Differences in Disease Severity but Similar Telomere Lengths in Genetic Subgroups of Patients with Telomerase and Shelterin Mutations

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Abstract

The bone marrow failure syndrome dyskeratosis congenita (DC) has been considered to be a disorder of telomere maintenance in which disease features arise due to accelerated shortening of telomeres. By screening core components of the telomerase and shelterin complexes in patients with DC and related bone marrow failure syndromes we have identified 24 novel mutations: 11 in the RNA component of telomerase (TERC), 8 in the reverse transcriptase component (TERT), 4 in dyskerin (DKC1) and 1 in TRF1-interacting nuclear factor 2 (TINF2). This has prompted us to review these genetic subtypes in terms of telomere length, telomerase activity and clinical presentation among 194 genetically characterised index cases recruited onto the registry in London. While those with DKC1 and TINF2 mutations present at a younger age and have more disease features than those with TERC or TERT mutations, there is no difference in telomere length between these groups. There is no difference in the age of onset and numbers of disease features seen in those with TERC and TERT mutations despite the fact that the latter show higher levels of telomerase activity in vitro. The incidence of aplastic anaemia is greater in patients with TERC or TINF2 mutations compared to patients with DKC1 mutations, and cancer incidence is highest in patients with TERC mutations. These data are the first to provide robust comparisons between different genetic subtypes of telomerase and shelterin mutations (the “telomereopathies”) and clearly demonstrate that disease severity is not explained by telomere length alone.

Introduction

Telomeres are nucleoprotein structures that protect chromosome ends, distinguishing them from double strand breaks that might occur elsewhere in the genome and need repair [1]. Telomeric DNA consists of a TTAGGG repeat sequence that is bound by a protein complex known as shelterin [2]. Because of the end-replication problem [3] telomeres shorten with each cell division and when they become critically short, a p53-dependent checkpoint is activated that leads to apoptosis or cell senescence [4–6]. It has been suggested that this mitotic clock has a tumour-suppressor function in long lived mammals, giving cells a limited lifespan and hence restricting the accumulation of DNA damage [7]. Telomere shortening has therefore been linked to the processes of both aging [8] and cancer [9]. In the germ line and in some stem cells, telomeric DNA can be replenished by telomerase, a ribonucleoprotein complex that involves an RNA template and a reverse transcriptase [10].

Dyskeratosis congenita (DC) is an inherited multi-system disorder classically characterized by a mucocutaneous triad of abnormal skin pigmentation, nail dystrophy and leuкоplakia [11]. DC patients frequently develop bone marrow failure and are at a high risk of developing cancer [12] as well as a variety of other features [13,14]. DC is clinically and genetically heterogeneous. Eight disease genes (DKC1, TERC, TERT, NOP10, NHP2, TINF2, C16orf57 and TCAB1) have been identified to date [15–22] and all but one are known to be involved in telomere maintenance. The four most common genetic subtypes of DC are those that involve either the core components of the telomerase complex [23], the RNA (TERC), the reverse transcriptase (TERT) and the accessory protein dyskerin (DKC1), or the shelterin component, the telomeric repeat binding factor 1-interacting nuclear factor 2 (TINF2).

It is not surprising therefore that DC has been considered to represent the clinical manifestation of defective telomere maintenance and indeed, very short telomeres are a hallmark of this disease [24–26]. However, there is a broad clinical spectrum associated with these mutations and patients can present with a number of overlapping disorders, characterised by one or more of a constellation of clinical features [7,27]. In addition to DC, these include the Hoyeraal-Hreidarsson (HH) syndrome [28], which is characterised by a variety of features including bone marrow failure, growth retardation, cerebellar hypoplasia, enteropathy and...
immunodeficiency. Other patients may present with only aplastic anaemia (AA) [29,30], myelodysplastic syndrome (MDS) [31,32], acute myeloid leukaemia (AML) [33] pulmonary fibrosis [34,35] and liver fibrosis [36], and collectively these can be referred to as the ‘telomereopathies’.

We have been screening telomere-related genes among patients referred, primarily with bone marrow failure, to our registry in London and in this paper we report on 24 novel mutations. The variable clinical presentation among these patients has prompted us to review the clinical features, telomere length and mutation status among 194 genetically characterised index cases. While there are clear differences between the genetic subgroups in terms of clinical presentation, we find that the range of telomere length is similar in each group. We conclude that telomere length alone does not account for variation in disease severity between the different genetic subtypes.

