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Clonal Hematopoiesis in Patients with Dyskeratosis Congenita

Nieves Perdigones, PhD[*,1], **Juan C. Perin, PhD**[2], **Irene Schiano, BSc**[1], **Peter Nicholas, BSc**[1], **Jaclyn A. Biegel, PhD**[3], **Philip J. Mason, PhD**[1], **Daria V. Babushok, MD PhD**[1,4], and **Monica Bessler, MD PhD**[1,4]

[1]Comprehensive Bone Marrow Failure Center, Division of Hematology, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, 19104, USA

[2]Department of Biomedical and Health Informatics, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, 19104, USA

[3]Department of Pathology and Laboratory Medicine, Children's Hospital Los Angeles and Keck School of Medicine at the University of Southern California, Los Angeles, CA, 90027, USA

[4]Division of Hematology-Oncology, Department of Medicine, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania, 19104, USA

Abstract

Dyskeratosis congenita (DC) is a rare inherited telomeropathy most frequently caused by mutations in a number of genes all thought to be involved in telomere maintenance. The main causes of mortality in DC are bone marrow failure as well as malignancies including leukemias and solid tumors. The clinical picture including the degree of bone marrow failure, is highly variable and factors that contribute to this variability are poorly understood. Based on the recent finding of frequent clonal hematopoiesis in related bone marrow failure syndromes, we hypothesized that somatic mutations may also occur in DC and may contribute at least in part to the variability in blood production. To evaluate for the presence of clonal hematopoiesis in DC, we used a combination of X-inactivation, comparative whole exome sequencing (WES) and single nucleotide polymorphism array (SNP-A) analyses. We found that clonal hematopoiesis in DC is common, as suggested by skewed X-inactivation in 8 out of 9 female patients compared to 3 out of 10 controls, and by the finding of acquired copy neutral loss-of-heterozygosity on SNP-A analysis. Additionally, 3 out of 6 independent DC patients were found to have acquired somatic changes in their bone marrow by WES, including a somatic reversion in *DKC1*, as well as missense mutations in other protein coding genes. Our results indicate that clonal hematopoiesis is a common feature of DC, and suggest that such somatic changes, though commonly expected to indicate malignancy, may lead to improved blood cell production or stem cell survival.

Keywords

dyskeratosis congenita; *DKC1*; clonal hematopoiesis; somatic mutation; somatic reversion

*Comprehensive Bone Marrow Failure Center, 3615 Civic Center Blvd., Philadelphia, PA, Room 302, Philadelphia, PA19104, USA; Phone:2158824951; Fax: 267-426-9892; nievich@gmail.com;.

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Introduction

Dyskeratosis congenita (DC, [MIM: 305000, 127550, 2243230]) is an inherited telomeropathy characterized by a combination of bone marrow failure, typical mucocutaneous findings, and a predisposition to malignancies[1]. The pathogenic defect in DC is an inability to maintain telomere ends, leading to progressive telomere attrition. DC is caused by inherited defects in genes involved in telomere maintenance (*DKC1* [MIM: 300126], *TERC* [MIM: 602322], *TERT* [MIM: 187270], *NHP2* [MIM: 606470], *WRAP53* [MIM: 612661], *NOLA3* [MIM: 606471], *USB1* [MIM: 613276], *CTC1* [MIM: 613129], *TINF2* [MIM: 604319], *RTEL1* [MIM: 608833], *ACD* [MIM: 609377] and *PARN* [2] [MIM: 604212]). The main causes of mortality in patients with DC are bone marrow failure (BMF), as well as malignant transformation to myelodysplastic syndrome (MDS, [MIM: 614286]) or leukemia[1].

A variety of somatic mutations have been associated with clonal expansion in hematologic malignancies[3], including recurrent mutations in *ASXL1* [MIM: 612990], *BCOR* [MIM: 300485], *GATA2* [MIM: 137295], *IDH1* [MIM: 147700], *IDH2* [MIM: 147650], *KMT2A* [MIM: 159555], *KRAS* [MIM: 190070], *NRAS* [MIM: 164790], *PHF6* [MIM: 300414], *PTPN11* [MIM: 176876], *RUNX1* [MIM: 151385], *SF3B1* [MIM: 605590], *SRSF2* [MIM: 600813], *STAG2* [MIM: 300826], *TET2* [MIM: 612839], *TP53* [MIM: 191170], *U2AF1* [MIM: 191317] and *ZRSR2* [MIM:300028]. The molecular profile of a given malignancy influences the clinical presentation, and can affect the prognosis and therapeutic response[4]. More recently, it has been recognized that clonal hematopoiesis is a common finding in the elderly[5, 6], as well as in other bone marrow failure syndromes (BMFS), acquired aplastic anemia[7, 8] (AA, [MIM: 609135]) and paroxysmal nocturnal hemoglobinuria[9] [MIM: 300818], where somatic mutations can be frequently seen in the absence of malignant transformation. Interestingly, clonal hematopoiesis has been described as a “natural therapy” in inherited BMFS, where patients with autosomal dominant forms of DC due to a heterozygous *TERC* mutation may undergo a mitotic gene conversion, where a wild type (WT) copy of TERC replaces the mutant *TERC* allele, leading to clonal expansion of cells carrying two WT copies and rescuing the bone marrow failure phenotype[10]. Thus, somatic mutations and clonal hematopoiesis are not always associated with malignancy, but may sometimes be beneficial.

