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Pathogenic *TERT* promoter variants in telomere diseases

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Purpose: The acquisition of pathogenic variants in the *TERT* promoter (*TERTp)* region is a mechanism of tumorigenesis. In nonmalignant diseases, *TERTp* variants have been reported only in patients with idiopathic pulmonary fibrosis (IPF) due to germline variants in telomere biology genes.

Methods: We screened patients with a broad spectrum of telomeropathies ($n = 136$), their relatives ($n = 52$), and controls ($n = 195$) for *TERTp* variants using a customized massively parallel amplicon-based sequencing assay.

Results: Pathogenic −124 and −146 *TERTp* variants were identified in nine (7%) unrelated patients diagnosed with IPF (28%) or moderate aplastic anemia (4.6%); five of them also presented cirrhosis. Five (10%) relatives were also found with these variants, all harboring a pathogenic germline variant in telomere biology genes. *TERTp* clone selection did not associate with peripheral blood counts, telomere length, and response to danazol treatment. However, it was specific for patients with telomeropathies, more frequently co-occurring with *TERT* germline variants and associated with aging.

Conclusion: We extend the spectrum of nonmalignant diseases associated with pathogenic *TERTp* variants to marrow failure and liver disease due to inherited telomerase deficiency. Specificity of pathogenic *TERTp* variants for telomerase dysfunction may help to assess the pathogenicity of unclear constitutional variants in the telomere diseases.

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INTRODUCTION

The *TERT* gene encodes the catalytic component of the telomerase complex required to elongate telomeres in stem and progenitor cells.[1,2] *TERT* is epigenetically silenced in normal somatic and nonproliferative cells, but aberrantly expressed in many human cancers.[3–6] Acquisition of pathogenic *TERT* promoter (*TERTp*) variants located upstream of the translation initiation site at positions −124C>T (chr5:1,295,228), −146C>T (chr5:1,295,250), and −57A>C (chr5:1,295,161) has been described as a mechanism of tumorigenesis in cancer cells.[3,6–9] These variants increase *TERT* expression and promote cell proliferation through recruitment of the transcription factor GABPA to the mutant allele.[10–12] Somatic *TERTp* variant clones recently have been found in a few patients with idiopathic pulmonary fibrosis (IPF) caused by pathogenic germline variants in telomere biology genes. These pathogenic *TERTp* variants appear to functionally compensate the deleterious impact of disease-causing germline *TERT* variants by increasing telomerase activity and cell proliferation.[13]

Germline variants in telomere-related genes are etiologic in a broader spectrum of diseases collectively named telomere diseases or telomeropathies,[14] including IPF but also affecting other organs, such as the bone marrow (aplastic anemia [AA] and dyskeratosis congenita [DC]) and the liver (cirrhosis and nonalcoholic steatohepatitis). We investigated the distribution of somatic pathogenic *TERTp* variants in a large cohort of patients with a spectrum of telomere diseases using a customized low-cost massively parallel sequencing assay optimized for identification and quantification of hematopoietic clones bearing the pathogenic −124 and −146 *TERTp* variants. We further assessed the association of these *TERTp* variants with telomere length (TL) and peripheral blood counts of patients.

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MATERIALS AND METHODS

Cohort

In this retrospective study, we screened blood leukocytes from 136 patients with telomeropathies (median age, 29 years; range, 1–76), 52 relatives (median age, 40 years; range, 8–72), and 195 controls for the pathogenic −124 and −146 *TERTp* variants (Table 1). Patients were primarily diagnosed with DC ($n = 21$), AA ($n = 86$), IPF with or without another telomeropathy-related phenotypes ($n = 18$), or other phenotypes ($n = 11$; Supplementary Table S1). Clinical diagnosis of DC and AA was defined according to previous criteria.[2,15,16] Briefly, DC patients had at least two of three manifestations of the clinical triad (dystrophic nails, patchy skin hyperpigmentation, and oral leukoplakia) and TL below the 1st percentile for age-matched controls, whereas AA patients presented with pancytopenia and hypocellular bone marrow without any evidence for myelodysplasia, myelofibrosis, or leukemia. Inclusion criteria were based on molecular diagnosis: TL below the 10th percentile of age-matched controls or a germline variant in a telomere biology gene classified as pathogenic, likely pathogenic, or variant of uncertain significance (VUS) by the Sherloc criteria, a framework that incorporates the American College of Medical Genetics and Genomics (ACMG) criteria[17,18] (Supplementary Tables S2–S3). The Sherloc criteria refined the ACMG guidelines to comprehensively assess variants' pathogenicity based on both clinical and functional evidence, attributing points to score each variant for pathogenicity. The point score thresholds for pathogenic and likely pathogenic variants are four (4P) and five points (5P), respectively (Supplementary Table S4). Patients' relatives were only studied if they harbored the same germline variant as the proband or had short telomeres, regardless of symptoms or evidence of disease. Enrolled individuals were seen in the Hematology Branch clinic of National Heart, Lung, and Blood Institute (NHLBI) or the bone marrow failure clinic in the Hospital das Clínicas, Ribeirão Preto School of Medicine, University of São Paulo (USP) between 2004 and 2017 (Supplementary Tables S2–S3). TL was measured by Southern blot (SB), quantitative polymerase chain reaction (qPCR), or flow fluorescent in situ hybridization (flow-FISH) according to protocols previously described.[19] TL measurements by qPCR were confirmed by SB or flow-FISH at the time of this study. Patients with acquired immune AA ($n = 70$), IPF without evidence of inherited disease and a telomere-related germline pathogenic variant ($n = 12$), other inherited bone marrow failure syndromes (IBMFS; Diamond–Blackfan anemia, $n = 4$; chronic neutropenia, $n = 3$), and acute myeloid leukemia (AML; $n = 106$) were studied as controls (Supplementary Table S2-S3).

