Mutations in TERT, the Gene for Telomerase Reverse Transcriptase, in Aplastic Anemia

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ABSTRACT

BACKGROUND
Mutations in TERC, the gene for the RNA component of telomerase, cause short telomeres in congenital aplastic anemia and in some cases of apparently acquired hematopoietic failure. We investigated whether mutations in genes for other components of telomerase also occur in aplastic anemia.

METHODS
We screened blood or marrow cells from 124 patients with apparently acquired aplastic anemia and 282 control subjects for sequence variations in the TERT, DKC1, NHP2, and NOP10 genes; an additional 81 patients and 246 controls were examined for genetic variations in TERT. Telomere lengths and the telomerase activity of peripheral-blood leukocytes were evaluated in patients carrying genetic variants. Identified mutations were transfected into telomerase-deficient cell lines to examine their effects and their mechanism of action on telomerase function.

RESULTS
Five heterozygous, nonsynonymous mutations (which cause an amino acid change in the corresponding protein) were identified in TERT, the gene for the telomerase reverse transcriptase catalytic enzyme, among seven unrelated patients. Leukocytes from these patients had short telomeres and low telomerase enzymatic activity. In three of these patients, the mutation was also detected in buccal mucosa cells. Family members carrying the mutations also had short telomeres and reduced telomerase activity but no evident hematologic abnormality. The results of coexpression of wild-type TERT and TERT with aplastic anemia–associated mutations in a telomerase-deficient cell line suggested that haploinsufficiency was the mechanism of telomere shortening due to TERT mutations.

CONCLUSIONS
Heterozygous mutations in the TERT gene impair telomerase activity by haploinsufficiency and may be risk factors for marrow failure.
IN APLASTIC ANEMIA, THE BONE MARROW contains very few hematopoietic cells and consists mainly of fat. The disease can be acquired or constitutional. In most acquired cases, the hematopoietic tissue is the target of an immune process dominated by oligoclonal expansion of type I cytotoxic T cells, which secrete interferon-γ and tumor necrosis factor α and cause hematopoietic cell death by apoptosis. Acquired aplastic anemia can be successfully treated by allogeneic bone marrow transplantation or immunosuppressive therapy.

Fanconi’s anemia and dyskeratosis congenita are the most common types of constitutional (congenital) aplastic anemia. X-linked dyskeratosis congenita (Online Mendelian Inheritance in Man [OMIM number 305000) is caused by mutations in the DKC1 gene, which encodes dyskerin, a small nucleolar ribonucleoprotein particle that associates with the telomerase complex. Involvement of this gene has implicated the telomere-repair complex in the pathophysiology of dyskeratosis congenita; indeed, cells from patients with this disease have strikingly short telomeres and low telomerase activity. Subsequently, mutations in the TERC gene, which encodes the RNA component of the telomerase complex, were identified in the autosomal dominant form of dyskeratosis congenita (OMIM number 127550).

Telomeres are structural elements that seal the ends of chromosomes, protecting them from recombination, end-to-end fusion, and recognition as damaged DNA. In human somatic cells, telomeres typically consist of more than 1000 tandem repeats of nucleotides (CCCTAA on one strand of DNA and TTAGGG in the other) and associated proteins. These repeats are gradually lost with cellular replication and aging, owing to the inability of DNA polymerase to fully replicate the 3’ end of DNA. The attrition of repeats eventually shortens telomeres critically; the result is arrested proliferation and senescence, shortened life span, apoptosis, or genomic instability of the cell. Maintenance of the integrity of telomeres requires the telomerase ribonucleoprotein complex, which consists of telomerase reverse transcriptase (TERT) and its integral RNA template (TERC), in addition to other proteins. TERT copies a short region of TERC into telomeric DNA to extend the 3’ end of the chromosome.

We and others have found short telomeres in leukocytes from approximately one third of patients with acquired aplastic anemia, especially those who do not have a response to immunosuppressive therapy. For this reason, we sought evidence of cryptic dyskeratosis congenita and TERC mutations in aplastic anemia. We discovered two families in which each proband had apparently acquired aplastic anemia. TERC mutations were present in the severely affected patients and multiple other family members, but physical stigmata of dyskeratosis congenita (abnormal skin pigmentation, nail dystrophy, and mucosal leukoplakia) were absent. Nevertheless, because TERC mutations are infrequent in acquired aplastic anemia, we hypothesized that mutations in genes corresponding to other components of the telomerase ribonucleoprotein complex could contribute to bone marrow failure.

