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Higher Percentage of In Vitro Apoptotic Cells at Time of Diagnosis in Patients with Chronic Lymphocytic Leukemia Indicate Earlier Treatment Requirement: Ten Years Follow Up

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SUMMARY

Introduction Chronic lymphocytic leukemia (CLL) has an extremely variable clinical course. Biological reasons for that wide variation in clinical course and survival rates in CLL patients are not fully understood.
Objective The aim of the study was to evaluate the value of spontaneous apoptosis of CLL cells *in vitro* determined at presentation of disease, in prediction of treatment requirements and evolution of the CLL.
Methods Malignant B cells were isolated from the whole blood of 30 newly diagnosed CLL patients and cultured for 24 hours in RPMI-1640 medium supplemented with 10% of serum obtained from the same CLL patient. Cells were later fixed and processed for embedding in Epon, or cell smears were prepared and stained with TUNEL technique.
Results Ten-year follow-up revealed that patients with lower percentage of cells in apoptosis at presentation of disease had significant longer time treatment initiation (log rank test $p<0.05$). On the contrary, apoptosis of CLL cells was not shown to have significant impact on survival of patients (Kaplan Meier log rank test $p>0.05$).
Conclusion The results of this study emphasize the importance of apoptosis of CLL cells at the time of the initial diagnosis in pathobiology of this disease.
Keywords: apoptosis; chronic lymphocytic leukemia; autologous serum; prognosis; survival

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most frequent type of leukemia in the Western world [1] and has an extremely variable clinical course with survival ranging from few months to decades [1, 2]. Biological reasons for wide variation in clinical course and survival rates of CLL patients are not fully understood; therefore, the search for prognostic factors contributing to proper management of patients with CLL has become important in the last two decades [2].

Up to now, a certain number of clinical and biological markers of prognostic relevance has been identified [3]. There are three traditional clinical prognostic factors: the clinical stage according to either Rai or Binet [4], the lymphocyte doubling time [5, 6] and bone marrow infiltration pattern [7, 8]. Two of the most reliable molecular prognostic markers are the immunoglobulin heavy chain variable (IGHV) gene mutational status and fluorescence *in situ* hybridization (FISH) detection of prognostically relevant genomic aberrations (e.g. 11q-, 13q-, +12 and 17p-) [9]. In addition to these markers, a myriad of additional biomarkers have been postulated as potential prognostic factors in CLL, proteins (e.g. CD38, ZAP70, TCL1), the RNA (e.g. LPL, CLLU1, microRNAs) and the genomic (e.g. TP53, NO, TCH1, SF3B1 and BIRC3 mutations) [9]. Recently, two new biological markers, the expression of CD38 antigen and ZAP-70 have shown independent significance in prediction of prognosis in CLL patients [10]; although these prognostic factors are used currently in practice, they are time-consuming and require standardization of laboratory protocol [11]. New prognostic factors are still needed.

The neoplastic population of cells in CLL is characterized by a very low proliferative rate. The accumulation of these cells in G0 phase of the cell cycle [12, 13, 14] cannot be explained only by cell proliferation but seems to be the result of prolonged survival of these cells as well [15, 16]. Despite of their long survival *in vivo* due to an increased capacity to resist normal apoptotic signals, *in vitro* CLL cells die rapidly by apoptosis [12]. These *in vitro* findings suggest that some factors (humoral or cellular), necessary for the survival of these cells, are missing from the culture media [12]. The results of recent study [13], suggested a relationship between higher *in vitro* spontaneous apoptosis before treatment in advanced-stage patients with poor outcome.

OBJECTIVE

The aim of this study was to evaluate whether spontaneous apoptosis of CLL cells *in vitro*

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determined at presentation of disease could predict the evolution of CLL.

METHODS

Thirty previously untreated patients with CLL, referred to the Institute of Hematology, Clinical Center of Serbia, University of Belgrade in 2000, were enrolled in this prospective study. They were followed up for 10 years, until 2010. This study was approved by the Ethics Committee of Medical Faculty Belgrade and the Institutional Review Board. The informed consent was obtained from all patients. Peripheral blood samples were obtained from CLL patients at the time of diagnosis, before the initiation of any treatment, and during regular diagnostic procedure. Diagnosis and clinical staging of CLL were based on the National Cancer Institute criteria (NCI) [4]. Bone marrow biopsies were available in 23 CLL patients. Evaluation of spontaneous apoptosis in CLL cells *in vitro* was performed at the Institute of Histology and Embryology, Medical Faculty of Belgrade. Patients were followed-up and treated according to the standard clinical protocol of the Institute of Hematology at the time of diagnosis (no Fludarabine based treatment). Data from medical documentation were reviewed in order to determine the time of the initiation of CLL treatment for every patient and other relevant clinical data.

