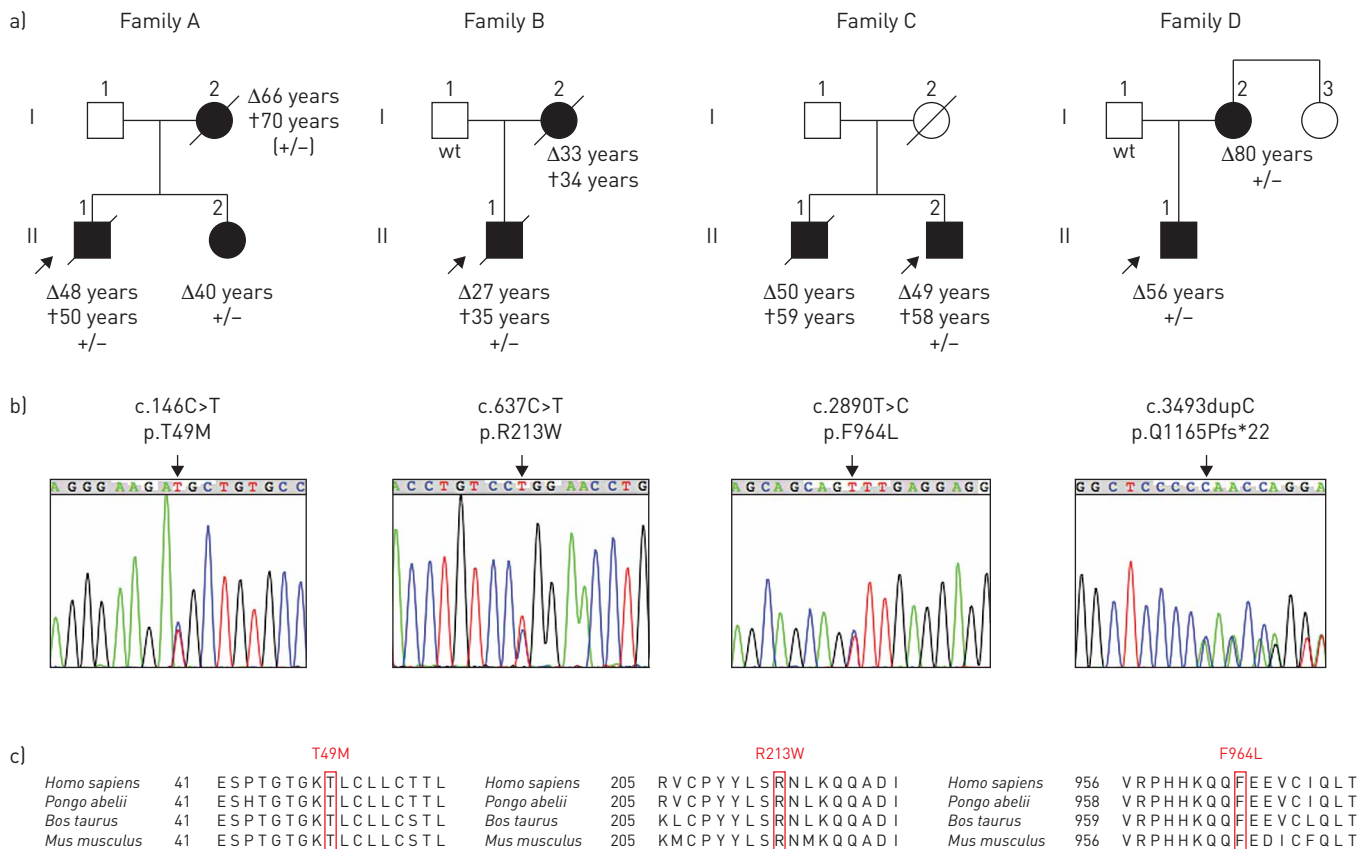


FIGURE 1 Segregation of regulator of telomere elongation helicase 1 (*RTEL1*) mutations in familial pulmonary fibrosis and conservation of altered *RTEL1* amino acids. a) Pedigree of four families with familial pulmonary fibrosis and heterozygous *RTEL1* mutation. Individuals with pulmonary fibrosis are indicated by black symbols. Heterozygosity for the mutation is indicated by +/- . The symbol +/- in parentheses indicates the obligate carrier. Δ: age at diagnosis; wt: wild-type status (not carrier of the mutation); †: age at death. b) Presence of *RTEL1* mutations confirmed by Sanger sequencing. c) Alignment generated with protein reference sequences: human (*Homo sapiens* RTEL1, RefSeq NP_116575.3/NP_057518.1); orangutan (*Pongo abelii* RTEL1, RefSeq NP_001124929); cow (*Bos taurus* RTEL1, RefSeq NP_001091044); mouse (*Mus musculus* RTEL1, RefSeq NP_001001882.3).