Approval was obtained from the Institutional Review Board of NHLBI and from the Comitê de Ética em Pesquisa do Hospital das Clínicas de Ribeirão Preto. Samples were collected according to the Declaration of Helsinki and written consent was obtained from all participants or their legal guardians.

Massively parallel amplicon-based sequencing assay for detection of *TERTp* variants

We customized a massively parallel amplicon-based sequencing assay that targeted the *TERTp* region in which pathogenic −124 and −146 *TERTp* variants were located (Supplementary Figure S1). Although primers were not optimized to cover the −57A>C *TERTp* position, we also detected this variant in some of our samples. *TERTp* variant

Table 1 The cohort screened for pathogenic *TERT* promoter variants by massively parallel amplicon-based sequencing assay

	Telomeropathies				Family members	Control group[a]	
	DC ($n = 21$)	AA ($n = 86$)	IPF (with or without AA, MDS, and cirrhosis) ($n = 18$)	Other phenotypes ($n = 11$)	($n = 52$)	AML ($n = 106$)	Acquired AA, IPF or other IBMFS ($n = 89$)
Median age (range)	13 (1–59)	28 (5–73)	54 (27–76)	27 (3–69)	40 (8–72)	50 (2–86)	29 (1–88)
Females/males	4/9	40/46	7/11	3/8	30/22	51/55	44/45
Patients with somatic *TERTp* (%)	0	4 (4.6%)	5 (28%)	0	5 (9.6%)	0	0
Patients with a germline *TERT* variant	3	37	14	5	34	0	0
Patients with a somatic *TERTp* and a germline *TERT* variant	0	3	5	0	5	0	0
Patients with somatic *TERTp* variants from the total of patients with *TERT* germline variants (%)	0	8.1	36	0	14.7	0	0

TERTp pathogenic *TERT*.

In this study, we screened 136 patients with telomeropathies that presented dyskeratosis congenita (DC, $n = 21$), aplastic anemia (AA, $n = 86$), idiopathic pulmonary fibrosis (IPF, $n = 18$), and other phenotypes that included myelodysplastic syndrome (MDS) or hypoplastic MDS (HypoMDS, $n = 7$), isolated thrombocytopenia ($n = 3$), and thrombocythemia ($n = 1$).

[a]Control group was composed of patients with acquired AA ($n = 70$; median age = 28 years), IPF ($n = 12$; median age = 62 years), other inherited bone marrow failure syndromes (IBMFS; Diamond–Blackfan anemia, $n = 4$; chronic neutropenia, $n = 3$), and acute myeloid leukemia (AML, $n = 106$; Supplementary Tables S2–S3).

screening was performed in patients, relatives, and controls using peripheral blood leukocytes collected at the time of first clinical evaluation. Whenever possible, testing was also performed in granulocytes separated by gradient centrifugation in parallel with the respective leukocyte samples.

Library preparation consisted of two rounds of PCR to amplify the *TERTp* region. A first PCR was designed to amplify the targeted region and a second PCR for addition of Illumina adapters (Illumina, San Diego, CA, USA) into fragments. In the first PCR, we used a set of four different forward and reverse primers (total of eight primers) that were pooled in equimolar amounts to amplify the *TERTp* region (Supplementary Table S5). PCR products were then subjected to a second PCR round for the addition of the full i5/i7 Illumina adapter/index sequences into the DNA fragments.

Up to 96 libraries were pooled in equimolar amounts and pair-end sequenced in 300 cycles on the MiSeq platform (Illumina). Median coverage depth on targets was 104×. Reads were aligned to the human genome reference (hg19) using Burrows–Wheeler Aligner (BWA)[20] and data quality was assessed using FastQC. Sequences were trimmed to remove the adapters as well as low-quality bases (-q 15 –minimum-length 35). Variants were called using VarDict and the following filtering criteria: -f 0.005 -v -c 1 -S 2 -E 3 -g 4 -th 8 (ref. [21]). For comparison, we also used SAMTools[22] and Genome Analysis Toolkit (GATK)[23] to call the variants. While VarDict called all *TERTp* variants that were further validated by droplet digital PCR (ddPCR; RainDance Technologies, Billerica, MA, USA), GATK and SAMTools only called variants with allele frequency >20% and >8%, respectively. Variants were annotated using Annovar[24] and variant allele frequency (VAF) was calculated by a ratio of variant minor allele counts and total reads.

