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Possible prognostic value of nucleolar morphology in pathologic cells of B-chronic lymphocytic leukemia

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B-cell chronic lymphocytic leukemia (B-CLL) represents a heterogeneous disease with a very variable outcome. The reliable prognosis of this disease at the time of initial diagnosis is difficult to predict. The purpose of this preliminary study was to utilize the nucleolar morphology and to investigate the incidence of main nucleolar types in leukemic lymphocytes in B-CLL patients to assess their possible predictive value for the disease outcome, in correlation with immunophenotype parameters. The evaluation of nucleolar morphology of pathologic lymphocytes was performed at diagnosis and during the course of disease. Median follow up period of patients was 16.4 months (range from 2 to 32 months) from diagnosis. The nucleoli were visualized by a simple cytochemical demonstration of RNA and the proportion of main nucleolar types in pathologic lymphocyte population infiltrating bone marrow of 84 patients suffering from B-CLL was analyzed. The presence of ring shaped and compact nucleoli in leukemic lymphocytes divided patients into two subgroups with different outcome of the disease. Malignant lymphocytes of the majority of patients (Group 1, 71 patients, 84.5%) mostly contained ring shaped nucleoli. These patients were in stable phase and did not require any treatment during the follow up. The population of leukemic cells of a small group of B-CLL patients (Group 2, 13 patients, 15.4%) was characterized by the presence of various proportions of pathologic lymphocytes with one large compact nucleolus.Different response to the therapy discriminated the B-CLL patients whose leukemic lymphocytes revealed evident compact nucleoli at presentation, to next two subsets. Four of these patients (Group 2, 4/13, 31%) appeared to be resistant to chemotherapy, others (9/13, 69%) showed response to therapy, though the response time was variable. Leukemic cells with compact nucleolus morphologically resembled prolymphocytes, but hematologically and immunophenotypically did not fulfill the diagnostic criteria for prolymphocyte population. None of our B-CLL patients had the signs of transformation to prolymphocytic or other type of B cell neoplasms during the follow up. Our results indicate the possibility of relationship between the presence of malignant lymphocytes with compact nucleoli and unfavorable outcome in patients with B-CLL. The simplicity and utility of the nucleolar test as a possible prognostic parameter may help to identify the subset of patients with early B-CLL disease that will run a more progressive course.

Key words: B-chronic lymphocytic leukemia; nucleolar morphology; ring shaped nucleolus; compact nucleolus; prognostic marker; immunophenotype

B-cell chronic lymphocytic leukemia (B-CLL) is a malignant hematological disease with a wide clinical spectrum, course, laboratory findings and prognosis [1, 2, 3]. Early stages of B-CLL which are most frequent at diagnosis have an extremely variable individual prognosis, as many patients survival for decades without requiring any treatment, however a subset of B-CLL patients develop more advanced stages and progressive forms of the disease less or more rapidly [4, 5, 6]. There are some difficulties in identifying these two different subgroups of B-CLL patients at the moment of diagnosis [2, 5, 7]. Prediction of B-CLL progression and outcome of disorder from its early stage is a key issue in the decision to initiate early treatment [8].

During the last decades many efforts have been made to identify prognostic features that may be predictive of decreased survival outcome, disease progression, and that may play a role in defining the subgroups of B-CLL patients with high risk of disease. The known prognostic markers include clinical staging system, genetic abnormalities of pathologic lymphocytes, mutational status of the immunoglobulin (Ig) heavy (H) chain variable (V) genes, disrupted p53 protein function, expression of intracellular ZAP-70 and cell surface CD38 antigen expression [6, 9, 10]. However, the predictive value of these markers is variable and in some cases limited [9]. The clinical staging system fail to accurately predict the

prognosis of individual patients, especially in early stages and therefore to identify the subset of patients whose disease may progress rapidly [1, 10]. Moreover, many of the methods to assess patient's prognosis may be expensive, a time consuming or technically and routinely unavailable to many hematological laboratories [9, 11].

The morphologic criteria of pathologic lymphocytes have also been identified permitting the prognosis of the course character of B-CLL. Variations in leukemic lymphocyte morphology, blood lymphocyte levels, degree of bone marrow infiltration among patients with CLL may reflect the biological diversity of the disease. There is strong evidence of a close association between atypical morphology and clinical features of this disease [12, 13, 14, 15, 16].

