Telomere length in inherited bone marrow failure syndromes

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ABSTRACT

Telomeres are long DNA repeats and a protein complex at chromosome ends that are essential for genome integrity. Telomeres are very short in patients with dyskeratosis congenita due to germline mutations in telomere biology genes. We compared telomere length in patients with Fanconi anemia, Diamond-Blackfan anemia and Shwachman-Diamond syndrome with telomere length in dyskeratosis congenita. Telomere length was measured in six leukocyte subsets by automated multicolor flow fluorescence *in situ* hybridization, and age-adjusted using Z-scores (-2.326 = 1st percentile) were created. We examined individual data, and used canonical variate analysis for group comparisons and outlier detection. Most dyskeratosis congenita telomere lengths were below the 1st percentile, while only 2 Fanconi anemia and one each Diamond-Blackfan anemia and Shwachman-Diamond syndrome were that low. However, Fanconi anemia, Diamond-Blackfan anemia and Shwachman-Diamond syndrome clustered in the bottom half of the normal range. Canonical variate analysis separated dyskeratosis congenita widely from the other three syndromes by the first canonical variable (89.7% of the variance); the second variable (10.0%) separated Diamond-Blackfan anemia, Shwachman-Diamond syndrome, and Fanconi anemia from each other. Overall, unlike in dyskeratosis congenita, telomere lengths in patients with non-dyskeratosis congenita inherited bone marrow failure syndromes were usually in the normal range, albeit shorter than in unaffected individuals. *clinicaltrials.gov identifier: 00027274*

Introduction

Telomeres are long nucleotide repeats and a protein complex at chromosome ends essential for chromosome integrity. Telomere biology provides the mechanism to address the end-protection problem during DNA replication.[1] Dyskeratosis congenita (DC) is a genetic disease associated with mutations in several genes whose RNA and protein products collaborate in the telomere maintenance pathway. Germline mutations in these genes result in failure of proper elongation of telomere repeats at the ends of chromosomes, which results in a loss of chromosomal integrity, and short telomeres compared with normal. At least 70% of patients with DC have mutations in telomere maintenance genes [*DKC1*, MIM 30126; *TERC*, MIM 602322; *TERT*, MIM 187270; *TINF2*, MIM 604319; *NHP2*, MIM 606470; *NOP10*, MIM 606471; *WRAP53* (protein TCAB1), MIM 612661; *CTC1* MIM 613129; and *RTEL1*, MIM 608833].[2] The major physical aspects of DC are the age-dependent diagnostic triad of nail dystrophy, lacey reticulated pigmentation, and oral leukoplakia; many patients lack one or more of the triad features but have bone marrow failure alone, or have other features such as epiphora, blepharitis, developmental delay, pulmonary fibrosis, liver cirrhosis or fibrosis, dental caries, esophageal strictures, early gray hair, hair loss, sparse eyelashes, hyperhidrosis, cerebellar hypoplasia, microcephaly, hypogonadism, urethral stricture, osteoporosis, and avascular necrosis.[3]

Dyskeratosis congenita is one of a family of inherited bone marrow failure syndromes (IBMFS) that may not always be clearly distinguished based on the combination of bone marrow failure and physical findings. The most common of the non-DC IBMFS include Fanconi anemia (FA, MIM 227650), Diamond-Blackfan anemia (DBA, MIM 105650), and Shwachman-Diamond syndrome (SDS, MIM 260400).[4] There are also patients who present with one or more cytopenias, with or without physical abnormalities characteristic of one of these syndromes. While there are diagnostic screening tests specific for some of the syndromes, e.g. chromosome breakage for FA,[5] the tests for others are not completely sensitive and specific (e.g. red cell adenosine deaminase for DBA[6] or serum levels of pancreatic enzymes for SDS).[7]

We have previously demonstrated the excellent performance characteristics for the diagnosis of DC by measurement of telomere length (TL) in leukocyte subsets by flow cytometry and fluorescence *in situ* hybridization (flow-FISH).[8,9] We determined that the most sensitive parameter for DC was TL less than the 1st percentile in at least four of six cell types (granulocytes, lymphocytes, naïve T cells, memory T cells, B cells, and NK cells), or at least three of four lymphocyte subsets (excluding granulocytes and NK cells).[9] The majority of patients with non-DC IBMFS had TLs within the range seen in more than 400 normal patients. Only 2 non-DC patients among 46 met the "DC" TL diagnostic criteria, although 4 had very short TL in total lymphocytes.[8]