Results

Novel mutations in telomerase and shelterin components

Through mutation screening in patients with DC and related bone marrow failure syndromes, we have identified 23 novel mutations in core components of telomerase: 11 in TERC, 8 in TERT and 4 in DKC1 (Table 1, Figures S1 and S2). Two additional TERT mutations were identified during the course of this study which have been reported elsewhere in unrelated families [15,37]. One mutation in the shelterin component TINF2 has been identified (reported also by Sasa et al [38] while this paper was in preparation) which is in addition to the 8 TINF2 mutations that we have recently reported elsewhere [39]. All TERC, TERT and TINF2 mutations were heterozygous, while the DKC1 mutations are hemizygous (X-linked). None have been reported previously and none are reported among previous screens of normal healthy individuals [15,16,20,30] or on dbSNP or the 1000 genomes database. The clear majority (8/11) of the TERC mutations disrupt base pairing in the pseudoknot region of the molecule and they look like classic disease causing mutations (Figure S1). Of all 45 disease-associated TERC mutations that are known to us, 30 are located in the pseudoknot. Also consistent with previous findings [30,36,37], the TERT mutations are spread throughout the molecule and show variable degrees of conservation across species (Figure S2). Polyphen conservation scores support the notion that most of these mutations are disruptive: 8/10 are predicted to be probably damaging (score = 0.949–1.0) and one is possibly damaging (Val56Leu, score = 0.433); the only one predicted to be benign (Arg972His, score = 0.059) is seen in combination with a second substitution, Gly1063Asp, which has predicted to be benign (Arg972His, score = 0.059) is seen in one is possibly damaging (Val56Leu, score = 0.433); the only one (Figure S2). Polyphen conservation scores support the notion that most of these mutations are disruptive: 8/10 are predicted to be probably damaging (score = 0.949–1.0) and one is possibly damaging (Val56Leu, score = 0.433); the only one predicted to be benign (Arg972His, score = 0.059) is seen in combination with a second substitution, Gly1063Asp, which has predicted to be benign (Arg972His, score = 0.059) is seen in combination with a second substitution, Gly1063Asp, which has.

Telomere Length and Disease Severity

TERC mutations cause lower telomerase activity than TERT mutations

The in vitro telomerase activity of the TERC and TERT mutations, as determined by the telomere repeat amplification protocol (TRAP assay) may act as a guide to the clinical penetrance of these different alleles. It is noticeable that for all but one of the TERC mutations, the TRAP activity is <12% of wild type (Table 1, Figure 1A). The exception is the TERC 242C>T mutation, which gives an activity of 47±11%; this mutation is located in a loop bordering the CR4-CR5 domain of the molecule, away from other disease-causing alleles. It is seen in a patient who has HH, consanguineous parents, and whose asymptomatic mother and sister are also heterozygous; this may therefore not be the pathogenic lesion in this family.

In contrast, only 2/7 of the TERT mutations give a TRAP activity of <10% wild type, while the others give an activity >15% normal (Table 1, Figure 1B). When we include mutations that we have analysed previously, (14 in TERC and 8 in TERT), we see that as a group, the TERC mutations give significantly lower telomerase activity than the TERT mutations (Figure 1C, Student’s t-test P-value = 0.04).

Disease severity varies between different genetic subtypes

These novel data have prompted us to review the clinical and biological manifestations of different mutations across a large series of patients referred to our registry in London. We have information relating to 194 index cases in which we have defined the genetic basis of the disease; 91 with DKC1, 56 with TINF2, 30 with TERC and 17 with TERT mutations. As indicators of disease severity, we have compared the age of report and the number of presenting disease features between the different subtypes (Figure 2). No difference is seen in either category between patients with TERC and TERT mutations. However, those with DKC1 mutations present at a significantly younger age compared to those with TERC mutations (Student’s t-test, P-value <0.0001) and have a significantly greater number of disease features than those with both TERC and TERT mutations (P-value <0.0001). Those with TINF2 mutations present at a younger age than those with both TERC and TERT mutations (P-value <0.0001 and 0.008, respectively) and have more disease features than those with TERC mutations (P-value = 0.027).

