Blood First Edition Paper, prepublished online February 8, 2012; DOI 10.1182/blood-2011-10-383182

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

Anna M. Aalbers[1,2,3], 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, USA
[2]Department of Pediatric Oncology/Hematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands
[3]Department of Immunology, Erasmus MC, Rotterdam, The Netherlands
[4]Present affiliation: Department of Internal Medicine, University of São Paulo at Ribeirão Preto Medical School, Ribeirão Preto, SP, Brazil

Running head: *TERC* promoter mutation causes telomeropathy
Section designation: Brief Report
Scientific category: Hematopoiesis and stem cells

Corresponding Author:
Neal S. Young, MD

10 Center Drive
Bldg 10-CRC/Rm 3-5140
Hematology Branch, NHLBI, NIH
Bethesda, MD 20892, USA

Phone: (301) 496-5093
Fax: (301) 496-8396
Email: youngns@mail.nih.gov

Abstract

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 include gene promoter regions for the diagnosis of telomere diseases. This clinical trial was registered at www.ClinicalTrials.gov, identifier: NCT00071045.

Introduction

Telomeres, the structures capping 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, using 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 bone marrow (BM) failure syndromes dyskeratosis congenita (DC) 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, leading to telomere disease.

Methods

Patients and controls

Diagnosis of AA was performed as previously described.[2] Blood samples were collected after written informed consent in accordance with the Declaration of Helsinki, according to protocols approved by the IRB of the National Heart, Lung, and Blood Institute, protocol 04-H-0012 (www.ClinicalTrials.gov identifier: 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 polymerase chain reaction (PCR) on leukocyte-genomic DNA (10 ng), followed by bi-directional sequencing. A total of 378 individuals served as controls. Telomere length was measured as described previously.[3,4]

Gel shift assay

The gel shift assay was performed using the LightShift Chemiluminescent EMSA kit (Pierce) with unlabeled (competitors) or 5'biotin end-labeled (probes) oligonucleotide duplexes (wild-type or mutant), covering the hTERC-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 polyacrylamide gel electrophoresis 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 ($2.5x10^5$), luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega).

RT-PCR for TERC expression

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

Results and discussion

Telomere disease was suspected in our 39-year old Caucasian index patient (III-3) who was diagnosed with AA and had a family history of AA and leukemia (Figure 1A). Her peripheral blood leukocyte telomere length was 5.4 kb, which is very short in comparison to age-matched healthy controls (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, 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 muco-cutaneous stigmata of DC. 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 -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 controls,[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 clearly causing 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 (PNH)[15] and with myelodysplastic syndrome (MDS).[16] Telomere lengths in these patients were not reported, and functional analyses were inconclusive. The PNH and MDS 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, spanning 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). Whereas wild-type unlabeled oligonucleotide out-competed 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 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, as compared to the wild-type construct (Figure 2C). Transfection of HeLa cells resulted in comparable patterns (Figure 2D). Co-transfection of wild-type and mutant constructs in a 1:1 ratio in HEK293T cells reduced luciferase activity by about 2-fold as compared to 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 as compared to unaffected individual IV-2 (TERC/actin relative expression +/- SEM: 0.00536+/-0.00130, 0.619+/-0.107, 1.00+/-0.232, respectively). TERC expression also was lower in skin fibroblasts from the proband in comparison to an unrelated healthy subject (0.781+/-0.0517, 1.00+/-0.0402, respectively). However, these results should be cautiously interpreted, as 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, suggesting 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, aplastic anemia, pulmonary fibrosis, to the more severe phenotype in infancy Revesz syndrome, all with different prognoses. We prefer to use the term dyskeratosis congenita for patients with the classical 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 etiologic 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, as with current approaches only in around half of inherited marrow failure cases a mutation can be identified.[18]

Acknowledgements

The authors thank Mr. JE Decker and Dr. T Winkler for kindly providing TERC expression data in fibroblasts. This research was supported in part by the NIH (NHLBI) Intramural Research Program. AMA was supported by the KiKa Foundation, Amstelveen, The Netherlands, and the René Vogels Foundation, Oirschot, The Netherlands.

