Telomerase activity and telomere length in AML-M3 patients

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Abstract

Introduction: The telomeric DNA together with its associated proteins protects the chromosome ends from degradation or aberrant recombination. Telomerase and telomere are closely associated with development of cancers. In this study we aim to investigate the significance of telomerase activity (TA) and telomere length (TL) in patients with acute promyelocytic leukemia (APL).

Methods: Peripheral blood samples were taken from 20 APL patients during the diagnosis and from 25 healthy normal individuals at different age ranges. Telomerase activity (TA) was assessed by TRAP- ELISA and -PAGE procedures. Genomic DNA isolated from patient mononuclear cells was digested with Rsa1 and Hinf1 restriction enzymes; electrophoresis was performed in 0.8% agarose gels, and telomere length (TL) was determined by southern analysis using a Chemiluminescence-based assay.

Results: As oppose to the normal individuals, telomerase activity was detected in peripheral blood mononuclear cells of all APL patients (P<0.001). Marked differences were observed in the sizes of the telomere length in the normal blood cells and APL leukemic cells. The leukemic cells of 18 of 20 (90%) patients with APL showed a significant reduction in the length of telomeric DNA, ranging from 2.3 to 6.7 kbp (median 3.5 kbp), while the telomere length in healthy normal individuals was 9.1 to 14.8 kbp (median 11.6 kbp) (P<0.000). In healthy individuals, the average TL was found to vary with age, and the rate of telomere shortening was age dependent.

Conclusions: Telomere length shortening and Telomerase up-regulation are closely associated with acute promyelocytic leukemia; therefore, they may be used as potential markers for diagnostic, prognostic and for therapeutic intervention in APL patients.

Keywords: Telomere length, Telomerase activity, acute promyelocytic leukemia, TRAP assay

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Introduction

Telomeres are repeated DNA sequences at the ends of chromosomes in eukaryotic cells, and they play a critical role in maintaining chromosomal stability.(1,2) Because DNA polymerase cannot replicate the ends of linear template as normal somatic cells divide, the telomeres become shorter with each cell division.(3,4) As the number of divisions increases, telomere length falls beyond a critical value, leading to senescence, a peculiar phase of cell life mostly characterized by growth inhibition.

During tumorigenesis, telomeres usually undergo progressive shortening until TA is restored to allow indefinite cell replication.(5) This late activation is now regarded as a critical step in tumor development.(5-7) Telomerase is a specialized ribonucleoprotein polymerase that functions in the maintenance of telomeres and is considered to be necessary for the indefinite proliferation of human cancer cells. Progressive shortening of telomeres and activation of telomerase have been considered to be one of the key mechanisms in chromosome structural integrity, cellular immortalization, and tumor progression.(8-10)

Numerous investigations have demonstrated the presence of telomerase activity in the vast majority of different cancer types– and in immortalized cells– but failed to detect telomerase in most normal tissues.(11) The maintenance of telomeres by telomerase has been observed in over 80% of solid tumors, suggesting that telomerase activity is necessary for the indefinite proliferation of cancer cells. Also, the levels of telomerase activity correlate with the stage of carcinogenicity,(12) thus, determination of telomerase activity has a great potential for use in clinical diagnosis of various malignancies. Tumor-specific expression pattern makes telomerase an attractive research target to study its potential as a marker for cancer

diagnosis/prognosis and for therapeutic intervention in a variety of human cancers. The aim of our study was to investigate the significance of TA and TL in APL disease.

Patients and Methods

Patients: A total of 20 APL patients undergoing treatment were enrolled in this study at the time of diagnosis. The diagnosis of APL (AML-M3) was established on the basis of clinical presentation, morphological criteria of the FAB classification, cytogenetic evaluation for t(15;17), and reversed transcriptase polymerase chain reaction (RT-PCR) analysis for PML-RARα transcripts. All patients' clinical and molecular analysis before and after treatment was conducted at the HORC-BMT.

Mononuclear cells (MNCs) were isolated from PB by density gradient centrifugation on Ficol-Hypaque. The study was preformed in accordance with local institution approved regulations, and informed written consent was obtained from each patient.

Measurement of Telomerase activity: Telomerase activity was measured by the telomerase PCR-ELISA Kit (Roche Diagnostics) according to the manufacturers' instructions. In brief, the cells were collected as pellets and lysed in 1,3-((3-cholamidopropy) dimethylammonio)- 1-propanesulfonic acid (CHAPS) buffer. Each of the TRAP reactions contained 1000 cell extract, 10 pM forward TS primer, and 10 mM dNTP, 2.5U Taq DNA polymerase, 10x buffer, and 20 mM MgCl2 to a final volume of 50 µl; the reaction mixture was incubated 30 min at 37ºC. Then 10 pM reverse CX primer was added before PCR. PCR was preformed by 30 cycles of 94ºC for 30 S, 50ºC for 30 S, 72ºC for 45 S. Samples were separated by 10% polyacrylamcle gel electrophoresis and stained with AgNO3.

Telomerase activity was calculated as the ratio of the intensity of telomerase ladders originated from each sample/ the intensity of peak area of the 36-bp standard in each assay]/[The intensity of telomerase ladders originated from positive control/ the intensity peak area of the 36-bp standard in positive control]×100 = TPG U.