We have also investigated the relative incidence of several clinically significant disease features: the presence of 2 or more of the classical mucocutaneous features, AA, cancer, pulmonary disease, cerebellar hypoplasia and short stature (Table 2). Again, no difference is seen in any category between patients with TERC and TERT mutations. However, mucocutaneous features are far less common in patients with either TERC or TERT mutations compared to DKC1 (Pearson’s chi-squared test, P-value <0.0001). Patients with TINF2 mutations are also less likely to have mucocutaneous features than the DKC1 patients (P-value = 0.008), but are more likely to have them than in either TERC or TERT patients (P-value = 0.014 and 0.019, respectively). The incidence of AA is less among patients with DKC1 mutations than either TERC or TINF2 patients (P-value = 0.007 and 0.006, respectively); this is despite the fact that the TINF2 patient group is younger than the DKC1 group, while the TERC group is older than the DKC1 group. Cancer incidence is higher among patients with TERC and TERT mutations, significantly so compared to the TINF2 group (P-value = 0.003 and 0.011, respectively); this may well reflect the fact that they are generally an older patient group.
Telomere lengths do not explain differences in disease severity between genetic subtypes

Despite the fact that we see significant differences in clinical severity between the different genetic subtypes of patients, both in terms of age of presentation and the number of disease features, we do not see any difference in telomere lengths between these groups of index cases. This is true whether we compare age adjusted (Figure 3A) or absolute (Figure 3B) telomere lengths as measured by Southern blot analysis, or when we compare telomere lengths determined by quantitative PCR (Figure 3C). Plots of telomere length against age (Figure 3D and 3E) are consistent with the idea that disease presents when telomeres shorten below a certain length, independent of age.

Taking all index cases together, there is no correlation between telomere length and either age of report (Figure 4A) or number of disease features (Figure 4B). The same is true if each subtype is analysed separately (Figures S3 and S4), although there is a trend toward earlier presentation with shorter telomeres among patients with \textit{\textsc{tinf2}} mutations (R² = 0.424). However, we do see that among patients with \textit{\textsc{dkc1}} mutations, those that present with the severe HH syndrome have shorter telomeres than those that present with more classical DC (P-value = 0.03, Figure 5A). Somewhat surprisingly, when we compare all patients who did and did not present with bone marrow failure, we do not see any difference in telomere length (Figure 5B).

| Age (years) | Disease | Additional disease features | FH | Relatives | Nucleotide | Location or amino acid | TRAP | Δtel |
|-------------|---------|-----------------------------|----|-----------|------------|------------------------|------|------|
| TERCA       | CAA     | dysphagia, skin pigmentation, low NK cells | No | AsM | 36C>T | Ps P1b | 11.3 |
|             | CAA     | skin pigmentation           | No | 67G>A | Ps P2a.1 | 4.6 |
|             | AA/MDS  | non-cirrhotic portal hypertension with oesophageal varices | No | 83T>G | Ps P2a | 4.3 | -2.52 |
|             | DC      | dental & hair loss, pulmonary disease, cancer | No | AsM, AsSo | 95.96delGC | Ps P3 | 0.6 | -2.82 |
|             | CAA     | microcephaly, aseptic necrosis of femoral head | No | AsF | 107G>T | Ps P3 | 0.9 | -4.30 |
|             | NSAA    | aseptic necrosis of femoral head | No | 126A>G | Ps P2a | 4.8 | -6.22 |
|             | AA      | aseptic necrosis of femoral head | No | 176A>C | Ps P3 | 0.5 | -3.58 |
|             | CAA     | nail dystrophy               | No | 182G>A | Ps P3 | 1.3 | -3.03 |
|             | HH      | microcephaly, cerebellar hypoplasia, hair loss, low NK cells | No | AsM, AsSi | 242C>T | Loop P5 | 46.8 | -0.57 |
|             | MDS     | mucocutaneous features, lung disease, abnormal LFT | No | 287C>G | CR4-CRS P66 | 8.1 | -0.57 |
|             | NSAA    | skin pigmentation, VSD       | Yes | 377A>G | H box |
| TERCT       | NSAA    | easy fracture                | No | AsM | 166G>C | Val56Leu | 4.3 | -3.48 |
|             | AA/MDS  | premature greying, telangiectasia | Yes | 5F, SGM | 248G>C | Arg83Pro | 43.1 |
|             | DC      | short digit                  | No | 1142G>C | Arg381Pro | 43.1 |
|             | CAA     | short digit                  | Yes | AsF, Ssi | 2147C>T | Ala716Val | 16.6 | -3.14 |
|             | DC      | MDS, IPF, hair loss, dental caries | Yes | SB, AsSi | 2152G>A | Asp718Asn | 44.1 | -3.61 |
|             | MDS     | liver cirrhosis, congenital hearing loss, bilateral nystagmus | No | AsM | 2581G>A | Gly861Arg | 1.0 |
|             | DC      | hyperidrosis                 | Yes | SB, SB | 2915G>A | Arg972His |
|             | DC      | hypogonadism                 | No | 3082A>C | Asn1028His | 16.7 | -2.20 |
|             | DC      | hyperidrosis                 | Yes | SB, SB | 3187G>A | Gly1063Ser | 29.0 |
|             | NSAA    | abnormal LFT                 | No | 3388A>G | Ile1130Val |
| DKC1        | HH      | microcephaly, cerebellar hypoplasia, low B cells | Yes | SB | 202C>T | His687Tyr |
|             | DC      | No                           | 227C>T | Ser76Leu | -2.66 |
|             | HH      | microcephaly, enteropathy, immunodeficiency | No | 1133G>A | Arg378Gln | -0.7 |
|             | DC      | No                           | 114C>G | Ile38Met |
| \textsc{tinf2} | AA | Intracranial calcification, ocular hemorrhage | No | 811C>T | Gln271X |

