Prognostic Significance of Telomere Length in newly diagnosed Chronic Lymphocytic Leukemia patients: measured By Flow-FISH technique

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published at 3/7/2017

Abstract:

Background: Telomeres are specialized nucleoprotein structures present at the ends of human chromosomes, and are constituted of a tandem of repeats TTAGGG. Telomere length measurement has been assessed by many previous studies In B-Chronic Lymphocytic Leukemia patients (B-CLL). Telomere shortening was found to correlate with disease progression. The aim of our study was to measure Telomere Length (TL) by Florescence In-Situ Hybridization coupled with Flow-cytometer (Flow-FISH) technique in newly diagnosed B-CLL patients. Explore its prognostic significance, through determination of the relationship between TL; with disease stage, other prognostic marker such as CD38 & ZAP-70 expression and patient response to treatment.

Methods: This study included 61 B-CLL patients in chronic phase and 67 normal healthy controls. Telomere Length (TL) was assessed in Lymphocytes by Flow-FISH technique.

Results: This work revealed a significant reduction of the Relative Telomere Length (RTL) in B-CLL patients than normal healthy control. RTL was significantly negatively correlated with CD38 and ZAP70 expression measured at patient diagnosis ***(p= 0.09& 0.017 respectively).*** Patients follow up was done by BMA after six months. We observed that patients who had higher TL, showed a better response to treatment ***(p> 0.001).***

Conclusion: Telomere length may have a prognostic relevance in B-CLL cases.

Introduction:

Telomeres are specialized nucleoprotein structures present at the ends of human chromosomes, and are constituted of a tandem of repeats TTAGGG. They play a crucial role in cellular homeostasis by maintaining genome stability and integrity. These telomeric functions cannot be attained unless telomere lengths are maintained at a level that allows telomeres to avoid chromosome end-to-end fusion, DNA cascade signaling, and genomic instability ***(lange et al., 2010)***.

The average length of telomeres can be assessed by using genomic DNA or cytological preparations ***(Samassekou, 2010)***. Both in-vitro and in-vivo studies have shown a negative correlation between telomere length

and cellular aging (***(Samassekou, 2013)***. In normal stem cells and germ lines, telomeres are maintained by telomerase enzyme, which counteracts the loss of telomeric sequences by adding telomere repeats at the 3' telomeric overhang ***(Abdallah et al., 2009)***.

B-cell chronic lymphocytic leukemia (B-CLL), one of the most common adult leukemia, is characterized by a highly heterogeneous clinical course. Some patients rapidly progress and die within a few months after diagnosis, whereas others live for several years with minimal or no treatment. A comprehensive prognostic characterization of patients with B-CLL and identification of reliable prognostic markers is essential for tailoring therapeutic strategies **(*Rampazzo et al., 2012).***

Disease progression in CLL patients is quiet variable according some factors. The Rai and Binet systems are two commonly accepted staging methods in CLL. The prognosis is related to the particular biological parameters such as cytogenetic abnormalities, mutational status of Immunoglobulin Heavy chain Variable region gene (IgVH), expression of ZAP-70 and CD38, serum markers CD23, thymidine kinase and beta2-microglobulin (B_2M), bone marrow examination and lymphocyte doubling time ***(Vural et al., 2014)***.

These prognostic markers fail to cover the complex heterogenicity of CLL. Because of this; new markers developed to explain and predict the outcome of an unknown part of CLL. One of the most promising markers is telomerase activity. It is related with poor clinical outcome in most cancer types. Consequently, the aim of our study was to explore the prognostic significance of Telomere Length (TL) in patients with B-chronic lymphocytic leukemia (B-CLL) and to analyze the relation of TL with disease stage as well as other prognostic markers; such as CD38 and ZAP-70 expression.

Material and Methods:

Sixty one adults CLL patients were included in our study, who presented to South Egypt Cancer Institute (SECI) from October 2014 to December 2016. Bone marrow and peripheral blood samples were collected from patients at the time of diagnosis and before beginning of treatment.

