

Expression Pattern of Telomerase Reverse Transcriptase (hTERT) Variants and Bcl-2 in Peripheral Lymphocytes of Systemic Lupus Erythematosus Patients

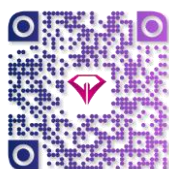
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KEYWORDS

Apoptosis,
Lymphocyte,
Systemic Lupus Erythematosus,
Telomerase

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Main Subjects:
Molecular Pathology

Received 09 July 2019;
Accepted 10 April 2020;
Published Online 21 Apr 2020;

doi:10.30699/ijp.2020.110994.2187

ABSTRACT

Background & Objective: It is not clear whether activated lymphocytes of patients with systemic lupus erythematosus (SLE) are more proliferative or less apoptotic. We aimed to delineate potential differences between B and T cells of SLE patients compared to healthy controls regarding the telomerase activity and apoptosis status.

Methods: In this cross-sectional case control study, Blood samples were taken from 10 SLE patients and 10 healthy controls. B and T cells were separated using magnetic cell sorting system. Telomeric repeat amplification protocol (TRAP) assay and real-time PCR were used to determine the telomerase activity and the expression of alternatively spliced variants.

Results: Four patients under treatment showed significant telomerase activity in their T cells. Four of the newly diagnosed patients showed telomerase activity in their B cells (20% of all patients and 40% of new onset patients). There was no specific pattern of human telomerase reverse transcriptase variant expression within the patients' lymphocytes. A significantly reduced expression of *Bcl-2* was detected in B cells ($P=0.018$) and a trend toward lower *Bcl-2* expression in T cells was seen in SLE patients compared to healthy controls.

Conclusion: Although not definitive, our results may suggest that B cells may have more active roles during the earlier phases of the disease attack, while T cells take over when the disease reaches its chronic stages.

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Introduction

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease that can cause dysfunction of multiple body organs such as the skin, joints, nervous system, kidneys, heart and hematopoietic system. Much evidence shows contribution of both adaptive and innate immunity in the pathogenesis of the disease, while T cell dependent B cell activation has a central role in this scenario (1,2).

Like other cells, proliferation and expansion of the lymphocytes is dependent on the activation of the cell cycle, for which stability of chromosomal telomere is critical. Maintenance of telomere by itself relies on the function of telomerase enzyme complex which is composed of an RNA subunit, human telomerase

RNA (*hTR*), and a protein component, human telomerase reverse transcriptase (*hTERT*) (3). Literature shows the existence of multiple alternative splicing variants for *hTERT* which take part in regulation of telomerase activity (4,5). Studies on different cell types have shown that spliced variants α deletion and β deletion are the most abundant variants of *hTERT* (6).

Subsequent to lymphocyte activation and proliferation, different mechanisms may act to subside the activation status of these cells and keep homeostasis. Apoptosis is one of the mechanisms which can be triggered by different pathways (7). External pathway initiates by interaction of the ligands

to death receptors of the cells, while the internal pathway originates from the mitochondria. Several factors contribute to the apoptosis process and control of the pathways. Within the intrinsic pathway, some members of *Bcl-2* family act as pro-apoptotic factors, while others work to halt the pathway (8).

We hypothesized that in SLE patients, the variation in factors controlling proliferation and survival of lymphocytes may contribute to improper activation of the lymphocytes and hence autoimmunity. Therefore, in this study we investigated the possible variation in the expression of the alternative splicing forms of *hTERT* and correlated them with the findings related to the expression of *bcl2*, anti-apoptotic factor in the same patients and compared them with the results obtained from healthy individuals.

Materials and Methods

Patients and Controls

Ten female patients who were admitted in Hafez hospital, Shiraz, Iran, from January to May 2015 with definite diagnosis of SLE and ten healthy volunteers were recruited in this study. Co-existence of malignancy was considered as the exclusion criteria. Of them, five patients were newly diagnosed SLE patients who had not received immunomodulatory medications. The other five patients were under treatment with medications. Healthy controls were women in the same range of age as the patients and had not received immunosuppressive medications during or before the study, or had any chronic inflammatory conditions such as atherosclerotic cardiovascular disease, Diabetes mellitus, and degenerative joint disorders. The study was explained for them and a written consent was taken from all the participants. The study was approved by the ethics committee of Shiraz University of Medical Sciences. Peripheral blood samples of 20 mL were taken by venipuncture with EDTA solution (10 μ L of 0.5 M stock) as anticoagulant.

