

Human Telomerase Reverse Transcriptase (hTERT) Expression and Telomerase Activity in Acute and Chronic Lymphocytic Leukemia Patients

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ABSTRACT

Telomerase activity is crucial for pathogenesis and progression of some hematological malignancies. Telomerase is composed of human telomerase reverse transcriptase (hTERT) and RNA component (hTR). Our aim was to determine hTERT expression and Telomerase activity in acute and chronic lymphocytic leukemia patients (ALL, CLL) and to study their correlation with clinical, hematological and available prognostic parameters. This case control study was conducted on 43 patients (24 B-ALL, 19 B-CLL), and 24 age and sex matched controls. Real-time quantitative polymerase chain reaction and PCR-based telomeric repeats amplification protocol were used for determination of hTERT expression and Telomerase activity, respectively. hTERT was expressed in 20/24 (83.3 %) ALL patients, 12/19 (63.15%) CLL patients with no significant difference in the incidence or the median expression of hTERT among both groups. ALL had a significantly higher telomerase activity compared to both CLL and controls, while the latter 2 groups showed no significant difference. Telomerase activity wasn't correlated with hTERT expression. hTERT expression was positively correlated with BM blast % in ALL patients and with the % of CD38 ($p = 0.001$) and ZAP 70 % ($p = 0.001$) in CLL patients. Telomerase activity was correlated positively with the absolute lymphocytic counts in CLL ($p = 0.021$) and negatively with the patient's age of both groups ($p=0.003$). Developing new targeted therapy directed against telomerase and/or hTERT could ultimately be of special benefit in patients with high telomerase activity and/or hTERT expression among ALL and CLL patients.

Keywords: Telomerase activity, hTERT expression, B-ALL/CLL, qRT-PCR; TRAP

INTRODUCTION

Telomeric protective sequences which are located at the ends of each chromosome, shorten after each cellular replication. When telomere shortens to a critical point, DNA damage pathways are turned on resulting in cellular apoptosis. Telomere erosion and replicative apoptosis is a tumor-suppressive machinery.¹

Telomerase enzyme which is responsible for telomeric ends expansion neutralizes the previous mechanism.² The two components of Telomerase module are; human telomerase reverse tran-

scriptase (hTERT) and RNA template named human telomerase RNA component (hTR). hTERT expression level are strongly associated with telomerase activity.³

The majority of malignant tumors endure high telomerase activity, empowering the cells to escape apoptosis and divide infinitely.⁴ Telomerase activity appears as a fundamental contributor in the pathogenesis and progression of some hematological malignancies whereas in other hematologic neoplasm telomerase activity is not the initiator of disease, meanwhile it is related to progressive disease, worse prognosis, or chemotherapy resistance.⁵

B-cell chronic lymphocytic leukemia (B-CLL), which is one of the most prevalent adult leukemias, uniquely manifests with extremely diverse clinical course, whereas a category of patients deteriorate and die within few months from diagnosis, others live for many years with minimal or no treatment and may die from a cause other than CLL. Detailed prognostic profile of B-CLL patients and identification of novel prognostic markers is crucial for individually tailored therapeutic approaches.⁶

Acute lymphoblastic leukaemia (ALL) is the most prevalent malignancy in childhood representing 80% of childhood leukaemia. Scarce studies have studied telomerase activity or hTERT in paediatric leukaemic malignancies.^{7,8} Although prognosis of ALL in childhood was enhanced with new evolved chemotherapeutics, still a category of ALL patients with failure of remission induction or early relapse represent a poor prognostic challenge. It is postulated that new prognostic markers and novel therapeutic targets might contribute to improved therapeutic policies.

The objective of this study was to determine hTERT expression and Telomerase activity in B-ALL and B-CLL patients and to study the correlation of hTERT expression and Telomerase activity with different clinical, hematological and available prognostic disease parameters.

PATIENTS AND METHODS

This study was conducted on 43 patients, 24 with B-acute lymphoblastic leukemia (B-ALL) and 19 patients with B- chronic lymphocytic leukemia (B-CLL), as well as 24 age and sex matched normal controls who attended the Out Patients Clinic of General Surgery for elective repair of hernia. These patients attended Kasr El Aini Hospital-Cairo University, to be diagnosed in the Clinical Pathology Department. Informed consent was obtained from each participant or his/her guardian before being enrolled in this study. The study was approved by the ethics committee of Cairo University. Detailed patients' characteristics are shown in Table 1.