Peripheral blood was collected using preservative-free heparin as the anticoagulant. Serum was withdrawn after sedimentation of coagulated blood samples obtained from all patients. Malignant B lymphocytes were isolated using sedimentation of the blood cells on Ficoll gradients (Lymphoprep®). Cells (5×10^6/ml) were cultured in RPMI-1640 medium (Sigma®) supplemented with 10% of serum obtained from the same CLL patient (autologous serum, AS) and incubated for 24 hours, 37° C, with 5% CO_2 and 98% humidity.

Immediately after separation (0 h) and after incubation (24 h), cells were fixed and processed for embedding in Epon (Agar Scientific®). One micrometer thick sections were stained with toluidine blue for light microscopy, where apoptotic cells were identified by the presence of nuclear condensation and/or fragmentation, based on previously defined morphological criteria [17-20]. Apoptotic cells were counted in 5 randomly chosen high power fields (100x oil immersion objective), and was expressed as a percentage of a total number of counted cells (apoptotic index, AI) (Figure 1). Thin sections were mounted on copper grids and stained with uranyl acetate and lead citrate and further examined by electron microscope (Morgagni 268D).

To identify fragmented DNA, a TdT-mediated dUTP nick-end labeling (TUNEL) assay was performed on CLL cell smears. After fixation in 4% paraformaldehyde and permeabilization with 0.2% Triton X-100, cells were treated with 3% bovine serum albumin and TUNEL reaction mixture. Converter for alkaline phosphatase was applied, and Fast red was used to visualize the signal for light microscopy. TUNEL positivity was seen within the apoptotic CLL cell nuclei as granular red nuclear staining (Figure 1). CLL cells were counted in 10 random high-power fields (x400) on each cell slide and the index of apoptosis was determined and expressed as percentage.

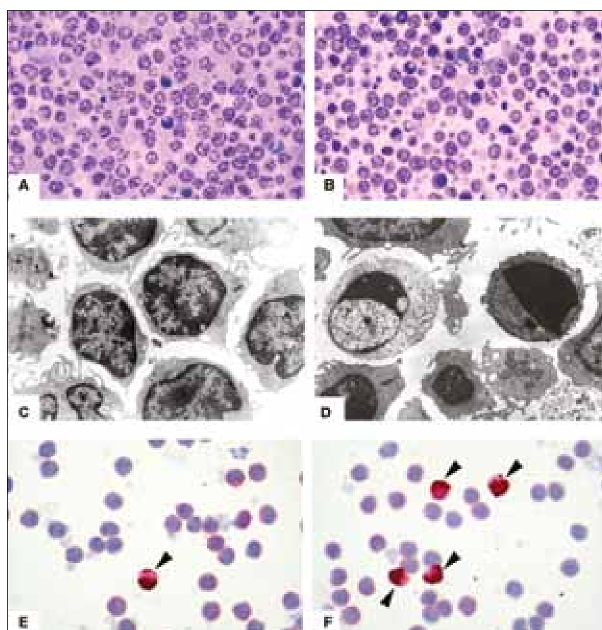

Figure 1. Freshly isolated CLL cells (A, C, E) and incubated for 24 hours (B, D, F). CLL cells on 1 μm thin section stained with toluidine blue (A, B), and viewed by electron microscopy (C, D). Number of CLL cells shows typical morphological features of apoptosis like condensation of cytoplasm and chromatin, with the formation of coarse granular chromatin masses after 24 hours incubation (B, D). TUNEL method performed on CLL cell smears, counterstained with Mayer hematoxylin (E, F). The presence of TUNEL-positive cells (nuclei stained red) is more frequent among cells incubated for 24 hours (F).

Statistics

All data were analyzed by the methods of descriptive parametric (mean, standard deviation and standard error, t-test and ANOVA) and nonparametric statistics (median, Chi square, Mann Whitney U test, Kruskall Wallis test) and also by survival analysis according to Kaplan Meier method together with Cox multivariate regression model and log rank test. All statistical tests were considered significant at level of at least $p<0.05$. Statistical analysis was performed using licensed STATISTICA 8.0 software (StatSoft Inc, Tulsa, USA).