METHODS

PATIENTS AND CONTROLS

Blood samples were obtained from 205 unrelated patients with apparently acquired aplastic anemia (age range, 2 to 83 years; median, 34) who were treated at a single institution (the Hematology Branch of the National Heart, Lung, and Blood Institute, National Institutes of Health). The diagnosis of aplastic anemia was based on the bone marrow and blood-count criteria of the International Agranulocytosis and Aplastic Anemia Study. The first group, consisting of 124 patients, was selected for study on the basis of one of the following: a lack of response to immunosuppressive therapy, a family history of hematologic abnormalities without physical anomalies characteristic of dyskeratosis congenita, or short telomeres in leukocytes, as previously observed. The second group consisted of 81 consecutive patients seen in the same clinic from January 2004 to July 2004 for evaluation and treatment of acquired aplastic anemia.

Of the 205 patients, 98 were female and 107 were male. Race or ethnic background, as reported by the patients or their guardians, was as follows: white, 137 patients (67 percent); black, 23 (11 percent); Hispanic, 29 (14 percent); Asian, 14 (7 percent); and Amerindian, 2 (1 percent). Patients came from the United States and from several Latin-American and Asian countries. Carriers of a TERC mutation were excluded from analysis. Patients or their guardians provided written informed consent for genetic testing, according to protocols approved by the institutional review board of the National Heart, Lung, and Blood Institute.
Samples from 282 healthy persons were studied as controls: 117 were white (94 from Human Variation Panel HD100CAU, Coriell Cell Repositories [http://locus.umdnj.edu/nigms/cells/humdiv.html], and 23 from SNP500Cancer [http://snp500cancer.ncbi.nlm.nih.gov]), 118 black (94 from Human Variation Panel HD100AA and 24 from SNP500Cancer), 23 Hispanic (from SNP500Cancer), and 24 Asian (from SNP500Cancer). The SNP500Cancer project aims to resequence reference samples from four ethnically diverse groups with the use of anonymous single-nucleotide polymorphisms . . . and other important classes of genetic variants of potential importance to molecular epidemiology studies of cancer and other diseases."27 Samples from an additional 246 anonymous healthy subjects of Hispanic origin (52 percent Peruvians, 28 percent Latin Americans, and 20 percent Pima and Maya Amerindians) were also examined as controls. In total, 1056 chromosomes from four major ethnic groups constituted the control group.

**Mutational Analysis**

Polymerase-chain-reaction (PCR) amplification of genes encoding the telomerase complex — namely, DKC1, NOP10, NHP2, and TERT — was performed with DNA samples extracted from peripheral-blood or bone marrow cells, as previously described.25 Primers and PCR conditions are listed in Table 1 of the Supplementary Appendix (available with the full text of this article at www.nejm.org). PCR products were purified with a QIAquick PCR purification kit (Qiagen), and direct sequencing was performed with BigDye Terminator version 3.1 (Applied Biosystems). Specific primers for sequencing were designed (Table 2 of the Supplementary Appendix), and sequencing products were analyzed in an automated genetic-sequence analyzer (ABI Prism 3100, Applied Biosystems). All sequences were determined in both directions, and mutations were confirmed by three separate PCR amplification products.

To assess microdeletion or microinsertion, PCR products were inserted directly into the pCR2.1-TOPO vector and transformed into competent Escherichia coli (TOP10F’ strain) with a TOPO TA cloning kit (Invitrogen). Patients had previously been screened for TERC mutations, as described elsewhere,25 and those positive for mutations were excluded from analysis.

**Functional Analysis**

The average length of telomere repeats at chromosome ends in individual peripheral-blood leukocytes was measured by flow fluorescence in situ hybridization, as previously reported.28 The telomerase activity of activated T cells was evaluated by the telomeric-repeat amplification protocol with the TRAPeze Telomerase Detection kit (Chemicon) in cells from patients and relatives carrying mutations at codon 202 or 1090 (cells from patients carrying mutations at codon 412, 694, or 772 were not available). Peripheral-blood cells were cultured in RPMI 1640 with L-glutamine and 10 percent fetal-calf serum in the presence of phytohemagglutinin (5 µg per milliliter) and interleukin-2 (40 IU per milliliter) for four days at 37°C with 5 percent carbon dioxide. An aliquot was stained with anti-CD3 and anti-CD4–phycoerythrin (BD Biosciences) for fluorescence-activated cell-sorter analysis in an LSR II flow cytometer (BD Biosciences). Cell-cycle and DNA-ploidy analyses were performed with a NuCycl kit (Exalpha), according to the manufacturer’s instructions. Protein was extracted and telomerase activity was assayed according to the manufacturer’s instructions, with slight modifications, as previously described.29