Short telomeres were previously reported in FA, DBA and SDS, using a variety of different cell types, and methods that are less sensitive than flow-FISH (Table 1).[10-20] Most studies indicated that average values for each group of patients were less than the average for age-matched controls. Teasing out numbers of individual patients below the normal range in the

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published reports suggests that only a minority of the patients within each group of those syndromes had shorter telomeres than expected.

We have continued to measure peripheral blood leukocyte subsets by flow-FISH in all of the IBMFS. We asked whether individual patients with non-DC IBMFS have telomeres in the diagnostic range for DC, i.e. below the 1st percentile for age, not just below the value of a single age-matched control. We then determined whether groups of patients with the non-DC IBMFS have telomeres below the normal length. We used several analytic methods, including age-adjustment with Z-scores, and canonical variates in order to combine data from all leukocyte subsets.

Methods

Participants enrolled in the National Cancer Institute Clinical Genetics Branch protocol 02-C-0052 (*clinicaltrials.gov identifier:00027274*), approved by the NCI Institutional Review Board (*www.marrowfailure.cancer.gov*). All participants or their guardians provided written informed consent in accordance with Health and Human Services regulation 45 CFR 46. Data were obtained between 2002 and 2013.

The diagnosis of DC depended on the presence of two of three components of the triad (dysplastic nails, lacy reticulated skin pigmentation, or oral leukoplakia), one of the triad plus bone marrow failure and/or other DC-associated physical findings, and/or a germline mutation in a DC gene.[4] FA, DBA, and SDS were diagnosed from clinical criteria and syndrome-specific laboratory tests;[4] chromosome breakage for FA,[5] red cell adenosine deaminase for DBA,[6] and serum pancreatic enzymes for SDS,[7] and confirmed with germline mutations when possible. Severe bone marrow failure (BMF), i.e. severe aplastic anemia (SAA), was classified as hemoglobin less than 80 g/L or below the normal limit for age, absolute neutrophils less than $0.5x10^9$/L, and platelets less than $30x10^9$/L; moderate (MAA) was below normal for age but not severe; and none, normal values for age.[8]

Blood was drawn in sodium heparin and shipped at room temperature, and automated multicolor flow-FISH for six leukocyte subsets (granulocytes, total lymphocytes, $CD45^+$ naïve T cells, $CD45^-$ memory T cells, $CD20^+$ B cells, and $CD57^+$ NK cells) was performed at Repeat Diagnostics, Inc. (Vancouver, BC, Canada).[8,21] "Very short" telomeres were below the 1st percentile for age derived from 400 normal control samples. Age-adjustment was provided by conversion of individual data into Z-scores; a Z-score of -2.326 is equivalent to the 1st percentile.[9]

We used standard descriptive analyses to visualize the distribution of Z-scores within and between patient groups. We used canonical variate analysis (CVA)[22] to visualize how Z-score values analyzed as a panel differed between patient groups. The first canonical variable explains the largest fraction of the variation between the group means; the second canonical variable is uncorrelated with the first and explains the second largest fraction. We plotted the group means and corresponding circular 95% confidence regions along the first two canonical variables, and the locations of each individual. We calculated how far away each patient's CVA values were from the group means, and classified each patient according to the closest group. All values were centered around the average of all patients. CVA analyses used data only from patients in whom all six leukocyte subsets were measured.

Analyses were performed with Microsoft Excel (Microsoft Office 2010), Stata 13.1 (StataCorp Release 13.1, College Station,

Table 1. Telomere reports in literature cases of non-DC IBMFS.

