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EVALUATION OF TELOMERASE ACTIVITY IN PATIENTS WITH CHRONIC B LYMPHOCYTIC LEUKEMIA VERSUS AGE MATCHED CONTROLS. CORRELATION BETWEEN TELOMERASE ACTIVITY AND BONE MARROW INFILTRATION

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A b s t r a c t: Telomerase is a ribonucleoproteic enzyme associated with cellular immortality and malignancy. This enzyme, besides the catalytic subunit bearing reverse transctiptase activity, contains an RNA template complementary to TTAGGG telomeric repeats, thus permitting *de novo* synthesis of telomeric DNA onto chromosomal telomeric ends. Increased telomerase activity has been reported in Chronic Lymphocytic Leukemia (CLL) by many authors. In order to investigate the telomerase activity in patients with CLL and its correlation to commonly used morphologic prognostic markers, 38 frozen blood lymphocyte samples from patients with CLL and 47 age-matched controls were investigated for telomerase activity using the Telomerase PCR ELISA-plus kit from Roche. Trepanobiopsies from the same patients were analysed for the type of bone marrow infiltration as well. Analysis showed highly variable Relative Telomerase Activity (RTA) in B-CLL patients, ranging from comparable or even lower than the mean RTA of controls (in Binet A stage patients) to manifold increase in the majority of patients with advanced stage disease. The sex and age of the patients showed no influence on RTA in CLL patients, in contrast to the control group, where the age influenced telomerase activity. We found a positive correlation between the RTA and disease stages (Binet), as well as between RTA and the type of BM infiltration.

Key words: telomerase, chronic lymphocytic leukemia, Binet, Bone, marrow Infiltration, RTA.

Introduction

Telomerase is a ribonucleoprotein enzyme associated with cellular immortality and malignancy. It is repressed in most normal somatic cells, but reactivated in most of the malignant tumour cells and immortal cell lines [8, 9]. There are variable numbers of tandem repeats of the hexanucleotide TTAGGG constituting chromosomal telomeres, present at the telomeric ends of the mammalian chromosomes. The telomeres stabilise chromosomal ends and prevent their fusion, rearrangements and chromosomal loss [13]. Progressive shortening of telomeres may be the major mechanism of cellular senescence and may result in chromosomal instability and cell death as well [2, 5, 8]. Telomerase, the enzyme that elongates telomeres, contains an RNA template complementary to TTAGGG repeats that permits *de novo* synthesis of telomeric DNA onto chromosomal telomeric ends [9]. Recent findings have suggested that activation of telomerase is one of the most common and fundamental steps in carcinogenesis, although reactivation, or telomerase up-regulation alone, might be insufficient for cells to proliferate indefinitely. Telomerase expression and consequent stabilisation of telomeres seem to be concomitant with the attainment of immortality of cancer cells [1, 9, 10, 14, 15, 20].

Telomerase itself is approximately 1000 kDa, and consists of at least 4 subunits. One of them is hTR (human Telomerase RNA) or hTERC (human Telomerase RNA Component), with a characteristic (5`-CUAACCCUAAC-3`) RNA sequence complementary to telomeric DNA. It is coded by a single copy gene located at 3q26.3, and most probably regulated by DNA methylation. However, binding sites for glucocorticoid, progesterone and androgen hormone receptors, as well as for several transcription factors involved in haematopoesis, have been found in the promoter region of this gene. Another subunit is hTERT (human Telomerase Reverse Transcriptase). Phylogenetically hTERT is much closer to reverse transcriptases encoded by a subclass of retrotransposons than to those encoded by retroviruses. That is why telomerase activity cannot be blocked with anti-retroviral drugs. The other two subunits, or more precisely telomerase associated proteins, are hTEP1 (a human homologue of an 80 kDa protein from Tetrahymena thermophyla) and a 95 kDa protein from Tetrahymena thermophyla, a homologue of which still has not been isolated in humans [9, 11]. Chronic lymphocytic leukemia (CLL) is a chronic lymphoproliferative disorder which is defined as a malignant neoplasm of the lymphoid cells. It is characterized by accumulation of long-living, nonfunctional, neoplastic cells, 95% of cases being of B-cell type [12, 17]. At the beginning of the ‘90s several authors compared the telomerase activity in normal lymphocytes with that in B-CLL cells, and with cells from other haematological malignancies [3, 4, 7, 18]. All the published data shows a significant increase in telomerase activity in CLL cells compared to normal lymphocytes. However, published data differs

regarding the degree of telomerase activity increase (from 2 times the normal telomerase activity to 8.6 times) [3, 18].