Isolation of B and T cells

Peripheral blood mononuclear cells (PBMC) were isolated using gradient density centrifugation on ficoll-Hypaque (Lymphodex, Inno-Train). The layer of mononuclear cells was isolated, and the number of isolated cells was determined using a hemocytometer.

CD3+ and CD19+ cells were purified from the PBMCs using magnetic micro-beads coated with anti-CD3 or CD19 antibodies, respectively, according to the manufacturer's instruction (Miltenyibiotec, Germany).

Flow Cytometry

Flow cytometry analysis was used for further characterization of the purified cells using Fluorescein

isothiocyanate (FITC) conjugated mouse anti-human monoclonal antibodies against CD3, CD4, CD45 and Phycoerythrin (PE) conjugated antibodies against CD8, CD14, CD16, CD19 and CD56, in different combinations. All antibodies were purchased from Abcam, UK. The cells were suspended in FACS buffer containing 2% fetal bovine serum and stained with appropriate amounts of antibodies at 4°C in dark. Mouse IgG1 and IgG2 isotopes antibody controls were used as negative control to determine the possible non-specific staining. A four color FACS Caliber instrument with Cell Quest Pro acquisition software (BD, US) was used to test the cells. Flow data were analyzed and graphically presented by Win MDI software.

Quantitative RT-PCR

The total RNA of the purified cells was extracted by Trizol reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. RNA samples were treated with DNase I (Fermentas, Vilnius, Lithuania) to remove the genomic DNA contamination. cDNA was synthesized from 5 μ g of the total RNA with the Revert Aid H minus First Strand cDNA Synthesis Kit (Fermentas, Lithuania).

The *hTERT* variant transcripts were measured by real time PCR, using specific primer sets for each variant (α -deletion, β -deletion, $\alpha\beta$ -deletion and Full length) (Table 1) and sybr green 1 (Applied Biosystems, USA) as reporter dye on Chromo4 Detector thermal cycler (Bio-Rad, USA). Primer-Blast online freeware (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) was used to design specific primers. Expression of β -actin was used as a housekeeping gene in analysis.

Figure 1 shows the location of deletions and the binding sites of the designed primers on *hTERT* mRNA.

PCR reactions were set up in a final volume of 20 μ L reaction. The mixture contained master mix, 0.5 μ g of cDNA and 100nM of each primer. The cycling program comprised of an initial denaturation at 95°C for 10 min and 45 cycles of denaturation at 95°C for 15s, annealing at 60°C for 50s, extension at 78°C for 34s. Fluorescence emission was collected at the end of the extension time. The specificity of amplifications was confirmed by melting curve analysis. Relative expression of *hTERT* variants was calculated by $2^{-\Delta\Delta Ct}$ formula.

Relative expression of *Bcl-2* was relatively quantified against 18sRNA housekeeping gene in the purified B cells and T cells of patients and healthy controls by real time PCR. Table 1 displays the sequence of forward and reverse primers used for analysis of expression pattern of the mentioned genes.

