Very short telomere length by flow FISH identifies patients with Dyskeratosis Congenita

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Short Title: Very short telomeres in Dyskeratosis Congenita

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Abstract

Dyskeratosis congenita (DC) is an inherited bone marrow failure syndrome in which the known susceptibility genes (DKC1, TERC, and TERT) belong to the telomere maintenance pathway; DC patients have very short telomeres. We used multicolor flow FISH analysis of median telomere length in total blood leukocytes, granulocytes, lymphocytes, and several lymphocyte subsets to: confirm the diagnosis of DC, distinguish DC patients from unaffected family members, identify clinically silent DC carriers, and discriminate between DC patients and those with other bone marrow failure disorders. We defined “very short” telomeres as below the 1st percentile measured among 400 normal controls over the entire age range. Diagnostic sensitivity and specificity of very short telomeres for DC were >90% for total lymphocytes, CD45RA-positive/CD20-negative naïve T-cells, and CD20-positive B-cells. Granulocyte and total leukocyte assays were not specific; CD45RA-negative memory T-cells and CD57-positive NK/NKT were not sensitive. We observed very short telomeres in a clinically normal family member who subsequently developed DC. We propose adding leukocyte subset flow FISH telomere length measurement to the evaluation of patients and families suspected to have DC, since the correct diagnosis will substantially affect patient management.
Introduction

The diagnosis of dyskeratosis congenita (DC) is usually made in individuals who have at least two features of the diagnostic triad: dyskeratotic nails, lacy reticular skin pigmentation, and leukoplakia. Since many patients with DC have aplastic anemia (AA), DC is classified under the rubric of inherited bone marrow failure syndromes (IBMFS). Family histories of DC patients are consistent with X-linked recessive (XLR, MIM 305000), autosomal dominant (AD, MIM 127550), and autosomal recessive (AR, MIM 224230) patterns of inheritance and, to date, pathogenic germline mutations have been identified in an XLR gene, $DKC1$, and in two AD genes, $TERC$ and $TERT$.1-4 The protein products of these genes are involved in the intricate telomere maintenance pathway.5 However, mutations have not been identified in approximately 60 percent of clinically diagnosed DC cases.6

Telomeres consist of TTAGGG nucleotide repeats and associated proteins at the ends of chromosomes. A minimum number of telomere repeats is required to protect chromosome ends from recombination and fusion. In most somatic cells telomere repeats are lost via several pathways,7 and telomerase partially compensates for such losses.5 Mutations in the genes involved in telomere maintenance are associated with failure to maintain telomere length. When one or more chromosomes contain telomeres that have reached a critically short telomere length, the cell can no longer divide and undergoes apoptosis or becomes senescent. Telomerase is able to maintain telomere length in germline cells, but continuous shortening of telomeres is a consequence of cell division in most somatic cells, and thus a feature of aging.8 In patients in families with mutations in telomere maintenance pathway genes telomeres are shorter with each generation9, and rapidly dividing hematopoietic cells from such patients provide a sensitive indicator of accelerated telomere shortening. The hematopoietic compartment may develop
genetic instability due to telomere erosion, resulting in aplastic anemia and increased risk of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).10

Previous reports suggested that average telomeres were shorter in granulocytes or mononuclear cells from some (but not all) patients with acquired AA than normal controls, as well as in granulocytes from patients with paroxysmal nocturnal hemoglobinuria.11-14 Short telomeres were also reported in some patients with inherited bone marrow failure disorders, including Fanconi Anemia (FA)11;15 and Shwachman-Diamond Syndrome (SDS).16 Correlations with the severity of the cytopenias or the presence of MDS or cytogenetic clones have been inconsistent.

Mitchell et al first observed short telomeres in lymphoblasts of patients with DC due to mutations in DKC1.17 Subsequently, short telomeres were reported in patients with DC due to mutations in DKC1, TERC, or TERT.3;9;18;19 For those patients in whom these genes are normal, the identification of short telomeres may be a useful surrogate for the diagnosis of DC.6

Most of the early studies used Southern blot analysis of DNA extracted from total leukocytes or mononuclear cells, and reported either mean telomere length in patients compared with age-matched controls, or the difference between the average telomere lengths of patients and controls (“deltaTEL”). The focus of those reports was the average telomere length in groups of patients compared with controls, rather than on the individuals whose telomeres were abnormally short. More recently, Lansdorp and colleagues developed and improved methods using flow cytometry and fluorescence in situ hybridization (FISH) to analyze telomere length in multiple peripheral blood cell types from the same individual, and to compare each result from patients with those from a large group of age-matched normal controls.20
We used automated multicolor flow FISH to assay telomere lengths in total leukocytes, granulocytes, total lymphocytes, and lymphocyte subsets. Participants included patients with DC, FA, Diamond-Blackfan Anemia (DBA), SDS, and other rare or unclassified bone marrow failure syndromes, as well as their first-degree relatives. We focused on identification of very short telomeres in individuals and in specific diagnostic groups. The performance characteristics of the assay were quantified for diagnosing DC as well as for distinguishing DC patients from their unaffected relatives and from patients with other marrow failure disorders (non-DC patients).

