Constitutional hypomorphic telomerase mutations in patients with acute myeloid leukemia

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Loss-of-function mutations in telomerase complex genes can cause bone marrow failure, dyskeratosis congenita, and acquired aplastic anemia, both diseases that predispose to acute myeloid leukemia. Loss of telomerase function produces short telomeres, potentially resulting in chromosome recombination, end-to-end fusion, and recognition as damaged DNA. We investigated whether mutations in telomerase genes also occur in acute myeloid leukemia. We screened bone marrow samples from 133 consecutive patients with acute myeloid leukemia and 198 controls for variations in *TERT* and *TERC* genes. An additional 89 patients from a second cohort, selected based on cytogenetic status, and 528 controls were further examined for mutations. A third cohort of 372 patients and 384 controls were specifically tested for one *TERT* gene variant. In the first cohort, 11 patients carried missense *TERT* gene variants that were not present in controls ($P < 0.0001$); in the second cohort, *TERT* mutations were associated with trisomy 8 and inversion 16. Mutation germ-line origin was demonstrated in 5 patients from whom other tissues were available. Analysis of all 3 cohorts ($n = 594$) for the most common gene variant (A1062T) indicated a prevalence 3 times higher in patients than in controls ($n = 1,110$; $P = 0.0009$). Introduction of *TERT* mutants into telomerase-deficient cells resulted in loss of enzymatic activity by haploinsufficiency. Inherited mutations in *TERT* that reduce telomerase activity are risk factors for acute myeloid leukemia. We propose that short and dysfunctional telomeres limit normal stem cell proliferation and predispose for leukemia by selection of stem cells with defective DNA damage responses that are prone to genome instability.

risk factor | telomere | dyskeratosis congenita | cancer

Telomeres are complex structures capping the ends of all eukaryotic cell chromosomes. In vertebrates, telomeres consist of thousands of double-stranded tandem T_2AG_3 nucleotide repeats shielded by various proteins that seal the DNA structure (1). Telomeres are essential for genomic stability, as they protect chromosomes from recombination, end-to-end fusion, and recognition as damaged DNA (double-stranded breaks). With each cell division, telomeric repeats are lost because DNA polymerases are unable to fully duplicate the very ends of linear chromosomes—the "end replication problem" (2)—and also because of C-strand degradation. Loss of repeats eventually produces critically short, dysfunctional telomeres unable to adequately cap chromosome ends, resulting in limited proliferative capacity, cell senescence, apoptosis, and genomic instability (3). To prevent critical telomere loss and to preserve their genome, germ-line and stem cells, as well as the majority of cancer cells, express telomerase, a telomere-specific reverse transcriptase (TERT) that catalyzes the addition of telomeric repeats to G-strand 3′ end by using the telomerase RNA component (TERC) as template (1).

Mutations in genes encoding components of the telomerase complex result in deficient telomerase function and telomere erosion, and they predispose to specific human diseases (4). In dyskeratosis congenita (DKC), an inherited syndrome characterized by marrow failure and mucocutaneous abnormalities, mutations in telomerase complex components are etiologic. The dyskerin gene *DKC1*, encoding a protein that associates with telomerase, is mutated in X-linked DKC (5), and *TERC*, *TERT*, and the telomere-binding protein TIN2 are mutated in autosomal dominant DKC (6–8). An autosomal recessive form of DKC is associated with mutations in *TERT* and *NOP10*, the latter encoding another telomerase complex partner (4). Mutations in *TERC* and in *TERT* also are risk factors for developing acquired aplastic anemia (4, 9–11). Idiopathic pulmonary fibrosis also is associated with telomerase mutations (12, 13); as many as 20% of DKC patients develop pulmonary fibrosis (14).

A predisposition to cancer, including acute myeloid leukemia (AML), is a feature of DKC, and clonal hematopoietic disorders, especially myelodysplastic syndrome (MDS) and AML, evolve from acquired aplastic anemia (15). Telomerase-mutant aplastic anemia patients often have a family history of MDS and AML (4). In one family that we have studied, the proband had progressive acquired aplastic anemia and was found to be heterozygous for the loss-of-function K570N *TERT* gene mutation; his father, who also carried the mutation, died at age 33 from MDS rapidly evolving to AML (16). These clinical observations suggested to us that telomerase deficiency may contribute to the development of hematopoietic malignancy.

We investigated whether constitutional telomerase complex mutations predisposed to AML. AML is a heterogeneous malignant disease of hematopoietic progenitor cells, causing abnormal proliferation and deficient maturation of leukemic cells (17). That genomic instability plays a crucial role in leukemogenesis is indicated by the development of AML after exposure to cytotoxic chemotherapy and to ionizing radiation, and by the presence of abnormal karyotypes in about half of AML cases, including recurrent chromosomal abnormalities in about one-fifth of patients (17).