TABLE 1 Clinical data in families with familial pulmonary fibrosis and patients with mutations in regulator of telomere elongation helicase 1 (*RTEL1*)

Family	<i>RTEL1</i> mutation [#]	Individual	Age at presentation years	Sex	Smoking pack-years/fibrogenic exposure or aerocontaminants	Pulmonary function at presentation FVC/ <i>D</i> _{LCO} % predicted	Chest CT scan diagnosis	Other manifestations	Follow-up months	Status
A	c.146C>T, p.(Thr49Met)	I.2 [¶]	66	F	0/no	86/56	UIP pattern	Cyclophosphamide-induced neutropenia	48	Dead
		II.1 ⁺	48	M	35/no	77/28	UIP pattern	Cyclophosphamide-induced neutropenia	21	Dead
		II.2 ⁺	40	F	5/no	94/61	Possible UIP pattern	Sarcoidosis in remission	12	Alive
B	c.637C>T, p.(Arg213Trp)	I.2 [¶]	33	F	0/no	NA	NA	No	30	Dead
		II.1 ⁺	27	M	2/mould	43/17	Possible UIP pattern	Osteopenia	69	Dead after lung transplantation
C	c.2890T>C, p.(Phe964Leu)	II.1 [¶]	50	M	30/no	72/67	Possible UIP pattern	Cholesteatoma	109	Dead
		II.2 ⁺	49	M	30/painter	87/NA	UIP pattern	Rheumatoid arthritis Lung cancer	107	Dead
D	c.3493dupC, p.(Gln1165Profs*22)	I.2 ⁺	80	F	0/no	60/NA	UIP pattern	Autoimmune hepatitis	64	Alive
		II.1 ⁺	56	M	30/mould	63/50	Possible UIP pattern	Finger agenesis Short left femur	52	Alive

FVC: forced vital capacity; *D*_{LCO}: diffusing capacity of the lung for carbon monoxide; CT: computed tomography; F: female; M: male; UIP: usual interstitial pneumonia; NA: not available. [#]: mutation nomenclature follows the recommended cDNA numbering (www.hgvs.org/mutnomen); the genomic locations of the p.(Thr49Met), p.(Arg213Trp), p.(Phe964Leu) and c.(3493dupC) mutations are Chr20 (Hg19/GRCh37) g.62292694, g.62298844, g.62324534 and g.62326565, respectively. [¶]: confirmation by Sanger sequencing impossible because DNA and consent were not available for deceased patients. ⁺: confirmation of the *RTEL1* mutation by Sanger sequencing.

previous patient [30]). Recently, biallelic mutations in *RTEL1* were reported in patients with HH syndrome, a severe variant of dyskeratosis congenita, characterised by early-onset bone-marrow failure, immunodeficiency and developmental defect associated with abnormally short telomeres [22, 30–33]. This clearly demonstrates the pathogenicity of the p.(Phe964Leu) mutation.

None of the four *RTEL1* mutations was present in the Exome Variant Server (>6500 individuals), in the 1000 Genomes (www.1000genomes.org) database or in our in-house database (4237 individuals). Moreover, the three *RTEL1* substitutions (p.(Thr49Met), p.(Arg213Trp) and p.(Phe964Leu)) were located in highly conserved regions (fig. 1c) and predicted to be highly deleterious by prediction tools for amino acid substitutions such as Polyphen-2 [34], SIFT [35] and MutationTaster [36]. Combined annotation-dependent depletion score analysis was also performed for each mutation and high scores were obtained in favour of a conclusion of pathogenic mutations (online supplementary material). Genomic evolutionary rate profiling to assess conservation of the regions targeted by the mutations showed that nucleotides targeted by missense mutations are very conserved (online supplementary material).

