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A novel homozygous *RTEL1* variant in a consanguineous Lebanese family: phenotypic heterogeneity and disease anticipation

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Abstract

Phenotypic heterogeneity is often observed in patients with telomeropathies caused by pathogenic variants in telomere biology genes. However, the roles of recessive variants in these different phenotypes are not fully characterized. Our goal is to describe the biological roles of a novel homozygous *RTEL1* variant identified in a consanguineous Lebanese family with unusual presentation of telomeropathies. A proband was screened for germline variants in telomere biology genes by whole exome sequencing. Leukocytes' telomere length was measured in the proband and eight relatives. We identified a novel homozygous p.E665K *RTEL1* variant in the proband, his mother, and seven siblings that associated with telomere shortening and a broad spectrum of clinical manifestations, ranging from mild unspecific findings to severe phenotypes. Consanguinity in at least three family generations led to increased frequency of the homozygous p.E665K variant in the youngest generation and progressive telomere shortening. The increased frequency of the homozygous *RTEL1* variant due to consanguinity in this Lebanese family allowed us to infer novel behaviors of recessive *RTEL1* variants, as the expressivity and penetrance of this gene are very heterogenous between inter- and intra-generations. Progressive telomere shortening was associated with disease anticipation, first reported in recessive autosomal

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telomeropathies. Both genetic testing and telomere length measurement were critical for the clinical diagnosis of this family with telomere diseases marked by phenotypic heterogeneity.

Introduction

Telomere diseases are characterized by a spectrum of clinical manifestations, classically affecting the bone marrow (BM), lungs, liver, and skin. The molecular causes of these diseases are defects in genes that encode proteins involved in telomere maintenance, such as *DKC1*, *TERT*, *TERC*, and *RTEL1* (Townsley et al. 2014; Niewisch and Savage 2019). Pathogenic variants in *RTEL1* have been described in patients with telomere diseases presenting with a broad spectrum of clinical manifestations including dyskeratosis congenita (DC), aplastic anemia (AA), and idiopathic pulmonary fibrosis (IPF) (Vannier et al. 2014). Initial reports have linked biallelic *RTEL1* germline variants to classical telomere-related phenotypes characterized by short telomeres and high penetrance and severity, such as DC and Hoyeraal-Hreidarsson (HH) syndrome (Ballew et al. 2013; Walne et al. 2013). Further, heterozygous variants were linked to IPF (Cogan et al. 2015), a disease of late onset. Recent studies have demonstrated that *RTEL1* dysfunction is also associated with variable penetrance and disease severity. Pediatric and adult *RTEL1* patients have been reported with isolated cytopenias, immunodeficiency, mild bone marrow failure (BMF), liver disease, and myelodysplastic syndromes (MDS)/acute myeloid leukemia (AML) (Touzot and Kermasson 2016; Cardoso et al. 2017; Speckmann et al. 2017; Ballew et al. 2013). In some cases, *RTEL1* dysfunction are not associated with telomere shortening (Marsh et al. 2018; Borie et al. 2019). This heterogeneity in clinical presentation is often attributed to differences in patients' mutational status (heterozygous, homozygous, or compound heterozygous), age, and telomere length (TL).

Here, we present a unique and complex consanguineous family with telomere diseases caused by a novel homozygous *RTEL1* variant. We also report the impact of this variant on telomere biology and patients' management. Our findings suggest that the novel *RTEL1* variant may be associated with disease anticipation, first described in an autosomal recessive telomere disease.

Methods

Human subjects

The study was approved by the Ethics Committee of Saint Joseph University, Beirut, Lebanon (Reference:FM327) and has been performed in accordance with Declaration of Helsinki. Informed consent was obtained from each participant of the study.