Authorship contributions

AMA, SK, RTC, NSY conceived and designed the experiments; AMA, SK, RTC performed the experiments; AMA, SK, RTC, NSY analyzed the data; RTC, NSY, VHJvdV, MMvdHE contributed reagents, materials and analysis tools; AMA, SK, RTC, NSY wrote the paper.

Disclosure of conflicts of interest

The authors have no conflicts of interest to declare.

References

1. Calado RT, Young NS. Telomere diseases. *N Engl J Med.* 2009;361(24):2353-2365.
2. Kaufman DW, Kelly JP, Levy M, Shapiro S. *The Drug Etiology of Agranulocytosis and Aplastic Anemia*. New York: Oxford University Press; 1991.
3. Scheinberg P, Cooper JN, Sloand EM, et al. Association of telomere length of peripheral blood leukocytes with hematopoietic relapse, malignant transformation, and survival in severe aplastic anemia. *JAMA.* 2010;304(12):1358-1364.
4. Cawthon RM. Telomere measurement by quantitative PCR. *Nucleic Acids Res.* 2002;30(10):e47.
5. Takeuchi J, Ly H, Yamaguchi H, et al. Identification and functional characterization of novel telomerase variant alleles in Japanese patients with bone-marrow failure syndromes. *Blood Cells Mol Dis.* 2008;40(2):185-191.
6. Dolfini D, Zambelli F, Pavesi G, Mantovani R. A perspective of promoter architecture from the CCAAT box. *Cell Cycle.* 2009;8(24):4127-4137.
7. Mantovani R. A survey of 178 NF-Y binding CCAAT boxes. *Nucleic Acids Res.* 1998;26(5):1135-1143.
8. Zhao JQ, Glasspool RM, Hoare SF, et al. Activation of telomerase rna gene promoter activity by NF-Y, Sp1, and the retinoblastoma protein and repression by Sp3. *Neoplasia.* 2000;2(6):531-539.
9. Zhao J, Bilsland A, Hoare SF, Keith WN. Involvement of NF-Y and Sp1 binding sequences in basal transcription of the human telomerase RNA gene. *FEBS Lett.* 2003;536(1-3):111-119.
10. Oner R, Kutlar F, Gu LH, Huisman TH. The Georgia type of nondeletional hereditary persistence of fetal hemoglobin has a C---T mutation at nucleotide-114 of the A gamma-globin gene. *Blood.* 1991;77(5):1124-1125.
11. Fucharoen S, Shimizu K, Fukumaki Y. A novel C-T transition within the distal CCAAT motif of the G gamma-globin gene in the Japanese HPFH: implication of factor binding in elevated fetal globin expression. *Nucleic Acids Res.* 1990;18(17):5245-5253.
12. Zertal-Zidani S, Merghoub T, Ducrocq R, et al. A novel C-->A transversion within the distal CCAAT motif of the Ggamma-globin gene in the Algerian Ggammabeta+-hereditary persistence of fetal hemoglobin. *Hemoglobin.* 1999;23(2):159-169.

13. Gilman JG, Mishima N, Wen XJ, et al. Distal CCAAT box deletion in the A gamma globin gene of two black adolescents with elevated fetal A gamma globin. *Nucleic Acids Res.* 1988;16(22):10635-10642.

14. Chen XW, Mo QH, Li Q, Zeng R, Xu XM. A novel mutation of -73(A-->T) in the CCAAT box of the beta-globin gene identified in a patient with the mild beta-thalassemia intermedia. *Ann Hematol.* 2007;86(9):653-657.

15. Keith WN, Vulliamy T, Zhao J, et al. A mutation in a functional Sp1 binding site of the telomerase RNA gene (hTERC) promoter in a patient with Paroxysmal Nocturnal Haemoglobinuria. *BMC Blood Disord.* 2004;4(1):3.

16. Ortmann CA, Niemeyer CM, Wawer A, et al. TERC mutations in children with refractory cytopenia. *Haematologica.* 2006;91(5):707-708.

