Complete sequence analysis of mitochondrial DNA and telomere length in aplastic anemia

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Abstract. The present study was primarily undertaken to examine the hypothesis that mitochondrial DNA (mtDNA) mutations and telomere length may be associated with aplastic anemia (AA). Our study included a single institution analysis of 40 patients presenting with AA first diagnosed at the Affiliated Hospital of Shandong, University of Traditional Chinese Medicine between 2010 and 2013. Bone marrow and oral epithelial samples were collected from patients with AA (n=40) for mtDNA mutation and telomere length determinations. Bone marrow specimens were collected from 40 healthy volunteers as controls for the examination of telomere length. The mitochondrial genome was amplified by polymerase chain reaction (PCR), and the products were used for sequencing and analysis. We detected 146 heteroplasmic mutations in 18 genes from 40 patients with AA, including 39 silent mutations and 28 frameshift mutations. We used the gamma globin gene (HBG) as the control gene in real-time PCR to survey the relative telomere length measurements of the patients with AA and the healthy volunteers. Telomere length was expressed as the relative T/S value. We observed a negative correlation between the mtDNA non-silent mutation and the white blood cell (WBC) count, hemoglobin and platelet count. Of note, there was a positive correlation between the relative T/S value and WBC count, hemoglobin and platelet count, and a negative correlation between the non-silent mutation and the relative T/S value. We conclude that the functional impairment of the mitochondrial respiratory chain induced by mutation and telomere length shortening may play an important role in the process of hematopoietic failure in patients with AA. Additionally, mtDNA mutations and telomere length shortening influenced each other.

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Introduction

Aplastic anemia (AA) is a bone marrow failure syndrome characterized by peripheral pancytopenia and bone marrow hypoplasia. The damage to the bone marrow may be triggered by environmental exposures, such as chemicals and drugs, or viral infections, and possibly endogenous antigens generated by genetically altered bone marrow cells. However, in approximately half of the cases, the cause is unknown. Based on our previous study (1), in the present study, we aimed to analyze the entire mitochondrial DNA (mtDNA) nucleotide sequences and telomere length of patients with AA.

While most of the genetic material of a cell is contained within the nucleus, the mitochondria possess their own circular DNA. Additionally, mitochondria have their own molecular machinery for protein synthesis and replicate by the process of fission, in the same way as bacteria. Mitochondria are considered to be the 'powerhouses of the cell', as they produce adenosine triphosphate (ATP) by systematically extracting energy from nutrient molecules (substrates) (2). Moreover, mtDNA is replicated with a high mutation rate, since it lacks protective histones and an effective DNA repair system.

mtDNA is located near the inner mitochondrial membrane, where it is far more likely to be exposed to oxygen-free radicals generated by the respiratory chain than is nuclear DNA (3,4). A number of studies have reported unexpectedly large numbers of somatic mutations in patients with leukemia and myelodysplastic syndromes (MDS) (5-7). Acquired deletions of mtDNA in the hematopoietic compartment have also been found to occur in association with severe pancytopenia and reticulocytopenia (8).

Among the etiologies which are thought to promote AA, exposure to radiation, and some drugs and chemicals appar-

ently give rise to mtDNA abnormalities (8,9). In the present study, we hypothesized that AA may be associated with mtDNA aberrations.

Telomeres were first measured more than a decade ago, and found to be short in approximately one third of patients with acquired AA. Those individuals with the shortest telomeres appeared to have a longer disease duration and more likely to develop late malignant clonal complications. Granulocytes were subsequently found to be mainly affected by telomere erosion in acquired marrow failure; patients with short telomeres were predominantly those who did not respond well to immunosuppression (10-12). Telomere shortening was thought to be the result of a 'stressed' hematopoietic stem cell, which over-proliferates in response to marrow failure.

Subjects and methods

Patients. Between September 2010 and February 2013, 40 patients, 23 males and 17 females (median age, 39.95 years; range, 11-64 years) were compared with 40 healthy controls, 17 males and 23 females (median age, 38.50 years; range, 15-68 years) (Table I). These patients were diagnosed at a single institution (Department of Hematology, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, China). The diagnosis of AA was based on the bone marrow and blood-count criteria of the International Agranulocytosis and Aplastic Anemia Study (13). These patients had no family history of hematologic disease, and the majority of the patients presented with fatigue and petechiae. Bone marrow and oral epithelial samples were collected from the patients with AA for the examination of mtDNA mutations and telomere length. Bone marrow samples were also collected from 40 healthy volunteers as the controls for the examination of telomere length. This study was approved by the Institutional Review Board of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Extraction of genomic DNA. Bone marrow cells were collected from the patients and oral epithelial cells were collected for normal tissue comparison. Bone marrow cells were collected from the healthy volunteers. Total DNA from the bone marrow of patients and healthy volunteers was extracted using an EasyPure Genomic DNA Extraction kit (Beijing TransGen Biotech Co., Ltd., China), and oral epithelial samples from patients were extracted using an TIANamp Swab DNA kit (Tiangen Biotech Co., Beijing, China). The extracted DNA was resuspended in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and stored at -20˚C prior to use.