A consanguineous Lebanese family was investigated for telomere diseases due to their clinical history. At their first clinical evaluation at the Lebanese American University Medical Center Rizk Hospital, a detailed clinical evaluation was recorded and peripheral blood was collected in EDTA tubes for genetic testing. As part of the clinical investigation for telomere diseases, the proband was assessed for blood, lung, and liver diseases; he underwent BM biopsy, a computerized tomography (CT) scan of chest and abdomen, and

pulmonary function tests. After confirmation of molecular diagnosis, his mother, six living siblings, and a cousin were also screened for blood disorders and underwent CT scans of the chest and abdomen. They refused BM biopsy due to financial reasons. Screened subjects denied tobacco use and alcohol consumption.

Whole exome sequencing and data analysis

The proband was screened for pathogenic variants in telomere biology genes by whole exome sequencing. Briefly, DNA was extracted from whole blood cells using a salting out method and the exome was captured using the SureSelect Human All Exons kit (Agilent Technologies, Santa Clara, CA). Samples were pooled and paired-end sequenced in the Illumina HiSeq2000. Reads were aligned to the hg19/b12637 reference genome using the Burrows-Wheeler Aligner (BWA) (Li and Durbin 2009). Variants were called according to the Genome Analysis Tool Kit (GATK) (McKenna et al. 2010) and were annotated with VarAFT (Desvignes et al. 2018). For analysis, we prioritized variants that could potentially impact protein functions (stop gain/loss, start loss, frameshifts, missense, canonical splice-site variants, in-frame indels affecting protein-coding regions, and variants within the intron–exon boundary) in the following telomere biology genes: *ACD*, *C16ORF57*, *CTC1*, *DKC1*, *Nola2*, *Nola3*, *PARN*, *RTEL1*, *TERC*, *TERT*, *TINF2*, and *WRAP53*. Variants with high frequency (> 1%) in the Lebanese population were filtered out based on an in-house database of more than 300 exomes from people with no genetic disease. Results were confirmed by Sanger sequencing and reported variants were classified as likely pathogenic, pathogenic, of uncertain significance, likely benign, or benign according to the Sherloc/ACMG criteria (Nykamp et al. 2017). Family members were screened for the same variant identified in the proband by Sanger sequencing. Primer sequences are available upon request.

Telomere length measurement

Telomere length was measured by Southern blot using DNA samples collected at diagnosis as previously described (Gutierrez-Rodrigues et al. 2014). In two family members treated with danazol, TL was measured in serial samples collected during their treatment. For age-matched analysis, patients' TLs were compared to distribution curves derived from best-fit analysis of TLs from 263 healthy controls that were also measured by SB. The 1st, 10th, 50th, 90th, and 99th percentiles were adjusted to the curve. TLs below the 1st or 10th percentile of age-matched controls were considered very short or short, respectively. An expected TL for age was obtained by a third-order polynomial regression analysis of controls' TLs. The extent of telomere shortening among patients was compared by calculating a mean deviation of their TLs from an age-adjusted median TL from controls (ΔTL) (Alder et al. 2018; Vulliamy et al. 2004; Armanios et al. 2005). ΔTL values are negative when patients' TLs are shorter than expected for their ages.

Results

Clinical findings and molecular diagnosis

Using whole exome sequencing, we identified a novel homozygous *RTEL1* variant (p.E665K, c.1993G > A, NM_001283009; Isoform 1300 amino acids) in a 31-year-old

Lebanese male (IV-3; Fig. 1a) suspected to have telomere disease. He was first admitted to hospital with severe dyspnea, hypoxemia, and thrombocytopenia; there was a 5-year history of progressive dyspnea, requiring oxygen supplementation. On physical examination, his hair was gray, there was facial skin hypopigmentation, and patchy hyperpigmentation over his knees and lower extremities associated with exposure to heat sources during winter. The CT scan of his chest and abdomen showed pulmonary fibrosis and portal hypertension. BM biopsy was 30% cellular with trilineage hematopoiesis and no excess of blasts (Table 1).