**Patients and methods**

Blood samples were obtained from 26 patients with DC, 54 of their first-degree relatives, 17 with FA (one after bone marrow transplant [BMT], three with hematopoietic somatic mosaicism21), 14 with DBA, five with SDS, 10 with other unclassified but possibly inherited conditions, and 35 clinically-normal family members (i.e., normal blood counts and physical examinations, with no stigmata of the proband’s syndrome) of these non-DC patients. Controls were 400 healthy persons ranging from birth to 100 years of age.3 Participants were enrolled in the Clinical Genetics Branch Inherited Bone Marrow Failure Syndromes protocol (www.marrowfailure.cancer.gov), which was approved by the NCI Institutional Review Board; all participants or their guardians provided written informed consent.

The diagnosis of DC was made if the patient had: (1) two or three components of the diagnostic triad (dyskeratotic nails, lacy reticular skin pigmentation, and leukoplakia); (2) one of the triad plus hypoplastic bone marrow and at least two less common physical findings (epiphora, developmental problems, pulmonary disease, short stature, dental caries, esophageal narrowing, hair loss, early gray hair, and others22,23); and/or (3) a deleterious germline mutation
in a known DC gene. Other family members were considered to have DC if they met those criteria, or had the same mutant gene as the proband. Patients were classified as the Hoyeraal-Hreidarsson variant (HH) if they had DC features plus intrauterine growth retardation, developmental delay, microcephaly, cerebellar hypoplasia, and immunodeficiency; and as Revesz Syndrome (RS) if they had exudative retinopathy in addition to the previous findings. Non-DC patients (FA, DBA, SDS, and others) were diagnosed if they met criteria for a specific IBMFS, or for none of the major IBMFS. The “other patients” included two siblings with unexplained neutropenia, two with thrombocytopenia absent radii (TAR), three patients with early-onset thrombocytopenia, two with unexplained aplastic anemia, and one with skin poikiloderma and macrocytic red blood cells. None of these had at least two of the classic DC triad, or two or more of the minor DC physical findings. Those with thrombocytopenia had wild type $DKC1$, $TERC$, and $MPL$ genes. Non-DC relatives were unaffected parents and siblings of patients with non-DC diagnoses.

Bone marrow failure (BMF) was defined according to clinical guidelines for the management of FA: severe = Hb <8 g/dL, absolute neutrophil count (ANC) <500/µL, platelets <30,000/µL, or on treatment; moderate = below normal for age but above criteria for severe; none = normal values for age. For single cytopenias, severe = on treatment; moderate = below diagnostic values for the relevant lineage (Hb below normal for age for DBA, ANC <1500/µL for SDS, platelets <140,000/µL for thrombocytopenias). Bone marrow data were not included since they were not available for many patients.

Blood was drawn in sodium heparin and shipped at room temperature to the telomere laboratory in Vancouver, British Columbia, Canada; processing was performed within 48 hours of phlebotomy. Details of the technical methods can be found in Baerlocher et al. Briefly,
preparation of leukocytes involved lysis of the red cells with NH₄Cl. Leukocytes were denatured in formamide using 87°C, hybridized with a fluorescein-conjugated (CCCTAA)₃ peptide nucleic acid probe and counterstained with LDS751 DNA dye. Analysis of fluorescence was then performed on a FACSCalibur instrument. The cell types analyzed included total leukocytes, granulocytes, total lymphocytes, CD45RA-positive/CD20-negative naïve T-cells (referred to as naïve T-cells), CD45RA-negative memory T-cells (memory T-cells), CD20-positive B-cells (B-cells), and CD57-positive NK/NKT-cells (NK/NKT-cells). “Very short” telomeres were defined as less than the 1st percentile for the age-matched normal control range, and “short” telomeres were from the 1st to less than the 10th percentile. The normal curves were derived from best-fit analysis of actual telomere length analysis for each subset in 400 normal individuals; the only standardization was the inclusion of bovine thymocytes with long telomeres in every test as an internal control. The laboratory studies and interpretations were performed on coded samples lacking personal and diagnostic identifiers.

Chromosome breakage studies for FA were done on T-lymphocytes or skin fibroblasts, using both diepoxybutane and mitomycin C (Oregon Health and Science University, Portland, OR). Red cell adenosine deaminase (ADA) for DBA was measured according to standard methods (Stanford University Medical Center, Palo Alto, CA). Mutations in DKC1, TERC, and TERT, as well as genes for FA, DBA, SDS, and MPL were identified by bi-directional sequencing of PCR-amplified fragments (GeneDx, Inc., Gaithersburg, MD).

Analyses were performed using Microsoft Excel (Microsoft Office Excel 2003) and Stata9 (StataCorp Release 9, College Station, TX). P-values are two-sided. Data are reported as odds ratios in favor of diagnosis of DC, 95% confidence intervals, sensitivity and specificity, and positive and negative predictive values (PPV and NPV), using the frequency of “very short”, or
“short plus very short” telomeres in each patient group. Eighteen of the total of 156 subjects did not have sufficient cells for analyses of one or more of granulocytes, B-cells, or NK/NKT-cells.

**Study Subjects:**

The features of the subjects are summarized in Tables 1-2. The DC patients were on average significantly younger than their relatives: 58% of DC patients were below age 18 (i.e. children), *versus* 20% of their unaffected family members (p <0.002, Fisher exact test). Similarly, the non-DC patients included 59% children *versus* 37% children in their relatives (p = 0.07). Fifteen classic DC patients had at least two features of the diagnostic triad, as did three HH and one RS patients; six were classified as DC based on a combination of the less common findings. One 11 year old patient with no signs or symptoms of DC had a germline mutation in *TERC* (see below).

