Mutations in telomerase catalytic protein in Japanese children with aplastic anemia

Juan Liang
Hiroshi Yagasaki
Yoshiro Kamachi
Asahito Hama
Kimikazu Matsumoto
Kouji Kato
Kazuko Kudo
Seiji Kojima

Recent studies indicate that a subset of patients with apparently acquired aplastic anemia (AA) have mutations in genes for telomerase ribonucleoprotein complex components. We looked for mutations in telomerase RNA (*TERC*) and telomerase reverse transcriptase (*TERT*) in 96 Japanese children with acquired AA and in 76 healthy controls. No mutations in *TERC* were found in any subjects. Novel heterozygous, non-synonymous mutations in *TERT* (T726M and G682D) were found in two patients with AA, neither of whom had clinical characteristics suggesting constitutional AA. This genetic difference does not explain the higher incidence of AA in Asian populations.

Key words: aplastic anemia, *TERC*, *TERT*, telomere length, children.

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From the Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan (JL, HY, YK, AH, KKu, SK); Division of Hematology and Oncology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan (KM, Ka).

Correspondence:
Seiji Kojima, MD, Department of Pediatrics, Nagoya University Graduate School of Medicine, 65, Tsurumai-Cho, Showa-Ku, Nagoya, 466-8550, Japan.
E-mail: kojimas@med.nagoya-u.ac.jp

Aplastic anemia (AA) is an uncommon but serious disorder characterized by pancytopenia resulting from non-function of the bone marrow. The incidence of acquired AA in Western countries is approximately 2 per million population per year and it is two to three times higher in East Asia.[1] The reason for this difference is not known. The two populations may have different AA-related genetic backgrounds. Recent studies indicate that in Western countries, a subset of patients with apparently acquired AA have mutations in genes for telomerase ribonucleoprotein complex components such as telomerase RNA (*TERC*)[2-5] and telomerase reverse transcriptase (*TERT*).[6] However, there have been no studies of the incidence of these mutations in Asian patients with AA. In the present study, to determine whether mutations of *TERC* and *TERT* genes are associated with AA in Asian patients, we analyzed the sequences of *TERC* and *TERT* genes of 96 Japanese children with acquired AA.

Design and Methods

We obtained blood samples from 96 Japanese children with AA (54 boys and 42 girls) and from 76 healthy individuals, and analyzed the samples for mutations of the *TERC* and *TERT* genes. The median age of the AA patients was 7 years (range, 0-16 years). Twenty-two patients had very severe AA, 39 patients had severe AA, and 35 patients had non-severe AA. The causes of AA were as follows: hepatitis in seven patients, viral infection in one patient, and unknown etiology in 88 patients. We also measured telomere length in a patient with a *TERT* mutation, the patient's parents, and 53 healthy Japanese individuals. Informed consent for blood sampling was obtained according to the protocol approved by the Institutional Review Board of the Nagoya University Graduate School of Medicine.

DNA samples were extracted from peripheral blood or bone marrow cells collected from the patients and normal controls. All exons and flanking introns of the *TERC* and *TERT* genes were amplified by polymerase chain reaction (PCR) using genomic DNA. Nineteen primer pairs were used for the PCR (1 *TERC*, 18 *TERT*).[3,6] PCR amplification of genomic DNA was performed using a High Fidelity Platinum Taq DNA polymerase kit (Invitrogen, Carlsbad, CA, USA). The thermal cycling of the PCR was as follows: pre-heating at 94°C for 2 minutes; 35 amplification cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and final extension at 72°C for 7 minutes. Each PCR product was amplified by a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The thermal cycling was as follows: initial denaturation at 96°C for 2 minutes; 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. The reaction products were analyzed using an ABI/ PRISM 3100 automated sequencer (Applied Biosystems). Telomere length was measured by flow-fluorescence *in situ* hybridization (flow-FISH), using a Telomere PNA kit (Dako Cytomation, Glostrup, Denmark).[7,8] The analysis was performed using a FACS caliber flow cytometer (Becton Dickinson Biosciences, Mississauga, Canada). After gating of diploid cells based on staining

with propidium iodide (PI), lymphocytes were isolated on the basis of size and granularity. Relative telomere length (RTL) was calculated as the ratio between the telomere signal of each sample and the telomere signal of the control cell line (cell line 1301). Because telomeres shorten with age, we obtained age-adjusted measurements for comparison. This was done by drawing a line of best fit through a plot of telomere length measurements against age for healthy individuals, yielding predicted telomere length measurements for any age.