The novel homozygous p.E665K *RTEL1* variant was classified as pathogenic by the Sherloc/ACMG criteria: the variant was absent in population databases, was predicted to affect a highly conserved amino acid as well as to be deleterious by the majority of in silico algorithms commonly used to predict pathogenicity (Richards et al. 2015), and segregated with the disease and telomere shortening in multiple affected family members. The p.E665K variant may directly impair the catalytic activity of RTEL1, as it is located at the helicase domain of the protein. The mutated amino acid is highly conserved from mammals to invertebrates, substantiating its functional importance (Fig. 1b). No other rare or novel variants in telomere biology genes were called using the same filtering analysis.

Proband's clinical features prompted a detailed family history. Notable were multiple consanguineous marriages (seven unions in total) and a variety of pulmonary and hematologic manifestations in the family. We further screened family members for the p.E665K *RTEL1* variant by Sanger sequencing. The variant was found in the proband's mother (III-1) and seven siblings (Fig. 1a): all were homozygous and had very short telomeres (Fig. 2a). The same variant was also identified in the proband's cousin (III-16), the only heterozygous carrier who had short telomeres but was asymptomatic (Figs. 1a, 2a). Other family members declined testing, except for a son of III-5 (not depicted in the pedigree) who had wild-type *RTEL1* and normal TL (Fig. 2b).

Phenotypic heterogeneity among homozygous individuals, including twin siblings, was marked. The proband presented with a classical phenotype associated with telomere diseases (early gray hair, skin hypopigmentation, IPF, hypocellular BM, and liver disease) while his relatives had different clinical manifestations (Table 1). His oldest siblings (IV-1 and IV-2) developed AML and MDS with excess of blasts RAEB-2, respectively; the twin siblings (IV-5 and IV-6) and IV-7 had mild cytopenias; the mother (III-1) only presented with macrocytosis; and two sisters were asymptomatic (IV-4 and IV-8; Table 1). Based on family reports, IV-2 and IV-4 also had avascular hip necrosis and underwent hip replacement. The father (II-8), an obligatory carrier, died of pulmonary fibrosis at age 50 years. The only heterozygous carrier in the family had a history of complicated pneumonia but was otherwise healthy. Multiple family members were reported to have neurological disorders, including intellectual impairment and progressive blindness (Fig. 1a). Developmental delay and retinopathy have been already linked to *RTEL1* dysfunction and other telomere diseases. However, due to multiple consanguineous marriages in the family and the lack of accurate clinical reports, these findings could be related to other unidentified genetic defects and/or environmental factors.

Both the proband and his sister IV-1 had hyperpigmentation involving the knees and areas exposed to heat sources, compatible with a diagnosis of erythema ab igne. We are uncertain if this finding is associated with patients' telomere diseases as the hypo/hyperpigmentation typically described in telomeropathies usually involve the face, neck, and sun exposed areas (Calado and Young 2009).

After confirmation of the molecular diagnosis, oral administration of danazol (800 mg daily) was initiated for the proband and two siblings with thrombocytopenia (IV-5 and IV-7). After 8 weeks of therapy, the proband succumbed to pneumonia due to respiratory failure, and no improvement in blood counts was observed. IV-5 showed a robust platelet response and longer telomeres than at diagnosis after 6 months of treatment, without drug toxicity (Fig. 2c). IV-7 did not respond to danazol after 6 months of treatment, but she was non-compliant to treatment (with side effects noted of amenorrhea and elevated serum liver enzyme levels). At this writing, the proband, his father, and two siblings are all deceased due to complications of BMF and/or pulmonary disease.

The impact of the homozygous *RTEL1* variant in disease anticipation

In this family, homozygous individuals from the fourth generation presented with an earlier onset of disease compared to those from the third generation, suggesting a mechanism of disease anticipation. In some cases (IV-1, IV-2, and IV-3), they also had more severe phenotypes. To investigate if disease anticipation was associated with progressive telomere shortening, we calculated patients' ΔTL as they represented the extent of telomere shortening adjusted for their ages. There was progressive telomere shortening through generations, as the median ΔTL of the proband and siblings was − 4.3 kb, whereas difference in the homozygous mother or the heterozygous carrier was − 3.3 kb or − 1.8 kb, respectively (Fig. 2b). Higher negative ΔTL values indicate that age-adjusted patients' TL are shorter.