Finally, we confirmed, by fluorescent sequencing, the cosegregation of the *RTEL1* mutations with the pulmonary fibrosis phenotype in the three families with affected available first-degree relatives (that were not first included in the exome sequencing project because they were not available at that time) (fig. 1a). The genetic hypothesis in family B was that the disease-causing mutation was dominantly transmitted by the affected mother (BI.2) but she could not be analysed because of a lack of biological material. As expected, the healthy father (BI.1) did not carry the p.(Arg213Trp) *RTEL1* mutation. Analysis of WES data for the four families carrying an *RTEL1* mutation confirmed the absence of mutations in *TERT* and *TERC*, as well as other genes involved in telomere maintenance and previously implicated in human diseases, such as *DKC1*, *TINF2* (encoding TIN2), *NOP10* (nucleolar protein 10), *NHP2* (encoding a ribonucleoprotein (RNP)), *WRAP53* (encoding telomerase and Cajal body protein 1) [14], *CTC1* (conserved telomere capping protein 1) [37] and *ACD* (encoding tripeptidyl peptidase 1) [38] (data not shown).

Clinical review

Clinical data were complete for eight out of nine patients with FPF from four families (fig. 1 and table 1). *RTEL1* mutation present in all six patients with available DNA samples.

Family A

The proband from family A was a 48-year-old male smoker with a diagnosis of IPF (fig. 2c). He initially received azathioprine, steroids and *N*-acetylcysteine. Azathioprine was well tolerated. An acute exacerbation of IPF was treated with steroids and cyclophosphamide. The patient presented with neutropenia ($<0.5 \text{ G}\cdot\text{L}^{-1}$) 1 week after the first infusion of cyclophosphamide, which resolved spontaneously. He died of pulmonary fibrosis progression 21 months after the initial diagnosis. His mother was a 66-year-old nonsmoker with a diagnosis of IPF. She received oral cyclophosphamide and steroids, and neutropenia developed ($<0.5 \text{ G}\cdot\text{L}^{-1}$), which resolved after cyclophosphamide was stopped. She received azathioprine without blood cytopenia and died of respiratory failure 48 months after the diagnosis. His sister was a 40-year-old smoker with stage 2 pulmonary sarcoidosis that was actually in remission but still presenting with interstitial pneumonia. The first CT scan of the sister showed micronodules with lymphatic distribution, which disappeared on further CT scanning, superimposed over basal subpleural reticulations and mild ground-glass opacities, which persisted on further CT scanning; an initial bronchial biopsy showed granulomas and bronchial biopsies were not further reassessed (fig. 2d). The patient was asymptomatic and received no treatment until 2 years of follow-up.

Family B

The proband from family B was a 27-year-old mild smoker with a diagnosis of IPF (possible usual interstitial pneumonia (UIP) pattern on CT, UIP pattern on lung biopsy). He received bosentan or placebo in the BUILD-3 trial. The patient had an acute exacerbation of IPF (fig. 2b) and underwent lung transplantation, then received combined therapy with mycophenolate mofetil, cyclosporin and corticosteroids. He developed chronic rejection and received a new transplant 19 months later, with rejection of the second graft. He died 51 and 69 months after the first lung transplantation and diagnosis, respectively. He did not have myelodysplasia. His mother was a 33-year-old nonsmoker, with, according to her family, lung fibrosis. Medical files were not available for review. The patient died of respiratory failure 30 months after the initial diagnosis.

Family C

The proband from family C was a 49-year-old male smoker with rheumatoid arthritis and lung involvement (UIP pattern on CT with emphysema in the upper lobes). He experienced a slow decline in lung function despite therapy with azathioprine and steroids, without cytopenia. Lung cancer (adenocarcinoma) developed. He died 107 months after the diagnosis of ILD.

