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Expression pattern of *hTERT* telomerase subunit gene in different stages of chronic myeloid leukemia

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Abstract Telomerase is activated in chronic myeloid leukemia (CML); however, it is not known whether the catalytic telomerase reverse transcriptase subunit (hTERT) is vital in the progression of this disease. This study involved patients with CML in the chronic phase (pre-treatment and after treatment), accelerated and blastic phase. Expression of the *hTERT* gene differed significantly among the four major groups ($p < 0.05$). We also compared *hTERT* expression according to demographic parameter such as age and sex, and found no significant differences ($p > 0.05$). Taken together, our findings suggest the importance of *hTERT* as a valuable molecular marker in the follow-up of patients with CML, which may have clinical implications for the prognosis.

Keywords Telomerase · hTERT · Chronic myeloid leukemia · Prognostic marker

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Introduction

Chronic myelogenous (or myeloid) leukemia (CML), also called chronic granulocytic leukemia, is a hematopoietic stem cell disorder that is genetically characterized by a reciprocal translocation of chromosomes 9 and 22, t (9; 22) (q34; q11) which results in the fusion of the *BCR/ABL* gene on the derivative chromosome 22, called the Philadelphia (Ph′) chromosome, in more than 90 % of cases. The expression of BCR–ABL tyrosine kinase results in increased cell proliferation and the inhibition of apoptosis [1]. Chronic myeloid leukemia occurs in all age groups, but is most common in the middle-aged and elderly (50–60 years). This disease represents about 7–20 % of all cases of adult leukemia and 15 % of all leukemia.

Chronic myeloid leukemia is often divided into three phases: chronic, accelerated and blastic. About 85 % of patients are diagnosed in the chronic phase (CP) of the disease, which lasts 2–7 years before turning into the accelerated phase (AP), and finally shifting to the blastic phase (BP), with a short survival time of 2–6 months [2]. The Ph′ chromosome is the hallmark of the chronic phase; other cytogenetic abnormalities associated with the blast phase of CML are 8^{+}, 19^{+}, Ph^{+} and iso-17q [3, 4].

Telomeres are specialized protein–DNA complexes that cap the termini of linear chromosomes in most eukaryotic organisms. Their function is to protect the ends of chromosomes from loss of genetic information, end-to-end fusion, senescence and apoptosis [5, 6]. Every time a chromosome is copied, 50-100 nucleotides are lost from the telomeric region. Telomerase is a ribonucleoprotein enzyme consisting of an integral RNA subunit (hTER), a telomerase-dependent subunit (TEP1, telomerase-associated protein) and a catalytic subunit (hTERT, telomerase reverse transcriptase) that adds DNA sequence repeats (TTAGGG) to the 3′ end of DNA strands in

the telomere region [7]. The enzyme complex remains fully active in specific germ line cells and cancer cells, but is undetectable in most normal somatic cells. Further erosion of the telomeres may impair their function in protecting the chromosome ends, resulting in genetic instability [8, 9].

Some researchers have tried to measure the expression of telomerase subunits instead of evaluating their activity. The expression of all telomerase subunits has no direct association with telomerase activity. About 85–95 % of tumors express *hTERT*, and this association identified *hTERT* as credible marker for the diagnosis and stage determination of breast cancer and other tumors [10].

In most advanced cancers (85 %), telomerase is reactivated and serves to maintain telomere length. Therefore, analyzing *hTERT* expression can provide clinically useful information. Several studies have shown that telomerase reactivation in hematological neoplasms has a notable influence on prognosis [11, 12]. Because telomerase activation is a key factor in cancer, in the present study we investigated the expression of the *hTERT* gene in the different stages of CML to determine the influence of the *hTERT* subunit on prognosis.

Materials and methods

Seventy-three samples from patients who gave their informed consent and met the criteria for CML were selected randomly from patients who were referred to Tehran Shariati Hospital.

The most important indication and inclusion criteria was Philadelphia chromosome-positivity CML(Ph+) or BCR–ABL-positivity. However, the phases were based mainly on the number of immature white blood cells—myeloblasts (blasts)—that were seen in the blood or bone marrow. Other inclusion criteria are listed below:

Chronic phase

- Patients in chronic phase typically had less than 10 % blasts in their blood or bone marrow samples.
- Mild symptoms (if any) and usually responsive to standard treatments.