Discussion

Significant clinical heterogeneity in the spectrum of telomere diseases, specifically with *RTEL1* dysfunction, has been described (Speckmann et al. 2017; Cardoso et al. 2017; Marsh et al. 2018). In general, biallelic *RTEL1* variants are associated with early disease onset and clinical features commonly seen in DC/HH, whereas heterozygous variants are more associated with variable penetrance (Gutierrez-Rodrigues et al. 2014; Cardoso et al. 2017; Borie et al. 2019). In this report, for example, III-16 was an asymptomatic heterozygous carrier with longer telomeres compared to homozygous individuals in the pedigree (Fig. 2a). However, we also showed that family members from the same generation with similar mutational status and very short telomeres had malignant diseases, multi-organ failure, non-specific hematologic findings, or no apparent disease (Table 1).

The phenotypic heterogeneity observed in our study has never been described in multiple individuals within a family. Most biallelic *RTEL1* patients have AA with or without any mucocutaneous, developmental, and neurological abnormalities in the first two decades of life (Walne et al. 2013; Vannier, Sarek, and Boulton 2014; Marsh et al. 2018). Also, MDS/AML and isolated cytopenias have only been reported in patients with pathogenic

heterozygous *RTEL1* variants (Marsh et al. 2018; Cardoso et al. 2017), and disease severity and onset of manifestations are usually similar among siblings with biallelic *RTEL1* variants (Cardoso et al. 2017; Speckmann et al. 2017; Marsh et al. 2018; Vannier et al. 2014; Ballew et al. 2013; Walne et al. 2013). Only one study has previously reported pediatric twin siblings with the same homozygous *RTEL1* variant and such different diseases: one with DC and the other with macrocytosis and NK-cell lymphopenia (Speckmann et al. 2017). Of note, biallelic *RTEL1* patients are often observed with a complex immunodeficiency that particularly affects the NK and B cells (Ballew et al. 2013; Touzot and Kermasson 2016; Speckmann et al. 2017).

The novel p.E665K *RTEL1* variant is located in the conserved catalytic domain of RTEL1, believed to be essential to the helicase activity. Loss of function variants in this domain are suggested to have a strong impact on protein function being clinically translated into severe phenotypes (Vannier et al. 2014; Le Guen et al. 2013; Ballew et al. 2013). Here, the p.E665K variant was associated with variable expressivity and incomplete penetrance, suggesting that this novel variant is hypomorphic. Indeed, the novel homozygous variant may not lead to the protein's loss of function as in vivo experiments have shown that knockout of *RTEL1* is embryonically lethal in mice (Ding et al. 2004). Our findings demonstrated the genetic complexity of *RTEL1* biology as well as the challenge for interpreting genetic data of recessive disorders caused by pathogenic variants in genes with variable penetrance and expressivity; it would be difficult to link such different phenotypes to the same genetic alteration if affected individuals were not related.