Droplet digital PCR

In samples that were available, the −124C>T and −146C>T *TERTp* variants identified by sequencing were validated by ddPCR (Supplementary Figure S2a), except for a single case that was confirmed by Sanger sequencing (Supplementary Figure S2b). *TERTp* variant clones were also tracked over time using this technique. In patient NIH46, the *TERTp* variant was quantified in the following blood cells' subpopulations by ddPCR: leukocytes after whole-blood ammonium chloride potassium (ACK) lysis, granulocytes separated by gradient centrifugation using Ficoll–Hypaque, peripheral mononuclear cells separated by gradient centrifugation using Ficoll–Hypaque, $CD14^{+}CD16^{-}$ monocytes, $CD3^{+}$ T cells, and mononuclear fraction depleted for $CD3^{+}$ and $CD14^{+}CD16^{-}$. The $CD14^{+}CD16^{-}$ monocytes were isolated from mononuclear cells using immunomagnetic negative selection (the EasySep™ Human Monocyte Isolation kit, Stemcell Technologies, Cambridge, MA, USA) and $CD3^{+}$ T cells were isolated from mononuclear cells by immunomagnetic positive selection (the EasySep™ Human CD3 Positive Selection kit II, Stemcell Technologies).

ddPCR was performed according to a protocol previously optimized with minor modifications to transfer the assay to the RainDance platform (RainDance).[25] To evaluate the linearity and limit of detection of the ddPCR assay, a variant control sequence (CCCCTTCCGG) was serially diluted into 300 ng of sheared normal human genomic DNA to have an expected variant target copy number. A mean frequency abundance of the variant template was plotted versus the target copies input to generate a standard curve. Linearity of the assay was high ($R^2 = 0.99$; Supplementary Figure S3a) and lower limit of detection was 0.17%. The highest mean frequency abundance obtained when genomic DNA from negative controls were used was 0.5%, and this value was used as the negative cut-off. The customized sequencing assay accurately detected pathogenic *TERTp* variants confirmed by ddPCR but did not identify false positives. The correlation between sequencing and ddPCR in quantification of *TERTp* variant clones was high ($R^2 = 0.97$; Supplementary Figure S3b). Agreement between techniques was evaluated by Bland–Altman analysis, a statistical tool to compare clinical assays.[26] Bland–Altman analysis evidenced a good agreement between these two methods (Supplementary Figure S3c), as the mean difference of VAF measurements was 0.95 and no measurements exceed the 95% confidence interval (CI) limits of agreement. The standard deviation (SD) between assays was 2.1 and 95% limits of agreement ranged from 5.15 to −3.25. Detailed protocols and primer sequences are available in Online Supplementary Data.

RESULTS

Pathogenic *TERTp* variants associated with different phenotypes from the spectrum of telomeropathies and patients' ages

Of 136 cases, nine unrelated patients (7%; median age, 39 years; range, 24–65; Fig. 1) were found with *TERTp* variants. Nine patients had the pathogenic −124C>T variant, including two patients who also had the −57A>C *TERTp* variant (NIH37 and NIH93; Table 2). Patients were clinically diagnosed with IPF (5/18; 28%) and moderate AA (MAA; 4/86; 4.6%) (Table 1 and Fig. 1). Of note, eight of them also presented other phenotypes related to telomeropathies (Table 3); co-occurrence with cirrhosis and MAA was frequent (Tables 2–3). Five relatives (10%; median age, 63 years; range, 17–72) had the −124C>T ($n = 4$) or the −146C>T ($n = 1$) *TERTp* variants: three were asymptomatic and two were diagnosed with MAA or a DC-like phenotype. (Table 2). Within the same family, a somatic *TERTp* variant was not observed in more than one subject (Supplementary Figure S4), suggesting that acquisition of these variants was not due to a genetic susceptibility caused by the telomere-related germline pathogenic variant identified in the family.