The morphology of nucleolus, a highly dynamic nuclear compartment, is a good model to investigate the cell characteristic and abnormality [17, 18, 19, 20, 21]. The nucleolus is the mirror of a series of metabolic changes that characterize tumor cells [20] and in cancer cells undergoes variations in size, shape, fine structure, and cytochemical composition [17]. Nucleoli are known as the important markers for tumor diagnosis and progression [18, 20]. The nucleolar hypertrophy is one of the most consistent cytological features of malignancy and independent predictive and prognostic parameter of a clinical outcome. In fact only enlarged nucleoli, defined as "prominent" nucleoli are easily visualized and analyzed. The nucleolar size may be a very useful parameter for the clinical outcome of the disease, and can be very precisely and inexpensively defined [20].

Main nucleolar types represent the morphologic expression of nucleolar ribosome biosynthetic activities and the incidence of functional dominant nucleolar types in pathologic cells reflects the various states of individual cells [18, 19]. Because the production of ribosomes is its major metabolic activity, the function of the nucleolus is tightly linked to cell growth and death, proliferation and cell cycle regulation [18, 20]. However, the importance of nucleolar changes in cancer cells is often underestimated in tumor pathology [20].

The visualization of nucleoli by a simple cytochemical procedure of the determination of RNA [22, 23] and evaluation of the proportion of different nucleoli types, ring shaped and/or compact may be applied even in clinical hematological laboratories [18].

The aim of this preliminary study was to determine the incidence of main types of nucleoli and the possible changes in their proportion in the leukemic lymphocyte population of B-CLL patients investigated at the time of diagnosis and during the course of disease. The additional purpose of this study was to ascertain whether the nucleolus type and its morphology may have some predictive value related with disease progression and thus whether makes it possible to identify those patients in early disease stage who probably will progress to a more advanced disease, and on the other hand, whether the nucleolar parameter may be used for prognostic purpose. In regard to assumed predictive value of distinct types of nucleoli in pathologic lymphocytes and their association with unfavorable outcome it is necessary to follow up the patients suffering from B-CLL longer time to determine whether nucleolar morphologic features persist and progress.

Materials and methods

Patients and leukemia samples. Eighty-four patients with B-cell chronic lymphocytic leukemias (B-CLL) were included in this study between 2006 and 2009. Patients were evaluated at the time of initial diagnosis, before receiving any treatment and during routine follow up. All cases were diagnosed as having classical B-CLL on the basis of clinical examination, peripheral blood (PB) and bone marrow (BM) count, morphology, <10% of prolymphocytes and immunophenotyping. The diagnosis of B-CLL required a persistent lymphocytosis of more than $10.0x10^9$/L, a CD5+, CD19+, CD23+, CD20+ and monoclonality of the κ or λ light immunoglobulin chain restricted immunophenotype. Cases were excluded if the findings were inconsistent with the diagnosis of B-CLL. Age of the patients ranged from 44 to 86 years. There were 48 males (57%) and 36 females (43%). Patients have been followed up at a median of 16.4 months (range, 2-32 months) since diagnosis. During the follow up analysis, 13 patients underwent chemotherapy.

Lymphocyte immunophenotyping. Fresh, heparin anticoagulated PB and BM samples were immunophenotyped at diagnosis and repeatedly during the course of disease. Analysis of leukemic cells was performed using an erythrocyte lysed-whole blood method with Optilyse as a lysing agent (Optilyse B, Immunotech, France). Direct immunofluorescence staining was performed with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated monoclonal antibodies (MoAbs) specific for: lymphocyte lineage (CD5, CD19, CD23, CD20, CD22, FMC7, CD3, CD10, and CD38) and cell immaturity/non lineage (HLA-DR, CD45). In addition, determination of kappa (κ) and lambda (λ) light immunoglobulin chain expression was performed. The CD11b as myeloid cell marker was also used. MoAbs were purchased from Immunotech, Beckman Coulter Company, Marseille, France. Flow cytometric analysis was performed on EPICS ALTRA flow cytometer (Beckman Coulter International S.A, USA) equipped by Expo 32 program for analysis. Cases with more than 30% of mononuclear cells reactive with an appropriate antibody were considered positive.

Identification of leukemic pathologic lymphocytes was performed using forward (FSC) versus side scatter (SSC) parameters and/or CD45 intensity versus SSC dot plots. Gating of cells was important to differentiate between pathologic and normal cells. The results of each antigen were expressed as percentage positivity (>30%) of stained cells within the gated pathological population.

In all cases, isotype-matched immunoglobulins with no reactivity to PB and BM cells were used as negative control.