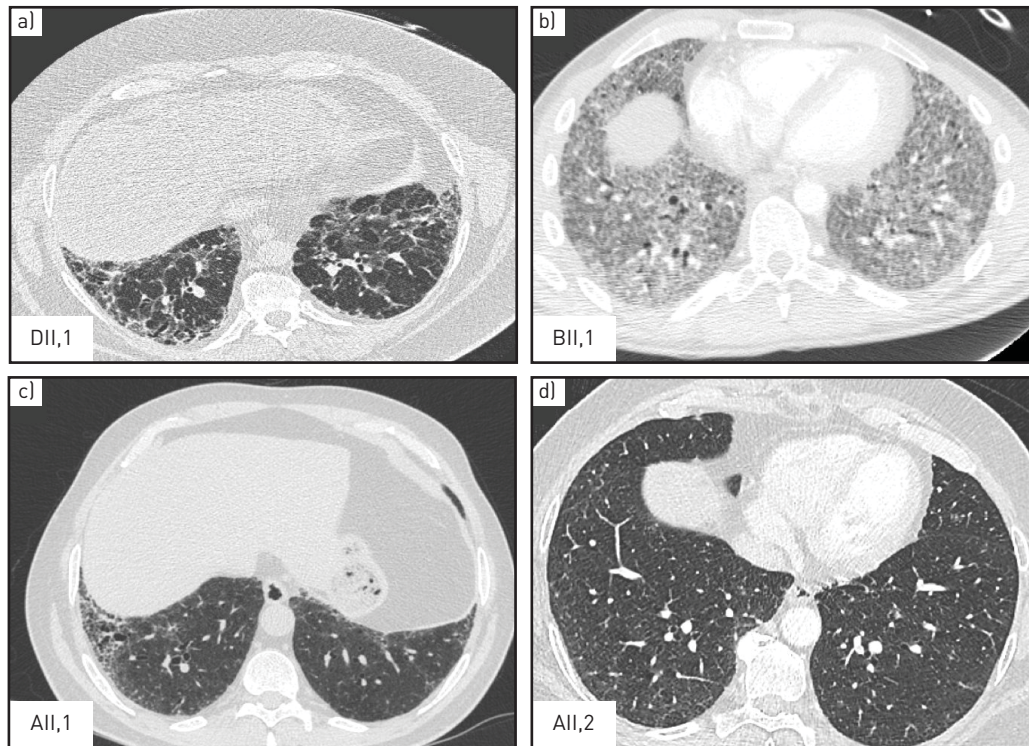


FIGURE 2 Thoracic computed tomography (CT) of four probands, heterozygous carriers of regulator of telomere elongation helicase 1 (*RTEL1*) mutations. a) Subpleural, basal predominance of reticular abnormalities without honeycombing, considered a possible usual interstitial pneumonia (UIP) pattern. b) CT image obtained during acute exacerbation showing new ground-glass infiltrates superimposed on reticular abnormalities, considered acute exacerbation of possible UIP. c) Subpleural, basal predominance of reticular abnormalities and honeycombing with traction bronchiectasis, classified as definite UIP pattern. d) Small bilateral micronodules, subpleural reticulations and ground-glass opacities without predominance, considered an atypical UIP pattern.

His brother was a 50-year-old smoker with lung fibrosis (possible UIP pattern on CT, lung biopsy not performed). He received corticosteroids and experienced a slow decline of lung function. He had an acute exacerbation but did not receive specific therapy and died 109 months after the diagnosis of lung fibrosis. Their father presented digital clubbing but his pulmonary status was unknown.

Family D

The proband from family D was a 56-year-old male smoker with lung fibrosis and a possible UIP pattern on CT scan (fig. 2a); lung biopsy was not performed. He presented skeletal abnormalities: agenesis of two fingers on the left hand and three fingers on the right hand, and a 10-cm shorter left femur. He experienced a slow decline of lung function despite receiving nintedanib or placebo in the TOMORROW trial, then pirfenidone. Lung transplantation was not considered because of coronary artery disease and obesity. He was alive 52 months after diagnosis. His mother was an 80-year-old nonsmoker. She had lung fibrosis with a UIP pattern on CT and autoimmune hepatitis. The diagnosis of autoimmune hepatitis was confirmed by cytolysis, the presence of autoantibodies (anti-RNP, anti-smooth muscle and anti-nuclear) and polyclonal hypergammaglobulinaemia. She received azathioprine and did not present with cytopenia but experienced a slow decline of lung function until the last follow-up 64 months after diagnosis. Neither the mother nor the two daughters of the proband, who all carried the mutation, presented skeletal abnormalities.

Predicted impact of *RTEL1* mutations on protein structure and function

RTEL1 belongs to the subclass of iron-sulfur cluster-containing DNA helicases within the SF2 DEAH subfamily including XPD (xeroderma pigmentosum), FANCI (Fanconi anaemia) and ChIR1 (Warsaw breakage syndrome) [39–42]. Common to all proteins of this family is an N-terminal Rad3-related DNA helicase core of four domains (two RecA-like motor domains; helicase domain (HD)1 and HD2; a 4Fe–4S cluster; and an ARCH domain) (fig. 3a). This catalytic core is followed by an *RTEL1*-specific C-terminal region that contains a tandem of harmonin-like domains that may have a critical role in interacting with yet-unidentified partners [43] as well as a RING finger domain, which is likely to have ubiquitin ligase

activity [22]. In addition, the C-terminal extension contains a proliferating-cell nuclear antigen (PCNA) interaction motif (PIP box) through which RTEL1 associates with the replisome [44].