The aims of this study were:

1. Evaluation of telomerase activity in lymphocytes from patients with B-CLL, as well as in lymphocytes from age-matched controls.

2. Determination of the difference in telomerase activity in different Binet stages of B-CLL.

3. Evaluation of a possible correlation between the telomerase activity and the type of bone marrow infiltration.

Material and methods

Blood samples from 38 patients suffering from B-CLL as well as blood samples from 47 age-matched controls were collected. Bone marrow biopsies from all patients were taken for diagnostic purpose previous to blood sampling. Biopsy samples were fixed in 10% neutral formalin, embedded in paraffin, sectioned and stained with H.E. and Giemsa. Immunohistochemical stainings for CD20, CD3 and LCA were also performed using the LSAB+ immunoperoxidase technique (DAKO) [16]. Biopsy findings were divided into two groups. The three pathomorphologic types of infiltration: interstitial, focal-patchy and diffuse infiltration (Fig. 1), were divided into non-diffuse and diffuse bone marrow infiltration, where the non-diffuse group encompassed the interstitial and focal-patchy type of BM infiltration.

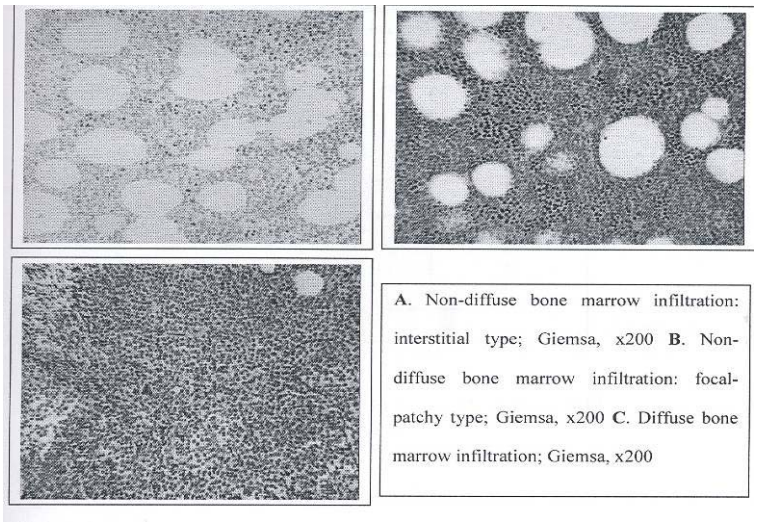


Figure 1 – Types of bone marrow infiltration
Слика 1 – Типови на инфилтрација на коскена срцевина

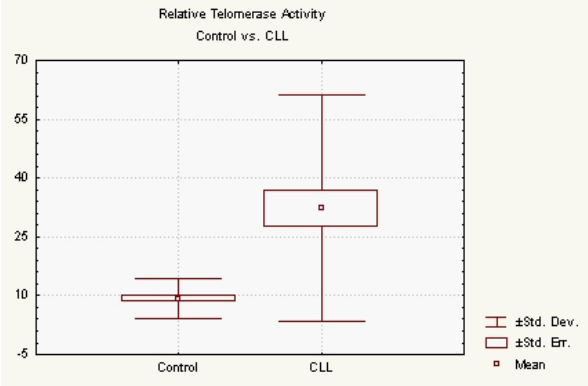
Blood samples, both from patients and the control group, were collected in portions consisting of 3 ml heparinised venous blood from 3–5 patients daily, and pre-processed immediately. The pre-processing step included isolation of mononuclear cells on ficol-triosyl 75 in a refrigerated centrifuge (4^{o}C) for 15 minutes and 300 x g, with subsequent re-suspension of the pellet and lysing of the remainder of erythrocytes with lysing solution (7 g NH_4Cl + 0,07 g NH_4HCO_3 ad 1000 ml *aqua destilata*) for 15 minutes with gentle shaking, followed by another centrifugation and 3 subsequent washings of the pellets with 0.9% NaCl. After the final washing, the quality of isolates was tested with a blood cell-counter. From samples containing more than 90% lymphocytes, less than 0.02 erythrocytes/L and no free haemoglobin, volume aliquots containing 2 x 10^5 cells were centrifuged in a refrigerated micro-centrifuge at 4^{o}C and 3000 x g for 10 minutes and pellets were stored for further telomerase activity analysis. Telomerase activity was analysed with a photometric immunoassay for quantification of telomerase activity using Telo TAGGG Telomerase PCR Elisa plus kits from Roche, based on the Telomeric Repeat Amplification Protocol – TRAP. We used a protocol fully identical to the one described in the manual provided with the kit, according to which, analysed telomerase activity is presented as a Relative Telomerase Activity (RTA) [19]. For description of the obtained results, methods from the descriptive statistics were used and the null hypothesis was tested using parametric and non-parametric tests by means of commercial software [23]. The normality of distribution was tested using the Kolmogorov-Smirnov D test, Lillefors test and Shapiro-Wilk's W test. For testing the mean RTA differences between the patients and the control group, t-test for independent samples was used, while the significance of differences between patient subgroups was evaluated by means of nonparametric tests for comparison of multiple independent samples (Kruskal-Wallis ANOVA by ranks and Median chi-square test). Correlations between parametric variables were tested with Pearson Product Moment Correlation (r), and for the non-parametric variables the Spearman's Rank Order Correlation (R) was used.

