Mutations in the telomere capping complex in bone marrow failure and related syndromes

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ABSTRACT

Dyskeratosis congenita and its variants have overlapping phenotypes with many disorders including Coats plus, and their underlying pathology is thought to be one of defective telomere maintenance. Recently, biallelic *CTC1* mutations have been described in patients with syndromes overlapping Coats plus. CTC1, STN1 and TEN1 are part of the telomere-capping complex involved in maintaining telomeric structural integrity. Based on phenotypic overlap we screened 73 genetically uncharacterized patients with dyskeratosis congenita and related bone marrow failure syndromes for mutations in this complex. Biallelic *CTC1* mutations were identified in 6 patients but none in either *STN1* or *TEN1*. We have expanded the phenotypic spectrum associated with *CTC1* mutations and report that intracranial and retinal abnormalities are not a defining feature, as well as showing that the effect of these mutations on telomere length is variable. The study also demonstrates the lack of disease-causing mutations in other components of the telomere-capping complex.

Introduction

Telomeres are nucleoprotein complexes required by the chromosome for stability. They protect the chromosome terminus from inappropriate nuclease and repair activities, and provide a mechanism to compensate for the inability of DNA polymerase to completely replicate the 5'end of the linear chromosome.[1] Protection of the telomere ends in vertebrates and yeast is broadly similar. Both possess a nucleoprotein cap that is comprised of double stranded DNA (dsDNA) with a single stranded DNA (ssDNA) overhang bound by dsDNA and ssDNA-binding proteins. In vertebrates, the shelterin complex (comprising TRF1, TRF2, TIN2, RAP1, TPP1 and POT1) protects telomeres from degradation. In budding yeast, although the double stranded region of the telomere is bound by Rap1 and two other associated proteins; these are not involved in telomere protection.[2] This function is provided by the CST complex comprising Cdc13, Stn1 and Ten1 which interact with the single stranded 3' overhang.[3] Due to the lack of identity with the shelterin complex, it was thought these were two distinct pathways that had evolved to perform a similar function in different organisms.[4] Mammalian homologs of the CST complex[5,6] have now been identified which are CTC1 (conserved telomere maintenance component 1, which is similar to Cdc13), human STN1 (originally identified as OB-fold-containing 1 [OBFC1]) and TEN1. It is now thought that both the CST and shelterin complexes have a role in protecting telomeric DNA against inappropriate repair mechanisms and recombination,[7] but their modes of action are different. CTC1 specifically has a role in promoting efficient DNA replication and maintaining telomere length.[8]

Two recent studies have identified mutations in the CTC1 component of the CST complex in patients with cerebroretinal microangiopathy with calcifications and cysts (CRMCC). This rare multisystem disorder is thought to encompass both Coats plus and leukoencephalopathy with calcifications and cysts (LCC).[9,10] The key characteristics of this disorder include intracranial calcifications and leukoencephalopathy, retinal vasculature abnormalities, skeletal abnormalities and recurrent gastrointestinal hemorrhage. Dystrophic nails, sparse hair and abnormal skin pigmentation have occasionally been observed in such patients.[11-13]

Dyskeratosis congenita (DC) is a complex bone marrow failure syndrome in which the principal pathology is defective telomere maintenance and it is often associated with short telomeres. The classical clinical manifestations include abnormal skin pigmentation, nail dystrophy, oral leukoplakia and bone marrow failure.[14] Seven of the genes mutated in DC are associated with either the telomerase holoenzyme (*TERT, TERC, DKC1, NOP10, NHP2* and *TCAB1*) or the shelterin complex (*TINF2*),[15] accounting for approximately 50% of DC patients. Phenotypically severe variants of DC have been identified, known as Hoyeraal-Hreidarsson (HH) and Revesz syndromes (RS), and share an overlapping genetic basis. In addition to short telomeres, similar genetics and many common clinical features, both can have cerebral abnormalities and, in the case of RS, present with retinopathy and intracranial calcifications. In the light of the clinical overlap between

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CRMCC (Coats plus and LCC) and DC (particularly in the severe variants), the aim of this study was to determine whether any of our patients with uncharacterized DC and related syndromes had mutations within the CST complex.