Two of the described missense *RTEL1* mutations are located in the Rad3-related DNA helicase core and the third is located within the first harmonin-like domain (fig. 3a).

p.Phe964 (p.F964) belongs to the fifth α -helix of the first harmonin-like domain (fig. 3b). It corresponds to a highly conserved position within harmonin-N-like domains, which is always occupied by an aromatic amino acid (phenylalanine or tyrosine [29]). It is conserved for structural reasons, because its side-chain is buried within the core of the domain. The substitution of this aromatic amino acid with leucine (p.F964L) may

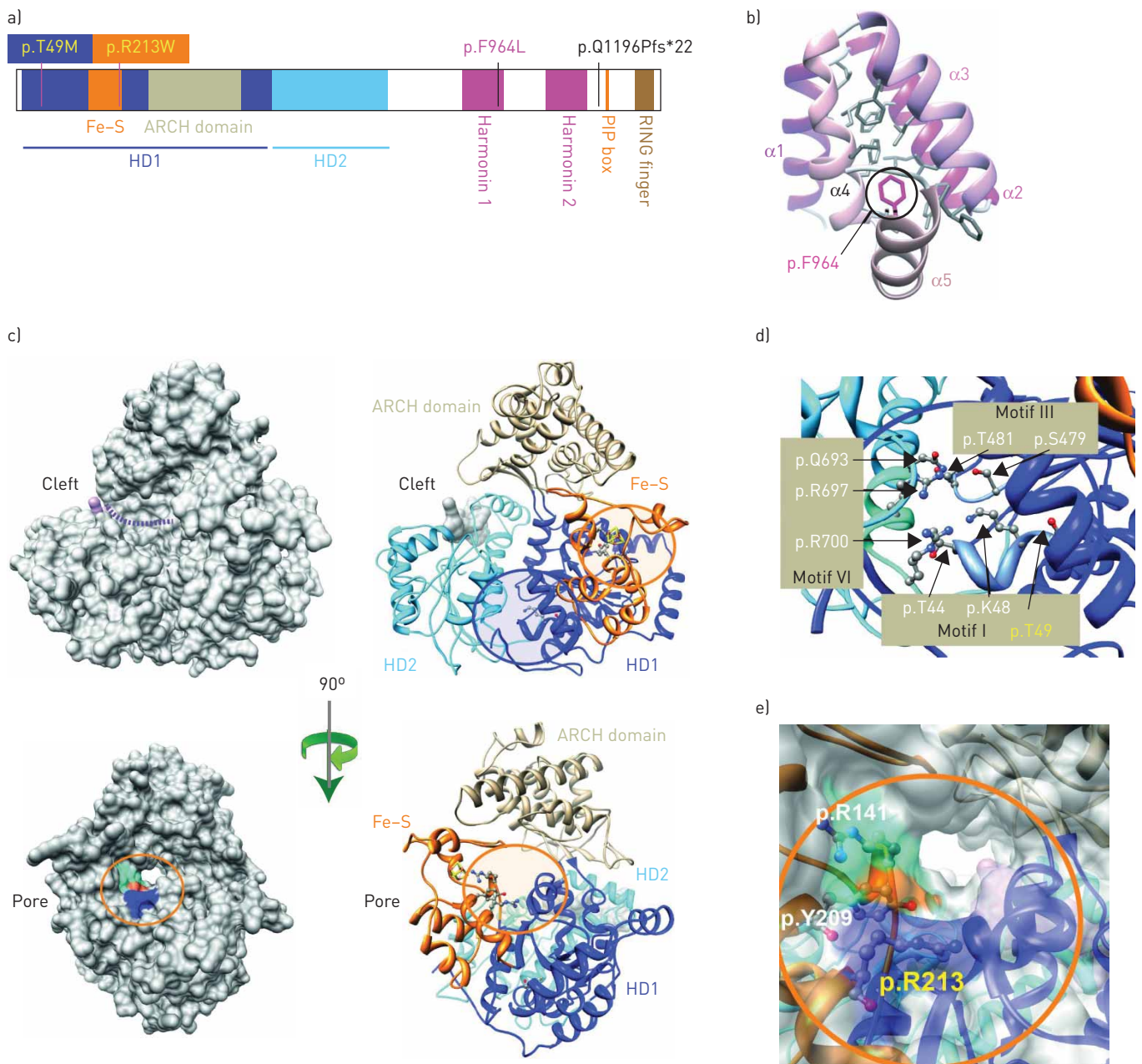


FIGURE 3 Molecular modelling of regulator of telomere elongation helicase 1 (*RTEL1*) missense mutations. a) Schematic representation of *RTEL1* protein, highlighting the domain architecture and the position of mutations. b) Position of p.F964 on the model of the three-dimensional structure of the first harmonin-N-like domain [29]. The amino acid side-chains shown form the hydrophobic core; the hydrophobic character is conserved across the whole harmonin-N-like family. c) Two orthogonal views of the solvent-accessible surface and ribbon representations of the *RTEL1* catalytic core, highlighting the positions of the two mutated amino acids. A short DNA strand is represented as a reference within its binding cleft. The predicted translocation path (groove) leading to the pore is indicated by a purple dashed line. d) Enlarged ATP binding domain at the interface between helicase domain (HD)1 and HD2 (p.T49 position). e) Enlarged pore exit (p.R213 position).