About 1000 cells from NB4 cell line served as a positive control and lysis buffer alone served as a negative control for the assay.

Measurement of Telomere length: Telomere length was measured by using a non-radioactive chemiluminesent assay, using the Telo TAAGGG Telomere Length Assay kit (Roche Diagnostics) according to the manufacturers' instructions. In brief, genomic DNA from patients' samples was extracted by salt out procedure using proteinase k. One to 1.5 µg genomic DNA was digested with 20 units of RsaI and HinfI for 2 h at 37ºC. Digested samples were resolved on a 0.8% agarose gel and gel was depurinated, denatured, neutralized, transferred to a positive charge nylon membrane (Roche Diagnostics) using 20% SSC, dried for 1 h at 70ºC, and hybridized with a digoxigenin-labelled telomeric probe (TTAGGG)4 at 65ºC for 16 h and washed in 2x SSC/0.1% SDS. Chemiluminescent detection was preformed according to the anti-DIG-AP Luminescent Detection kit (Roche Diagnostics). Telomeric smears were visualized by exposing the membranes to a Lumi-Film Chemiluminescent Delection Film (Roche Diagnostics). Telomere peak valves were measured by estimating the band size corresponding to the point with the highest absorbance. The mean terminal restriction fragment (TRF) lengths were calculated and recorded as telomere lengths.

Results

APL Patients and healthy individuals: Twenty five healthy individuals consisted of 14 men and 11 women; aged from 15 to 50 years (average: 43 yr). Twenty APL patients consisted of 10 men and 10 women; aged from 20 to 50 years (average: 45 yr). According to the FAB classification, all of the patients were AML-M3. Cytogenetic analysis was performed on bone marrow aspirates at diagnosis and all had t(15;17) translocation. At diagnosis, the median white blood count (WBC) in APL patients was 2250 cells/µl (ranged 550-5000), and the percentage of leukemic cells ranged from 70 to 99% (medium 87%) of the circulating WBC.

Telomerase activity in APL patients and healthy individuals: All PBMC samples taken from healthy individual exhibited undetectable or very low telomerase activity (< 1 TPG). All samples obtained from APL patients had detectable levels of TA. The average TPG in PB of APL patients ranged between 3 to 70 TPG and the median was 7 TPG (Figure 1). At diagnosis, APL cells revealed a seven-fold increased TA (ranged 30-70 fold) in comparison to normal MNCs ($p<0.005$) (Figure 1).

Telomere length in healthy individuals: In healthy individuals, the average TRF ranged between 9.1 and 14.8 kbp and the median TRF was 11.6 kbp. We used linear regression analysis to determine the TL as a function of age in samples obtained from healthy individuals (Figure 2). The average TL in PBMN cells declined with age: TL=13.15–0.050xA (R^2=0.44; $p<0.005$), where TL is the TRF in kbp

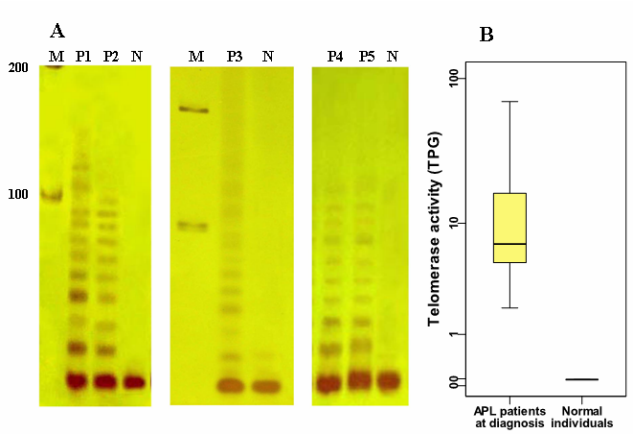

Figure 1. Telomerase activity in peripheral samples of APL patients and normal individuals. **(A)** Telomerase activity in PB cell from representative APL patients at diagnosis (P) and from health individuals (N) were detected by TRAP assay and visualized on 8% PAGA. M indicate molecular weight marker; 36 bp, internal standard. **(B)** Box plot of telomerase activity level in 20 APL patients vs healthy normal individuals. The APL patients had significantly higher TA level than the normal individuals ($P < 0.001$).

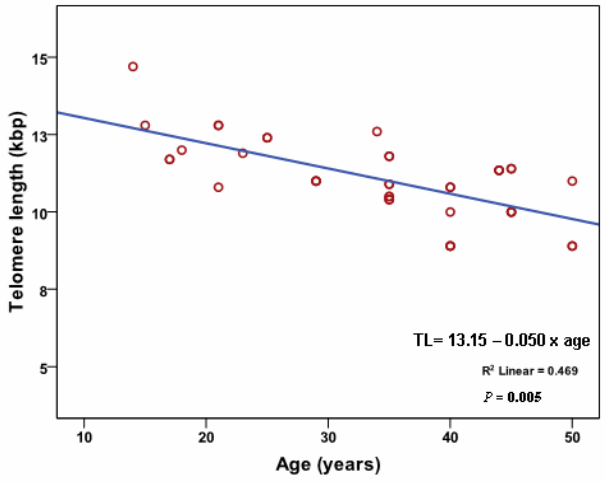

Figure 2. Telomere length in peripheral blood mononuclear cells obtained from normal individuals at various ages. Linear regression analysis was used to determine the TL as a function of age in samples obtained from healthy individuals. The average TL in PBMN cells declined with age: TL=13.15–0.050 x A (R^2=0.44; p<0.005), where TL is the TRF in kbp and A is age in years.

and A is age in years. TL reduction was on average of 50 bp per year.