Results

The patient group consisted of 26 males and 12 females with a mean age 64.5 years (min = 37; max = 81; SD = 10.28), while the control group consisted of 34 males and 13 females with a mean age 63.9 years (min = 40; max = 80; SD = 9.58). In the first group there were 15 patients in Stage A according to Binet (8 males and 7 females), 11 patients in stage B (10 males, 1 female), and 12 patients in stage C (8 males and 4 females).

According to the type of BM infiltration, the distribution of patients was as follows: 25 patients had non-diffuse bone-marrow infiltration (17 males; 8 females), and 13 patients had a diffuse type of infiltration (9 males; 4 females).

Analysis showed highly variable telomerase activity with overall RTA increase in the group of patients (mean RTA = 32.26; min = 2.24; max = 115.89; SD = 28.88), compared to the control group (mean RTA = 9.2; min = 2.14; max = 24.22 SD = 4.99) ($p < 0.01$) (Fig. 2). This increase was attributable solely to the mean RTA values of the patients in more advanced stages of the disease, since the patients in Binet stage A had no significantly increased RTA compared to the control group. We found no significant difference among the mean RTA in males and females in either the control group or the patients. However, there was a strong negative correlation (Pearson correlation –0.543; $p < 0.01$) between the age and RTA in the control group, which was not the case in the group of patients, where RTA showed no correlation to age (Fig. 3).



T-test for large independent samples: t-value: 5.365; df = 83; $p < 0.01$

Figure 2 – Mean RTA in B-CLL patients and control group
Слика 2 – Средна РТА кај испитаниците со Б-ХЛЛ и кај контролната група

As far as the clinical stages were concerned, the greatest mean RTA was observed in patients in stage C according to Binet (mean RTA = 55.76; min = 17.26; max = 115.89; SD = 33.36), followed by the mean RTA in stage B patients (mean RTA = 37.39; min = 9.81; max = 58.72; SD = 18.42), and the mean RTA in stage A patients (mean RTA = 9.69; min = 2.24; max = 26.22; SD = 6.89) (Fig. 4). The statistical evaluation of the inter-group differences showed that there were significant differences ($p < 0.01$) between the mean RTA of the patients in Binet stages B and C compared to the control group, as well as between stage C compared to stage A, but there was no significant difference of mean RTA between patients in stage A compared to the control group ($p > 0,05$); as also between stages B and C, and between stages A and B ($p > 0.05$) (Fig. 4).