Mutations were introduced into the pCL-TERT plasmid with use of the QuikChange site-directed mutagenesis kit (Stratagene) and were verified by DNA sequencing. Two micrograms of either wild-type or mutant pCL-TERT DNA was transfected into telomerase-deficient VA13+TERC cells (at 60 percent confluence), as previously described.29 VA13+TERC is a line of human-lung fibroblasts that do not express telomerase activity, owing to the absence of TERT expression; instead the cells adopt an alternative mechanism for telomere maintenance, the ALT (alternative lengthening of telomeres) pathway.30 In studies of cotransfection into VA13-TERC cells, two ratios of wild-type to mutant pCL-TERT DNA were used: 1 µg to 1 µg or 1 µg to 3 µg, respectively, which totaled 2 µg or 4 µg, respectively. Subsequent manipulations of the cell extracts were carried out as described previously.29 Mutations other than the codon 412 mutations were tested at least twice in the transfection experiments. Total cellular RNA was also extracted with Trizol reagent (Invitrogen), and TERT expression assayed by Northern blotting with the random-primed probes to the TERT coding sequence.29

Peripheral-blood mononuclear cells from mutation carriers and controls were assessed in methyl-
cellulose medium for the number of hematopoietic progenitors with the use of recombinant cytokines (StemCell Technologies), according to the manufacturer’s instructions. Myeloid and erythroid colonies were counted 10 days after triplicate sample plating.

**Statistical Analysis**

Differences in the frequencies of coding-sequence variations between samples from patients with aplastic anemia and those from controls were evaluated by means of Fisher’s exact test. The Kruskal–Wallis nonparametric test, followed by Dunn’s multiple-comparison test, was used to compare differences in the number of colonies in hematopoietic-progenitor assays.

**Results**

**Mutations**  
Of the 205 patients with aplastic anemia, 5 carried a heterozygous TERC mutation and were excluded from analysis. Among the remaining 200 patients, five novel nonsynonymous mutations (i.e., mutations that introduce an amino acid change in the corresponding protein) in TERT, all heterozygous, were identified in 7 patients with apparently acquired aplastic anemia (Tables 1 and 2). A mutation in codon 202, which replaced alanine with threonine in the N-terminal region of TERT (codon 202 Ala/Thr), was found in two unrelated patients; another mutation, codon 412 His/Tyr, also in the N-terminal of TERT, was identified in two other, unrelated patients. Codon 694 Val/Met and codon 772 Tyr/Cys were located within the reverse transcriptase domain, and codon 1090 Val/Met was located in the C-terminal of TERT (Fig. 1A and 1B). No mutations were found in DKC1, NOP10, and NHP2, but nonsynonymous, single-nucleotide polymorphisms were found in all the genes analyzed, at similar overall allele frequencies in both the patients and the controls (Table 1). Additional synonymous, single-nucleotide polymorphisms were identified in TERT and in the box H/ACA-related genes (where box H/ACA refers to RNA involved in RNA modification) (Tables 3 and 4 of the Supplementary Appendix).

The germ-line origin of the TERT mutations was established by the detection of the mutations in DNA from buccal mucosa specimens obtained from three of the patients affected by aplastic anemia and in DNA from blood cells obtained from two of these patients’ family members. However, none of these patients or their family members showed physical signs of dyskeratosis congenita. The bone marrow cells of all the patients carrying TERT mutations