1AA: aplastic anemia, \textit{\textsc{CAA}}: constitutional AA, \textit{\textsc{NSAA}}: non-severe AA, \textit{\textsc{MDS}}: myelodysplastic syndrome, HH: Hoyeraal Hreidarson syndrome.

2NK: natural killer cells; VSD: ventricular septal defect; IPF: idiopathic pulmonary fibrosis; LFT: liver function test.

3FH: family history.

4Relatives are listed where they were available and tested positive for the mutation. As = asymptomatic, S = symptomatic, M = mother, F = father, So = son, Si = sister, B = brother, Gr = grandmother.

5Location: domain of \textit{\textsc{terc}}: Ps = pseudoknot.

6TRAP: telomere repeat amplification protocol, % wild type.

7Δtel: age-adjusted telomere length measurement.

8Also published in another family elsewhere [15,37].

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Another factor that can be taken into consideration is the number of asymptomatic relatives (both younger and older) that exist in each of the subtypes: while we know of very few asymptomatic relatives with TINF2 or DKC1 mutations, this is a common feature of families with TERC and TERT mutations. It has been proposed that this is at least in part due to disease anticipation in families with both TERC and TERT mutations, and that this is associated with progressive telomere shortening through the generations. We now know of 27 parent-child combinations from 15 different families in which both are TERC heterozygotes. In all but 4 of these combinations, the affected child has a shorter age-adjusted telomere length than their affected parent (Figure 5C). As a group, the children have significantly shorter telomeres than the parents (P value = 0.0026) with a median difference in Δtel between parent and child (Δtel, child-Δtel, parent) of −1.53 kb.

Discussion

This is the first study to systematically review the relationship between telomere length and disease severity in a large cohort of

![Figure 1.](image1.png)

**Figure 1. TERC mutations have lower residual telomerase activity than TERT mutations.** Telomerase activity of (A) novel TERC mutations and (B) novel TERT mutations were determined using the TRAP assay and are expressed as a % of wild-type activity. (C) A comparison of all TERC mutations (n = 23, blue circles) and TERT mutations (n = 15, pink diamonds) that we have studied shows that as a group, the TERC mutations give significantly lower residual telomerase activity (*P value = 0.04). Black bars indicate median values.