Our study also included 67 healthy control samples. Peripheral blood samples were collected from healthy control donor. Cord blood samples were enrolled in our study as external positive control sample used for the calculation of the Relative Telomere Lengths (RTL). Both bone marrow and peripheral blood samples were collected in Ethylene Diamine Tetra Acetic acid (EDTA) vacutainer tubes. The study protocol was approved by the Institutional Review Board (IRB). Informed consent from patients was written.

All patients were subjected to history taking and clinical examination, with careful assessment of clinical signs as lymphadenopathy and/or organomegaly. Bone marrows aspirate (BMA), Bone marrow biopsy (BMB) and Immunophenotyping (IPT) were performed for diagnosis of the patients. All patients were treated according to European Society for Medical Oncology (ESMO) Guidelines for diagnosis, treatment and follow up ***(Bo¨ttcher et al., 2012).***

Follow-up was done for all patients to know their response to treatment, by immunophenotyping and monitoring the presence of Minimal Residual Disease (MRD) of the malignant cells population after six months of continuous treatment.

Cell preparation:

One ml of blood samples was transferred to 15ml conical tubes prefilled with10 ml Phosphate Buffer Saline (PBS) with 0.2 % Bovine Serum Albumin (BSA). Samples incubated at 4^{o} C for at least 10 minutes, and then centrifugation at 450 xg for 4 minutes at room temperature was done. White blood cells (WBCs) were obtained by osmotic lysis of Red blood cells (RBCs) by adding 1ml freshly prepared ice-cold NH4CL, and then incubated on ice for 10 minutes. Centrifugation of the samples at 290xg for 5 minutes at 4^{o} c was done. Finally, the obtained purified WBCs were washed by containing 0.2 % BSA ***(Baerlocher et al., 2002).***

Transfer $1x10^{6}$ cells in two 1.5-ml V-bottom tubes was performed, one for the cells to be hybridized to Peptide Nucleic Acid

(PNA) probe and labeled test tube and the other was treated identically but without the PNA probe and labeled auto control tube. The latter was required to measure the level of autofluorescence in cells of interest and to enable telomere length to be calculated from specific PNA hybridization.

Phosphate Buffer Saline was added and the tubes centrifuged at 300xg for 30 seconds. The pellets were resuspended in 100ul fixation reagent (Code no. 554722, BD. USA) and incubated for 15 minutes at room temperature. An aliquot of 1 ml PBS added to each tube, and after centrifugation, the pellets were resuspended in 100 ul in permeabilization reagent (Code no. 554722, BD. USA). Washing of cells twice was performed using PBS after incubation 15 minutes.

Denaturation and Hybridization & DNA Counter staining:

Cells in two tubes were resuspended in 300ul of hybridization mixture: (Tris-Buffered-Saline pH 7.5, 1% BSA and Pre-Treatment solution which contain a proteolytic enzyme) with or without 0.3 μg/mL of PNA probe ***(Telomere PNA FISH Kit/FITC, Code No.K5325, DAKO, Carpinteria, CA).***

After incubation for 10 minutes at room temperature, samples were incubated for 15 minutes at 87^{o} C and then 10 minutes at room temperature. The tubes were placed in the dark at room temperature overnight (15–20 h). To remove the excess unbound or non-specifically bound Probe; samples were washed twice with wash and rinse solutions provided by the manufacturer kit.

Finally, cells were resuspended in 0.3 ml of PBS containing 0.1% BSA, RNase (10ug/ ml), then DNA counterstain containing five ug/ml of Propidium Iodide (PI) was used. Incubation for at least 10 minutes at 4C in the dark before the acquisition was performed.

Sample acquisition and analysis on Flow-cytometry:

Cells were analyzed using FACSCalibur flow-cytometry apparatus (Becton Dickinson, BD, USA).Using the FL1 channel for detection of fluorescein signal of PNA for Telomere and the FL2 channel for PI. Ten thousands cells in each experiment were acquired and analyzed using CELL-Quest software ***(Baerlocher et al., 2006)***.