17. Marrone A, Walne A, Tamary H, et al. Telomerase reverse-transcriptase homozygous mutations in autosomal recessive dyskeratosis congenita and Hoyeraal-Hreidarsson syndrome. *Blood.* 2007;110(13):4198-4205.

18. Tsangaris E, Klaassen R, Fernandez CV, et al. Genetic analysis of inherited bone marrow failure syndromes from one prospective, comprehensive and population-based cohort and identification of novel mutations. *J Med Genet.* 2011;48(9):618-628.

Figure Legends

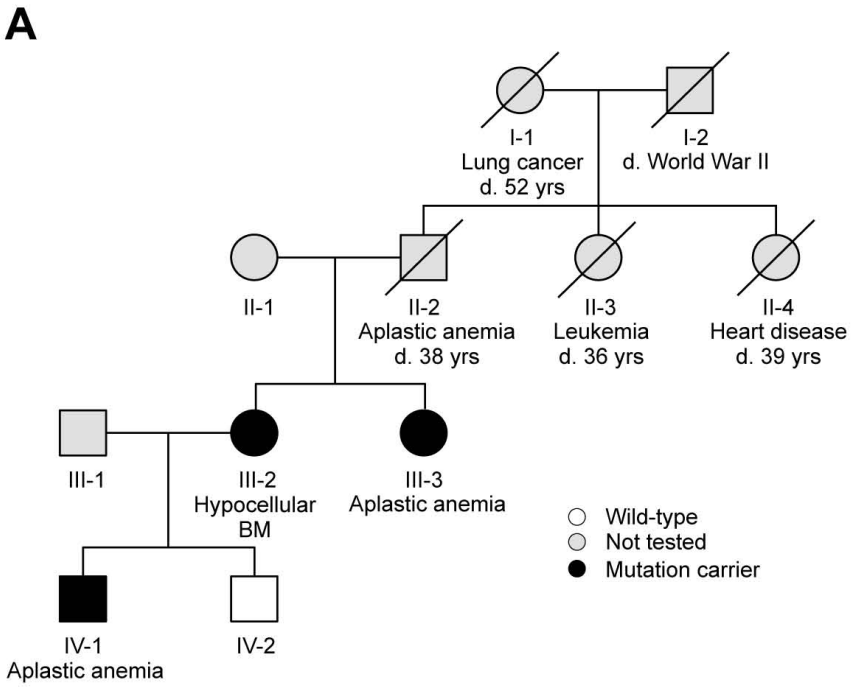
Figure 1. Pedigree, telomere length, and BM histology.

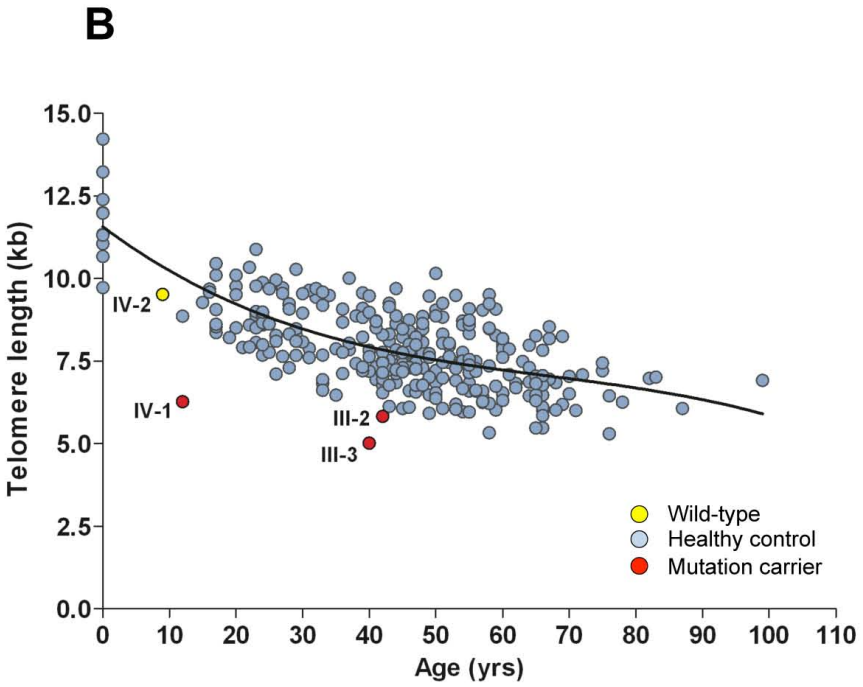
(A) Pedigree of proband (III-3; indicated by an arrow). Individuals II-2 and II-3 are suspected mutation carriers. Slashed symbols indicate deceased (d.) individuals. Neither the proband, her sister, or affected nephew showed abnormal pigmentation of the skin, nail dystrophy, or oral leukoplakia, nor was there evidence of pulmonary or immunological 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 (kb) as a function of age. Mutation carriers have very short telomeres in peripheral blood leukocytes. The curve marks the 50^{th} percentile of telomere length for control subjects derived from 298 healthy NIH 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 (kb). BM biopsy showing profound hypocellularity in the proband (III-3) (C), and hypocellular BM and eosinophilic ground substance in her sister (III-2) (D).