Design and Methods

Patients

Patient samples analyzed in this study were selected from the collection of bone marrow failure samples that comprised in part the dyskeratosis congenita registry (DCR) located at Barts and The London School of Medicine and Dentistry. All samples were obtained with informed consent and with the approval of our local ethics committee. Seventy-three patients were selected for analysis, 55 of whom were registered on the DCR (with sufficient features to be classified as DC[14]). The remaining 18 had features overlapping with DC, specifically the HH phenotype but not enough to be entered into the registry, or had bone marrow failure and retinal abnormalities. Eight of 73 patients had either retinopathy, Coats plus or RS. All patients had been previously screened for mutations in the commonly occurring DC-associated genes.

Gene screening for the CST complex

Primers (available on request) were designed to amplify the entire coding region of *CTC1* (NM_025099.5), *STN1* (NM_024928.4) and *TEN1* (NM_001113324.2). PCR products for each exon were pooled pair-wise and analyzed by denaturing high performance liquid chromatography (dHPLC) using the Transgenomic Wave DNA fragment analysis system. All abnormal elution patterns were re-amplified and confirmed by direct sequencing using ABI BigDye v3.1 to identify which specific DNA harbored the mutation of interest.

Telomere length measurement using monochrome multiplex quantitative PCR (MM-qPCR)

Telomere lengths were measured using a multiplex real-time PCR method.[16] Poor quality DNAs as assessed by gel electrophoresis were excluded from analysis. All samples were measured in triplicate as previously described.[17]

Results and Discussion

Identification of mutations in CTC1

Compound heterozygous mutations in *CTC1* were observed in 6 patients, (3 of them fulfilling the diagnostic criteria for DC,[14] Table 1), resulting in the identification of nine heterozygous mutations. Four of these mutations are novel and predicted to be probably damaging by PolyPhen2 analysis[18] (c.1389_1390insGTTAGGA, p.Leu464Valfs*66; c.740T>C, p.Leu247Pro; c.833G>T, p.Gly278Val and c.1270T>G, p.Cys424Gly). Full or partial segregation was only possible in 2 out of the 5 families due to sample availability; this enabled us to confirm the genotype of a second affected sibling in Family 4 as well as demonstrating an autosomal recessive pattern of inheritance (Figure 1A). The patient in Family 5 (F5: II-3) has been previously reported[9] but this was only determined