Cytometer stability and sensitivity were checked by using the Quantum™24 premixed fluorescein isothiocyanate molecules of equivalent soluble fluorochrome (FITC MESF) beads (Bangs Laboratories, Fishers, IN; formerly Flow Cytometry Standards Corporation, San Juan, PR) ***(Hawley T.S. and Hawley R.G., 2004)***.

The first step in the subsequent analysis was to identify cells using forward light and side scatter in a bivariate dot plot. The Region around target cells (malignant) was drawn as (R1). In external healthy control sample the; we gated on Lymphocyte population. Exclusion of all debris and dead cells from the gate was done.

For each blood sample two tubes were analyzed: The first was for the cells hybridized to the peptide nucleic acid (PNA) probe and the second was treated identically but without the PNA probe. The latter was required to measure the level of autofluorescence in cells of interest and to enable telomere length to be calculated from specific PNA hybridization. Fluorescence histograms of the indicated cell populations were obtained, and used for subsequent calculations of Relative Telomere Length (RTL)**.** Relative Telomere length (RTL) was estimated based on the ratio between mean fluorescence intensity (MFI) of the test sample (PNA-MFI), and the PNA-MFI of a positive control sample, after subtraction of the auto fluorescence of the blank from each of them ***(Baerlocher et al., 2006)***.

Relative Telomere Length (RTL) =

$$\frac{\text{PNA MFI (Test) – Auto Fluorescence (blank)}}{\text{PNA MFI (positive control)) – Auto Fluorescence (blank)}}$$