Author	Year	N. of patients	Patient age, range (years)	N. "short"	Cell type	Method	N. of controls	Controls age, range (years)	Authors' interpretations
Fanconi anemia									
Ball[10]	1998	6	4-18	4	Total leukocytes	Southern, TRF	60	1-90	<Normal range for age
Leteurtre[11]	1999	45	3-47	9	PBMC	Southern, TRF	42	1-55	<Normal range for age
Hanson[12]	2001	16	0-11	15	Fixed lymphocytes	Q-FISH	16	Match to patients	Loss of telomere signal
Adelfalk[13]	2001	6	1-14	Group	Fibroblast lines	Southern	5	1-38	Increased shortening per cell division
Callen[14]	2002	9	3-14	Group	T lymphocytes	Q-FISH	9	6-14	Shorter TL
Li*[15]	2003	71	4-47	47	PBMC	Southern	51	0-55	Shorten >200 bp/yr
Pavesi[16]	2009	9	2-33	2	PB	Q-PCR	95	0-92	<1st percentile
Diamond-Blackfan anemia									
Pavesi[16]	2009	45	2-33	1	PB	Q-PCR	95	0-92	<1st percentile
Du[17]	2009	41	0.2-62	5	PBMC	Flow-FISH	234	0-94	≤1st percentile
Shwachman-Diamond syndrome									
Thornley[18]	2002	12	1-18	7	PB PMN	Southern	41	0.5-18	TRF, <normal range for age
Calado[20]	2007	2	<10	0	PB lymphocytes	Flow-FISH	400	0-100	≤1st percentile
Pavesi[16]	2009	1	20	0	PB	Q-PCR	95	0-92	<1st percentile
Du[17]	2009	5	2-41	1	PBMC	Flow-FISH	234	0-94	≤1st percentile
Myers[19]	2014	1	5	1	PBMC	Flow-FISH	400	0-100	≤1st percentile

*N: number; PBMC: peripheral blood mononuclear cells; PMN: polymorphonuclear cells; TRF: mean telomere restriction fragment length; TL: telomere length; Bp/year: base pairs per year. *Includes the 45 patients previously reported by Leteutre et al.[11]*

TX, USA), and MATLAB.[23] Computations were performed in MATLAB using the MANOVA function, which provides a statistical test of significance that can determine how many canonical variates are needed to discriminate between group means. We calculated how many cases from each population were assigned to its own group *versus* other groups, in order to quantify how tightly the observations clustered around their group means. Comparison of TL Z-scores in groups according to severity of bone marrow failure was made using Kruskall-Wallis. $P<0.05$ was considered significant.

Results

Participants included 100 patients with DC, 34 with DBA, 30 with FA, and 14 with SDS (Table 2). The ages were similar among the syndromes. There was an excess of males in the patients with DC, and females in those with FA. Five of the patients with FA had hematopoietic somatic mosaicism.[24] Germline mutations were identified in 67% of DC, 53% of DBA, 88% of FA, and 80% of SDS patients.

The individual results for TL *versus* age in the six leukocyte subsets are shown in Figure 1A and B. It is apparent that TL in patients with DC were generally well below the 1st percentile in all leukocyte lineages, while the majority of the results from non-DC patients were within the normal range. The number of patients whose TL was below the 1st percentile in each cell type are shown in *Online Supplementary Table S1*, in which those with DC are com-