directly affect the folding or fold stability, even though the hydrophobic character is not lost. The p.(Phe964Leu) mutation was reported in an HH patient, which demonstrates its deleterious impact on RTEL1 function [30].

p.Thr49 (p.T49) belongs to conserved helicase motif I (Walker A) within HD1 (figs 3c and d). This amino acid is located within the ATP-binding pocket near the magnesium ion, one amino acid downstream of p.K48, which binds the ATP γ -phosphate. Thus, the p.T49M mutation likely disrupts the catalytic activity of the helicase domain.

p.Arg213 (p.R213) is located at the end of the 4Fe–4S cluster, within the helix α 8. In the crystal structure of taXPD, this basic residue (corresponding to p.170K) is predicted to form a hydrogen bond with a sulfate molecule, together with the conserved p.Y209 and p.R141 (p.Y166 and p.R88, respectively, in taXPD). As observed in our 3D model of the RTEL1 catalytic core (figs 3c and e), p.R213, together with p.Y209 and p.R141, occupies a critical position within the basic groove at the exit of the pore created by the ARCH domain, HD1 and the Fe–S domain, which was suggested to be part of the path for the translocated DNA [27]. The taXPD p.K170A mutation was associated with a large reduction (\sim 30-fold) in DNA binding, even when artefacts due to misfolding were ruled out [28]. This finding suggests that p.R213 in RTEL1 plays an active role within the path followed by single-stranded DNA, which stretches over the complete length of the protein from a cleft to the pore exit [28].

Patient DII.1 carried a mutation leading to a frameshift and a premature stop codon (p.Gln1165Profs*22). This mutation results in the expression of a truncated RTEL1 protein lacking the PIP box and RING finger domain [22]. Although the function of this RING domain is still unknown, a homozygous substitution mutation in this domain causes the HH syndrome, demonstrating its relevance for RTEL1 activity [33]. The importance of the PIP box has been demonstrated by accelerated senescence, replication fork instability, reduced replication fork extension rates and increased origin usage in mouse cells disrupted for RTEL1–PCNA interaction (PIP mutant) [44].

Overall, the structural analysis supported that the *RTEL1* mutations identified in FPF patients strongly affected the function and/or stability of the protein.

RTEL1-mutated FPF patients show shortened telomeres

Knowing that RTEL1 participates in telomere maintenance [15], and that patients with HH syndrome and biallelic *RTEL1* mutations show shortened telomeres, we assessed the telomere length in FPF patients carrying a heterozygous *RTEL1* mutation. A TRF assay of DNA from whole-blood cells revealed shorter telomeres in *RTEL1*-mutated FPF patients than in 13 healthy age-matched controls (fig. 4a and b). The heterozygous *RTEL1* mutations identified in FPF patients may be associated with defective telomere maintenance.

Discussion

We performed WES analysis in a cohort of 47 patients from 35 families with unexplained familial FPF and found four (11%) out of 35 families to have heterozygous *RTEL1* mutations. All four *RTEL1* mutations identified in FPF patients were predicted to be highly detrimental to the function and/or stability of the protein. Indeed, one mutation leads to a frameshift and a premature stop codon, and three others modify highly conserved residues in functional domains of the protein. The p.(Phe964Leu) mutation identified in a heterozygous status in the two FPF patients from family C was recently reported in an homozygous status in two siblings with HH syndrome [30], demonstrating its pathogenicity. Moreover, the telomeres were shorter in *RTEL1*-mutated FPF patients than in controls, a phenotype previously observed in some heterozygous carriers of *RTEL1* mutations from families with HH syndrome [31, 32]. This finding confirms that RTEL1 tightly regulates telomere length [15, 18], and that *RTEL1* haploinsufficiency is sufficient to lead to telomere shortening and predispose to pulmonary disease. DENG *et al.* [32] reported pulmonary fibrosis in a 58-year-old male carrying a heterozygous *RTEL1* mutation and COGAN *et al.* [17] identified heterozygous mutations of *RTEL1* in nine FPF families.