Based on recent finding of clonal hematopoiesis in related bone marrow failure syndromes, we hypothesized that clonal hematopoiesis may also occur in DC, where it may not be limited to gene conversion, but instead can occur due to a number of somatic events, and likely contributes at least in part to the variability in blood production. Here, we comprehensively evaluated 16 DC patients using a combination of the classical X-inactivation analysis, together with comparative whole exome sequencing (WES) and single nucleotide polymorphism array (SNP-A) genotyping to discover frequent clonal hematopoietic expansion in DC.

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Subjects and Methods

Subject Enrollment

Patients with DC were recruited from the hematology clinics of the Children's Hospital of Philadelphia (CHOP), the Hospital of the University of Pennsylvania (HUP), and Washington University at St. Louis (WUSTL), in accordance with the procedures approved by the Institutional Review Boards of the respective institutions. The control population was recruited from healthy volunteers at CHOP and HUP. Written informed consent from all participants or their legal guardians was obtained in accordance with the Declaration of Helsinki prior to study participation. The diagnosis of DC was made according to the established guidelines[11]. Briefly, individuals were considered to have DC if they had median telomere lengths below the first percentile in several leukocyte subsets, as well as the characteristic clinical findings such as classical mucocutaneous changes and bone marrow failure, or a family history of DC with a confirmed genetic diagnosis[12, 13]. Patients who progressed to hematologic malignancies were excluded from this study. Medical histories, median lymphocyte telomere lengths and bone marrow histologies are shown in Table I.

Telomere Length Measurement

Five patients had telomere length measurements performed by fluorescence in situ hybridization coupled with flow cytometry (flow-FISH) as a part of the clinical diagnostic evaluation by a CLIA-certified testing center (Repeat Diagnostics, Inc., North Vancouver, Canada). Eleven patients, who did not have a TL measurement performed clinically during an evaluation at our center, had TL measured on a research basis using the FITC-conjugated $(C_3TA_2)_3$ peptide nucleic acid probe, as previously described [14]. Relative TL were obtained by comparison to a control cell line (GM03671C; Coriell Institute, Camden, NJ), which was assigned a TL of 100%.

HUMARA analysis

HUMARA assays were performed as previously described[15]. Briefly, undigested and *HhaI*-digested DNA was subjected to polymerase chain reaction (PCR) amplification of the first exon of the *AR* [MIM; 313700] locus using fluorochrome-coupled primers, followed by analysis on the ABI PRISM 3100 Automatic Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Allele calling and the area under the curve (AUC) were determined using Peak Scanner™ software v1.0 (Applied Biosystems, Foster City, CA, USA) and used to calculate the skewing from X chromosome inactivation (XCI). The XCI ratio of the digested fraction was corrected with that of the undigested fraction to allow for preferential amplification of the shortest allele. Skewing was considered to be present when the percentage of the predominant allele exceeded 74%[16].

Tissues and Sample Preparation

Bone marrow mononuclear cells were collected by Histopaque-1077 (Sigma-Aldrich, St Lois, MO, USA) gradient separation of whole bone marrow aspirates. Fibroblasts from skin biopsies were cultured using standard techniques in MEM-α media (Life Technologies, Grand Island, NY), supplemented with 10% FBS, 1% Penicillin/Streptomycin, and 1% L-

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Glutamine at 37°C and 5% CO_2 for no more than three passages. One of the patients had an established induced pluripotent stem cell line, which was used as a constitutional control due to the unavailability of primary skin fibroblasts. Peripheral blood was obtained from vein puncture. DNA was extracted using QiAmp DNA Mini Kit (Qiagen, Maryland, USA) according to the manufacturer's protocol.

Whole Exome Sequencing

Exome libraries from bone marrow aspirate or peripheral blood DNA, as well as from the corresponding skin fibroblast DNA were prepared with SureSelect Human All Exon V4+UTR Kit (Agilent Technologies, Santa Clara, CA, USA) and sequenced on the HiSeq2000 (San Diego, CA, USA) at 150x coverage at the BGI@CHOP High Throughput Sequencing Core, according to the manufacturer's protocol. Following alignment with NovoAlign (Novocraft Technologies, Jaya Selangor, Malaysia), somatic variant discovery was performed using VarScan2[17] with parameters –min-coverage 4,–min-var-freq 0.08,–p-value 0.05,–strand-filter 1–min-avg-qual 20. Variant calling files were analyzed using SNP & Variation Suite v8.0 (Golden Helix, Inc., Bozeman, MT). All putative somatic mutations were subsequently visualized in Integrative Genomics Viewer[18] and validated using PCR amplification and bi-directional Sanger sequencing using standard techniques. All genome coordinates were based on hg19 (NCBI build 37).