Two patients with DC and three siblings with HH had mutations in *DKC1*, four patients had mutations in *TERC*, and two in *TERT*19. The 11 year old silent carrier inherited a mutation in *TERC* from his symptomatic mother. During four years of follow-up he developed mild thrombocytopenia, decreasing bone marrow cellularity, and a stable cytogenetic clone; he is now considered clinically affected. Fifteen of the 26 patients with DC had no detectable DC gene mutations. Half of the DC patients had severe cytopenias and were receiving transfusions, androgens, and/or granulocyte-colony stimulating factor (G-CSF); six were considering hematopoietic stem cell transplantation at the time of the telomere length study.

Three mothers and one sister of male patients with mutations in the X-linked *DKC1* gene shared the proband’s mutation; one mother did not. Two brothers (who had no physical or hematologic DC stigmata) were being evaluated as hematopoietic stem cell donors for their sister with severe aplastic anemia; their father and his twin brother had died from complications of DC,
and several additional relatives had signs of DC. All other relatives of DC patients were well and lacked the family mutation and any DC-associated clinical findings.

All seventeen FA patients had increased chromosome breakage in blood T-lymphocytes or in fibroblasts; their relatives had normal chromosome breakage. One FA patient was three years post-bone marrow transplant from his sister, and three were hematopoietic somatic mosaics with normal blood counts (skin fibroblasts confirmed the FA diagnoses).\textsuperscript{21,25} Results from the transplanted FA patient are included in the graphs but not in the statistical analyses. Six FA patients had severe pancytopenia and were receiving transfusions and/or androgens and/or G-CSF, while four had abnormal values in at least one cell line, usually platelets.

Fourteen patients were clinically diagnosed with DBA. Four belonged to one family with an $RPS19$ mutation\textsuperscript{26}; this gene was wild type in the others. Eleven were receiving transfusions or corticosteroids, two had mild macrocytic anemia, and one patient with an $RPS19$ mutation had normal blood counts. Five patients with exocrine pancreatic insufficiency and neutropenia had clinical SDS\textsuperscript{27}; four had mutations in $SBDS$.\textsuperscript{28} The ten “other” patients included those with single or multiple cytopenias, and/or abnormal fingernails or skin pigmentation, sufficient to warrant considering the diagnosis of DC. Although not every patient was directly examined by the research team, clinical summaries, laboratory results, and high-quality photographs were available, and the consensus was that these patients did not have DC.

Results

Comparison of DC Patients with DC Relatives:

Telomere length was reported as the median value in kb for each cell type. The results for DC patients and their relatives are shown in Figure 1 and summarized in Table 3 and Table S1
Because the DC patient group was significantly younger than the unaffected DC relatives, the median telomere lengths cannot be directly compared (supplemental Table S1). Total leukocyte telomeres were shorter than the age-matched 1st percentile of control cells in all 26 patients whose DC diagnosis preceded the telomere study. Five clinically unaffected relatives also had total leukocyte telomeres shorter than the 1st percentile; however, two relatives had telomeres in all cell subsets that were as short as in the majority of the known patients, suggesting that they might be silent carriers. The total leukocyte telomere assay was 100% sensitive, but only 91% specific (Table 3). Assay performance characteristics were improved when several leukocyte subsets were examined individually. Granulocytes were sensitive (96%) but not specific (84%) for distinguishing DC patients from their relatives. However, lymphocytes, naïve T-cells, and B-cells all had sensitivities and specificities >90%, while memory T-cells and NK/NKT-cells were less sensitive (85% and 72% respectively). The combinations of very short telomeres in granulocytes and lymphocytes, granulocytes plus naïve T-cells plus B-cells, and lymphocytes plus naïve T-cells plus B-cells were 92% sensitive and 96% specific. The PPV was ≥92% for lymphocytes, memory T-cells, NK/NKT-cells, and all the combinations mentioned, but was only 75% for granulocytes, 84% for total leukocytes, and 89% for naïve T-cells. The NPV was very high in all cell types. Relaxing the cut-off to include very short and short telomeres, i.e. <10th percentile of normal, improved the sensitivities and NPVs for all cell types, but markedly reduced the specificities and PPVs (supplemental Table S1).

Two DC patients did not have universally very short telomeres (Figure 1). A 39 year old TERC mutation-positive patient with mild pancytopenia associated with hypocellular MDS had telomeres that were below the 1st percentile in total leukocytes and granulocytes, between the 1st and 10th percentile in total lymphocytes, naïve T-cells, memory T-cells and B-cells, and above
the 10th percentile in NK/NKT-cells. A 31 year old patient from a large, mutation-unknown
dominant family with classical DC had leukoplakia, dysplastic nails, mild thrombocytopenia, and
a hypocellular marrow; telomeres were at or below the 1st percentile in all cell types except
lymphocytes, memory T-cells, and NK/NKT-cells. All other DC patients had very short
telomeres in at least six different subsets.

Two clinically-normal DC relatives (members of the large family mentioned above) had
very short telomeres in all cell types; they may be silent carriers of a mutation in a new DC gene.
Seven relatives from other families had very short telomeres in one to three leukocyte subsets,
particularly granulocytes and total leukocytes.

In DC patients there was no correlation between telomere length and severity of bone
marrow failure, presence of the diagnostic triad, or less common physical findings (data not
shown). Telomeres were shorter in DC patients lacking a mutation in the known DC genes than
in those with mutations (data not shown).

