Telomerase activity and telomere length in patients with acute promyelocytic leukemia: indicative of proliferative activity, disease progression, and overall survival

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Background: The progressive shortening of telomeres and the activation of telomerase have been considered to be one of the key mechanisms in cellular immortalization and tumor progression.

Patients and methods: About 300 sequential samples were collected from 40 patients during the course of acute promyelocytic leukemia (APL) disease. Telomerase activity (TA) and terminal restriction fragment (TRF) length were assessed by TRAP and Southern blot analyses, respectively. PML-retinoic acid receptor α (RARα)/glucose-6-phosphate dehydrogenase transcripts were quantified by real-time PCR.

Results: About 90% of the patients had a significant reduction in telomere length (TL) relative to the control (median 3.5 versus 11.37 kbp; \(P<0.001\)). A significant positive correlation between TL and PML-RARα expression was found \((P=0.001)\). Telomerase was activated in all patients; however, TA level was significantly higher in the group of relapsed patients than patient with newly diagnosed. The group of patients with shortened TRF and elevated TA had a significantly poorer overall survival.

Conclusions: The shortened TL and elevated TA in APL patients are mainly indicative of extensive proliferative activity and they correlate with disease progression and relapse; thus, they may serve as prognostic factors for a subset of APL patients with more aggressive disease and poor outcome, those who may not respond favorably to arsenic therapy.

Key words: acute promyelocytic leukemia, arsenic trioxide, NB4, telomerase activity, telomere length

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]. During tumorigenesis, telomere length (TL) usually undergoes progressive shortening until telomerase activity (TA) is restored [2]. Thus, this allows the telomeres to be stabilized at a constant length. The stabilized telomeres, by telomerase or some times by an alternative mechanism called alternative lengthening of telomere [3], confer cell immortalization and have the ability to proliferate indefinitely. The progressive shortening of telomeres and the activation of telomerase have been considered to be one of the key mechanisms in chromosome structural integrity, cellular immortalization, and tumor progression [4].

Acute promyelocytic leukemia (APL), an acute myelogenous leukemia subtype (AML-M3), is characterized by a specific t(15;17) genotype that results in a reciprocal translocation between the PML gene and the retinoic acid receptor α (RARα) gene, a distinct morphologic picture, and a clinical coagulopathy that contributes to the morbidity and mortality of this disease [5]. The current treatment of APL with all-transretinoic acid and more recently with arsenic trioxide (As2O3) has increased long-lasting complete remissions (CRs) and apparent cure rates to >70% [6, 7]. However, a proportion of patients continue to die eventually as a result of disease recurrence. The identification of reliable prognostic markers is essential in APL. Some pretreatment characteristics have been identified that are correlated with an increased risk of relapse [8]. Our previous result shows that the minimal residual disease levels are the only significant independent parameters for identification of patients who will undergo a relapse [9]. Recently, considerable interest is being focused on the applications for the detection and evaluation of cancer by TA and TL. Their potential use as a diagnostic and prognostic marker and also for the development of telomerase-based therapies has been evaluated in several malignancies [10]. The significance of telomerase and telomere status has been evaluated extensively in solid tumors [11–16], and, on a smaller scale, in hematopoietic malignancies [17, 18]. Few studies have evaluated the same issue in AML [18–21], and...
none have investigated in APL. The aim of our study was to investigate the significance of TA and TL in a clinical course of APL disease with respect to the therapeutic effects of arsenic trioxide.

**patients and methods**

**patients**

A total of 40 APL patients undergoing treatment were enrolled in this study at the time of diagnosis. Their median age was 35 years (range 14–50), 17 were male and 23 female, 32 were newly diagnosed patients (primary APL), and eight were relapsed (secondary APL) patients. The diagnosis of APL (AML-M3) was established on the basis of clinical presentation, morphological criteria of the French-American-British (FAB) classification, cytogenetic evaluation for t(15;17), and reversed transcriptase polymerase chain reaction (RT–PCR) analysis for PML-RARα transcripts. Patients received standard intensive remission induction and courses of consolidation therapy with arsenic trioxide as described previously [7, 9]. The study was carried out in accordance with our local institution-approved regulations, and informed written consent was obtained from each patient.

Following diagnosis, all patients were monitored for TA, TL, and PML-RARα transcript. Serial peripheral blood (PB) samples (300 samples) were collected at diagnosis, during induction and consolidation at weekly intervals, and after therapy at 3-month intervals. Mononuclear cells (MNCs) were isolated from PB by density gradient centrifugation on Ficoll–Hypaque. Human APL cell line, NB4, that harbors t(15;17), was used as a positive control.