SNP array (SNP-A) genotyping

Illumina Infinium SNP-A genotyping was performed on DNA extracted from bone marrow aspirates or peripheral blood DNA at the CHOP Center for Applied Genomics according to the manufacturer's protocol. Due to the manufacturer's technology upgrades during this study period, genotyping was performed with Illumina Quad610, Illumina Human Omni1-Quad, or Human Omni2.5 beadchips (Illumina, Inc., San Diego, CA). All SNP-A data were analyzed using GenomeStudio (Illumina), which allows direct visualization of the B allele frequency (BAF) and log R ratio (LRR).

Antibodies and Western Blotting

Western blotting was performed using standard SDS-PAGE technique using 20 μg total protein, with band density measured with ImageJ 1.48v (National Institutes of Health, USA). The following antibodies were used: TATA binding protein (anti-TBP ab74222, Abcam, Cambridge, MA, USA) and a previously published in-house antibody to human dyskerin[19].

Real-Time RT-PCR

cDNA was obtained by reverse-transcribing 1 μg of RNA with the SuperScript III First Strand Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Real-time (RT)-PCR analysis was performed using 60ng of cDNA on the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems, Woolston, WA, UK) with the following cycling conditions: 2 min at 50°C (1 cycle), 10 min at 95 °C (1 cycle), followed by 45 cycles of 95 °C for 15 sec and 60 °C for 1min. The following oligonucleotide sequences were used: *GAPDH*—forward

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AGCCACATCGCTCAGACAC and reverse GCCCAATACGACCAAATCC; *TERC*—forward TCTAACCCTAACTGAGAAGGGCGTAG and reverse GTTTGCTCTAGAATGAACGGTGGAAG. Messenger RNA levels were calculated according to the 2- CT method[20].

Statistical Analysis

Comparison of X-inactivation skewing between DC patients and controls was performed using Fisher's exact test (one-tailed) using the Epidat v3.1 software (Dirección Xeral de Saúde Pública (DXSP) de la Consellería de Sanidade (Xunta de Galicia), Organizacion Paramericana de la Salud OPS/OMS, Galicia, Spain). Age distribution of patients and controls was compared using a two-tailed t-test, and RNA and protein levels were compared using a one-tailed t-test with Microsoft Excel. For all comparisons, a significant p-value of 0.05 was used.

Results

Clinical Characteristics

A total of sixteen DC patients were included in this study; patients were divided into two different subpopulations according to sample availability (Table I). One population, used for HUMARA analysis, consisted of ten female DC individuals, with ages ranging from 1 to 53 years old, all with telomere lengths below the first percentile of age-matched healthy controls (Table I). Genetic information was available for nine of these patients: seven were heterozygous for mutations in *TERC* and two were compound heterozygous for mutations in *TERT*. Based on sample availability, one individual from this group (CHOP 60.02) was also chosen for additional genetic studies with SNP-A and WES.

The population used for comparative WES analysis consisted of six male DC patients, ranging in age from 9 to 53 years, four of whom had available SNP-A data (Table I). All these patients had telomere lengths below the first percentile (Table I). Genetic information was available for five of the six patients: three were hemizygous for mutations in *DKC1*, one was heterozygous for mutant *TERC* and one was compound heterozygous for mutant *TERT*.

A novel pathogenic mutation in *DKC1*

Of the six male patients subjected to WES analysis, one patient, presenting with classical clinical features of dyskeratosis, was not found to carry any known pathogenic mutations in genes associated with DC. This patient (CHOP 398.01), a 21 year old male, presented with a constellation of characteristic findings including nail dystrophy, oral leukoplakia, abnormal skin pigmentation, thrombocytopenia and a hypocellular bone marrow, as well as diarrhea and a congenital endocardial cushion defect. Clinical evaluation revealed very short median telomere lengths at under the 1st percentile of telomere lengths for age-matched healthy controls (Figure 1B), as well as a family history of similar clinical findings of severe bone marrow failure in his brother who died from complications of a bone marrow transplant at the age of 14 years (Figure 1A). WES of the patient's skin fibroblast DNA revealed a novel germline mutation in a conserved nucleotide c. -35 G>A (chrX: 153,991,206 G>A based onGRCh37/hg19 assembly) within the 5′UTR region of *DKC1*, an X-linked gene (Figure

1G). Genotyping of the patient's parents revealed that the patient inherited the mutation from his asymptomatic mother, who was heterozygous for the c. -35 G>A *DKC1* allele (Figure 1E). Importantly, this mutation was not found among the known polymorphisms in the Database of Single Nucleotide Polymorphisms (dbSNP[21]) and 1000Genomes project[22] suggesting that it may be pathogenic in this family.