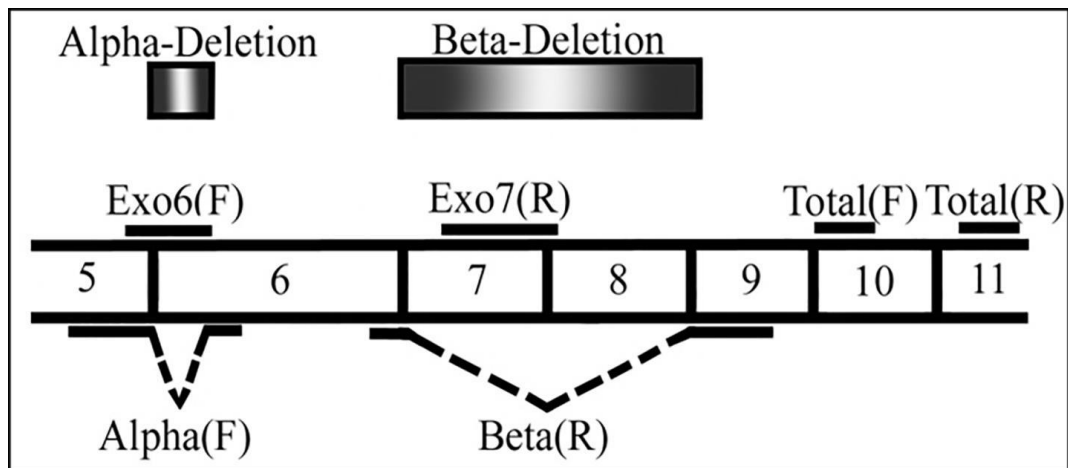


Fig. 1. Position of the primers on the hTERT mRNA and deletion sites of different variants of hTERT

Table 1. The sequence of primers used for Real Time PCR in relative quantitation of *hTERT* variants in purified cells and apoptotic factor (*Bcl-2*) in purified lymphocytes

Position	Sequence
<i>exo6</i> (F) primer	5'- TTG TCA AGG TGG ATG TGA CG -3'
Alpha (F) primer	5'- CTT TGT CAA GGA CAG GCT CA -3'
<i>exo7</i> (R) primer	5'- ATG TAC GGC TGG AGG TCT GT -3'
<i>beta</i> (R) primer	5'- GGA CGT AGG ACG TGG CTC T -3'
β - <i>actin</i> (F)	5'- ACA GAG CCT CGC CTT TGC CG -3'
β - <i>actin</i> (R)	5'- CAC CAT CAC GCC CTG GTG CC -3'
18sRNA (F)	5'-CGA ACG TCT GCC CTA TCA ACT T-3'
18sRNA (R)	5'-ACC CGT GGT CAC CAT GGT A-3'
<i>bcl-2</i> (F)	5'-ACG GAG GCT GGG ATG CCT TT-3'
<i>bcl-2</i> (R)	5'-CAA GCT CCC ACC AGG GCC AA-3'

Quantitative Determination of Telomerase Activity

To quantify telomerase activity in B and T cells, the telomeric repeat amplification protocol (TRAP) assay was performed using the TeloTAGGG Telomerase PCR ELISA kit (Roche, Germany), according to the manufacturers' instructions.

Statistical Analysis

The "one sample Kolmogorov-Smirnov" test was used to determine the distribution of data. Student *t*-test or by Mann-Whitney post-test was used to analyze the differences between the results based on data distribution. P value <0.05 was considered as statistically significant.

Results

Patient Data

SLE diagnosis of the patients who participated in this study was confirmed by clinical and laboratory

data. [Table 2](#) contains the demographic data and laboratory test results of the patients.

Purity of the Magnetic Cell Separation of T and B Lymphocytes

Magnetic beads and column were used to purify B and T lymphocytes from fresh blood samples. Flow cytometry analysis was used to assess the purity of the separated cells. Results showed that the mean purity of the isolated T cells was $98.4 \pm 0.8\%$, while $72.6 \pm 0.6\%$ of them were CD4+ cells and $20.6 \pm 0.5\%$ were CD8+ cells. CD19, CD16 and CD14 positive cells were all less than 0.5%. B-cell isolation also reached a similar purity and $87.4 \pm 0.9\%$ of the purified cells were found CD19+. There were less than 0.5% of CD16 and CD14 positive cells in those preparations. However, a small fraction of CD3+ cells ($8.4 \pm 0.4\%$) could be detected in some preparations ([Figure 2](#)).