As observed here and in general, the main impact of consanguinity is increased rates of autosomal recessive disorders (Hamamy et al. 2011). The novel p.E665K *RTEL1* variant described in this family was found in the two independent family groups from the first generation, suggesting a common ancestor. Nevertheless, the pathogenic role of the p.E665K variant only became evident when homozygous patients required medical care for severe disease manifestations. The increased prevalence of homozygosity in the family was also associated with disease anticipation, a phenomenon in which progressive telomere shortening is linked to severity and earlier onset of disease. Anticipation has been described in few families with autosomal dominant telomeropathy caused by *TERC*, *TERT,* or *RTEL1* pathogenic variants (Armanios et al. 2005; Vulliamy et al. 2004; Kannengiesser et al. 2015). To our knowledge, this is the first report that supports the mechanism of disease anticipation in the context of a recessive autosomal telomere disease. Despite clinical and molecular data were only available for proband's mother, we observed that both parents, including an obligated carrier, had mild phenotypes later on in life compared to their children. Our findings were consistent with previous reports in which disease anticipation was observed in few individuals from the same family and onset of disease was two decades earlier in the proband and siblings compared to their mother (Vulliamy et al. 2004; Armanios et al. 2005; Kannengiesser et al. 2015). Further studies are required to establish convincing mechanisms of disease anticipation in telomere diseases, as current data suggest that this phenomenon is variable, unpredictable, and likely modulated by each individual's genetic background and environment. We did not identify any other rare or novel variant in telomere biology genes that could explain the phenotypic heterogeneity in affected individuals, and the somatic profiles of individuals that developed hematologic

malignancies are unknown. We also screened patients for pathogenic variants in the *TERT* promoter region according to methodology previously described (Gutierrez-Rodrigues et al. 2019); however, no pathogenic variants were found. Further, the presence of a cryptic disease in organs affected in telomere diseases is possible in individuals with normal blood counts, pulmonary function, or liver enzyme levels. Other molecular studies, such as 3′ overhangs measurement and transcriptional profiles, was not performed due to the lack of clinical samples.

In telomere diseases, patients are often treated with supportive therapy, steroid sex hormones, or organ transplantation (Townsley et al. 2016; Khincha et al. 2014). Both hematologic improvement and telomere elongation have been described after danazol in patients with AA and DC (Ziegler et al. 2012; Kirschner et al. 2018), and in our recent clinical trial with a small cohort of telomeropathies patients (Townsley et al. 2016). In this study, only IV-5 had a platelet response and slight telomere elongation following danazol treatment. However, androgen therapy has also been reported to improve hematologic counts independently of telomere elongation (Khincha et al. 2018). Of note, the optimal dose, duration of treatment, adverse reactions, and long-term risks and benefits of danazol treatment are still not known.

In summary, this study highlights some biological concepts about the complex genetic architecture associated with *RTEL1* dysfunction. The unique demographic and social history of this Lebanese family with multiple p.E665K homozygous individuals was an optimal opportunity to study the variable expressivity and penetrance of recessive *RTEL1* phenotypes between inter and intra-generations of this family. Both genetic testing and TL measurement were critical for molecular diagnosis and should be recommended for relatives of families suspected of inherited BMF syndromes as it is important for genetic counseling. Increased surveillance needs to be encouraged for all carriers, regardless of the lack of classical clinical manifestations of telomere diseases.

