

# The relationship between DNA methylation and telomere length in dyskeratosis congenita

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## Summary

The regulation of telomere length (TL) is a complex process, requiring the telomerase enzyme complex and numerous regulatory proteins. Epigenetic regulation may also be important in telomere maintenance. Specifically, methylation at subtelomeres is associated with changes in TL *in vitro* and in mouse models. Dyskeratosis congenita (DC) is an inherited bone marrow failure syndrome characterized by exceedingly short telomeres and mutations in telomere biology genes. To understand the interaction between methylation and TL in humans, we measured LINE-1, pericentromeric (NBL2), and subtelomeric (D4Z4) methylation in peripheral blood DNA derived from 40 patients with DC and 51 mutation-negative relatives. Pearson's correlation coefficient and linear regression models were used to evaluate the relationship between age-standardized lymphocyte TL measured by flow FISH and % DNA methylation. No differences in % subtelomeric, LINE-1, or pericentromeric methylation between patients with DC and relatives were noted except for an increase in % subtelomeric methylation in DC patients with a telomerase-complex mutation (*TERC*, *TERT*, *DKC1*, or *TCAB1*) (63.0% in DC vs. 61.8% in relatives,  $P = 0.03$ ). Positive correlations between TL and DNA methylation at LINE-1 ( $r = 0.39$ ,  $P = 0.01$ ) and subtelomeric ( $r = 0.32$ ,  $P = 0.05$ ) sites were present in patients with DC. The positive correlation between TL and % LINE-1 methylation was restricted to *TINF2* mutations. In contrast, statistically nonsignificant inverse correlations between TL and % LINE-1 ( $r = -0.17$ ), subtelomeric ( $r = -0.20$ ) were present in unaffected relatives. This study suggests an interaction between TL and both subtelomeric and LINE-1 methylation, which may be altered based on mutation status of telomere biology genes.

**Key words:** dyskeratosis congenita; epigenetic; human; methylation; telomere.

## Introduction

Human telomeres consist of long (TTAGGG)<sub>n</sub> nucleotide repeats and an associated protein complex at chromosome ends that are essential for maintaining chromosomal stability. They shorten with each cell division because of the inability of DNA polymerases to replicate the ends of linear DNA. Thus, telomeres are markers of aging and cellular replicative history (Aubert & Lansdorp, 2008). Telomeres are elongated by the telomerase ribonucleoprotein complex that consists of the telomerase reverse transcriptase (*TERT*), its RNA component (*TERC*), and other regulatory proteins such as dyskerin (*DKC1*). In addition, telomere length and structure are maintained, in part, by six core proteins called the 'shelterin' complex (Palm & de Lange, 2008). Epigenetic regulation also appears to be an important component of telomere maintenance. Mammalian telomeres are enriched with histone markers but lack CpG islands and thus are not methylated. In contrast, subtelomeres, the adjacent regions of repetitive DNA, are rich in both histone markers and CpG islands, reviewed in Blasco (2007).

Mouse models and *in vitro* studies suggest that histone modifications and/or DNA methylation patterns at the subtelomere may be important in telomere length (TL) regulation (Schoeftner & Blasco, 2009). Subtelomeric hypomethylation and telomere elongation via the recombination-based alternative lengthening of telomeres (ALT) pathway were noted in DNA methyltransferase (DNMT)-deficient mice (Gonzalo *et al.*, 2006). This suggests a negative regulatory role of DNA methylation on TL. In contrast, studies on lymphoblastoid or fibroblast cell lines derived from patients with immunodeficiency, centromeric region instability, and facial anomalies (ICF) syndrome – caused by a germline mutation in the DNA methyltransferase 3b (*DNMT3b*) gene – showed that the DNA hypomethylation status, particularly in subtelomeric DNA repeats, did not activate ALT and was associated with short telomeres. Telomere repeat-containing RNA (TERRA) was overexpressed in these cell lines (Yehezkel *et al.*, 2008; Deng *et al.*, 2010).

Gradual telomere shortening was associated with a reduction in levels of subtelomeric DNA methylation in *TERC*-deficient mice (Benetti *et al.*, 2007). However, it is important to recognize that murine telomeres are significantly longer than human telomeres; murine phenotypes because of defects in telomere biology genes are often not seen until late generations of knockout mice are created (Riethman, 2008). In a panel of human cancer cell lines, a negative correlation between subtelomeric methylation and TL was observed (Vera *et al.*, 2008). A subsequent study suggested that the inverse relationship between subtelomeric methylation and TL was chromosomal dependent (Lee *et al.*, 2009).