The frequency of pathogenic *TERTp* variants was much higher in IPF patients compared with AA cases (28% vs. 4.6%; Fisher's exact test, $P = 0.007$; Table 1). Because some patients presented different phenotypes from the spectrum of telomeropathies, we then investigated whether pathogenic

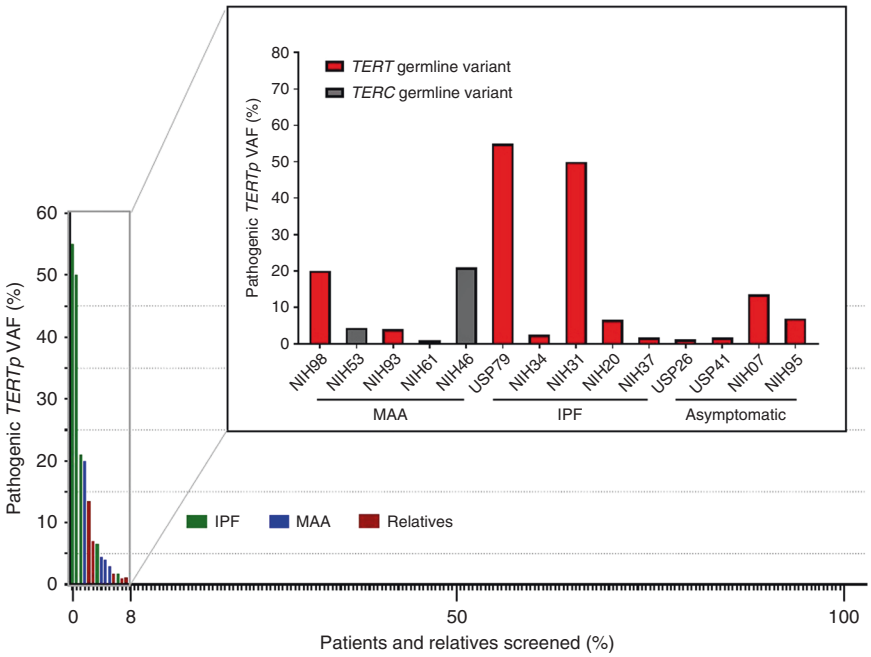

Fig. 1 Clinical association of somatic pathogenic *TERT* promoter (*TERTp*) variants and telomere diseases. Frequency of pathogenic *TERTp* variants in patients and relatives screened in the study. The size of the *TERTp* clone is represented by the variant allele frequency (VAF) and shown for each individual screened according to their primary diagnosis: idiopathic pulmonary fibrosis (IPF), moderate aplastic anemia (MAA), and relatives. Of 188 patients and relatives, 14 had the −124 or −146 *TERTp* variants (8%). An additional graph shows in detail the pathogenic *TERTp* clone size, initial diagnosis, and the telomere biology gene in which a germline variant was identified from the patients/relatives with pathogenic *TERTp* variants. Four relatives were asymptomatic.

TERTp variants were more frequent in the setting of pulmonary disease compared with marrow failure or liver disease. No difference in frequency of disease phenotypes (IPF vs. marrow failure or liver disease) was observed among patients with a pathogenic *TERTp* variant (36% vs. 50% or 42%, respectively; χ^2 test, $P > 0.05$; Table 3), suggesting that these variants occurred in all these clinical presentations. Pathogenic *TERTp* variant clones positively correlated with age, as they were only present in individuals older than 18 years old and more frequent in those 60 to 80 years old (Fig. 2a). Six of 86 individuals ranging in age from 21 to 40 (7%), 3 of 44 individuals ranging in age from 41 to 60 years (6.8%), and 5 of 18 patients older than 61 years (27.8%) had pathogenic *TERTp* variants. The median age of patients with *TERTp* variants and a primary diagnosis of IPF and MAA was 57 and 27 years, respectively.

Pathogenic *TERTp* variants were found in telomeropathy patients who had a germline variant in telomere biology genes but not in controls (median age, 29 years; range, 1–88) or in patients with very short telomeres without a germline variant in telomere biology genes (median age, 25 years; range, 5–63; Table 1). We also confirmed the absence of pathogenic *TERTp* variants in the granulocytic fractions from controls in which materials were available (Supplementary Table S2–S3).

Pathogenic *TERTp* variants more frequently co-occurred with germline *TERT* variants in myeloid cells

The customized sequencing assay detected pathogenic *TERTp* clones at VAF as low as 1.2%, which was confirmed by ddPCR. Overall, *TERTp* clones were more frequent in individuals with germline *TERT* variants (12/14 cases); only two patients harbored a germline variant in *TERC*. The germline variants identified in telomere biology genes were classified as pathogenic or likely pathogenic ($n = 8$), and VUS ($n = 6$; Table 2) and, except for the *TERT* R696C found in USP26, they were heterozygous. All variants classified as VUS had some evidence for being pathogenic (all had a Sherloc score of 3.5P) but insufficient to meet Sherloc criteria for pathogenicity; all were predicted as deleterious in silico, absent in control populations, and associated with a family history and phenotype of telomere diseases (Supplementary Table S4).