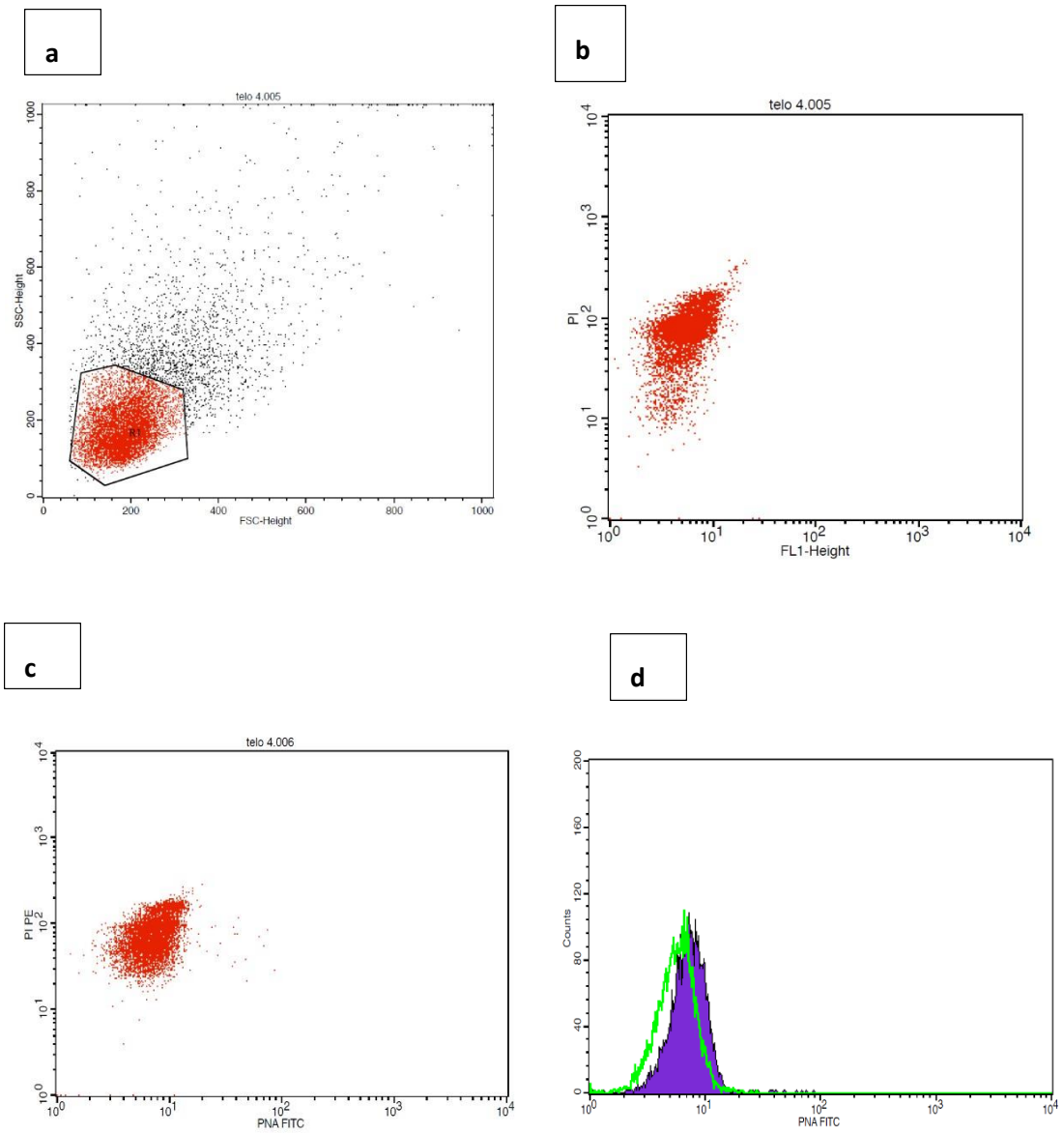
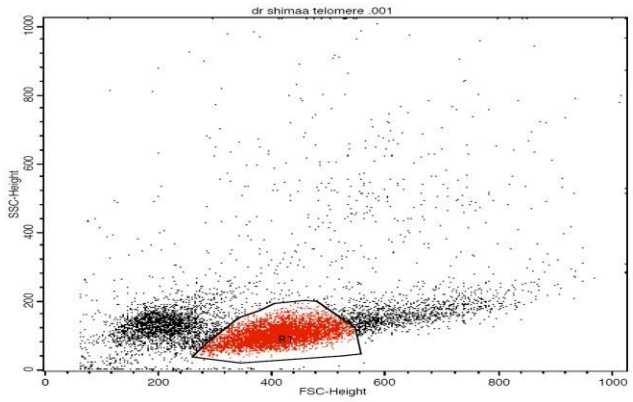


Figure (16): Example of flow FISH data analysis of blood cells from patient with B- chronic lymphocytic leukemia B-CLL. For each blood sample two samples are analyzed: one in which the cells were hybridized to the peptide nucleic acid (PNA) probe (c) and one that was treated identically but without the PNA probe (b). The latter is required to measure the level of auto fluorescence in cells of interest and to enable telomere length to be calculated from specific PNA hybridization. Cells are counterstained with the DNA dye Propidium Iodide (PI) before the acquisition. The first step in the subsequent analysis is to identify cells using forward light and side scatter in a bivariate dot plot (a), target cell population can be distinguished as R1 which represent mononuclear cells. Fluorescence histograms (d), green peak represents the auto fluorescence and violet peak represent the fluorescein signal of the PNA probe for the telomere. This histogram was used for subsequent calculations of Relaive Telomere Length.