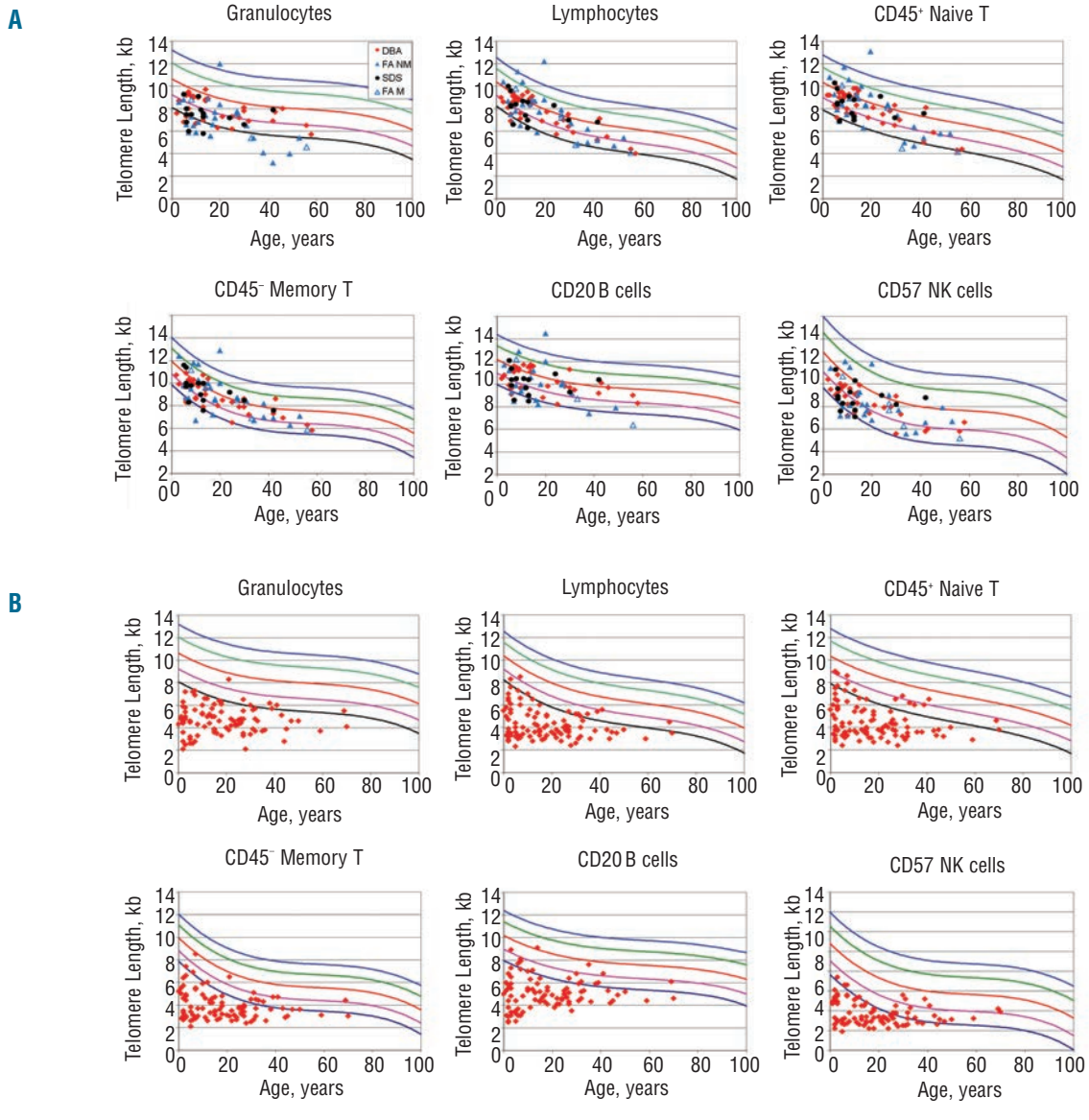

Figure 1. Telomere length according to age in patients with dyskeratosis congenita (DC), Diamond-Blackfan anemia (DBA), Fanconi anemia (FA), and Shwachman-Diamond syndrome (SDS). The vertical axis represents telomere length in kilobases (kb). The curved lines in the figures indicate the 1st, 10th, 50th, 90th, and 99th percentiles of results from 400 normal controls. Symbols represent patients. (A) 34 DBA (red diamond), 25 non-mosaic FA (closed blue triangle), 5 mosaic FA (open blue triangle), 14 SDS (black circle). (Top panels) Granulocytes, lymphocytes, and CD45RA-positive/CD20-negative naïve T cells. (Bottom panels) CD45RA-negative memory T cells, CD20-positive B cells, and CD57-positive NK cells. (B) 100 patients with DC (red diamond).

pared with those with the non-DC syndromes. There were more patients in all syndromes with very short TL in granulocytes than in any other lineage; that is the lineage with the lowest specificity for the diagnosis of DC.[8,9] The results are consistent with our previous data that very short TL in at least three cell types among total lymphocytes, naïve and memory T cells, and B cells were the most sensitive and specific criteria for the diagnosis of DC. Those criteria were met by only one patient with DBA, 2 with FA, and one with SDS. We concluded from this analysis that only very rare patients with a non-DC IBMFS had "very short" telomeres.