Results and Discussion

No *TERC* mutations were found in any of the 96 patients with AA. Two novel heterozygous, non-synonymous *TERT* mutations were identified (one mutation in each of two patients): exon 5, n2045G→A; exon 6, n2177C→T (Figure 1). These base substitutions caused the amino acid changes G682D and T726M, respectively. Neither of the two patients had clinical characteristics of constitutional bone marrow failure syndrome.

The T726M substitution was identified in a 9-year-old girl with very severe AA who did not respond to immunosuppressive therapy. We found the T726M mutation in the patient's asymptomatic father, but not in her mother or younger brother. Figure 2 shows the telomere lengths of the patient and her father and mother plotted against age, and compared to those of normal individuals. The patient's lymphocytes had very short telomeres, compared with those of age-matched controls, but her father's lymphocytes did not have shortened telomeres.

The G682D substitution was identified in a 10 year old boy with non-severe AA who had not required medication for AA for 8 years. Unfortunately, we could not obtain his peripheral blood sample to measure the length of his telomeres. The samples from his family were also unavailable for study. No *TERC* or *TERT* mutations were detected in any of the 76 healthy controls. We sequenced all of the exons and flanking introns of *TERC* and *TERT* and all sequence alterations were compared with the *ENSEMBL SNP* database (*www.ensembl.org/index.html*) (Table 1). In *TERC*, we found an n514G→A substitution at the 3' downstream region in 57 of the 96 patients (59.4%) and in 41 of the 76 healthy controls (53.9%). Six polymorphisms of TERT exons were identified. Three (n915G→A, n2097C→T, n3039C→T) had been previously reported.[6,9] Five polymorphisms of *TERT* introns were also identified in the 96 patients. Among them, only two substitutions (n915G→A, IVS4+143G→A) were listed in the *ENSEMBL SNP* database. Vulliamy *et al.* first reported an association between acquired AA and mutations in *TERC*. They identified the n58G→A mutation in two out of 17 patients with idiopathic AA.[2] However, the n58G→A mutation has been found in 4 to 10% of healthy African subjects,[3] and it has no effect on telomerase activity in a telomerase reconstitution assay.[10] In a study by the National Institute of Health (NIH), non-synonymous mutations in *TERC* (n305G→A, n450G→A) were found in two out of 150 patients with AA. The patient with the n305G→A variant had a family history of hematopoietic disease. The patient with the n450G→A variant had normal telomere length

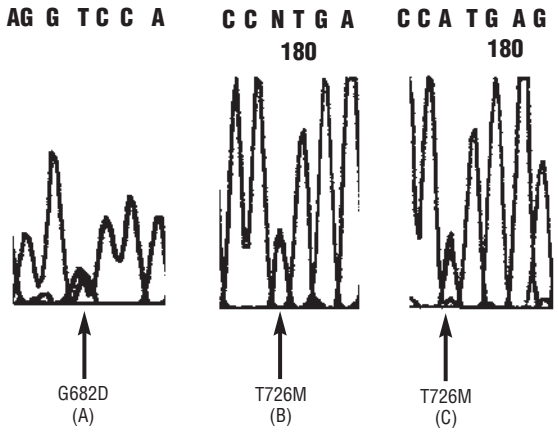

Figure 1. Identification of the *TERT* mutations (sequence of complementary strand). (A) Mutation in a 10-year old boy (G682D); (B) mutation in a 9-year old girl (T726M); (C) mutation in the girl's asymptomatic father (T726M).

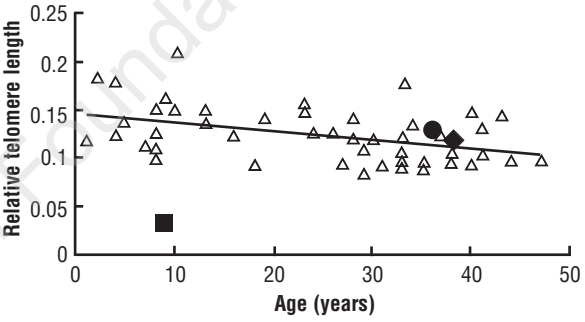

Figure 2. Measurement of relative telomere length (RTL) in peripheral blood lymphocytes. Telomere lengths were expressed as the RTL, which is the ratio between the telomere signal of each sample and the telomere signal of control cell lines. RTL-values were calculated using the following formula:

$$\frac{(\text{mean FL1 sample cells with probe} - \text{mean FL1 sample cells without probe}) \times \text{DNA index of control cells}}{(\text{mean FL1 control cells with probe} - \text{mean FL1 control cells without probe}) \times \text{DNA index of sample cells}}$$