Accelerated phase

- More than 10 % but fewer than 20 % blasts in the bone marrow or blood samples.
- Basophil count making up at least 20 % of total white blood cells, which did not decrease with treatment.
- Very high or very low platelet counts which were not caused by treatment.
- In addition, patients in the accelerated phase may have symptoms such as fever, poor appetite and weight loss. They had not responded as well to treatment as patients in the chronic phase.

Blast phase (acute phase)

- Bone marrow and/or blood samples from a patient in this phase had more than 20 % blasts.
- These patients often had fever, poor appetite and weight loss.

Blood samples were obtained from 26 patients in the chronic phase before treatment and 3 months after treatment, 9 patients in the accelerated phase and 12 patients in the blastic phase.

Mononuclear cells were isolated from peripheral blood by Ficoll and density-gradient centrifugation. Total RNA extraction from mononuclear cells was performed as described by Trizol (Sigma, Munich, Germany). The final volume used for cDNA synthesis was 20 μL; the mixture contained 1 μg extracted RNA, random primers (Table 1), MuLV enzyme, RNase Inhibitor (Fermentase, Vilnius, Lithuania) and dNTP Mix (Cinagen, Tehran, Iran).

The amplification reaction for reverse-transcriptase polymerase chain reaction (RT-PCR) for *hTERT* and *ABL* (as a housekeeping gene) was carried out in a final volume of 25 μL.

When PCR was completed, the amplified fragments were analyzed by electrophoresis on 3 % agarose gel. A negative control (water instead of the DNA template) and a positive control (cDNA from the NB4 cell line) were included in each reaction. Statistical analyses were done with Fisher's exact test in SPSS v. 15 for Windows®.

Results

Thirty-three (73 %) patients were male and 12 (27 %) were female. Mean age of our patients was 38 years. In participants who were positive for *hTERT* expression, we detected the 144-bp fragment (Fig. 1). The five *ABL* gene products (200-bp fragment) detected with RT-PCR are shown in Fig. 2. Among the 73 samples, 43 were *hTERT*-positive (58.9 %) and 30 were negative (41.1 %). Figure 3 shows the proportion of *hTERT*-positive samples in different stages of CML (CP: 69.2 %; AT: 38.5 %; AP: 55.6 %; BP: 83.3 %). The expression of *hTERT* differed significantly between stages of CML ($p = 0.036$). However, we observed no significant differences ($p = 0.451$) in *hTERT* expression between the chronic phase and the accelerated or blastic phase, and between expression after treatment and the accelerated phase (although the

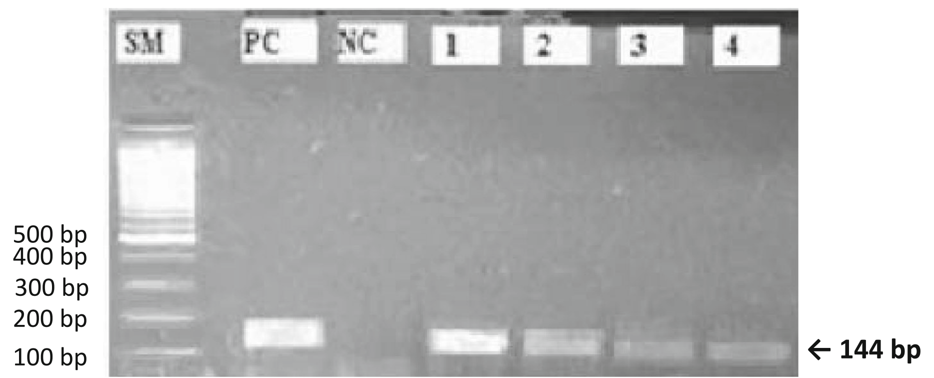

Fig. 1 Gel analysis of *hTERT* RT-PCR products. *SM* size marker; *PC* positive control; *NC* negative control