Morphological and cytochemical evaluation. Peripheral blood and/or bone marrow smears were analyzed at diagnosis

and during routine follow up with May-Grünwald-Giemsa stain as well as with the cytochemical demonstration of RNA. The nucleoli of the pathologic lymphoid cells were stained by a simple but sensitive cytochemical method for the demonstration of RNA, which facilitates the visualization of the nucleolar distribution of this nucleoli acid at the light microscopic level. Dry peripheral blood and bone marrow smears, not older than 24 hours, were stained without previous fixation with the diluted methylene blue buffered with Mc Ilwaine buffer at pH 5.3 for 15 min. at room temperature [22, 23]. For each patient the percentage of nucleolar type, ring shaped and compact were determined. According to the presence or absence of compact nucleolus in pathologic lymphocytes, the patients were subdivided into two distinct groups. Leukemic cells in patients formed Group1 (71 patients, 84.5%) displayed only ring shaped nucleolus. Among the eighty-four patients, a small group of patients (13 patients, 15.4%) was characterized, besides the presence of ring shaped nucleoli, by the presence of different proportions of pathologic cells with compact nucleolus (Group2). The influence of the presence or absence of these two different types of nucleoli in malignant lymphocytes on a future course of disease was worthy of remark.

The controls were subjects who underwent BM aspiration for clinical diagnosis, but whole BM and PB contained no pathologic cells.

Statistical analysis. Student's t-test for equal and unequal variance was used to analyze the statistical significance of the results. P values <0.05 were considered significant.

Results

In the cohort of 84 B-CLL patients involved in the present study, 48 were males (57%) and 36 were females (43%). Age of patients ranged from 44 years to 86 years. PB and, in a substantial number of cases, bone marrow specimens were examined. The BM aspirates revealed high degree of lymphocyte infiltration (range, 77%-96% pathologic lymphocytes). Although a bone marrow aspirate is generally not required to make the diagnosis of B-CLL, nevertheless it is appropriate to investigate BM as a major site of pathologic cells involvement. As there were no considerable differences in evaluated parameters between PB and BM samples, the presented results are derived from BM analysis.

The detailed morphologic, cytochemical and immunophenotypic analysis of malignant lymphocytes at diagnosis and during the course of disease was made. The most pronounced differences between patient's leukemic lymphocytes were observed when their nucleolar morphology and cytochemistry was carefully evaluated. On the ground of these differences, it was allowed the discrimination of patients into two distinct B-CLL subgroups. Seventy-one patients comprised Group 1 (38 males, 33 females) and 13 patients were included into Group 2 (10 males, 3 females). Patient's main characteristics are summarized in Table 1.

Table l. Characteristics of B-CLL patients at diagnosis

Variable		range	P- value
Number of patients	84		
Sex			
Males	48 (57%)[a]		
Females	36 (43%)		
Age (years)			
Group1 (71 patients, 84.5%)	62.0±9.8[b]	(44-86)	
Group2 (13 patients, 15.4%)	59.6±7.1	(47-72)	0.3500
WBC (x 10^9L)			
Group1	66.1±68.5[c]	(13-308)	
Group2	146.5±120.8	(38-450)	0.0426
% of pathologic cells by FC			
Group1	84.7±5.6[d]	(77-95)	
Group2	86.3±4.8	(82-96)	0.3600
% of pathologic cells by cytochemistry			
Group1			
with ring shaped nucleolus	79.2±5.0[e]	(70-89)	
with compact nucleolus	3.0±1.5	(0-6)	< 0.0001
Group2			
with ring shaped nucleolus	65.2±4.5	(58-70)	
with compact nucleolus	22.8±5.7	(13-32)	< 0.0001
% of healthy lymphocytes by FC			
Group1	7.2±3.4	(1-18)	
Group2	4.6±2.4	(1-8)	0.0022
% of prolymphocytes (May-Grünwald-Giemsa staining)			
Group1	4.2±2.1[f]	(0-8)	
Group2	2.3±1.4	(0-5)	0.0014
% of CD38+ leukemic cells			
Cutt-off 30% positive cells			
Group1 (7/71 patients, 9.8%	51.1±15.6	(30-77)	
Group2 (5/13 patients, 38.4%)	48.4±17.1	(35-82)	0.8743

[a] number of patients (%)
[b] median ± SD (range)
[c] WBC – white blood cell count
[d] % of pathologic cells by immunophenotyping (flow cytometry)
[e] % of pathologic cells by cytochemical demonstration of RNA
[f] % of prolymphocytes stained with May-Grünwald-Giemsa