RNA isolation and cDNA preparation

Total RNA was purified using the RNeasy Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's protocol.

The integrity and the relative amount of RNA were evaluated through agarose gel electrophoresis and spectrophotometric measurements, respectively. RNA was retro-transcribed into cDNA using Superscript™ First-Strand Synthesis Systems for RTQ-PCR (Life Technologies) according to the manufacturer's instructions. Synthesized cDNA was stored at -80°C till used.

Determination of hTERT expression by real-time quantitative polymerase chain reaction (qRT-PCR)

QRT-PCR was performed for hTERT and the endogenous control (GAPDH) on Applied Biosystems 7300 thermocycler (Qiagen, CA, USA) using specific primers and fluorogenic probes as described before.⁹ In brief, the 20 µL amplification reactions contained 7 µL cDNA, 10 µL of the Universal Taqman 2X PCR mastermix (Applied Biosystems) and 1.0 µL of each of the specific primers and probe. The thermal cycling conditions were set as follows; 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 minute. All reactions were performed in duplicate and the average cycle threshold (CT) was obtained. PCR controls without reverse transcriptase was routinely used as negative control. Results were provided as normalized hTERT expression, using the $\Delta\Delta C_t$ method (the amount of target, normalized to an endogenous reference (GAPDH) and relative to the reference), target amount = $2^{-\Delta\Delta C_t}$ where $\Delta\Delta C_t = [C_t(\text{hTERT target}) - C_t(\text{GAPDH target}) - C_t(\text{hTERT reference}) - C_t(\text{GAPDH reference})]$ ⁹.

Determination of telomerase activity by PCR-based telomeric repeats amplification protocol (TRAP)

Peripheral blood mononuclear cells were tested for the presence of telomerase activity using telomerase PCR-ELISA (Roche Diagnostic GmbH) as reported previously.¹⁰ In short, each TRAP reaction contained 25 µl reaction mixture (Tris-buffer, containing telomerase substrate, primers, nucleotides, and Taq polymerase), 2 µl cell extract (corresponding to 2×10^3) and sterile water to make 50 µl. The reaction mixture was incubated at 25°C for

Table 1. Clinical and laboratory data of the studied patients

Variable	ALL (24)	CLL (19)	Total
Sex, Number (%)			
Male	17 (58.6)	12 (41.4)	29 (67.4)
Female	7 (50.0)	7 (50.0)	14 (32.6)
Age, years			
Median, Range	24.0 (13-60)	60 (40-79)	31.0 (13-79)
Splenomegaly, Number (%)			
Yes	10 (55.6)	8 (44.4)	18 (41.9)
No	14 (56.0)	11 (44.0)	25 (58.1)
Hepatomegaly, Number (%)			
Yes	3 (75.0)	1 (25.0)	4 (9.3)
No	21(53.8)	18 (46.2)	39 (90.7)
Lymphadenopathy, Number (%)			
Yes	6 (50.0)	6 (50.0)	12 (27.9)
No	18 (58.1)	13 (41.9)	31 (72.1)
Platelet X 10 ⁹ /L (Median,Range)	129 (18-292)	123 (10-1116)	129 (10-1116)
Hemoglobin, g/dl			
Mean ± S. D.	10.5± 2.4	9.7± 2.7	10.2± 2.5
TLC, x10 ³ /μl (Median, Range).	7.0 (0.7-817)	48.7 (9.3-626)	15.3 (0.7-817)
Newly diagnosed	13(54.2 %)	9 (100 %)	-
Relapsed	5 (20.8 %)	0	-
Remission	6 (25 %)	0	-
PB blast % (Median, Range)	24.0 (0-100)	-	-
BM blast % (Median, Range)	86.0 (0-100)	-	-
Lymph% (Median, Range)	-	83.0 (57.0-98)	-
ALC x 1000 (Median, Range)	-	29.4 (5.3-330.2)	-
Zap70 (Median, Range)	-	40.0 (4-80)	-
CD38 (Median, Range)	-	40.0 (2-80)	-
Quantitative hTERT			
Median, Range	2.2 (0-61)	2.6 (0-48.1)	2.6 (0-61)
Qualitative hTERT			
Yes	20 (62.5)	12 (37.5)	32 (74.4)
No	4 (36.4)	7 (63.6)	11 (25.6)
Telomerase activity			
Median, Range	39.5 (0-348)	19.5 (0-1132)	29 (0-1132)