Table 2. Demographic data and laboratory test results of the SLE patients

Patient no	Sex	Age	WBC	Lymph	Plt (×1000)	dsDNA	ANA	proteinuria	Clinical characteristics	Under treatment	Duration of treatment
1	F	36	2600	1200	180	+	+	+	Lower extremity edema	N	N/A
2	F	32	2800	1250	160	+	+	-	Oral lesion, rash	N	N/A
3	F	28	6400	3100	190	+	+	-	Poly arthritis, rash, photosensitivity	N	N/A
4	F	30	3200	1100	200	+	+	-	Rash, photosensitivity	Hydroxychloroquine, MTX	9 months
5	F	37	2500	1000	130	+	+	-	Poly arthritis, rash, photosensitivity	N	N/A
6	F	27	2000	950	140	+	+	-	Oral lesion, rash, photosensitivity	N	N/A
7	F	30	4800	2000	250	+	+	+	Lower extremity edema, photosensitivity, rash	Hydroxychloroquine, Prednisolone	3 months
8	F	41	3500	1550	200	+	+	-	Poly arthritis, photosensitivity	Hydroxychloroquine, prednisolone	3 months
9	F	36	4200	1950	170	+	+	-	Poly arthritis, rash, photosensitivity	Hydroxychloroquine, MTX	12 months
10	F	31	3000	1200	140	+	+	-	Oral lesion, rash	Hydroxychloroquine, MTX	16 months

Abbreviations: N, No; N/A, Not Applicable, MTX, Methotrexate

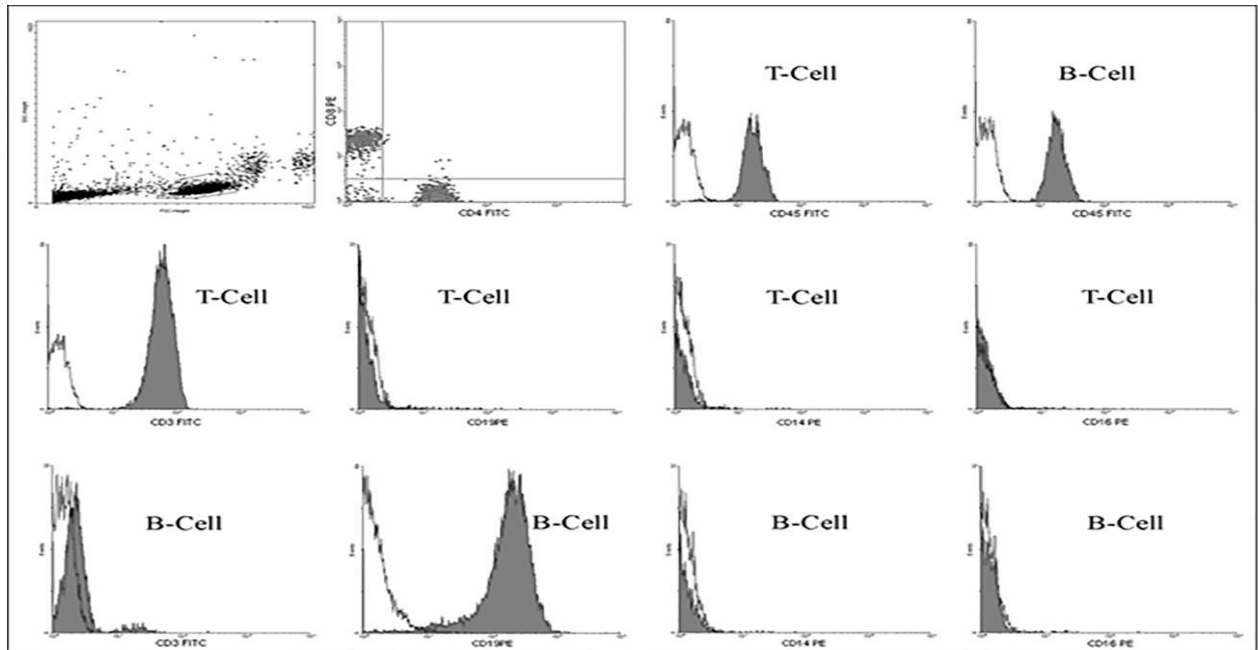


Fig. 2. FACS assessment of purity of B cells and T cells isolated with MACS. Mean purity of the isolated T cells is 98.4±0.8%, while 72.6±0.6% of them are CD4⁺ cells and 20.6±0.5% are CD8⁺ cells. CD19, CD16 and CD14 positive cells are all less than 0.5%. CD19⁺ cells constituted 87.4±0.9% of the purified B cells. There were less than 0.5% of CD16 and CD14 positive cells in B cell sample. A small fraction of CD3⁺ cells (8.4%±0.4%) could be detected in B cell sample.