Results

Mutations. In the first cohort of 133 consecutive AML patients, we found 11 who carried a missense *TERT* gene variant (Table 1; Fig.

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Table 1. *TERT* gene variants resulting in amino acid changes in patients with acute myeloid leukemia and controls

Location of variation	Patients with acute myeloid leukemia		Controls	
	Consecutive samples, USP*, Brazil, $n = 133$	Selected samples MD Anderson, Texas, $n = 89$	Brazilian-matched controls, $n = 198$	Controls†, $n = 528$
Exon 1, codon 65 CCC/GCC (Pro/Ala)	1; 0 (0.004)‡	0	0	0
Exon 2, codon 299 GTG/ATG (Val/Met)	0	1; 0 (0.005)‡	0	0
Exon 2, codon 412 CAC/TAC (His/Tyr)	1; 0 (0.004)	0	0	0
Exon 2, codon 441 (Glu) deletion	0	0; 1 (0.005)	0	1; 0 (0.001)‡
Exon 2, codon 522 AGG/AAG (Arg/Lys)	1; 0 (0.004)	0	0	0
Exon 15, codon 1062 GCC/ACC (Ala/Thr)	7; 1 (0.034)	2; 0 (0.010)	0	7; 0 (0.007)
Total	10; 1 (0.045)	3; 1 (0.027)	0	8; 0 (0.008)
	13; 2 (0.034)		8; 0 (0.006)	

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†528 healthy controls were analyzed as previously reported (9) and consisted of 117 self-described Caucasians (94 from human variation panel HD100CAU and 23 from SNP500Cancer), 118 African ancestry (94 from human variation panel HD100aa and 24 from SNP500Cnacer), 24 self-described Asians (from SNP500Cancer), 23 Hispanic (from SNP500Cancer), and 246 anonymous Hispanic subjects (52% Peruvian, 28% Latin American, and 20% Pima and Maya Ameridians).
‡No. of heterozygotes; homozygotes (allele frequency).

1): One was homozygous and seven heterozygous for A1062T *TERT* gene variant, one patient was heterozygous for a unique P65A *TERT* gene mutation, one patient was heterozygous for H412Y, and one was heterozygous for a unique R522K *TERT* mutation. None of these genetic variants were found among 198 gender-, age-, and ethnicity-matched controls (Fisher's exact test, $P < 0.0001$). Silent SNPs and intronic SNPs showed similar allele frequencies in patients, their matched controls, our previously reported controls (9), and the SNP database from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/snp ref.cgi?locusId = 7015), indicating a comparable genetic background among these groups [supporting information (SI) Tables S1 and S2]. No *TERC* mutations were observed in either patients or controls. Germline origin of mutations was established by analysis of nonhematopoietic tissues obtained from biopsy specimens in patients B (transbronchial lung biopsy to diagnose pneumonia), D (fine-needle liver biopsy for posttransplant hepatic complications), and H (buccal mucosa biopsy for oral lesion investigation); for patient H, the mutation also was detected in a relative from which peripheral blood cells were already available. None of the *TERT*-mutant patients had physical signs suggestive of DKC.

Cytogenetic analysis was available for 7 of the 11 *TERT*-mutant patients and was abnormal in all of them (Table 2), including trisomy 8, inversion 16, t(15;17), and complex rare karyotypes. About 55% of patients in this cohort would be predicted to have an abnormal karyotype, based on cytogenetic results in large series of AML patients (17), including those from Brazil (18); therefore, we inferred that mutation status could be associated with abnormal cytogenetics and that short and dysfunctional telomeres caused by telomerase mutations might contribute to genomic instability. In particular, trisomy 8 is usually found in 9% and inversion 16 in only 7% of AML patients (17).

To investigate the possible association between telomerase mutations and abnormal karyotype, we examined an additional 89 AML patients from a second referral institution (MD Anderson Cancer Center), who were selected based on the presence of cytogenetic abnormalities (9) (see Table 1). Again, we found a significantly higher allele frequency of missense *TERT* gene variants in patients, in comparison to 528 healthy controls in whom we had previously sequenced *TERT* ($P = 0.028$). Patient L was heterozygous for a unique mutation in codon V299M, patient M was homozygous for a codon 441E deletion, and patients N and O were heterozygous for an A1062T *TERT* gene variant (see Table 2). There was a statistically significant association between mutation status and trisomy 8 ($P = 0.02$) and a marginal association with inversion 16 ($P = 0.08$). Germ-line origin of mutations also was confirmed in 2 patients (L and O) from whom remission bone marrow slides (less than 5% blast cells) were available for genetic analysis; the other 2 patients never achieved remission and no leukemia-free sample was available for testing.