Sequencing of mtDNA and data analysis. For the direct sequencing of the entire mtDNA genome, we used 8 primer pairs based on a modification of a published protocol to obtain 8 partially overlapping segments. The amplified mtDNA polymerase chain reaction (PCR) products were directly sequenced using the BigDye Terminator v3.1 ready reaction kit and the ABI Prism 3100 Genetic Analyzer (both from Applied Biosystems, Foster City, CA, USA). The sequencing primers used and the electropherogram of 8 fragments were described in our previous study (1). Experimentally obtained mtDNA sequences were compared with the revised Cambridge Reference Sequence (rCRS) (found at http://www.mitomap.org/mitomap/mitoseq.html) (14) and using the Blast2 program (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) and the database search tool MitoAnalyzer (www.cstl.nist.gov/biotech/strbase/mitoanalyzer.html) (15) to determine which polymorphisms and mutations differed from the rCRS, and to determine whether the differences caused amino acid changes in the resultant polypeptides. Nucleotide changes that were present in both the bone marrow and the oral epithelial cells of the same patient were counted as polymorphisms or homoplasmic mutations. Those that had not already been included in the databases (MITOMAP, mtDB or GenBank) were considered as novel polymorphisms. Changes that were only present in the bone marrow were counted as mutations or heteroplasmic mutations.

Measurement of telomeres. The method used for determining relative telomere length by real-time PCR has been previously described (16), and is hereafter referred to as Tel-PCR. For the PCR assay, 2 μl of each DNA dilution were prepared in a total reaction volume of 20 μl vol using the Ultra SYBR Two-Step qRT-PCR kit (Beijing CoWin Biotech Co., Ltd., Beijing, China). The final telomere primer concentrations were as follows: tel 1, 270 nM; and tel 2, 900 nM. In the control gene, the master mix primers, gamma globin gene (HBG)1 and HBG2, were used at a concentration of 400 nM. The primer sequences were as follows: tel 1, 5'-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3'; tel 2, 5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3'; HBG1, 5'-GCTTCTGACACAACTGTGTTCACTAGC-3'; and HBG2, 5'-CACCAACTTCATCCACGTTCACC-3'.

Table I. Comparison of patient characteristics between groups.

Characteristic	Normal group (n=40)	AA group (n=40)
Age (mean ± SD, years)	38.50±14.47	39.95±11.46
Gender (male/female)	17/23	23/17
WBC count (x10^9/l)	-	2.18±0.46
Hemoglobin count (g/l)	-	67.25±11.56
Platelet count (x10^9/l)	-	48.98±11.72
mtDNA mutations (without silent mutation)	-[a]	2.68±2.31
Relative T/S value	1.26±0.31[b]	1.06±0.38

[a]As regards the mitochondrial DNA (mtDNA) polymorphisms, we wished to determine which polymorphisms and mutations differed from the rCRS; thus, we studied patients with aplastic anemia (AA) and collected the oral epithelial cells from these patients as controls. The nucleotide changes present in both the bone marrow and the oral epithelial cells in the same patient were counted as polymorphisms or homoplasmic mutations. Therefore, we did not analyze the mtDNA sequences of the normal (healthy) group. [b]There were significant differences between the 2 groups ($P<0.05$). SD, standard deviation; WBC, white blood cell.

Table II. Pearson correlation coefficient between the groups.

	Age (mean ± SD, years)	Male	Female
Relative T/S value in normal group	-0.438[a]	1.29±0.38	1.23±0.25
Relative T/S value in AA group	-0.204	1.13±0.42	0.96±0.31

The relative T/S value in the normal group varied, as previously described (6). [a]The overall relative T/S values were shorter with increasing age ($P<0.05$; R^2 linear =0.192). SD, standard deviation; AA, aplastic anemia.