<table>
<thead>
<tr>
<th>Type of Variation</th>
<th>Gene</th>
<th>Location of Variation</th>
<th>Patients with Aplastic Anemia (N=200)</th>
<th>Controls (N=528)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>no. of patients (allele frequency)</td>
<td></td>
</tr>
<tr>
<td>Polymorphism</td>
<td>DKC1</td>
<td>Exon 14, codon 487 CCG/CTG (Pro/Leu)</td>
<td>11 (0.09)*</td>
<td>37 (0.13)*</td>
</tr>
<tr>
<td></td>
<td>NOP10</td>
<td>Exon 1, codon 12 GAT/CAT (Asp/His)</td>
<td>2 (0.02)*</td>
<td>1 (0.01)*</td>
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<tr>
<td></td>
<td>NHP2</td>
<td>Exon 4, codon 118 GCA/ACA (Ala/Thr)</td>
<td>1 (0.01)*</td>
<td>2 (0.01)*</td>
</tr>
<tr>
<td></td>
<td>TERT</td>
<td>Exon 2, codon 279 GCC/ACC (Ala/Thr)</td>
<td>6 (0.03)</td>
<td>10 (0.02)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon 2, codon 441 (Glu) deletion†</td>
<td>1 (0.005)</td>
<td>1 (0.002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon 15, codon 1062 GCC/ACC (Ala/Thr)</td>
<td>5 (0.025)</td>
<td>7 (0.01)</td>
</tr>
<tr>
<td>Mutation</td>
<td>TERT</td>
<td>Exon 2, codon 202 GCC/ACC (Ala/Thr)</td>
<td>2 (0.01)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon 2, codon 412 CAC/TAC (His/Tyr)</td>
<td>2 (0.01)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon 5, codon 694 GTG/ATG (Val/Met)</td>
<td>1 (0.005)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon 7, codon 772 TAC/TGC (Tyr/Cys)</td>
<td>1 (0.005)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon 15, codon 1090 GTG/ATG (Val/Met)</td>
<td>1 (0.005)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Gene mutations were screened in 122 patients and 282 controls only. Polymorphisms in DKC1, NOP10, NHP2, and TERT had similar allele frequencies in patients and controls, but the cumulative frequency of mutations in TERT was significantly greater in patients than in controls (P=0.000012, by Fisher’s exact test).  
† Nucleotides GGA 1378 to 1380 (GenBank accession number NM_003219) were deleted, abolishing codon 441.
had a normal karyotype. Detailed family histories revealed hematopoietic disorders in relatives of four of the seven affected patients (Table 2).

TELOMERE LENGTH

Compared with a reference group of 400 normal subjects (unpublished data), the length of telomeres in peripheral-blood leukocytes of patients carrying TERT mutations was markedly shortened (Fig. 1C). In contrast, among patients with aplastic anemia with polymorphisms in the TERT gene, telomere lengths were within the 90 percent confidence interval of the normal reference group. In the family of a patient with a codon 202 Ala/Thr mutation, one of his daughters, his two brothers, and one sister also carried the same heterozygous mutation. The leukocytes of these family members had short telomeres, but to date, they have not been found to have hematologic abnormalities in the blood; the leukocytes of one daughter and one sister without the mutation had normal telomeric length (Fig. 1D). Three genetic variants in TERT were considered polymorphisms because they were observed in normal donors and were not associated with telomere shortening; they included a change in codon 441 in exon 2 that resulted in the deletion of glutamine, a nonsynonymous single-nucleotide polymorphism in codon 279 Ala/Thr in exon 2, and a nonsynonymous single-nucleotide polymorphism in codon 1062 Ala/Thr in exon 15. Moreover, the observed allele frequencies for the two nonsynonymous single-nucleotide polymorphisms were similar in patients with aplastic anemia and controls (Table 1).

TELOMERASE ACTIVITY OF CULTURED CELLS

Peripheral-blood mononuclear cells from patients and healthy family members carrying codon 202 Ala/Thr and codon 1090 Val/Met mutations and from healthy controls were cultured. After expansion, more than 90 percent of the cells were viable, according to a dye-exclusion assay; on flow cytometry, the cells were mainly T cells (either CD4+ or CD4−) and had similar proliferation rates, as determined by cell-cycle analysis (data not shown). Telomeric-repeat amplification analysis of cell lysates from the patients showed that telomerase activity was reduced by approximately 50 percent as compared with that of healthy, unrelated controls (Fig. 2A).