Western blotting for human dyskerin confirmed low levels of dyskerin protein in the patient's skin fibroblasts, similar to the low dyskerin levels in the DC patient carrying the known pathogenic p.Gln31Glu in *DKC1*[23], and significantly lower than dyskerin levels in healthy male controls (p=1.4E-05; Figure 1C). The family history and genetic analysis were consistent with a classical X-linked inheritance (Figure 1 A and E), and, in agreement with prior reports of destabilization of *TERC* RNA due to *DKC1* alterations[24], the patient's *TERC* RNA was lower than in non-DC individuals (p=0.02) and similar to other X linked DC patients (Figure 1D).

Finally, because female carriers of *DKC1* mutations frequently develop biased X-inactivation due to the growth advantage of WT *DKC1*-expressing cells[25], we performed the X chromosome inactivation analysis with the HUMARA[15] assay to look for evidence of skewed X-inactivation in peripheral blood of the patient's mother. Taking advantage of the microsatellite allele of the human androgen receptor shared between the patient and his mother as a marker of co-segregating mutant *DKC1*, we found that the mother's X-inactivation was strongly biased towards expression of the WT *DKC1* allele (Figure 1F).

Together, our observations strongly suggest that c.-35G>A mutation in *DKC1* is the causative mutation in this DC family.

The Majority of Female DC Patients Have Clonal Hematopoiesis as Evidenced by Skewed X-Inactivation

Because X-inactivation occurs randomly early in embryogenesis, approximately half of an individual's cells normally have an active maternal X-chromosome and half have an active paternal X chromosome; the silenced chromosome remains inactive for the lifetime of the cell and all its descendants. Thus, when a cell acquires a mutation that leads to increased survival or growth and subsequent clonal expansion, X-inactivation ratio changes from 1:1 with increasing skewing overtime. Taking advantage of skewed X-inactivation as a marker of clonal hematopoiesis, we first used the classical X chromosome inactivation analysis with HUMARA in 10 female DC patients and 10 healthy controls to evaluate for clonal hematopoiesis in DC (Table II, Supplemental Table S1). Of the ten female DC patients, nine patients had informative HUMARA locus, while one patient was not informative and was excluded from subsequent analysis. DC patients ranged in age from 3 to 53 years old while the control group had ages ranging from 6 to 46 years (p=0.26). DC patients were significantly more likely to have biased X-inactivation than healthy controls, with 8 out of 9 of patients having more than 74% skewing of X-inactivation in peripheral blood, as compared to 3 out of 10 in healthy individuals (p=0.015) (Table II).

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Clonal Hematopoiesis with Somatic Mutations is Common in DC Patients and Includes Somatic Reversion of Mutant *DKC1*

In order to evaluate for acquired copy number variants and somatic mutations in DC, we evaluated six additional patients using comparative WES. Based on sample availability four of the six patients were further evaluated using SNP-A (Supplemental Table II). Comparative WES revealed non-synonymous coding single nucleotide variants (SNV) and small insertion/deletions (Indels) (Supplemental Table III). Orthogonal validation using Sanger sequencing confirmed somatic mutations in three patients (Table III). Two patients carried a single coding missense mutation: a p. Pro3298Ser in Titin (*TTN* [MIM: 188840]) with a clone size of 66%, and another, a G397R in Latrophilin-1 (*LPHN1* [MIM:616416]) with a clone size of 32% (Figure 2A-B). It is of interest that the *LPHN1* p. Gly397Arg somatic mutation corresponds to a known polymorphic site (rs200537037) with a minor allele frequency of 0.02% in the general population. Direct visualization of the original etiologic mutations led to the detection of a somatic reversion in *DKC1* gene in the third patient, where the patient with the germline mutation in the 5′UTR of *DKC1* (c.-35 G>A), discussed above, was found to carry a 14% somatic reversion with a clone size of 14% (Figure 2C). SNP-A analysis, performed to detect acquired loss of heterozygosity, detected a copy number neutral loss of heterozygosity (CN-LOH) of the long arm of chromosome 1 in another patient (Figure 2D).

Discussion

Our work demonstrates that, akin to several other BMF syndromes, clonal hematopoiesis is common in DC. Taking advantage of a combination of X-inactivation studies, comparative WES and SNP-A genotyping in two independent patient subpopulations, we have shown that most DC patients have clonal expansion of hematopoietic cells. First, our data demonstrate that a striking majority of female DC patients have skewed X-inactivation, a rate significantly higher than expected based on age[26] or than observed in our control population. Second, we have demonstrated clonal expansion of cells bearing nonsynonymous coding somatic mutations in half of DC patients investigated by comparative WES.