a



b

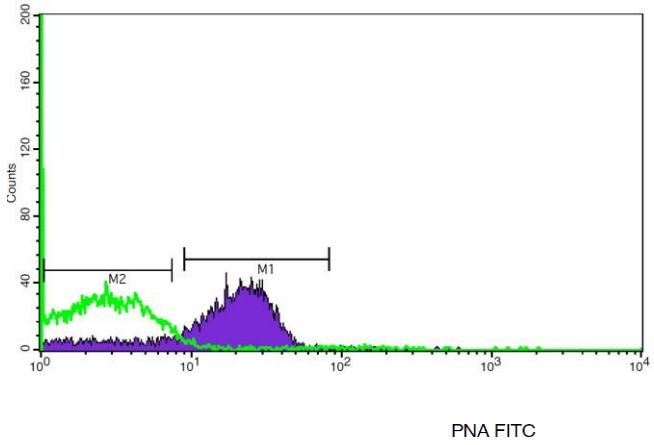


Figure (17): Example of flow FISH data analysis of cord blood sample (as a positive control). The first step in analysis is to identify cells using forward light and side scatter in a bivariate dot plot (a), one cell population can be distinguished as R1. Fluorescence histograms (b), green line represent the auto fluorescence, and blue line represent the fluorescence of the indicated cell populations.

Statistical analysis:

Statistical analysis was performed using the Statistical package for Social Sciences (SPSS), version 22. Mean and standard deviation (mean ±SD) were used for continuous variables and percentages for categorical variables. Independent-*t* test was used for two continuous variables. Pearson correlation coefficient was used to measure the linear correlation between two variables X and Y. ANOVA was also used to calculate *p values* in comparisons of more than two continuous variables. *P value* of ≤ 0.05 was considered statistically significant.

Results:

Diagnosis of CLL was usually made following a routine blood test that reveals a high white blood cell count and the presence of at least 5×10^9 lymphocytes /Liter peripheral blood, small mature lymphocytes in blood or bone marrow smears, and clinical observation (organomegaly and/or lymphadenopathy).The characteristic of CLL patients and the Healthy controls are shown in Table 1.

Staging of the cases was performed according to the Standard Scoring System ***(Modified Rai staging).*** Seven patients were low risk (stage A), 24 were an intermediate risk (stage B) and 30 were high risk (stage C).

The classification was done depending on routine blood count (hemoglobin and platelet values) and clinical observation to assess as well as the presence of organomegaly and lymphadenopathy.

Immunophenotyping of cases was done. The characteristic phenotype of B-CLL was; cell surface co-expression of CD19, CD5, CD23 and weak expression of surface IgM and CD79b. We also investigated some prognostic factors in CLL, such as expression of CD38 and ZAP-70 measured by flow cytometry.

CD 38 was expressed in (57.4%) of CLL patients. The mean value of CD38 expression on malignant lymphocytes was ***(34.4% ± 3.4%).*** Zap70 was expressed in (47.5%) of CLL cases. The mean value of ZAP-70 expression on malignant lymphocyte was ***(22.7% ±2.95%).***

Expression of CD38 and ZAP-70 in different disease stage:

There was a significant direct relation between the expression of CD38 and ZAP70 and modified Rai stages as the more advanced disease stage (stage C), the higher expression of these markers ***(Figure 1) (Table 2).***

Relative Telomere Length in B-CLL patient and Healthy control group:

Relative Telomere Length in B-CLL patients (0.16+/- 0.07) was significantly lower than the control group (0.66 ± 0.15) **(*p* < 0.001)** ***(Figure 2).***

Correlation between Relative Telomere Length and some diagnostic and prognostic parameters in B-CLL patients:

There was a negative correlation between RTL with some studied diagnostic parameters of CLL patients, but the results were not statistically significant. On the other hand, there were significant negative correlations between RTL and CD38 & ZAP-70 expression on malignant lymphocytes of CLL patients {**(r=-0.28*) (p=0.02) &***

(r=-0.4) (p=0.002)} *respectively* ***(Table 3).***

Relative Telomere length and its relation to disease stage:

There was significant inverse relationship between RTL and disease stage (stage A, B or C). There was more reduction in the RTL with more advanced disease stage ***(P=0.001).*** Mean Fluorescence intensity (MFI) of telomere was higher in CLL patients in stage A than others in stage B and C ***(0.21± 0.07, 0.17±0.081, 0.12±0.04 respectively) (Table 4).***