As A1062T *TERT* was the most common gene variant among

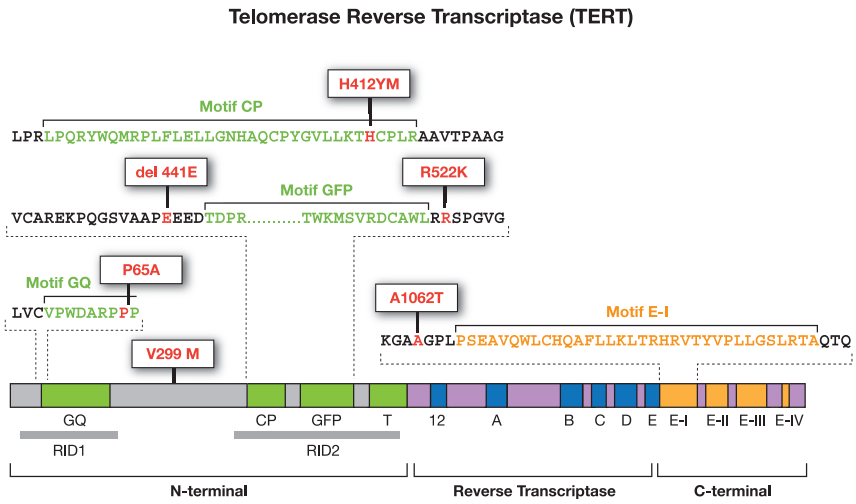

Fig. 1. *TERT* mutations in AML. Schematic domain structure of TERT, indicating 3 major regions: N-terminal, reverse transcriptase motifs, and C-terminal. RID denotes RNA-interaction domain and T telomerase-specific motif. Mutation codon locations and amino acid substitutions caused by mutations are shown. Abbreviations for amino acid residues: A, alanine; E, glutamic acid; H, histidine; K, lysine; M, methionine; R, arginine; T, threonine; V, valine.

Table 2. Clinical profile of acute myeloid leukemia patients carrying *TERT* gene variants

Patient	Mutation	Age, yrs	Sex	Ethnicity	FAB classification	Cytogenetics	Follow-up
First cohort of consecutive acute myeloid leukemia patients, USP, Brazil							
A	Codon P65A	21	F	White	M3	t(15;17)	Lost follow-up
B	Codon H412Y	35	M	White	M0	Inversion 16	Relapse 5 months after remission induction
C	Codon R522K	43	M	White	M0	Inversion 16	Death caused by infection before achieving recovery after remission induction
D	Codon A1062T (homozygous)	31	M	Mixed-race	M2	t(5;11)(q35;q13), del(10)(p15)	Refractory to remission induction; relapse after allogeneic BMT
E	Codon A1062T	39	M	White	M2	ins(2;?)(p11;?), del(4)(q25), del5(q13q33), del (7)(q22), trisomy 8	Refractory to remission induction
F	Codon A1062T	56	F	Mixed-race	M1	N.D.	Persistent pancytopenia after allogeneic BMT
G	Codon A1062T	60	F	White	M1	N.D.	No follow-up
H	Codon A1062T	51	M	White	M4	N.D.	Refractory to remission induction
I	Codon A1062T	45	F	White	M4	N.D.	No follow-up
J	Codon A1062T	N/A	F	White	M3	t(15;17)	Died from bleeding before therapy
K	Codon A1062T	26	F	White	M3	t(15;17)	Died from bleeding before therapy
Second cohort of selected acute myeloid leukemia patients, MD Anderson, Texas							
L	Codon V299M	22	M	Hispanic	M1	Inversion 16	In remission
M	Codon 441E deletion (homozygous)	72	F	White	Unknown	Trisomy 8; trisomy 13	Refractory to remission induction
N	Codon A1062T	69	M	White	M2	Trisomy 8	Refractory to remission induction
O	Codon A1062T	66	M	White	M4Eo	Inversion 16	In remission
Third cohort of selected acute myeloid leukemia patients, University of British Columbia Cancer Center, Vancouver							
P	Codon A1062T	35	M		M2	t(8;21)	In remission
Q	Codon A1062T	27	F		M3	t(15;17)	Relapse after 2 years and salvage with arsenic trioxide with auto hematopoietic SCT; in remission
R	Codon A1062T	44	M		M3	Normal	Allogeneic SCT in first relapse with second relapse within 90 days and death 4 months later
S	Codon A1062T	37	F		M4Eo	Inversion 16; trisomy 22	Allogeneic SCT in complete remission with secondary graft failure. Died after second allogenic SCT with multiorgan failure.
T	Codon A1062T	52	F		M1	Trisomy 8	Allogeneic SCT after relapse, in second complete remission
U	Codon A1062T	51	F		M5	Normal	In remission
V	Codon A1062T	28	F		M4	Normal	In remission
W	Codon A1062T (homozygous)	63	F		M3	t(15;17)	In remission
X	Codon A1062T	25	F		M3	t(15;17)	In remission
Y	Codon A1062T	63	M		Unknown	Normal	Died during induction therapy

BMT, bone marrow transplant; F, female; FAB, French-American British morphologic classification of myeloid neoplasms; M, male; M0, minimally differentiated AML; M1, AML without maturation; M2, AML with maturation; M3, acute promyelocytic leukemia; M4, acute myelomonocytic leukemia; M4Eo, acute myelomonocytic leukemia, eosinophilic variant; M5, acute monocytic leukemia; N/A, not available; N.D., not done; SCT, stem cell transplant; t, translocation.