ALC, absolute lymphocytic count

30 minutes and heated at 94°C for 5 minutes. Then PCR amplification for 30 cycles was performed. Each cycle consisted of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and polymerization at 72°C for 90 seconds, followed by extension at 72°C for 10 minutes. After PCR, 5 μl of the PCR product was incubated with 20 μl of denaturation reagent at room temperature for 10 minutes, vortexed briefly with 100 μl hybridization buffer. One hundred μl of the mixture was transferred to each well of the precoated microplate, then incubated at 37°C on a shaker for 2 hours,

washed 3 times and 100 μl Anti-Dig- POD were added. After washing substrate solution was added and incubated at room temperature for 20 minutes. Using an ELISA reader, the absorbance values of the samples were measured at 450 nm within 30 minutes after 100 μl of stop reagent was added. The mean absorbance of the negative controls was subtracted from those of the samples. Samples are regarded as telomerase- positive if the difference in absorbance (Δ A) is higher than 0.2. Negative samples were repeated.

Table 2. Comparison of different parameters in association with the hTERT expression pattern among the studied CLL patients

CLL group variants	Negative hTERT expression (7)			Positive hTERT expression (12)			p
	Median	Minimum	Maximum	Median	Minimum	Maximum	
Age, years	60.0	50.0	70.0	58.0	40.0	79.0	0.482
Hb g/dl	8.5	4.0	12.3	10.6	4.8	12.9	0.494
TLC x 1000	49.5	16.0	626.0	40.2	9.3	355.0	0.616
Plat x 1000	47.0	10.0	298.0	136.5	10.0	1116.0	0.385
Lymph%	76.5	60.0	98.0	85.0	57.0	93.0	0.340
ALC x 1000	35.5	11.2	212.7	32.0	5.3	330.2	0.616
Zap70%	9.0	4.0	53.0	50.0	5.0	80.0	0.964
CD38%	10.5	2.0	68.0	59.0	2.0	80.0	0.020*
Telo A	14.5	0.0	1132.0	21.0	5.8	130.0	0.098

ALC= Absolute lymphocytic count, Telo A= Telomerase Activity, *= p< 0.05 statistically significant

RESULTS

hTERT was found to be expressed in 20 out of the 24 (83.3%) ALL patients and 12 out of 19 (63.15%) CLL patients with no significant difference in the incidence of expression among the ALL and CLL groups ($p= 0.722$). The median expression of hTERT was 2.2 (range 0.0-61) in the ALL group, and 2.6 (range 0.0-48.1) in the CLL group with no significant difference ($p= 0.132$). Control group did not show any expression of hTERT.

ALL patients showed a significantly higher median telomerase activity (39.5, range 0.0-348) compared to both CLL patients (19.5, range 0.0-1132) and controls (13, range 1.2-64), ($p< 0.001$), while the latter 2 groups showed no significant difference regarding telomerase activity.

Among CLL patients, telomerase activity values weren't correlated with hTERT expression values ($p= 0.923$, $r= 0.024$), while for ALL patients this was just on the cutoff ($p= 0.05$, $r= 0.40$).

Comparing between CLL patients with positive hTERT expression versus those with negative expression regarding different clinical and laboratory variants, showed a significantly higher percent of CD38 in hTERT positive CLL group ($p= 0.02$). No other significant difference could be elicited regarding (patient's age, hemoglobin percent, platelets count, total leucocytic count, lymphocyte %, absolute lymphocytic counts or ZAP 70 %) between the 2 groups, despite the median Telomerase

activity was higher among the hTERT positive group, however this couldn't reach a statistical significance ($p= 0.098$) may be due to small sample size (Table 2).

The previous comparative study couldn't be applied for the ALL patients group as the number of ALL patients with negative hTERT expression was too small for statistical analysis.

Comparing different categories of ALL or CLL patients regarding the level of hTERT expression as well as the level of telomerase activity, didn't show any significant difference, this was elicited for the following categories (males/females, patients with organomegaly/patients with no organomegaly, patients with lymphadenopathy/ patients with no lymphadenopathy) (Table 3).