Dyskeratosis congenita (DC) is an inherited bone marrow failure and cancer predisposition syndrome caused by defects in telomere biology (reviewed in Savage & Alter, 2009; Walne & Dokal, 2009). This disorder is defined clinically by the presence of the diagnostic triad of lacy reticular skin pigmentation, nail dystrophy, and oral leukoplakia. Multiple other medical complications, such as esophageal stenosis, developmental delay, pulmonary fibrosis, or liver disease, may also be present. Patients with DC have very short telomeres (typically less than 1st percentile for their age) (Vulliamy *et al.*, 2001; Alter *et al.*, 2007; Du *et al.*, 2009b). Germline mutations in genes important in telomere biology have been identified in about 60% of patients with DC. All forms of inheritance have

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been noted in DC families: X-linked recessive (*DKC1*), autosomal dominant (*TERC*, *TERT*, or *TINF2*), and autosomal recessive [*TERT*, *NOP10*, *NHP2*, or *WRAP53* (protein: TCAB1)] (Savage & Alter, 2009; Walne & Dokal, 2009; Zhong *et al.*, 2011).

To better understand the role of methylation in TL regulation, we studied the relationship between TL and subtelomeric, global (LINE-1), and pericentromeric DNA methylation levels in patients with aberrant telomere biology because of DC and their mutation-negative, unaffected relatives.

## Results

### Characteristics of study participants

Patients with DC were significantly younger than their unaffected relatives [median age in years (range); 14.4 (1.3–71.1) vs. 45.6 (4.9–87.1), respectively,  $P < 0.0001$ ], more likely to be males ( $P < 0.001$ ), and had very short telomeres for their age ( $P < 0.0001$ ). The median age-standardized TL (measured by Z score) for relatives was  $-0.5$  standard deviation from the population mean of the same age (range =  $-4.1$  to  $1.5$ ) compared with a median of  $-4.5$  in patients with DC (range =  $-7.9$  to  $-1.5$ ) (Table 1). As expected, we observed a statistically significant inverse correlation between age and TL in the unaffected relatives ( $r = -0.59$ ,  $P < 0.0001$ ). The inverse correlation between age and TL in patients with DC was present but not significant ( $r = -0.17$ ,  $P = 0.30$ , data not shown). No statistically significant ( $P < 0.05$ ) correlations between age and % subtelomeric, LINE-1, or pericentromeric DNA methylation were observed in patients with DC or relatives (data not shown).

### The relationship between subtelomeric methylation, disease status, and TL

Overall, the level of subtelomeric methylation in patients with DC was not different from that of their healthy relatives (median = 61.9% vs. 61.8%,  $P = 0.5$ ). Stratification of patients with DC by genotype subgroup showed that the median % subtelomeric methylation was higher in patients with a telomerase-complex mutation (*TERC*, *TERT*, *DKC1*, or *TCAB1*) than in unaffected relatives (all cases 63.0% vs. 61.8% in rela-

tives,  $P = 0.03$ ) (Table 1). The relationship between telomerase-complex mutations in DC and the presence of subtelomeric hypermethylation remained statistically significant after adjusting for age and gender and accounting for the correlation between family members [odds ratio (OR) = 1.15, 95% confidence interval (CI) = 1.01–1.30,  $P = 0.03$ ]. Within the telomerase-complex group, there was a trend toward higher levels of subtelomeric methylation in *TERT* mutations (67.2%), than in *DKC1* (63.3%) or *TERC* (62.8%) (data not shown). However, these gene-specific differences were not statistically significant.

We further evaluated the correlation between subtelomeric methylation and TL in patients with DC and relatives separately. In DC, subtelomeric methylation and TL were positively correlated ( $r = 0.32$ ,  $P = 0.05$ ) (Fig. 1A). This suggests that within DC, subtelomeric hypermethylation is associated with relatively longer telomeres (although still very short for age). The positive relationship between TL and subtelomeric methylation remained statistically significant after accounting for the correlation between affected family members and adjusting for gender and age ( $\beta = 0.07$ , 95% CI = 0.02–0.13,  $P = 0.01$ ). Furthermore, this positive relationship did not vary by genotype group in patients with DC ( $r = 0.23$  and 0.33, in *TINF2* and telomerase complex, respectively) (Fig. 2A). In contrast, there was an inverse correlation between TL and subtelomeric methylation ( $r = -0.20$ ,  $P = 0.20$ ), although not statistically significant, in the unaffected relatives (Fig. 1A).