AML patients and its allele frequency was 5 times higher in patients than in controls, we further screened 372 unselected AML patients from a third cohort diagnosed at the Leukemia/Bone Marrow Transplant Program (British Columbia Cancer Agency) specifically for the A1062T *TERT* gene variant. In this third cohort, the A1062T *TERT* allele was found in 10 patients: one was homozygous for this gene variant whereas the others were heterozygous. Among mutant patients, 6 had abnormal cytogenetic reports, including 1 with trisomy 8, 1 with inversion 16 and trisomy 22, 1 with t(8;21), and 3 (including the homozygous patient) with t(15;17). Trisomy 22 is usually found in only 3%, t(8;21) in 6%, and t(15;17) is found in 7% of AML cases (17). When the 3 cohorts were analyzed together ($n = 594$ patients), the A1062T *TERT* allele frequency was 3-fold higher in patients than in 1,110 controls ($P = 0.0009$) (Table 3).

Telomere Length and Telomerase Enzymatic Activity. Measurement of telomere length of *TERT*-mutant patients by Southern analysis indicated that telomeres were very short in most blast cells analyzed, independent of mutation status; mean telomere length was 3.1 kb (range, 2.4–5.9 kb) for mutant blast cells, slightly shorter that the mean observed for nonmutant blast cells (3.8; range 3.0–8.0 kb; $P = 0.10$; data not shown).

To test whether mutations decreased telomerase activity, vectors containing mutagenized *TERT* genes with AML-associated variants were transfected into WI38-VA13 cells along with *TERC*-containing vectors. The capacity of telomerase to elongate telomeres (telomerase enzymatic activity) was measured in transfected cell lysates. AML-associated *TERT* gene variants resulted in sig-

Table 3. Prevalence of A1062T *TERT* gene variant in acute myeloid leukemia patients and controls

	AML Patients, $n = 594$	Controls, $n = 1,110$	P value, χ-square test
Homozygous	2	0	
Heterozygous	18	13	
Minor allele frequency	0.019	0.006	0.0009