Relation of Relative Telomere Length and response to therapy in CLL patients:

In our study, we did follow up of B-CLL patients, after 1st induction therapy, by doing Immunophenotyping and monitoring the MRD of malignant lymphocytes which still show co-expression of CD5, CD19, and CD23 in the bone marrow aspiration sample. Twenty-eight patients (42.8%) were entered in remission and were negative for MRD after 1st induction therapy. TL was significantly shorter in patients who were positive for MRD after 1st induction therapy than others ***(p=0.04*) (*Table).***

Discussion:

The interest for investigating telomere length as a potential clinical biomarker has grown noticeably in recent years and it has been proposed as a prognostic indicator in a plethora of disorders, both malignant and benign. In our study, we found that there was a reduction in RTL in B-CLL cases compared to healthy control samples. This indicated the occurrence of telomere dysfunction in B-CLL cases.

In agreement with our work, previous two studies were done to explore the prognostic significance of TL in CLL patients. They determined the relative TL by using a real-time fluorescent quantitative polymerase chain reaction (qPCR). They showed that the

telomere length in CLL patients was lower than normal control samples ***(Yan-Qiu et al., 2010; Billy et al., 2013).***

We observed that, there was significance difference between RTL as regard disease stage (Modified Rai Staging), as more lowering in TL occurred with advanced disease stage. It was more obvious in stage B and C. Another study also proved that TL was significantly correlated with disease stage (Binet staging) ***(Yan-Qiu et al., 2010)***. These results, ours and others may indicate that the TL could be used as another risk factor in CLL patients. More researches and studies are needed to verify this point.

There was a negative correlation between TL and CD38 and ZAP-70 expression. As the more lowering in RTL, the higher expression of CD38 and ZAP-70. ***In concordance with our result; Brezinova et al., Elizabeth et al.*** evaluated TL by terminal repeat fragment method in patients with B-CLL to ascertain whether telomere shortening is associated with CD38 and ZAP-70 expression and their results were similar to our finding (***Brezinova et al., 2010; Rampazzo et al., 2012; Elizabeth et al., 2012)***. Reversely, another study done at ***2010*** showed that TL was not related to ZAP-70 protein and CD38 expression ***(Yan- Qiu et al., 2010)***. That study used smaller sample size than our study; this may be the cause of discrepancy.

We also noticed that B-CLL patients with lower RTL might have a worse outcome, by having higher expression of residual B-CLL phenotype cells in the BMA after six months of continuous treatment. ***Billy Michael*** and colleagues also determined telomere length in 55 CLL patients, and 20 normal B-cell samples using Q-PCR to study its association with disease progression and outcome. A significant correlation between short telomeres and poor prognostic subgroups was confirmed. There was a trend toward an increased number of genomic aberrations with shortening of telomeres in B-CLL ***(Billy et al., 2013)***.

At 2015, Zhang and his colleagues did a meta-analysis of thirty-three independent articles. They showed that short telomere predicted poor prognosis in CLL and it is considered an independent predictor of prognosis. This association appeared to be more obvious in an older population, though it was still significant when adjusted for age. Their meta-analysis suggested that TL might serve as a useful cancer prognostic biomarker and a potential therapeutic target for cancer treatment. In conclusion, their study provided evidence for an inverse association between TL and risk of cancer survival ***(Zhang et al., 2015)***.

Conclusion:

Telomere length may have a prognostic relevance in B-CLL cases. Measurement of both Telomerase level and Telomere length may help the clinician in the management of B-CLL patients, as short Telomere B-CLL will progress more rapidly and might require therapy earlier than those with long Telomere.

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Table (1): The base line characteristic of CLL patients and the Healthy controls.