**Non-DC Patients and their Relatives:**

Telomere lengths were generally above the 1st percentile in the relatives of non-DC
patients (Figure 2). Only three relatives had very short telomeres in up to three cell types.
However, several of the non-DC IBMFS patients had very short telomeres, particularly in total
leukocytes (6/44 patients) and granulocytes (11/42 patients). Only two of these patients had very
short telomeres in at least five cell subsets, and only one in the combination of lymphocytes plus
naïve T-cells plus B-cells.

**DBA:** One transfusion-dependent patient had very short telomeres in all six analyzed cell
types; telomeres were also very short in the granulocytes and memory T-cells of his transfusion-
dependent fraternal twin. *RPS19, DKC1*, and *TERC* were wild type in the first twin (with
microcephaly, developmental delay, and growth retardation but no components of the DC diagnostic triad), who might have the HH variant of DC. One other DBA patient had very short telomeres only in granulocytes.

**FA:** Seven patients had very short telomeres in at least one cell type. Granulocytes were very short in one *FANCF* and one *FANCA* patient. Two patients had very short telomeres in granulocytes and total leukocytes (*FANCA*) and granulocytes and memory T-cells (*FANC unclassified*). Two patients (*FANCA* and *FANCC*) had very short telomeres in three cell types, and one *FANCA* patient with hematopoietic somatic mosaicism (no chromosome breakage in peripheral blood; positive breakage in fibroblasts) surprisingly had very short telomeres in total leukocytes, granulocytes, lymphocytes, and naïve T-cells. This patient had previously received full-dose radiation therapy for head and neck squamous cell carcinoma, as had one of the *FANCC* patients with very short telomeres for vaginal squamous cell carcinoma, which might have affected the rate of division of hematopoietic stem cells during recovery from radiation-induced suppression.

**SDS and Others:** One SDS patient had unexplained very short telomeres in granulocytes, lymphocytes, memory T-cells, B-cells, and total leukocytes. One “other” patient with pancytopenia had very short telomeres only in B-cells; red cell- and platelet transfusion-dependent bone marrow failure has spontaneously improved. None of the other patients in this group had very short telomeres in any leukocyte subset.

**Comparison of DC Patients with Non-DC Patients:**

While the DC and non-DC IBMFS patients were similar in age (Tables 1-2), the telomere lengths in each cell type were significantly shorter in the DC patients (Figure 3, Table 4 and supplemental Table S2). The performance characteristics of the telomere length assay in
distinguishing DC patients from non-DC patients were similar to those distinguishing DC patients from their own relatives; sensitivities for each cell type exceeded 90% for all cell types except memory T-cells and NK/NKT-cells. However, the specificities and PPVs were lower in granulocytes, B-cells, and total leukocytes. The combinations of cell types which performed well for DC patients versus their relatives also did so for DC patients versus non-DC patients, i.e. very short granulocytes and lymphocytes, granulocytes plus naïve T-cells plus B-cells, and lymphocytes plus naïve T-cells plus B-cells were all 92% sensitive and 93-98% specific, with PPVs of 88% to 96%, and NPVs of 95%.

**Telomere length versus Age:**

The cross-sectional patterns of age-related telomere lengths (Figure 4) in the non-DC IBMFS patients as well as the DC and non-DC relatives showed the expected decreasing median telomere lengths with increasing age.\(^8\) In contrast, telomeres were very short in DC patients of all ages, slightly increasing with age, which reached statistical significance in the B-cells (p for slope = 0.008). These unexpected cross-sectional results may not reflect individual changes with age, but the distinctive trend in DC is tantalizing. The median intercept with age zero for telomere length in lymphocytes was 3.7 kb for DC patients, 9.7 kb for non-DC patients, and 8.3 and 8.8 kb in the respective relatives. At age 40, the comparable telomere lengths were 4.2, 6.0, 6.7, and 7.0 kb, respectively.

**Discussion**

We addressed two major questions: 1. Can we distinguish DC patients from their relatives on the basis of telomere length, and can we identify clinically silent carriers of DC in the absence of a mutation in a known DC gene? 2. Can we diagnose DC in patients with an
IBMFS that does not meet diagnostic criteria for one of the defined IBMFS, or in patients with apparently sporadic AA, including those who fail to respond to immunosuppressive therapy?31

Nearly all of the known DC patients in our series had very short telomeres in the majority of their leukocyte subsets (Tables 3-5), clearly distinguishing them from the other groups, and demonstrating that very short telomeres are a surrogate marker for DC. Management decisions in families without mutations in known DC genes must be made without genetic identification of the specific family members at risk, complicated by variable expression and age-related penetrance of the DC clinical phenotype; analysis of telomere length may be informative. Silent carriers may be at risk of subsequently developing DC-related complications such as bone marrow failure, MDS, AML, premalignant leukoplakia, or solid tumors. They may benefit from appropriate genetic counseling regarding their own future health risks and the risk of DC in their children. If very short telomeres represent a reliable indicator of DC, then designating such persons as affected can markedly enhance the statistical power of linkage analysis aimed at identifying new DC genes in mutation-negative families. It is also essential to exclude as BMT donors family members who appear well but have very short telomeres, as their cells may fail to engraft.32

Identification of individuals with very short telomeres within mutation-positive DC families would alter the risk/benefit assessment with regard to whether to undergo clinical germline mutation testing. Confirmation of DC in silent carriers identified with very short telomeres will expand our understanding of the clinical and hematologic spectrum and the natural history of DC, and facilitate insights into gene/gene or gene/environment interactions which result in bone marrow failure or malignant progression in only some DC patients.
The correct attribution of bone marrow failure, MDS, AML, or a solid tumor (upper or lower gastrointestinal tract carcinomas) to DC has major management implications. DC-related aplastic anemia is more likely to respond to treatment with androgens or hematopoietic growth factors, and unlikely to respond to immunosuppressive therapy.\textsuperscript{23,31,33} The protocol for BMT may require modification, since patients with DC are at particular risk of pulmonary and hepatic fibrosis and other transplant-related complications.\textsuperscript{34} Treatment of leukemia or solid tumors may also require modification, since all cells share the defect in telomere maintenance and associated genomic instability. The failure to recognize DC in patients (particularly older patients) with marrow failure or typical cancers may be more common than generally appreciated, due to its rarity, variable clinical phenotype and variable age at onset.