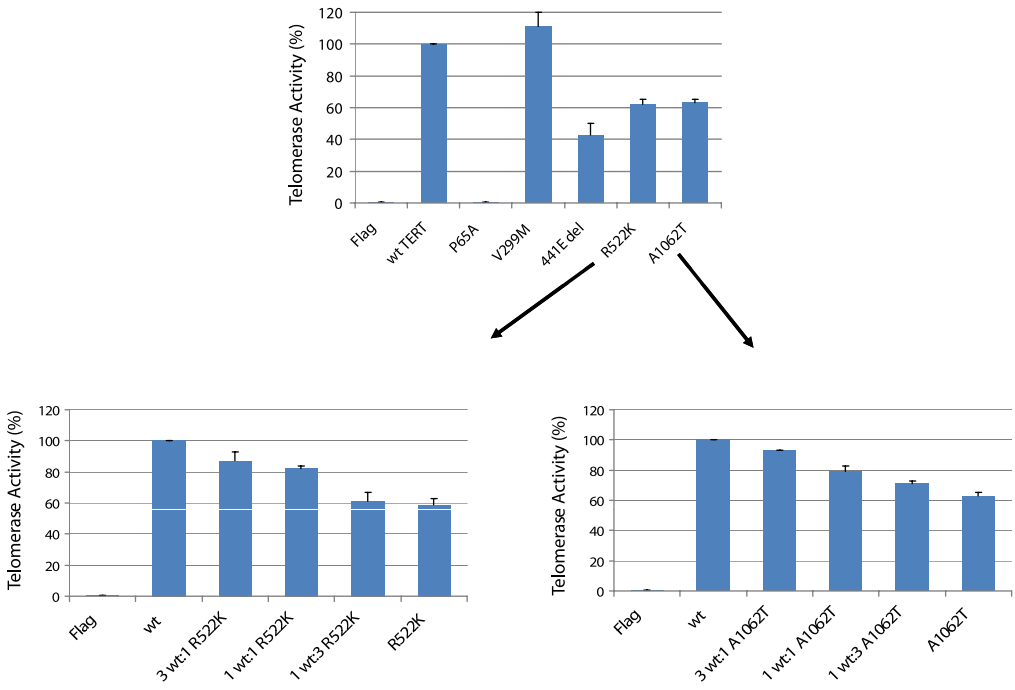

Fig. 2. Analysis of *TERT* gene variants' telomerase enzymatic activity. Telomerase activity of cell lysates made from reconstitution of the wild-type or mutated *TERT* expression vectors in the telomerase-negative VA13 cell line cotransfected with *TERC*-containing vector was measured by the fluorescent telomeric repeat-amplification protocol (TRAP) assay. Telomerase activity was considered 100% for the wild-type. Each bar (*blue*) for each vector represents a different transfection experiment and the bar on the top (*black*) denotes the standard deviation for all experiments for each vector. Telomerase activity in each experiment was corrected for *TERT* mRNA levels as measured by RT PCR. Cotransfection of wild-type *TERT* and mutant *TERT* (R552K *Bottom Left* and A1062T *Bottom Right*) at different ratios (indicated below bars) show that telomerase activity is reduced by haploinsufficiency.

nificantly reduced telomerase enzymatic function in comparison to wild-type *TERT* (Fig. 2). Cotransfection of mutant and wild-type *TERT* into VA13 cells at various ratios showed that mutations cause reduced telomerase activity by haploinsufficiency (see Fig. 2). In our previous study of 205 patients with apparently acquired aplastic anemia, two were found to be heterozygous for the *TERT* codon H412Y mutation and to have short-for-age telomeres (9). One of these patients had a family history of myelodysplasia. We also have previously demonstrated that this mutation reduces telomerase activity to ≈50% of wild-type *TERT*, also by haploinsufficiency (9). That *TERT* codon H412Y mutation is a hypomorphic variant impairing telomerase activity by haploinsufficiency has been reported by others in a family with DKC (19). In our experiments, the unique *TERT* codon V299M mutation did not appear to modulate telomerase's ability to elongate telomeres in vitro. However, this mutation is located in the N-terminal region, believed to be responsible for protein folding, and it is possible that telomerase interaction with its substrate or with other protein partners might be affected by this mutation. Telomerase also plays additional functions other than telomere elongation; for example, telomerase induces cell proliferation through transcriptional control of a Myc- and Wnt-related developmental program (20).

Discussion

We report association between constitutional telomerase gene hypomorphic variants and risk of developing AML. *TERT* abnormalities appeared to underlie leukemia in individuals with no clinical evidence of DKC or preceding bone marrow failure. Striking is the presence of 3 mutant homozygotes (two for A1062T and one for 441E del) among AML patients, whereas none was found in 1,110 control individuals. Based on the A1062T allele frequency observed in our 1,110 controls (0.006), the expected frequency for homozygosity is approximately 1 in 30,000. Hypomorphic *TERT* gene variants appear to have low penetrance and a wide spectrum of clinical phenotype, which may be partly explained if short telomeres rather than mutation status mediate disease development. Disease anticipation, in which clinical manifestations are more severe or appear at younger ages with succeeding generations, is observed in DKC as a result of inheritance of a deficient telomerase gene with a set of chromosomes with short telomeres (21). In addition, a few critically short or uncapped telomeres in a cell, rather than the average short telomere length per cell, may be responsible for triggering cell senescence (clinically translated into marrow failure) or genomic instability (clinically resulting in cancer). Homozygosity for *TERT* in some patients, heterozygous mutations in others, and the high prevalence of a hypomorphic polymorphism (whereas it is common that silent SNPs have similar frequencies between patients and controls), suggest a spectrum of genetic variation—a complex genomic "architecture" even in respect to the single *TERT* gene—by which variants occurring at different frequencies may have distinct differences in the effect that their contribution to the etiology of a disease.

Because most cancer cells over-express telomerase to maintain their proliferative capacity, that telomerase deficiency was associated with leukemia development may appear paradoxical. However, telomere attrition has been hypothesized as a molecular mechanism promoting genomic instability and predisposing to cancer development (22, 23). In vitro, extremely short telomeres can be recognized as DNA double-stranded breaks, activating the DNA-repair machinery. These ends then become the targets of nonhomologous end-joining or homology-directed repair, facilitating aneuploidy, inversions, translocations, and deletions. Homology-directed repair may recombine a telomere sequence from one chromosome with more centromeric sequences from another chromosome, producing inversions, translocations, and terminal deletions. Nonhomologous end-joining creates covalent bonds between chromosomes, disrupting proper chromosome segregation during mitosis and causing breakage-fusion-bridge cycles. Chromosomal rearrangements and aneuploidy are thought to play important roles in the early stages of leukemogenesis, and the detection of specific chromosomal rearrangements is clinically useful to monitor response to therapy, to detect minimal residual disease, and to predict relapse (17). Chromosomal abnormalities may not be sufficient for leukemia development, and additional genomic alterations and mutations undoubtedly accumulate during leukemia development, such as the contribution of activating FLT3 internal tandem duplications to inversion 16 leukemia (24). In the current work, an abnormal karyotype was present in 18 of 21 patients who were mutant in *TERT* (and in whom chromosomes were tested in a clinical laboratory), a much higher

proportion than expected by chance alone based on ≈50% frequency of abnormal cytogenetics in AML in general. However, the correlation of *TERT* mutations and cytogenetic findings will need to be confirmed in a larger study.

