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Brief report

Human telomere disease due to disruption of the CCAAT box of the *TERC* promoter

Anna M. Aalbers,[1-3] Sachiko Kajigaya,[1] Marry M. van den Heuvel-Eibrink,[2] Vincent H. J. van der Velden,[3] Rodrigo T. Calado,[1,4] and Neal S. Young[1]

[1]Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD; [2]Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam, The Netherlands; [3]Department of Immunology, Erasmus MC, Rotterdam, The Netherlands; and [4]Department of Internal Medicine, University of São Paulo at Ribeirão Preto Medical School, Ribeirão Preto, São Paulo, Brazil

Mutations in the coding region of telomerase complex genes can result in accelerated telomere attrition and human disease. Manifestations of telomere disease include the bone marrow failure syndromes dyskeratosis congenita and aplastic anemia, acute myeloid leukemia, liver cirrhosis, and pulmonary fibrosis. Here, we describe a mutation in the CCAAT box (GCAAT) of the *TERC* gene promoter in a family in which multiple members had typical features of telomeropathy. The genetic alteration in this critical regulatory sequence resulted in reduced reporter gene activity and absent binding of transcription factor NF-Y, likely responsible for reduced TERC levels, decreased telomerase activity, and short telomeres. This is the first description of a pathogenic mutation in the highly conserved CCAAT box and the first instance of a mutation in the promoter region of *TERC* producing a telomeropathy. We propose that current mutation-screening strategies should include gene promoter regions for the diagnosis of telomere diseases. This clinical trial was registered at www.clinicaltrials.gov as #NCT00071045. (*Blood*. 2012;119(13):3060-3063)

Introduction

Telomeres, the structures that cap the ends of linear chromosomes, consist in vertebrates of hundreds to thousands of TTAGGG repeats. To prevent critical telomere shortening, highly proliferative cells express telomerase (encoded by *TERT*), a reverse transcriptase that adds TTAGGG repeats to telomeres, with TERC as its RNA template. Constitutional loss-of-function mutations in telomerase complex genes result in deficient telomere maintenance and accelerated telomere attrition. Telomere disease in humans manifests clinically as a spectrum of the BM failure syndromes dyskeratosis congenita and aplastic anemia (AA), acute myeloid leukemia, cirrhosis, and pulmonary fibrosis.[1] Sequencing strategies for the diagnosis of telomere disease are based on screening exons and their flanking regions of telomerase genes for mutations. Pathogenic mutations in the promoter region of *TERT* or *TERC* have not been firmly established. Here, we report a mutation in the CCAAT box of the *TERC* promoter region that leads to telomere disease.

Methods

Patients and control subjects

Diagnosis of AA was performed as described previously.[2] Blood samples were collected after written informed consent was obtained in accordance with the Declaration of Helsinki, according to protocols approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute, protocol 04-H-0012 (www.clinicaltrials.gov #NCT00071045).

Sequence analysis and telomere length measurement

A *TERC* promoter/gene region (−1661 through +502; +1 is defined as the transcriptional start site of *TERC*) was amplified by PCR on leukocyte genomic DNA (10 ng), followed by bidirectional sequencing. A total of 378 people served as control subjects. Telomere length was measured as described previously.[3,4]

Gel shift assay

The gel shift assay was performed with the LightShift Chemiluminescent EMSA kit (Pierce) with unlabeled (competitors) or 5′-biotin end-labeled (probes) oligonucleotide duplexes (wild-type or mutant), which covered the *TERC*-CCAAT box and adjacent regions (nucleotide −63 through −39). Anti–NF-YA antibody (Rockland Immunochemicals) was used for supershift assay. DNA-protein complexes were analyzed by PAGE and the Chemiluminescent Nucleic Acid Detection Module (Pierce).

Plasmid construction, transfection, and luciferase activity assays

Plasmids carrying wild-type or mutant *TERC* promoter regions (starting at positions −798, −436, −272, −107, and −42; ending at a position +62) were constructed based on the pGL4.18[luc2P/Neo] luciferase vector (Promega). After plasmid transfection into HEK293T or HeLa cells (2.5×10^5), luciferase activity was measured with the Dual-Glo Luciferase Assay System (Promega).

RT-PCR for TERC expression

TERC expression levels were determined on total RNA isolated from PBMCs or skin fibroblasts with the RNeasy Mini Kit (QIAGEN). RT-PCR for actin and TERC was performed with the OneStep RT-PCR Kit (QIAGEN) and custom-made primers and probes.