This study is the first to systematically examine leukocyte telomere length by flow FISH in patients with a non-DC IBMFS. While we agree with earlier reports of “short telomeres” in groups of patients with FA and SDS, our analysis of multiple leukocyte subsets in individuals, and use of a larger cohort of age-matched controls led us to identify important differences in the pattern of telomere abnormality in DC \textit{versus} other IBMFS. We found that very short telomeres (explicitly defined as below the 1\textsuperscript{st} percentile of normal) were restricted to a small subset of patients in the non-DC IBMFS group (Figure 2, Table 5), and were primarily observed in total leukocytes and granulocytes. Only one of 14 DBA patients had very short telomeres in all leukocyte subsets; while he met standard diagnostic criteria for DBA, he also had some soft features of DC, such as microcephaly and developmental delay. It is theoretically possible that he has both genetic conditions. Identification of new genes for both these disorders may resolve this diagnostic dilemma.
One of five SDS patients also had very short or short telomeres in five white cell subsets. This patient has both clinical and molecular evidence of SDS, and no DC phenotype; sequencing of known DC genes is underway. Three of 16 FA patients without prior BMT had very short telomeres in three or four white cell subsets. Two of these patients had received radiation therapy for cancer (which might have affected their hematopoietic cell cycle kinetics), while the third had mild neutropenia and thrombocytopenia and required no treatment. One of the patients with prior radiation therapy had somatic hematopoietic mosaicism; excessive cell division due to clonal expansion of a genetically corrected stem cell might have resulted in short telomeres. However, the other two mosaic patients had normal telomeres.

There are several possible explanations for very short telomeres in some non-DC IBMFS patients, in addition to the coincidence of two very rare conditions. Unlike the pattern in DC, telomere length was not equally short in all cell types among the non-DC IBMFS patients. Very short telomeres were more frequent in total leukocytes and granulocytes than in lymphocytes or lymphocyte subsets in this patient subset. Very short telomeres in DC patients support an etiologic role for mutations in genes in the telomere maintenance pathway. In contrast, very short telomeres in non-DC patients may be a consequence of hematopoietic failure or stress, or a result of treatment (e.g. radiation).

The unexpected rise in age-related telomere length in DC (Figure 4) contrasts with the expected decrease in the non-DC patients and in all the relatives. Interpretation is complicated by the cross-sectional nature of the data. DC patients may be born with hematopoietic stem cells with very short telomeres and it is possible that cells with telomeres below a specific length do not survive. Younger DC patients were clinically more severe (this younger group includes the four patients with HH and the four with RS phenotypes) than older DC patients, and had the
shortest telomeres. Patients who were identified as DC when they were older may have milder forms of DC. Thus, the aberrant pattern of telomere length in successive subgroups of DC patients by age may reflect the type of patients available for analysis at each age.

The analysis of leukocyte subsets within the same blood sample by flow FISH is a powerful tool for addressing our clinical and experimental questions. The median value for telomere length in each cell type is plotted on a graph which contains the range for a large number of normal, age-matched controls, permitting easy comparison of the patient’s results with those of the controls. The definition of abnormal, or very short, as less than the 1st percentile is arbitrary, but provides good sensitivity and specificity for the important distinction between DC patients and their unaffected relatives. The performance characteristics for distinguishing between DC and non-DC IBMFS patients were slightly less satisfactory, with good sensitivity but less specificity in individual leukocyte subsets. However, the combination of very short telomeres in the combination of lymphocytes, naïve T-cells, and B-cells, was similar in the distinction of DC patients from their relatives or from non-DC patients, with sensitivity 92%, specificity 96-98%, PPV 92-96% and NPV 95-96%. In our experience, all but two patients with DC had very short telomeres in at least five white cell types, while the two DC relatives with this result were presumably silent carriers; all other relatives had very short telomeres in less than four cell types (most had no subsets with very short telomeres). Only four patients with non-DC disorders had very short telomeres in more than three leukocyte subsets; most had no cells with very short telomeres (supplemental Figure S1).

Using intact cells in the highly-sensitive and reproducible flow FISH system provides a more precise measure of telomere length than Southern blots, which estimate median telomere length from an electrophoretic pattern. Although no single cell type was perfectly sensitive and
specific, the most suitable cell types appear to be total lymphocytes, naïve T-cells, and B-cells, alone, or particularly, in combination. NK/NKT-cells and memory T-cells lack sensitivity and granulocytes and total leukocytes lack specificity. Total leukocytes are a heterogeneous cell population, with the proportions of each cell type specific to each patient, thus providing less consistency than the individual analyses of defined leukocyte subsets. For example, in acquired aplastic anemia the telomere length in granulocytes may be very short whereas the telomere length in lymphocytes is less affected \(^{13}\). Most likely this reflects sustained destruction of hematopoietic stem cells (HSC) resulting in an increased turnover of the remaining HSC. However, DC patients have short telomeres in hematopoietic cell subsets from birth; thus, even long-lived cells with physiologically slow turnover, such as lymphocytes, are diagnostic. Eleven of the non-DC IBMFS patients had very short granulocyte telomeres, and six had short total leukocyte telomeres, contributing to the lack of specificity in these cells.