(△) RTL of lymphocytes from 53 healthy controls (age range, 1- 47 years). (■) RTL of lymphocytes from the patient with the T726M *TERT* mutation. (◆) RTL of lymphocytes from the patient's father. (●) RTL of lymphocytes from the patient's mother. As shown in this figure, the RTL of controls decreased significantly with age (r=-0.48), and the patient with the T726M *TERT* mutation had very short telomeres, whereas her father and mother had normal telomere length, compared with age-matched controls.

and exhibited sustained remission of AA after immunosuppressive therapy.[3] A functional assay has shown that the n450G→A variant is a polymorphism of *TERC* with no functional effect on telomerase activity.[11] Therefore, functional assays are essential to confirm the pathogenic role of mutations. Researchers in Brazil found no mutations in *TERC* in any of the 42 Brazilian AA patients.[12] Although no *TERC* mutations were identified in our study, our data do not exclude the possible role of *TERC* mutations in the pathogenesis of aplastic anemia. On the other hand, we identified two heterozygous, non-synony-

Table 1. Polymorphisms in *TERT* and *TERC*.

Gene	Location	Frequencies in AA patients (n=96)	Frequencies in controls (n=76)
TERT	915 G→A (A305A)	38(39.6%)	39(51.3%)
	2097 C→T (A699A)	3(3.1%)	4(5.3%)
	2520 G→A (L840L)	1(1.0%)	2(2.6%)
	2946 T→C (C982C)	1(1.0%)	1(1.3%)
	3039 C→T (H1013H)	57(59.4%)	43(56.6%)
	3366 G→A (T1103T)	2(2.1%)	2(2.6%)
	IVS4+143 G→A	42(43.8%)	36(47.4%)
	IVS9+11 C→T	3(3.1%)	3(3.9%)
	IVS13+45 C→T	7(7.3%)	4(5.3%)
	IVS15+136 G→A	1(1.0%)	2(2.6%)
	IVS16+81 C→T	1(1.0%)	2(2.6%)
TERC	514 G→A	57(59.4%)	41(53.9%)

mous *TERT* mutations in two out of 96 Japanese children with apparently acquired AA. Both of the two present mutations have been located in the RT domain. Mutation of residues within the RT domain of TERT can abolish telomerase activity, indicating that the RT domain comprises the catalytic core of telomerase.[13] The present patient with the T726M mutation had markedly short telomeres and did not respond to immunosuppressive therapy. Although a functional assay is necessary to establish the pathogenic role of the T726M mutation, the following observations suggest that it is not a polymorphism: (i) it was found in only one patient and her family; (ii) the patient with the mutation had very short telomeres; (iii) the patient's father had the same mutation; (iv) the affected amino acid is located in the functional domain. The asymptomatic father of the patient with the T726M mutation had the same mutation, but his lymphocytes did not have shortened telomeres. In families with dyskeratosis congenita (DC) caused by a *TERC* mutation, disease anticipation is associated with progressive telomere shortening. Some patients with DC remain asymptomatic well into adulthood. The severity of the disease appears to be increased with telomere shortening through the generations.[14] Recently, Armanios *et al*. described a family with autosomal dominant DC, disease anticipation and telomere shorting.[15] The present family with the *TERT* mutation may represent a similar example of disease anticipation. In a NIH study, five non-synonymous *TERT* mutations were identified in seven out of 200 patients with acquired AA. Four of the seven patients had a family history of hematologic abnormalities, but none of them had physical anomalies characteristic of DC. The other three patients had no signs of constitutional AA, and were diagnosed with idiopathic AA. The telomeres of all seven patients were markedly shorter than those of age-matched controls. A telomerase reconstitution assay showed less than 1% activity compared with that of a lysate containing the wild-type *TERT* gene in four of five samples. A 50% reduction was seen in one sample. Thus, there are at least five *TERT* mutations that can cause dysfunction of telomerase and predispose patients to AA.[6] Vulliamy *et al*. screened 80 patients with constitutional or acquired AA for *TERT* mutations. They found two non-synonymous mutations in two patients with constitutional AA, but did not find mutations in any of the patients with idiopathic AA.[9]

In summary, we found that a small proportion of the present patients with apparently acquired AA had mutations in the gene for telomerase ribonucleoprotein complex components. These patients should be regarded as having cryptic DC as a result of gene mutations. Although the incidence of AA is higher in Asian populations than in Western populations, the incidence of *TERC* and *TERT* mutations is not higher in Asian populations.

All persons designated as authors qualified for authorship by contributing to the design and development of the study as well as the interpretation of data. All of them approved the final version of the manuscript. The authors declare that they have no potential conflicts of interest.

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