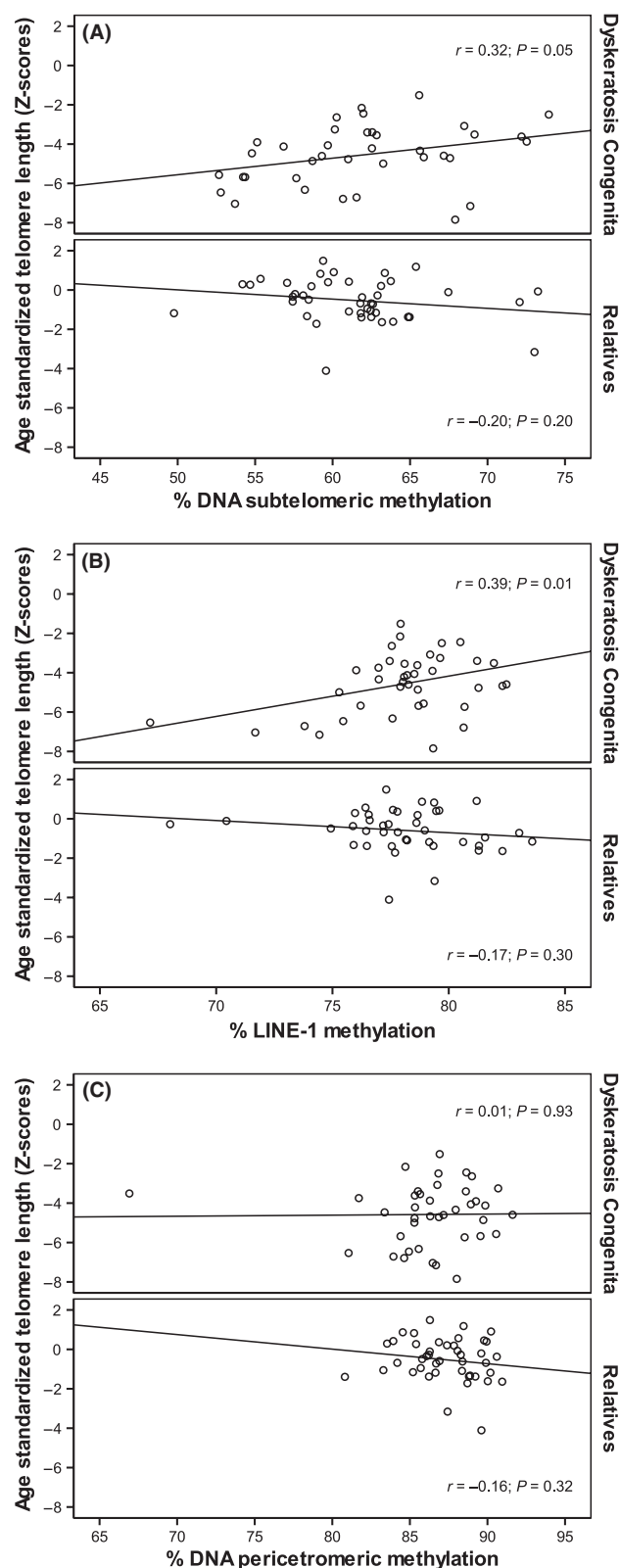
### The relationship between LINE-1 methylation, disease status, and TL

Methylation at LINE-1 sites was determined as a measure of global methylation. Overall, LINE-1 methylation was not different between patients with DC and their relatives (78.2% vs. 77.9, respectively,  $P = 0.7$ ). DC patient genotype was not associated with LINE-1 methylation. We next evaluated the correlation between LINE-1 methylation and TL to evaluate whether methylation throughout the genome has an effect on TL. Within the setting of very short telomeres in DC, we found that higher levels of LINE-1 methylation correlated with having relatively longer telomeres ( $r = 0.39$ ,  $P = 0.01$ ) (Fig. 1B). This positive correlation was restricted to individuals with *TINF2* mutations; no correlation between TL and % LINE-1 methylation was noted in patients with telomerase-complex mutations