RESULTS

Patient characteristics

Our cohort of patients with CLL consisted of 18 men (66.7%) and 12 women (43.3%). At the time of diagnosis, the mean age of patients was 61.5±8.7 years, median 62.5 years, and ranged from 42 to 81 years. At presentation of disease, the majority of patients (80%, 24 patients) was in the early phase of disease defined as low Rai stages (clinical stage 0 in 8 patients, I in 9 patients, and II in 7 evaluated

patients), while 6 patients (20%) had advanced Rai stages (3 patients had stage III and 3 patients had stage IV disease). The difference in absolute lymphocyte count between early ($35.3\pm56.4\times10^9$/L) and advanced stage CLL ($56.9\pm59.7\times10^9$/L) was noted, but this difference was not statistically significant. Bone marrow biopsy was performed in 26 patients, but quality specimen was obtained in 23 patients. Diffuse infiltration pattern was found in 6 patients. Lymphocyte doubling time (LDT) was defined according to well-defined criteria and LDT <12 months was observed in 12 patients (42.9%), and LDT>12 months was noted in 16 patients (57.1%) while there were no accurate medical records for 2 patients.

In ten-year follow up, two patients remained in indolent stage of disease and did not require any treatment, while the rest of patients required therapy either immediately after diagnosis and staging of CLL or up to 8 years of observation (in 5 of 6 patients with diffuse pattern of bone marrow, infiltration therapy was initiated in the first year of disease).

Detection of apoptosis in CLL cells at presentation of disease

Freshly isolated CLL cells from peripheral blood (0h) showed low number of apoptotic cells (AI_{0h} 3.8±1.7%). Apoptotic index of CLL cells cultured for 24 hours with autologous serum (AI_{24h} 17.7±9.7%) was significantly higher ($p<0.05$) than in freshly isolated cells. No significant difference in the percentage of spontaneous apoptosis of CLL cells was noticed when apoptosis was measured using TUNEL technique or toluidine blue stained sections (AI_{24h} 17.7±9.7% and 17.1±9.1%, respectively). Data obtained using TUNEL technique was used further in the text.

There was no significant difference in apoptotic index (AI_{0h} and AI_{24h}) between different Rai stages (ANOVA $p>0.05$), although somewhat higher values in advanced stages of CLL were found. In addition, difference of the increment of apoptosis defined as a rise in percentage from 0h to 24h (ΔAI) was tested among these groups of patients, but no significant difference was found. Higher values of ΔAI in advanced Rai stages was also noticed (Graph 1).

Considering that there is a biological difference in CLL behavior between early and advanced Rai stages, we also performed the same analysis by grouping the patients with early CLL phase (Rai 0, I, II) and advanced CLL phase (Rai III and IV). Apoptotic index, AI_{0h} was significantly higher (5.8±0.4%) in the advanced stage patients (ASP) compared to the early stage patients (ESP) (3.1±1.4%) ($p<0.05$). Difference in the AI_{24h} between these two groups of patients was also observed (AI_{24h} in ASP was 27.1±7.1%; and in ESP was 15.2±8.7%), but this difference was not proven to be significant due to high variability of data. Early stage CLL patients had lower increase in ΔAI (12.2±8.9%) than late stage patients (21.2±7.2%) but this difference was not significant (Graph 2).

Apoptotic index of CLL cells obtained from the patients with diffuse infiltration of bone marrow did not vary significantly from that determined in patients with other types of infiltration, either in freshly isolated CLL cells (AI_{0h} 3.6±1.6%, 3.9±1.8% respectively), or in cells incubated for 24h (AI_{24h} 16.1±2.6% and 16.9±9.0%).

In patients with short doubling time, LDT<12 months, AI_{0h} was higher (4.0±1.9%) than in the cells obtained from patients with longer LDT (3.4±1.5%) (Mann Whitney U test, $p<0.10$). AI_{24h} was also different between respective groups of patients with LDT <12 months (19.0±10.5%) and LDT>12 months (14.3±6.3), but this difference was not significant ($p>0.05$) (Graph 3).

Time to treatment initiation (TTI)

During 10-year follow up of our CLL patients, 22 patients received therapy at certain time, 5 patients did not receive therapy at all and 3 patients were lost in the follow up. Early stage CLL patients had significantly longer TTI time (median was 4 years) compared to advanced stage patients (median less than 1 year; log rank test $p<0.01$). Patients with LDT >12 months had also significantly longer TTI than those with LDT <12 months ($TTI_{50\%}$ is 5 versus 1 year; log rank test $p<0.05$).