All sequences were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Telomere sequences were amplified in an ABI 7300 real-time PCR system (Applied Biosystems) under the following conditions: 95˚C, 10 min, to activate Taq polymerase; 35 cycles of denaturation at 95˚C, 15 sec, and annealing/extension, which was carried out at 54˚C, 2 min. The conditions for the amplification of the HBG gene were as follows: 95˚C, 10 min; 40 cycles at 95˚C, 15 sec, and 58˚C, 1 min. ABI Prism 7300 SDS software was used for the data analysis. The telomere length (x) for each sample was based on the telomere to single copy gene ratio (T/S ratio), which was based on the calculation of the ΔCt [Ct(telomeres)/Ct(HBG)].

Telomere length was expressed as the relative T/S value, which was normalized to the average T/S value of the reference sample defined as $[2^{-(\Delta Ctx-\Delta Ctr)} = 2^{-\Delta\Delta Ct}]$, which was used for the construction of the standard curve, as the reference sample, and as the validation sample. To make the results obtained from different plate runs comparable, the results of each plate were approved only if the relative T/S ratios of the validation reference sample fell within a 3% variation. Laboratory personnel conducting the telomere length assay were blinded to the clinical outcomes of the patients prior to statistical analysis.

Statistical analysis. Pearson correlation coefficient was used for the analysis of an association between the variables. We analyzed the association between the data such as mtDNA mutations (without silent mutation), relative T/S value and the white blood cell (WBC)/hemoglobin/platelet counts. Paired t-test was used to assess the differences between the variables. The tests were performed at the 0.05 level of significance. SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

Results

Heteroplasmic mutations of mtDNA. We detected mutations in all 40 entire mtDNA genomes obtained from both bone marrow and oral epithelial cells of the same patient. Overall, we detected 146 mutations in 18 genes, including 39 silent mutations (39/26.7%) and 28 frameshift mutations (28/19.2%). The non-silent mutations included NADH dehydrogenase (ND)2; 34/31.8%), *ND4* (16/11.1%), *ND5* (8/5.56%), cytochrome b (Cytb; 8/5.56%), cytochrome *c* oxidase subunit 1 (*COX1*; 7/4.86%), *ND6* (6/4.17%), *ND1* (6/4.17%), RNA, ribosomal cluster 1 (*RNR1*; 4/2.78%), *ND3* (4/2.78%), RNA, ribosomal cluster 2 (*RNR2;* 3/2.08%), *TRNE* (3/2.08%), *TRNG* (2/1.39%), *ND4L* (1/0.69%), cytochrome *c* oxidase subunit 2 *COX2*; 1/0.69%), cytochrome *c* oxidase subunit 3 (*COX3*; 1/0.69%), *TRNY* (1/0.69%) and *TRNA* (1/0.69%). These data are presented in our previous study (1). Mitochondrial sequencing indicated that these mutations were found in the coding region closely related to the mitochondrial oxidative respiratory chain, covering *ND1-6*, *ND4L*, *Cytb* and other related genes. We found that the mutation levels were particularly high in *ND2* and *ND4*.

mtDNA polymorphisms in patients with AA. In this study, we detected 424 polymorphisms in 15 genomes. These polymorphisms were found in the D-loop region (264/62.3%), *RNR1* (47/11.08%), *RNR2* (21/4.95%), *ND1* (5/1.18%), *ND2* (16/3.77%), *COX1* (16/3.77%), *COX2* (1/0.24%), *COX3* (2/0.47%), *ATP6* (2/0.47%), *ND3* (6/1.42%), *ND4L* (6/1.42%), *ND4* (5/1.18%), *ND5* (8/1.88%), *ND6* (6/1.42%) and *Cytb* (19/4.48%). Of note, the majority of these polymorphisms were found in the D-loop region, where numerous mutations are found to be involved in leukemia and MDS. However, in this study, no novel polymorphisms were found.

Relative T/S value in patients with AA as compared with the healthy individuals. We compared the relative T/S value in DNA obtained from the healthy volunteers with those found in the patients with AA (Tables I and II). The results presented herein and those obtained from healthy volunteers indicated that there were significant differences between the relative T/S values. Additionally, whereas the relative T/S value in the normal (healthy) group varied, overall, the relative T/S values were shorter as a function of increasing age ($P<0.05$). In the patients with AA, there was no obvious association between the relative T/S value and age, and there was no obvious association between the relative T/S value and gender in each group.