Table 2. Clinical Characteristics and Laboratory Profiles of Patients Carrying TERT Mutations.*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutation</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Race or Ethnic Group†</th>
<th>Hemoglobin (g/dl)</th>
<th>Absolute Neutrophil Count (×10³/mm³)</th>
<th>Platelet Count (×10³/mm³)</th>
<th>Bone Marrow Cellularity (%)</th>
<th>Diagnosis</th>
<th>Family History</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Codon 202 Ala/Thr</td>
<td>41</td>
<td>M</td>
<td>Hispanic</td>
<td>8.3</td>
<td>1.3</td>
<td>6</td>
<td>20</td>
<td>Severe aplastic anemia</td>
<td>—</td>
<td>Transient response to immunosuppression</td>
</tr>
<tr>
<td>B</td>
<td>Codon 202 Ala/Thr</td>
<td>75</td>
<td>F</td>
<td>Hispanic</td>
<td>11.4</td>
<td>3.0</td>
<td>21</td>
<td>20</td>
<td>Moderate aplastic anemia</td>
<td>Brother with MDS</td>
<td>No treatment</td>
</tr>
<tr>
<td>C</td>
<td>Codon 412 His/Tyr</td>
<td>56</td>
<td>M</td>
<td>White</td>
<td>11.7</td>
<td>2.3</td>
<td>140</td>
<td>50</td>
<td>Moderate aplastic anemia</td>
<td>Mother with MDS</td>
<td>Response to granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>D</td>
<td>Codon 412 His/Tyr</td>
<td>31</td>
<td>M</td>
<td>Hispanic</td>
<td>3.1</td>
<td>2.0</td>
<td>22</td>
<td>&lt;10</td>
<td>Severe aplastic anemia</td>
<td>—</td>
<td>No response to immunosuppression</td>
</tr>
<tr>
<td>E</td>
<td>Codon 694 Val/Met</td>
<td>34</td>
<td>M</td>
<td>White</td>
<td>12.1</td>
<td>2.2</td>
<td>86</td>
<td>15</td>
<td>Moderate aplastic anemia</td>
<td>Sister with MDS evolving to AML</td>
<td>No response to immunosuppression</td>
</tr>
<tr>
<td>F</td>
<td>Codon 772 Tyr/Cys</td>
<td>53</td>
<td>M</td>
<td>White</td>
<td>12.6</td>
<td>1.1</td>
<td>100</td>
<td>10–20</td>
<td>Moderate aplastic anemia</td>
<td>Sister with anemia</td>
<td>No treatment</td>
</tr>
<tr>
<td>G</td>
<td>Codon 1090 Val/Met</td>
<td>64</td>
<td>F</td>
<td>Hispanic</td>
<td>10.1</td>
<td>0.3</td>
<td>3</td>
<td>&lt;10</td>
<td>Severe aplastic anemia</td>
<td>—</td>
<td>No response to immunosuppression</td>
</tr>
</tbody>
</table>

* MDS denotes myelodysplastic syndrome, and AML acute myeloid leukemia; dashes indicate that there was no family history of blood diseases or hematologic abnormalities.
† Race or ethnic group was self-reported by the patients.
HEMATOPOIETIC FUNCTION

We measured the number of erythroid and myeloid colonies in peripheral-blood specimens from two healthy siblings of Patient A (who had the codon 202 Ala/Thr mutation; one sibling was a carrier and one a noncarrier) and from two siblings of Patient G (who had the codon 1090 Val/Met mutation; one sibling was a carrier and one a noncarrier) and in blood specimens from healthy controls. The total number of progenitors was significantly lower among carriers than among noncarriers or controls (mean [±SD] colonies, 41±9, 116±45, and 118±30, respectively; P<0.05), indicating that hematopoietic function was reduced in carriers of a TERT mutation.

TELOMerase ACTIVITY OF CELLS TRANSFECTed WITH VECTORS CONTAINING A TERT MUTATION

Aplastic anemia–related TERT sequence variants were created in a TERT expression vector, which was then transfected into VA13+TERC cells. As shown in Figure 2B, the aplastic anemia–associated TERT preparations showed varying degrees of deficiency in telomerase enzymatic activity in these reconstituted cells. Whereas cell lysates carrying TERT mutations in codons 202, 694, 772, and 1090 showed less than 1 percent telomerase activity as compared with lysate containing the wild-type TERT gene (lanes 1 through 15), mutation of codon 412 resulted in approximately 50 percent telomerase activity (lanes 19 through 24). Loss of enzymatic function in the transfected cells was not due to altered transcription of the mutated TERT gene or instability of the messenger RNA, since RNA expression levels were found to be similar to those in the normal, wild-type TERT sample, as determined by Northern blot analysis (Fig. 2D).