Clonal hematopoiesis has been classically associated with malignancy, where a number of recurrent mutations have been found[3]. Although DC patients have a high risk of developing MDS or acute myeloid leukemia (AML, [MIM: 601626]), patients in our WES study did not carry somatic mutations in genes associated with myeloid malignancies. Notably, our study identified several noncoding somatic mutations, including a somatic reversion of the *DKC1* gene. Although X-linked form of dyskeratosis congenita[27] is most commonly caused by single amino acid substitutions in the *DKC1* gene, disease-causing mutations in the 5′ UTR region of *DKC1* have also been described[28, 29]. Our study uncovered a novel, inherited, pathogenic mutation in the 5′UTR of *DKC1* (c.-35 G>A), 35 base pairs upstream of the start codon, which was associated with a lower level of dyskerin, and, in agreement with known effect of dyskerin on stabilization of *TERC* RNA[24, 30], with a lower level of *TERC* RNA. Interestingly, the patient was found to have a clonal reversion of this mutation in 14% of cells in the patient's bone marrow, with an expected

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normalization of dyskerin and telomere maintenance in cells bearing the reversion. Clonal expansion of revertant cells further supports our finding that the new underlying *DKC1* gene mutation is indeed pathogenic.

Importantly, clonal hematopoiesis has been reported in a context of "natural therapy" in other inherited BMF syndromes. In Fanconi Anemia (FA [MIM: 227650]), functional mosaicism, frequently involving the correction of the original etiologic mutation[31-33], is seen in 10-25% of patients[34], and is associated with higher blood counts and clinical improvement[32]. Clonal hematopoiesis has also been observed in Shwachman Diamond Syndrome (SDS, [MIM: 260400]), where patients with compound heterozygosity involving a null and a hypomorphic mutation can acquire isochromosome i(7)(q10) encompassing the hypomorphic *SBDS* allele, and leading to clinical improvement[35, 36]. More recently, first evidence of clonal rescue in DC was provided with the observation that carriers of pathogenic *TERC* mutations who did not have BMF all showed LOH on chromosome 3 leading to cells with two copies of the wild type *TERC* allele at the expense of the mutated allele[10]. Our study expands the current understanding clonal hematopoiesis in inherited BMF by demonstrating frequent clonal hematopoiesis in an unselected, "all-comer" cohort of DC patients, where it does not appear to be driven by malignancy-associated mutations, but instead may be a product of genetic changes that may modulate or restore hematopoiesis as seen with somatic reversion in *DKC1*.

There were three other acquired genetic changes identified in our patient cohort: acquired CN-LOH for chromosome arm 1q, and two somatic missense mutations—titin (*TTN)* p. Pro3298Ser and latrophilin-1 (*LPHN1*) p. Gly397Arg. Intriguingly, alterations of the long arm of chromosome 1 involving a gain of genetic material have been recurrently observed in other inherited and acquired BMFs as well as in blood-related malignancies. For example, duplications of 1q has been observed in AA[37], FA, MDS including refractory anemia (with or without excess blasts), AML as well as other myeloid and lymphoid malignancies (for a review see[38]). Interestingly, in FA, trisomy 1q can be found years prior to the development of AML/MDS. In this context, Quentin *et al.* proposed that trisomy 1q might provide hematopoietic rescue of bone marrow failure in FA, partially or completely, but would not be protective against progression to MDS and leukemia[39]. Given the similarities between these inherited bone marrow failure syndromes, it is intriguing to consider that FA and DC may share a bone marrow environment that selects for changes in 1q.

Finally, two other patients carried somatic missense mutations in their bone marrow: titin (*TTN*) p. Pro3298Ser and latrophilin-1 (*LPHN1*) p. Gly397Arg. *TTN* encodes for a protein called titin, expressed and localized typically in the striated muscle. Titin has also been proposed to have a role in chromosomal structure[40]. Somatic mutations in *TTN* have been recurrently detected in different cancer types, with a frequency over 5%[41]. Our study detects a missense mutation in *TTN* (p. Pro3298Ser; NM_003319), located in a very conserved region and predicted by *in silico* functional prediction algorithm Sorting Tolerant From Intolerant[42] (SIFT) to be damaging (SIFT scores: p=0.029 and a median of 3.15). The missense mutation we found in *LPHN1* (p. Gly397Arg; NM_001008701), corresponds to SNP rs200537037 with a minimum allele frequency (MAF) of 0.02% in the general population. SIFT prediction of this variant has a score of p<0.05 and a median of 3.29.

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LPHN1, also known as *ADGRL1*, encodes the latrophilin-1 precursor/calcium-independent receptor for α-latrotoxin, a member of the latrophilin subfamily of G-protein coupled receptors (GPCR) and may function in both cell adhesion and signal transduction. Gene expression assays have linked high expression of *LPHN1* in low invasion lung cell lines[43].