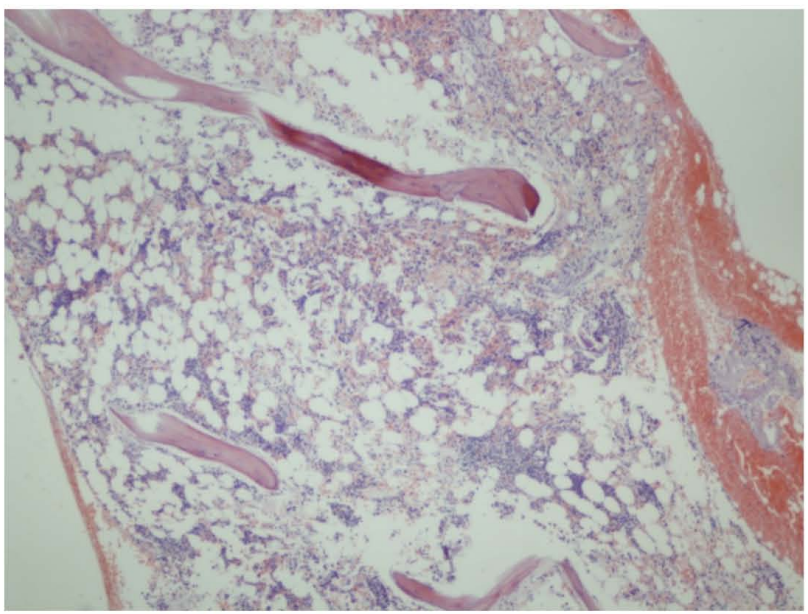
Figure 2. Functional analysis of wild-type and mutant CCAAT boxes of the *TERC* promoter.