**measurement of TA and TL**

TA was determined by Telo TAGGG Telomerase PCR ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturers' instructions.

TL [terminal restriction fragment (TRF)] was measured by using a nonradioactive chemiluminescent assay, using the Telo TAAGGG Telomere Length Assay kit (Roche Diagnostics), according to the manufacturers’ instructions.

**quantification PML-RARα by real-time PCR**

Quantification of PML-RARα and glucose-6-phosphate dehydrogenase transcripts were carried out on MNC by LightCycler Instrument using LightCycler t(15;17) Quantification Kit (Roche Diagnostics) as described previously [9].

**statistical analysis**

The Wilcoxon rank sum test was used to compare the differences in TL and TA between case patients and control, pre- and post-treated, and relapsed and new case patients. All statistical tests were two sided. Survival distributions were presented using the Kaplan–Meier method and were compared using the log-rank test. The statistical analyses were carried out using SPSS 13 software (SPSS, Chicago, IL).

**results**

**patient characteristics**

According to the FAB classification, all the patients were AML-M3. Cytogenetic analysis was carried out 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 2100 cells/μl (ranged 500–4300), and the percentage of leukemic cells ranged from 66% to 99% (medium 86.5%) of the circulating WBC. After a course of induction/consolidation therapy with arsenic trioxide, all patients achieved a clinical CR with the medium time of 30 days, and all achieved molecular remission as detected by conventional RT–PCR.

**telomerase activity**

All peripheral blood mononuclear cell samples taken from 20 healthy individuals exhibited undetectable TA. However, TA was detectable in all APL patients' samples. APL patients could be divided into two groups according to the TA level at the time of presentation, the group of patients (nine patients) with significantly high TA (>20 total product generated units (TPG U)) versus the patients (31 patients) with low TA (<20 TPG U). As shown in Figure 1, with patient response to the treatment, TA was decreased 70%–90%. The decrease in TA during the therapy with As2O3 was highly correlated with the reduction of leukemic cells (PML-RARα+) and the attainment of normal cells (PML-RARα−) in PB.

**Figure 1.** TRAP analysis of telomerase activity in PB samples of acute promyelocytic leukemia (APL) patients during treatment with As2O3. Mononuclear cells were collected and telomerase activity (TA) was measured for 1000 cells equivalent per lane using PCR-based TRAP assay. (A) TA was assayed in three APL patients during the arsenic treatment. (B) TRAP ladder was resolved on a 10% polyacrylamide gel electrophoresis and visualized by silver staining. The lowest band is related to internal control with 36 bp. The relative level of TA was expressed by total product generated units.
telomere length
We studied TL in PB MNC samples of 20 healthy adult individuals at ages ranging from 15 to 50 years. The average TRF ranged between 9.7 and 14.7 kbp and the median TRF was 12 kbp. We used linear regression analysis to determine the TL as a function of age in samples. TL in PBMN cells declined with age: TL = 13.15 − 0.050 × A (R² = 0.44; P < 0.005), where TL is the TRF in kbp and A is age in years. A decline in TL with age (~50 bp/year) was found in PBMN cells from healthy individuals.

Representative examples of TRF length analysis at the time of diagnosis and CR are shown in Figure 2. Patient-by-patient comparison of samples during diagnosis showed that 36 of 40 (90%) had significant reduction in the TRF length relative to the age-matched control or to that at the time of CR from the same patients. No significant differences in TRF length were apparent between patients at CR and healthy individuals. The median TRF obtained from patients at the time of diagnosis was 3.5 kbp and ranged from 2.3 to 10.4 kbp (Figure 2B). The TRF obtained from the same patients at the time of CR ranged 8.9 and 14.7 kbp and the median was 11.4 kbp. In only four (10%) patients long telomeres were observed, with a median TRF difference of 0.8 kbp (range 0–1.5 kbp) between diagnosis and CR. In 90% of the patients, the median TRF