There were no significant differences between the age 62±9.8 (range, 44-86 years) for Group 1 and 59.6±7.1 (range, 47-72 years) for Group2, respectively (P=0.3500). Initial WBC counts between Group 1 and Group 2 were significantly distinct

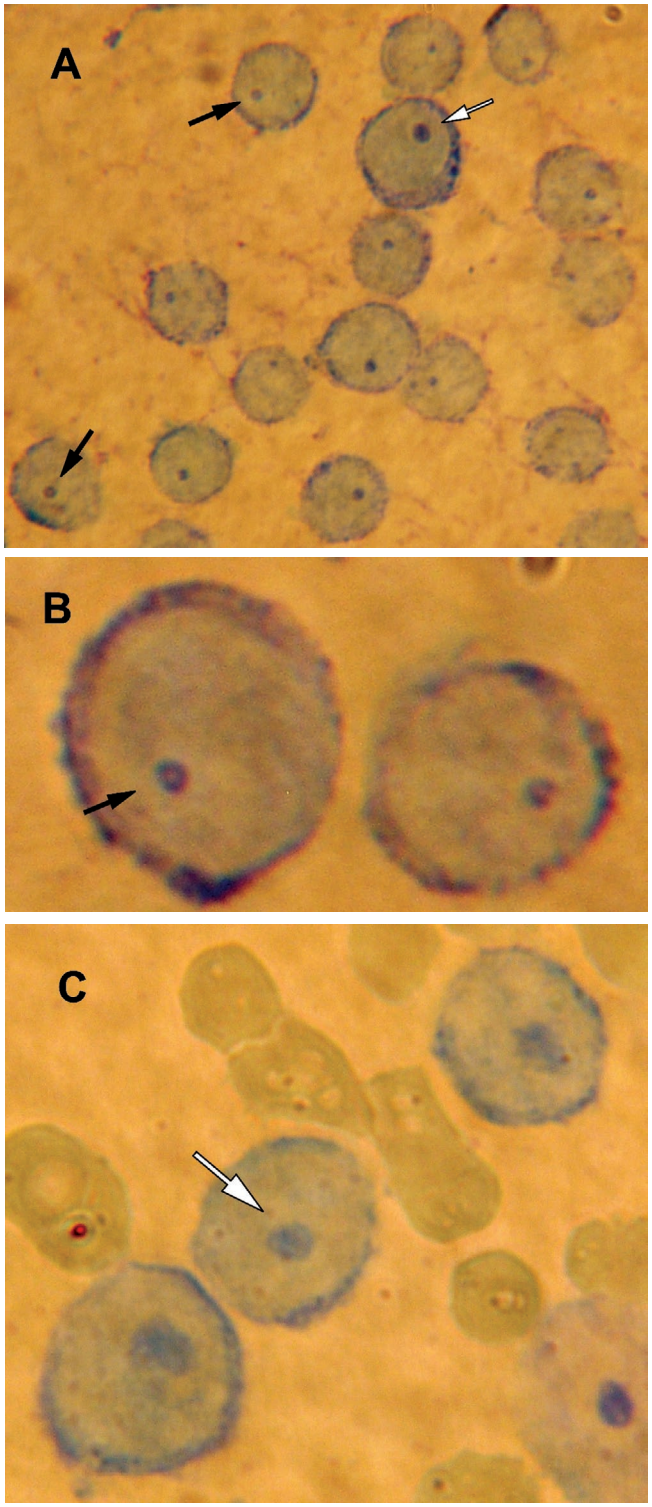

Figure 1. Leukemic lymphocytes in the bone marrow smear of B-CLL patients. Staining for RNA. A: Note the leukemic cells with ring shaped nucleoli (black arrows) and lymphocyte with compact nucleolus (white arrow). B: Pathologic lymphocytes containing a ring shaped nucleolus with RNA in the peripheral part of nucleolus (black arrow). C: Leukemic cells with prominent dense nucleolus with a uniform RNA distribution (white arrow). Original magnification 1 200 x.

(P=0.0426). The median WBC count of patients in Group 1 was 66.1±68.5 x 10^9/L (range 13 to 308 x 10^9/L). The median WBC count of patients from Group 2 was 146.5±120.8 x 10^9/L (range, 38-450 x 10^9/L). The marked lymphocytosis exceeding 100.0 x 10^9/L was noted in 11 patients from Group 1 and in 7 cases of Group 2. There were no significant differences between B-CLL subgroups in the percentage of pathologic lymphocytes analyzed by flow cytometric immunophenotyping. The median percentage of leukemic lymphocytes was 84.7±5.6% (range, 77%-95%) for Group 1 and 86.3±4.8 (range, 82%-96%) for Group 2, respectively (P=0.3600). Only a few normal, healthy lymphocytes demonstrated by immunofluorescence in both groups were found.