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![Figure 2.](image2.png)

**Figure 2. Differences in clinical presentation between genetic subgroups of patients.** (A) Age at report of patients with different telomerase or shelterin mutations, as indicated below the panel. (B) The number of disease features observed in the same group of patients. The numbers of subjects (in brackets) differ slightly, reflecting the fact that some information is missing. The disease features that were scored included: abnormal skin pigmentation, nail dystrophy, leucoplakia, AA/cytopenia, immunodeficiency, cancer, oesophageal stenosis/dysphagia, interopathy, liver disease, splenomegaly, pulmonary disease, epiphora, ear abnormality, deafness, dental decay/loss, hair greying/loss, retinopathy, microcephaly, cerebellar hypoplasia/ataxia, learning disabilities/developmental delay, abnormal facies, intrauterine growth retardation/low birth weight, short stature/growth retardation, aseptic necrosis, osteoporosis, renal disease, gonadal abnormality, phimosis, hyperhiderosis and cardiac disease.

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unrelated patients with mutations in different telomere-related genes. While clear phenotypic differences exist between the genetic subtypes, there is no difference in the range of telomere lengths observed. This conclusion may seem somewhat surprising as several previous studies have indicated that disease severity may be related to telomere length. These include the phenomenon of

| Gene | Number of cases | Aplastic anaemia (%) | 2 or more mucocutaneous features (%) | Cancer (%) | Pulmonary disease (%) | Cerebellar hypoplasia (%) | Short stature (%) |
|------|-----------------|----------------------|-------------------------------------|------------|----------------------|--------------------------|------------------|
| DKC1 | 91              | 44 (48.4)            | 60 (65.6)                           | 7 (7.7)    | 6 (6.6)              | 15 (16.5)                | 19 (20.8)        |
| TINF2| 56              | 40 (71.4)            | 24 (42.9)                           | 1 (1.8)    | 3 (5.5)              | 6 (10.7)                 | 12 (21.4)        |
| TERC | 30              | 23 (76.7)            | 5 (16.7)                            | 12 (17.6)  | 9 (13.3)             | 1 (3.3)                  | 3 (10)           |
| TERT | 17              | 12 (70.6)            | 2 (11.8)                            | 3 (13.3)   | 3 (17.6)             | 1 (5.8)                  | 2 (11.8)         |
| All  | 194             | 119 (61.3)           | 91 (46.9)                           | 17 (8.8)   | 16 (8.3)             | 23 (11.9)                | 36 (18.6)        |

*Homozygotes excluded.

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**Figure 3. Telomere length measurements in patients with telomerase and shelterin mutations.** Different genetic subgroups of patients with telomerase and shelterin mutations, shown as purple circles (DKC1 mutations), blue diamonds (TERC mutations), pink triangles (TERT mutations) and inverted green triangles (TINF2 mutations) are compared to healthy controls (open grey circles). Solid bars indicate median values and the dotted bars indicate the 10th centiles of the healthy control measurements. (A) Telomere lengths were measured by Southern blot analysis and adjusted for age to give a Δtel value. N = normal healthy controls (n = 176), DK = patients with DKC1 mutations (n = 56), TR = patients with TERC mutations (n = 26), RT = patients with TERT mutations (n = 10), TN = patients with TINF2 mutations (n = 26). (B) Absolute telomere lengths, measured as terminal restriction fragments for the same group of patients as shown in panel A. (C) Telomere lengths measured as a T/S ratio, determined by monochrome multiplex quantitative PCR for a subset of patients shown in panel A (73 healthy controls and 29, 19, 10 and 23 patients with DKC1, TERC, TERT and TINF2 mutations, respectively). (D) Absolute telomere lengths (terminal restriction fragments) plotted against age in years, for the patients and healthy controls shown in panel A. A line of best fit is drawn for the healthy controls. (E) Telomere lengths measured by monochrome multiplex quantitative PCR analysis plotted against age in years for all patients in panel C. A line of best fit is drawn for the healthy controls.

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disease anticipation associated with telomere shortening in families with TERC and TERT mutations [17,40,41], shorter telomeres among patients with TINF2 mutations [42], and patients with severe disease caused by DKC1 mutations having shorter telomeres than those with mild disease [43].

There are several differences between previous studies and the one we present here. Firstly, we are now looking at a considerably larger number of patients with telomere defects, representing the largest series published to date. Secondly, we have focused on the index cases only to avoid the issues relating to asymptomatic individuals with telomerase/shelterin mutations, particularly in the context of disease anticipation. Thirdly, we are comparing different genetic subtypes of the disease, rather than looking within a single subtype.