Figure 2. Telomere length (TL) in the acute promyelocytic leukemia (APL) patients at diagnosis and complete remission. (A) Measurement of telomere restriction fragment (TRF) length in PB samples of four representative patients with APL at diagnosis (Dig) and complete remission (CR). The shorter TRF peak most likely represents the leukemic telomere; the longer peak corresponds in size to the normal TL. (B) The TL at diagnosis (the median TRF of 3.5 kbp; range 2.3–10.4 kbp) was significantly shorter than at CR (the median TRF of 11.4 kbp; range 8.9–14.7 kbp) in the APL patients. The average TL in the APL cells was ~71% (range 43%–77%) shorter than at remission (P < 0.001 by Mann–Whitney test). (C) Patient-by-patient comparison of TRF length from samples obtained from 20 representative APL patients at the time of diagnosis and during CR. In 90% of the APL patients, the median size of TL at diagnosis was significantly shorter than at CR. TA, TA (1+ is about ≤20 total product generated). SD, status at the time of disease presentation (N, newly diagnosed or primary APL; R, relapsed or secondary APL). Overall survival (OS) during a 40-month follow-up (C, complete remission; D, deceased), PN, patient number.
length at the time of diagnosis was 7.8 kbp (range 4.7–11.2 kbp) shorter than at CR; that indicates, 71% (range 45%–77%) reduction ($P < 0.001$). The TRF length in APL patients at diagnosis was independent of age.

During the treatment, the median TRF length tended to increase as patients responded to the arsenic therapy and underwent CR. Figure 3A illustrates a typical example of such cases with a rapid kinetic of incline during the course of induction therapy. By analyzing the follow-up sample during the CR, the relapse was even detectable as the TL decreased (Figure 3B).

correlation of TL with PML-RARα expression

In order to determine whether the increase in TRF length observed during the arsenic treatment was due to a shift from PML-RARα+ to PML-RARα- cells in the PB of these patients, we compared the intensity of the shorter TRF peak in Southern blot with the result of quantitative RT–PCR obtained from the same patients’ samples. Significant positive correlation between telomere with a smaller TRF peak and PML-RARα expression were found in the APL patients ($P < 0.001$; Figure 4), indicating that shortened TL was specific to APL cells.

TL, TA and survival

Since TL varies with age and has been found to vary considerably in human PBL from individuals of the same age, we used two parameters for survival analysis: TL ratio of leukemic to nonleukemic cell and the TL size difference of leukemic from nonleukemic in the same patient. The Kaplan–Meier survival curves for TL ratio and TL size difference were determined by log-rank statistics according to classification and regression trees technique using a cut-off value of 0.3 and 8.0 kbp, respectively. The survival curve in Figure 5A illustrates that the patients with TL size difference >8 kbp (11 of 40 patients) had a significantly poorer overall survival (OS) (7 of 11 died, 13-month survival of 36.5%) compared with a 40-month survival rate of 100% for 29 patients with TL size difference <8 kbp ($P < 0.001$). The patients with TL ratio <0.3 (15 of 40 patients) also had a significantly poorer OS (7 of 15 died, 13-month survival of 53.3%; $P < 0.001$).

The only other independent prognostic factor for OS was TA. The patients with high TA (>20 TPG U; 9 of 40 patients) also had a significantly poorer OS (seven of nine died, 13-month survival of 22%) compared with a 40-month survival of 100% for 31 patients with TA level <20 TPG U ($P < 0.001$) (Figure 5B). Most of the patients with high TA level had significantly longer TL size difference (seven of nine; $P = 0.006$) (Figure 5C).

TL and TA in relapsed versus newly diagnosed patients

Of the 40 APL patients enrolled in this study at the time of disease presentation, 32 were newly diagnosed (primary APL), and eight were relapsed patients (secondary APL). Four patients were in their first relapse (R1), three in R2, and one in R3. In the group of relapsed patients, the TL size difference

![Figure 3.](image-url)
numbers every 106 copies of glucose-6-phosphate dehydrogenase. Of 100%; diagnosed patients (0 of 32 died; 40-month survival estimate 13-month OS estimate of 12.5%) compared with the newly had significantly shorter survival (seven of eight died; than the newly diagnosed patients. The relapsed patients only a few patients (10%) showed TRF length within the specimens compared with their age-matched healthy donors; reported that TA level was increased 18-fold in AML malignancies, Xu et al. [20] reported that TA level was elevated in 77% of the primary AML; Engelhardt et al. [12] reported that TA level was increased 18-fold in AML specimens compared with their age-matched healthy donors; however, in their study, there was none or only one patient with AML-M3 subtype.