Different Patterns of Telomerase Activity in SLE Patients

Telomerase activity in the purified B and T lymphocytes was studied by TRAP assay. Based on that, there was no considerable telomerase activity in the B and T lymphocytes purified from healthy controls. However, among the patients, four patients who were receiving therapy had telomerase activity in their T cells (equivalent to 20% of all patients or 40% of patients under treatment), while no detectable activity was found in their B cells. On the other hand, telomerase activity was seen only in B cells, but not T cells, newly diagnosed SLE (2 patients; equivalent to 20% of all patients or 40% of newly diagnosed patients).

Expression of *hTERT* Variants by Purified Lymphocytes

Real time PCR was used to analyze the *hTERT* variants' expression in the separated cells. Expression of *hTERT* was only observed in the samples that

showed telomerase activity, i.e. B cell samples from two recently diagnosed patients and T cells from two other patients who were receiving immunosuppressant medication for a while. Although a variable expression pattern of four variants of *hTERT* was demonstrated by telomerase positive samples, dominant variants could be distinguished in each cell type on the basis of lower Ct values. The dominant variants in B cells with positive telomerase activity were β -variant and α/β -variant, while in two T cell samples with evident telomerase activity, the variant of no deletion and β -variant were dominant forms.

Expression of *Bcl-2* as Anti-apoptosis Factor

Comparison of *Bcl-2* expression showed significantly reduced expression of *Bcl-2* in the B cells purified from SLE patients ($P=0.018$). Although T cells also showed decreased expression of *Bcl-2*, the difference was not statistically significant compared to healthy individuals ($P=0.55$) (Figure 3).

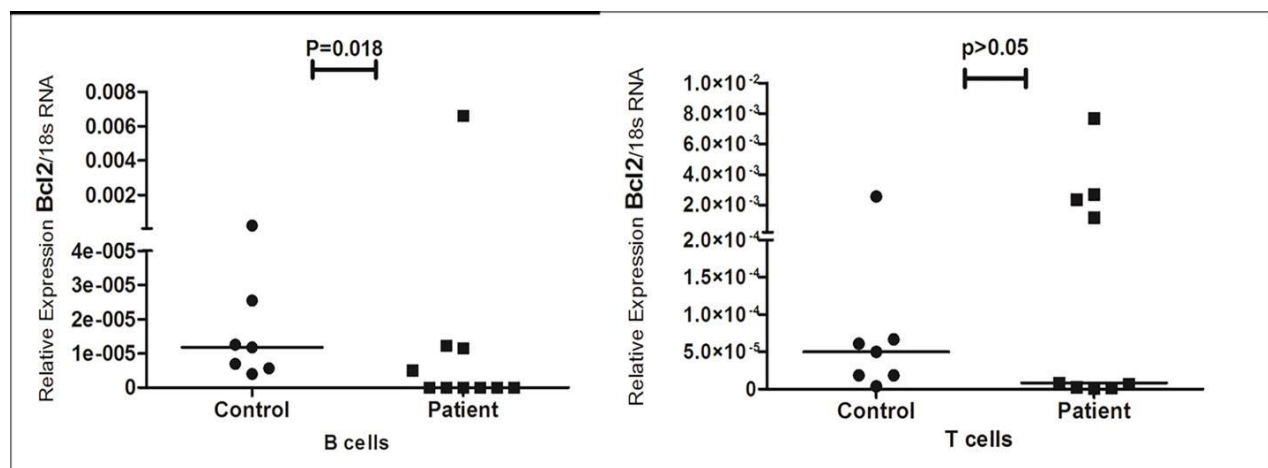


Fig. 3. *Bcl-2* expression in B cells and T cells of the control and SLE patients. *Bcl-2* expression in the B cells of SLE patients is reduced in comparison to healthy controls ($P=0.018$). Median *Bcl-2* expression in T cells of SLE patients is less than healthy controls although the whole expression is not significantly different.