A limitation of our analysis is the lack of mutations in known genes in 60%, 70% and 20% of the DC, DBA and SDS patients, respectively. Although the diagnosis of FA is definitive in the presence of an abnormal chromosome breakage test, diagnosis of the other disorders in the absence of a deleterious mutation is less certain. Furthermore, we have no clear diagnoses for some of the “other” patients. Strengths of our study include the classification of disease status in patients and relatives prior to performance of the telomere length assay, the large number of patients with DC and other IBMFS, the large number of normal relatives and controls, and the evaluation of multiple leukocyte subsets at the individual level.

We propose that telomere length measurement by automated multicolor flow FISH in leukocyte subsets has great potential in the clinical evaluation of patients in whom DC is suspected. The result can be obtained rapidly, and the assay is particularly useful in families
without mutations in the known DC genes. Asymptomatic family members with very short telomeres can be excluded as transplant donors due to anticipated failure of engraftment\textsuperscript{32,35}, and patients with bone marrow failure of unclear etiology may be classified as DC, or DC may be excluded, thus permitting syndrome-appropriate management. Our data suggest that the diagnostic algorithm for marrow failure patients can now be expanded: chromosome breakage for FA, red cell ADA for DBA, serum trypsinogen and isoamylase for SDS, and telomere length of leukocyte subsets by automated multicolor flow FISH for DC.

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Authorship

Contribution: B.P.A., G.M.B., and P.M.L. participated in designing and performing the research; G.M.B. and P.M.L. interpreted the telomere results blindly; S.A.S. and S.J.C. performed analyses of DC genes; B.B.W. and J.P.W. referred atypical important patients; N.G. and J.A.P. provided clinical and genetic counseling support to all patients in the study. B.P.A., G.M.B., and P.M.L. wrote the paper; and all authors checked the final version of the manuscript.

Conflict-of-interest disclosure: P.M.L. is a founding shareholder in Repeat Diagnostic Inc., a company specializing in leukocyte telomere length measurements using flow FISH.
Reference List


### Table 1: DC Subjects

<table>
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<td>Age, years Median</td>
<td>23</td>
<td>7.5</td>
<td>4.5</td>
<td>11</td>
<td>13</td>
<td>38</td>
</tr>
<tr>
<td>Range</td>
<td>3-47</td>
<td>3-13</td>
<td>1-13</td>
<td>-</td>
<td>1-47</td>
<td>0-87</td>
</tr>
<tr>
<td>N&lt;18 yo</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>15 (58%)</td>
<td>11 (20%)+</td>
</tr>
<tr>
<td>DC Triad N</td>
<td>15</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Mutations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DKC1</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>-</td>
<td>5</td>
<td>4*</td>
</tr>
<tr>
<td>TERC</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>TERT</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>9</td>
<td>1</td>
<td>4</td>
<td>-</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>BMF None</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>54</td>
</tr>
<tr>
<td>BMF Moderate</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>-</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>BMF Severe</td>
<td>10</td>
<td>1</td>
<td>4</td>
<td>-</td>
<td>15</td>
<td>0</td>
</tr>
</tbody>
</table>


*3 mothers and 1 sister of males with DKC1 mutations were heterozygous carriers of this X-linked recessive gene, 1 mother was not a carrier.

**1 silent carrier son of an affected mother with TERC mutation.

+A larger proportion of DC patients was less than 18 years old compared with DC relatives (p <0.002, Fisher exact).
Table 2: Non-DC Subjects

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>FA</th>
<th>BMT</th>
<th>Mosaic</th>
<th>FA Total</th>
<th>DBA</th>
<th>SDS</th>
<th>Other</th>
<th>Non-DC Patients</th>
<th>Non-DC Relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>13</td>
<td>1</td>
<td>3</td>
<td>17</td>
<td>14</td>
<td>5</td>
<td>10</td>
<td>46</td>
<td>35</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>18</td>
<td>15</td>
<td>27</td>
<td>18</td>
<td>13.5</td>
<td>12</td>
<td>5.5</td>
<td>15</td>
<td>31</td>
</tr>
<tr>
<td>Range</td>
<td>5-42</td>
<td>-</td>
<td>27-33</td>
<td>5-42</td>
<td>3-43</td>
<td>8-42</td>
<td>1-21</td>
<td>1-43</td>
<td>5-72</td>
</tr>
<tr>
<td>N&lt;18 yo</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>7 (41%)</td>
<td>8 (57%)</td>
<td>4 (80%)</td>
<td>8 (80%)</td>
<td>27 (59%)</td>
<td>13 (37%)*</td>
</tr>
<tr>
<td>Mutations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FANCA</td>
<td>8</td>
<td>1</td>
<td>3</td>
<td>12</td>
<td>RPS19</td>
<td>4</td>
<td>SBDS 4</td>
<td>0</td>
<td>Mutations 20</td>
</tr>
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<td>0</td>
<td>0</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>FANCF</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>Unknown</td>
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<td>0</td>
<td>2</td>
<td>10</td>
<td>1</td>
<td>10</td>
<td>23</td>
<td>NA</td>
</tr>
<tr>
<td>BMF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3</td>
<td>1*</td>
<td>3</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>2**</td>
<td>7</td>
<td>35</td>
</tr>
<tr>
<td>Moderate</td>
<td>4</td>
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<td>0</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>6</td>
<td>30</td>
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<tr>
<td>Severe</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>11</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

FA, Fanconi Anemia. NA, not applicable. DBA, Diamond-Blackfan Anemia. SDS, Shwachman-Diamond Syndrome. BMT, bone marrow transplant. BMF, bone marrow failure.