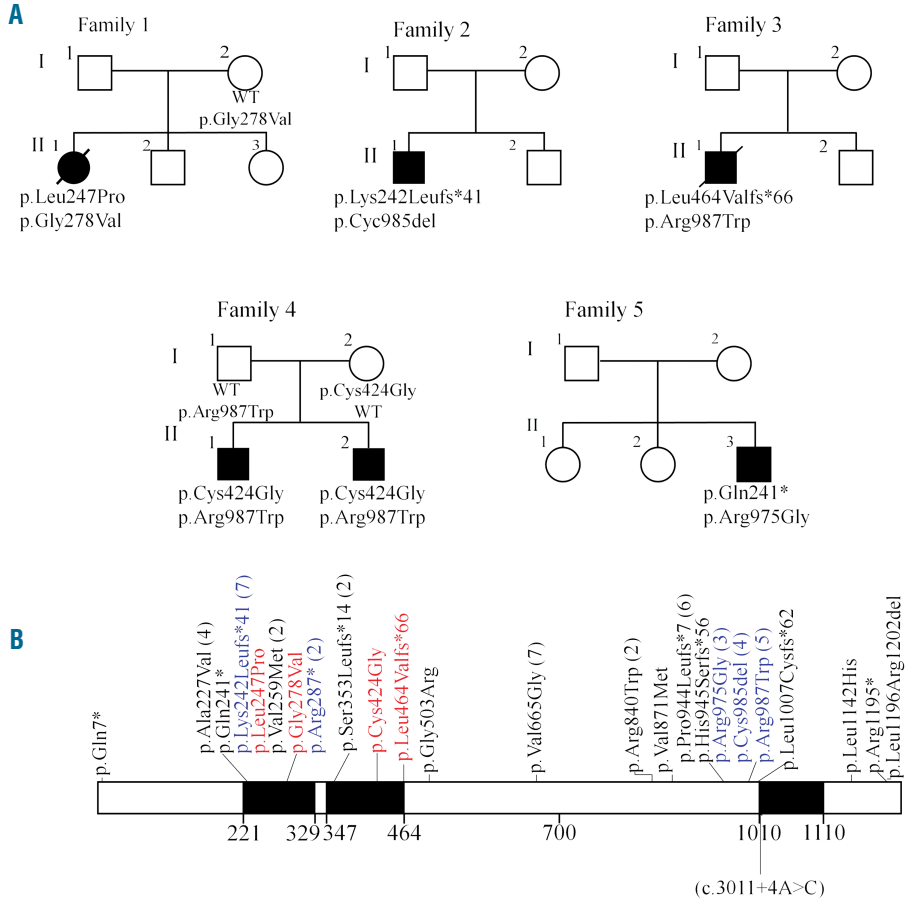

Figure 1. Segregation and relative position of mutations in *CTC1* in patients with DC and associated phenotypes. (A) Pedigrees associated with *CTC1* mutations. Filled symbols indicate affected individuals. Identified mutations at the protein level are shown below each genotyped individual. "WT" denotes wild type with no mutation. (B) Schematic structure of CTC1 protein showing the relative location of all reported mutations. The black boxes show the predicted oligosaccharide-binding (OB) folds; the N-terminal 700aa region has been shown to be involved in DNA binding whereas the C-terminal region is involved in STN1 binding. (Number) indicates the number of families in which each mutation has been observed. Novel mutations identified in this study in red. Mutations seen in this study and previously reported in blue. Mutations seen in other reports in black.

Table 1. Summary of clinical features and mutation status in affected individuals with compound heterozygous CTC1 mutations.

Family	1	2	3	4	4	5*
Individual	II-1	II-1	II-1	II-1 13	II-2	II-3[9]
CTC1 mutation	*p.Leu247Pro* *p.Gly278Val*	*p.Lys242Leufs*41* *p.Cys985del*	*p.Leu464Valfs*66* *p.Arg987Trp*	*p.Cys424Gly* *p.Arg987Trp*	*p.Cys424Gly* *p.Arg987Trp*	*p.Gln241** *p.Arg975Gly*
Gender	F	M	M	M	M	M
Age at presentation	6 yrs	7 yrs	15 yrs	9 mo	12 mo	14 yrs
Current age (age at death)	(22 yrs)	12 yrs	(26 yrs)	29 yrs	25 yrs	24 yrs
In DCR	yes	yes	no	no	no	yes
Feature						
Abnormal skin pigmentation	+	+	+/–	–	–	+
Nail dystrophy	–	+	–	+/–	+/–	+
Oral leukoplakia	–	–	–	–	–	–
Bone marrow failure (age at 1st report)	Pancytopenia/ NSAA (12 yrs)	Leukopenia/ NSAA (8 yrs)	Pancytopenia/ NSAA (13 yrs)	–	Pancytopenia/ NSAA (12 yrs)	Pancytopenia/ NSAA (14 yrs)
Retinal abnormalities	–	–	+	+	+	+
Intracranial cysts or calcifications	+	–	+	+	+	–
Microcephaly	+	–	–	–	–	–
Developmental delay	+	–	–	–	–	+
Ataxia	+	–	–	+	+	–
Intrauterine growth retardation	+	–	–	+	+	+
Sparse hair	+	–	–	–	–	–
Premature graying	+	–	+	+	+	–
Skeletal abnormalities	-	–	+	+	+	+
Gastrointestinal abnormalities	-	–	+	–	–	–

*+ feature present; - feature absent; +/- some suggestion; F1-F5 families 1-5; mo: months; yrs: years; *Family previously reported with CTC1 mutations; DCR: Dyskeratosis Congenita Registry, patients within the DCR have sufficient features to be classified as DC; NSAA: non-severe aplastic anemia.*

after screening as he was an uncharacterized case of DC in our registry. No mutations were identified in *STN1* or *TEN1* in this analysis.

Clinical presentation

Mutations in *CTC1* are usually described in the context of CRMCC either with or without Coats retinopathy,[9,10] but more recently one patient presenting with DC has been reported.[19] Two patients (F1: II-1 and F2: II-1) in this study lacked retinopathy, whereas F2: II-1 and F5: II-3 had no reported brain abnormalities. These observations show that although most patients with *CTC1* mutations usually have retinal abnormalities and intracranial calcifications, there is a subset lacking either or both of these features (Table 1). These findings suggest biallelic CTC1 mutations produce a spectrum of phenotypes which could come under the heading of "CTC1-related diseases" rather than CRMCC or Coats retinopathy.