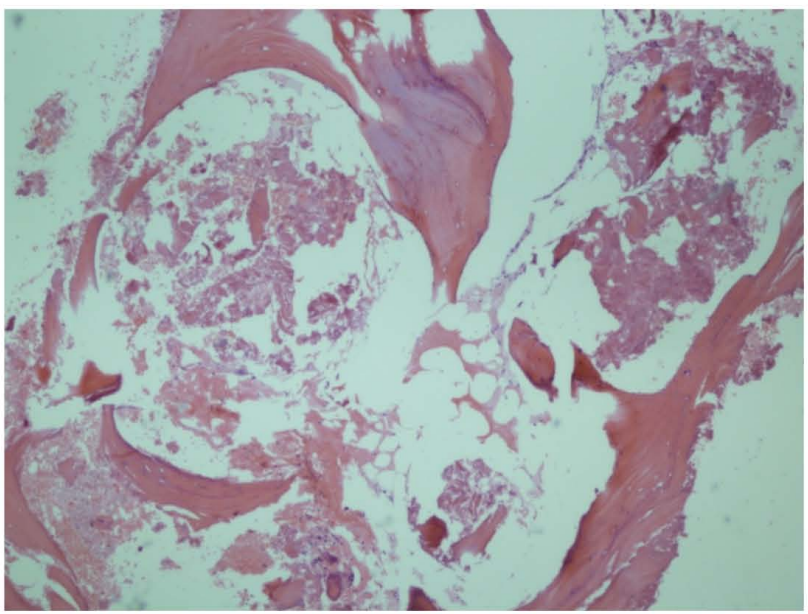
(A) Gel shift and supershift assays. Gel shift assay was performed using HeLa nuclear extract with wild-type probe (wt, 5'-Bio/cttggccaatccgtgcggtcgg-3'), a mutant probe (mt1, 5'-Bio/cttgg**g**caatccgtgcggtcgg-3'), and additional mutant control probes (mt2, 5'-Bio/cttgg**agtc**tccgtgcggtcgg-3'; mt3, 5'-Bio/cttggcca**t**tccgtgcggtcgg-3'): bold and underlined letters indicate mutated nucleotides. Anti-NF-YA antibody was used for supershift assay. Arrows show bands which are shifted and supershifted with the wild-type probe, but not with the mt1 probe (-58C>G) and additional mutant control probes (mt2 and mt3). (B) 200-fold molar excess of unlabeled mutant competitor (mt1, mt2, or mt3) does not compete with wild-type binding, whereas wild-type competitor does (indicated with an arrow). Mutant promoter activity in HEK293T (C) or HeLa cells (D) is reduced as compared to 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 ends 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 three (HEK293T) or one (HeLa) independent experiment(s) performed in duplicate. Error bars indicate standard error of the mean. Detailed methods including sequences of primers and probes used in this study will be provided upon request.

1

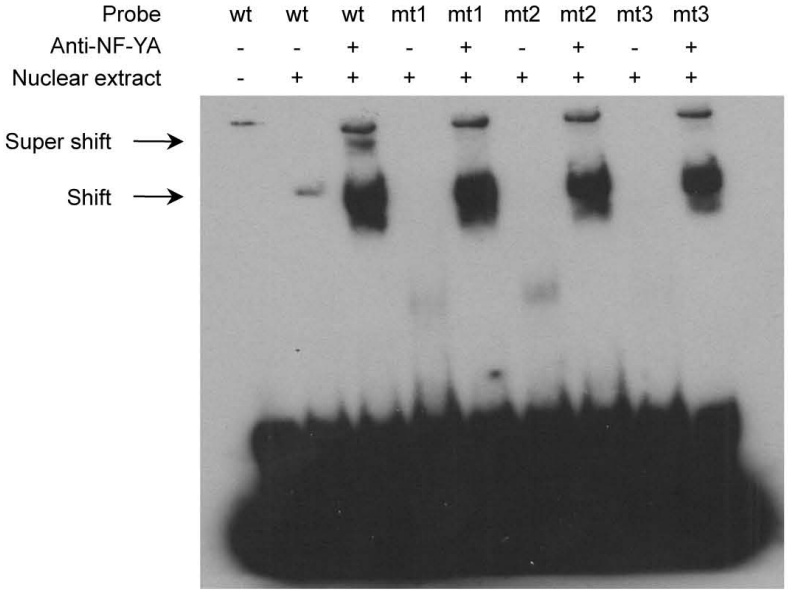

A
I-1
Lung cancer
d. 52 yrs
I-2
d. World War II
II-1
II-2
Aplastic anemia
d. 38 yrs
II-3
Leukemia
d. 36 yrs
II-4
Heart disease
d. 39 yrs
III-1
III-2
Hypocellular
BM
III-3
Aplastic anemia
IV-1
Aplastic anemia
IV-2
Wild-type
Not tested
Mutation carrier


B
Telomere length (kb)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0
10
20
30
40
50
60
70
80
90
100
110
Age (yrs)
IV-2
IV-1
III-2
III-3
Wild-type
Healthy control
Mutation carrier