*History of severe BMF pre-BMT. Not included in subsequent statistical analyses, but data included in figures. Severity defined in Methods.

**Two patients with physical findings (one sibling of a subject with thrombocytopenia absent radii, one with skin poikiloderma and macrocytic red cells) and normal blood counts.

The trend was for a larger proportion of non-DC subjects less than 18 years old compared with non-DC relatives (p = 0.07, Fisher exact).
Table 3: Telomere Lengths in DC Patients compared with DC Relatives: Very Short = <1st Percentile

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>DC Patients</th>
<th>DC Relatives</th>
<th>OR</th>
<th>95% CI</th>
<th>Sens</th>
<th>Spec</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes</td>
<td>24/25</td>
<td>8/51</td>
<td>129</td>
<td>15-5419</td>
<td>96%</td>
<td>84%</td>
<td>75%</td>
<td>98%</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>24/26</td>
<td>2/54</td>
<td>312</td>
<td>34-3694</td>
<td>92%</td>
<td>96%</td>
<td>92%</td>
<td>96%</td>
</tr>
<tr>
<td>CD45RA+ / CD20- naïve T-cells</td>
<td>25/26</td>
<td>3/54</td>
<td>425</td>
<td>37-17734</td>
<td>96%</td>
<td>94%</td>
<td>89%</td>
<td>98%</td>
</tr>
<tr>
<td>CD45- memory T-cells</td>
<td>22/26</td>
<td>2/54</td>
<td>143</td>
<td>21-1443</td>
<td>85%</td>
<td>96%</td>
<td>92%</td>
<td>93%</td>
</tr>
<tr>
<td>CD20+ B-cells</td>
<td>24/26</td>
<td>4/54</td>
<td>150</td>
<td>22-1508</td>
<td>92%</td>
<td>93%</td>
<td>86%</td>
<td>96%</td>
</tr>
<tr>
<td>CD57+ NK/NKT-cells</td>
<td>18/25</td>
<td>1/50</td>
<td>129</td>
<td>14-5347</td>
<td>72%</td>
<td>98%</td>
<td>95%</td>
<td>88%</td>
</tr>
<tr>
<td>Total Leukocytes</td>
<td>26/26</td>
<td>5/54</td>
<td>∞</td>
<td>--</td>
<td>100%</td>
<td>91%</td>
<td>84%</td>
<td>100%</td>
</tr>
<tr>
<td>Granulocytes, Lymphocytes</td>
<td>23/25</td>
<td>2/51</td>
<td>282</td>
<td>30-3349</td>
<td>92%</td>
<td>96%</td>
<td>92%</td>
<td>96%</td>
</tr>
<tr>
<td>Granulocytes, naïve T-cells, B-cells</td>
<td>23/25</td>
<td>2/51</td>
<td>282</td>
<td>30-3349</td>
<td>92%</td>
<td>96%</td>
<td>92%</td>
<td>96%</td>
</tr>
<tr>
<td>Lymphocytes, naïve T-cells, B-cells</td>
<td>24/26</td>
<td>2/54</td>
<td>312</td>
<td>34-3694</td>
<td>92%</td>
<td>96%</td>
<td>92%</td>
<td>96%</td>
</tr>
</tbody>
</table>

N, number. Abnormal, below 1st percentile. Numerator and denominator indicated the number abnormal/number assayed for each cell type. OR, odds ratio in favor of being a DC patient. CI, confidence interval. Sens, sensitivity, the proportion of those with disease who were correctly identified. Spec, specificity, the proportion of nondiseased people who were correctly identified as negative. PPV, positive predictive value, the proportion of those who test positive who actually have the disease. NPV, negative predictive value, the proportion of those who test negative who do not have the disease. **Bold** indicates the leukocyte subsets with the best performance characteristics.
Table 4: Telomere Lengths in DC Patients compared with Non-DC Patients: Very Short = <1st Percentile