Telomere length in APL patients: Depending on the leukemic cell burden in PB of APL patients at the time of diagnosis, one or two distinct populations of cells in APL samples were detected: a normal cell population as the larger peak and a leukemic cell population as the shorter peak. Figure 3 illustrates the patients with a shorter peak only (P1, P2 and P5) and patients with two distinct peaks (P3 and P4).

Result showed that median telomere length at the time of diagnosis was significantly shorter in APL patients than in healthy individuals (p<0.001) (Figure 3). The average TRF in PB of APL patients ranged between 2.3 to 10.4 and the median was 3.5 kbp (Figure 3). About 18 of 20 patients (95%) had significant telomere reduction in the length of telomeric DNA relative to age-matched control, with the median TRF difference of 7.8 kbp (range 4.5 to 11.4 kbp) (Figure 3). In only two (5%) patients with APL long telomere (>9.5kbp) were observed, both patients had telomerase activity levels comparable to those of other patients with APL.

Discussion

Detection of TA and with lesser extend TL have been applied to discriminate benign from malignant tissue including solid tumors and hematologic malignancies in several studies. Their potential use as a diagnostic and prognostic marker and also for the development of telomerase-based therapies has been evaluated in several malignancies.(14) The significance of telomerase and telomere status has been evaluated extensively in solid tumors,(12-21) and, on a smaller scale, in hematopoietic malignancies.(22,23) Few studies have evaluated the same issue in AML(21,23,24) and non in APL. To obtain information about TA in APL, we evaluated PB samples obtained from APL patients at the time of diagnosis and compared them to TA in healthy individuals, by TRAP-ELISA and –PAGE assays. In this study, our results illustrate that telomerase activity is highly up regulated in PB samples of all APL patients at initial presentation, but very low or mostly no TA was detected in healthy individuals. At diagnosis, APL cells revealed a seven-fold increased TA (ranged 30-70 fold) in comparison to normal MNCs (p<0.005). A high level of TA has been reported in both AML and ALL.(21) Ohyashiki et al(20) reported that about 75% of the patients with acute leukemia showed elevated levels of TA. In our study, the expression of TA was not related to age or sex of the patients, WBC, or history of disease. These data may indicate that telomerase activation commonly occurs in APL. Therefore, the detection of telomerase activity holds promise as a diagnostic marker in the detection of APL.

We also studied telomere length (terminal restriction fragment [TRF]) in PB leukocyte samples of the APL patients and 25 age-related

healthy individuals, who ranged in age from their fifteen to fifty. No significant variation was observed in the size of the TL in the normal population. The average TRF in healthy individuals ranged between 9.1 and 14.8 kbp and the median TRF was 11.6 kbp. We used linear regression analysis to determine the TL as a function of age in samples. TL in healthy individuals showed to be age dependent with a TL reduction of 50 bp per year.

On the other hand, the telomere lengths were more heterogeneous in APL cells, with the difference in sizes range from 2.3 to 10.4 kbp. In 95% of leukemic cells, there was significant shortening of the telomeric DNA at the time of diagnosis, and they were 40% to 80% (median 72.5%) shorter compare to the telomere length of samples obtained from the healthy individuals.

In this group of APL patients, only 10% of the APL patients had excessively longer telomeres (>9.0 kbp) than the most patients with shorter mean TL. These leukemic cells with long telomeres may use a non-telomerase mechanism, the alternative lengthening of pathway (ALT), to maintain their telomere, involving intertelomeric homologous recombination.(25) The ALT is reported in approximately 10% or less of tumors.(25,26) All of our candidate ALT patients had telomerase activity levels comparable to those of other patients with APL. The coexistence of ALT pathway (non-telomerase) with telomerase-induced telomere stabilization mechanisms has been documented in immortalized human cells,(27) multiple myeloma patients(28) and in knockout mutation (mTR-/-) mice (…).

Shorter telomeres found in APL cells are indicative for extensive cell proliferation and population doubling. These data show that in APL cells the extensive proliferation and telomere shortening occurs prior to activation of telomerase. However, telomerase up regulation finally occurs at a critical point of telomere shortening in leukemia, stabilizing the telomere and preventing the onset of crisis and proliferative senescence.

In conclusion, our result indicates that telomerase is activated in APL cells; however, TA was very low or undetectable in normal cell. On the other hand, the average telomere length in majority of APL cells was well below that of normal cells. These differences of TA and TL in leukemic cells versus normal cells make them potential markers for diagnostic and prognostic and for therapeutic intervention in APL patients.

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