Morphology assessment and cytochemistry. All patients at presentation and during routine follow up underwent nucleolar test. Nucleoli of pathologic lymphocytes were visualized using standard May-Grünwald-Giemsa staining as well as by a simple cytochemical procedure for the demonstration of RNA containing structures. At least 300 lymphocytes were counted in each BM smears.

With the standard May-Grünwald-Giemsa stain, patients of both groups revealed typical B-CLL lymphocyte morphology without differences between them. The majority of malignant lymphoid cells were presented by small lymphocytes without nucleoli. Only a very small proportion of leukemic cells (4.2±2.1, range, 0%-8% in Group 1 and 2.3±1.4, range, 0%-5% in Group 2, respectively, P=0.0014) were larger and presented by a visible nucleolus (prolymphocytes). On the other hand, the visualization of nucleoli using cytochemical demonstration of RNA showed the nucleolar distinctions, morphologically expressed by the presence of two main types of nucleoli in pathologic lymphocytes. The majority of leukemic lymphocytes in Group 1 (79.2±5.0%, range, 70%-89%, Table 1) were usually characterized by the presence of ring shaped nucleolus with RNA only in their nucleolar peripheral part, with the minimal occurrence of compact nucleoli (Table 1, p<0.0001, Figure 1A, B). The presence of prominent compact, dense nucleolus with a relative uniform distribution of RNA in the proportion of pathologic lymphocytes of patients in Group 2 distinguished them from malignant population in Group1 (Table 1, Figure 1C). The medium percentage of leukemic cells with compact nucleolus was 22.8±5.7%, range from 13 to 32. The difference in the percentage of lymphocytes with compact nucleolus between Group 1 and Group 2 was evident. Leukemic cells with compact nucleolus were larger and reminiscent of prolymphocytes, though hematologically and immunophenotypically did not fulfill the diagnostic criteria for prolymphocyte population. In some respect, these cells had an immature blast-like appearance.

Immunophenotyping by flow cytometry. The malignant cells from both groups displayed typical B-CLL phenotype with the antigen expression of CD5+, CD19+, CD23+, CD22+, CD20+, HLA-DR+, FMC7-, CD3-, CD10- and CD11b- (Figure 2). Immunoglobulin light chain restriction was demonstrated in 67 patients of 84 cases of B-CLL (78%) with 35 patients (52%)

expressing kappa and 29 (43%) expressing lambda epitopes. Three patients (5%) showed neither kappa nor lambda restriction of Ig light chain. Interestingly, prevalence of lambda light chain expression was seen in patients of Group2 (6/13, 46%).

The immunophenotypic evaluation failed to show significant differences in the main antigenic profile of the pathologic lymphocytes of both B-CLL groups at presentation (Figure 2).

Concerning the CD38 antigen, its expression at diagnosis was observed in 27 patients with wide variation (range, 1%-77% leukemic cells) of Group 1 as well as 8 patients (range, 9%-82% leukemic cells) in Group 2. Choosing the cut-off value 30%, 7 of 71 patients in Group1 (9.8%) was CD38 positive (range, 30%-77% leukemic cells). On the other hand, the CD38 antigen was expressed in 5 of 13 patients in Group 2 (38.4%, range, 35%-82% pathologic cells). Comparison between the Group 1 and Group 2 revealed remarkable higher frequency of CD38 expression in patients with compact nucleolus in leukemic cells compared to patients with ring shaped nucleolus in pathologic cells (Table 1).

On the basis of morphologic and cytochemical distinctions between Group 1 and Group 2 at diagnosis and for purpose to determine possible importance and the effect of these differences on the future outcome of the disease, the patients were followed up at different time interval since diagnosis. The outcome of patients from Group2 was quite different from the outcome of cases involved in Group1. It was observed, that B-CLL patients whose pathologic lymphocyte population was presented by the proportion of leukemic cells with compact nucleolus (Group 2) appeared the signs of progression and relapse, in contrast to patients that malignant cells revealed only ring shaped nucleolus (Group 1). During the follow up period, 71 patients (Group1) did not require any treatment,