Although we were fortunate to assemble a moderately-sized cohort of patients with this rare disease with sufficient tissue material to perform a comprehensive genetic analysis, future studies with larger numbers of patients are needed to more fully explore the nature and implications of clonal hematopoiesis in DC. Additionally, it is possible that some of the somatic changes in this study may not be the primary driver of clonal hematopoietic expansion, but instead may be "passengers" or modifiers. In this case a driver could be an undiscovered genetic change outside of WES capture region or below the sensitivity of our analysis; alternatively, there may be an epigenetic driver. Although formally stem cell attrition can contribute to clonal hematopoiesis by limiting the number of available stem cells, our findings of CN-LOH of 1q, a region recurrently involved in BMF, as well as the discovery of somatic reversion of mutant *DKC1*, argue against simple clonal depletion as a primary mechanism causing clonal emergence in DC.

In sum, we have shown for the first time that clonal hematopoiesis is common in DC. We have found genetic changes including a somatic reversion in *DKC1*, missense mutations in *TTN* and *LPHN1* and CN-LOH of chromosome arm 1q as the most plausible drivers of clonality. Future studies with a larger patient population and longitudinal surveillance of clonal evolution will help to better define clinical heterogeneity and prognosis in this rare and devastating genetic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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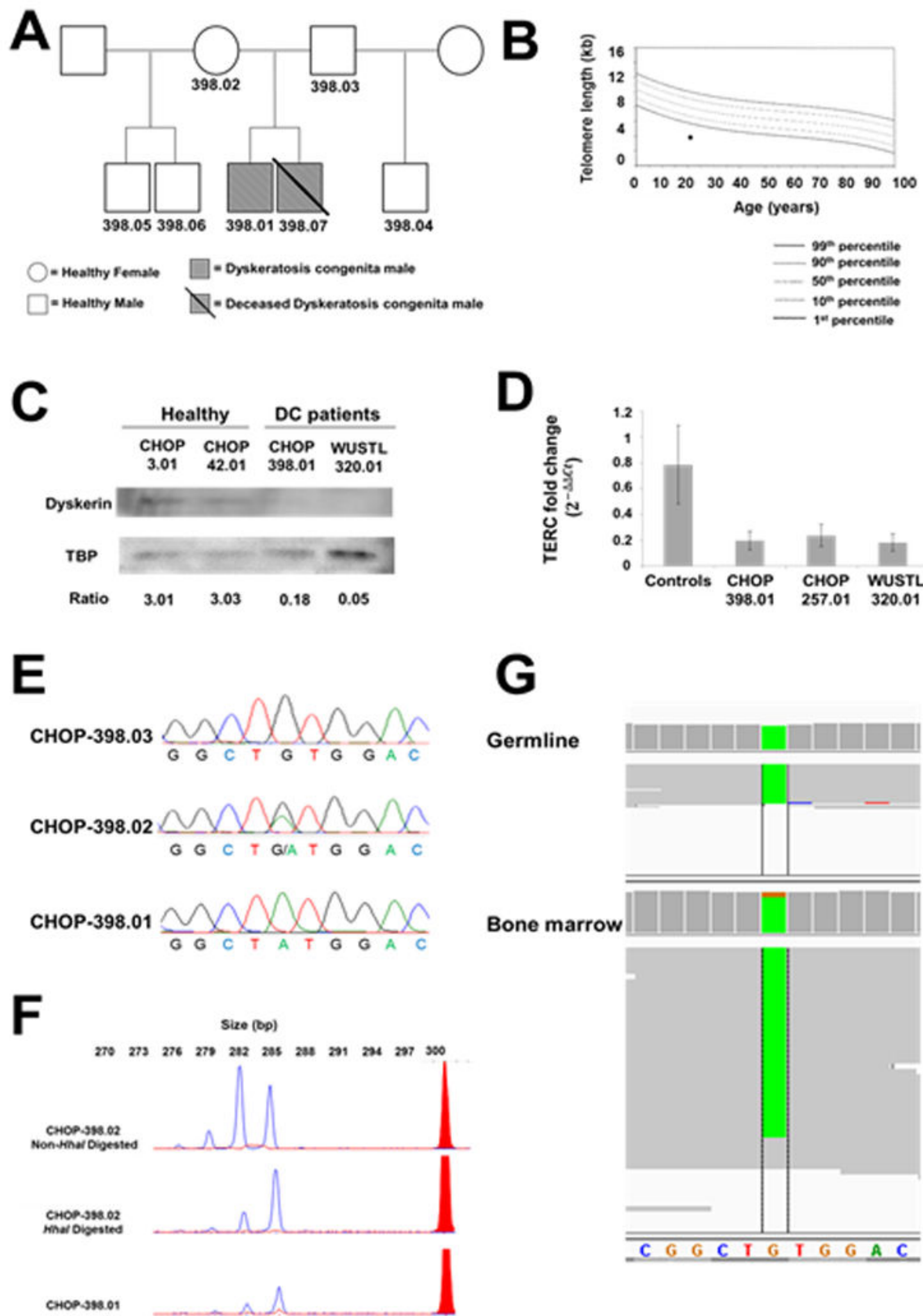

Figure 1.