In telomerase-deficient animal models, telomere erosion contributes to genomic instability. Cells from late generation *mTerc*-null mice display chromosome ends lacking telomere repeats, aneuploidy, and chromosomal abnormalities, including end-to-end fusions (25). Telomere shortening in these mice also associates with increased rates of cancer and promotes nonreciprocal translocations and epithelial neoplasms (23). In budding yeast, recent data indicate that telomerase deficiency increases the rate of spontaneous translocations (26). In this model, absent telomerase function correlates with Rad52 accumulation in telomeres, decreasing the cell's ability to repair DNA double-stranded breaks in other areas of the genome, thus favoring homologous recombination. In human cancer cells, telomere dysfunction coincides with the progression of breast cancer from ductal hyperplasia to ductal carcinoma in situ (27). The length of peripheral blood lymphocytes' telomeres was shorter in patients with head and neck, lung, renal cell, and bladder cancers than in controls (28).

Telomerase-deficient mice with very short telomeres develop a variety of cancers, indicating that absent telomerase does not preclude tumor formation in mice (25). Telomerase activity also was found to be absent or low in at least half of AML cases, suggesting that leukemic cells may use telomerase-independent mechanisms to maintain their telomeres (29). Thus, reduced telomerase activity is not inconsistent with an indefinite proliferative capacity of leukemic cells.

We infer that constitutional hypomorphic telomerase mutations provide a genetic basis for a molecular mechanism of telomere shortening as a predisposition to human leukemia. However, other nongenetic causes for excessive telomere erosion also might contribute to genomic instability. Smoking, a known risk factor for leukemia, and even psychological stress are associated with telomere shortening (30). Iatrogenic causes, such as stem cell transplantation or chemotherapy, induce accelerated telomere attrition, and short telomeres after intensive chemotherapy have been associated with a greater risk of developing secondary myelodysplasia (31). Our results broaden the phenotypes associated with mutations in telomerase component genes and provide evidence that short telomeres in the hematopoietic compartment may contribute to genomic instability and predispose to leukemia.

Methods

Patients and Controls. Frozen bone marrow samples were obtained at time of diagnosis from 100 consecutive patients with AML (excluding acute promyelocytic leukemia) who were diagnosed at a single institution (Division of Hematology, University of São Paulo at Ribeirão Preto Medical School, Ribeirão Preto, São Paulo, Brazil) between February 2004 and March 2006. AML was diagnosed when patients presented with ≥20% bone marrow myeloid blasts, according to World Health Organization criteria (32). AML cases were further categorized according to the FAB classification (33–35). Forty-nine patients were male and 51 female; median age was 50 years, ranging from 2 to 86 years old. Eighty-two percent of patients self-reported as White, 5% as Black, and 13% as mixed-race. Peripheral blood samples from 198 age-, sex-, geographic-, and ethnicity-matched healthy volunteers (blood donors at the Centro Regional de Hemoterapia) also were obtained as controls. Samples were collected and frozen at the time of diagnosis as part of a blood and bone marrow sample bank regulated by protocols approved by the local ethics committee; informed consent was waived by the ethics committee, and use for genetic analysis was approved by both local and Brazilian National Ethics Committee in Research (Comissão Nacional de Ética em Pesquisa, CONEP process number 25000.098750/2006–38). DNA was extracted by using the DNeasy blood kit (Qiagen). A second group of 89 patients with AML, diagnosed at MD Anderson Cancer Center and selected based on cytogenetic abnormalities were further studied. Diagnostic criteria were similar. Samples were collected after informed consent was obtained, according to the local institutional review board. Fifty patients were male and 39 were females; median age was 55 years, ranging from 18 to 84 years. Seventy percent of patients self-reported as White, 13% as Black, and 17% as Hispanic. According to cytogenetic studies, 9 patients were diploid, 10 had trisomy 8, 12 had monosomy 7, 7 had both monosomy 5 and monosomy 7, 8 had monosomy 5, 11 had t(8;21), 23 had inversion 16, and 9 had $11q^-$. Frozen cell lysates were homogenized in DNA STAT-60, and DNA extracted according to the manufacturer's instructions (IsoTex Diagnostics, Inc.). Blood or bone marrow samples from a series of 372 newly diagnosed adult AML patients from the Leukemia/Bone Marrow Transplant Program of British Columbia were obtained after informed consent was obtained with approval of the Clinical Research Ethics Board of the University of British Columbia. Diagnostic criteria and cytogenetic analysis were as described for the previous cohorts. Peripheral blood samples from 384 healthy subjects were obtained as controls. Samples consisted of anonymized leftover peripheral blood samples (age and sex available only) from individuals/patients over the entire age range for blood clotting tests at the University of British Columbia Cancer Center. Individuals had to have normal clotting results and no other illnesses for their sample to be included, and presumably represent a cross section of the normal population in Vancouver.