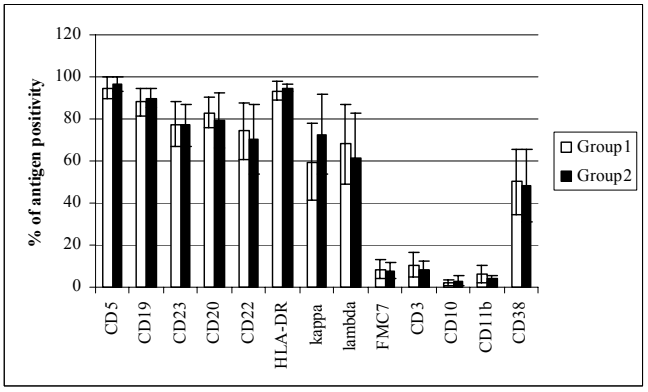

Figure 2. Comparison of the surface membrane antigens expression in two subgroups of B-CLL patients at diagnosis.

however, 13 patients (Group2) required early chemotherapy. Median follow up of patients was 16.4 months (range, 2 to 32 months). Interesting, the high percentage (77%-97%) of pathologic lymphocytes persisted for a long time (7-29 months) (Table 2).The further development of the disease of patients included in Group 1 (only with ring-shaped nucleolus in leukemic cells) appeared to be favorable. All the patients were frequently (3-7 times) investigated morphologically, cytochemically and immunophenotypically.

Moreover, the different response to the therapy discriminated the B-CLL patients whose leukemic lymphocytes revealed compact nucleolus at presentation, to next two subsets. Four of patients (Group 2, 4/13, 31%, cases 1, 2, 3, 4, Table 2) appeared to be resistant to chemotherapy. Moreover, the WBC counts were increased with time after diagnosis (130 $x10^9$/L, 400 $x10^9$/L, 96 $x10^9$/L and 259 $x10^9$/L, respectively) in compari-

Table 2. Distribution of pathologic cells and of leukemic cells with compact nucleoli at diagnosis and during the follow-up (months)

Patients	At diagnosis		Follow-up from initial diagnosis (months)											
Group2 (n=13)	% of pathol. cells (FC)	% of pathol. cells with c.n.	% of pathologic cells (FC)						% of pathologic cells with compact nucleolus (cytochemistry)					
1	88	25	95(4)	93(8)	93(10)	88(13)	97(16)	97(18)	27(4)	31(8)	26(10)	29(13)	24(16)	26(18)
2	90	28	92(8)	90(29)					26(8)	18(29)				
3	84	18	87(7)	89(9)					25(7)	20(9)				
4	86	32	95(5)	92(7)					30(5)	29(7)				
5	83	13	85(5)	82(22)	0(25)	0(32)			19(5)	14(22)	0(25)	0(32)		
6	78	27	65(10)	62(14)	77(18)	0(20)	0(22)	0(25)	21(10)	18(14)	13(18)	0(20)	0(22)	0(25)
7	80	17	72(14)	24(20)	25(24)				22(14)	5(20)	3(24)			
8	91	31	25(4)	32(8)	7(12)				29(4)	0(8)	4(12)			
9	96	26	93(3)	0(6)	0(10)	0(17)	31(21)		17(3)	0(6)	0(10)	0(17)	12(21)	
10	82	22	16(7)	13(12)					13(7)	0(12)				
11	86	23	25(6)	0(18)					20(6)	0(18)				
12	86	16	70(3)	16(5)					15(3)	0(5)				
13	92	19	27(3)						4(3)					

c.n. compact nucleolus
FC flow cytometry

son with WBC counts observed in these patients at the time of initial diagnosis (50 x10^9/L, 209 x10^9/L, 94 x10^9/L and 132 x10^9/L, respectively (data not shown). In addition, no differences were noticed either in morphology of pathologic cells stained with the standard May-Grünwald-Giemsa method or in immunophenotype at diagnosis and during the course of disease. The long lasting persistence of the malignant cells with compact nucleolus stained for RNA was worth of note during the follow up time in this group of patients (Table 2). Although the leukemic cells with compact nucleolus reminded of prolymphocytes, the picture was not typical of prolymphocyte leukemia. None of our B-CLL patients had the signs of transformation to prolymphocytic or other type of B cell neoplasms.

The disease outcome of other patients from Group 2 (9/13, 69%, cases 5-13) was somewhat different. Patients showed response to therapy, though the response time was variable (Table 2). They were all exposed to repeated chemotherapy cycle. As a result of therapy, the sequential disappearing of leukemic cells containing compact nucleolus and increasing in the percentage of cells with ring shaped nucleolus, together with decreasing of the total number of pathologic cells was clearly evident. However, it was observed, that bone marrow of one of patients (case 9) was again infiltrated with leukemic cells containing compact nucleolus after 21 months of follow up (Table 2). The micronucleoli occurred occasionally in some lymphocytes. During the follow up period from two to thirty two months, patients showed successive return the WBC counts to normal levels as well as slow decrease in percentage of B-CLL characteristic membrane antigens. The BM of the majority of patients was not infiltrated with cells of pathologic phenotype. A very slow restitution of normal bone marrow cells indicated the development of the phase of remission (data not shown). Notwithstanding, all patients remained under the longterm surveillance.