It was observed that patients with lower percentage of AI_{0h} (<5%) required therapy later (median 5 years), while those with higher AI_{0h} (>5%) demanded therapy earlier (median 1 year). By evaluating TTI time according to this threshold, it was found that patients with lower AI_{0h} had significantly longer TTI time (log rank test $p<0.05$; Graph 4). Although patients with ΔAI at threshold less than 15% had longer TTI period (median 4 years) than those with ΔAI >15% (median was 2 years), rise in apoptosis had no significant influence on TTI (Kaplan Meier log rank test $p>0.05$).

Ten-year survival period

Early stage CLL patients had, as expected, significantly longer overall survival (median OS was 10 years) than advanced stage patients (median was 2 years) (Kaplan Meier log rank test $p<0.01$). Patients with LDT>12 months had longer survival than those with shorter LDT, <12 months ($OS_{50\%}$ 10 y vs. 5 years, Kaplan Meier log rank test $p<0.10$).

CLL patients with AI_{0h}<5% had longer survival ($OS_{50\%}$ 10 years), while those with AI_{0h}>5% had shorter ($OS_{50\%}$ 7 year), but AI_{0h} at this threshold was not shown to have significant impact on survival of patients (Kaplan Meier log rank test $p>0.05$). Cox multivariate analysis model with lymphocyte doubling time (LDT), AI_{0h} and increase in percentage of apoptosis during 24h (ΔAI) revealed that AI_{0h} behaved as an independent factor for time to treatment initiation, TTI ($p<0.05$). When distinction of stage (early/advanced) was added in Cox multivariate regression model, only clinical stage of disease behaved as an independent factor (variable) for both time to treatment initiation ($p<0.05$) and survival ($p<0.05$), while apoptosis and LDT were not independent variables any more.

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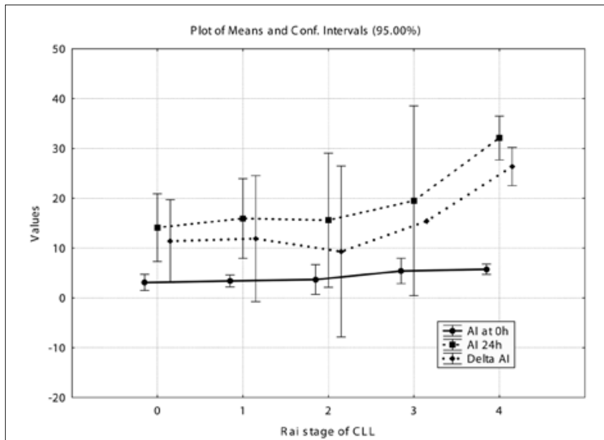
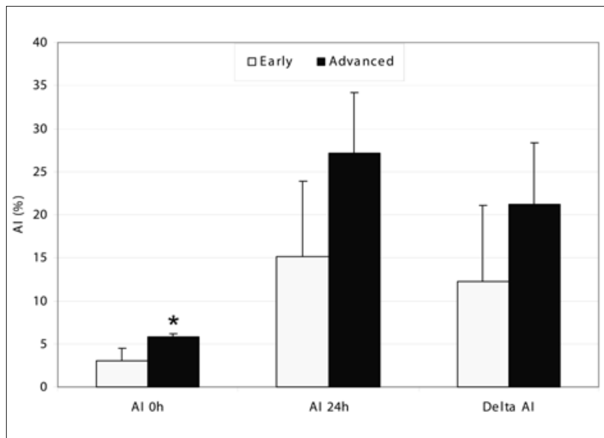


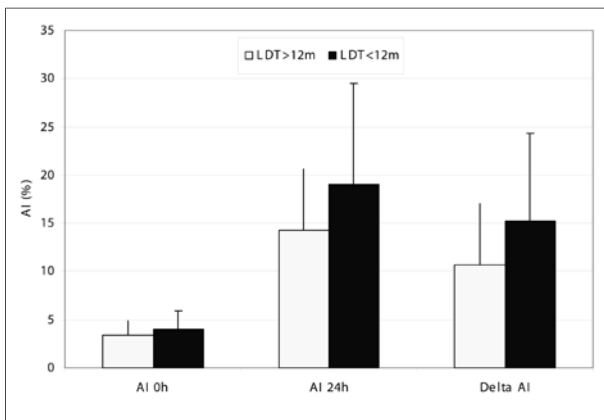
Graph 1. Apoptotic index (AI) of freshly isolated CLL cells (AI_{0h}), after 24h of incubation with autologous serum (AI_{24h}), and difference in AI (ΔAI), defined as rise in percentage of apoptosis from 0h to 24h, in different Rai groups of patients