Pathogenic *TERTp* variant clone sizes varied from 1.2% to 50% in total leukocytes and clones were found at higher allele frequencies in the granulocytic fractions in four patients (Table 2 and Fig. 2b). In NIH93, the −124C>T *TERTp* was identified in total leukocytes and granulocytes by both sequencing and ddPCR at VAF as low as 6%. However, in NIH61, pathogenic *TERTp* clones were only detected in granulocytes by sequencing due to very low VAF in total leukocytes. Results were similar when peripheral blood cell subpopulations from NIH46 were separated by magnetic selection for screening of the −124C>T *TERTp* variant by ddPCR. The −124C>T *TERTp* variant was found enriched in the granulocyte fraction and mononuclear cells depleted for $CD3^+$ and $CD14^+$ cells (Fig. 2b). Clonal dominance was not observed in most individuals with pathogenic *TERTp* variants; nine had pathogenic *TERTp* clones at VAF lower than 10%.

Table 2 Mutational and clinical profile of patients with telomeropathies and somatic pathogenic *TERT* promoter variants

	Age	TL	Clinical diagnosis	Germline variant	Sherloc germline variant classification	Somatic *TERTp* variant	Leukocyte VAF (%)	Bone marrow cellularity	Hb (g/dL)	MCV (fL)	Plt ($\times 10^3$/μL)	ANC ($\times 10^3$/μL)	WBC ($\times 10^3$/μL)
Patients													
USP79	46	<1st	IPF/cirrhosis	***TERT***, T937A c.2809A>G	VUS	−124C>T	50	NA	16.9	107	117	2	3.5
NIH20	61	<1st	IPF/cirrhosis	***TERT***, V170L c.508G>A	P	− 124C>T	6.6	40%	13.5	103	117	3.9	7
NIH31[a]	57	<10th	IPF/MAA/cirrhosis	***TERT***, A1009G c.3026C>A	VUS	−124C>T	50	30–40%	9	99	55	1.37	2.3
NIH37[a]	65	<1st	IPF	***TERT***, P59S c.175C>T	VUS	− 124C>T − 57C>T	5 4.5	40–50%	13	111	104	3.0	5.4
NIH43	24	<1st	MAA	***TERT***, V1025F c.3073G>T	P	−124C>T	3	<10%	8.7	89	21	0.86	1.7
NIH46[b]	39	<1st	IPF/MAA	***TERC***, r.433G>C	VUS	−124C>T	21	<5%	8.9	107	65	1.32	2.7
NIH53	30	<1st	MAA/cirrhosis	***TERC***, r.107G>C	P	−124C>T	4.4	<5%	9.2	92	31	0.72	2.5
NIH93[b]	38	<1st	MAA/cirrhosis	***TERT***, A130V c.389C>T	VUS	−124C>T −57C>T	5/6[d] 6/5[d]	<20%	11.4	101	27	0.32	0.6
NIH98	25	Normal	MAA	***TERT***, R537C c.1609C>T	VUS	−124C>T	20	10%	10.6	112	83	1.0	3.3
Relatives													
USP26	18	<1st	Asymptomatic but with DC-like phenotype	***TERT***, R696C c.2086C>T[c]	P	−124C>T	1.2	NA	10.4	86	87	2.0	4.0
USP41	63	Normal	Asymptomatic	***TERT***, R865H c.2594G>A	P	−124C>T	1.8	NA	15.4	105	163	2.8	6.2
NIH07	71	<1st	Asymptomatic	***TERT***, K570N c.1710G>C	P	−124C>T	13.5	NA	12.1	85	305	4.0	4.8
NIH95	72	<1st	Asymptomatic	***TERT***, R1084P c.3251G>C	P	−124C>T	7	NA	12.6	104	245	2.0	4.7
NIH61[b]	44	<1st	MAA	***TERT***, L864P c.2591T>C	LP	−146C>T	3[e]	15%	6.7	110	39	0.3	1.6

Patients who underwent danazol treatment and were [a]off study or [b]responders.
[c] The only pathogenic germline variant in *TERT* or *TERC* identified in homozygosity.
[d]*TERTp* clones were detected in both leukocyte and granulocytic fractions. Clone sizes in granulocytes are described after the VAF observed in leukocytes.
[e]*TERTp* VAF in the granulocytic fraction. Clones were not detected in leukocytes.
ANC absolute neutrophil count, *DC* dyskeratosis congenita, *Hb* hemoglobin, *IPF* idiopathic pulmonary fibrosis, *LP* likely pathogenic, *MAA* moderate aplastic anemia, *MCV* mean corpuscular volume, *NA* not available, *P* pathogenic, *Plt* platelets, *TERTp* pathogenic *TERT* promoter, *TL* telomere length, *VAF* variant allele frequency, *VUS* variant of uncertain significance, *WBC* white blood cell count. TLs below the first percentile of age-matched controls (<1st) were considered very short and below the tenth percentile (<10th) were considered short.