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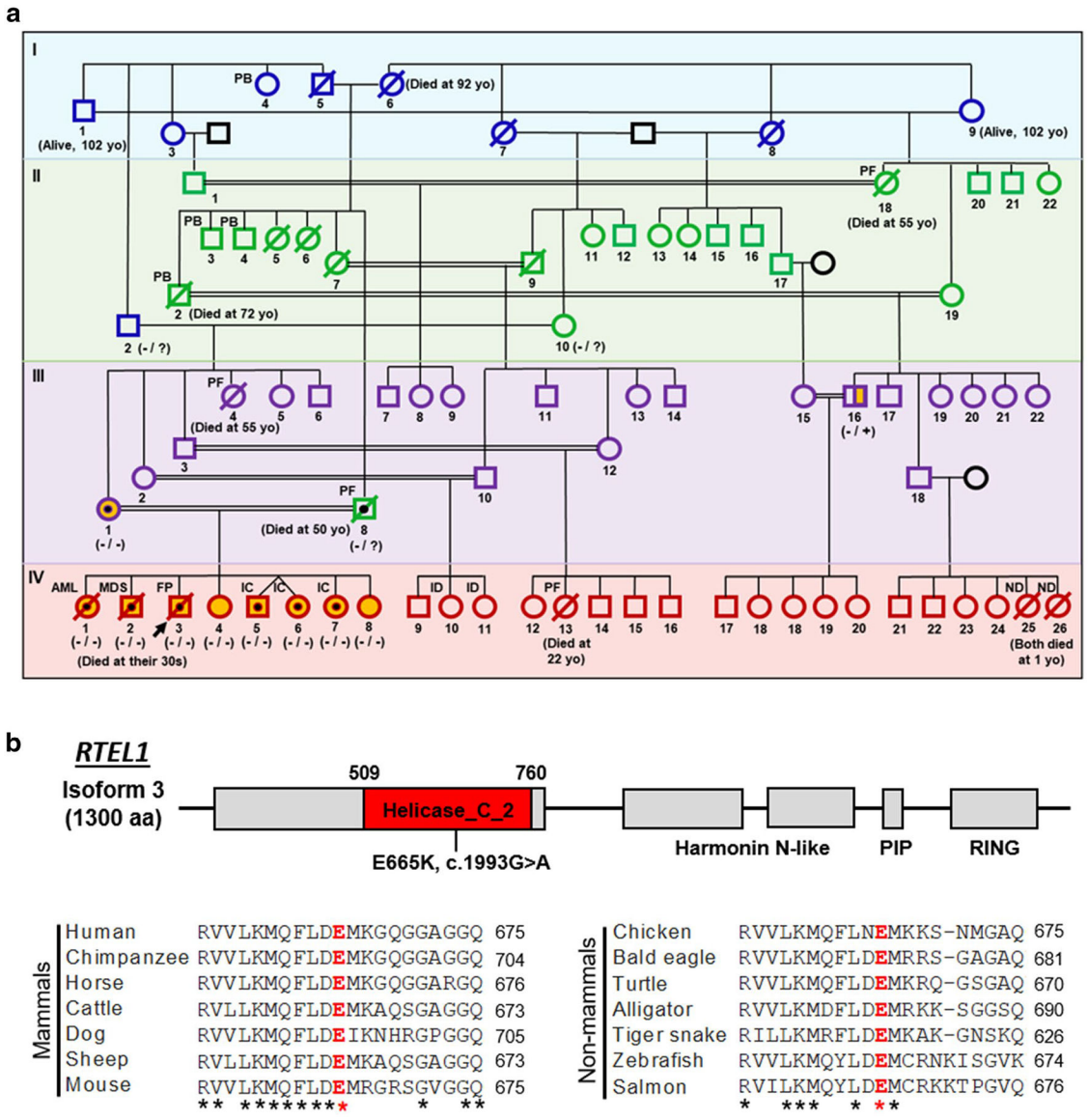

Fig. 1.

A Lebanese family with a novel *RTEL1* homozygous p.E665K (c.1993G > A) pathogenic variant. **a** Complex pedigree representing four generations of the family. Individuals from the first, second, third, and fourth generations are colored in blue, green, purple, and red, respectively. Consanguineous marriage was observed at seven instances in this family. Individual I-2 from first generation (colored in blue) got married to individual II-10 from second generation (colored in green) and individual II-8 from second generation (colored in green) got married to individual III-1 from third generation (colored in purple). An arrow indicates the proband of the family. Proband's mother (III-1), six siblings (except IV-4), and a cousin (III-16) were the only individuals in this family examined for marrow, lung, and liver diseases. Although an extensive pedigree is represented, only nine subjects were screened for *RTEL1* variants. Other relatives were not investigated as they refused clinical care due to financial and geographic reasons. Clinical manifestations reported by the