The data are mean ± SD values.
p>0.05



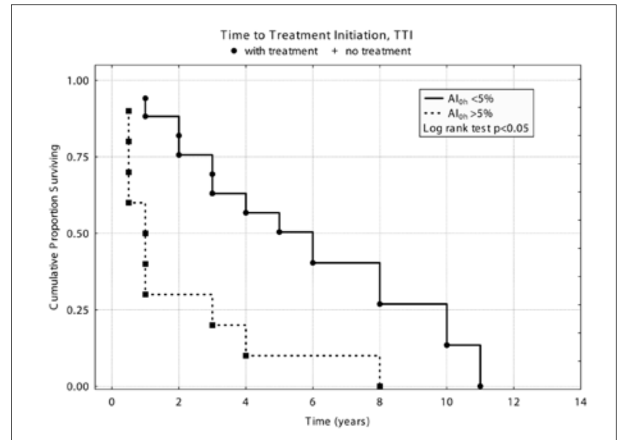
Graph 2. Differences in Apoptotic index (AI) of freshly isolated CLL cells (AI_{0h}), after 24h of incubation with autologous serum (AI_{24h}), and rise in percentage of apoptosis from 0h to 24h (ΔAI) between early-staged and advanced-staged CLL patients

The data are mean ± SD values.
* p<0.05



Graph 3. Differences in Apoptotic index (AI) of freshly isolated CLL cells (AI_{0h}), after 24h of incubation with autologous serum (AI_{24h}), and difference in AI (ΔAI), between patients with lymphocyte doubling time less than 12 months (LDT<12 m), and patients with lymphocyte doubling time more than 12 months (LDT>12 m).

The data are mean ± SD values.
p>0.05



Graph 4. Time to treatment initiation (TTI) in patients with Apoptotic index (AI) of freshly isolated CLL cells (AI0h) higher than 5% (>5%) and lower than 5% (<5%)

DISCUSSION

A large variety of clinical manifestations and variable survival rates are well documented in CLL patients [2, 3]. In our study, groups of patients with the advanced-stage and patients with the early-stage disease at the enrollment were in concordance with recently published epidemiological estimations describing that in the first year of CLL, 20% of patients would be in the advanced stage [20]. Other traditional adverse prognostic factors like diffuse infiltration of bone marrow and LDT less than 12 months were present in minority of patients. Similar observations were found in other studies [13, 21].

The fact that CLL cells die by the spontaneous apoptosis in cell cultures supplemented with fetal calf serum is well documented [22]. We tested whether autologous serum contains some factors which may alter the survival time of CLL cells *in vitro*. Autologous serum failed to protect CLL cells from spontaneous apoptosis. Previously published data are in concordance with our results [21]. We have observed substantial variation in the level of spontaneous apoptosis of CLL cells in different patients. Variations in the level of spontaneous apoptosis in CLL was noticed before and is believed to be the result of cell-intrinsic differences in CLL cells susceptibility to apoptosis, or occur due to variable levels of protective components in the serum of different patients [22]. Activation of the PI3K/Akt cytoprotective-signaling pathway by plasma may contribute to its anti-apoptotic action [23]. Since autologous serum failed to prevent apoptosis *in vitro*, variation in the level of spontaneous apoptosis of CLL cells seen in different patients is possibly related to differential expression of apoptosis regulating proteins [15] rather than to variable levels of protective components in the serum of different patients. Our data confirm a model in which the longevity of CLL cells is determined by the interplay between the intrinsic disease-related genetic changes and survival signaling by environmental factors [15].

In our study, higher percentage of apoptosis was noticed to be present in the advanced-staged patients and patients with short doubling time (LDT<12 mo). It has

been published recently that patients showed significantly better *in vitro* response to purine analogues when they were in the initial stages of the disease, with low beta(2) microglobulin serum level, with long LDT, with few cells expressing CD38, with normal karyotype or no p53 deletion, whereas there was no correspondence with ZAP-70 expression [24]. It has been also published before that the expression of apoptosis regulating protein Mcl-1 significantly correlates with LDT and with the stage of disease [25]. Bone marrow infiltration was the only classical prognostic factor examined that was not found to be related to apoptosis of leukemic cells. There are no previously published data regarding correlation between spontaneous apoptosis of CLL cells and bone marrow infiltration.