Table 3 Spectrum of phenotypes observed in patients with telomeropathies and pathogenic *TERTp* variants

	Age	Sex	TL	Primary diagnosis	Spectrum of phenotypes related to telomeropathies			
					Pulmonary fibrosis	Marrow failure	Liver disease	Isolated cytopenia
Patients								
USP79	46	M	<1st	IPF	x		x	
NIH20	61	M	<1st	IPF	x		x	
NIH31	57	F	<10th	IPF	x	x	x	
NIH37	65	F	<1st	IPF	x			
NIH43	24	F	<1st	MAA		x		
NIH46	39	M	<1st	IPF	x	x	x[a]	
NIH53	30	M	<1st	MAA		x	x	
NIH93	38	M	<1st	MAA		x	x	
NIH98	25	F	Normal	MAA		x		
Relatives								
USP26	17	M	<1st	Asymptomatic				x
USP41	63	M	Normal	Asymptomatic				
NIH07	71	M	<1st	Asymptomatic				
NIH95	72	F	<1st	Asymptomatic				
NIH61	44	F	<1st	MAA		x		
				Frequency (%)	**36**	**50**	**42**	**7**

AA aplastic anemia, *DC* dyskeratosis congenita, *F* female, *IPF* idiopathic pulmonary fibrosis, *M* male, *MAA* moderate AA, *TL* telomere length below the first (<1st) or tenth (<10th) percentile of age-matched controls.
[a]Patient with mild steatosis.

Pathogenic *TERTp* variant clones expanded over time but did not associate with telomere elongation or response to danazol treatment

The clonal dynamics of pathogenic *TERTp* variants were assessed in serial samples from five patients over a period as long as four years; three had a pathogenic or likely pathogenic germline variant, and two had a germline VUS. In all cases, the *TERTp* variant clone size expanded over time (Fig. **2c**), suggesting a selective growth advantage in comparison with unmutated hematopoietic cells.

Pathogenic *TERTp* variants were not associated with changes in patients' TLs or improvement in blood counts; most subjects with a pathogenic *TERTp* variant, which is known to upregulate *TERT* expression, nevertheless had short or very short telomeres (12/14 individuals). Two individuals presented with normal TL (NIH98 and USP26). Despite her normal TLs, NIH98 had short 3' overhangs as previously reported.[27]

Twenty-one patients and three relatives from the cohort were enrolled in a clinical trial for treatment with danazol for two years (Supplementary Table S3) (ref.[16]). Five had pathogenic *TERTp* variant clones at diagnosis; three were responders and two were off-study after 3–6 months (Table **2**). NIH93 and NIH61 achieved a hematologic response at 3 months of treatment and NIH46 showed a response at 6 months. In these patients, an average size of pathogenic *TERTp* clone in myeloid cells was 4%, except for NIH46 who had a clone of 21%. Patients NIH31 and NIH37 responded to danazol at 3 months of treatment but were withdrawn from the study due to organ complications not involving the marrow. NIH31 had a large pathogenic *TERTp* variant clone (VAF of 50%) and NIH37 had two different variants at VAFs <5% (Table **2**).

Chronological analysis of pathogenic *TERTp* variants during androgen treatment was assessed using serial samples that were available for NIH46 and NIH61 (Fig. **2d**). In both, the size of pathogenic *TERTp* clones decreased during danazol treatment while blood counts improved. After treatment, the VAF of these pathogenic *TERTp* variant clones increased. These data suggest that hematologic response observed in these patients was mostly attributed to danazol and not to the presence of pathogenic *TERTp* variants, which appeared diluted in peripheral blood by treatment response. Indeed, pathogenic *TERTp* variants did not predict response to danazol, because patients with and without these somatic variants responded to treatment at 3–6 months and were not off-study (with *TERTp* variants vs. without *TERTp* variants, 3/5 vs. 8/12; Fisher's exact test, $P > 0.5$).

DISCUSSION

We have expanded the spectrum of nonmalignant diseases associated with pathogenic *TERTp* variants to MAA and cirrhosis. Our data indicated that the emergence of pathogenic *TERTp* variants correlates with chronologic aging and may be clonal evidence of a telomere disease in 8% of patients and relatives presenting these clinical phenotypes.

Pathogenic *TERTp* variants have been reported only in cancer or IPF but are rare in hematologic malignancies.[4,5] Indeed, we did not find these variants in 106 AML patients screened by our sequencing assay. Lack of association between pathogenic *TERTp* activation and hematologic diseases has