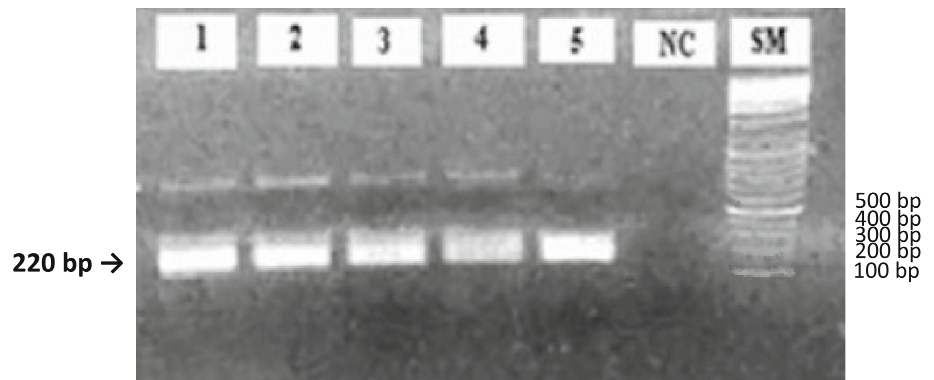

Fig. 2 Gel electrophoresis of five *ABL* gene products

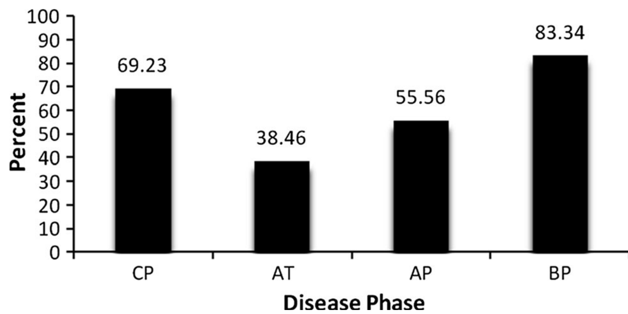

Fig. 3 *hTERT*-positive samples in different stages of chronic myeloid leukemia. *CP* chronic phase; *AT* after treatment; *AP* accelerated phase; *BP* blastic phase

Table 1 Forward and reverse sequences of *hTERT* and *ABL* primers

hTERT		
	5′ CGGAAGAGTGTCTGGAGCAA 3′	Forward
	5′ GGATGAAGCGGAGTCTGGA 3′	Reverse
ABL		
	5′ CGGCTCTCGGAGGAGACGATGA 3′	Forward
	5′ CCCAACCTTTTCGTTGCACTGT 3′	Reverse

Table 2 Laboratory variables and indicators in different phases of chronic myeloid leukemia

Phase	WBC (per mL)	Hb (g/dL)	Blasts (%)	Platelets (1,000/mL)
Chronic	120,600.00	10.69	1.88	366.96
After treatment	2,465.038	12.42	1.11	210.00
Accelerated	27,600.00	9.73	4.11	283.22
Blastic	25,880.83	9.27	11.41	148.75
Total	59,390.13	10.95	3.45	264.86

expression in each phase was higher than the former phase), but expression differed significantly between the posttreatment and blastic phase ($p = 0.010$). No significant differences in *hTERT* expression were found according to demographic parameters such as age and sex ($p = 0.72$).

We also analyzed blood indices such as WBC, RBC and platelet counts, hemoglobin and percentage of blasts. The highest WBC counts were seen in the chronic phase (120,600/mL) and the lowest counts in the accelerated phase (24,650/mL). Platelet count tended to be lower in the chronic phase (367,000/mL) and higher in the blastic phase (149,000 mL). The percentage of blasts was highest in the blastic phase (11 %) and lowest in accelerated phase (1 %). Table 2 shows the laboratory variables and indicators in the different phases of CML.

Comparisons of the laboratory variables with the Kruskal–Wallis test revealed statistically significant differences ($p < 0.0001$). We found no significant relationship between

WBC counts or blast percentage and *hTERT* expression ($p > 0.05$). However, platelet counts and hemoglobin levels differed according to gene expression ($p = 0.001$). In other words, platelet count was higher in *hTERT*-positive than in *hTERT*-negative patients, whereas hemoglobin concentration was lower in *hTERT*-positive than in *hTERT* negative patients.

Discussion

A study by Dawei et al. in patients with acute myelogenous leukemia and by Bie′che et al. in patients with breast cancer showed that 85 % of cancer cells have increased telomerase activity [13, 14]. A wide range of studies in different solid tumors (for example, Kirkpatrick et al. in breast cancer) reported a significant association between *hTERT* expression and telomerase activity [15].

Research by Ohyashiki et al. showed that shorter telomere length and greater telomerase activity are always associated with disease severity in hematological neoplasms such as recurrent leukemia and high-grade lymphoma [7]. In addition, an analysis of telomerase activity by Ghaffari and colleagues in patients with acute promylocytic leukemia found that telomere length and telomerase activity were significantly different in the group with recurrent disease compared to patients with recently diagnosed APL. In other words, the telomeres in patients with recurrent disease were significantly shorter, and survival rate was lower [16, 17].