Mutational Analysis. *TERT* sequencing was performed as previously described (9). Dot blot hybridization was performed as described previously (36). Briefly, PCR products were generated across exon 15 of hTERT from genomic DNA of AML patients and normal donors, and 3 to 100 ng per kilobase of each amplicon was spotted onto Hybond-N^+ membranes (Amersham) to produce 96-well format dot blots. Allele specific oligonucleotides were end-labeled with ^{32}P and hybridized sequentially to the dot blots in tetramethylammonium chloride (Sigma) hybridization solution at 53 °C. Following posthybridization washes, the dot blots were exposed to Kodak Blue-XB1 X-ray film.

Southern Blot for Telomere Length. Telomere length of blast cells was determined by analysis of terminal restriction fragments obtained by digestion of genomic DNA with *Hinf*I and RsaI restriction enzymes by using the *Telo*TAGG Telomere Length Assay (Roche Applied Science) following the manufacture's instructions. Briefly, one microgram of genomic DNA was digested at 37 °C overnight; DNA fragments were separated by 0.8% agarose gel electrophoresis and transferred to a nylon membrane by Southern blotting overnight. The blotted DNA was hybridized with a digoxigenin-labeled probe specific for telomeric sequences and incubated with anti-digoxigenin-specific antibody coupled with alkaline phosphatase and incubated with alkaline phosphatase highly sensitive chemiluminescent substrate. Average terminal restriction fragment lengths were determined by comparing to the molecular weight standard by using the ImageQuantTL digital analyzer (Amersham).

Vector Preparation. The mutations to encode *TERT* V299M, R522K, K570N, and A1062T were introduced into the pcDNA3-FLAG-TERT plasmid by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the Advantage-GC2 PCR kit (Clontech). Primers were designed by using the manufacturer's guidelines (see *SI Appendix*). Each 50-μl reaction contained 10-μl 5X GC2 PCR Buffer, 10-μl GC-Melt, 1-μl 50X GC2 Polymerase Mix, 5 nmol of each dNTP (Invitrogen), 1.25 ng of each primer, and 100-ng plasmid DNA. After amplification, DNA from each reaction was purified by using a PCR Purification Kit (Qiagen) following the manufacturer's instructions, except that DNA was eluted with 50-μl 1X New England Digestion Buffer 4 (New England Biolabs). To each eluate, 40 units DpnI (New England Biolabs) was added, and the reaction mixture was incubated at 37 °C for at least an 1 h. The plasmids were amplified in TOP-10 cells (Invitrogen) according to the manufacturer's protocol. Colonies were harvested and grown in liquid culture media (Luria-Bertani Broth) from which DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen). To sequence the insert, the DNA was amplified by using the Advantage-GC2 PCR Kit as described above, except 10 pmol of a forward and a reverse primer were used (see *SI Appendix*); the amplicon was purified by using ExcelaPure 96-Well UF PCR Purification plates (Edge BioSystems) and direct sequencing was performed with BigDye Terminator version 3.1 (Applied Biosystems). Sequencing products were purified in sephadex G-50 (Sigma-Aldrich) columns (0.45-μm, 96-filter plate, Pall Corporation) for excess dye extraction. Specific primers for sequencing were designed (see *SI Appendix*), and sequencing products were analyzed in an automated genetic-sequence analyzer (ABI Prism 3100, Applied Biosystems). Plasmids with only the desired nonsynonymous mutation were purified (HiSpeed Plasmid Maxi Kit, Qiagen), and the clone sequence was confirmed. All PCRs were performed in an MJ Research PTC-200 Thermocycler (Global Medical Instrumentation). For *TERT* codon P65A and 441E deletion, wild-type vector was mutagenized by Mutagenex, sequence was confirmed by direct sequencing of the whole insert, and plasmids were purified by using the HiSpeed Plasmid Maxi Kit.

pcDNA3-Flag-TERC was kindly provided by Dr. Hinh Ly (Emory University). The sequence was confirmed, the plasmid was transformed into TOP-10 cells, and purified by using the HiSpeed Plasmid Maxi Kit.