Discussion

The clinical heterogeneity and biologic diversity of B-cell chronic lymphocytic leukemia (B-CLL) necessitates precise investigation by using such prognostic markers that should be able to identify subsets of B-CLL patients in early clinical disease stage, which will progress to a more advanced disease requiring early therapy or to predict probable an favorable outcome of the disease at the time of diagnosis [4, 24].

Among various predictive factors [6, 9, 10], the examination of the typical and/or atypical morphology of pathologic lymphocyte population infiltrating the bone marrow of B-CLL patients may also be of prognostic relevance. Atypical morphology usually is associated with poor clinical outcome of the disease [13, 14, 15, 16]. In addition, the investigation of nucleolus, the very active and variable nuclear compartment, and its morphologic and functional changes assumed its usefulness in predicting the early disease progression probability [20]. One important difference between cancer and normal cells is hyperactivity of the nucleolus [17, 20]. The fine structure, size, shape and cytochemical composition of a nucleolus is largely determined by its activity in ribosome biogenesis that is the major metabolic effort of proliferating cells [17, 20, 21]. Main nucleolus types may be distinguished according to the distribution of RNA [17, 18]. More importantly, the number of proliferating cells and the rapidity of cell proliferation may be the most important prognostic factors in malignancy [20]. Thus the investigation and identification of the distinct types of nucleoli in pathologic lymphocyte population in early B-CLL patients may be useful as a prognostic marker helping to determine the risk category of patients at diagnosis.

In our study, the evaluation of nucleolar morphology of pathologic lymphocytes was performed at diagnosis and during the course of the disease.

The results of this preliminary study indicate that according to the presence of distinct nucleolar type in leukemic lymphocytes, at least two different subgroups of our B-CLL patients were observed. The presence of ring shaped and compact nucleoli in leukemic lymphocytes divided patients into two subgroups with different outcome of the disease. Malignant lymphocytes of the majority of patients (Group 1, 71 patients, 84.5%) mostly contained ring shaped nucleolus. On the other hand, the population of leukemic cells of a small group of B-CLL patients (Group 2, 13 patients, 15.4%) was characterized by the presence of different proportions of pathologic lymphocytes with large compact nucleolus. Moreover, the different response to the therapy discriminated the B-CLL patients whose leukemic lymphocytes revealed evident compact nucleoli at presentation, to next two subsets. Four of these patients (Group 2, 4/13, 31%) appeared to be resistant to chemotherapy, other patients (9/13, 69%) showed response to therapy, though the response time was variable.

Response to therapy may be also an important prognostic parameter by itself, whereby the higher response the longer the survival [9].

The structural organization of the nucleolar components as visualized by light microscopy as well as electron microscopy is constantly characterized by the presence of ring shaped nucleolus with RNA only in the nucleolar peripheral part surrounding one large fibrillar centre, compact nucleoli with a relative uniform distribution of RNA and the type of micronucleoli, in which the distribution of RNA containing structures are not distinguisable [18, 19, 20]. Each type of nucleoli is restricted to cells with a different rate of ribosome biogenesis [17, 20]. There is evidence that lymphocytes, which nucleoli have ring shaped appearance, can be considered mature, reversibly sleeping with low level of ribosome biogenesis [18]. By contrast, the presence of large compact nucleoli rich in nucleolar ribonucleoprotein particles in leukemic lymphocytes indicates that these cells are rapidly synthesizing ribosomal precursors, and may represent the population of proliferating or less differentiated cells [18]. Proliferating cells have a higher demand for ribosome synthesis [21]. Our results are in line with these considerations. The subgroup (Group1) of B-CLL

patients whose leukemic lymphocyte population revealed the presence only ring shaped nucleoli did not require any therapy during thirty two months of follow up. However, the appearance of lymphocytes with large compact nucleoli in the portion of pathologic leukemic population at initial B-CLL diagnosis (Group 2) was accompanied with unfavorable course of the disease and its progression.