Concerning ALL patients; comparison between newly diagnosed, relapsed and those in remission as regards expression of hTERT and telomerase activity, showed no statistically significant difference ($p= 0.079$, 0.64) respectively, despite the median hTERT expression was evidently lower among the remission group, however this couldn't reach a statistical significance ($p= 0.079$) (Table 3).

hTERT expression was positively correlated with blast % in bone marrow (BM) of ALL patients ($p= 0.044$), with the % of CD38 ($p= 0.001$) and ZAP 70% ($p= 0.001$) in peripheral blood (PB) of CLL patients, otherwise hTERT expression was not correlated with any other variable. Telomerase activ-

Table 3. Comparison of different categories of ALL and CLL patients regarding the level of hTERT expression as well as the level of telomerase activity

	No	hTERT expression			p	Telomerase Activity			p	
		Med	Min	Max		Med	Min	Max		
Sex	Female	14	3.0	0.1	61.0	0.264	32.5	0.0	153.0	0.678
	Male	29	1.3	0.0	52.7		29.0	0.0	1132.0	
Hepatomegaly	Y	4	3.4	0.0	42.0	0.702	25.3	0.0	58.0	0.732
	N	39	1.8	0.0	61.0		29.0	0.0	1132.0	
Splenomegaly	Y	18	3.0	0.0	61.0	0.204	23.5	0.0	153.0	0.398
	N	25	1.0	0.0	50.2		30.0	0.0	1132.0	
Lymphadenopathy	Y	12	3.0	0.0	52.7	0.243	26.0	0.0	348.0	0.698
	N	31	1.2	0.0	61.0		29.0	0.0	1132.0	
ALL group	Newly diagnosed	13	3.0	0.0	61.0	0.079	39.0	0.0	153.0	0.638
	Relapsed	5	3.0	0.0	50.2		48.0	25.0	348.0	
	Remission	6	0.5	0.0	2.6		35.5	12.2	48.0	

Y= Yes, N= No, Med= Median, Min= Minimum, Max= Maximum

ity was positively correlated with the absolute lymphocytic counts among CLL group ($p= 0.021$) and negatively correlated with the patient's age of both leukemia groups ($p= 0.003$), otherwise it was not correlated with any other variable in both ALL and CLL patients, in addition no correlation was elicited between Telomerase activity and % of CD38 or ZAP 70 among CLL patients) (Table 4).

DISCUSSION

Telomerase regulation is essential component in cellular aging and cancer. hTERT is crucial controlling subunit of telomerase in various cells. In this study we analyzed both Telomerase activity and hTERT expression in context with clinical, hematological as well as prognostic parameters among two groups of ALL and CLL patients.

The current study encountered hTERT expression in 83.3% of ALL patients and 63.15% of CLL patients, and none of the controls, with no difference in the incidence of expression among the two leukemia groups. In agreement, Tchirkov et al reported nearly the same incidence as ours (59%) for the expression of hTERT gene among 90 B-CLL patients.⁹ Meanwhile Terrin et al, reported a higher

incidence (74.6%) of hTERT expression in 134 B-CLL patients.¹¹ Also, a recent study by Abdelrahman et al, reported that hTERT mRNA was found to be expressed in all studied BCLL patients and none of the normal controls.¹² These variant results may be referred to different amplified hTERT transcript as it has been established that hTERT has alternate splicing sites.¹³

As for ALL, considering the scarce studies that has analyzed hTERT expression in context with the ALL phenotype, Kleideiter et al. reported a near-by incidence of expression as ours (90%, 18/20) among T-ALL patients.¹⁴ On the other hand Nowak et al¹⁵, reported the expression of hTERT in all the ALL pediatric patients in their study, and stated that majority of healthy individuals did not show any expression of telomerase components.

Our data showed no significant difference in telomerase activity between CLL patients and controls, while ALL patients had a significantly higher telomerase activity compared to both groups. All our reviewed studies have established that telomerase activity was elevated in ALL patients compared to healthy individuals.¹⁵⁻¹⁷ As for CLL patients, in support to our findings, Trentin et al reported that unlike most cancer cells, telomerase activity is not

Table 4. Correlative study between different parameters of ALL/CLL patients in association with the level of hTERT expression as well as the level of telomerase activity

		q-hTERT expression		Telomerase Activity	
		r	p	r	p
ALL, CLL groups	Age, years	-0.149	0.340	-0.442	0.003
	Hb g/dl	0.150	0.341	-0.160	0.311
	TLC X 1000	0.017	0.916	-0.026	0.871
	Plat X 1000	-0.109	0.492	-0.215	0.172
ALL group	Blast % PB	0.374	0.079	0.255	0.240
	Blast %BM	0.423	0.044	0.246	0.259
CLL group	Lymph %	0.107	0.662	0.276	0.253
	ALC X 1000	-0.018	0.943	0.526	0.021
	Zap70	0.739	0.001	0.126	0.629
	CD38	0.712	0.001	-0.174	0.503

up regulated in CLL patients.¹⁸ Also it is demonstrated that telomerase activity is not vital for the origination of B-CLL, but it is essential for its maintenance.¹⁹