The four common genetic subtypes of DC (caused by mutations in the DKC1, TINF2, TERC, and TERT genes) can be divided into three groups: (i) the inherited autosomal dominant mutations in the telomerase-specific components, TERC and TERT; (ii) the X-linked recessive mutations in DKC1, encoding a protein that acts as a pseudouridine synthase in H/ACA small nuclear ribonucleoprotein (snoRNP) complexes [44], predominantly involved in ribosomal RNA processing [45], as well as a core component of telomerase; and (iii) the predominantly de novo mutations in TINF2, encoding a core component of the telomere-binding protein complex, shelterin [46,47]. Mutations in any of these genes can clearly impact dramatically on the ability to maintain telomere length and result in a multisystem disease. However, the fact that...
three different pathways are affected in the different genetic subtypes may explain why telomere length alone does not correlate with disease severity. This suggests other biological defects are likely to be important in the disease pathology and the resulting overall clinical phenotype. Although there is no clear evidence of a defect in pseudouridylation in patients with DKC1 mutations [48,49], several studies have indicated that in mice they can have a significant impact beyond telomere maintenance, affecting snoRNA accumulation [50], the DNA damage response [51] and IRES-mediated translation [52].

No significant differences were observed in clinical presentation of patients with TERC and TERT mutations in our cohort. This is despite the fact that as a group, the TERC mutations gave lower residual telomerase activity than TERT mutations in the in vitro TRAP assay. No equivalent comparison has been made previously although a trend toward higher residual TRAP activity of TERT mutations has been apparent in other studies [26,30,32]. In the largest series of TERT mutations published to date [37], the mean age of 134 carriers was 51 years; 50% of these heterozygotes had pulmonary disease but only 16% had haematological abnormalities. This highlights the variable penetrance as well as the diverse and heterogeneous nature of the disease caused by TERT mutations. A similar spectrum of disease severity can be observed in families with TERC mutations, although these appear more likely to present with haematological abnormalities than pulmonary disease. Nevertheless, the disease seen in patients with both TERC and TERT mutations clearly contrasts dramatically with the severe early onset of DC, the HH syndrome, and/or AA that can be seen in patients with mutations in either the shelterin component, TIN2 or the snoRNP component, DKC1.

It is interesting to consider how the rate of telomere shortening differs between the genetic subtypes, even though this is very difficult to measure empirically in individual patients. However, in a typical patient with a TIN2 mutation (e.g. the recurrent Arg282His substitution), this will have arisen de novo and presentation, with very short telomerces occurs in the early years of life. A similar rapid shortening of telomeres will have occurred in approximately 1/3 of patients with DKC1 mutations (e.g. the recurrent Ala353Val substitution) in which the mutation will also have occurred de novo; in patients that inherit a DKC1 mutation, they do so from an asymptomatic heterozygous mother, who will show a highly skewed pattern of X-inactivation in peripheral blood. Both situations are completely different to the typical presentation of TERC and TERT mutations which, although dominant, are usually present in the family for at least one asymptomatic generation. The rate of telomere shortening here appears to be much slower, with critically short telomeres manifesting in disease at an older age and after transmission through the germ line.

One confounding factor in the comparison of different mutations within and between genetic subtypes is the diversity of the mutations themselves. Some mutations are clearly and recognizably pathogenic because they recur (such as Ala353Val in DKC1 and Arg282His in TIN2) or because they abolish telomerase activity (as seen in TERC and TERT). However with private missense mutations in DKC1 and TIN2 or with mutations that only partially reduce telomerase activity in TERC and TERT, it is more difficult to be sure that they are disease causing, rather than bystander mutations.

The significant clinical differences observed between the genetic subgroups (such as the early onset of disease and greater number of disease features in DKC1 and TIN2 patients compared to TERC and TERT patients and the higher incidence of cancer in patients with TERC mutations) will facilitate more informed decisions in the clinic. Equally these findings serve to highlight that whilst these genetic subtypes are unified by having a defect in telomere biology (the “telomereopathies”) the clear differences they exhibit suggest other factors (inherited and acquired) contribute to the overall clinical phenotype. Finally, as a major subgroup of the novel mutations were observed in patients who did not fulfill the criteria to be classified as DC, this study highlights the importance of screening the telomerase and shelterin genes in patients with a spectrum of clinical phenotypes in addition to DC.