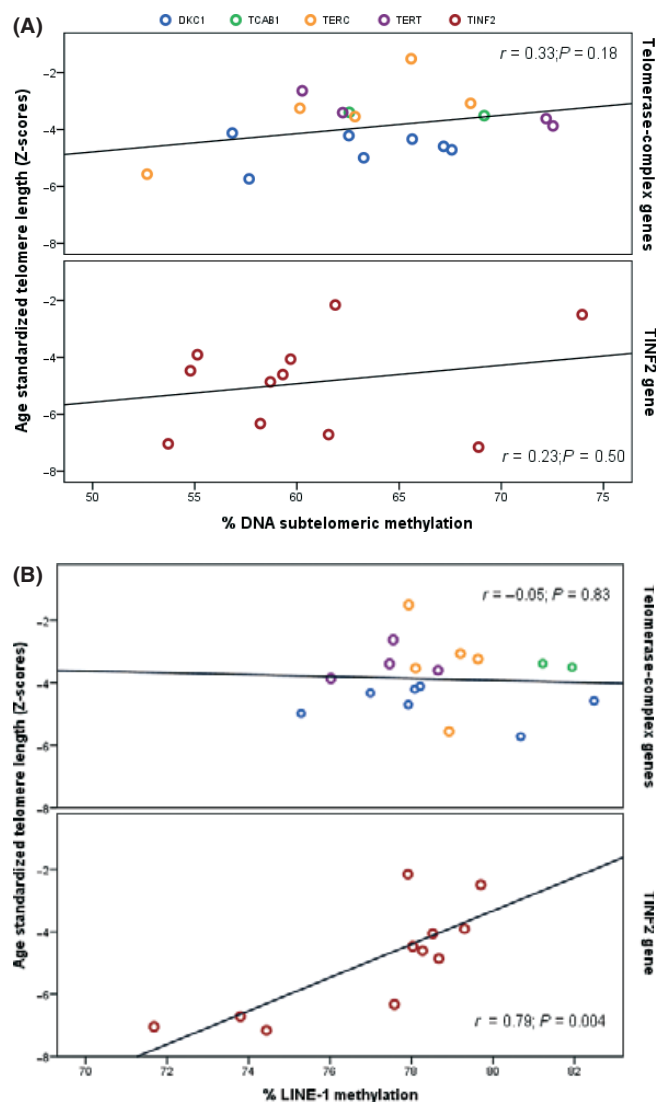
**Table 1** Demographic characteristics and telomere length of patients with dyskeratosis congenita by genotype and their unaffected, mutation-negative family members

	Unaffected relatives ( <i>N</i> = 51)	Patients with dyskeratosis congenita by genotype ( <i>N</i> = 40)		
		Mutations in telomerase-complex genes ( <i>N</i> = 18) <sup>†</sup>	Mutations in <i>TINF2</i> ( <i>N</i> = 11)	Unknown gene ( <i>N</i> = 11)
Age	45.6 (4.9 to 87.1)	24.3 (3.04 to 47.76)	21.2 (3.4 to 71.05)	7.9 (1.32 to 16.9)
Median (range)				
Gender	23:28	13:5	9:2	9:2
Male:female				
Absolute telomere length (kb)	6.4 (3.4 to 10.3)	4.0 (3.2 to 6.0)	3.4 (3.0 to 5.2)	3.8 (2.8 to 6.6)
Median (range)				
Age standardized (Z scores)	$-0.5$ ( $-4.1$ to $1.5$ )	$-3.8$ ( $-5.7$ to $-1.5$ )	$-4.6$ ( $-7.1$ to $-2.2$ )	$-5.7$ ( $-7.8$ to $-2.4$ )
Median (range)				
% LINE-1 methylation	77.9 (68.0 to 83.6)	78.1 (75.3 to 82.5)	78.0 (71.7 to 79.7)	79.1 (67.2 to 82.3)
Median (range)				
% subtelomeric methylation	61.8 (49.8 to 73.2)	63.0 (52.7 to 72.5)	59.3 (53.7 to 73.9)	61.0 (52.8 to 73.5)
Median (range)				
% pericentromeric methylation	87.4 (59.4 to 90.9)	86.9 (66.9 to 91.6)	86.7 (83.4 to 89.7)	85.3 (81.0 to 89.5)
Median (range)				

<sup>†</sup>Four patients with *TERT* mutations, five with *TERC*, seven with *DKC1*, and two with *TCAB1*.



**Fig. 1** Correlation between age-standardized telomere length and methylation in patients with dyskeratosis congenita (DC) and their mutation-negative, unaffected relatives. (A) % subtelomeric, (B) % LINE-1, and (C) % pericentromeric DNA methylation in patients with DC (upper panel) and their unaffected relatives (lower panel). Telomere length was age standardized using Z scores;  $r$  values represent the Pearson's correlation coefficient between telomere length and methylation.