We found significant differences in the PCR results between the four stages of CML ($p < 0.05$). Although a difference was found between the blastic (83.3 %) and chronic phase (69.2 %), this difference was not statistically significant. A possible explanation for the lack of significance is the low number of samples in the blastic phase. Verstovsek et al., unexpectedly, were unable to find significant telomerase activity in the blastic and accelerated phases and suggested small sample size as a likely explanation [18].

The significant difference between the chronic phase and the posttreatment group in the present study ($p < 0.05$ %) suggests that treatment reduced the percentage of *hTERT* PCR positivity. This important finding may have several explanations. First, malignant cells disappeared after treatment, and as a result mRNA content decreased to levels that were undetectable by RT-PCR. Normal cells lack *hTERT* mRNA, or if it is present, it appears only at very low levels. In this connection, Yamada et al. reported that *hTERT* gene expression in the K562 cell line was markedly decreased after treatment with imatinib, a finding consistent with our results. It should be noted that the main treatment protocol for all patients was imatinib.

A significant relationship in *hTERT* expression between the blastic and accelerated phases is a hallmark for *hTERT* mRNA in malignant cells. As drug-resistant cells increase, PCR positivity also increases in the blastic phase, with even greater increases than in the chronic phase. Thus RT-PCR positivity after treatment is likely to be a prognostic marker for the progression of CML [20–22].

It is important to note that a number of studies found that in patients with CML, the telomeres are shorter than in controls. For example, Bagheri et al. reported that telomere loss as the blastic phase begins may be a marker of disease severity and may thus provide information of significance for the prognosis. Moreover, both telomerase activity and telomere length play an important role in the progression of CML, so measuring these features in recently diagnosed patients can provide valuable prognostic information [23].

The high capacity for cell proliferation in chronic phase CML may induce progressive telomere shortening, which can be caused by cell division in hematopoietic cells without a clear increase in telomerase activity. Furthermore, in the blastic phase *hTERT* mRNA levels are significantly elevated; one possible reason is progressive telomere shortening at the critical point, which helps cells with unregulated telomerase activity to survive, as confirmed by Ohyashiki et al. [24]. Additionally, high levels of *hTERT* can be resistant to treatment with imatinib.

For example Yamada et al. reported that K562 leukemia cells with increased telomerase activity were resistant to this drug [22]. This finding and other studies [12, 21, 25] suggest that the evaluation of *hTERT*, telomerase activity and telomere length during CML disease progression could be used as a prognostic factor in the pursuit of better treatments for the disease in clinical practice.

Other notable findings in our study were the mean WBC and platelet counts, percentage of blast cells and hemoglobin content in our patients. These indicators were significantly elevated in different stages of the disease (chronic, after treatment, accelerated and blastic), and are associated with *hTERT* PCR-positivity in these phases. The differences between phases were not statistically significant. Moreover, our results confirmed the finding by Verstovsek et al. that there was no significant relationship between telomerase activity in the chronic phase and age, hemoglobin, platelet, WBC or blast counts [21]. In addition, mean WBC count and the percentage of blast cells did not show a statistically significant relationship in the PCR results for the entire sample ($p < 0.05$), i.e. there were no significant differences in mean WBC count or the percentage of blast cells between the total sample and PCR-positive or -negative samples.

However, the differences in hemoglobin and platelets were significant: PCR-positive individuals had a higher mean platelet count and lower hemoglobin level than PCR-

negative patients. In this connection, a study by Jin Huh et al. showed that *hTERT* mRNA expression was unrelated to sex, WBC count or the percentage of blasts, but was associated with age in patients with AML [19]. In the present study we found no significant correlation between the PCR results and patients' age or sex. It is noteworthy that the percentage of women with PCR-positive results (75.0 %) was higher than in men, despite the smaller number of women than men in our sample; this may reflect a positive effect of estrogen on *hTERT* expression [26].

Conclusion

The expression of *hTERT* differed significantly among the four phases of CML. Therefore, *hTERT* expression is a potentially valuable prognostic marker for the follow-up of patients with CML, although more studies are needed with larger sample sizes. Furthermore, the *hTERT* subunit can act as a potential therapeutic target in different stages of CML.

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