Transfection Assay. Two micrograms of pcDNA3-Flag-TERC and 2 μg of either pcDNA3-Flag, wild-type pcDNA3-Flag-TERT, or mutant (P65A, V299M, R522K, K570N, A1062T) pcDNA3-Flag-TERT DNA were transfected into telomerase-deficient WI-38 VA13 cells (ATCC) at 60% confluence in each well of 6-well polystyrene dishes by using Superfect Transfection Reagent (Qiagen), according to manufacturer's instructions. VA13 cells were plated the day before transfection and grown in RPMI 1640 media (Mediatech) containing 10% FBS and antibiotics. This cell line, derived from human-lung fibroblasts, is telomerase-negative and maintains telomeres by a recombination-based pathway called ALT (alternative lengthening of telomeres). In cotransfection studies, wild-type and mutant pcDNA3-Flag-TERT were used in ratios of 3:1, 1:1, and 1:3 with a total of 2-μg pcDNA-Flag-TERT (wild-type and mutant) for each well. Subsequent manipulations of the cell extracts were carried out as described previously (16). Briefly, cells were scraped off the dishes 48 h after transfection, washed 3 times in PBS, and lyzed with CHAPS buffer. Total cellular RNA was extracted with TRIzol reagent (Invitrogen), treated with DNaseI (Roche Molecular Biochemicals), and purified with TRIzol reagent. Transfections were carried out at least twice and in triplicates for each mutation.

TERT and TERC Expression by RT PCR. RNA concentrations were estimated by absorbance at 260 nm (Nanodrop). Expression of TERT, TERC, and β2-microglobulin (β2M) were assayed by RT PCR (TaqMan Gene Expression Assays, Applied Biosystems) according to the manufacturer's instructions with the exceptions listed below. A standard curve of 10-fold serial dilutions from 2.5×10^8 to 250 copies pGRN145-TERT (ATCC) or TERC cDNA was run in parallel with 50-ng RNA from each treatment group. For TERT, a primer and probe premix were used (TaqMan, Applied Biosystems). For TERC, each reaction contained 900 nM of each primer and 250 nM probe (see *SI Appendix*). To more accurately quantify the amount of amplification-capable RNA for each sample, expression of B2M was assessed. A standard curve of 10-fold serial dilutions from 10^6 to 1 copy pCMV6-XL5-B2M (Origene) and 50 ng of sample RNA (by A260) were run in parallel by using a probe and primer premix (Applied Biosystems). All standards and unknowns were run in triplicate, and each sample was run in the same position of the plate for measuring expression of TERT, TERC, and β2M. All plates were run in duplicate. A single PTC-200 Thermal Cycler (MJ Research) with a Chomo4 Fluorescence Plate Reader (MJ Research) was used for all of the aforementioned plates; the results were analyzed with MJ Opticon Monitor Analysis Software (Bio-Rad). All standard curves were linear (R^2>0.99); all nontemplate controls were negative; the pcDNA3-FLAG transfected control was negative for TERT and TERC expression, confirming that the VA13 cells were not expressing telomerase; and all experimental values fell within the linear range. TERT:β2M ratio for each well was calculated, and the normalized, mean ratio for each transfection group was used to correct telomerase activity for variation in transfection efficiency.

Protein Concentration. Protein concentrations were assayed in triplicate twice for each sample according to the DC Protein Assay (Bio-Rad) instructions on a Victor3 (PerkinElmer) except that absorbance was taken at 650 nm instead of 750 nm. A standard curve of 2, 1, 0.5, 0.25, and 0 mg/ml BSA in CHAPS was run in parallel with samples to be run on the same telomerase activity plate. All standard curves were linear ($R^2 > 0.99$), and all experimental values fell within the linear range.

Telomerase Activity by Telomerase Repeat Amplification Protocol. The fluorescent telomerase repeat amplification protocol (TRAPeze XL, Chemicon) was used to measure telomerase activity as described by the manufacturer. We used 250 ng of protein per unknown reaction; PCR was performed in a GeneAmp PCR System 9700 (PerkinElmer); and fluorescence was measured in a Victor3. Each standard, control, and unknown was run in triplicate. This procedure was performed twice for each transfection group each time the transfection was performed. All standard curves were linear ($R^2 > 0.95$) and all experimental values fell within the linear range. No negative controls, including CHAPS and RNA from the pcDNA3-FLAG-transfected group, showed more than 0.03% wild-type telomerase activity. Telomerase activity was corrected for the level of TERT expression in each transfection group.

Statistical Analysis. Differences in the frequencies of coding-sequence variations between samples from patients and those from controls were evaluated by means of Fisher's exact test. For A1062T *TERT* gene variant, because of the large number of individuals analyzed in both groups, differences in minor allele frequency between patients and controls were determined by χ-square test. Differences in Telomere lengths between mutant and nonmutant patients were compared by using the nonparametric Mann–Whitney *U* test and considering a *P* value <0.05 as statistically significant.

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