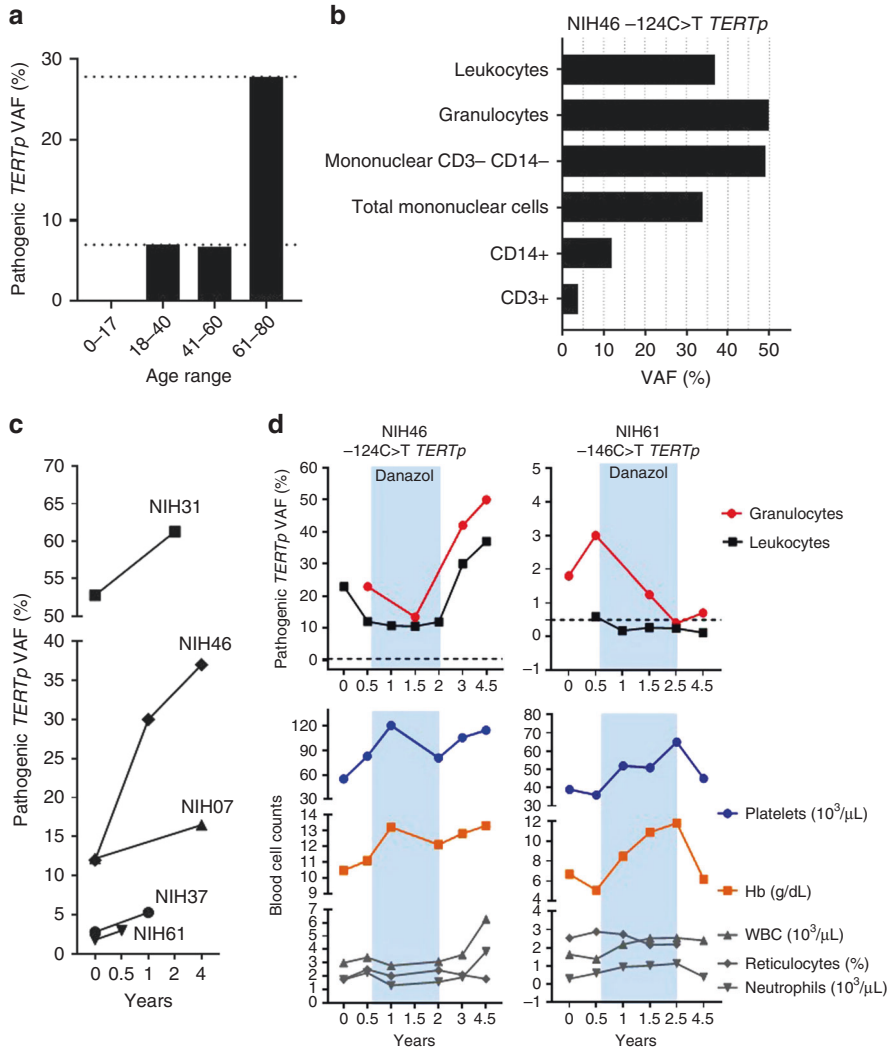

Fig. 2 Molecular and clinical characteristics of individuals with pathogenic *TERT* promoter (*TERTp*) variants. (**a**) Frequency of pathogenic *TERTp* variants in the different groups classified by age range. Pathogenic *TERTp* variant frequency increased with aging. (**b**) The −124C>T *TERTp* variant allele frequency (VAF) in blood cells' subpopulations from patient NIH46 quantified by droplet digital PCR (ddPCR). (**c**) Chronological pathogenic *TERTp* variant dynamics detected by ddPCR. In serial samples, clones bearing the −146C>T or −124C>T pathogenic *TERTp* variants expanded over time for all patients evaluated. (**d**) Chronological analysis of the pathogenic *TERTp* VAF and hematologic blood counts from two patients during danazol treatment. During the androgen therapy, *TERTp* clone sizes decreased in both total leukocytes and granulocytes as blood counts improved, especially the platelet counts and hemoglobin levels. A blue bar represents the time frame in which patients were under the danazol treatment. Pathogenic *TERTp* clones were tracked by ddPCR and dashed lines represent the lower limit of detection (0.5%) of this technique. *Hb* hemoglobin, *WBC* white blood cell count.

been attributed to constitutively high *TERT* expression in hematopoietic stem cells.[4,12] Consistent with this hypothesis, clones bearing pathogenic *TERTp* variants in our study were positively selected in the bone marrow when patients had an inherited telomerase deficiency. No pathogenic *TERTp* variants were found in our control group or previously reported cohorts including more than 2,500 healthy subjects (mean age of 50 ± 11 years) and 132 elderly individuals (range, 98–108 years).[13]

Germline pathogenic variants in 12 genes that impair telomere biology have been linked to telomere diseases.[14] TL measurement and genetic screening have been used for differential diagnosis of telomeropathies. Both commercial and in-house targeting sequencing panels are preferred due to lower cost and a known subset of genes recurrently mutated in these diseases. However, an accurate interpretation of genetic testing is often complicated by the heterogeneity in telomere diseases' penetrance and presentation. In this work, seven patients had a germline VUS; all were novel and found in *TERT* and *TERC* genes, both commonly mutated genes in telomere diseases and not tolerable to either missense or loss-of-function variants according to ExAC metrics (http://exac.broadinstitute.org/). Despite the strong clinical evidence, the identified VUS did not meet criteria for pathogenicity using either ACMG or Sherloc classifications due to lack of segregation data and specific functional assays. Specificity of pathogenic *TERTp* variants for telomerase dysfunction may be additional evidence for pathogenicity of germline VUS and

may help identify patients with telomeropathies that lack classical phenotypes.