COEXPRESSION OF WILD-TYPE AND MUTATION-CONTAINING TERT VECTORS

Telomerase functions as a multimeric complex consisting of at least two TERT enzyme proteins and two TERC RNA templates.31-36 Because primary cultures of cells from patients carrying TERT mutations had reduced telomerase activity, we investigated whether the reduction was due to haploinsufficiency or a dominant negative mechanism of action of the mutated TERT on the wild-type sequence. We cotransfected equal amounts of vector containing wild-type TERT and the individual mutations into VA13+TERC cells. As shown in Figure 2C, lysates from cells that were cotransfected with wild-type TERT and TERT containing codon 202 Ala/Thr, codon 694 Val/Met, codon 772 Tyr/Cys, or the codon 412 His/Tyr mutations (at either 1:1 or 1:3 ratios, based on plasmid concentration) also showed an approximately 50 percent reduction in the enzymatic activity of telomerase (lanes 28, 29, 30, 34, 35, 36, 43, 44, 45, and 49 through 54) in comparison with samples that were transfected with wild-type TERT and vectors containing either the codon 441 polymorphism (lanes 46, 47, and 48) or the control vector expressing wild-type TERT (lanes 25, 26, 27, 31, 32, 33, 40, 41, and 42). That wild-type telomerase activity was only partially reduced by mutated TERT suggested a mechanism of haploinsufficiency.

FIGURE 1 (facing page). TERT Mutations in Aplastic Anemia.

Panel A shows the linear structure of the TERT gene, which encodes human telomerase reverse transcriptase, and aplastic anemia–associated mutations. The segments represent exons. Nonsynonymous mutations were found in exons 2 (codon 202 Ala/Thr and codon 412 His/Tyr), 5 (codon 694 Val/Met), 7 (codon 772 Tyr/Cys), and 15 (codon 1090 Val/Met). Panel B shows the linear structure of TERT and aplastic anemia–associated mutations relative to known functional domains. DAT denotes disassociated activities of telomerase, L1 and L2 nonessential linker regions, and T telomerase-specific motif. Panel C shows the telomere length in peripheral-blood leukocytes from patients with aplastic anemia and TERT gene mutations. Telomere lengths were measured by flow fluorescence in situ hybridization analysis. Lines represent the 1st, 10th, 50th, 90th, and 99th percentiles of telomere length in age-matched controls’ granulocytes and lymphocytes, based on a reference group of 400 persons. The telomere length in granulocytes from six of the seven patients was below the 10th percentile (the exception was Patient C, with a granulocyte telomere length above the 15th percentile), whereas the lymphocyte telomere length from five patients was at or below the 10th percentile. Panel D shows the pedigree of Patient A, who carries the codon 202 Ala/Thr mutation in TERT; the pedigree suggests that the mutation associates with short telomeres. Telomere length is described as normal, low, or very low, according to the age-adjusted telomere length of normal controls, where very low indicates a length at or below the 1st percentile of normal age-matched controls; low, at or below the 10th percentile; and normal, between the 10th and 90th percentiles. No abnormalities in peripheral-blood cell counts were present in carriers, except for the proband, who was pancytopenic.

DISCUSSION

In this study, we found nonsynonymous mutations in the TERT gene, which encodes telomerase reverse
TERT MUTATIONS IN APLASTIC ANEMIA

A  TERT Mutations, According to Exon

<table>
<thead>
<tr>
<th>Exon Number</th>
<th>Codon 202 Ala/Thr</th>
<th>Codon 412 His/Tyr</th>
<th>Codon 694 Val/Met</th>
<th>Codon 772 Tyr/Cys</th>
<th>Codon 1090 Val/Met</th>
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<tbody>
<tr>
<td>2</td>
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<td>7</td>
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<td>15</td>
<td></td>
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</table>

B  TERT Mutations, According to Domain

<table>
<thead>
<tr>
<th>N-Terminal Region</th>
<th>Reverse-Transcriptase Motifs</th>
<th>C-Terminal Region</th>
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<tbody>
<tr>
<td>Codon 202 Ala/Thr</td>
<td>Codon 412 His/Tyr</td>
<td>Codon 694 Val/Met</td>
</tr>
<tr>
<td>Codon 694 Val/Met</td>
<td>Codon 772 Tyr/Cys</td>
<td>Codon 1090 Val/Met</td>
</tr>
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</table>

C  Telomere Length (kb) vs. Age (yr)