Figure 4. Serial measurement of telomere restriction fragment (TRF) peak intensity, telomerase activity level, and PML-retinoic acid receptor α (PML-RARα) transcript level during treatment of an acute promyelocytic leukemia patient with arsenic trioxide. Telomere values are expressed as a percent intensity of the peak with a shorter TRF length (most likely represents the leukemic TRF) in comparison with the intensity of a longer TRF peak (corresponds in size to the normal TRF length). Telomerase values are expressed as in total product generated units. The values for PML-RARα transcript are expressed as PML-RARα transcript copy numbers every 10⁶ copies of glucose-6-phosphate dehydrogenase.

was significantly longer (the median 50 versus 7.5 TPG U; P < 0.001) (Figure 5D) and the TA level was significantly higher (the median 50 versus 7.5 TPG U; P < 0.001) (Figure 5E) than the newly diagnosed patients. The relapsed patients had significantly shorter survival (seven of eight died; 13-month OS estimate of 12.5%) compared with the newly diagnosed patients (0 of 32 died; 40-month survival estimate of 100%; P < 0.001).

discussions

We investigated TL and TA in APL patients at the time of presentation, during the treatment with arsenic trioxide and after the induction of CR. Telomerase is consistently activated in the PB samples of all APL cases at the initial presentation; however, telomerase level varied among the patients at presentation and patients could be divided into two groups: low and high. The high level of TL is found mostly in the relapsed patients. The activation of telomerase has been found in a wide variety of cancers and hematological malignancies and is believed to be an important step in the progression of almost all human malignancies [22]. In hematological malignancies, Xu et al. [20] reported that TA level was elevated in 77% of the primary AML; Engelhardt et al. [12] reported that TA level was increased 18-fold in AML specimens compared with their age-matched healthy donors; however, in their study, there was none or only one patient with AML-M3 subtype.

The TL in APL patients varied in size at presentation, and only a few patients (10%) showed TRF length within the normal range, and the majority of patients (90%) had a significant shortened TRF length (median 3.5 kbp) relative to the age-matched control or TRF at the time of CR (median 11.37 kbp). The results indicate that the average TL at diagnosis had ~71% reduction (median size difference of 7.87 kbp) in 90% of the APL patients (P < 0.001) (Figure 2). This difference in size is much larger than what has been reported for most cancers such as colorectal carcinoma [14], head and neck cancer [16], prostate, colon, sarcoma [11], and thyroid cancer [15], compared with adjacent normal tissues, with a median size range differences of 0.9–3.17 kbp. It is also larger than what has been reported for most hematological malignancy such as chronic myelogenous leukemia (CML), AML, acute lymphoblastic leukemia (ALL), and multiple myeloma with a median size range differences of 2.0–5.5 kbp [17]. The short telomere seen in APL might be due to a rapid and extensive cell proliferation and population doublings that occur before activation of telomerase. Then, the telomerase activation stabilizes the telomere at constant length and confers to cell the ability to proliferate indefinitely. These data may support the hypothesis that telomerase-mediated telomere stabilization is achieved late in tumorigenesis after extensive cell proliferation and telomere shortening has already taken place [12].

It has been reported that because of the difficulties of longitudinal studies in a clinical setting, the comparison of cancer and adjacent noncancer tissue is a useful model to investigate carcinogenesis-related changes. This comparison may illustrate the individual differences between cancer cells and noncancer cells that serve as a representative from which leukemia might have started [14]. In this study, we used the TL ratios of leukemic to nonleukemic and also the TL size difference of leukemic from nonleukemic cells which in our study, the later parameter proved to be a better independent prognosis factor. We also studied TL in healthy individuals which showed to be age dependent with a TL reduction of 50 bp/year. The median TL in samples from patients in complete molecular remission was also age dependent and was not any different from the healthy individuals. The increase in TL in which we observed during the arsenic therapy was the normalization of previously shortened TL arguing against a preexisting TL deficit in normal hematopoietic stem cells form patients with APL at the time of malignant transformation.

In current study, we sought to determine whether the increase in TL observed during the treatment was due to a shift from PML-RARα+ to PML-RARα− cells in response to the arsenic treatment in the PB of APL patients; the intensity of peak with a shorter TRF in Southern blot was compared with the result of quantitative real-time RT–PCR. A significant positive correlation between the intensity of telomere peak with a shorter TRF and PML-RARα expression was found in APL patients (P < 0.001; Figure 4). The follow-up of patients during the treatment shows that as patient respond to the treatment, the intensity of a shorter TRF peak, reflecting the leukemic population, is decreased; its length is increased and returns to within the normal range during the CR, almost to the level of a healthy donor within 15–60 days after treatment. The increase in TL.
During the course of induction/consolidation therapy with As$_2$O$_3$ was highly correlated with the reduction of leukemic cells (PML–RAR$^+$ with shorter telomeres) and the emergence of normal hematopoietic cells (PML–RAR$^-$ with longer telomeres) in PB. With a longer follow-up of patients during the CR, clinical relapse was even detectable by reappearance of a shorter telomere, correlating with the increase of PML–RAR$^+$ cells and progression of a relapse. Our data provide evidence that TL analysis at diagnosis and its correlative increase during the course of treatment could be a useful marker in the management of APL disease.