Parameter	**Patients (n= 61)**	**Control (n= 67)**	**P value**
Age (years)	60.4 ± 11.30	50.96 ± 9.15	**…..**
SEX (M/F)	**44/17**	**50/17**	**…..**
Hemoglobin (gm/dL)	10.64 ± 0.22	12.01 ± 1.17	0.007
Platelets(10^9/L)	150.00 ± 8.80	256.00 ± 93.42	<0.001
WBCs(10^9/L)	96.6 ± 17	6.91 ± 2.41	<0.001
PB lymphocytes (%)	73.00 ± 3.00	33.30 ± 6.01	<0.001
RTL	0.16±0.07	0.66±0.15	>0.001

Independent samples t-test, Significant *P* value < 0.05, Data expressed as mean ± SD *WBC: White blood cell; PB: Peripheral blood; BM: Bone marrow.* RTL: Relative Telomere Length.

Table (2): Association between Modified Rai stage, CD38 and ZAP-70 expression in B-CLL patients:

Parameter	***Stage A (n=7)***	***Stage B (n=24***	***Stage C (n=30)***	***P value***
CD38% (mean±SD)	18.3% ± 4.2%	34.4% ± 4.8%	44.7% ± 6.2%	0.009
ZAP-70% (mean±SD)	10.6% ± 1.3%	21.3% ± 4.0%	31.7% ± 5.9%	0.017

Table (3): Correlation between Telomere length some diagnostic and prognostic parameters in B-CLL patients:

Parameter	***Relative Telomere Length(RTL)***	
WBCs x10^9/L	r=-0.122	*P=0.355*
P.B Lymphocytes (%)	r= -0.13	*P=0.324*
B.M Lymphocytes (%)	r=-0.141	*P=0.281*
CD38%	r= -0.28	*P=0.020*
ZAP-70%	r= -0.39	*P=0.002*

P.B: Peripheral Blood, B.M: Bone Marrow, WBCs: White Blood Cells

Table (4): Differences in Telomere Length in different disease stages:

Parameter	***Stage (A) (n=7)***	***Stage (B) (n=24)***	***Stage (C) (n=30)***	***P value***
RTL	***0.21± 0.07***	***0.17±0.081***	***0.12±0.04***	***0.001***

RTL: Relative Telomere Length.

Table (5): Association between the Relative Telomere Length and response to therapy in B-CLL patients:

Patient's response to therapy			***P value***
RTL	Remission (n=28)	Not in remission(n=33)	>0.04
	0.20 ± 0.08	0.14 ± 0.05	

Independent samples t-test, Significant *P* value < 0.05

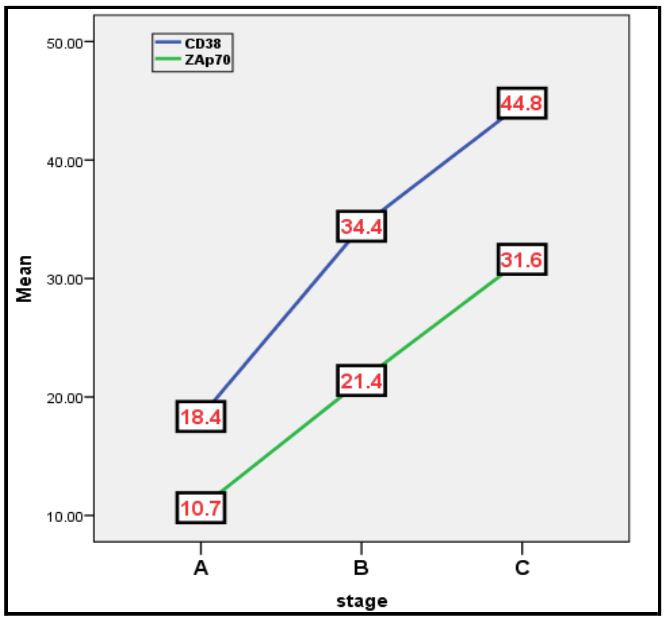

Figure (1): Expression of CD38 and ZAP-70 in different Modified Rai stage in CLL patients.