- Granulocytes
- Lymphocytes

D  Telomere Length Distribution

<table>
<thead>
<tr>
<th>Telomere Length</th>
<th>Low</th>
<th>Low</th>
<th>Very Low</th>
<th>Normal</th>
<th>Normal</th>
<th>Low</th>
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<tbody>
<tr>
<td>White-cell count (x10^3/mm^3)</td>
<td>8.8</td>
<td>6.7</td>
<td>8.1</td>
<td>5.7</td>
<td>8.2</td>
<td>6.2</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>16.5</td>
<td>16.3</td>
<td>13.3</td>
<td>7.3</td>
<td>13.9</td>
<td>13.6</td>
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<tr>
<td>Platelet count (x10^3/mm^3)</td>
<td>240</td>
<td>165</td>
<td>345</td>
<td>6</td>
<td>373</td>
<td>193</td>
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<tr>
<td>Age at time of study (yr)</td>
<td>51</td>
<td>49</td>
<td>11</td>
<td>41</td>
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</table>
transcriptase, in patients with apparently acquired aplastic anemia. That these mutations are functionally important is indicated by their association with very short telomeres and reduced telomerase activity in primary cultures of the patients’ leukocytes. In cell-culture experiments, the aplastic anemia–associated TERT mutations resulted in decreased telomerase activity by a mechanism of haploinsufficiency; that is, the remaining normal allele was insufficient for the production of adequate amounts of the enzyme.

We identified mutations in all three major domains of the TERT protein: the N- and C-terminal telomerase-specific domains and the conserved reverse-transcriptase domain. Primary cultures of leukocytes from patients and relatives carrying TERT mutations yielded lower telomerase enzymatic activity than did cultures of leukocytes from healthy persons. When we cotransfected vectors containing the wild-type TERT and TERT with the individual mutations into a telomerase-negative cell line, the telomerase activity of the mutated forms of the TERT proteins was only partially reduced, indicating that these mutations may act by haploinsufficiency and not by a true dominant negative mechanism. Haploinsufficiency is also the main mechanism by which dyskeratosis congenita and aplastic anemia–associated TERC mutations decrease telomerase activity.

Generally, a 50% reduction in enzyme expression in heterozygotes does not influence the phenotype, but expression of the telomerase complex is tightly regulated, and a partial reduction in its activity is sufficient to disturb the maintenance of telomere length. In murine models, Tert is a limiting factor, and loss of one copy of mTert also results in telomere shortening intermediate between telomere lengths in wild-type and mTert-null embryonic stem cells.

Our data are also consistent with an effect of the TERT gene deletion in patients with the cri du chat syndrome, in which the distal portions of chromosome 5p in one of the alleles, including the entire coding region of the TERT gene, are deleted. Reduced TERT expression and low telomerase activity have been observed in some patients with the cri du chat syndrome, suggesting haploinsufficiency as the mechanism of action in this syndrome as well. Since patients with aplastic anemia and TERT mutations do not have physical anomalies, TERT deletion alone is unlikely to be responsible for the complete phenotype of the cri du chat syndrome. That patients with this syndrome do not have marrow failure may be explained by “disease anticipation,” as occurs in dyskeratosis congenita; in that condition, the symptoms and signs become apparent at progressively younger ages in successive generations, and telomere shortening progresses over several generations.

Published mutations in genes of the telomere-repair complex in patients with bone marrow failure are summarized in Figure 3. Nucleotide alterations in the DKC1 gene and some mutations located

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**Figure 2 (facing page). Functional Analysis of TERT Mutations.**

Panel A shows the enzymatic activity of telomerase (measured by the telomeric-repeat amplification assay) in the lysates made from primary T cells collected from two patients who are heterozygous for a TERT mutation; the total protein concentrations of the cell lysates in each sample were 1.0 µg, 0.5 µg, and 0.1 µg. Samples from healthy persons were used as positive controls. These cells were expanded in tissue culture under the same conditions as those applied to the disease-associated samples. Experiments were repeated at least twice. A minus symbol indicates the heat-inactivated telomerase ribonucleoprotein of the positive control sample at a concentration of 1 µg; a plus symbol indicates a positive control for the polymerase chain reaction (PCR) with a DNA template containing the artificial telomeric repeats provided in the telomerase-detection kit. IC denotes PCR products amplified from a DNA template used as internal control for the efficiency of PCR amplification in each reaction. Panel B shows the results of telomeric-repeat amplification assays of cell lysates made from reconstitution of the mutated TERT expression vectors (2 µg per transfection reaction) in the telomerase-negative VA13+TERC cell line, which normally expresses only the TERC RNA component of the telomerase ribonucleoprotein. Serial 5x dilutions of each sample (at a maximal cell number of 20,000) were carried out to ensure the linearity of the PCR amplification reactions. Experiments were repeated at least twice, except for codon 412 His/Tyr. Panel C shows the results of analysis of coexpression of wild-type TERT and TERT with aplastic anemia–associated mutations or (at codon 441) a polymorphism. Equal concentrations of the wild-type TERT expression vector and the plasmid that contained the individual mutation (at 1:1 or 13:1 ratios, based on the plasmid concentration in micrograms) were cotransfected into the VA13+TERC cell line. For cells expressing a wild-type copy of the TERT gene alone, a total of either 2 µg or 4 µg of DNA was used. Cell lysates were prepared and assayed as outlined for Panel B. Panel D shows the results of Northern blot analysis of TERT RNAs from VA13+TERC cells transfected with a TERT expression vector expressing either the wild-type sequence or various disease-related mutations. “Empty vector” refers to cell lysate made from VA13+TERC cells transfected with an empty pcI vector (i.e., a vector that is devoid of TERT expression).
TERT MUTATIONS IN APLASTIC ANEMIA