Data were transformed into Z-scores, in order to account for age differences and compare within and across syndromes. Dot plots for the Z-scores of each leukocyte subset (Figure 2) show that patients with DC had telomeres far below the lower limit of the normal range (below -2.326 SD). Those with FA, DBA, and SDS typically had telomeres that were shorter than normal for age, but not as short as in DC. Most Z-score values in the non-DC patients were within the lower half of the normal range indicated by the dashed reference lines (between the mean and -2.326 SD from the mean). For any given subset, there was considerable overlap of the values for FA, DBA, and SDS, and there was also a small amount of overlap with the upper range for DC. The same combination of subsets described above (total lymphocytes, naïve and memory T cells, and B cells) was used as for the raw data, leading to identification of one patient with DBA, and 2 each with FA and SDS to be in the DC range (*Online Supplementary Table S2*). For each subset the median values for the Z-scores of FA, DBA, and SDS were not nearly as short as among patients with DC (*Online Supplementary Figure S1*). Specifically, the confidence intervals for the DC medians were significantly below the medians for each of the other groups ($P<0.001$), while TL in FA, DBA, and SDS were

Table 2. Subjects in cohort

	DC	N.	DBA	N.	FA total	FA non-mosaic	FA mosaic	SDS	N.
Number	100		34		30	25	5	14	
Male: female	70:30*		22:12		8:22†	8:17	0:5	6:8	
Age median (range)	15 (0-70)		14 (2-58)		18 (3-56)	17 (3-53)	27 (6-56)	12 (5-42)	
Genes	*DKC1*	13	RPS19	6	*FANCA*	16	4	*SBDS*	12
	RTEL1	10	RPS24	3	*FANCC*	2			
	TERC	9	RPS26	2	*FANCF*	1			
	TERT	11	RPS7	1	*FANCI*	2			
	TINF2	17	RPL11	2	*FANCJ*	1			
	WRAP53	2	RPL35A	2	*FANCD2*				
	Other	5							
	Unknown	33	Unknown	18	Unknown	3	1	Unknown	2
BMF**									
None	17		9		10	5	5		2
Moderate	21		5		10	10	0		9
Severe	48		20		10	10	0		3

*Ages are not significantly different. *Excess of males. †Excess of females. **Data on BMF not available in 14 patients with DC.*

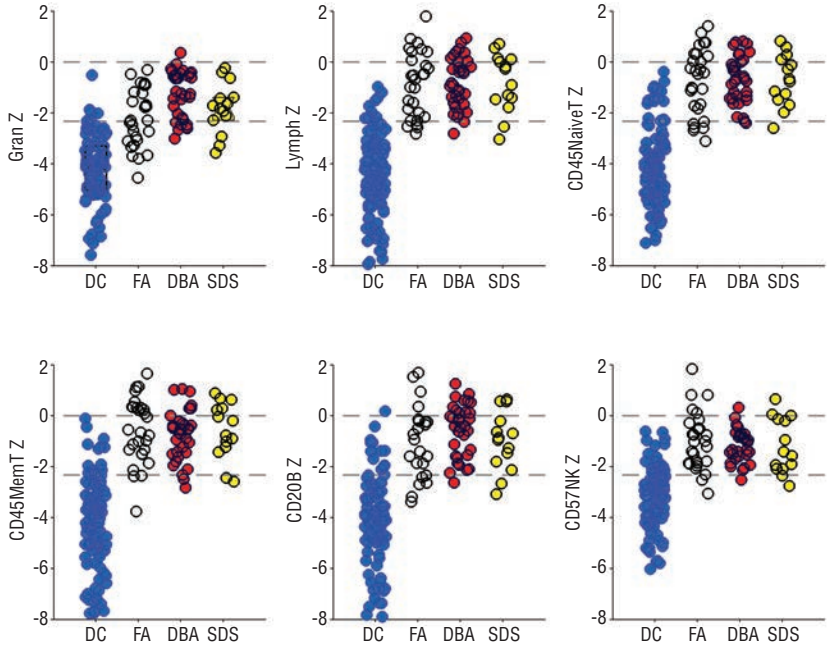

Figure 2. Telomere length Z-score individual data in patients with DC, FA, DBA, and SDS. Dashed lines represent the mean and +2.326 or -2.326 standard deviations (SD) from the mean (equivalent to the 1st percentile) for normal individuals.

similar to each other (P>0.05). *Online Supplementary Figure S1* also indicates that TL Z-scores for DC were well below the normal -2.326 line, and that the other 3 syndromes were within the normal range, albeit in the bottom half.