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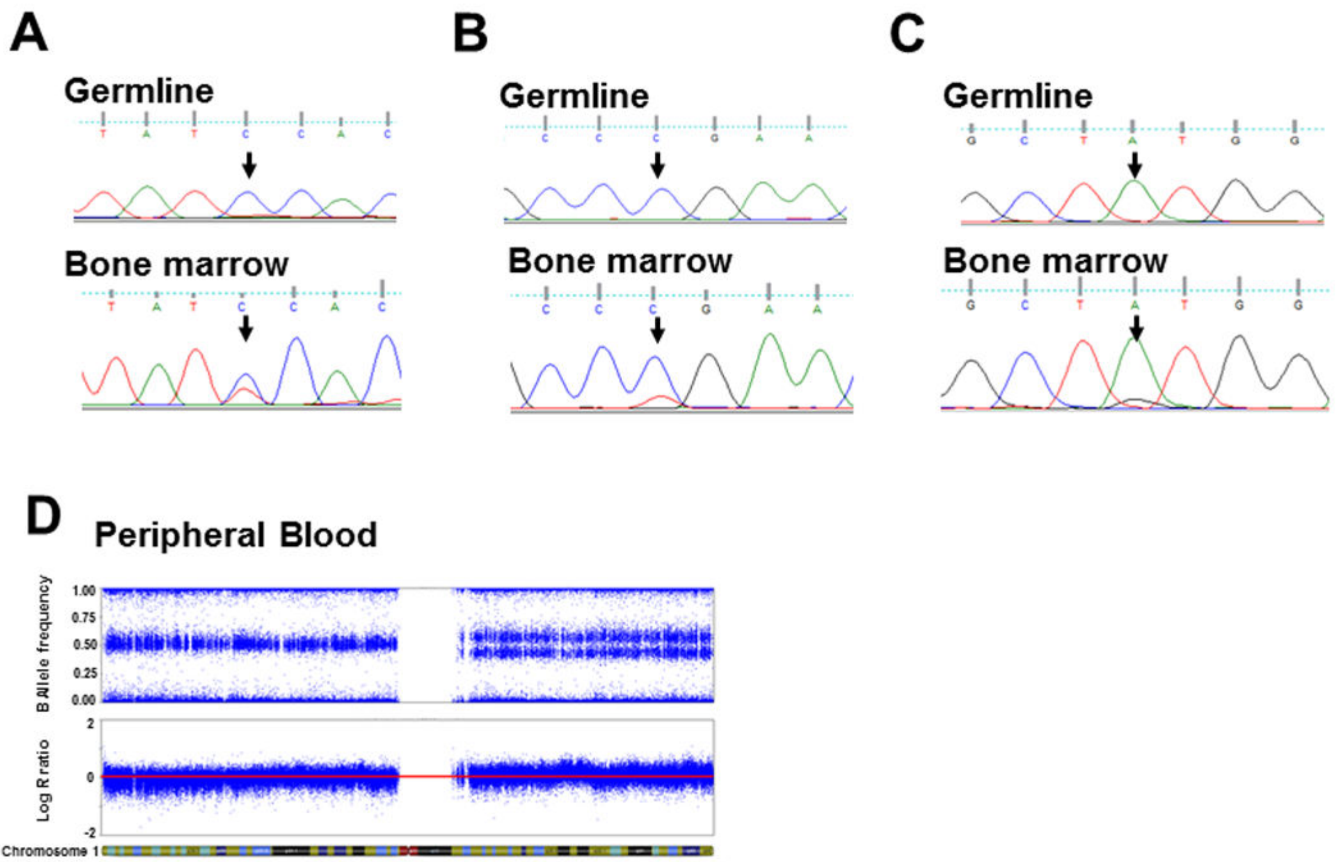

Figure 2.

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Table 1

Clinical, epidemiological and genetic features of patients with dyskeratosis congenita