**Fig. 2** Correlation between age-standardized telomere length and methylation in patients with dyskeratosis congenita (DC) by genotype. (A) % subtelomeric, (B) % LINE-1 methylation in patients with DC by genotype group; telomerase-complex genes (upper panel) and the *TINF2* gene (lower panel). Telomere length was age standardized using Z scores;  $r$  values represent the Pearson's correlation coefficient between telomere length and methylation.

(*TINF2*  $r = 0.79$ ,  $P = 0.004$  vs. telomerase complex  $r = -0.05$ ,  $P = 0.83$ ,  $P$  interaction  $<0.0001$ ) (Fig. 2). In unaffected relatives, we observed a weak statistically nonsignificant negative correlation between TL and levels of LINE-1 ( $r = -0.17$ ,  $P = 0.30$ ) (Fig. 1B),

### The relationship between pericentromeric methylation, disease status, and TL

The level of pericentromeric methylation was measured as a negative control, since it is not expected to have a direct effect at the telomeres. Levels of pericentromeric methylation were very similar in patients with DC and their relatives (86.7 vs. 87.4, respectively,  $P = 0.4$ ). There were no genotype-specific associations. There was no correlation between levels of pericentromeric methylation and TL in patients with DC ( $r = 0.01$ ,  $P = 0.93$ ) or in their relatives ( $r = -0.16$ ,  $P = 0.32$ ) (Fig. 1C).

## Discussion

The complex process of telomere maintenance is typically thought to primarily require the telomerase holoenzyme complex and numerous interacting proteins. However, several studies suggest that epigenetic regulation, in the form of DNA methylation and/or histone modification, is also very important in telomere biology (reviewed in Schoeftner & Blasco, 2009). We sought to better understand the relationship between DNA methylation and telomere biology in humans by evaluating the relationship between methylation levels at subtelomeric, LINE-1, and pericentromeric sites in a well-characterized cohort of patients with the telomere biology disorder DC and their healthy, mutation-negative relatives.

Our case-control comparison of DNA methylation at subtelomeric, LINE-1, and pericentromeric sites did not identify methylation differences in patients with DC overall compared with their healthy, mutation-negative relatives. We did find that DC patients with telomerase-complex mutations had elevated levels of subtelomeric methylation compared with DC patients with *TINF2* mutations or with the relatives. The exact reason for this difference is not clear, but it could be related to differences in the expression level of the telomere repeat-containing RNA (TERRA). TERRA expression was suggested to mediate subtelomeric methylation in telomerase-positive cancer cells (Ng *et al.*, 2009), as well as in lymphoblastoid and fibroblast cells isolated from ICF patients (Yehezkel *et al.*, 2008). However, the role of TERRA in DC has not yet been elucidated.

The inverse correlations between TL and subtelomeric, LINE-1, or pericentromeric methylation in mutation-negative healthy relatives observed in our study are in agreement with previously reported relationships in a panel of more than 20 different human cancer cell lines (Vera *et al.*, 2008). Our study provides additional data suggesting a negative regulatory mechanism of DNA methylation on TL (Blasco, 2007). Notably, we found that DC-associated telomere biology abnormalities appear to change the direction of this regulatory mechanism. In patients with DC, TL and methylation at subtelomeric and LINE-1 sites were positively correlated. In other words, elevated levels of methylation were associated with longer telomeres (albeit still exceedingly short for age) in DC. This correlation between subtelomeric hypermethylation and relatively longer telomeres in DC was independent of genotype.

Several studies suggest that telomere shortening caused by *TERT* or *TERC* mutations is less severe than what has been observed in patients with *TINF2* mutations (Walne *et al.*, 2008; Du *et al.*, 2009a; Vulliamy *et al.*, 2010; Sasa *et al.*, 2011). It is possible that the relatively longer telomeres (although still very short for age) in patients with DC who have mutations in telomerase-complex genes can be explained, at least partially, by the subtelomeric hypermethylation observed in those patients.

Surprisingly, the positive relationship between % LINE-1 methylation and TL was observed only in patients with *TINF2* mutations. A recent study suggests that heterochromatin protein 1 $\gamma$  (HP1 $\gamma$ ) binds to the C-terminal domain of the *TINF2* protein (TIN2) and that DC-associated *TINF2* mutations disrupt this binding (Canudas *et al.*, 2011). The extra-telomeric functions of TIN2 have yet to be elucidated, but it is theoretically possible that interactions between TIN2 and heterochromatin proteins are related to changes in methylation across the genome.