Canonical variate analysis of the Z-scores achieved statistically significant discrimination between the patient populations on the basis of two canonical variables (Figure 3A). The first canonical variable captured 89.7% of the total variance and separated DC from FA, DBA, and SDS. The distance between DC and the other groups is very large and significant (P=0.003). The second canonical variable captured 10.0% of the variance and separated DBA from FA and SDS. A hypothetical normal Z-score is shown with an asterisk in Figure 3A, and all four patient population averages were distinct from this point. As expected, DC was by far the most distant from normal. DBA was closest to normal, followed by SDS and FA. FA was significantly different from DBA, but the precise position of SDS was unclear because small numbers resulted in relatively large confidence regions.

Figure 3B shows scatter plots of the canonical variables for individual patients. Based on these two canonical variables, the majority of DC patients (71 of 85; 84%) were closer to the mean for DC than to any other group (Figure 3B, upper left panel). In contrast, patients with FA, DBA, and SDS frequently had canonical values closer to the mean for another syndrome than to the mean for their own syndrome. Importantly, relatively few patients with FA, DBA, or SDS were closest to DC: just 2 of 23 (9%) with FA (Figure 3B, upper right panel), one of 30 (3%) with DBA (Figure 3B, lower left panel), and one of 14 (7%) with SDS (Figure 3B, lower right panel). Three of the 4 patients with TL closest to DC had pathogenic mutations in the disease to which they were assigned; one of those with FA has not yet been confirmed but clearly met clinical criteria for FA. None of these patients had a DC phenotype.

Short telomeres in lymphocytes were associated with severity of BMF in DC, as we had found previously (*Online Supplementary Figure S2*).[9] The shorter TL with increasing BMF severity was only significant for DC (P=0.003), and not for FA, DBA, or SDS. Combining MAA with SAA into a category of "any AA" did not change the results. These results indicate that TL in patients with the non-DC syndromes is independent of the degree of marrow function.

Discussion

Our results show that patients with FA, DBA, and SDS on average had telomeres that were more or less in the lower half of the normal range. This finding was not specific: for any given leukocyte subset, there was considerable overlap of TL Z-scores between FA, DBA, and SDS, and there was also some overlap of these syndromes with the upper range for DC. However, in the majority of cases, TLs among patients with these syndromes were not nearly as short as among patients with DC.

We were able to draw additional conclusions with 'optimal' combinations of Z-scores calculated using CVA. CVA shows that, as a group, patients with DC were quite distinct from those with FA, DBA, or SDS. In addition, TLs among patients with FA were significantly different from DBA, and both groups were significantly different from normal, with DBA being significantly closer to normal than FA. This result incorporates information about the correlations between the Z-scores in the panel, and is not apparent in standard analyses of individual leukocyte subsets. Using CVA, only 9% of patients with FA, 3% of those with DBA, and 7% of those with SDS had TLs that would make one consider DC, if, in fact, the patient's diagnosis was uncertain. Conversely, sometimes DC patients had TLs above the 1st percentile for age; in fact, using CVA, 16% of patients with DC had values closer to the mean for another group than the mean for DC. Thus, patients with FA, DBA, and SDS had short telomeres, but