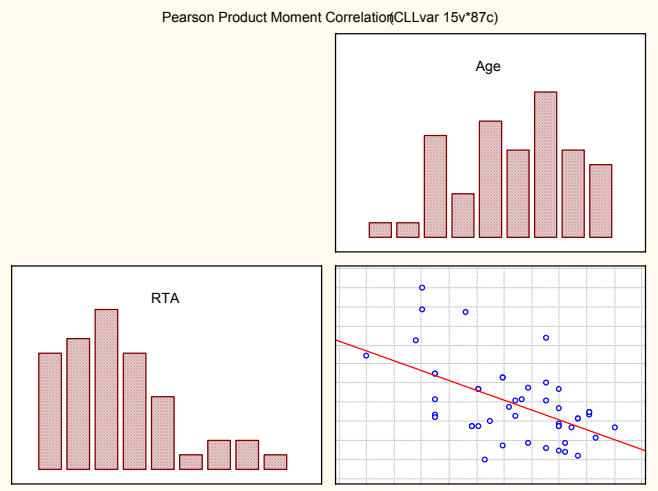
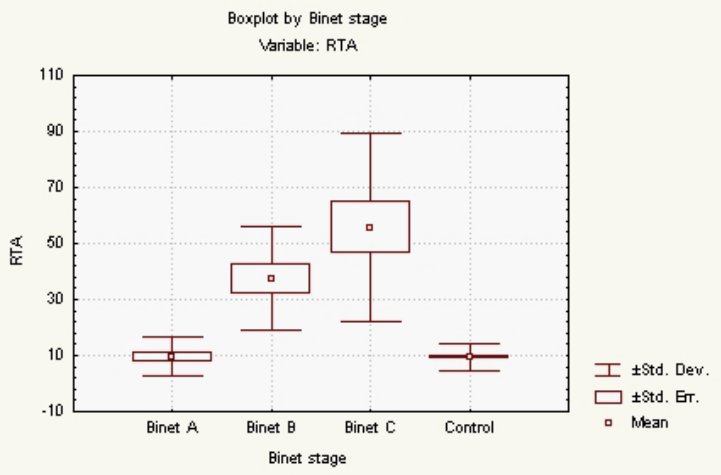


Figure 3 – Correlation between age and RTA in control group
Слика 3 – Корелација помеѓу возраста и RTA во контролната група

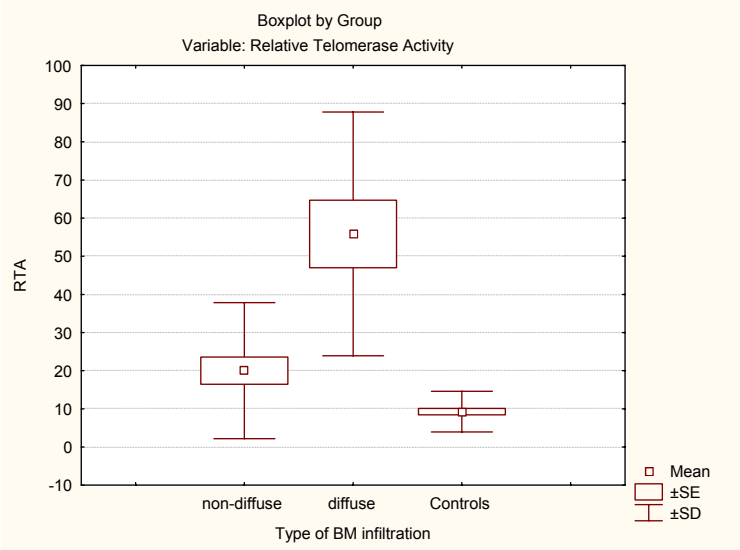


Kruskal-Wallis test: H (3; N = 78) = 41.79; p < 0.01
Chi-square = 27.43; df = 3; p < 0.01

Figure 4 – Mean RTA in different Binet stages
Слика 4 – Средна RTA во различни стадиуми на болеста според Бинет

The mean RTA of the patients distributed according to the type of bone-marrow (BM) infiltration was as expected. The mean RTA was significantly higher in patients showing diffuse BM infiltration (mean RTA = 55.837; SD = 31.945; min = 17.26; max = 115.89), compared to the patients having non-diffuse types of BM infiltration (mean RTA = 19.999; SD = 17.83; min = 2.24;

max = 58.72). The Mann-Whitney U test for evaluation of the significance of inter-group differences showed that there was a significant difference between the RTA of patients with non-diffuse types of BM infiltration compared to those having diffuse type of BM infiltration ($p < 0.01$). There was also a significant difference ($p < 0.01$) between mean RTAs of patients expressing diffuse BM infiltration compared to the control group, and significant difference ($p < 0.05$) between the patients with non-diffuse BM infiltration compared to the mean RTA of the control group (Fig. 5). We found a strong positive correlation between the mean RTA and the type of BM infiltration (+0.589; $p < 0.01$).