Higher percentage of spontaneous apoptosis in advanced-stage patients and higher proliferation of leukemic cells and poor outcome was suggested by Sieklucka M et al. [13]. Contradictory data on the relationship between proliferative markers and disease progression in CLL were published [21]. Increased proliferation, measured by Ki67 and sCD23 [26, 27] was described in CLL cells of patients with the advanced-stage disease. Vrhovac et al. [28], on the contrary, reported a high expression of cell cycle inhibitor p27 in progressive CLL disease, suggesting that CLL cells expressing low p27 levels could be characterized by higher levels of apoptosis. Ricciardy et al. [19] found that CLL cells in patients with progressive disease had more quiescent status and reduced susceptibility to apoptosis. The subdivision of CLL, to progressive and stable disease, does not always match the clinical staging, and indeed the authors noted that four patients considered having progressive disease have been staged as Rai 0 or I [21]. The difference in grouping of samples might be a reason for different results obtained in the study of Ricciardy et al. [21] compared to our study and the study of Sieklucka et al. [13].

During 10-year follow up of our cohort of CLL patients, it was noticed, as expected, that patients with negative prognostic factors like advanced stage disease and LDT less than 12 months, required therapy earlier, and had shorter overall survival time. It was also noted that patients with higher percentage of spontaneous apoptosis of CLL cell also required therapy earlier. Apoptotic index of freshly isolated cells was shown to have significant influence on TTI in our study, but did not have significant influence on survival of patients. Apoptosis of freshly isolated cells behaved as an independent factor for TTI period when other variables, like LDT and ΔAI, were included in Cox multivariate regression model. But, with addition of stage of disease (early/advanced) to Cox multivariate analysis model, the apoptosis was not an independent factor any more. The main independent prognostic factor in CLL is stage of the disease.

Our results after ten-year follow up of CLL patients are somehow consistent with the baseline findings when the patients were seen for the first time. Relationship between high levels of apoptosis and poor prognosis was seen both at the beginning and at the end of study. At the beginning of study, high levels of apoptosis were detected in patients presenting with bad prognostic factors, like advanced Rai stage and short LDT. At the end of this study, higher levels of apoptosis were found in patients with shorter therapy free period. Relationship between high levels of apoptosis and poor prognosis is thus consistent in this study.

CONCLUSION

The results of this study draw attention to the relation of high levels of apoptosis among CLL cells at the time of the initial diagnosis and poor prognosis, emphasizing the importance of apoptosis in pathobiology of the chronic lymphocytic leukemia.

ACKNOWLEDGMENTS

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Висок проценат апоптотичних ћелија *in vitro* у тренутку постављања дијагнозе хроничне лимфоцитне леукемије указује на ранију потребу за лечењем особа с овом болешћу: десетогодишње испитивање

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КРАТАК САДРЖАЈ

Увод Хронична лимфоцитна леукемија (ХЛЛ) има изузетно променљив клинички ток. Биолошки разлози који доводе до великих варијација у клиничком току и степену преживљавања болесника са ХЛЛ нису потпуно разјашњени.

Циљ рада Циљ студије је био да се утврди важност одређивања спонтане апоптозе ХЛЛ ћелија *in vitro* на презентацију болести ради прогнозе тока ове болести и потребе за лечењем болесника са ХЛЛ.

Методе рада Леукемијски лимфоцити изоловани су из периферне крви 30 новодијагностикованих болесника и инкубирани 24 часа у РПМИ-1640 медијуму обогаћеним са 10% серума добијеног од истог болесника са ХЛЛ. Ћелије су касније фиксиране и калупљене у Епону или су од њих прављени ћелијски размази који су бојени *TUNEL* техником.

Резултати Током десетогодишњег периода испитивања откривено је да су болесници с мањим процентом апоптотичних ћелија на презентацији болести имали дужи период до започињања терапије (*log-rank* тест, $p<0,05$). Апоптоза ХЛЛ ћелија, међутим, није имала значајан утицај на преживљавање болесника (Каплан–Мајеров *log-rank* тест, $p>0,05$).

Закључак Резултати ове студије указују на значај апоптозе ХЛЛ ћелија у тренутку дијагнозе болести у патогенези хроничне лимфоцитне леукемије.

Кључне речи: апоптоза; хронична лимфоцитна леукемија; аутологни серум; прогноза; преживљавање

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