Methods

Subjects

All individuals included in this study have given written informed consent in accordance with the Declaration of Helsinki and our study design, approved by the East London and The City Research Ethics Committee. The novel mutations reported in this study were identified among 732 patients referred to our registry over a period of 69 months. They comprised 318 patients with AA, 99 patients who had AA in combination with a family history and/or other somatic disease features (constitutional AA), 81 patients with MDS, 123 patients who had features overlapping those of DC, but insufficient for a clinical diagnosis to be made and 111 who were classified as having DC on clinical grounds. The latter were defined as having either (i) the diagnostic triad of mucocutaneous features, or (ii) 1 or more of these features combined with a hypoplastic bone marrow and at least 2 other features known to occur in DC, or (iii) at least 4 of the 6 features commonly associated with the HH syndrome (intrauterine growth retardation, developmental delay, microcephaly, cerebellar hypoplasia, immunodeficiency and bone marrow failure). From this same group of patients we have previously reported on 4 families with MDS/AML and mutations in TERC or TERT [53], as well as 33 families with mutations in the TIN2 gene [39,42] and have also identified 14 families with recurrent DKC1 mutations and 3 families with recurrent TERC mutations.

Mutation analysis

Genes were scanned for mutation as previously described [43]. Briefly, fragments encompassing all coding exons and flanking intronic sequences of each gene were amplified from genomic DNA by PCR. These fragments were then subjected to denaturing HPLC analysis (in the presence of equal amounts of wild-type DNA fragments in the case of the X-linked DKC1 gene). Where abnormal patterns of elution were identified, the fragments were re-amplified and sequenced directly using the BigDye™ chain termination method and a 3130xl genetic analyzer (Applied Biosystems).

Telomere length measurement

Telomere terminal restriction fragment lengths were determined by Southern blot analysis using the sub-telomeric probe pTelBam8 in DNA extracted from total peripheral blood white cells, as previously described [16]. Age adjusted lengths (Δtel) for any one individual were determined by subtracting the expected telomere length, obtained from a regression line drawn from 176 healthy controls, from the observed telomere length as described [54].

We have also used a monochrome multiplex quantitative PCR method to measure telomere length [55] in which the amount of telomeric DNA (T) and the amount of a single copy gene (S) are quantified using standard curves established by titration of a reference genomic DNA sample. All samples were measured in triplicate. T/S ratios, which are proportional to the telomere
length [55], were then normalised against a second reference DNA sample that was run on every plate. We have adapted the method for use on a LightCycler 480 real time thermocycler (Roche) as described previously [21].

Telomerase repeat amplification protocol (TRAP) assay

The TRAP assay was performed as previously described [53]. Briefly, mutations identified in the TERC or TERT genes were introduced into plasmids harbouring the wild-type gene using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). Plasmids were then transfected into WI-38 VA13 cells and after 48 hours the telomerase activity was measured using the TRAPEze RT Telomerase Detection Kit (Millipore/Chemicon).

Statistical methods

Student’s t-tests were performed in Excel and the Pearson’s chi-squared test was performed using VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html).

Supporting Information

Figure S1 Sequence traces and location of novel TERC mutations. Arrows indicate the heterozygous base change named beneath each panel. Their location is shown on a sketch of the TERC molecule.

Figure S2 Sequence traces and conservation of novel TERT mutations. Arrows indicate the heterozygous base change named beneath each panel. Alignment of the human, mouse, chicken, frog, yeast and plant TERT protein sequences (generated by muscle at the NCBI) indicates the degree of conservation of the affected amino acid, shown in bold font. (PPT)

Figure S3 No correlation between age at report and telomere length. Age at report versus telomere length of index cases in different genetic subtypes, as indicated on each panel. (PPT)

Figure S4 No correlation between number of disease features and telomere length. Number of disease features versus telomere length in index cases with different genetic subtypes, as indicated on each panel. (PPT)

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Author Contributions

Conceived and designed the experiments: TJV MJK AW ID. Performed the experiments: TJV MJK RB UH CB AR. Analyzed the data: TJV MJK AR ID. Contributed reagents/materials/analysis tools: JM ID. Wrote the paper: TJV AW ID.

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