Kruskal-Wallis test: H (2; N = 78) = 29.39; $p < 0.01$
Chi-square = 19.76; df = 2; $p < 0.01$

Figure 5 – Mean RTA in patients with different types of bone marrow infiltration
Слика 5 – Средна РТА кај пациентите со различен тип на инфилтрација на коскената срцевина

We also tested the correlation between mean RTA values and the type of BM infiltration to the Binet stage. There was a strong positive correlation between the mean RTA and the Binet stage (Spearman's R +0.75; $p < 0.01$), as well as between the type of the BM infiltration and the Binet stage (+0.847; $p < 0.01$).

Discussion

Our study showed a mean RTA increase in advanced stages B-CLL patients compared to the control group ($p < 0.01$), which is similar to the results

from other studies [3, 18]. Bechter *et al*. (1998), in a study encompassing 58 B-CLL patients, found significantly increased telomerase activity, as well as a significant correlation of telomerase activity to shortened survival [3]. Inappropriate and irregular telomerase activity was also found by Counter *at al*. [7].

Our statistical analysis showed far more cohesive values of RTA (with lower standard deviation) in the control group compared to the B-CLL patients, which could be explained by the great variation of RTA at different Binet stages.

The mean age of the patents was 64.5 years, and their sex distribution showed that males were twice as much affected as females, which correlates to the age and sex distribution in other studies [6, 12, 17].

The control group was constructed according to the age and sex distribution of the patient group. From that point of view, we were able to evaluate the mean RTA differences between sexes, both in the control group and the group of B-CLL patients. We found that there was no significant difference in mean RTA between males and females in both groups. This finding cannot be compared with the other studies cited, because these authors have not published such results. However, we found a significant negative correlation (-0.543; $p < 0.01$) between the age and the RTA in the control group, which was not the case in the group of patients. This was clearly a result of disease-influenced telomerase activation in patients, and the fact that the disease stages are age-independent. Concerning the possibility of age influencing RTA, our initial speculations were the opposite of the obtained results. For healthy individuals we initially expected that if there was a correlation between RTA and age, it should be positive, taking into consideration the probably shorter telomeres (thus activated telomerase) in older individuals. However, our results showed the opposite. We were unable to compare these results with similar ones from the literature, but we speculate that this could be attributable to the overall state of senescence in older individuals where borderline bone-marrow hypocellularity is not an infrequent finding, while telomerase reactivation is clearly a pathophysiological event, not seen in physiological aging tissue homeostasis, independently of how severe the selective pressure (possible shortness of telomeres) on the cells might be. Another speculative explanation, for which we are unable to offer either solid background or references, could be that after the isolation and recovery of the telomerase from peripheral blood mononuclear cells, our samples were possibly boosted with some serum-derived residual telomerase activity originating from highly turn-overing epithelial tissues, which is more pronounced in younger individuals. The possibility of a biased sample, as another explanation, cannot be completely overruled either.

As far as Binet stages are concerned, we found that patients with more advanced disease expressed higher RTA, which correlates with the findings of other authors [1, 3, 7, 18, 21]. The mean RTA in Binet stage A patients showed no significant increase compared to the control group, which is also consistent

with the findings of other authors [7] who also found an insignificant increase of telomerase activity in early stages of B-CLL, but opposite to the findings obtained by Bechter *et al*. [14] and Trentin *et al*. [17]. This could be a result of different sensitivity of the methods used (Bechter *et al*. used bone marrow cellular substrate, instead of peripheral lymphocytes), as well as a result of "by chance" different random selection of the patients (i.e. some Binet A stage patients – presumably those with higher telomerase activity – progress more rapidly to more advanced stages, while others do not). However, we found a highly significant difference in mean RTA between Binet B and Binet C patients, compared to the control group ($p < 0.01$ and $p < 0.01$, respectively), as well as between mean RTA in Binet A compared to Binet C patients ($p < 0.01$), which was consistent with the findings of Bechter *et al*. [14]. Unlike the latter author, we found no significant difference between the mean RTA in Binet A compared to Binet B patients ($p > 0.05$).