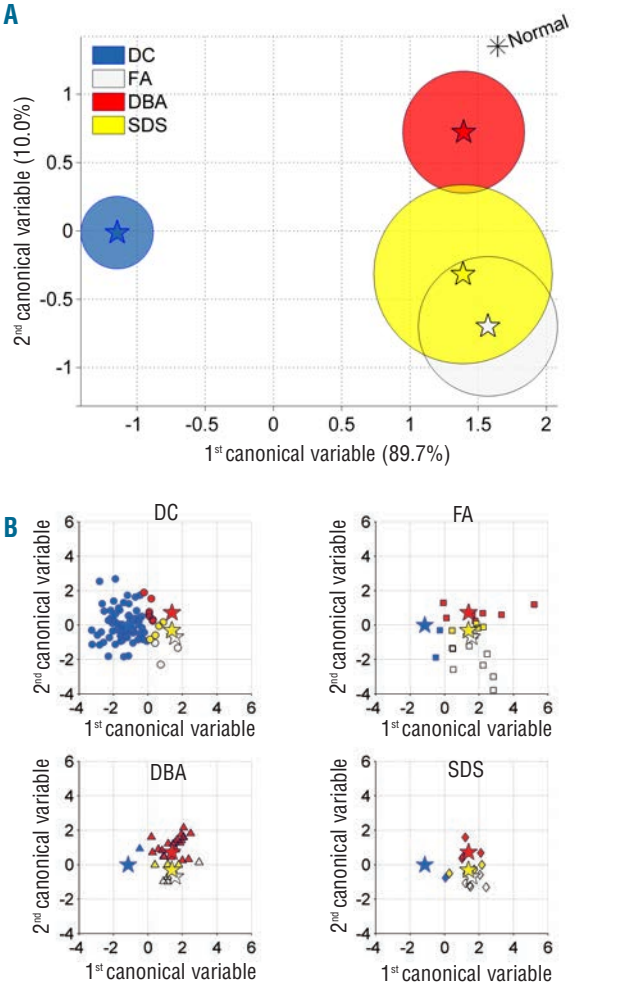

Figure 3. (A) Telomere length Z-scores of IBMFS groups analyzed by canonical variate analyses. Patients were included only if they had data for all six leukocyte subsets. There were 85 with DC, 23 with FA, 30 with DBA, and 14 with SDS. According to the first canonical variable, the DC group was distinct from the 3 non-DC patient groups, which were similar to normal (marked with *). According to the second canonical variable, FA and SDS were low, while DBA was the closest to normal. The stars are the means, and the circles represent the 95% confidence intervals. The first canonical variable accounted for 89.7% of the difference, while the second accounted for 10%. (B) Telomere length Z-scores of IBMFS individuals analyzed by canonical variate analyses. The stars are the group means (see Figure 3A). The symbols represent individuals and indicate how close they are to other types of IBMFS. (Top left) Position of DC patients compared with DC, FA, DBA, and SDS. (Top right) Position of FA patients. (Bottom left) Position of DBA patients. (Bottom right) Position of SDS patients. (All panels) blue: close to DC; gray: close to FA; red: close to DBA; yellow: close to SDS.

on average they were not 'DC-short'. At the same time, although a majority of patients with DC had profoundly short telomeres, well below the 1st percentile for age, around one in 7 patients with DC had telomeres that were not outside the normal range, but were below the 10th percentile for age. Utilization of multiple methods of analysis, as we have done in this paper, allows variability and extremes of TL within each syndrome group to be described. Further studies may determine whether this variability is random, or associated with genetic or epigenetic modifiers of TL. The non-DC patients with the shortest TLs might even have mutations in DC genes, but those were not investigated in this study.

The strengths of this study are the large number of patients with DC (n=100), and the relatively large number of patients with any non-DC syndrome (n=78). The majority of the patients were well characterized; all met diagnostic criteria for their syndrome, and the majority were confirmed by identification of germline mutations. We presented raw data, as well as data age-adjusted by Z-scores, and introduced canonical variate analysis in order to utilize results from all leukocyte subsets. The use of Z-scores facilitated comparisons within and across syndromes, using box plots and CVA analyses. Limitations include the relatively small numbers of patients with each individual non-DC syndrome (34 with DBA, 30 with FA, and 14 with SDS). All data points were from single assays performed in duplicate. The Z-scores were calculated from the mean and SD of the normal data at each age, under the assumption that these data were normally distributed. Overall, our data indicate that most individuals with non-DC IBMFS (FA, DBA, and SDS) did not have extremely short telomeres, and that TL was associated with the severity of BMF only in DC. There was very little overlap between TL in DC compared with non-DC IBMFS. As groups, however, the telomeres in the non-DC patients fell in the bottom half of the normal range (Z-scores generally between -2.326 and 0), and thus were different from true normal individuals, whose Z-scores are presumably normally distributed between -2.326 and +2.326 SD. There is growing evidence suggesting that DNA repair and ribosome biogenesis may be connected to telomere biology, but not to the same degree as the telomere biology of DC.

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