A Primary Cells

B TERT Expression

C TERT Coexpression

D Northern Blot
within the 3’ region of TERC in box H/ACA and the CR7 domain result in dyskeratosis congenita,\textsuperscript{9,12} with presentation of pancytopenia in the first decades of life and associated physical anomalies. Mutations in the 5’ region of TERC (in the so-called pseudoknot and CR4–CR5 domains) and in TERT are more frequently associated with aplastic anemia later in life and are not usually related to the physical stigmata of dyskeratosis congenita.\textsuperscript{24,25} TERT and the 5’ region of TERC are required for telomerase enzymatic activity and for assembly of the telomerase ribonucleoprotein complex, whereas the box H/ACA–related proteins (dyskerin, NOP10, and NHP2) and 3’ region of TERC affect the stability of the complex and its maturation.

More patients should be analyzed genetically to determine whether there is an association between phenotypic features of the disease (e.g., the severity of pancytopenia, the age at the onset of clinical manifestations, and physical anomalies) and the location of genetic lesions in specific regions of functional activity in the genes encoding the telomere repair complex. Small nucleolar RNA with the box H/ACA motif participates in pseudouridylation of ribosomal RNA and other small RNAs,\textsuperscript{45} but there are no data yet to suggest that TERC

![Figure 3. Schematic Structure of the Telomere-Repair Complex and Location of Mutations Associated with Syndromes of Bone Marrow Failure.](image)

TERC, TERT, dyskerin, NOP10, NHP2, and GAR1 constitute the telomerase ribonucleoprotein complex. Mutations described in patients with classic dyskeratosis congenita associated with physical anomalies are shown in red; mutations in patients with isolated marrow failure, who usually present later in adult life and without physical stigmata, are shown in blue. Amino acids are denoted by their single-letter codes.
RNA or telomerase reverse transcriptase also has a role in post-transcriptional modification of ribosomal RNA.

Although the number of patients in our study is too small to allow us to draw a definite conclusion, none of the patients with TERT mutations had a response to immunosuppressive therapy. In our previous series, patients with TERC mutations also did not have an adequate response to immunosuppression. It is possible that specific mutations in one or more genes could influence the choice of therapy — the choice of specific drug regimens or the decision to undergo stem-cell transplantation. For this reason, measurements of telomere length in blood leukocytes and genetic testing for telomerase gene mutations could be useful in the management of acquired aplastic anemia.

Remarkably, humans with deficient telomerase activity, as well as telomerase knock-out mice, may appear to be phenotypically normal and have minimal or no apparent hematologic abnormalities. However, because their hematopoietic proliferative capacity is limited, affected persons and animals may be especially susceptible to environmental insults to the bone marrow (such as those caused by drugs or viruses), and patients’ reduced number of stem cells may limit their response to immunosuppressive therapies. Certain histocompatibility antigens and cytokine-gene polymorphisms are more prevalent in the immune-mediated marrow failure syndromes; these genetically determined immunologic characteristics affect the recognition of specific antigens and the nature of the immune response. For the hematopoietic target cells, TERC and TERT mutations provide a further genetic explanation for the rare, seemingly idiosyncratic appearance of aplastic anemia. Additional genetic variants should be sought in an effort to characterize possible modifiers of outcome and to explain differences in the timing of diagnosis as well as disease pene trance. Mutations in genes of the telomere repair complex reduce the size of the hematopoietic stem-cell compartment and the regenerative capacity of the marrow, making carriers of gene mutations susceptible to the development of marrow failure and affecting the course of aplastic anemia once it develops.

TERT and TERC mutations may be viewed as genetic risk factors for human hematopoietic failure. Defects in the maintenance of telomere length result in a reduced hematopoietic stem-cell compartment that may be especially vulnerable to environmental insults.

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