It is interesting that the only overlap between combinations of mutations seen in our patients and those previously reported is patient F2: II-1 and the DC patient seen by Keller *et al.*[19] for the mutations p.Lys242Leufs*41 and p.Cys985del. Although both patients present with DC, the patient in this study does not have retinal disease or intracranial calcifications. This suggests other factors affect the phenotype in addition to the *CTC1* status.

Reviewing all the literature detailing biallelic *CTC1* mutations, genetic analysis has been performed on 125 patients. Twenty-five different mutations have been identified in 30 patients from 26 families. Clustering of these mutations appears to be occurring between amino acids 227-287 (7 mutations) and 944-987 (5 mutations, Figure 1B). Twenty-six patients present with the CRMCC spectrum of disease whereas 4 present with a DC phenotype. One individual has been classified as both CRMCC disease spectrum and DC on the basis of its clinical presentation, whereas another with late onset intracranial calcification and retinopathy, amongst other characteristics, has been diagnosed as DC but could be viewed as a Coats plus variant. This emphasizes the degree of overlap between the two groups and highlights the difficulty of making an accurate clinical diagnosis.[20] The degree of retinopathy in patients with DC is different in our group compared with previously published accounts. Retinal changes were noted in 4 of 55 DC patients screened (7.4%) compared with 21% observed by Tsilou *et al.*[21] Overall, in all the previous reports of *CTC1* mutations, all patients except one had retinal abnormalities. In our study group, 8 of 73 patients had retinal abnormalities whereas only 3 patients with retinal abnormalities were found to have *CTC1* mutations. In our screening of 55 DC patients, mutations in *CTC1* are seen in less than 6% of this group. Overall these data suggest that any patient who presents with intracranial abnormalities either with or without retinal abnormalities should be screened for *CTC1* mutations regardless of their actual clinical diagnosis.