This study found no correlation between hTERT expression and telomerase activity in ALL or CLL patients. In accordance, Nowak et al, opposed any correlation between the degree of hTERT amplification and telomerase expression or activity among ALL patients.¹⁵ In contrast, it is reported that levels of hTERT mRNA significantly correlated with telomerase activity in ALL¹⁴ and CLL.²⁰ To our explanation these discordant results between expression of hTERT and telomerase activity could be explained by transcriptional control of hTERT. As hTERT is alternatively spliced in heterogenous sites, these variant transcripts might create controlling machinery for telomerase activity, to be noted that spliced variants lacking a and b regions which carry the reverse transcriptase domains, can't encode a functional protein.¹³ It is worth mentioning that differential expression of hTERT splice variants is encountered in ALL at diagnosis as well as in remission.¹⁶

We encountered a significantly higher percent of CD38 in hTERT positive expression CLL group, with no significant difference regarding age, total leucocyte count, lymphocyte %, absolute lymphocytic counts or ZAP 70 between hTERT positive and negative expression groups. In confirmation to our findings, hTERT expression values were signif-

icantly higher in the CD38 positive CLL samples, meanwhile the same study reported that hTERT levels were higher in ZAP-70 positive cases than in the ZAP-70 negative cases despite being statistically insignificant.¹¹

The present work didn't encounter any significant difference either in expression of hTERT or in telomerase activity among ALL patients groups (newly diagnosed, relapsed and remission). To our conclusion, the evidently lower hTERT expression in ALL patients who were in remission couldn't reach a statistical significance owing to the small number of the remission group (6 patients). Nowak et al, reported that telomerase activity in ALL patients in remission was at the level equivalent to that of normal individuals, and they explained the severe reduction of telomerase activity in remission by the elimination of blasts cells by chemotherapy.¹⁵

hTERT expression was positively correlated with BM blast % of ALL patients as well as with CD38 and ZAP 70 among CLL patients. Telomerase activity was positively correlated with the absolute lymphocytic counts among CLL group and negatively correlated with the patient's age of both leukemia groups, otherwise neither hTERT expression nor Telomerase activity shows correlation with any other studied variable. Vural et al, stated that the expression levels of hTERT was higher in ZAP-70 positive CLL patients however this wasn't statistically significant and added that no significant correlation between hTERT with CD38 or ZAP70 was

reported, only significant correlation with lymphocyte count and age of the patients.²¹ Also, hTERT mRNA was not correlated with hematological parameters or ZAP-70, as reported before.¹² In concordance to the current study, telomerase activity was correlated significantly with white blood cell and lymphocyte count among but not with bone marrow cellularity or other patient characteristics in CLL patients, as signified by Verstovsek et al.¹⁹

The limitation of the current work is the small patients' number, as we expect some correlative findings may prove to be significant if applied on larger patients' numbers. We recommend performing larger studies including full prognostic profile of patients that could establish sequential serial detection of hTERT expression and Telomerase activity starting from diagnosis, treatment, remission/resistance and follow up phases as this would help in identifying the actual role of these parameters in disease progression and prognosis.

One of the insights for future researches, is to evaluate hTERT expression and Telomerase activity in context with chemo-resistance in different groups of acute and chronic lymphocytic leukemia as par example; ALL patients who show poor response to hyper-CVAD, being the most frequently prescribed protocol for ALL adult patients.²²

As telomerase activity tends to decline obviously during ALL remission phase, consequently it could be further evaluated in larger studies as one of the surrogate parameters for monitoring remission in ALL patients.

As CLL patients with high hTERT expression was reported to have poorer response to usual lines of therapies.¹¹ Developing hTERT targeted new therapeutic approaches could ultimately be of special benefit in these patients. Larger studies are still needed to validate the prognostic significance of hTERT expression in different malignancies and open the possibility of the potential success of hTERT targeted therapy. Also the established higher telomerase activity among certain malignancies lightens a rationale for targeting telomerase therapeutically in these diseases.

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