Discussion

Our results indicated the existence of potential differences between SLE patients (at different disease stages) and healthy individuals regarding telomerase activity and *Bcl-2* expression in the T-B lymphocytes, in the absence of obvious correlations with specific *hTERT* splice variants. Although not definitive, our results may suggest that in SLE patients, B cells may have more active roles during the earlier phases of the disease attack, while T cells take over when the disease reaches its chronic stages. Higher apoptosis rate of the lymphocytes may be responsible for prolonged exposure of auto-antigens and participate in induction of autoimmunity. These suggestions need further evaluation.

Inappropriate activation and consequently survival of different subsets of the lymphocytes can result from turbulence in the homeostasis status of the immune system which causes autoimmunity. In general, resting peripheral blood lymphocytes like other somatic cells

have very low to undetectable telomerase activity (9,10). However, upon activation, the lymphocytes up-regulate the telomerase activity (9,11). In this regard, several lines of evidence showed elevated telomerase activity in the peripheral blood lymphocytes of SLE patients (12-14). Similarly, in this study, we were able to show higher telomerase activity and more mRNA expression of *hTERT* in B cells from patients with new onset of SLE. In the same way, T cells from the patients under treatment for SLE had apparently higher telomerase activity.

When we studied the variants of *hTERT* in telomerase positive cells, we could not show the correlation between the telomerase activity and the expression of the alternative variants of *hTERT* in the B cells and T cells purified from SLE patients. This contrasts with what Jalink *et al.* showed where higher telomerase activity in activated normal T cells was in association with expression of full-length *hTERT* variant (15).

Having telomerase activity in the B cells, but not T lymphocytes, from newly diagnosed patients and more telomerase activity in only T cells from patients with chronic disease may raise suggestions about the discrimination of these two phases of the disease. Probably, B cells may have more active roles during the earlier phases of the disease attack, while T cells take over when the disease reaches its chronic stages or after therapy, despite the undeniable role of T cells in activation of B cells in early stages of SLE. Continuous activation of T cells and multiple rounds of cell division during the course of the disease cause shortening of the telomere in spite of the telomerase activity (16). Shorter telomere length in T cells, and not B cells, from SLE patients compared to healthy individuals supports such speculation (17,18).

The inability of the immune system to eliminate the auto-reactive lymphocytes through apoptosis has been considered as one possible mechanism of induction of autoimmunity (19). In this regard, some studies correlated the prolonged T cell life span of SLE patients to increased *bcl-2* expression, as one of the main hallmarks of anti-apoptosis pathways (20,21). However, some studies showed increased apoptosis in the lymphocytes, particularly T cells from SLE patients (22-25), but with deficiency in the removal of the apoptotic debris (8,26,27). This can increase the chance of more exposure of the immune system to auto-antigens and induction of autoimmunity. We studied the expression of *Bcl-2* in the purified B and T cells of SLE patients vs. healthy controls by real time PCR. Our results showed a substantial reduction in the expression of *Bcl-2* gene in B cells and a non-significant reduction in T cells purified from SLE patients in comparison to healthy controls. These results are in line with the latter hypothesis of increased apoptosis of the lymphocytes in SLE patients. However, more detailed information is needed to reveal the malfunction of elements involved in this mechanism.

Our study had some limitation. The most important one is the small sample size. Results might be, however, useful as a platform for further evaluations in larger studies.

Conclusion

In conclusion, the altered pattern of telomerase activity in the B and T lymphocytes of SLE patients in addition to abnormal pattern of apoptosis or clearance of auto-antigens all together can make a platform of induction of auto-reactivity. Identification of defective elements in this pathway may provide potential therapeutic targets to restore the problem.

Acknowledgements

The authors would like to thank all the staff at the Institute for Cancer Research, Shiraz, IR Iran for their technical assistance.

Conflict of Interest

The authors declared that there is no conflict of interest regarding the publication of this article.

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Attar, A., Khosravi Maharlooei, M., Nazarnia, M., Hosseini, A., Bajalli, Z., Moeini, Y., Monabati, A., Amirmoezi, F., Jaberipour, M., Habibagahi, M. Expression Pattern of Telomerase Reverse Transcriptase (hTERT) Variants and Bcl-2 in Peripheral Lymphocytes of Systemic Lupus Erythematosus Patients. *Iranian Journal of Pathology*, 2020; 15(2): 225-231. doi: 10.30699/ijp.2020.110994.2187