A defect in telomere maintenance has been consistently observed in sporadic pulmonary fibrosis and FPF. Up to 25% of IPF patients and 40% of FPF patients exhibit shortened telomeres, only half of the FPF patients being carriers of *TERT* or *TERC* mutations [45, 46]; patients' short leukocyte telomeres are associated with worse survival in IPF [47]. In addition, four genes involved in telomere length regulation (*TERT*, *TERC*, *TINF2* and *DKC1* [7–10]) have been implicated in monogenic FPF. These genes were primarily identified as responsible for dyskeratosis congenita, a disease of bone-marrow failure associated with short telomeres. Pulmonary disease is found in about 20% of dyskeratosis congenita patients [10, 14]. Recently, homozygous mutant mice expressing a p53 lacking the C-terminal domain exhibited increased p53 activity and downregulation of *RTEL1*, had short telomeres, and suffered from aplastic anaemia and pulmonary fibrosis [48]. Our findings show that *RTEL1* deficiency could explain half of the unexplained FPF patients with shortened telomeres without identified genetic causes.

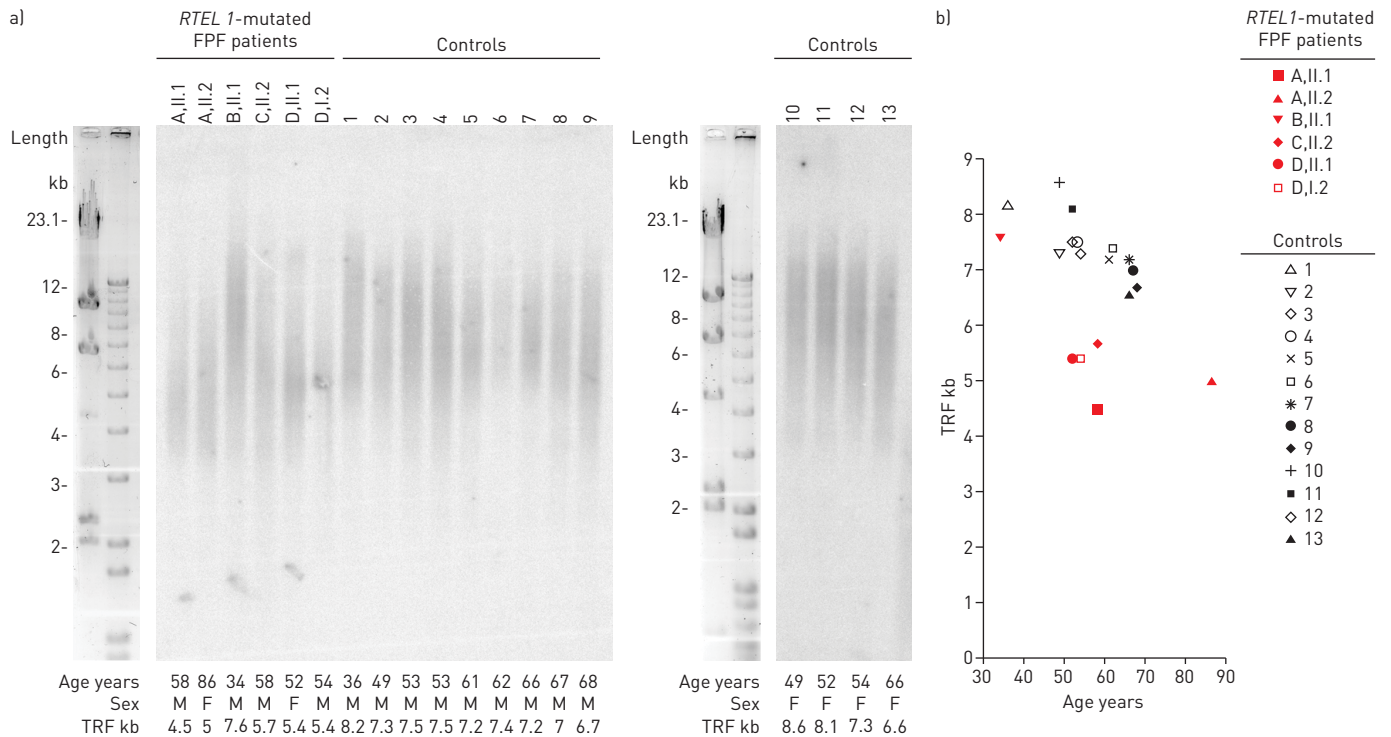


FIGURE 4 Telomere length in whole-blood cells from regulator of telomere elongation helicase 1 (*RTEL1*)-mutated patients with familial pulmonary fibrosis (FPF) and age-matched controls. a) The mean telomere length of whole-blood cells from patients with FPF and *RTEL1* mutations (AII.1, AII.2, BII.1, CII.2, DII.1 and DII.2) and 13 age-matched controls (1–13; 36–68 years old) estimated by the telomeric restriction fragment (TRF) method. The estimated mean TRF length is indicated, as is the sex and age of the individuals. b) TRF length by age of subjects.