Analysing the mean RTA of the patients grouped by the type of BM infiltration, we found significantly lower mean RTA in patients with non-diffuse type BM infiltration, compared to those with diffuse type BM infiltration (M-W U test $Z = -4.46$; $p < 0.01$). There were also significant differences between the mean RTAs of patients with both non-diffuse and diffuse BM infiltration compared to the control group ($p < 0.05$ and $p < 0.01$, respectively). The degree of correlation between the type of BM infiltration and the mean RTA was +0.589 ($p < 0.01$). These results are contrary to those of Bechter *et al*. [14], who did not find any significant correlation between the telomerase activity and the type of bone marrow infiltration. The fact that Bechter *et al*. measured the telomerase activity in bone-marrow cellular substrate, plus the fact that there is a lower number of cells in the S-phase of the cell cycle in the peripheral blood compared to the bone-marrow [22], could explain the disproportion of the results. In this context the influence of the telomerase activity from the normal haematopoetic cells in the bone-marrow should not be overlooked either.

Conclusions

1. Significantly higher mean RTA is encountered in more advanced Binet stages (B and C) compared to healthy controls ($p < 0.01$).

2. There is significant difference ($p < 0.01$) between mean peripheral lymphocyte RTA in Binet B compared to Binet C stage.

3. There is no significant mean RTA increase in peripheral lymphocytes from B-CLL patients in Binet stage A, compared to healthy controls.

4. The age and sex of the patients with B-CLL does not influence the mean peripheral lymphocyte RTA.

5. The age of the healthy persons shows a moderate negative correlation to the mean peripheral lymphocyte RTA (-0.55; $p < 0.01$).

6. Peripheral blood lymphocyte RTA in B-CLL patients shows a strong positive correlation (+0.589; $p < 0.01$) to the type of the bone-marrow infiltration.

7. There is significantly increased telomerase activity in peripheral lymphocytes from B-CLL patients with diffuse type bone marrow infiltration compared to patients exhibiting non-diffuse bone marrow infiltration patterns ($p < 0.01$).

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Резиме

ЕВАЛУАЦИЈА НА ТЕЛОМЕРАЗНА АКТИВНОСТ КАЈ ПАЦИЕНТИ СО ХРОНИЧНА В ЛИМФОЦИТНА ЛЕУКЕМИЈА ВО СПОРЕДБА СО СООДВЕТНИ КОНТРОЛИ СПОРЕД ВОЗРАСТ. КОРЕЛАЦИЈА ПОМЕЃУ ТЕЛОМЕРАЗНАТА АКТИВНОСТ И ИНФИЛТРАЦИЈАТА НА КОСКЕНИОТ МОЗОК

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Теломеразата е рибонуклеопротеински ензим, асоциран со клеточна бесмртност и малигнитет. Овој ензим, покрај каталитичката субединица, која е носител на реверзно-транскриптазна активност, содржи и матрична РНК која има комплементарна секвенца со секвенцата на теломерите (TTAGGG).

Ензимски активната субединица од теломеразата, синтетизира нови хексануклеотидни повторувачки секвенци на краевите од теломерите по принцип на реверзна транскрипција, користејќи ја оваа РНК матрица. Зголемената теломеразна активност е феномен кој е утврден од повеќе автори при хронична лимфоцитна леукемија (ХЛЛ). Со цел да ја испитаме теломеразната активност кај пациенти со ХЛЛ и да ја одредиме нејзината корелација со вообичаените морфолошки прогностички маркери, анализиравме 38 примероци крв од болни со ХЛЛ и 47 примероци од контролна популација со слична полова и возрасна дистрибуција. Анализата на теломеразната активност ја изведовме со помош на комплет за одредување теломеразна активност "Telomerase PCR ELISA-plus kit; Roche". Од истите пациенти беа анализирани и коскени биопсии, кај кои посебно се анализираше типот на инфилтрација. Анализите покажаа висока варијабилност на теломеразна активност (РТА) во периферните лимфоцити кај болните од ХЛЛ, од вредности блиски или дури и пониски од средната вредност на контролните примероци (пациентите во Бинет А стадиум), до многукратно повисока РТА кај повеќето пациенти во понапреднатите стадиуми. При тоа полот и возраста на пациентите не покажаа корелација со РТА, за разлика од контролната група, каде што добивме негативна корелација на РТА со возраста. Најдовме и позитивна корелација помеѓу РТА и некои стадиуми (според Бинет) на болеста, како и со типот на инфилтрација на коскената срцевина.

Клучни зборови: теломераза, хронична лимфоцитна леукемија, Бинет, коскена срцевина, РТА.

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