The lymphocytes with visible and large nucleoli, resembling prolymphocytes, are by some authors considered to be immature and/or proliferating [18, 19, 20] and correlated with a poor prognosis [18, 19, 20]. Interestingly, Criel et al. [13, 14] showed that certain patients even without an increased prolymphocyte population might have an accelerated poor clinical outcome. The immunophenotypic picture of the pathologic population with lymphocytes containing compact nucleoli in our group of patients, even though they looked like prolymphocytes, was characteristic for B-CLL but neither for prolymphocytic leukemia (PLL) nor transformation to this type of leukemia. The membrane phenotype of the B-PLL is quite distinct from that of B-CLL The expression of CD5+, CD19+, CD23+and the absence of FMC7 surface markers are routinely used as diagnostic criteria in patients with B-CLL [15, 25]. In addition, the absence of expression of FMC7 is one of the most reliable markers that differentiated B-CLL from other B cell neoplasms [25] and, moreover, CD5 negativity can help distinguish between B-CLL and B-PLL [26]. The CD23 antigen negatively correlates with prolymphocyte infiltration of the BM and PB lymphocyte counts [27]. Also, the CD23 negativity is rare in classical B-CLL [28]. The immunologic repertoire of both subgroups of our cases of B-CLL showed high CD5 and CD23 antigen positivity and FMC7 negativity.

We observed, in accordance with other studies [18, 29], that the incidence of lymphocytes with large active nucleoli properly characterized the subgroup of B-CLL patients with poor outcome and was only slightly influenced by the chemotherapy as compared to patients without lymphocytes with compact nucleoli. Also, it was found, in agreement with Kusenda et al. [30] that the therapy did not affect in a significant level the expression of surface markers characteristic for B-CLL.

In view of our results arise the question whether the leukemic lymphocytes with compact nucleoli in our B-CLL represent the proliferating fraction and thus if they may be responsible for the progression of the disease? Messmer et al [31] demonstrated that B-CLL is not a stable disease that results simply from accumulation of long-lived lymphocytes, but a disease where a dynamic process in which cells proliferate and die, often at appropriate level. This finding is in conflict with dogma that B-CLL is a disease characterized almost exclusively by cell accumulation due to a defect in apoptosis. Chiorazzi and Ferrarini [32] examined the contribution of proliferation to the progression of B-CLL, supported the existence of a proliferative compartment in B-CLL and clearly documented the role of proliferating cells in the progression of B-CLL. The existence of a distinguishable proliferating pool of leukemic cells in B-CLL has clinical relevance [32]. To characterize the proliferating component, Bennett et al. [33] assessed the expression of proliferation-associated protein Ki67 to characterize the proliferating component in CLL cells. Messmer et al [31] in their study used deuterium, a nonradioactive isotope to label newly synthesized DNA of dividing B-CLL cells.

The activity of nucleoli may reflect the proliferating activity of the cells [17, 18, 20]. It is supposed that the relationship between the changes of nucleolar morphology due to increased demand for ribosome biogenesis and proliferation may explain the feasible progression of B-CLL. In fact, the number of proliferating cells and the rapidity of cell proliferation are major factors in determining the growth rate of a tumor cell population, which in turn is one of the most important prognostic factors in oncology [20]. Furthermore, the CD38 antigen positive subpopulation in B-CLL was finding to be linked to cell activation and labeling the proliferative component defined by Ki67 expression [34, 35]. B-CLL cases with ≥ 30% CD38+ cells are associated with poor prognosis [35]. Moreover, the close association between increased proliferative activity and the presence of CD38 positive subpopulation may serve as a prognostic marker in B-CLL patients [34]. In addition, the study by Ghia et al [36] showed that CD38 expression is higher in bone marrow when compared with peripheral blood. Therefore, it seems likely that remarkable higher frequency of CD38 expression in our patients whose bone marrow leukemic cells contained compact nucleoli might help explain the reason for disease progression, in comparison to patients with ring shaped nucleoli in pathologic cells.

We assumed that subtyping our B-CLL patients by nucleolar morphology enables the identification of two groups of cases, each characterized by a different outcome and prognosis. In the light of these findings, we conclude that the presence of a proportion of leukemic lymphocytes with compact nucleoli might be useful indicator for predicting disease progression and thus regarded as an unfavorable prognostic marker in B-CLL.

Whether the rapid, relatively simple and inexpensive nucleolar test will be sufficient evidence and helpful approach for the evaluation of its prognostic validity in predicting the progression of B-CLL at diagnosis have to be verified by further studies on larger cohort of patients and longer follow up period. However, the correlation with the others hematological laboratory procedures is necessary.

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