In cancer cells, current models propose that pathogenic *TERTp* acquisition only occurs in cells with very short telomeres due to selective pressure for cell immortalization.[6,28–30] However, two of our patients with pathogenic *TERTp* variants had normal TLs (NIH98 and USP41) in which the germline *TERT* variant pathogenicity is supported by eroded 3' overhangs, functional assays, and segregation data.[27,31] We did not observe longer TL in patients with pathogenic *TERTp* variants nor telomere elongation in screened serial samples. In agreement with our findings is the report that *TERT* upregulation driven by *TERTp* pathogenic variants does not prevent telomere erosion but rather sustains cell proliferation.[29] Also, a pathogenic *TERTp* variant increased cell proliferation and telomerase activity in Epstein–Barr virus (EBV)-transformed lymphoblastoid cells derived from an IPF patient despite his very short telomeres.[13] In fibroblasts from DC patients, *TERT* expression prevents premature senescence and extends cells' proliferative lifespan but is insufficient to maintain TL.[32,33] In different cancers, *TERT* reactivation has not been widely associated with telomere elongation but rather with sustained proliferative capacity.[8,34,35]

The *TERTp* variant most commonly identified in our cohort was the pathogenic -124C>T (C228T) variant. However, we found rare −124 ($n = 2$) and −146 ($n = 1$) *TERTp* variants with a C>A transition instead of the frequently found C>T substitution in two DC patients (NIH39 and USP02) and one IPF patient (NIH34; Supplementary Figure S5). The two DC patients harbored pathogenic germline variants in *RTEL1* or *DKC1* whereas the IPF patient harbored a germline VUS in *TERT*. The −124 C>A also creates a putative ETS motif[11,36] and has been previously observed in melanoma and meningioma.[36,37] The −146 C>A has been previously found in a single case of thyroid cancer[38] but fails to increase *TERT* expression in luciferase reporter assays and does not create a novel ETS motif.[11] Even though these variants have been reported in other studies, the −124C>A and −146C>A variants identified by our sequencing assay could be artifacts because they were not validated by either ddPCR or Sanger sequencing: the ddPCR probes were specific for the C>T transition; clones were very small to be detected by Sanger; and amounts of patients' samples were limited.

In our study, the frequency of pathogenic *TERTp* variants in IPF patients was higher than previously observed in a cohort of 200 IPF patients with heterozygous germline variants in *TERT*, *TERC*, *PARN*, or *RTEL1* (28% vs. 5%) (ref.[13]). This difference may be explained by the distinct methodologies used to detect the *TERTp* variants. Our customized amplicon-based sequencing assay detected clones as small as 1.2% VAF, as opposed to Sanger sequencing, which detects clones at VAF of 20% or higher. Also, our IPF cohort was relatively small ($n = 18$).

Our study has limitations. First, we did not evaluate whether pathogenic *TERTp* variants were in *cis* or *trans* to the wild-type allele due to insufficiency of clinical samples. Although these somatic variants have been described preferentially in *cis* with the wild-type allele,[13] one of our patients with a homozygous pathogenic *TERT* germline variant also harbored the pathogenic −124 *TERTp* variant that was not associated with clinical worsening. Lack of serial and myeloid cell–enriched samples also hampered analysis of *TERTp* variant clones' chronological dynamics and detection of pathogenic *TERTp* variants at low allele frequency in leukocytes. Second, the natural history of these variants in telomeropathies and the clonal dynamics under androgen treatment need to be assessed by functional studies. Nevertheless, the mechanism for *TERT* reactivation by androgens appears to be different from pathogenic *TERTp* variants, because the hormone does not bind to the region in which the somatic *TERTp* variants are located.[10,39] Based on our preliminary results, cells without pathogenic *TERTp* variants may be more responsive to danazol compared with pathogenic *TERTp* variant clones, which would lead to a dilution of *TERTp* variant clones during treatment. Also, these variants may not affect patients' responses to androgen therapy, because patients responded to treatment regardless of pathogenic *TERTp* clones.

Conclusion

Our findings indicate that pathogenic *TERTp* clones were positively selected in nonmalignant diseases, in the setting of telomerase-deficiency bone marrow cells. *TERTp* clones' selection appeared to be age-dependent and random, and clones might be selected as an attempt to restore telomerase activity in patients with telomere diseases. Pathogenic *TERTp* variants may be good evidence of an inherited bone marrow failure driven by telomerase impairment; although these somatic variants have been found in only 8% patients and relatives, the addition of *TERTp* region in targeted panels may further support the pathogenic role of germline variants identified in telomere biology genes.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (https://doi.org/10.1038/s41436-018-0385-x) contains supplementary material, which is available to authorized users.

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DISCLOSURE

The authors declare no conflicts of interest.

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