Association between non-silent mutations, relative T/S values and white blood cell (WBC), hemoglobin and platelet count. We then compared the non-silent mutations, relative T/S value, WBC, hemoglobin and platelet counts in the same patients with AA. We found that there was a negative correlation between the non-silent mutations and the WBC, hemoglobin and platelet counts obtained. Additionally, there was a positive correlation between the relative T/S value and the WBC, hemoglobin and platelet counts obtained. Finally, there was also a negative correlation observed between the non-silent mutations and the relative T/S value (Fig. 1).

Discussion

In this study, we analyzed entire mtDNA nucleotide sequences and telomere lengths of patients with AA. To the best of our

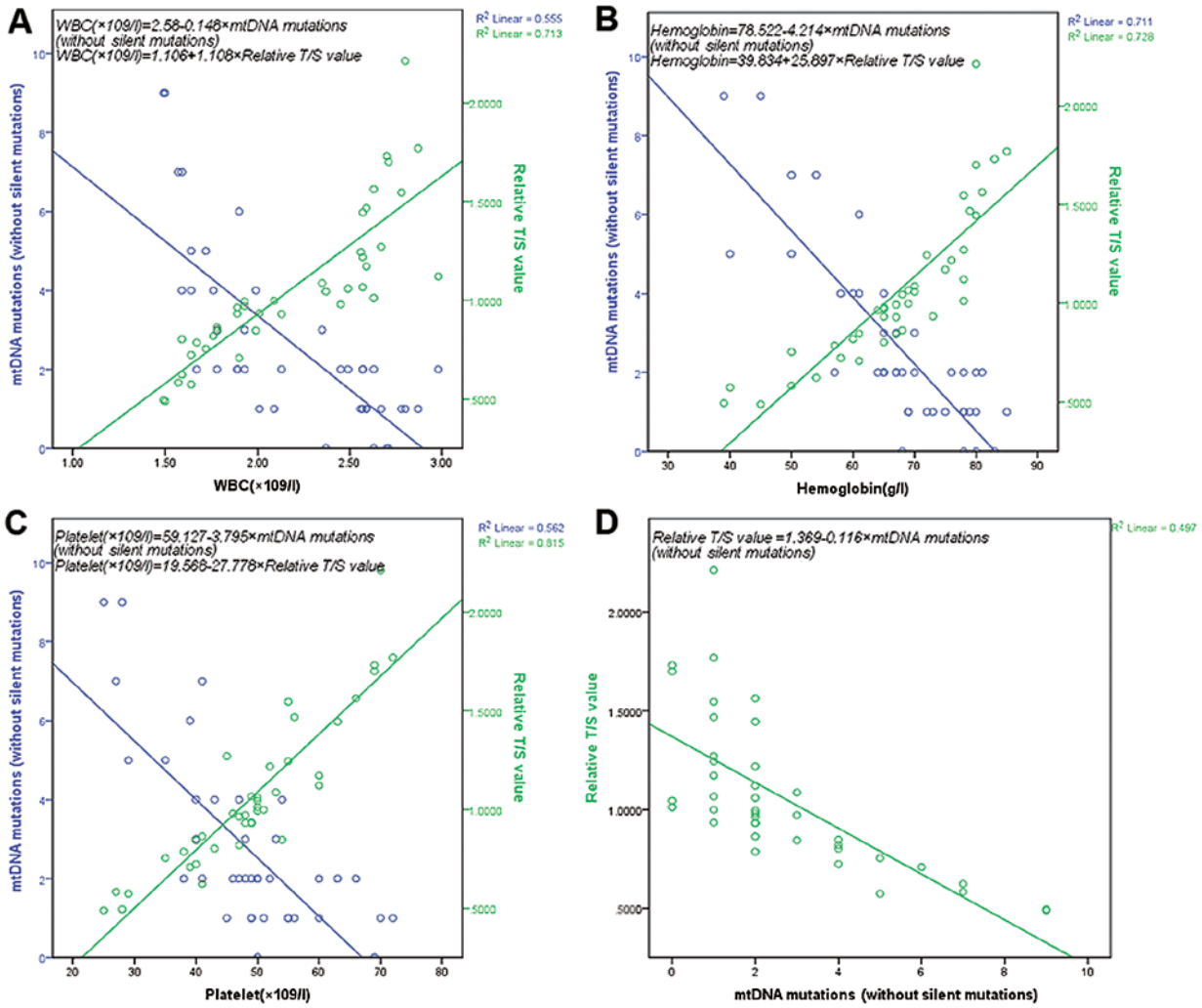

Figure 1. (A-C) Scatter plot showing the levels of mitochondrial DNA (mtDNA) mutations (without silent mutation) on the primary vertical axis and relative T/S value on the secondary vertical axis vs. the white blood cell (WBC)/hemoglobin/platelet counts on the horizontal axis from the same patient with AA. We found that WBC/hemoglobin/platelets were organized by decreasing levels of mtDNA mutations (without silent mutation), and WBC/hemoglobin/platelets were organized by increasing levels of the relative T/S value. The correlation between these data was highly significant (P<0.001). (D) Scatter plot showing the levels of mtDNA mutations (without silent mutation) on the vertical axis vs. the relative T/S value on the horizontal axis. We found that the negative correlation between mtDNA mutations (without silent mutation) and the relative T/S value was highly significant (P<0.001).

knowledge, this is the first study indicating that there is an association between telomere length and mtDNA mutations in patients with AA. We also found that there was a negative correlation between the non-silent mutations and the WBC, hemoglobin, platelet counts obtained and the relative T/S value.