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>DC Patients</th>
<th>Non-DC Patients</th>
<th>OR</th>
<th>95% CI</th>
<th>Sens</th>
<th>Spec</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes</td>
<td>24/25</td>
<td>11/42</td>
<td>68</td>
<td>8-2867</td>
<td>96%</td>
<td>74%</td>
<td>69%</td>
<td>97%</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>24/26</td>
<td>4/44</td>
<td>120</td>
<td>17-1215</td>
<td>92%</td>
<td>91%</td>
<td>86%</td>
<td>95%</td>
</tr>
<tr>
<td>CD45RA+/-CD20- naïve T-cells</td>
<td>25/26</td>
<td>3/45</td>
<td>350</td>
<td>31-14665</td>
<td>96%</td>
<td>93%</td>
<td>89%</td>
<td>98%</td>
</tr>
<tr>
<td>CD45- memory T-cells</td>
<td>22/26</td>
<td>4/45</td>
<td>56</td>
<td>11-325</td>
<td>85%</td>
<td>91%</td>
<td>85%</td>
<td>91%</td>
</tr>
<tr>
<td>CD20+ B-cells</td>
<td>24/26</td>
<td>5/41</td>
<td>86</td>
<td>13-855</td>
<td>92%</td>
<td>88%</td>
<td>83%</td>
<td>95%</td>
</tr>
<tr>
<td>CD57+ NK/NKT-cells</td>
<td>18/25</td>
<td>0/42</td>
<td>1/∞</td>
<td>--</td>
<td>72%</td>
<td>100%</td>
<td>100%</td>
<td>86%</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>26/26</td>
<td>6/45</td>
<td>∞</td>
<td>--</td>
<td>100%</td>
<td>87%</td>
<td>81%</td>
<td>100%</td>
</tr>
<tr>
<td>Granulocytes, Lymphocytes</td>
<td>23/25</td>
<td>3/42</td>
<td>150</td>
<td>19-1592</td>
<td>92%</td>
<td>93%</td>
<td>88%</td>
<td>95%</td>
</tr>
<tr>
<td>Granulocytes, naïve T-cells, B-cells</td>
<td>23/25</td>
<td>1/41</td>
<td>460</td>
<td>33-19338</td>
<td>92%</td>
<td>98%</td>
<td>96%</td>
<td>95%</td>
</tr>
<tr>
<td>Lymphocytes, naïve T-cells, B-cells</td>
<td>24/26</td>
<td>1/41</td>
<td>480</td>
<td>35-20144</td>
<td>92%</td>
<td>98%</td>
<td>96%</td>
<td>95%</td>
</tr>
</tbody>
</table>
Table 5: Percent of Patients with Very Short or Short Telomeres in Each Leukocyte Subset*

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Granulocytes</th>
<th>Lymphocytes</th>
<th>Naïve T-Cells</th>
<th>Memory T-Cells</th>
<th>B-Cells</th>
<th>NK/NKT-Cells</th>
<th>Leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC Patients</td>
<td>96</td>
<td>4</td>
<td>92</td>
<td>8</td>
<td>96</td>
<td>4</td>
<td>85</td>
</tr>
<tr>
<td>DC Relatives</td>
<td>16</td>
<td>22</td>
<td>4</td>
<td>15</td>
<td>6</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>FA**</td>
<td>40</td>
<td>33</td>
<td>13</td>
<td>20</td>
<td>13</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>DBA</td>
<td>21</td>
<td>21</td>
<td>7</td>
<td>36</td>
<td>7</td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td>SDS</td>
<td>20</td>
<td>40</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>Other</td>
<td>13</td>
<td>13</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>All Non-DC Patients</td>
<td>26</td>
<td>26</td>
<td>9</td>
<td>23</td>
<td>7</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>Non-DC Relatives</td>
<td>3</td>
<td>20</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>17</td>
<td>0</td>
</tr>
</tbody>
</table>

*Data are percentage of patients in the diagnostic group who had telomeres <1st percentile, or between 1st and <10th percentile. Telomeres that were normal or longer than normal (ie 10th percentile or above) are not included.

**FA includes 1 patient after bone marrow transplant whose telomeres were normal, and 3 with hematopoietic somatic mosaicism.

**Bold** indicates the important groups for this study: DC patients, DC relatives, and all non-DC patients.
Figure Legends

Figure 1: Telomere length according to age in DC patients and their relatives. The vertical axis represents telomere length in kb. Lines in the figures indicate the 1st, 10th, 50th, 90th, and 99th percentiles of results from 400 normal controls. Symbols represent subjects: 17 DC patients (●), 4 HH (▲), 4 RS (◆), 1 silent carrier (■), and 54 relatives (□). Top panels show granulocytes, lymphocytes, and CD45RA-positive/CD20-negative naïve T-cells. Bottom panels show CD45RA-negative memory T-cells, CD20-positive B-cells, and total leukocytes.

Figure 2: Telomere length according to age in non-DC patients and their relatives. The vertical axis represents telomere length in kb. Lines in the figures indicate the 1st, 10th, 50th, 90th, and 99th percentiles of results from 400 normal controls. Symbols represent subjects: 13 FA patients (●), 1 FA post-BMT (●), 3 FA mosaics (●), 14 DBA (▲), 5 SDS (◆), 10 other (■), and 35 relatives (□).

Figure 3: Telomere length according to age in DC and non-DC patients. The vertical axis represents telomere length in kb. Lines in the figures indicate the 1st, 10th, 50th, 90th, and 99th percentiles of results from 400 normal controls. Symbols represent subjects: 26 DC patients (●), 46 non-DC patients (▲).

Figure 4: Regression lines for telomere length according to age. The vertical axis represents the median telomere length in kb. Lines in the figures indicate the 1st, 10th, 50th, 90th, and 99th percentiles of results from 400 normal controls. DC patients (——), DC relatives (-----), Non-DC patients (-----), and non-DC relatives (- - - -).
Figure 1
Figure 2

![Graphs showing telomere length in granulocytes, lymphocytes, naïve T-cells, memory T-cells, B-cells, and leukocytes across different age groups and conditions. The graphs depict the relationship between age and telomere length for each cell type, highlighting variations across different patient groups such as FA, FA BMT, FA Mosaic, DBA, SDS, Other, and Rels.]
Figure 3
Figure 4
Very short telomere length by flow FISH identifies patients with Dyskeratosis Congenita

Blanche P. Alter, Gabriela M. Baerlocher, Sharon A. Savage, Stephen J. Chanock, Babette B. Weksler, Judith P. Willner, June A. Peters, Neelam Giri and Peter M. Lansdorp

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