Results and discussion

Telomere disease was suspected in our 39-year-old white index patient (III-3) who was diagnosed with AA and had a family history

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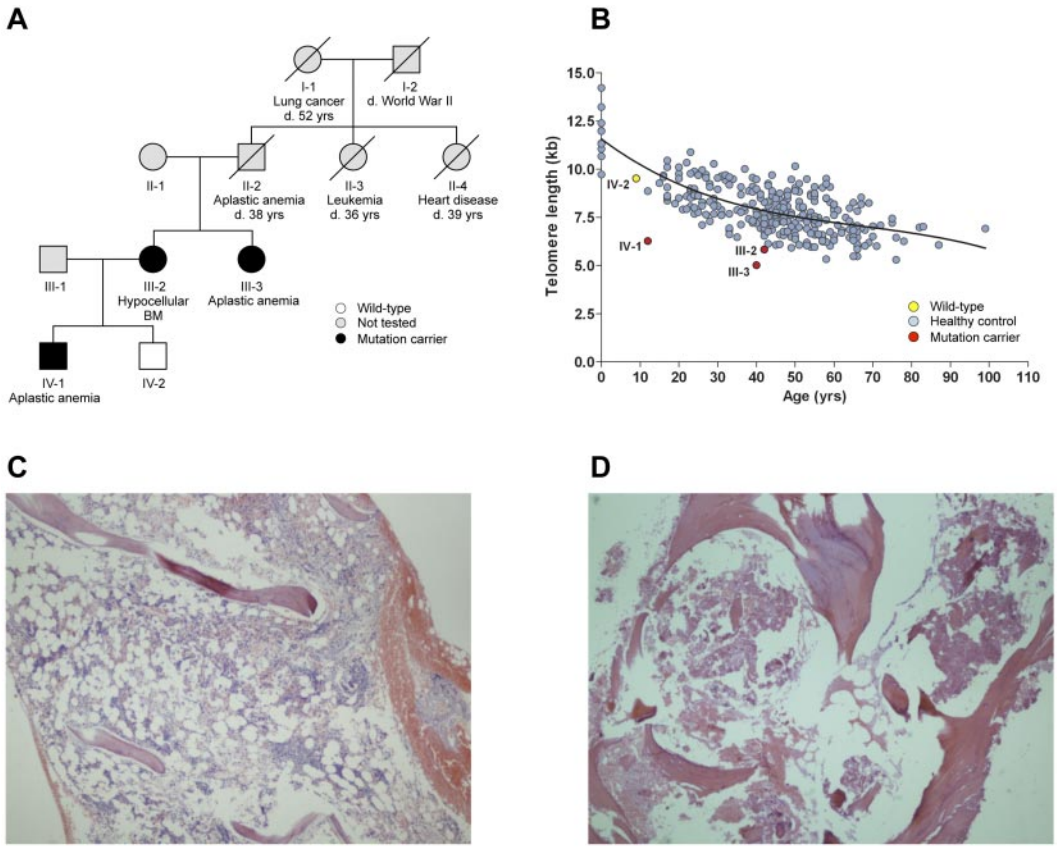

Figure 1. Pedigree, telomere length, and BM histology. (A) Pedigree of proband (III-3). Individuals II-2 and II-3 are suspected mutation carriers. Slashed symbols indicate deceased (d.) individuals. Neither the proband, her sister, or affected nephew showed abnormal pigmentation of the skin, nail dystrophy, or oral leukoplakia, nor was there evidence of pulmonary or immunologic problems, growth retardation, developmental delay, or microcephaly. During cholecystectomy, an enlarged liver was noticed in III-2; however, this finding was not further evaluated after surgery. (B) Blood leukocyte telomere length (in kilobases) as a function of age. Mutation carriers have very short telomeres in peripheral blood leukocytes. The curve marks the 50th percentile of telomere length for control subjects derived from 298 healthy National Institutes of Health blood bank donors. A sample's telomere length was expressed as a telomere to single-copy gene (T/S) ratio, which was converted to kilobases. BM biopsy showed profound hypocellularity in the proband (III-3; C) and hypocellular BM and eosinophilic ground substance in her sister (III-2; D).