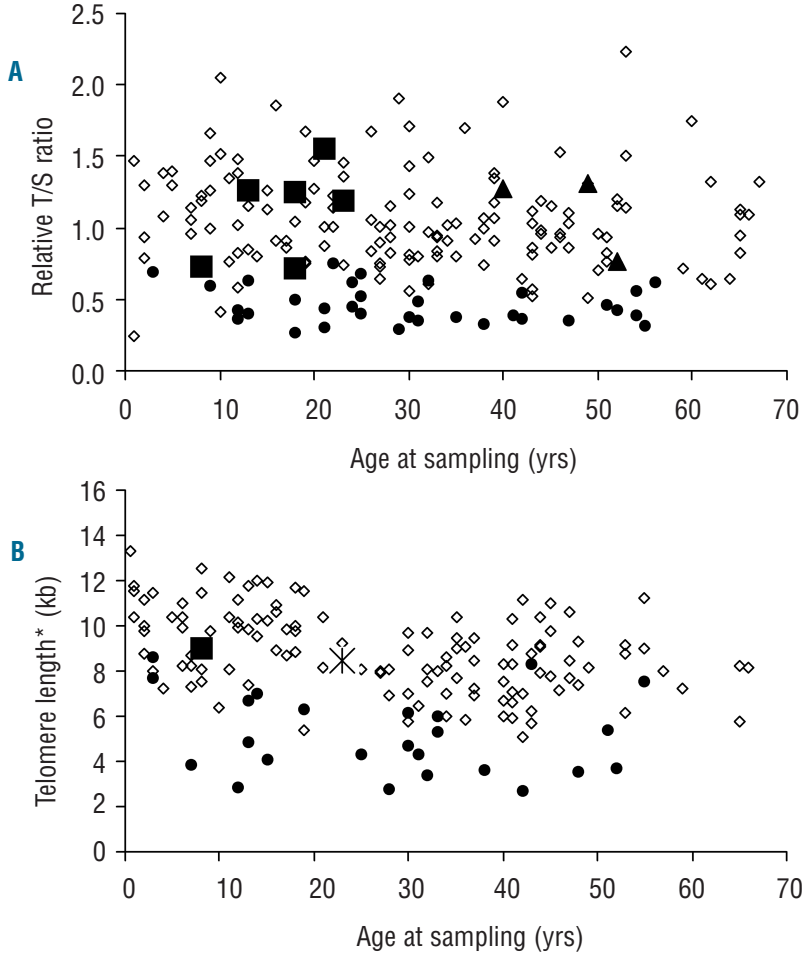

Figure 2. Telomere lengths are not significantly short in our patients with *CTC1* mutations as measured by two different methods. (A) Telomere lengths measured by MMqPCR expressed as a T/S ratio in bone marrow failure affected patients, heterozygous parents with *CTC1* mutations and control individuals. There was no significant difference when either of the sample groups was compared with controls, either as the whole control set or when segregated out to match according to age. Black squares indicate patients with biallelic *CTC1* mutations; black triangles heterozygous parents of patients with *CTC1* mutations; open diamonds controls; black circles patients with *TERC* mutations. (B) Telomere length measured by Southern blotting Genomic DNA was digested with *BamH1* and analyzed by Southern blot using a 0.75% agarose gel and the subtelomeric probe pTelBam8. [*] Telomere lengths were measured as the size of the fragment of peak signal intensity and adjusted to exclude the 7.8Kb of subtelomeric DNA. Sizes were determined with reference to the same standards run on each gel using Image Quant software. In both graphs, patients with known mutations in *TERC* are included as examples of short telomeres. Black square indicates patient F2: II-1; black star patient F3: II-1; open diamonds controls; black circles patients with *TERC* mutations.

Telomere lengths are not significantly reduced in patients with biallelic CTC1 mutations

Due to the recognized association of short telomeres in patients with DC/HH, and the disagreement between telomere lengths observed in CRMCC patients associated with *CTC1* mutations in the two main studies in the literature,[9,10] we measured telomere length using a quantitative PCR-based method. Telomere lengths were measured in the 6 patients and 3 parents with *CTC1* mutations as well as 143 controls. T/S ratios from 33 patients with known *TERC* mutations are included as examples of short telomeres (Figure 2A). In this study, there was no significant difference in the T/S ratios between patients and controls. In the three reports published, two different methods have been used to measure telomere lengths with contradictory results. Anderson *et al.*[9] and Keller *et al.*[19] used flow-FISH and showed that telomeres are below the 1st percentile which is considered by some to be diagnostic of DC.[22] Polvi *et al.* used a qPCR-based method, and showed there was no difference in telomere length in patients with *CTC1* mutations compared to controls; agreeing with our observations. The difference observed may be due in part to the different methods used,[23] but qPCR is an ideal method to use with archived samples with limited DNA quantity. From previous work we had telomere length data for F2: II-1 and F3: II-1 measured by Southern blotting using a pTelBam8 probe and again these 2 individuals did not have short telomeres compared with 124 controls or 24 patients with known *TERC* mutations (Figure 2B). F2: II-1 is the DC patient who has the identical mutations as the DC patient reported by Keller *et al.*[19] but in our patient the telomere lengths are within the normal range. This observation by a second method suggests that short telomeres are not a hallmark of patients with biallelic *CTC1* mutations. This is in contrast to the short telomeres that are observed by both methods in patients with heterozygous *TERC* mutations. Functionally the effects of *CTC1* mutations are not well understood. It has been shown that in a conditional knockout mouse model a null is initially viable and pathogenic features appear with age.[8] *Ctc*$^{-/-}$ mice have severe bone marrow failure just before death with a complete absence of trilineage hematopoiesis. This could account for the bone marrow failure observed in some patients with *CTC1* mutations. Its involvement in telomere length regulation is more complex. Gu *et al.*[8] suggest that CTC1 is involved in restarting stalled replication forks at telomeres thereby promoting efficient and complete replication but it is not involved directly in the protection of telomeres from adverse DNA damage response mechanisms. The differences seen in the mouse model compared with patients is that in the mouse the mutation is effectively homozygous and produces a severely truncated product. In patients, mutations are only observed as compound heterozygote, which suggest that a severe homozygous mutation would be non-viable. Furthermore, two truncat-

ing mutations are not observed in any patient to date suggesting such a combination would be non-viable.

In summary, we have shown in a large series of patients with DC and related bone marrow failure syndromes that compound heterozygous *CTC1* mutations are a rare cause of DC (<6% of uncharacterized DC cases; <2% of all cases in DCR) and related diseases and suggest there is no involvement of the other 2 members (TEN1 and STN1) of the CST complex in this group of diseases. The effect of *CTC1* mutations on telomere length is more variable than has been suggested previously. Patient phenotype is also more variable and this report highlights that intracranial and retinal abnormalities are not prerequisite features for the presence of mutations in *CTC1*.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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