We studied survival analysis using Kaplan–Meier survival curve for TL and TA in PB samples of the APL patients. We have demonstrated that the APL patients at presentation could be separated into two groups according to their TA level and/or TL size difference of leukemic from nonleukemic by log-rank statistics. Most of the patients with a high TA level had a significantly longer TL size difference ($P = 0.006$) (Figure 5C) and these patients had a significantly poorer OS; whereas, those with a low TA and a shorter TL size difference had more favorable outcome ($P < 0.001$) (Figure 5A and 5B). Our results are consistent with the reports of several previous studies that in certain malignancies, TA level is closely related with prognosis [20, 23–25]. Some studies have also observed associations between short TLs and worse outcomes in certain malignancies [23, 26, 27]. Thus, our data further suggest that in APL patients, high TA and short TL may point to a poor prognosis and severity of disease.

**Figure 5.** Telomere length size difference and telomerase activity in PB samples of acute promyelocytic leukemia (APL) patients in relation to patient overall survival and patient status at the time of presentation (relapsed versus newly diagnosed). (A) Kaplan–Meier survival curve in 40 APL patients stratified by telomere length (TL) size difference of leukemic from nonleukemic (8.0 kbp). (B) Kaplan–Meier survival curve in 40 APL patients stratified by telomerase activity (TA) [low <20 total product generated units (TPG U) versus high >20 TPG U]. A cut-off value of 8.0 kbp and 20 TPG U was determined by log-rank statistics using the classification and regression trees techniques. (C) Box plot of TL size difference of leukemic from nonleukemic in 40 APL patients stratified by TA (low and high). The patients with a high TA level had a significantly longer TL size difference ($P = 0.006$). (D) Box plot of TL size difference of leukemic from nonleukemic in 40 APL patients stratified by status at the time of presentation (relapsed versus newly diagnosed). The relapsed patients had a significantly longer TL size difference than the newly diagnosed APL patients (median 8.40 versus 7.30 kbp; $P < 0.001$). (E) Box plot of TA level in 40 APL patients stratified by status at the time of presentation (relapsed versus newly diagnosed). The relapsed patients also had significantly higher TA level than the newly diagnosed patients (median 42.5 versus 7 TPG U; $P < 0.001$).

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P = .001

100 29 Patients with TL size difference < 8.0 kbp; 0 died

100 31 Patients with TA < 20 TPG U; 0 died
In further analyses, when we separated the patients into primary (newly diagnosed) and secondary (relapsed) APL at presentation, most of the patients with short TL and high TA were relapsed patients. The difference in telomere sizes and telomerase levels seen in the APL patients at relapse versus at diagnosis could be a consequence of increased proliferative activity and further cell divisions undergone before the relapse occurred. Ladetto et al. [28] speculated that significant difference in TRF length at relapse compared with at diagnosis may occur after chemotherapy only in the presence of a marked cytoreduction. This might allow the selection of specific tumor populations with TRFs lengths different from those observed at diagnosis. On the basis of the correlation of high TA with a relapse and poor outcome, as we observed in the APL patients, it has been speculated that tumor cells might be continuously selected for incrementally higher levels of TA as they proliferate and acquire genetic changes associated with invasive cancer [29]. In this regard, all our patients underwent a major degree of cytoreduction by arsenic therapy before relapse and almost all the patients exhibited further shortened TL and elevated TA at the time of relapse. On the basis of these results, we proposed a model of telomere and telomerase biology in arsenic-treated APL patients as represented in Figure 6. We speculate that during the course of disease, a subset of cell population may arise with shorter TL, higher TA, and highly aggressive which may not respond favorably to arsenic therapy.

In conclusion, the shortened TL and elevated TA in APL patients are mainly indicative of extensive proliferative activity and they correlate with disease progression and relapse. Therefore, these parameters may serve as prognosis factors for a subset of APL patients, with more aggressive disease and worse outcome, those who may not respond favorably to the arsenic therapy. We are suggesting that these patients may be good candidates for the therapeutic intervention with a telomerase inhibitor.

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**references**