Acquired mtDNA deletions in hematopoietic diseases have been observed in association with severe pancytopenia and reticulocytopenia (17). In this study, we detected 146 mutations in 18 genes, including 39 silent mutations (39/26.7%) and 28 frameshift mutations (28/19.2%). The mutation rate was particularly high in *ND2* and *ND4* of the mtDNA genome of the patients with AA, with the notable exception of these silent mutations.

These genes are closely linked to oxidation in the respiratory chain. We also found that there was a negative correlation between the non-silent mutations and the WBC, hemoglobin and platelet counts obtained. *ND1-6*, *ND4L* and *Cytb* are important components of the NADH-ubiquinone oxidoreductase (complex I) system, and the ubiquinone cytochrome *c* oxidoreductase (complex III) system. Mitochondrial injury is reflected by mtDNA damage and by a decrease in the levels of mtRNA transcripts, protein synthesis and mitochondrial function. A decrease in these complex activities may result in decreased cellular energy, disruption of cell signaling and interference with cellular differentiation and programmed cell death or apoptosis. Furthermore, deficient mitochondrial ATP production due to mutations in mtDNA may promote chromosomal instability (18). Cells with an inadequate ATP supply may also have difficulty in correctly segregating their chromosomes during mitosis. These factors may also result in decreased energy metabolism, which will affect the self-renewal and differentiation of hematopoietic stem cells. Of note, the genes affected were involved in oxidative phosphorylation. In contrast to the mutations involved in hematologic malignancies,such as acute leukemia (AL) and MDS, these mutations were mainly found in the D-loop (19,20). In these studies, the majority of nucleotide alterations were detected in the D-loop region in patients with AL and MDS, suggesting that the D-loop is a mutational hotspot in human cancer.

It was previously found that the mitochondria of leukemic cells utilize glycolysis more vigorously than oxidative phosphorylation (5,17,20-22). This may be caused by the