proband in any relative are abbreviated in the figure above the affected individuals. Open circles and squares represent females and males, respectively. Half or full yellow circles and squares show individuals who are heterozygous (−/+) or homozygous (−/−) for the *RTEL1* variant, respectively. Obligatory carriers (−/?) are also shown in the pedigree. A line-through indicates individuals who deceased. Individuals with any clinical manifestations related to telomere diseases are represented with black small circles. Abbreviations: *yo* years old, *PB* progressive blindness, *PF* idiopathic pulmonary fibrosis, *AML* acute myeloid leukemia, *MDS* myelodysplastic syndrome, *IC* isolated cytopenia, *ID* intellectual disability, *ND* neurological disease. **b** Linear representation of *RTEL1* with the novel homozygous p.E665K c.1993G > A. The novel variant is located in the catalytic domain of *RTEL1*, the helicase domain. The E665 amino acid is completely conserved among 68 different species evaluated, including mammals and non-mammals. Representative panels with the conserved E665 residue are shown in the figure. Asterisks indicate well-conserved amino acids

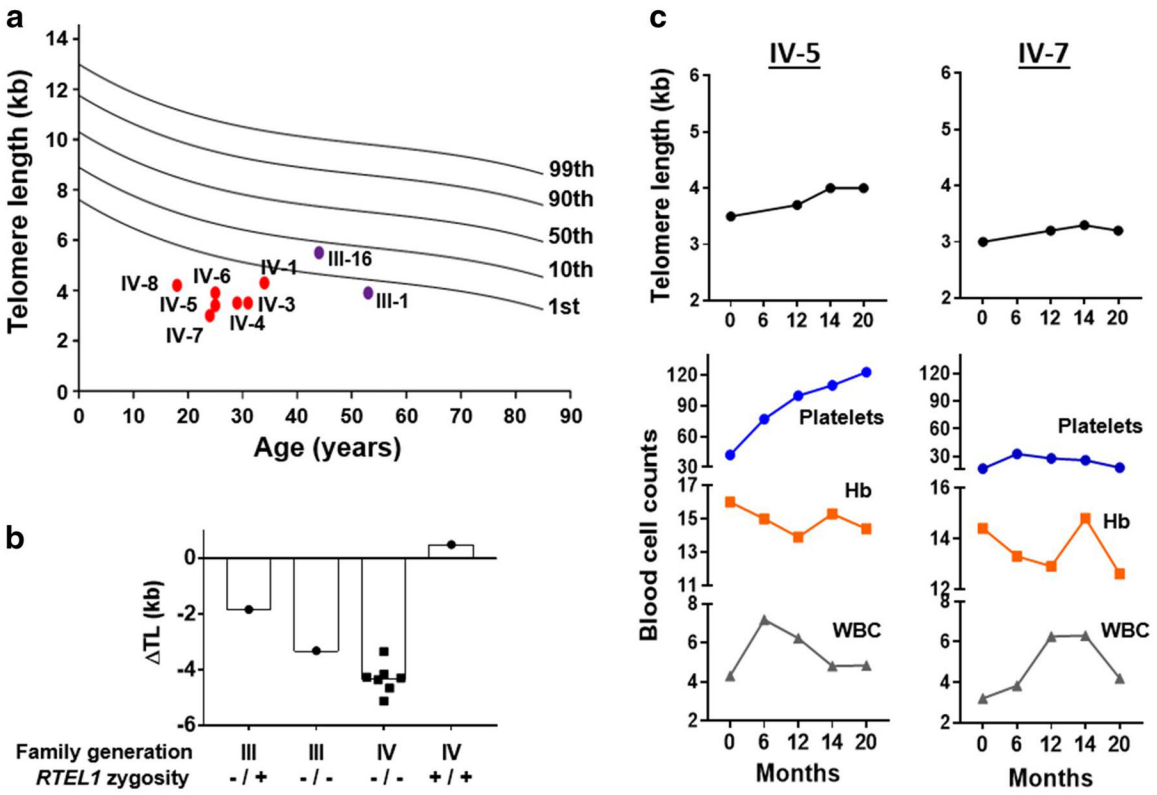

Fig. 2.