Patient ID	Sex	Age (yrs)	Race/Ethnicity	Etiological mutation	Median Telomere Length (kb)*	BM cellularity (%)	Clinical history and Physical Exam Findings	Study of Clonality	Family History	Skew XCI>74%
CHOP-12.02	F	40	W	Unknown	Very Low (**)	n/a	n/a	HA	DC with marrow failure and very low telomeres (<1st percentile)	Yes
CHOP-103.03	F	20	W	*TERC* (n.35C>T)	Very Low (**)	n/a	Absent	HA	DC with marrow failure	ni
CHOP-103.04	F	32	W	*TERC* (n.35C>T)	Very Low (**)	n/a	n/a	HA	DC with marrow failure	Yes
CHOP-103.05	F	17	W	*TERC* (n.35C>T)	Very Low (**)	n/a	Dysplastic nails	HA	DC with marrow failure	No
CHOP-103.09	F	29	W	*TERC* (n.35C>T)	Very Low (**)	n/a	n/a	HA	DC with marrow failure	Yes
CHOP-146.01	F	11	n/a	*TERT* (c.2593C>G/c.2683C>G, p. Arg865Gly/p.Leu895Phe)	Very Low (**)	n/a#	Bone marrow failure, requiring BMT	HA	DC	Yes
CHOP-373.02	F	40	W	*TERC* (n.173A>G)	Very Low (4.0)	30	Leukoplakia	HA	DC, with aplastic anemia, congenital malformations	Yes
CHOP-373.04	F	7	W	*TERC* (n.173A>G)	Very Low (6.2)	40	Hyperkeratosis, nail dystrophy, skin hypopigmentation	HA	DC, with aplastic anemia, congenital malformations	Yes
WUSTL-420.01	F	1	AA	*TERT* (c.2638G>A/c.1589C>T, p.Ala880Thr/p.Pro530Arg)	Low(**)	<5	Developmental delay, microcephaly, cerebellar hypoplasia, mucocutaneous findings	HA		Yes
CHOP-60.02	F	53	W	*TERC* (n.96_97 del CT)	Very Low(**)	n/a	Osteoporosis, pulmonary disease	HA, WES, SNP-A	DC, with pulmonary fibrosis, cervical cancer, aplastic anemia, MDS, ureteral stenosis	Yes
CHOP-9.01	M	16	H	*TERT* (c. 1610G>A/c.2173_2187del15insACAG, p.Arg537His / p. Leu725_Ile729delfs *39)	Very Low(**)	5-10	Pancytopenia, poor weight gain, short stature, oral leukoplakia, poor dentition, restrictive lung disease	WES, SNP-A	Bone marrow failure	ns
CHOP-328.01	M	9	H	*DKC1* (c.838 A>C, p.Ser280Arg)	Very Low (5.5)	40-50	Developmental delay, short stature, pancytopenia, pulmonary disease s/p lung transplant	WES, SNP-A	Premature graying	ns
CHOP-373.01	M	12	W	*TERC* (n.173A>G)	Very Low (5.7)	20-30	Nail dystrophy, pancytopenia, IUGR, learning disability	WES, SNP-A	DC, with aplastic anemia, congenital malformations	ns
CHOP-398.01	M	21	W	*DKC1* (c.-35G>A)	Very Low (3.8)	15	Aplastic anemia, reticular hyperpigmentation, leukoplakia, nail dystrophy, cardiac cushion defect, learning disability	WES, SNP-A	DC with aplastic anemia, coats retinopathy, cerebral calcifications	ns
WUSTL-141.03	M	24	W	*DKC1* (c. 1058C>T, p.Ala353Val)	Very Low(**)	n/a	Died of colorectal cancer	WES	DC with aplastic anemia, cardiac malformation, solid tumors	ns

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Patient ID	Sex	Age (yrs)	Race/Ethnicity	Etiological mutation	Median Telomere Length (kb)*	BM cellularity (%)	Clinical history and Physical Exam Findings	Study of Clonality	Family History	Skew XCI>74%
WUSTL-320.01	M	53	W	*DKC1* (c. 91C>G, p.Gln31Glu)	Very Low(**)	20	Leukoplakia, squamous cell carcinoma of tongue, pancytopenia	WES		ns

F, female; M, male; yrs, years; W, White; AA, African American; H, Hispanic; BM, bone marrow; HA, HUMARA; WES, whole exome sequencing; SNP-A, single nucleotide polymorphism array; n/a, not available; ni, not informative; ns, not suitable for HUMARA (male patients); Skew XCI>74%, significant skewing of X-inactivation as measured by HUMARA assay.

*Median Telomere Length (kilobases), compared to age-matched normal controls: Low (between 1st and 10th percentile), Very Low (<1st percentile). Patients whose telomere measurement was referenced to a standardized cell line, as previously described, are indicated with (**).

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Table II

X chromosome inactivation (XCI) is significantly skewed in dyskeratosis congenita patients compared to healthy controls

Population	Informative Individuals (n)	Median Age, Years (range)	Individuals with XCI> 74%, N (%)	P-value
Dyskeratosis Congenita patients	9	24.5 (3-53)	8 (88.8)	0.015
Healthy controls	10	36 (6-46)	3 (30)	

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Table III

Somatic mutations in patients with dyskeratosis congenita

Patient ID	Sex	Age	Gene	Change Position (DNA, Protein)	WES Allele Frequency (%)	Clone size (%)
WUSTL-320.01	M	53	*TTN*	c.10030C>T, p. Pro3298Ser	33	66
CHOP-9.01	M	16	*LPHN1*	c.1189G>A, p. Gly397Arg	16	32
CHOP-398.01	M	21	*DKC1*	c.-35A>G	14	14

M, male; WES Allele Frequency, percent of WES reads containing the mutation; Clone size, WES Allele Frequency corrected for autosomal or X-linked status.