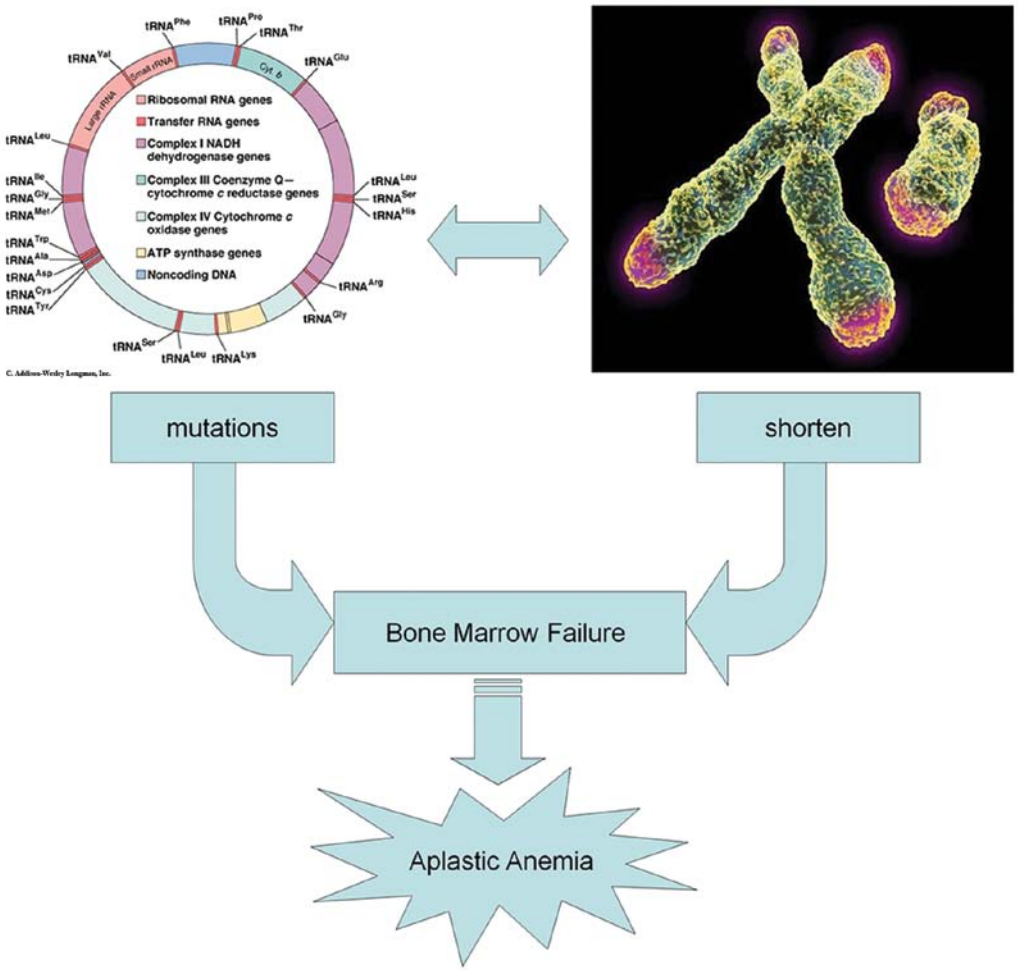

Figure 2. Diagram illustrating that mitochondrial DNA (mtDNA) mutations and shortening of the telomere length influence each other, and do so in such a manner, provoking the failure of bone marrow. We hypothesize that this may be an important pathogenic mechanism in aplastic anemia.

differences observed between benign and malignant hematologic disorders.

In this study, we used HBG as the control gene in real-time PCR analysis to survey relative telomere length measurement in patients with AA and healthy volunteers. In patients with AA, there was no obvious association between the relative T/S value and age. We then compared the relative T/S values and WBC count, hemoglobin and platelet counts in the same patients with AA. We found that there was a positive association between the context of the relative T/S value and WBC, hemoglobin and platelet counts. In addition, a negative association was found between the non-silent mutation and relative T/S value. Critically, short telomeres promote apoptosis, cell senescence and chromosomal instability in both tissue culture and animal models (23-25).

The major reverse transcriptase-incompetent splice variant of the human telomerase protein inhibits telomerase activity but protects cells from apoptosis. It was also found that the telomere lengths in granulocytes from patients with AA were significantly shorter than those in age-adjusted controls (12). In a large series of patients undergoing immunosuppressive therapy (n=183), patients with shorter telomeres (mostly without any telomerase mutation) had a higher possibility of relapse, an increased risk to evolve to MDS (particularly the most feared monosomy 7) and AML, coupled with a poorer overall survival in comparison to patients with longer telomeres (26). Bone marrow cells of short-telomere patients with AA also present increased chromosomal instability *in vitro* (27). In addition to absolute reticulocyte and lymphocyte counts (27), telomere length is likely to be critical in therapy decision making (28). Moreover, telomere length has been found to be related to the risk of relapse, clonal evolution and overall survival in severe AA (29).

In this study, we also compared non-silent mutations and the relative T/S value, and found that there was a negative correlation by this comparison. In conclusion, the mutations occurred in the region that influences the replication and transcription of mtDNA, and thus are likely to have significant effects on function, increasing the permeability of the mitochondrial inner membrane and destroying the mitochondrial membrane potential, which would then trigger caspase activity and, eventually, cell death by apoptosis. Therefore, functional defects caused by mtDNA mutations may be the primary cause of bone marrow failure in AA. In addition, since there was a negative correlation between the non-silent mutations and the relative T/S value, we suggest that mtDNA mutations and the shortening of telomere length may influence each other and cause the failure of the bone marrow. It is formally possible that our previously described supposition may represent an

important pathogenic mechanism in AA (Fig. 2). In future studies, we aim to increase the number of cases to prevent bias and then survey the related indicators of the oxidative respiratory chain and telomerase in an attempt to determine the pathogenesis of AA.

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