Telomere length (TL) and hematologic blood counts under the danazol treatment. **a** TL measurement by Southern blot analysis. Individuals from the third and fourth generations are shown with purple and red circles, respectively. Lines represent the 1st, 10th, 50th, 90th, and 99th percentiles of TL from 263 healthy controls. **b** Progressive telomere shortening according to *RTEL1* zygosity and family generations. For each individual, an expected age-adjusted TL was obtained by a third-order polynomial regression analysis with TL measurements from controls. The differences between the observed and expected age-adjusted TLs (ΔTL) in kilobases (kb) are plotted in the graphic for heterozygous (−/+) and homozygous (−/−) individuals from the third (III) and fourth (IV) family generations. A median ΔTL of controls was −0.004 and are represented by a black line. In the third generation, TL was longer in the heterozygous carrier (III-16) compared to the homozygous subject (III-1). All individuals from the fourth generation had shorter TLs for their ages compared to their mother III-1. The observed TL of the son of III-5 (not depicted in the pedigree), found to be wild-type for *RTEL1* (+/+) and with normal TL for his age (26 years old and TL of 8.5 kb), was similar to his expected age-adjusted TL. **c** Chronological analysis of TLs measured by Southern blot and hematologic blood counts from two individuals during danazol treatment (20 months of treatment). First measurements were before androgen therapy. During treatment, the IV-5 platelet count increased from 42 to 123 × 10^6/ml and TL slightly elongated (3.4–4 kb) in total leukocytes. In IV-7, we found no differences in the TL [differences in TLs were within a variability range intrinsic to the

technique (Gutierrez-Rodrigues et al. 2014)]. Abbreviations: *Hb* hemoglobin, *WBC* white blood counts

Table 1

Clinical and molecular features of individuals with the novel *RTEL1* variant

	IV-3	III-1	IV-1	IV-2	IV-4	IV-5	IV-6	IV-7	IV-8	III-16
RTEL1 E665K, c.1993 G > A	Hom	Hom	Hom	Hom	Hom	Hom	Hom	Hom	Hom	Het
Sex	M	F	F	M	F	M	F	F	F	M
Age	31	53	34	32	29	25	25	24	18	44
Telomere length (Kb)	3.5	3.9	4.3	n/a	3.5	3.4	3.9	3	4.2	5.5
Hematologic counts										
Hb (g/dL)	15.5	13.5	12.5	10.6	n/a	15.8	11.5	14.4	13.3	14.8
WBC (× 10^3/ml)	5.25	5.1	7.53	3.4	n/a	4.3	6.45	5.78	5.33	6.52
Platelets (× 10^3/ml)	41	172	244	38	n/a	42	185	17	224	224
ANC (× 10^3/ml)	3.15	1.9	3.9	1.87	n/a	2.07	3.71	3.02	2.15	3.27
MCV	107	102	100	110	n/a	97	87	106	90	83
BM cellularity (%)	30%	n/a	n/a	60%	n/a	n/a	n/a	n/a	n/a	n/a
Clinical findings										
AML/MDS	–	–	+	+	–	–	–	–	–	–
Pulmonary fibrosis	+	–	–	–	–	–	–	–	–	–
Portal hypertension	+	–	–	–	–	–	–	–	–	–
Isolated cytopenia	+	–	–	+	–	+	+	+	–	–
Hip necrosis	–	–	–	+	+	–	+	–	–	–
Treated with danazol	Yes	No	No	No	No	Yes	No	Yes	No	No

Individual IV-3 is the proband of the family. The IV-4 was not clinically assessed at our institution but she consented to the using of her sample for genetic testing. Telomere length measurement of individual IV-2 was not performed due to lack of a clinical material. His blood counts and clinical manifestation were evaluated before study during his treatment for AML in the same institution. Individuals under danazol treatment refused bone marrow biopsy due to financial reasons

Hom homozygous, *Het* heterozygous, *F* female, *M* male, *Hb* hemoglobin, *WBC* white blood counts, *ANC* absolute neutrophil counts, *MCV* median corpuscular volume, *BM* bone marrow, *NA* not available, *AML* acute myeloid leukemia, *MDS* myelodysplatic syndrome