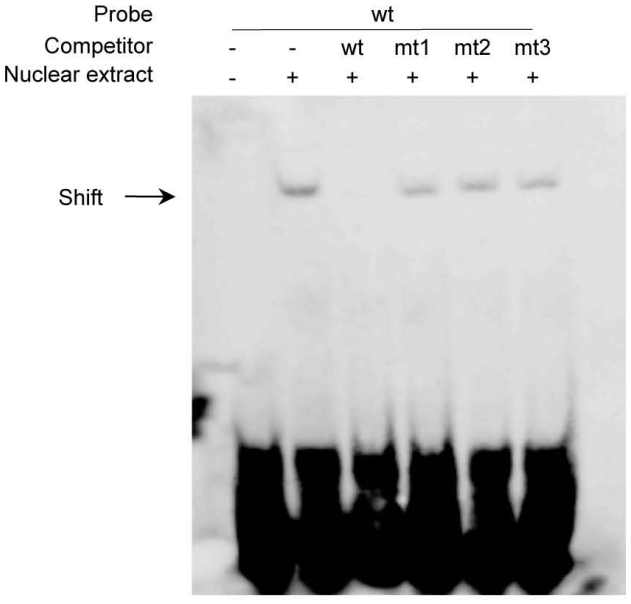

C


D

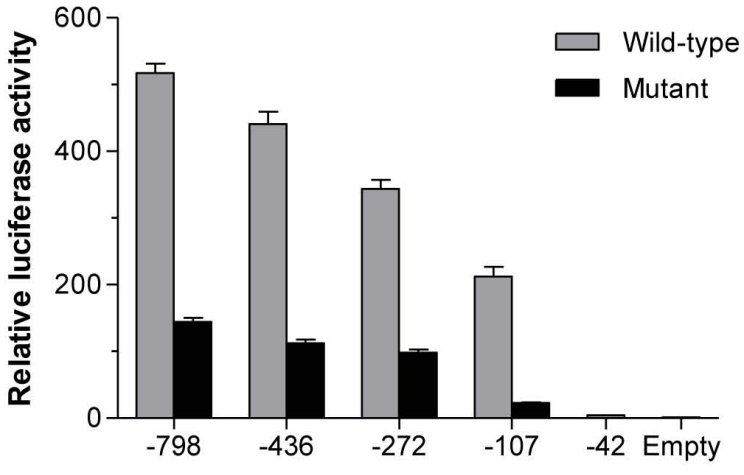
2 A


Probe wt wt wt mt1 mt1 mt2 mt2 mt3 mt3
Anti-NF-YA - - + - + - + - +
Nuclear extract - + + + + + + + +
Super shift
Shift

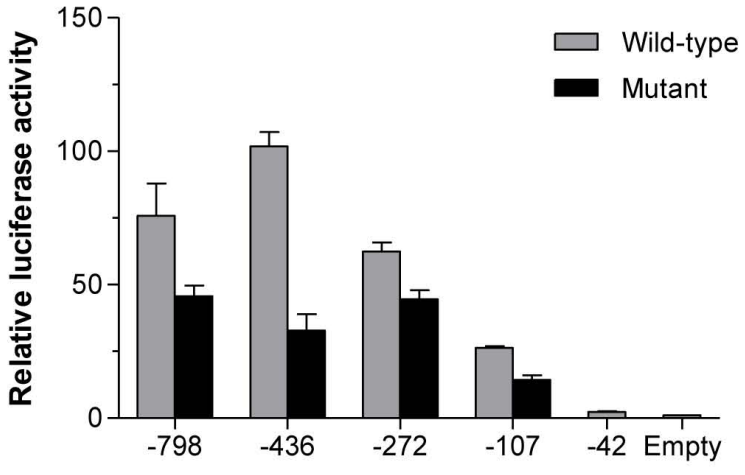
B


Probe wt
Competitor - - wt mt1 mt2 mt3
Nuclear extract - + + + + +
Shift

C


Relative luciferase activity
600
400
200
0
Wild-type
Mutant
-798
-436
-272
-107
-42
Empty

D


Relative luciferase activity
150
100
50
0
Wild-type
Mutant
-798
-436
-272
-107
-42
Empty