of AA and leukemia (Figure 1A). Her peripheral blood leukocyte telomere length was 5.4 kb, which is very short compared with age-matched healthy control subjects (Figure 1B). Her sister (III-2), who had normal blood cell counts but a severely hypocellular BM (Figure 1C), and her nephew (IV-1), diagnosed with AA at age 11 years, also had very short leukocyte telomeres, but an unaffected nephew (IV-2) had normal telomere length (Figure 1B). Neither the proband, her sister, or affected nephew showed classic mucocutaneous stigmata of dyskeratosis congenita. No mutations were found in the coding region of *TERC*, *TERT,* or exon 6a of *TINF2*. However, the proband, her sister, and her affected nephew, but not the unaffected nephew, carried a heterozygous mutation in the CCAAT box positioned at −58 to −54 (CCAAT > GCAAT) of the *TERC* core promoter region. The mutation was absent in 378 healthy subjects of various ethnic backgrounds. A −714 C insertion, present in 16.7% of healthy control subjects,[5] was found in the index patient, her sister, and her affected nephew on the same allele as the −58C>G. The remaining upstream region of the *TERC* promoter was wild type to base −1661.

The CCAAT box is frequently present in promoter regions of RNA polymerase II–transcribed genes, located preferentially −60 to −100 nucleotides from the transcriptional start site.[6] NF-Y, a nuclear protein composed of NF-YA, NF-YB, and NF-YC, binds with high affinity to the CCAAT box.[7] Binding of NF-Y to the CCAAT box of the *TERC* promoter region is crucial for *TERC* promoter activity.[8,9]

In humans, a few CCAAT box mutations have been reported, but none that clearly cause disease. In hereditary persistence of fetal hemoglobin, a benign condition that benefits sickle cell anemia and β-thalassemia patients, mutations in the CCAAT boxes of the *HBG1* and *HBG2* promoters have been reported.[10-13] In β-thalassemia intermedia, a mutation in the CCAAT box of the *HBB* promoter was described, but no meaningful functional data supported the hypothesis that this mutation was pathogenic.[14]

A *TERC* promoter mutation (−99C>G) was present in a patient with paroxysmal nocturnal hemoglobinuria[15] and in a patient with myelodysplastic syndrome.[16] Telomere lengths in these patients were not reported, and functional analyses were inconclusive. The paroxysmal nocturnal hemoglobinuria and myelodysplastic syndrome phenotypes were not explained by this mutation.

To elucidate the effect of the −58C>G mutation in the CCAAT box on *TERC* promoter function, we performed gel shift and reporter gene assays and determined TERC expression in primary cells. The gel shift assay showed a shifted band of wild-type biotinylated probe, which spanned bases −63 through −39 of the *TERC* promoter region; addition of anti-NF-YA antibody resulted in supershift of the band. Mutant probe did not bind to NF-Y (Figure 2A). Although wild-type unlabeled oligonucleotide outcompeted binding of wild-type probe to NF-Y, mutant unlabeled oligonucleotide did not (Figure 2B). To investigate the effect of loss of NF-Y binding to the mutant box on *TERC* promoter activity, we generated wild-type and mutant *TERC* promoter–luciferase