As described in telomerase disease [49], all *RTEL1*-mutated FPF families showed a decrease in the age of onset of pulmonary disease through generations (genetic anticipation). Similar to *TERT* mutation carriers, in whom the median age at diagnosis is 57 years, the median age at diagnosis of pulmonary fibrosis for families A, C and D (excluding family B) was 56 years [50], whereas the mean age of patients with sporadic IPF is 65–70 years [1]. The diagnosis was made at a younger age in patients from family B than those from the other families. It cannot be excluded that other genetic factors may contribute to earlier disease occurrence. For example, digenism explains some Mendelian diseases [51].

Interestingly, six out of nine patients were smokers and/or were exposed to inhaled aerocontaminants. A similar high rate of exposure to smoking and fibrogenic contaminants has been reported in patients with lung fibrosis associated with *TERT* mutations [20, 50], and may insult the alveolar epithelium, initiating the fibrotic process. Hence, pulmonary disease in *RTEL1* heterozygous carriers may result from the combination of a genetic defect and environmental factors such as smoking.

At diagnosis, none of the patients in this series had comorbidities commonly associated with the telomere syndrome observed in *TERT*, *TERC* and *DKC1* mutation carriers, such as cryptogenic liver disease, thrombocytopenia, macrocytosis or hyperpigmented rash with an erythematous dyskeratotic rash [13, 52, 53]. In family D, we could not exclude that the liver disease would be related to *RTEL1* deficiency. However, early hair greying was not specifically assessed. Two patients had early reversible neutropenia after cyclophosphamide treatment, whereas azathioprine was well tolerated. *RTEL1* is a helicase involved in DNA repair. *Rtel1*-deficient mouse embryonic stem cells show higher sensitivity to DNA-altering agents such as methyl methanesulfonate and ultraviolet light [16]. These data suggest that some drugs, and particularly alkylating agents, should be used with caution in patients with *RTEL1* mutations. Myelodysplasia secondary to immunosuppressants was recently reported in patients with *TERT* or *TERC* mutations [54, 55].

Three patients presented with an autoimmune disease (autoimmune hepatitis and rheumatoid arthritis) or sarcoidosis, thus excluding idiopathic interstitial pneumonia. This could suggest that fibrosis results from the combination of a monogenic defect, environmental factors and autoimmune diseases in *RTEL1* heterozygous carriers. Determining whether mutations of genes associated with telomere maintenance may be connected with the development of lung fibrosis in autoimmune conditions requires further study because this finding may be due to chance. Alternatively, telomere shortening may promote the development of autoimmune diseases with ageing [56].

One patient had skeletal abnormalities, which could possibly be related to *RTEL1* mutations. Osteoporosis and osteonecrosis have been reported to be associated with *TERT* or *DKC* mutations [50, 57]. No skeletal phenotype was described in the *Rtel1*-deficient mouse, despite the lethality of the homozygous mutation in mice [15].

The phenotype–genotype cosegregation is complete in this study. However, identification of more *RTEL1* carriers is needed to evaluate the penetrance of pulmonary fibrosis in *RTEL1* mutation carriers. Penetrance may depend upon several factors, including environmental exposures, as described for pulmonary fibrosis caused by *TERT* and *TERC* mutations [50]. Interestingly, families of Ashkenazi Jewish ancestry had a recurrent *RTEL1*-associated HH mutation, at a high frequency of 1% [33, 58]. *RTEL1* mutation carriers in these families could be followed up to determine whether they are at risk of developing early-onset pulmonary disease. Despite these limitations, our results have major implications for genetic counselling of FPF and HH families with mutations in *RTEL1*. *RTEL1* mutation carriers may warrant follow-up by a pulmonologist and should be advised to avoid all of the aforementioned environmental exposures.

In conclusion, our study identifies heterozygous *RTEL1* mutations as a novel major genetic cause of FPF and broadens the clinical spectrum of *RTEL1* deficiency. Mutations in genes implicated in telomere maintenance may collectively account for up to 20–25% of cases of FPF.

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