In conclusion, the genetic and phenotypic heterogeneity of DC which occurs as a consequence of telomere biology defects provides an excellent human model in which to explore the epigenetic regulation of telomeres. Subtelomeric methylation in patients with DC is positively correlated with TL independent of genotype whereas the converse is true in healthy individuals. In addition, the correlation between LINE-1 methylation and TL was only present with *TINF2* mutations. Our results suggest

that the relationship between TL and both LINE-1 and subtelomeric methylation is dependent upon telomere biology gene function.

## Experimental procedures

### Study participants

This study included 40 patients with DC and 51 unaffected mutation-free relatives who are enrolled in the National Cancer Institute's Institutional Review Board approved protocol 02-C-0052 (NCT00056121, <http://www.marrowsfailure.cancer.gov>) (Alter *et al.*, 2010). Patients were classified as patients with DC in the current analyses if they had a mutation in one of the known genes or if they had at least two features of the diagnostic triad and other clinical findings consistent with DC, such as hematologic or neoplastic complications. All patients had telomeres <1st percentile for age in lymphocytes as measured by automated multicolor fluorescence *in situ* hybridization combined with flow cytometry (flow FISH) (Alter *et al.*, 2007). All 40 patients with DC were tested for mutations in the known DC genes, *DKC1* (males only), *TERC*, *TERT*, *TINF2*, *NOP10*, *NHP2*, and *TCAB1*. Throughout the manuscript, we refer to patients with DC who do not have a mutation in one of the known genes as patients with unknown gene. Patients with DC were stratified according to their affected gene based on the gene biological function into: (i) telomerase-complex mutations: patients with a mutation in one of the genes critical to telomerase function (*TERC*  $n = 5$ , *TERT*  $n = 4$ , *DKC1*  $n = 7$ , and *TCAB1*  $n = 2$ ); (ii) patients with a mutation in the shelterin gene *TINF2* ( $n = 11$ ); and (iii) unknown gene patients ( $n = 11$ ). The healthy relatives were tested for the mutation associated with disease in their family and included as controls only if they did not carry the mutation. Family members of patients in whom the causative gene is not yet known were excluded from the analyses to avoid possible misclassification bias.

### Methylation assays

DNA was extracted from peripheral blood cells of patients and relatives by manual Gentra Puregene procedure (Qiagen Inc., Valencia, CA). Lymphocyte TL was measured by automated multicolor flow FISH, as previously described (Baerlocher *et al.*, 2006). Genomic DNA was bisulfate converted, and methylation at LINE-1, subtelomeric, and pericentromeric sites was quantified using PCR pyrosequencing (EpigenDx, Worcester, MA, USA), as described (Tost & Gut, 2007). Pericentromeric methylation was determined at the NBL2 locus (chromosomes 9, 13, 14, 21), and subtelomeric methylation was measured at the D4Z4 repeat sequences of chromosomes 4 and 10. The level of DNA methylation is presented as the mean percentage of methylated CpGs across all sequenced alleles to the total number of CpGs.

### Statistical analyses

Pearson's correlation coefficient ( $r$ ) was used to evaluate the strength of association between TL and levels of DNA methylation at different sites. Multivariable linear regression models were used to adjust for potential confounders and test for interactions. All models were adjusted for age and gender. Interaction terms between participant disease status (DC vs. unaffected relative) and methylation level were included in the models to test whether the relationship between TL and levels of DNA methylation was modified by DC status. The robust variance estimator (Zeger & Liang, 1986) was used to account for the correlations between observations from participants of the same family. TL in this study was age standard-

ized using Z scores. The Z scores standardize each subject's absolute TL to the population mean and standard deviation for TL within the same age and are calculated as follows:

$$Z = \frac{\text{Absolute TL} - \text{population mean TL for that age}}{\text{Standard deviation of TL in the population for that age}}$$

Population estimates were derived from TL measurement on 400 normal individuals who ranged in age between 0 and 100 years (Alter *et al.*, 2007).

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## Author contributions

SAS and SMG conceived and designed the experiments. SMG, HAK, and FMS analyzed the data. SAS, NG, and BP evaluated patients and collected clinical data. SMG and SAS wrote the paper. All authors reviewed, edited, and approved the final manuscript.

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