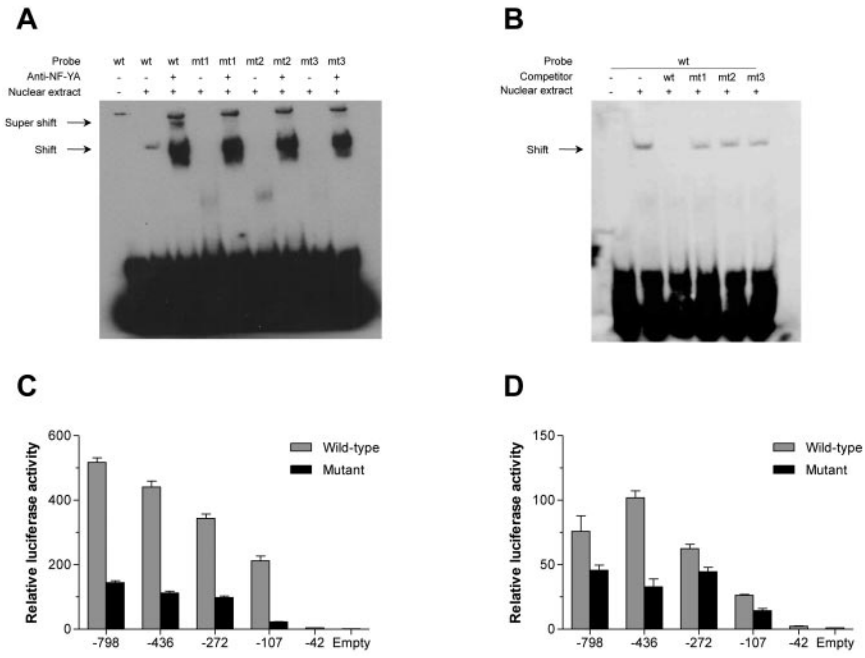

Figure 2. Functional analysis of wild-type and mutant CCAAT boxes of the *TERC* promoter. (A) Gel shift and supershift assays. Gel shift assay was performed with HeLa nuclear extract with wild-type probe (wt, 5′-Bio/cttggccaatccgtgcggtcgg-3′), a mutant probe (mt1, 5′-Bio/cttgg**g**caatccgtgcggtcgg-3′), and additional mutant control probes (mt2, 5′-Bio/cttgg**agtc**tccgtgcggtcgg-3′; mt3, 5′-Bio/cttggcca**tt**ccgtgcggtcgg-3′); bold and underlined letters indicate mutated nucleotides. Anti-NF-YA antibody was used for supershift assay. Arrows show bands that were shifted and supershifted with the wt probe but not with the mt1 probe (−58C>G) or additional mutant control probes (mt2 and mt3). (B) A 200-fold molar excess of unlabeled mutant competitor (mt1, mt2, or mt3) did not compete with wild-type binding, whereas wild-type competitor did (indicated with an arrow). Mutant promoter activity in HEK293T (C) or HeLa cells (D) was reduced compared with wild-type in reporter gene assays. Depicted on the x-axis is the 5′ position from the transcriptional start site of *TERC* for each *TERC* promoter-luciferase construct; each construct ended at nucleotide +69. Luciferase activity is reported as relative fold increase compared with the empty vector pGL4.18[luc2P/Neo] (given an arbitrary value of 1), normalized to protein concentration. Results shown are means of 3 (HEK293T) or 1 (HeLa) independent experiment performed in duplicate. Error bars indicate SEM. Detailed methods that include sequences of primers and probes used in the present study will be provided on request.

reporter plasmids of different lengths and transiently transfected HEK293T cells with these constructs. The relative luciferase activity of mutant constructs was decreased 3.5- to 9-fold compared with the wild-type construct (Figure 2C). Transfection of HeLa cells resulted in comparable patterns (Figure 2D). Cotransfection of wild-type and mutant constructs in a 1:1 ratio in HEK293T cells reduced luciferase activity by approximately 2-fold compared with the wild-type construct alone (data not shown). TERC expression was determined by RT-PCR in PBMCs and was lower in affected individuals III-2 and IV-1 than in unaffected individual IV-2 (TERC/actin relative expression ± SEM: 0.00536 ± 0.00130, 0.619 ± 0.107, and 1.00 ± 0.232, respectively). TERC expression also was lower in skin fibroblasts from the proband than in an unrelated healthy subject (0.781 ± 0.0517 and 1.00 ± 0.0402, respectively). However, these results should be interpreted cautiously, because TERC expression is highly variable within individuals.[17]

In conclusion, we show absent binding of NF-Y and reduced reporter gene activity with a mutant CCAAT box (GCAAT) in vitro, which suggests that the CCAAT-box disruption may lead to reduced TERC levels, lower telomerase activity, short telomeres, and human telomere disease. We believe that the terms *telomere disease* or *telomeropathy* are more adequate than *dyskeratosis congenita* to describe the phenotype observed in this family. First, these terms are descriptive of the underlying molecular defect. Second, telomeropathies are a large spectrum of phenotypes, from no clinical manifestations to macrocytosis, AA, pulmonary fibrosis, or the more severe phenotype in infancy Revesz syndrome, all with different prognoses. We prefer to reserve the term *dyskeratosis congenita* for patients with the classic clinical presentation and not for all subjects with a telomerase mutation.

We provide the first example of a *TERC* promoter mutation producing a telomeropathy and the first instance of a mutation in a CCAAT box that is causative in human disease. Mutation-screening strategies for the diagnosis of telomere diseases should include promoter regions of major genes related to telomere biology. Similar strategies may also be helpful for marrow failure syndromes such as Diamond-Blackfan anemia or Shwachman-Diamond syndrome, because with current approaches, a mutation can be identified only in approximately half of inherited marrow failure cases.[18]

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Authorship

Contribution: A.M.A., S.K., R.T.C., and N.S.Y. conceived and designed the experiments, analyzed the data, and wrote the paper; A.M.A., S.K., and R.T.C. performed the experiments; and M.M.v.d.H.E., V.H.J.v.d.V., R.T.C., and N.S.Y. contributed reagents, materials and analysis tools.

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Correspondence: Neal S. Young, MD, 10 Center Dr, Bldg 10-CRC/Room 3-5140, Hematology Branch, NHLBI, NIH, Bethesda, MD 20892; e-mail: youngns@mail.nih.gov.

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