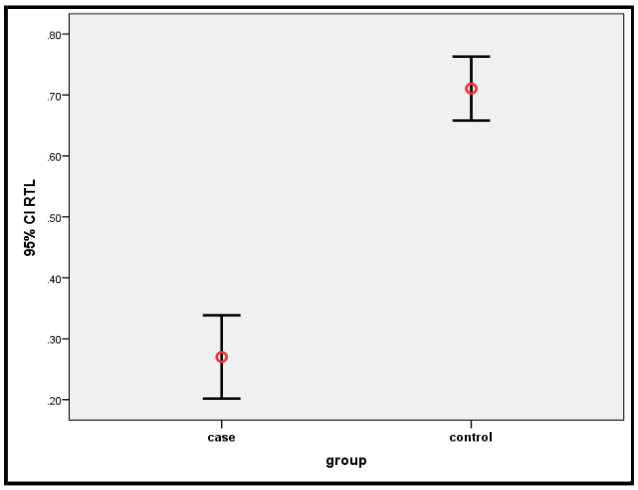

Figure (2): RTL in CLL patients and the healthy control sample

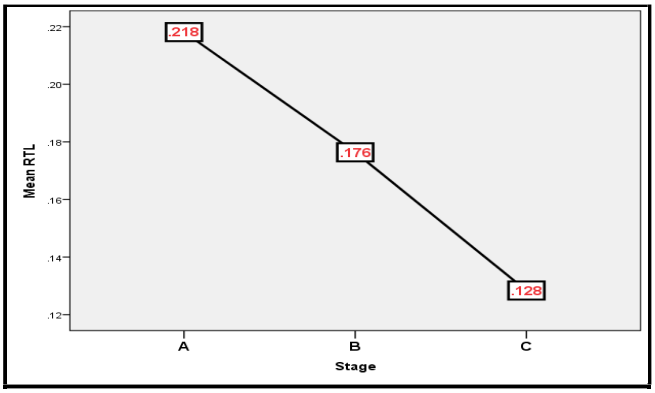

Figure (3): RTL and Modified Rai Disease Staging in CLL patients

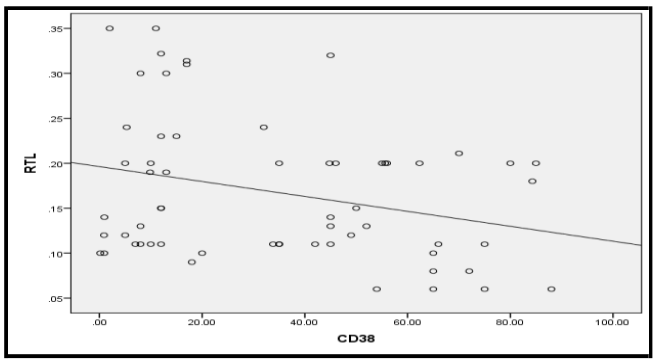

Figure (4): Correlation of Relative Telomere Length and expression of CD38 in CLL patients.

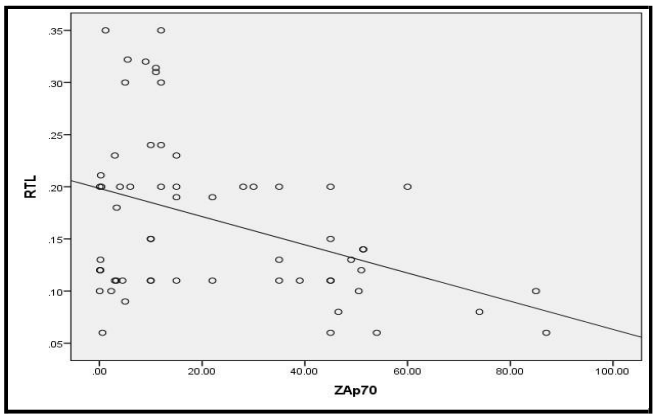

Figure (5): Correlation of Relative Telomere Length and the expression ZAP-70 in CLL patients